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June 5, 2002

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National Organic Standards Board
c/o Robert Pooler, Agricultural Marketing Specialist
USDA/AMS/TM/NOP
Room 2510-So.
Ag Stop 0268
P.O. Box 96456
Washington, DC 20090-6456

Petition for Evaluation of Egg White Lysozyme for Inclusion on the National List of Substances Allowed and Prohibited in Organic Production and Handling

Dear Mr. Pooler:

The Enzyme Technical Association ("ETA"), a trade association comprised of enzyme manufacturers and distributors in North American, hereby submits this petition pursuant to 7 U.S.C. § 6518(n) and 65 Fed. Reg. 43259 (July 13, 2000) (Proposed Rule) for the addition of egg white lysozyme to the National List of Substances Allowed and Prohibited in Organic Production and Handling (the "National List") as a nonagricultural substance allowed in processed products labeled as "organic" or "made with organic" ingredients.

The National Organic Standards Board ("NOSB") previously considered, but did not recommend, the inclusion of egg white lysozyme on the National List. This petition seeks a reconsideration of the board's recommendation in light of "significant new information" that was not available to the NOSB at the time of its prior evaluation and which clearly impacts on the basis of the board's decision. See 65 Fed. Reg. at 43260. It is our understanding, based on a review of the applicable meeting minutes and our attendance at the board's meeting, that the NOSB's prior recommendation for egg white lysozyme was based solely on the board's mistaken belief that the enzyme is not Generally Recognized As Safe ("GRAS") under the applicable provisions of the Federal Food, Drug, and Cosmetic Act ("FDCA") and Food and Drug Administration ("FDA") regulations. This petition contains new information that unequivocally demonstrates that lysozyme derived from egg whites is GRAS. As such, we request reconsideration of the NOSB's prior recommendation in light of the information contained herein.

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This petition is formatted in accordance with the required elements noted in the U.S. Department of Agriculture's ("USDA's") proposed rule on National List petitions. See id.

Category for Which Petitioner Seeks Inclusion of its Substance:

Nonagricultural (nonorganic) substances allowed in or on processed products labeled as "organic" or "made with organic (specified ingredients)"

1. **Common name of the substance:**

   Egg White Lysozyme (or Lysozyme)

2. **Petitioner's name, address, and telephone number:**

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   1800 Massachusetts Ave, N.W.
   Second Floor
   Washington, DC 20036-1221
   Tele: (202) 778-9273
   Fax: (202) 778-9100

   Contact Person: Gary L. Yingling

3. **Intended or current use of the substance**

   Processing Aid / Preservative

4, **Activities for which the substance will be used -- mode of action**

Egg white lysozyme is a naturally occurring enzyme derived from egg whites. Enzymes are proteins that are produced by animals, plants, and microorganisms that function as biochemical catalysts to facilitate specific chemical reactions. In the case of lysozyme, the enzyme produces an antibacterial effect by catalyzing the hydrolysis of the structural polysaccharide peptidoglycan contained in the cell walls of certain bacteria. Simply put, it kills bacteria by creating holes in the cell walls that hold bacteria together.

Egg white lysozyme has proven to be an economically viable alternative to synthetic chemical preservatives in a number of foods, most notably cheese and wine. Furthermore, the antimicrobial effect of lysozyme is selective for certain gram-positive bacteria that cause food spoilage, while not interfering with a number of organisms that are considered beneficial to human health.

In the cheese industry, lysozyme has been found to be particularly beneficial because it selectively destroys the vegetative forms of many clostridia, particularly *Clostridia tyrobutyricum* bacteria. These bacteria survive the normal heat treatment of milk used in the production of cheese and later propagate to cause "late blowing." Late blowing is the formation of gases and butyric acid from lactic acid in the course of cheese maturation. The unwanted gas formation can cause faults in the texture of the cheese as well as an undesirable taste and smell. If the level of gas reaches a sufficiently high level, the cheese block can completely break apart. The use of egg white lysozyme in the milk culture kills the bacteria that produce the gas and therefore eliminates all "late blowing" concerns. The only alternatives to lysozyme are more harsh preservatives such as formaldehyde, nitrate, nisin or hydrogen peroxide.

According to Bottazzi (20) 10% of all the « natural » spores present in milk are resistent against lysozyme in cheese. This bring the number of active spores back to one tenth those present. When the cheese milk contains 0.27 spores/ml. 2.5 g lysozyme/100 l appeared to be effective. Then only 0,027 spores/ml are active.

A level of 0.027 spores/ml is very near to the level at which Gouda cheese can be made without nitrate or any other addition to prevent butyric acid fermentation. These considerations could also indicate the reason that lysozyme (386 U/ml) could prevent butyric acid fermentation by *C. tyrobutyricum* BZ 15, possibly because this strain is susceptible to lysozyme. Further experiments have to be carried out in order to elucidate these problems.

References

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5) BOTTAZZI V. and BATTISTOTTI B.: Scienza e Tecnica Lattiero-Casearia 29 (1978) 313.
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7) GHITTI C., MOSCA G., LAVEZZARI D. and BIANCHI-SALVADORI B.: Industria del Latte 19 (1983) 49.
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15) ELGERSMA R.H.C., SEN N.P., STEPHANY R.W., SCHULLER P.L., WEBB K.S. and GOUGH T.A.: Neth. Milk Dairy J. 32 (1978) 125.
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18) SHUGAR D.: Biochem. Biophys. Acta 8 (1952) 302.
19) VAN BEYNUM J. and PETTE J.W.: Versl. Landbouwk. Onderz. 46 (1940) 397.
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Additional information concerning lysozyme is provided in the attached portion of the book entitled Natural Food Antimicrobial Systems (Exhibit 1).

5. Source of the substance / manufacturing process

The food-grade lysozyme that is the subject of this petition is naturally derived from chicken egg whites. A food-grade inert material (a polymer resin) that specifically binds to lysozyme is used to extract the enzyme from the egg whites. After the binding process, the lysozyme is stripped from the resin, concentrated, purified, and dried. The extraction process requires the use of the following chemicals: citric acid, salt (NaCl), and hydrochloric acid / sodium hydroxide for pH adjustment. There are no solvents used in the manufacturing of the enzyme and the final dry product is nearly 100% pure egg white lysozyme.

The lysozyme content of egg whites is approximately 0.3%. Upon completion of the extraction, the egg whites remain virtually unaffected and can be subsequently consumed in other food uses.

A flow chart of the typical lysozyme manufacturing process is attached as Exhibit 2[1].

6. Summary of previous reviews

Egg white lysozyme was reviewed by the NOSB for inclusion on the National List during the NOSB's November 2000 meeting. The enzyme was evaluated as part of a petition that requested the listing of all non-genetically modified animal enzymes. Instead of considering animal enzymes as a group (as was done with microbial and plant derived enzymes), the board singled out and evaluated the following six specific animal derived enzymes: rennet, catalase (bovine liver), lipase, pancreatin, pepsin, trypsin, and lysozyme. The NOSB voted to consider all of these animal enzymes "non-synthetic," and to include all of them except lysozyme on the National List. According to the meeting minutes, the sole reason for the NOSB's decision not to recommend lysozyme for inclusion on the National List was the board's mistaken belief that lysozyme lacked a "final GRAS status from FDA" (see Nov. 2000 NOSB meeting minutes, attached as Exhibit 4). As discussed below in section 7 of this petition, the NOSB's recommendation with respect to lysozyme was based on a misunderstanding of FDA's policy concerning the GRAS provisions of the FDCA. As such, a reconsideration of the NOSB's prior recommendation is clearly warranted.

7. EPA, FDA, and State Regulatory Information

Egg white lysozyme is GRAS, as that term is defined in the applicable provisions of the FDCA and FDA regulations. Furthermore, we are unaware of any EPA or State regulatory limitations on the use of the egg white lysozyme in food.

Because the prior NOSB recommendation relied heavily on the board's perception of the GRAS status of lysozyme, we thought it would be helpful to provide a brief history and explanation of

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[1] The manufacturing information contained in Exhibit 2 consists of commercial confidential information provided to ETA by one of its members for the limited purpose of inclusion in this petition. A Commercial Confidential Information ("CBI") statement addressing this information is attached as Exhibit 3. A "CBI-deleted" copy of Exhibit 2 is provided with this petition.

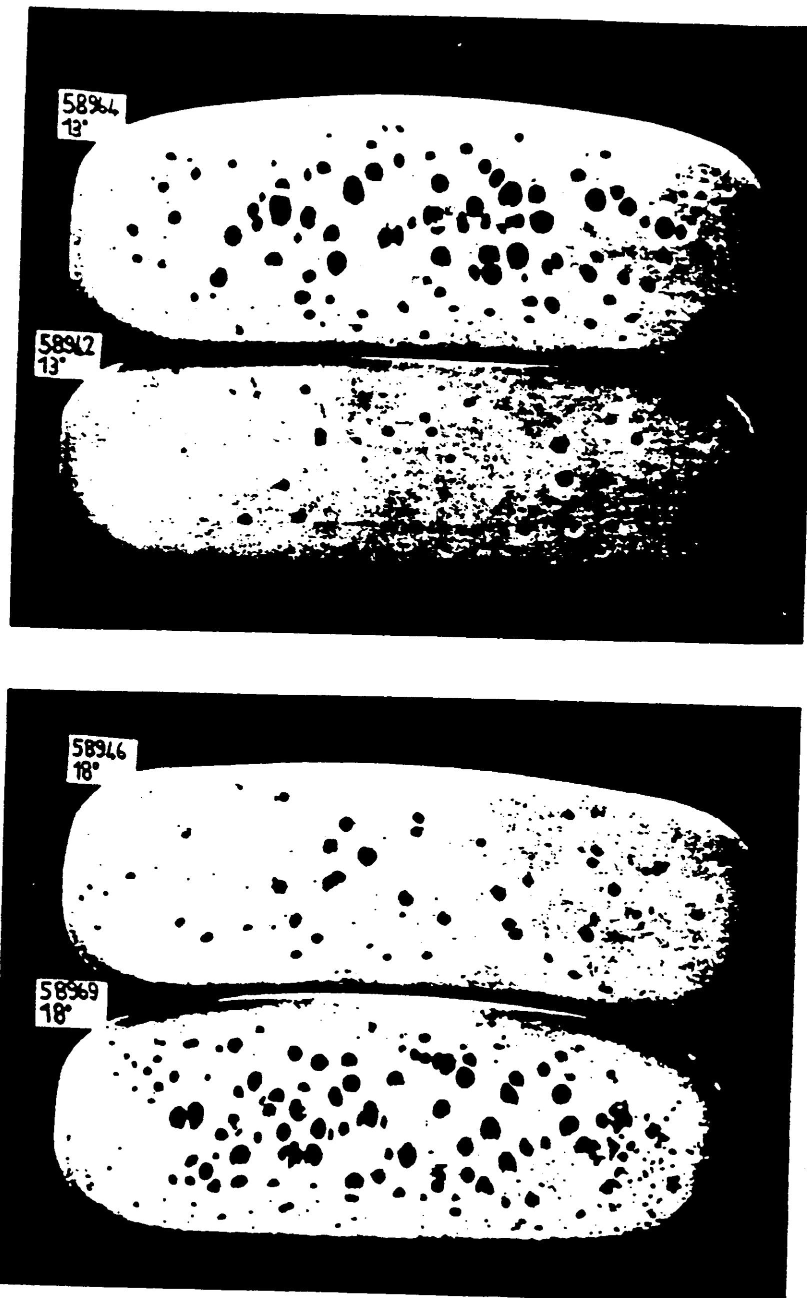

Fig. 3 - Effect of adding lysozyme to cheese milk on the occurrence of butyric acid fermentation. The spore numbers of the cheese milk were adjusted to 13 spores/ml in the milk of the first three vats and to 1.7 spores/ml in that of the fourth vat. Addition to the four vats was per 100 litres: 15 g $NaNO_3$, 3.0 g, 6.0 g and 3.0 g lysozyme respectively. Cross section are shown of the cheese after eight weeks; above cheeses at 13°C, below cheeses stored for four weeks at 13°C, thereafter at 18°C.

58793/58800 milk with 13.0 spores/ml + 15 g $NaNO_3$/100 l
58814/58813 milk with 13.0 spores/ml + 3 g lysozyme/100 l
58942/58946 milk with 13.0 spores/ml + 6 g lysozyme/100 l
58964/58969 milk with 1.7 spores/ml + 3 g lysozyme/100 l

the GRAS process in subsection A, below. In subsection B, we discuss the application of the GRAS process to lysozyme.

A. FDA's GRAS Process

In 1958, in response to public concern about the increased use of chemicals in foods and food processing, Congress enacted the Food Additives Amendment (the "1958 Amendment") to the FDCA. The basic purpose of the 1958 amendment was to require that, before a new additive could be used in food, its producer demonstrate the safety of the additive to FDA. The 1958 Amendment defined the terms "food additive" (FDCA § 201(s)) and "unsafe food additive" (FDCA § 409(a)), established a premarket approval process for food additives (FDCA § 409(b) through (h)), and amended the food adulteration provisions of the FDCA to deem adulterated any food that is, bears, or contains any food additive that is unsafe within the meaning of section 409. See FDCA § 402(a)(2)(C).

When enacting the 1958 Amendment, Congress recognized that many substances intentionally added to food would not require a formal premarket review by FDA to assure their safety. For example, the safety of some substances could be established by a long history of use in food or by virtue of the nature of the substances, their customary or projected conditions of use, and information generally available to scientists. Therefore, Congress enacted a two-step definition of "food additive." See FDCA § 201(s). The first step broadly includes any substance, the intended use of which results or may reasonably be expected to result, directly or indirectly, in its becoming a component or otherwise affecting the characteristics of food. The second step, however, excludes from the definition of "food additive" substances that are **generally recognized**, among experts qualified by scientific training and experience to evaluate their safety ("qualified experts"), as having been adequately shown through scientific procedures (or, in the case of a substance used in food prior to January 1, 1958, through either scientific procedures or through experience based on common use in food) **to be safe** under the conditions of their intended use. See id. This exception to the food additive definition came to be known as the GRAS exemption. Many substances that are commonly used in foods (e.g., vinegar, vegetable oil, baking powder, and many salts, spices, flavors, gums, and preservatives) are legally marketed under the GRAS exemption.

One of the key elements of the GRAS exemption is that a substance that is GRAS for a particular use may be lawfully marketed for that use **without FDA review or approval**. Nevertheless, many manufacturers have found it necessary to have a statement from FDA agreeing with the manufacturer's GRAS determination. Initially, FDA issued informal "opinion letters" concerning the GRAS status of substances. The "opinion letters," however, were issued only to the specific person requesting the letter and therefore did not provide industry-wide notification of the agency's GRAS decision. To address this and other concerns, FDA adopted the GRAS affirmation petition process. See 21 C.F.R. § 170.35. This was a **voluntary** administrative process whereby manufacturers could petition FDA to affirm that a substance was GRAS under certain conditions of use. If FDA agreed with the petitioner's GRAS determination, a regulation was published in the Code of Federal Regulations affirming the GRAS status of the substance. The GRAS affirmation petition process was intended to provide a mechanism for official recognition of lawfully made GRAS determinations. To the extent that a person elected to submit a GRAS affirmation petition, the process facilitated an awareness, by FDA as well as the domestic and international food industry, of lawful independent GRAS determinations.

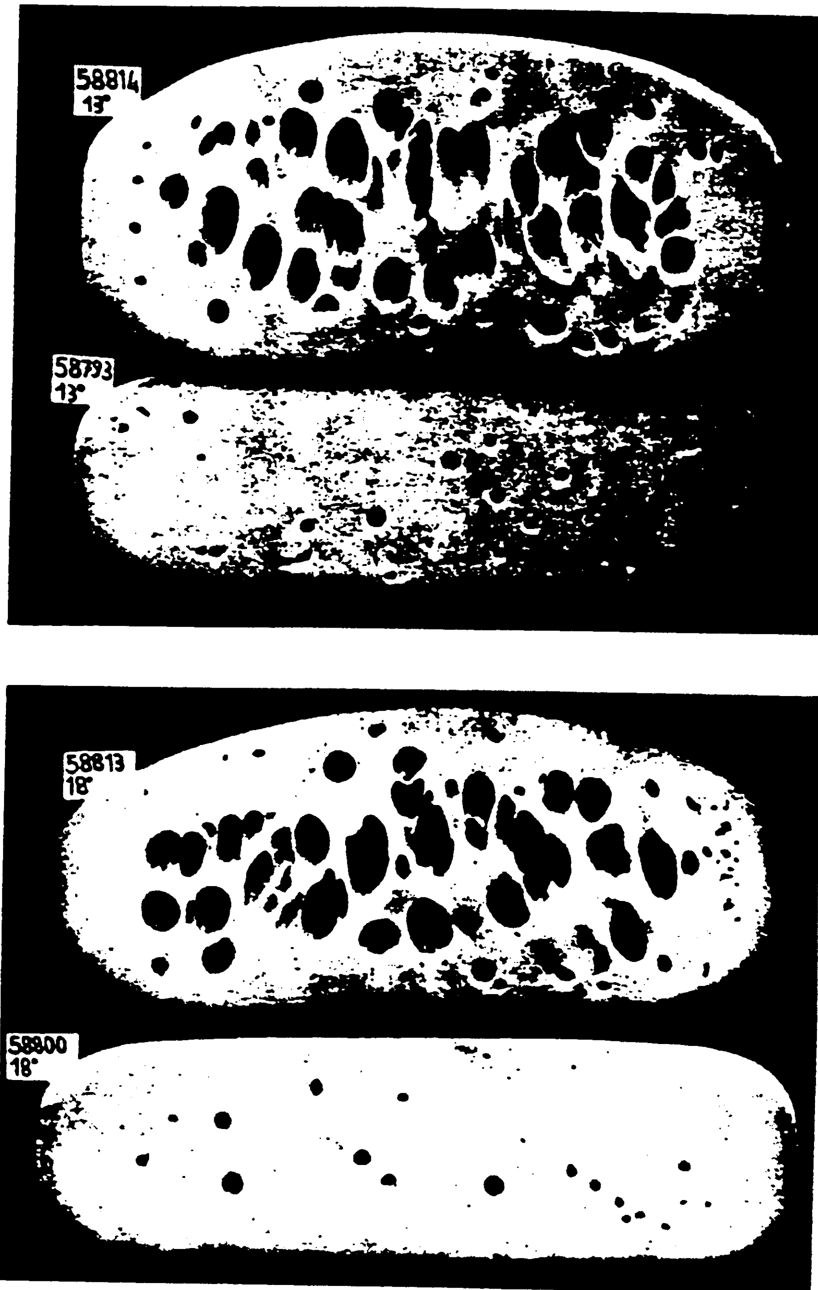

Fig. 3

The addition of 6 g of lysozyme/100 l cheese milk did not inhibit the growth and acid production of the mesophilic starter used (BD-starter Bos).

The results obtained are not directly in accordance with those observed by others (see references 2, 3, 6 and 7). Gouda cheese may be susceptible to butyric acid fermentation but also Swiss and Italian cheese varieties are equally susceptible.

However, the GRAS affirmation petition process turned out to be extremely resource-intensive. Not only did the process involve a comprehensive review of each petition, it also required going through the very cumbersome rulemaking process for each substance affirmed as GRAS. As a result, GRAS petitions languished at the agency for years, even decades, without the publication of a final regulation. Over time, the food industry began to consider the publication in the Federal Register acknowledging FDA's acceptance of a GRAS affirmation petition for filing as an acknowledgement that the agency did not have any significant objections to the petitioner's GRAS determination. In fact, it became common practice for manufacturers to reference those Federal Register notices as supporting evidence of their GRAS determinations.

As a result of the problems encountered with GRAS petition process, FDA proposed the "GRAS notification" procedure on April 17, 1997. 62 Fed. Reg. 18937. This procedure was intended to replace the GRAS affirmation petition process. Under the GRAS notification procedure, FDA evaluates whether a GRAS "notice" provided by a manufacturer provides a sufficient basis for a GRAS determination and whether information in the notice or otherwise available to FDA raises issues that might lead the agency to question whether use of the substance is GRAS. Within 90 days of receipt of the notice, FDA responds in writing as to whether it has identified a problem with the notice. To provide the industry with information on prior GRAS notices, FDA publishes a list of all submitted GRAS notices, along with the agency's response, on the FDA website. See http://www.cfsan.fda.gov/~rdb/opa-gras.html.

Although the GRAS notification regulation has never been finalized, FDA has wholeheartedly adopted the procedure as a replacement for the GRAS affirmation petition process. Since the publication of the GRAS notification proposed rule, the agency has received 105 GRAS notices. During that period, the agency has strongly discouraged the filing of (and even refused to accept) GRAS affirmation petitions. At the same time, the agency has been encouraging those persons with pending GRAS affirmation petitions to convert their petitions to GRAS notices. For example, FDA recently suggested to ETA that it could transfer the pending portions of a GRAS affirmation petition originally submitted by ETA's predecessor in 1973 into GRAS notices.

B. The GRAS Status of Lysozyme

The very nature of lysozyme made it a perfect fit for the GRAS exemption. Any safety concerns were minimal. The enzyme occurs naturally in numerous organisms and has been thoroughly characterized. The three-dimensional structure, mechanism of action, and substrate specificity of the enzyme are all well documented in the public literature. Likewise, the source of the enzyme, egg whites, has been safely consumed by humans throughout recorded history, and the enzyme is entirely inactivated in the stomach and intestines by proteolytic enzymes (e.g., pepsin).

A GRAS affirmation petition was filed in 1989 for the use of lysozyme in prevention of "late-blowing" of cheese (GRASP 9G0355). FDA published notice of receipt of the petition in the October 27, 1989 edition of the Federal Register. See 54 Fed. Reg. 43861. However, the agency did not formally respond to the petition until March 13, 1998, at which time it published a "tentative final rule" affirming that egg white lysozyme was GRAS. See 63 Fed. Reg. 12421 (attached as Exhibit 5). In that publication, FDA stated that (1) "the enzyme component of egg white lysozyme preparation is unaltered from the lysozyme found in commonly consumed food, eggs," (2) "the manufacturing process will not introduce impurities into the preparation that may render its use unsafe," and (3) the enzyme will "achieve its intended technical effect of preventing late blowing of cheese contaminated with *C. tyrobutyricum*." See id. Accordingly,

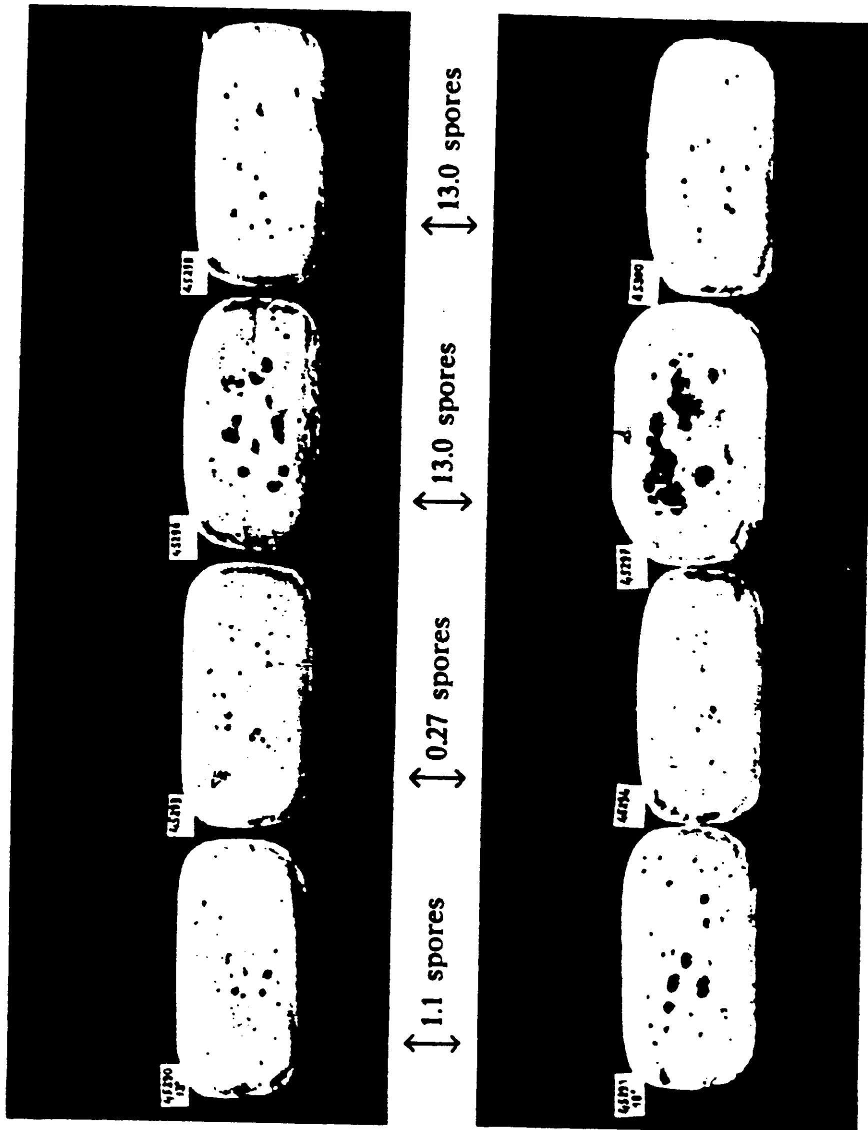

Fig. 2 - Effect of adding lysozyme to cheese milk on the occurrence of butyric acid fermentation. The spore numbers of the cheese milk were adjusted to 1.1, 0.27 and 13.0 respectively. The control cheese was made from milk with 13.0 spores/ml; 15 g $NaNO_3$/100 l was added instead of 2.5 g lysozyme. Cross sections are shown of the cheese after 24 weeks; above cheeses stored at 13°C, below cheeses stored for four weeks at 13°C, thereafter at 18°C.

45290/45291 milk with 1.1 spores/ml	+ 3 g lysozyme/100 l
45293/45294 milk with 0.27 spores/ml	
45296/45297 milk with 13.0 spores/ml	
45299/45300 milk with 13.0 spores/ml	+ 15 g $NaNO_3$/100 l

FDA "tentatively" concluded that egg white lysozyme was "GRAS for use by the general population in preventing late blowing in cheese." Id.

FDA's conclusion was termed "tentative" not because there was an unresolved issue with respect to the inherent safety of the enzyme, but rather to allow the agency to receive additional public comments on the labeling of the enzyme. There is clearly no safety concern with the consumption of egg whites by the general population. However, the agency was concerned that persons who are allergic to egg protein may not know that lysozyme could potentially (although extremely unlikely) contain a sufficient amount of egg protein to illicit an allergic reaction. As such, FDA proposed adding a condition to its GRAS affirmation that the enzyme be labeled as "egg white" lysozyme in order to be considered GRAS. The agency did not believe that it could impose such a condition without first receiving public comment on the issue. Thus, the rule was published in "tentative" form and the agency sought additional comment on the labeling issue from "interested persons."

Although the deadline for the submission of comments on the "tentative final rule" lapsed on May 27, 1998, FDA has not yet issued a "final" rule on lysozyme. In fact, FDA's rapid move away from GRAS affirmation petitions and towards the GRAS notification process makes it very unlikely that the agency will ever publish a "final" rule on the matter. As a result, the food industry has accepted the "tentative" final rule as the equivalent of an affirmation of the GRAS status of lysozyme for use in preventing late blowing in cheese, provided the enzyme is labeled with the "egg white" qualifier.

The GRAS status of egg white lysozyme was recently confirmed by the FDA response to a GRAS notice for the use of the enzyme as an antimicrobial agent in frankfurter casings and ready-to-eat cooked meat and poultry products. See Response Letter to GRAS Notice No. GRN 000064 (Apr. 2, 2001) (attached as Exhibit 6). In the response to the notice, FDA states that it "has no questions . . . regarding [the submitter's] conclusion that egg white lysozyme is GRAS under the intended conditions of use, provided that the ingredient statement of food products that contain egg white lysozyme contain the name 'egg white lysozyme' to identify the source of the protein." Id. This statement is as close to an FDA affirmation of the GRAS status of lysozyme as is currently possible under the current regulatory scheme.

In sum, FDA has been aware of the use of lysozyme as a processing aid in the food supply for over 13 years. Yet, the agency, which is tasked with ensuring the safety of the nation's food supply, has raised no objections to use of the enzyme. Not only has FDA allowed its continued use, the agency has specifically reviewed a comprehensive GRAS affirmation petition describing the substance and concluded that it is GRAS, provided it is labeled as derived from egg whites.[2] Additionally, FDA raised no questions when a GRAS notice was recently submitted for lysozyme. Given the data and information that FDA has reviewed on lysozyme, it is inconceivable that the agency would allow its continued use as a GRAS ingredient if it believed there were any question as to its GRAS status.

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[2] With respect to the FDA's condition that lysozyme be labeled as "egg white lysozyme" in order to be considered GRAS, we note that such labeling issues fall squarely within FDA's jurisdiction and are independent of the NOSB's assessment under the Organic Foods Production Act.

Table 1 - The effect of lysozyme on the degree of butyric acid fermentation in cheeses made from differently contaminated milk. Lysozyme preparation 2.

Number of spores of butyric acid bacteria ml cheese milk	Addition per 100 l cheese milk	Degree of butyric acid fermentation[2] after... weeks: cheese stored at 13°C					cheese stored four weeks at 13°C thereafter 18°C				
		5	6	8	10	24	5	6	8	10	24
1.1	2.5 g lysozyme[1]	–	–	–	+	+	n.d.	n.d.	n.d.	++	+++
0.27	2.5 g lysozyme	–	–	–	–	–	–	–	–	–	±
13.0	2.5 g lysozyme	+	++	++++	++++	++++	+	+++	++++	++++	++++
13.0	15 g $NaNO_3$	–	–	–	–	–	–	–	–	–	–

n.d. = not done

[1] 2.5. g lysozyme corresponding to 475 U/ml

[2] ± = fermentation doubtful
+ = fermentation slight
++ = fermentation clear
+++ = fermentation strong
++++ = fermentation very strong

Table 2 - The effect of lysozyme on the degree of butyric acid fermentation in cheeses made from differently contaminated milk. Lysozyme preparation 3.

Number of spores of butyric acid bacteria/ml cheese milk	Addition per 100 l cheese milk	Degree of butyric acid fermentation[1] after... weeks: cheese stored at 13°C				cheese stored four weeks at 13°C thereafter 18°C			
		5	6	7	8	5	6	7	8
13.0	15 g $NaNO_3$	–	–	–	–	–	–	–	±
13.0	3.0 g lysozyme[2]	++	++++	++++	++++	+++	++++	++++	++++
13.0	6.0 g lysozyme[2]	–	–	–	±	–	±	±	+
1.7	3.0 g lysozyme[1]	–	++	++	+++	++	+++	+++	+++

[1] see note 2, Table 1
[2] 3.0 g lysozyme, corresponding to 600 U/ml; 6.0 g corresponding to 1200 U/ml.

8. The Chemical Abstract Service (CAS) number and labels of products that contain the substance

CAS No. 9001-63-2
I.U.B. No. 3.2.1.17
International Union of Biochemistry systemic name: peptidoglycan N-acetylmuramoylhydrolase

9. Physical properties / mode of action

A. Chemical interactions with other substances – Egg white lysozyme produces a antimicrobial effect when added to foods. The enzyme is inactivated by other enzymes present in the human gastrointestinal tract. No other interactions are known.

B. Toxicity and environmental persistence – As indicated in the published literature and in FDA's GRAS reviews, lysozyme exhibits very low toxicity. Given the natural presence of lysozyme in numerous organisms, the use of the enzyme in foods would present little or no environmental concern.

C. Effects on human health – The potential effects on human health have been extensively reviewed by FDA and found to be insignificant. Additionally, the use of lysozyme in lieu of other synthetic alternatives provides a definite benefit to human health.

10. Material Safety Data Sheet

A Material Safety Data Sheet (MSDS) is attached as Exhibit 7.

11. Published Literature

A bibliography of published literature concerning lysozyme is attached as Exhibit 8. Copies of particularly relevant articles are provided in Exhibit 9.

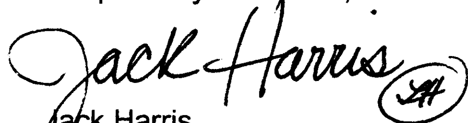
12. Petition Justification Statement

The action requested in this petition does not require a "Petition Justification Statement." See 65 Fed. Reg. at 43260-1.

13. Commercial Confidential Information Statement

The required "CBI Statement" is enclosed as Exhibit 3. A "CBI-deleted" copy with the CBI information redacted accompanies this petition.

Respectfully submitted,



Jack Harris
Chair, Enzyme Technical Association

In the first experiment four vats were made. The number of spores was adjusted to 1.1, 0.27 and 13.0 spores/ml cheese milk respectively. To the cheese milk 2.5 g lysozyme/100 l cheese milk (475U/ml) was added. Preparation 2 was used. Control cheeses were made from the cheese milk with 13 spores/ml; 15 g $NaNO_3$ was added per 100 l instead of lysozyme. The results are given in Table 1 and cross sections of the cheeses are shown in Figure 2. It is clear from the results that by the use of lysozyme butyric acid fermentation in the cheese made from milk with 13.0 spores/ml could not be prevented. In the cheese stored continuously at 13 °C as well as in those stored later on at 18 °C, a very strong butyric acid fermentation was observed. In cheeses made from milk 1.1 spores/ml a strong fermentation was observed after 24 weeks storage at 18 °C, after previous storage at 13 °C during 4 weeks. When the cheeses were stored continuously at 13 °C, the fermentation was only slight. No butyric acid fermentation was observed in the cheeses made from milk with 0.27 spores/ml.

In a second cheese experiment carried out in the experimental dairy also four vats of cheese were made. In one part of the milk (three quarters of the total volume required) the number of spores were adjusted to 13.0/ml in the other part (one quarter) to 1.7/ml. The first part was divided in three portions to which per 100 litres 15 g $NaNO_3$, 3.0 g and 6.0 g lysozyme were added respectively. The milk with 1.7 spores/ml received 3.0 g lysozyme. Lysozyme preparation n. 3 was used. The main results of this experiment are shown in Table 2 and Figure 3. Also from these results it is clear that the addition of 3 g lysozyme/100 l cheese milk could not prevent butyric acid fermentation in the cheese made from it. Even in cheeses made from the milk with 1.7 spores/ml the fermentation is quite serious. Only when 6.0 g lysozyme/100 l was added, the butyric acid fermentation was nearly completely prevented during a ripening time of eight weeks.

From the results shown in this report it appears that the usual amount of lysozyme added to cheese milk (500 U/ml) can only prevent butyric acid fermentation in the Gouda cheese made from it, if the number of spores of butyric acid bacteria does not exceed a 0,3 spores per ml of cheese milk. This number is normal in the cheese milk produced during summer time. In most European countries the number of spore in winter cheese milk amonuts at least to a level of a spores/ml. Doubling the amount of the added lysozyme to 6 g per 100 l was more effective, also in cheese made from milk with 13 spores/ml.

13 °C butyric acid fermentation was not observed during seven months of storage.

At the continuation of the experiments on a small scale use was made of a suspension of spores made by extracting heavily contaminated grass silage. Now less favourable results were obtained. The number of spores in the « bactofugated » cheese milk was adjusted to 10/ml by adding a calculated amount of the extract. To one portion of 180 litres cheese milk 0.65% ovalbumen (corresponding to 500 U lysozyme/ml) was added; to a second portion 0.22% ovalbumen was added. Both portions were made into cheese. Figure 1 shows cross sections of the cheese after four months ripening at 13 °C. When 0.65% ovalbumen had been added to the cheese milk serious gas hole formation was shown. At a reduction of the added amounts of enzyme to one third the cheeses showed a very pronounced butyric acid fermentation. Further, two cheese experiments carried out in the experimental dairy will be described.

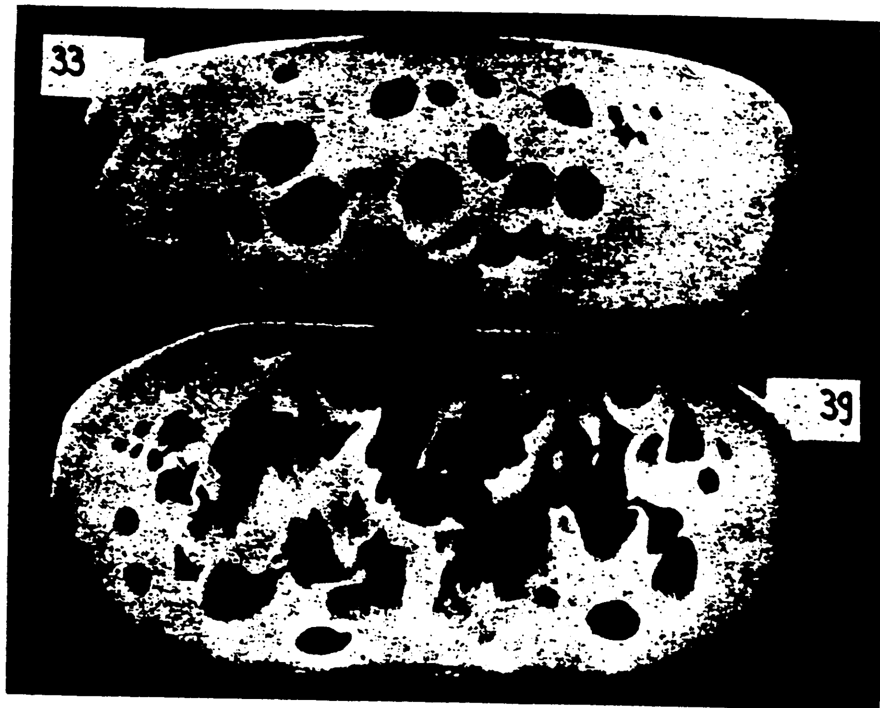


Fig. 1 - Effect of adding varying quantities of lysozime on the occurrence of butyric acid fermentation in cheese.
33: 0.65% ovalbumen added (corresponding to 500 U of lysozyme/ml cheese milk).
39: 0.22% ovalbumen added.
Cross sections are shown of the cheeses after 4 months of ripening at 15°C.

Natural Food Antimicrobial Systems

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13 °C butyric acid fermentation was not observed during seven months of storage.

At the continuation of the experiments on a small scale use was made of a suspension of spores made by extracting heavily contaminated grass silage. Now less favourable results were obtained. The number of spores in the « bactofugated » cheese milk was adjusted to 10/ml by adding a calculated amount of the extract. To one portion of 180 litres cheese milk 0.65% ovalbumen (corresponding to 500 U lysozyme/ml) was added; to a second portion 0.22% ovalbumen was added. Both portions were made into cheese. Figure 1 shows cross sections of the cheese after four months ripening at 13 °C. When 0.65% ovalbumen had been added to the cheese milk serious gas hole formation was shown. At a reduction of the added amounts of enzyme to one third the cheeses showed a very pronounced butyric acid fermentation. Further, two cheese experiments carried out in the experimental dairy will be described.

Fig. 1 - Effect of adding varying quantities of lysozime on the occurrence of butyric acid fermentation in cheese.
33: 0.65% ovalbumen added (corresponding to 500 U of lysozyme/ml cheese milk).
39: 0.22% ovalbumen added.
Cross sections are shown of the cheeses after 4 months of ripening at 15 °C.

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6

Lysozyme

I. INTRODUCTION

Lysozymes were discovered and named by Alexander Fleming (1922). Later Alderton et al. (1945) identified lysozyme in hen's egg albumen and found it to be the same as a previously known protein, globulin G1, discovered by Longworth et al. (1940) using moving-boundary electrophoresis. The lysozymes of avian egg albumen, which is the most plentiful source, shell and viteline membrane, belong to a class of enzymes that lyse the cell walls of gram-positive bacteria by hydrolyzing the β-1,4 linkage between *N*-acetylmuramic acid (NAM) and *N*-acetyl-glucosamine (NAG) of gigantic polymers $(NAM\text{-}NAG)_n$ in the peptidoglycan (murein). They are sometimes termed muraminidases or more precisely *N*-acetylmuramideglycanohydrolases (EC 3.2.1.17). 'Lysozyme' is the recommended common name. Lysozymes are ubiquitous in both the animal and plant kingdoms, and play an important role in the natural defense mechanism. Lysozyme is attractive as a natural food preservative because it is endogenous to many foods, specific to bacterial cell walls, and harmless to humans. There are microbial, viral, phage, insect, plant, and animal tissue lysozymes. Body fluids such as tears, saliva, urine and human milk contain 2.6, 0.13, trace, and 0.2-0.4 mg/ml of lysozyme, respectively (Grossowicz & Ariel, 1983). The lysozyme content in cow's milk was reported to be much lower (<1/100) than the above values in human milk (Packard, 1982).

II. OCCURRENCE

A. Sources

Egg-white lysozyme is the classic representative of the lysozyme family and the related enzymes are called *c* type (chicken- or conventional-type) lysozymes. One egg contains about 0.3-0.4 g of lysozyme. Until now, eggs have been the easiest and most economical source for recovering lysozyme. The presence of a radically different lysozyme, called *g* type after the Embden goose, was discovered. Three of only four lysozyme *g* sequences known to date have been reported. There is no evidence for the occurrence of lysozyme *g* or its gene in living systems other than birds (Prager & Jolles, 1996).

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2.3. The number of spores of butyric acid bacteria in cheese milk was estimated according to a modified procedure of Van Beynum and Pette (19). A MPN (Most Probable Number) — method was applied using mostly five tubes in each of four successice 1 ml — samples of milk and its decimal dilutions. For the experiments in the experimental dairy the same experiments was carried out; however fifty tubes with 0.1 ml milk were used instead of five.

2.4. In some experiments the milk was « bactofugated ». For the pilot plant experiments use was made of a modified Westfalia centrifuge type MN 1254. For the experiments in the experimental dairy the Westfalia « Entkeimungsseparator » type CNB 130 was used.

2.5. The number of spores of butyric acid bacteria was adjusted to the desired levels.

In the pilot plant experiments use was made of a suspension of spore of the pure culture of *Clostridium tyrobutyricum* BZ 15 or of an extract made from heavily contaminated grass silage. *C. tyrobutyricum* BZ 15 was grown for 3 weeks at 37 °C in AC-broth (composition: Proteose-peptone no. 3: 2%; Bacto-beef extract: 0.3%; Bacto-yeast extract: 0.3%; Malt extract: 0.3%; Bacto dextrose: 0.5%; ascorbic acid: 0.02%). The broth contained after incubation about i $\times$ 10^6 spore/ml.

In the experimental dairy the procedure applied to adjust the number of clostridial spores was as follows. Milk was heated by thermization, standardized (fat adjusted), cooled and stored at 5-6 °C. The number of spores of butyric acid bacteria was estimated (MPN). Four days later the number was known. A calculated part of the cheese milk was bactofugated. The concentrate obtained which contains about thirty times more spores than the milk, was used to adjust the number of spore in the cheese milk to the desired level.

3. Results and discussion

In a series of cheese experiments on pilot plant scale the effect of lysozyme (preparation 1) was studied. A half percent of preparation 1 (385 U/ml) was added to four portions of 200 l « bactofugated » cheese milk. Spores of *C. tyrobutyricum* BZ 15 were used. So much was added to the milk that the numbers in the four portions amounted to 0.2, 1.0, 5.0 and 25.0/ml respectively. In the cheese made from it and stored at

```
        1        10        20        30        40       50
Avian   KVFGRCELAAAMKRHGLDNYRGYSLGNWVCAAKFESNFNTQATNRNT-DGS
Human      E     RTL  L M G   I  A  M L  W  GY  R   Y AG R
Bovine   K Q     RTL KL   G K    A  L LTKW  SY  K   Y PSSE

        51       60        70        80        90      100
Avian   TDYGILQINSRWWCNDGRTPGSRNLCNIPCSALLSSDITASVNCAKKIVS
Human        F     Y     K   AV A HLS     QDN ADA A   RV R
Bovine       F    K      K  NAVDG HVS  E MEND AKA A   H

        101      110       120    129
Avian   DGNGMNAWVAWRNRCKGTDVQAWIRGCRL
Human    PQ IR         QNR  RQYVQ  GV
Bovine  E-Q IT     KSH RDH  SSKVE  T
```

FIGURE 1. Amino acid sequences of lysozymes of chicken, human and cow. Sites 1-40 of bovine are from cow's milk.

this problem, separation of lysozyme from egg white by ultrafiltration, ion exchange or affinity chromatography has been suggested (Ahvenainen et al., 1980). A variety of cation exchangers were compared and a macroporous weak acid type resin Duolite C-464 was selected on the basis of a high lysozyme recovery of 90-95%, retention of whipping and gelling properties of the lysozyme-free egg white, and ease of column manipulation (Li-Chan et al., 1986). The purity of lysozymes separated by salting-out crystallization, ultra-filtration and cation exchange were compared by Kijowski et al. (1999) using electrophoresis, calorimetry and amino acid analysis. Polymerization, including the formation of active or inactive reversible dimers, upon denaturation especially heating was also reported by the same authors.

B. Physico-chemistry

The molecular weight of chicken lysozyme computed from the amino acid sequence of 129 residues is 14,307 and the isoelectric point is 10.7. Molecular weight obtained by matrix assisted laser desorption/ionization mass spectrometry (MALDI-TOF-MS) is 14,308 (Yang et al., 1998). The precision of MALDI-TOF-MS is 0.05%.

The isoelectric points of lysozymes are not always high; those of lysozymes secreted in the stomach are much lower with pH values of 6-8. This variation is due to the difference of arginine residues in lysozyme molecules in the range of 3-14. Chicken lysozyme has a higher extinction coefficient $E^{1\%}$ at 280 nm (26.4 vs. 4-15) than most proteins because of higher contents of aromatic amino acids in the lysozyme molecule. There is no sequence homology of chicken lysozyme (129 residues) with *g* type lysozyme (185 residues). The *g* type is three times more active than *c* type (Canfield & McMurry, 1967). The amino acid sequences of chicken, human and cow lysozymes are shown in *FIGURE 1.* The latter two are mammalian lysozymes, and therefore, more homologous to each other than to chicken lysozyme.

Human lysozyme is about four times more active in bacteriolysis than chicken lysozyme although the hydrolytic activity against glycol chitin is about the same. Human lysozyme has more arginine residues (14 vs. 11) than chicken lysozyme, especially near *Asp52* (*FIGURE 1*), but has a lower K_a of 10000 vs. 71400 (M^{-1}) (Imoto, 1996). This fact might imply that there is no need of an excessively strong binding ability with substrates

the critical number is higher (11). Nitrate is an effective means to prevent butyric acid fermentation in Gouda cheese. Low amounts are sufficient to prevent this undesirable fermentation. The contribution of nitrate in cheese to the human daily intake of nitrate is negligible (12). Also no indication is found that nitrate in cheese induces the formation of N-nitrosocompounds (13, 14, 15, 16). Nevertheless some conutries require the presence of only low amounts of nitrate in cheese when imported.

It was therefore investigated in the Netherlands Institute for Dairy Research (NIZO) at Ede how far lysozyme could prevent butyric acid fermentation in Gouda cheese. The experiments are still going but the results obtained justify already some important conclusions.

2. Material and methods

2.1. The usual method for making Gouda cheese from pasteurized milk was applied. On pilot plant scale 6 Kg Gouda cheeses were made from 180 l portions of cheese milk. In the experimental dairy 10-12 kg Gouda cheeses were made from 2000 litres of cheese milk. As a control mostly cheese was made from milk to which 15 g $NaNO_3$ was added. In making the experimental cheeses lysozyme was added instead of nitrate. The BD-starter Bos was used in all experiments described. The cheeses were stored at 13 °C. After four weeks a part of the cheeses was further stored at 18 °C. During storage the gas formation in the cheeses was checked by sounding the cheese each week and by making X-ray photographs (17).

2.2. The strength of the lysozyme preparates was estimated according to Shugar (18).

The following preparations of lysozyme were used:

2.2.1. Ovalbumen from NIVE, Harderwijk, the Netherlands (preparation 1). The strength of this rough preparation was only 77 U/mg.

2.2.2. Afilact from CODIPI, Levallois Perret, France (preparation 2). We found a stength of 19 000 U/mg in this preparation.

2.2.3. The lysozyme preparation from SPA Società Prodotti Antibiotici SPA Milan, Italy (preparation 3). We found a strength of 20 000/mg in this preparation.

We used a Boehringer preparation (22 000 U/mg) as a standard.

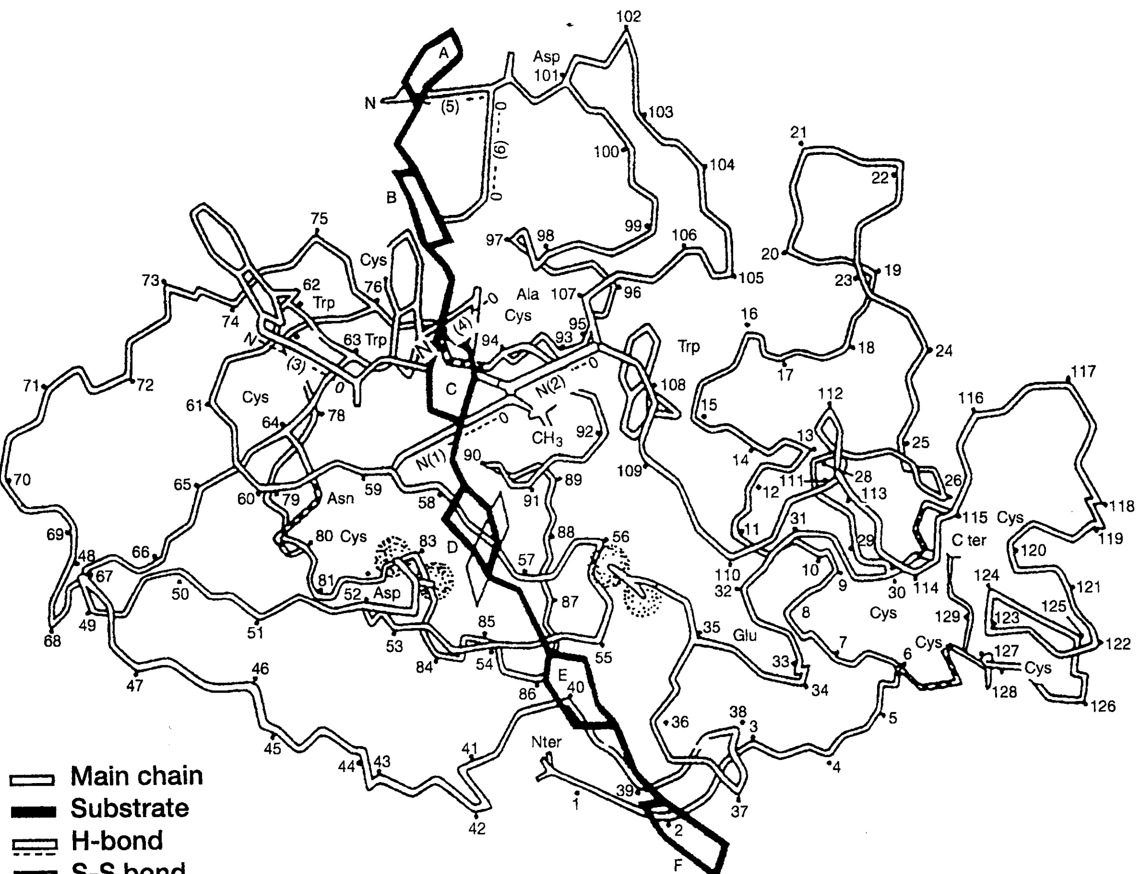

Figure 3. Estimated structure of lysozyme-(NAG)$_6$ complex by X-ray analysis [reprinted with permission from Imoto (1996); copyright Birkhäuser].

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The use of lysozyme for the prevention of butyric fermentation in Gouda cheese. The limited effect of the enzyme

1. Introduction

NIZO - EDE - NEDERLANDS

Pulay (1) detected that by use of lysozyme butyric acid fermentation in cheese was prevented. Wasserfall and Prokopek (2) studied the use of this enzyme more in detail. Thereafter many literature data came available on the effect of lysozyme (3, 4, 5). Carini and Lodi (6) confirmed the antiblowing effect of lysozyme in Grana and Montasio cheeses made experimentally from milk containing 0.25-2.3 clostridial spores per ml cheese milk. Also Ghitti et al. (7) claimed that the addition of 25 ppm lysozyme hydrochloride to the cheese milk prevented butyric acid fermentation in Grana Padano cheese when the clostridial spore counts in milk amounted to 2/ml. Losi and Chiavari (8) reported however that application of lysozyme was effective only when the spore content of the cheese milk was low.

Another question is how the use of lysozyme affects the development of the starter bacteria (lactic acid bacteria) or the organoleptic properties of the cheese. Lodi et al. (9) found that in most cases lysozyme up to 250 ppm to the cheese milk has non effect on growth of the starter bacteria, their acid production and proteolysis in cheese. *Lactobacillus helveticus*, however, was inhibited to some extent and the proteolytic activity diminished. Battistotti et al. (10) observed some change in organoleptic properties in Grana cheese when made from milk to which lysozyme had been added.

Gouda cheese is very susceptible to butyric acid fermentation. In a recent paper (11) we mentioned the critical numbers of spores of butyric acid bacteria per ml of cheese milk starting to cause butyric acid fermentation in Gouda cheese. When no nitrate is added to the cheese milk, this number is about one or two clostridial spores per 200 ml of cheese milk (0.005-0.01/ml). When nitrate is added to the cheese milk

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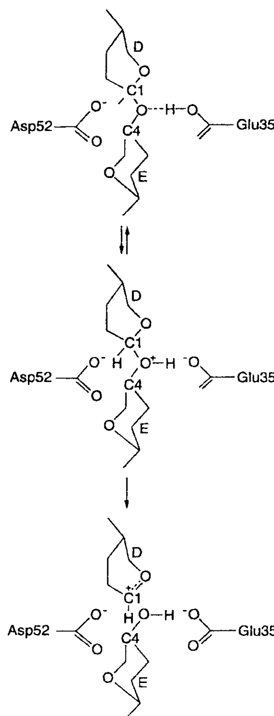

FIGURE 4. Reaction mechanism of *c* type lysozyme [reprinted with permission from Imoto (1996); copyright Birkhäuser].

bind six sugar residues (Imoto, 1996). The six subsites along the active site cleft position the catalytic groups Glu-35 and Asp-52 between subsites D and E.

IV. ANTIMICROBIAL ACTIVITY

A. Mechanism of action

Lysozyme promotes catalysis by inducing steric stress in the substrate. The reaction mechanism of lysozyme is illustrated in *FIGURE 4* (Imoto, 1996). *Asp52* and *Glu35* are the precise amino acids that participate in the catalysis. *Glu35*, which lies in a hydrophobic environment, participates in catalysis in the protonated form, and *Asp52*, which lies in a hydrophilic environment, does so in the dissociated form. The bond between this oxygen and C1 on the D sugar is cleaved. A carbonium ion is formed on the D sugar and this is stabilized by the formation of an oxocarbonium ion. The distortion of the D sugar from the chair form to the sofa form favors this process. The negative charge on *Asp52* stabilizes the formation of a positive charge on the D sugar. *Glu35*, which participates in catalysis in the protonated form, has an abnormally high pKa of 6.1 (normally 4.3), thus enhancing the catalytic efficiency. This abnormality is produced by the negative charge of *Asp52* and by the surrounding hydrophobic environment, especially of *Trp108* (Inoue et al., 1992a; 1992b). Site-directed mutagenesis studies in which *Asp52* and

TABLE N.3 (cont.ed)

TEST N.	LYSOZYME		SENSORIAL ANALYSIS			
			Packed cheeses		Waxed cheeses	
	Type	Concentration U/ml	e.i.c.s.t.(1)	Butyric acid test	e.i.c.s.t.	Butyric acid test
13 a	(Nitrate) Egg white	515	5 4 4 4 4 5 4 4 4 4	- -	5 4 5 4 4 5 5 5 4 4	- -
b	(Nitrate) Egg white	527	5 4 4 4 4 5 4 4 4 4-	- -	5 5 5 5 5 5 5 5 5 5	- -
c	(Nitrate) Albumine	489	5 5 5-4 4 1 1 3[b] 1 1	- vw	5 5 5 5 5 3 3 4[a] 3 3	- Vw

1) Internal aspect classification is as follows:

a= holings too large and/or too numerous
b= holing under development
c= optycal holing

In Gram-negative bacteria, the peptidoglycan that makes only 5-10% of the cell wall lies beneath the outer membrane of the cell envelope. The lipopolysaccharide layer of the outer membrane acts as a barrier against macromolecules and hydrophobic compounds. The lipid component of the inner core of the lipopolysaccharide molecules through their content of phosphate and carboxyl groups and their electrostatic interactions with divalent cations reinforce the stability of gram negative bacteria cell walls towards microbicidal agents such as lysozyme.

The antiviral activity of lysozyme is not associated with its lytic activity and was reported to be associated with the positive charge of lysozyme (Cisani et al., 1984). Addition of negative charges on lysozyme molecule, by succinylation, did not induce antiviral activity against influenza virus (Schoen et al., 1997). It is possible that a purely electrostatic effect of a protein does not suffice for antiviral activity. Possibly, a much more specific interaction is involved.

Recently, Düring et al. (1999) unexpectedly discovered that heat denatured T4 lysozyme as well as hen egg white lysozyme, in which the enzymatic activity was abolished, preserved its antimicrobial activity. The membrane perturbing activity of denatured lysozyme was demonstrated on bacterial, fungal, and plant cells. The amphiphatic C-terminal domains of the lysozymes seem to have mediated the bactericidal and fungicidal activities of the denatured lysozymes. A synthetic peptide, with sequence homology to the amphiphatic C-terminal of T4 or hen egg white lysozyme, showed bactericidal and fungicidal activities similar to the microbicidal activity of the C-terminal domain of heat denatured T4 and hen egg white lysozyme. This finding suggests that lysozyme has both enzymatic and non-enzymatic microbicidal activity in native and denatured state, respectively. Therefore, the antimicrobial activity of lysozyme may not be limited by heat treatment encountered during food processing operations.

B. Spectrum of activity

Lysozyme is most effective against some specific gram-positive bacteria including *B. stearothermophilus, Clostridium thermosaccharolyticum,* and *Clostridium tyrobutyricum*. The spectrum of activity of lysozyme is easily broadened to other spoilage and pathogenic organisms and even to some gram-negative bacteria when lysozyme is used in combination with other compounds. Johnson (1994) demonstrated that lysolecithin enhanced lysozyme inhibitory activity against yeasts. Gram-negative bacteria became susceptible to lysozyme after the outer membrane of the bacteria has been disrupted by compounds such as EDTA, aprotinin, organic acids or when lysozyme was conjugated to carbohydrates (Johnson, 1994; Pellegrini et al., 1992). It has also been shown that within some species, lysozyme may have different inhibitory activity against bacteria. Lysozyme was shown to be effective against some *S. aureus* and ineffective against some others (Johnson, 1994). *Salmonella senftenberg* 775 W is sensitive to lysozyme while *S. typhimirium* is not. The spectrum of antimicrobial activity of lysozyme against gram-negative and gram-positive bacteria is shown in *Table 1 and Table 2*, respectively. Results are mostly from *in vitro* studies and have been compiled from different sources (Cisani et al., 1984; Carini et al., 1985; Johnson, 1994).

T A B L E N.3

SENSORIAL TEST OF TYPE EDAM CHEESE OBTAINED WITH THE ADDITION OF LYSOZYME INSTEAD OF NITRATE AFTER 5 WEEKS RIPENING AT 15°C AND FURTHER 5 WEEKS STORAGE AT 8°C.

TEST N.	LYSOZYME		SENSORIAL ANALYSIS			
			Packed cheeses		Waxed cheeses	
	Type	Concentration U/ml	e.i.c.s.t. 1)	Butyric acid test	e.i.c.s.t.	Butyric acid test
9 a	Egg white	408	5 4 5 4 4		5 4 5 5 5	
		816	5 4 5 4 5		5 4 5 4 5	
b		402	5 4 5 4 5		5 4 5 5 5	
		703	5 4 5 5 5		5 4 5 4 5	
c		533	5 4 5 4 5		5 4 5 4 5	
		853	5 4 5 4 4		5 4 5 4 5	
10a	Egg white	415	5 3[a]5 4 4		5 4 5 5 4	
		622	5 4 5 3 4		5 4 5 5 4	
b		397	5 4 4 3 3		5 4 5 3 3	
		696	5 4 5 4 4		5 4 5 5 4	
c		508	5 3[a]4 3 3		5 3[a]5 4 4	
		711	5 4 5 4 4		5 4 5 4 4	
11a	Crystallized Lysozyme	400	5 5 5 4 4		5 5 5 5 4	
		700	5 4-5 4 4		5 4-5 4 4	
b		300	5 4 5 4 4		5 4 5 4 4	
		600	5 4 5 4 4		5 5 5 4 4	
c		400	5 3[a]5 4-3		5 3[a]5 4 4	
		700	5 5-5 4 3		5 5-5 4 4-	
12a	Albumine	392	3 2[b]4 3 3-	vw	5 3[b]4 4 3	-
		491	3 2[b]4 3 3	vw	5 3[b]4 4 3	vw
b		392	3 2[b]4 3 3-	w	5 3[b]4 4 3	w
		589	3 2[b]4 3 3-	vw	5 3[b]4 4 3	w
c		392	5 3[c]4 4 3		5 4[c]4 4 4	
		491	4 3[c]4 4 3		5 4[c]4 4 4-	

./..

TABLE 2. Antimicrobial spectrum of lysozyme against Gram-negative bacteria

Family	Species	Sensitivity
ENTEROBACTERIACEAE	*Escherichia coli*	-/ndr/+
	Salmonella typhimurium	-/+
	Shigella	+
	Proteus vulgaris	-
	Serratia marcescens	-
	Erwinia spp.	+
	Yersinia enterocolitica	m+
PSEUDOMONADIACEAE	*P. aeruginosa*	+
	P. fluorescens	ndr
	Achromobacter spp.	-
PASTEURELLA	*Pasteurella spp.*	+
NEISSERIACEAE	*Neisseria spp.*	+
VIBRIONACEAE	*Vibrio cholerae*	-
CAMPYLOBACTERIACEAE	*Campylobacter jejuni*	m+
KLEBSIELLEAE	*Klebsiella pneumoniae*	-

+ = sensitive, - = resistance, m+= moderately sensitive, nds= not demonstrated sensitivity, ndr = not demonstrated resistance (modified from Cisani et al., 1984; Carini et al., 1985; Johnson, 1994).

known to undergo any adverse chemical or organoleptic changes that would be of concern under normal food processing conditions.

Lysozyme has proven to be an economically viable alternative to chemical preservatives in a number of food applications, most notably in cheese making.

Lysozyme is selective, at the level used industrially, for certain gram-positive spoilage bacteria and may not interfere with a number of organisms that are considered to be beneficial for human health.

B. Physiological advantage to host

Since its discovery, lysozyme has never lost its role in the control of bacterial infection and the modification of host immunity (Sava, 1996). Lysozyme added to foods is destroyed in the human stomach, making it harmless to humans. Lysozymes of both human and avian origin kill gram-positive bacteria, binds to most of the lipopolysaccharides (LPS) produced by various gram-negative bacteria, independent of variations in the structure of lipid A and /or polysaccharide portions of the LPS molecule, with a high binding affinity to produce a complex, LPS-lysozyme, which results in lipopolysaccharide detoxification and lysozyme inactivation. As a consequence of the detoxification, the biological activities of LPS such as the mitogenic and tumor necrosis factor (TNF) production are significantly reduced by the LPS-lysozyme complex. The biological activity of LPS, such as the mitogenic activity of murine splenic B lymphocytes and the TNF production of RAW264.7 cells *in vitro* and of mice *in vivo,* were significantly reduced by lysozyme (Takada et al., 1994).

Lysozyme, orally administered, has proven to be an effective immunostimulant, antiviral and anti-inflammatory in cancer patients and individuals with herpetic lesions (Inoue, 1987; De Douder & Marias, 1974; Satoh et al., 1980).

T A B L E N.2 (cont.ed)

TEST N.	Weinzirl test on raw milk	LYSOZYME		SENSORIAL PROPERTIES				
		Concentration U/ml	Type	Control cheeses after 5 weeks			Lysozyme cheeses after 10 weeks	
				e.i.c.s.t.	Butyric acid test (1)	Positive Weinzirl test	e.i.c.s.t.	Butyric acid test
11 b	6	0		4 3 4 3 3		9		
		300	Cryst.Lysozyme				5 4 5 3 3	
		600	" "				5 4 5 3 3	
c	8	0		4 2 4 2 2		10		
		400	Cryst.Lysozyme				$4\ {}^{a}2\ {}^{c}2\ 2\ 0$	
		700	" "				5 5-3 3 2	
12 a	8	0		3 2 5 3 3	s	10		
		392	Albumine				$2\ {}^{a}1\ {}^{c}4$ 3-2	w
		491	"				$2\ {}^{a}1\ {}^{c}4$ 3-2	w
b	9	0		2 1 5 2 2	s	10		
		392	Albumine				$2\ {}^{a}1\ {}^{c}4$ 3-2	w
		589	"				$2\ {}^{a}1\ {}^{c}4$ 3-2	w
c	9	0		5 3 5 3 3		10		
		392	Albumine				$4\ 3\ {}^{d}4$ 3 3	
		491	"				$3\ {}^{a}2\ {}^{c}4$ 3 3	vw
13 a	8	0		3 2 3 3 3	vw	10		
		Nitrate					5 4 4 4 4-	
		515	Egg white				5 4 4 4 4-	
b	8	0		3 2 3 3 3	vw	9		
		Nitrate					$5\ 3\ {}^{b}3$ 4-4-	
		527	Egg white				$3\ {}^{a}2\ {}^{c}3$ 3 2	vw
c	7	0		1 0 3 3 3	w	10		
		Nitrate					$5\ {}^{a}5\ {}^{c}4$ 4 4	
		489	Albumine				1 1 3 1 1	w

LEGENDA: 1): s = strong; w = weak; vw = very weak

2): a = altered aspect; b= holing too large or too numerous; c = holing under development; d = atypical holing

3): No determination was possible owing to the presence of extraneous cultures

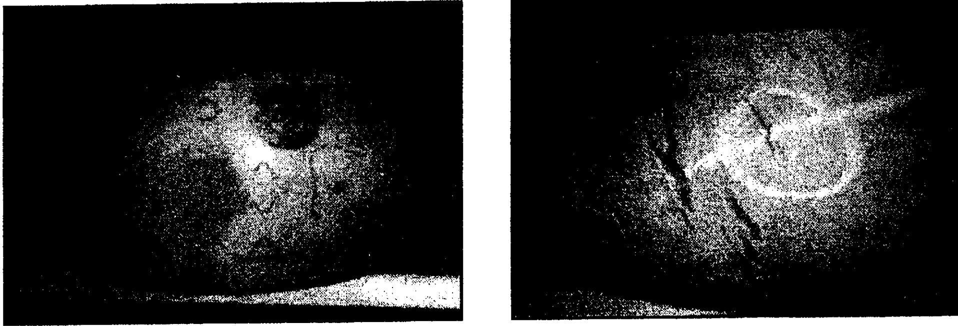

FIGURE 6. Comparison between good Grana cheese and *C. tyrobutyricum* contaminated Grana cheese [reprinted with permission from Canadian Inovatech, Inc. (1999); copyright Inovatech, Inc.].

1989). Lysozyme at 20 to 35 mg/L successfully replaced commonly used preservatives such as formaldehyde, nitrate, nisin, and hydrogen peroxide in protecting cheese against *C. tyrobutyricum.* Formaldehyde, nitrate, and nisin may be inhibitory to the starter and secondary cultures required for the ripening of cheese. There was no significant difference, organoleptically, between lysozyme stabilized cheese and nitrate stabilized cheese. The effectiveness of lysozyme in providing a natural enzymatic protection against *C. tyrobutyricum* is directly associated to its specificity. In general, lysozyme inhibits the spoilage organism, while not interfering with the starter culture. The activity of four starter cultures, in Gouda cheese, was not inhibited by lysozyme at concentration up to 2500 units/ml while growth of coliform isolates was inhibited by 1000 units/ml of lysozyme (Bester & Lombard, 1990). In specific cases the tolerance level of the starter culture may be somewhat lower and special care must be taken to inhibit spoilage without inhibiting the culture. *Lactobacillus helveticus* in milk was inhibited by a lysozyme concentration higher than 50 ppm (Makki & Durance, 1996).

Several patents claim the effectiveness of lysozyme at concentration as low as 50 ppm to prevent the development of undesirable microorganisms in butter and cheese for more than 24 months. A patent obtained by Dell'Acqua et al. (1989) reported enhancement of lysozyme activity around pH 5.2 in milk. Large volumes of lysozyme, about 100 tons, are used annually by the cheese industry to prevent the growth of *C. tyrobutyricum* from germinated spores.

Listeria monocytogenes is of greatest concern in soft cheese and milk (Farber & Peterkin, 1991; Schuchat et al., 1992). Lysozyme, at 20 to 200 mg/L delayed the growth of all four strains of *L. monocytogenes* isolated during a food poisoning outbreak (Johnson, 1994). The sensitivity of the pathogens to lysozyme depended mostly on the physiological state of the microbe and on the growth medium. Milk and dairy products have contributed the most to the outbreak of listeriosis. The rate of survival of *L. monocytogenes* in milk is very high and the resistance to lysozyme has been associated with the presence of minerals or mineral-associated components. In Camembert cheese, lysozyme was bacteriostatic but did not rid the cheese of viable *L. monocytogenes* (Hughey et al., 1989).

*2. **Alcoholic beverages:*** Microbes commonly found in wines are non-pathogenic because of the alcohol content and pH of wines. The most common bacteria and yeast

T A B L E N.2

CORRELATION BETWEEN ANAEROBIC SPORIGENES TEST IN RAW MILK AND SENSORIAL PROPERTIES OF CONTROL CHEESES AFTER 5 WEEKS RIPENING AND THOSE OF CHEESES OBTAINED BY ADDITION OF LYSOZYME AFTER 10 WEEKS RIPENING AT 15°C.

TEST N.	Weinzirl test on raw milk	LYSOZYME		SENSORIAL PROPERTIES				
				Control cheeses after 5 weeks			Lysozyme cheeses after 10 week	
		Concentration U/ml	Type	e.i.c.s.t.	Butyric acid test (1)	positive Weinzirl tests	e.i.c.s.t. 2)2)	Butyric acid test
9 a	9	0		1 1 - - -		10		
		408	egg white				5 4 5 4 4	
		816	" "				- - - - - 3)	
b	10	0		2 2 - - -		10		
		402	egg white				5 4 5 4-4	
		703	" "				5 4 5 4 4	
c	10	0		2 2 - - -		10		
		533	egg white				5 4 5 4 3	
		853	" "				5 4 5 4 3	
10a	10	0		1 0 - - -		10		
		415	egg white				4 3[b] 5 4 4	
		622	" "				5 4 5 4 4	
b	8	0		1 0 - - -		7		
		397	egg white				3[a] 2[c] 4 2 3	
		696	" "				5 3[b] 5 4 4	
c	10	0		2 1 - - -		10		
		508	egg white				5 3[b] 4 3 4	
		711	" "				5 4 5 4 3	
11a	7	0		4 3 4 3 3		9		
		400	Crystallized Lysozyme				5 5 5 5 4-	
		700	" "				5 4-5 4-3	

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disease outbreaks mostly in the US. Outbreak data have linked *Salmonella, Shigella, E. coli O157:H7, Enterotoxigenic E.coli, Cryptosporidium parvum, Cyclospora, Bacillus cereus*, hepatitis A virus, and *L. monocytogenes*. Fruits and vegetables are low-acid foods and are very susceptible to spoilage by *C. botulinum* strains and *L. monocytogenes*. Three US patents obtained by Dell'Acqua et al. (1989), Johnson et al. (1991), and Ueno et al. (1996) report on bacterial decontamination of vegetables using hen egg white lysozyme. The synergistic effect of chelators, in combination with lysozyme, delayed toxin production in potato suspensions (Cunningham et al., 1991). Fruits and vegetables may also be contaminated by non-pathogenic bacteria and fungi that may alter the quality of the product. Fresh vegetables, tofu, kimchi, Japanese potato salad, sushi, Chinese noodles, and creamed custards have been preserved using lysozyme or a combination of lysozyme and amino acids (Cunningham et al., 1991). Patents filed, in Japan, by Eisai (1971; 1972a; 1972b) report the use of lysozyme in combination with glycine to preserve fruit juice, uncooked noodles, bean jam, and custard cream. Fruit and vegetables were preserved by a combination of lysozyme chloride, lower fatty acid monoglyceride, phytic acid and /or sodium acetate (Q.P. CORP., 1985).

5. Chewing gum and toothpaste: Lysozyme, in chewing gum formulations, was effective against periodontitis-causing bacteria, and gingivitis associated inflammation and bleeding (Sava, 1996). Lysozyme is used in toothpaste, in combination with chloride, fluoride, thiocyanate, and bicarbonate to lyse *Streptococcus mutans* (Goodman et al., 1981). A US patent reports the use of lysozyme in combination with EDTA to control gum infections (Rabussay, 1982).

C. Synergism - the hurdle effect

Lysozyme is not totally effective against all gram-positive bacteria and is ineffective against most gram-negative bacteria. Hurdle technology has been defined as a combination of existing and novel preservation techniques in order to establish a series of preservative factors (hurdles) that any organism present in the food system should not be able to overcome (Leistner et al., 1995). Each preservative factor individually affects the growth and survival of microorganisms and concertedly controls microbial spoilage and food poisoning and makes it unlikely for any microorganism to survive. The hurdles may be a combination of temperature, water activity, pH, redox potential, enzyme antimicrobial, salt, sugar, pasteurization, bacteriocin, ultra high pressure treatment, edible coatings, gas packaging, bioconservation, etc. The preservative factors may disturb several or just one of the homeostatic mechanisms of microorganisms, such as pH habituation of resistant cells, and as a result the microorganism will not multiply but instead remain inactive or even die. Hurdle technology as applied to lysozyme has shown significant improvement in lysozyme activity against a wide range of bacteria. The combination of natural preservatives such as nisin, lactoferrin, glycine, organic acids, egg white, trypsin, aprotinin, gelatin, ultrahigh pressure, and electroporation has in most cases performed better against a wide range of bacteria than lysozyme used singly (Hauben et al, 1996; De Douder & Marias, 1974).

Lysozyme and nisin, used singly or in combination with EDTA, was incorporated into the structure of biodegradable packaging films made from corn zein or soy protein isolate and was evaluated for inhibition against *Lactobacillus plantarum* NCDO 1752 and *E. coli* ATCC 25922 (Padgett et al., 1998). Packaging films were made using the heat-

T A B L E   N.1   (cont.ed)

TEST N.	MILK ANALYSIS: RAW MILK: Germs number	RAW MILK: Coli number	RAW MILK: Weinzirl	PASTEURIZED MILK: Pasteuriz. effect %	PASTEURIZED MILK: Coli number	LYSOZYME CONCENTRATION U/ml	CHEESE ANALYSIS: Coli number after 5 weeks	Coli number after 10 weeks	Weinzirl test after 10 weeks	Sensorial analysis after 5 weeks e.i.c.s.t.	Sensorial analysis after 1[illegible] weeks e.i.c.s.[illegible]
7 a	$197x10^4$	10^{-4}	8(10)	93.35	1.0-	283	10^{-5}	10^{-3}	0(5)	4 3 5 4 4	0 0 - -
						566	10^{-3}	10^{-3}	0(1)	5 4 5 4 4	5 4-3 3
b	$238x10^4$	10^{-4}	5(9)	99.12	1.0-	306	10^{-4}	10^{-3}	3(5)	4 3-4 4 4	3 1 - -
						918	10^{-3}	10^{-2}	2(3)	5 4+4 5 4	5 3 4 3
c	$96x10^4$	10^{-5}	10(10)	98.32	1.0-	628	10^{-2}	10^{-1}	0(3)	5 4-5 5 4+	5 3 5 3
						943	10^{-3}	10^{-1}	0(1)	5 4+5 5 4+	5 4 5 4-
8 a	$81x10^4$	10^{-4}	4(9)	94.94	1.0-	399	10^{-3}	10^{-2}	3(6)	5 4 4 4 4	5 5 5 4
						598	10^{-3}	10^{-2}	4(7)	5 5 4 5 5	5 5 5 4
b	$114x10^4$	10^{-4}	2(10)	96.23	1.0-	423	10^{-1}	10^{-1}	0(4)	4 5 4 5 5	4 4 4 3
						847	10^{-4}	10^{-3}	3(4)	5 4 5 5 4	5 3 5 3
c	$221x10^4$	10^{-5}	8(10)	98.24	1.0-	621	10^{-4}	10^{-3}	0(1)	5 5 4 5 5-	5 3 5 4-
						828	10^{-2}	10^{-2}	0(1)	5 4 4 5 5	5 4 5 4-

LEGENDA:
e = external aspect
i = internal aspect
c = consistence
s = smell
t = taste

According to Weinzirl tests, the numbers outside bracket are the positive tests after three days incubation; the numbers inside brackets are the positive tests after 7 days incubation
In the test n. 6 the purified and three-times crystallized Lysozyme by Roth Company was used; in the other tests it was used Lysozyme as egg white

D. Potential applications

1. Meat products: Decontamination of meat and poultry carcasses during or at the end of the production line is a recommended procedure as part of the HACCP approach to help food processing facilities minimize the risk of contamination. In Europe, chemical or physical treatment of carcasses are not allowed, while these treatments are approved in the USA (Bolder, 1997). There is no report of meat carcass decontamination using hen egg white lysozyme. The use of lysozyme as natural sanitizer to prevent bacterial load on meat surfaces would fit the trend towards utilizing natural preservatives for safety in foods. The shelf life of raw meat, following removal from carbon dioxide storage, is always threatened by food pathogens and spoilage bacteria such as *L. monocytogenes*, *Pseudomonas*, *Lactobacillus*, *Brochothrix thermosphacta*, and *E.coli*. Lysozyme delayed the growth of *L. monocytogenes* in fresh pork sausage but did not eliminate the bacteria (Hughey et al., 1989). *L. monocytogenes* has been implicated in the contamination of pâté, sausages and frankfurters (De Douder & Marias, 1974). Extensive work carried out in Johnson's laboratory at the University of Wisconsin, USA, has demonstrated that lysozyme, alone, was less effective in controlling *L. monocytogenes* in pork sausage (bratwurst), pork, beef, or turkey frankfurters than lysozyme plus EDTA. It was suggested that lysozyme may have inhibitory activity against *L. monocytogenes* in meat products, but more conclusive work needs to be done. The synergistic effect of chemical preservatives such as EDTA, polyphosphates, phytic acid, parabens, benzoic acid, sorbic acid, hydrogen peroxide, and *p*-hydroxybenzoate was essential to delay growth and kill a significant number of *L. monocytogenes* cells in minimally processed meat products mentioned above. Nisin, in combination with lysozyme, was more effective than lysozyme alone on meat products. *In vitro* studies have also shown delayed growth of *L. monocytogenes* cells but not a total growth inhibition. Lysozyme alone or in combination with other biological preservatives offers an additional and acceptable processing parameter for improving the safety and assuring the quality of meat products. Cunningham et al. (1991) reported that vienna sausages were best preserved by a combination of dipping the casings in 0.05 % lysozyme plus phosphate buffer at pH 6.5, adding 0.055% lysozyme to the cured meat, and dipping the sausage in 0.05% lysozyme in phosphate buffer at pH 6.5 after cooking.

Lysozyme contains a pentadecapeptide sequence I-V-S-D-G-N-G-M-N-A-W-V-A-W-R (amino acids 98-112), obtained by enzymatic hydrolysis using clostripain at pH 7.5 and 37 °C. The pentadecapeptide induced non-catalytic bacterial death, against *E.coli, Klebsiella pneumoniae, Serratia marcescens* and *Streptococcus zooepidemicus*, while the cell membranes of these bacteria were not enzymatically attacked by native lysozyme (Pelligrini et al., 1997).

2. Wine spoilage: The potential application of lysozyme as a partial replacement of SO_2 to control malolactic fermentation in wines has reached industrial trials as the enzyme has shown its inhibitory effect on the growth of lactic acid bacteria (Green et al., 1995a; 1995b; 1995c; Amati et al., 1995). Two other enzymes considered as effective alternatives for reducing SO_2 use in wines are glucose oxidase and catalase. Glucose oxidase and catalase are safe and effective anti-oxidizing systems. Glucose oxidase has been used successfully to stabilize flavor and color in bottled white and rosé wines (Gomez et al., 1995; Pickering, 1998). The addition of lysozyme alone or in combination with an

TABLE N.1

ANALYSIS OF MILK AND CHEESE OBTAINED WITH ADDITION OF LYSOZYME

TEST N.	MILK ANALYSIS					LYSOZYME CONCENTRATION U/ml	CHEESE ANALYSIS				
	RAW MILK			PASTEURIZED MILK			Coli number after		Weinzirl test after 10 weeks	Sensorial analysis	
	Germs number	Coli number	Weinzirl	pasteuriz. effect %	Coli number		5 weeks	10 weeks		after 5 weeks e.i.c.s.t.	after 10 weeks e.i.c.s.
4 a	$72x10^5$	10^{-4} ml	9 (10)	99.47	1.0ml-	210	10^{-5}g	10^{-4}g	10(10)	5 3-4 4 4	3 1 3 -
						525	10^{-5}	10^{-4}	0(2)	5 4 4 5 4	5 3 3 3
b	$36x10^5$	10^{-5}	5 (10)	98.81	1.0-	210	10^{-4}	10^{-3}	8(9)	5 3 4-3 4	3 2 3 -
c	$135x10^4$	10^{-4}	10 (10)	97.93	1.0-	1050	10^{-5}	10^{-4}	1(1)	5 5 3 4 4-	5 5 4 4
						494	10^{-4}	10^{-4}	0(0)	5 4 4 4-4	5 4 4 4-
						987	10^{-3}	10^{-3}	0(0)	5 3 4 4 4	5 4 4 4-
5 a	$60x10^5$	10^{-5}	10 (10)	99.98	1.0+	200	10^{-2}	10^{-1}	3(9)	4 2 3 3 2	4 2 - -
						500	10^{-2}	10^{-1}	1(8)	5 5 3 4 3	5 3 4 4
b	$230x10^4$	10^{-4}	9 (10)	98.26	1.0-	210	10^{-3}	10^{-2}	1(9)	3 3 4 3 3	3 1 - -
						1050	10^{-4}	10^{-4}	2(4)	5 4 4 4 4	5 4 5 3
c	$66x10^4$	10^{-4}	9 (9)	94.82	1.0-	528	10^{-2}	10^{-2}	0(0)	5 4 5 5 3	5 5 5 4
						1056	10^{-3}	10^{-4}	1(2)	5 5 5 4 4	5 5 4 4
6 a	$178x10^4$	10^{-5}	10 (10)	98.74	1.0-	200	10^{-1}	10^{-1}	10(10)	4 2 4 3 2	2 1 - -
						500	10^{-2}	10^{-1}	1(2)	5 4 5 3 3	5 3 5 3
b	$150x10^4$	10^{-4}	8 (10)	99.33	10^{-1}+	200	10^{-2}	10^{-1}	10(10)	5 3 3 4 3	0 0 - -
						1000	10^{-3}	10^{-2}	1(3)	5 4 5 4 4	4 4 5 4
c	$127x10^4$	10^{-3}	10 (10)	98.39	1.0-	500	10^{-2}	10^{-2}	1(3)	5 4 5 3 3	5 4 5 3
						1000	10^{-4}	$1C^{-4}$	1(2)	5 4 4 4 4	5 3 4 4

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has been shown to increase the apparent heat resistance of spores of non-proteolytic *C. botulinum*. A 6-D inactivation could not be achieved with a heat treatment equivalent to 19.8 min at 90 °C and growth of *Clostridium* spores was detected in less than 93 days as compared to samples containing no lysozyme, which did not show presence of *Clostridium* for at least 93 days (Peck et al., 1993; 1992). Peck and Fernandez (1995) suggested that for processed foods containing lysozyme at concentration up to 50 μg/ml prior to heating, with an intended shelf-life of no more than 4 weeks and temperature of exposure approaching 12 °C, incubation at temperature <12 °C would be required to ensure safety with respect to non-proteolytic *C. botulinum*.

Lysozyme is probably not used extensively in processed cheese making because the processing temperature was thought to be typically high enough to cause a significant reduction in enzyme activity. However, the finding by Düring et al. (1999) on lysozyme will probably find applications where food is processed at temperature above lysozyme denaturation temperature.

Cost has been another factor that has limited the use of lysozyme in applications where the use of standard chemical preservatives, such as potassium sorbate, and nitrates, is less expensive. However, as consumer demands for more natural and healthy food products increase over time this hurdle may be overcome as the 'nutraceutical attributes' of lysozyme alone or in combination with other antimicrobials and preservatives become more evident.

VII. SAFETY/ TOLERANCE AND EFFICACY

A. Metabolism: Physiological turnover/clearance

Lysozyme, like many other low molecular proteins, is easily filtered through the kidney, reabsorbed by the proximal tubular cells and catabolized in the lysosomes. This property has opened the door to using lysozyme as anti-infective and carrier for renal delivery of various drugs including anti-bacterial agents (Haas et al., 1997; Meijer et al., 1996).

B. Cell culture and animal studies

Egg-white lysozyme bound to LPS from *E.coli* 0111:B4 suppressed TNF-α production from macrophage-like cell lines resulting in a reduction of the lethal toxicity associated with LPS (Kurasawa et al., 1996). The binding of lysozyme to LPS reduces the mitogenic activity and TNF production by LPS and may be an important therapeutic modulator of inflammatory response during sepsis and septic shock (Takada et al., 1994).

Immunization of Balb/c mice with hen egg white lysozyme stimulated T-cell activation while a conjugate lysozyme-PEG lowered the T-cell activating capacity of hen egg white lysozyme (So et al., 1996).

C. Clinical trials

Clinical trials of lysozyme have been reported with mixed results. Yamada et al. (1993) reported clinical studies that showed the development of specific IgE antibody titers to hens's egg white lysozyme in children allergic to egg. A careful examination of several publications, such as by Urisu et al. (1997), Bernhisel-Broadbent et al. (1994), Aabin et al. (1996), Holen et al. (1990), Picher and Campi (1992), Anet et al. (1985), and

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Wasserfall, F.: Unveröffentlichte Daten (1975)

A drawback of the above controlled Maillard reaction is the very slow reaction rate. As an alternative to the atmospheric treatment, it was found that shortening of the reaction time was successful by using a high-pressure treatment of a solution (150 Mpa, 60 °C for 1 h). A remarkable 200% increase in the lysozyme activity compared to the untreated lysozyme may have been due to a decrease in heat denaturation of the enzyme protein rather than the promotion of reaction rate. As a result, high solubility was maintained for lysozyme without melanoidin formation (Nakamura et al., 1997). The same group later found that from a lysozyme/dextran mixed solution treated at pH 4.5 with NaCl (0.1 M) under 192 MPa and 19.3 °C, 10.4% of the conjugate was obtained after 88-min treatment. This yield should still need improvement compared to the dry-powder process with higher than 70% yield. The high-pressure treated conjugate exhibited an increased antimicrobial activity against *E. coli* and *S. typhimurium.* Much higher activity against gram-negative bacteria was revealed by a lysozyme-lactoferrin complex conjugated with dextran (unpublished result).

Through chemical modification of lysozyme by linking a saturated fatty acid or hydrophobic peptide (*PhePheValAlaPro*) to the C-terminus, the activity against gram-negative bacteria was detected (Ibrahim et al., 1991; 1997). The gram-negative cells have an additional membrane outside the cell wall. The cells manipulate the biochemistry of the wall in a way that causes a local change in the biophysical parameter analogous to surface tension. A possible explanation of the inactivity of lysozyme is that the enzyme is entrapped in the outer membrane through specific binding to the LPS and hence becomes arrested at this site. The specific hydrophobic binding and the resultant inactivation of lysozyme by LPS isolated from a variety of gram-negative organisms were reported (Ibrahim et al., 1994).

*3. **Genetic engineering:*** Kumagai and Miura (1989) were successful in enhancing the cell-lysing activity of chicken lysozyme three-fold by simultaneously mutating N37G, W62Y and D101G, despite the fact that individual mutants did not show increased lysing activity.

High-level expression of lysozyme (550 mg/L) in *Pichia pastoris* was reported (Digan et al., 1989). It is desirable to produce the enzyme with different pH optimum, altered specificity, improved activity and stability (Imoto, 1996). Frequently, mutations that boost enzyme activity occur in unpredictable sites for simultaneous mutation of more than one site (Rawls, 1999). The application of a regulated random optimization for site-directed mutagenesis is recommended (Nakai et al., 1998). Selection of amino acid residues to replace at site, which are randomly chosen, is made by using amino acid scales for hydrophobicity, propensities for helix and strand or bulkiness. The Windows computer program written for genetic optimization could accommodate any new amino acid scale, if it is effective in searching for important functions of proteins.

It is worth noting that only one amino acid substitution (*Ile55Thr* or *Asp66His*) turned human lysozyme to an amyloid fibril protein (Pepys et al., 1993). Most proteins may have an inherent ability to form fibrils like those found in the brains of Alzheimer's disease victims (Borman, 1999).

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In most cases these holings are either too large or too numerous. The reason of this is unknown.
For clarification, to answer the question if lysozyme influences the alteration of blowing germs activity and/or other unwished bacteria responsible of ripening (coliforms, anaerobic sporogenes) with a modality till now unknown, further tests are needed.
The lab-tests, up to today carried out to answer this question, show that, contrary to the experiments made in Rotholz (1970) in the Gouvernment Cheesemaking Research Institute, the lysozyme, in the above established concentrations, can considerably delate the development of clostridia and also influence the development and acidification capacity of Str. lactis.

From the curdling time, determined with the help of a lactodinamograph, it is possible to establish that the suitable concentration to prevent the blowing, i.e. 500 U of lysozyme/ml of milk, shortens or delates only a little the curdling time and, consequently, has no influence on the curdling of milk.
The verification made with the lactodinamograph revealed always the same degree of consistence of the curd.

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on the contrary, frequent in milks of poorer quality.

The established concentration of lysozyme suitable to avoid the blowing seems feasible only with the use of fresh egg white or purified and crystallized lysozyme. By adding albumine, unexpected results were obtained: additions of lysozyme in higher concentrations than the minimum could not prevent the blowing. This result was confirmed in further tests, with the use of similar milk batches, by checking the anti-blowing effect of albumine against egg white. The cause of such unexpected results is unknow. It can be assumed that, after drying, not only the lysozyme content increased, as revealed by the tests carried out with Micrococcus lysodeiktycus as substrate, but also other substances developed to influence the enzyme activity on blowing germs.

With a lysozyme concentration higher than the minimum, no improvement of the cheese quality can be obtained.

There is no significative correlation between the number of positive tests, according to Weinzirl, on raw milk quality and the growth of the blowing in control cheeses.
Notwithstanding 10 positive results on 10 parallel tests, in the test n. 60 there is no blowing, even after 10 weeks ripening at 15°C. As consequence, it can be stated that the number of positive tests, according to Weinzirl, is not to be considered a ground as regards the contamination of milk with blowing spores; therefore, it can be asserted that the Weinzirl test to determine the presence of anaerobic sporogenic bacteria in raw milk is useful to locate the proteolitic and lactose fermenting germs but not to recognize the blowing responsible germs.
Owing to this and from the results obtained, time by time, it is evident that the milk, only with 0-5 positive Weinzirl tests determined with the above method, in general, does not cause the late blowing (Wasserfall, 1975).

The cheeses obtained with the addition of little quantities of lysozyme and that blowed after 10 weeks ripening and storage at 15°C generally have a number of anaerobic sporogenes higher than those where the blowing was prevented with lysozyme addition, as clearly shown by Weinzirl test on substrate of glucose solution (table 1).

According to these data, in need of confirmation, it is possible to state that the cheeses obtained with a large quantity of lysozyme as egg white or crystallized enzyme, are not different if compared to the nitrate cheeses type EDAM. It can be stated also that the cheeses obtained with lysozyme addition do not show any typical holing.

EXHIBIT 2 (2 Pages)

CBI DELETED

Moreover, compared to the packed cheeses, they were also, apparently, more tasty because of the ripening made without any packing and of the higher evaporation and, therefore, they were more "compact".

3.4. Influence of lyscozyme on curdling

To determine the influence of different quantities of added lysozyme on curdling, it was checked the curdling time (see 2-4) of control-milk without any addition and test-milk with additions in order to avoid the influence of milk inconstant curdling times. The average curdling time of control milk was of about 38 minutes (range 29-46 minutes).

By adding nitrate or albumine, the curdling times were shortened of 2,5 and 4,4 minutes rispectively; by adding egg white or crystallized lysozyme, such times were increased of 5,6 and 1,8 minutes rispectively. Furthermore, the addition of egg white caused an increase of curdling time in accordance with its concentration: with 300-500 U/ml (as egg white) the increase of curdling time was of about 2,3 minutes and with 600-900 U/ml was of about 10,1 minutes.

4. EVALUATION OF RESULTS AND CONSEQUENCES

The previous works purpose was to establish if the nitrate, till now used in cheesemaking to prevent the late blowing, could be replaced by lysozyme present, in large quantities, in the egg white. On the basis of tests carried out till now, it is possible to state that the minimum suitable concentration of lysozyme is about 500 U/ml; such concentration is higher than the one established by other investigators, i.e. almost more than twice.
Since the production standards followed by said investigators in their experiments are not revealed and, above all, there are no data concerning the bacteriological quality of milk used to this purpose, it is difficult to establish why the lysozyme was added in concentrations higher than the suitable ones. It can be only supposed that the milk used in Hungary, Poland and Norway was, as regards the anaerobic spores of BAB responsible of late blowing, of better quality than the milk used in the above mentioned tests.
This fact is not surprising as the above milk was stored in the farm and then collected the day before manufacture and stored again at 8°C for one day prior to use. As consequence, it could be ascertained that the milk used for this production was unusually highly contaminated from the microbiological point of view.

Although, in general, the cheese manufacturers may employ a raw milk bacteriologically better, it is impossible, by a minimum concentration of lysozyme, to avoid the hazard of blowing that is

All control cheeses were containing an high number of anaerobic sporogenes that, according to Weinzirl, gave 7-10 positive results on 10 tests, after 7 days incubation (table 2) and, except for some cases, were more or less highly blowed after 5 weeks ripening, with a score of 1-3 for external aspect and 0-2 for internal aspect. Excepting the control cheeses of test n. 11, that were showing a poor blowing (4 points for the external aspect, 2-3 points for the internal aspect and 2-3 points for smell and taste) and excepting also the control cheeses of test n. 12C, that were showing no evident blowing after 5 weeks ripening, the typical phenomena of late blowing appeared in the cheeses made with 400-500 U of lysozyme as albumine after an abnormal ripening of 10 weeks at 15°C.
Said blowing phenomena, more or less evident, appears in these prolunged ripenings also in the other cheeses obtained with albumine (tests n. 12A and B).

The results of sensorial tests on lysozyme cheeses stored for 3 weeks at 8°C - tests series 9-13 - are reported in the table n.3. Excepting the cheeses obtained with albumine (tests n. 12-13C) it can be stated that the production of cheeses without blowing is possible with a concentration of 300 U of lysozyme/ml of cheesemilk, when the raw milk has only a poor potential blowing (test n. 11B). Anyway, with an higher concentration of germs (higher number of positive results in Weinzirl test) also the use of concentrations of either 400 U/ml or 500 U/ml is not sufficient, since in the EDAM cheese the holes appear in heavy way (test n. 10A and 10C).

Notwithstanding the above results seem, apparently, of no account, the lysozyme, as egg white or crystallized product, can be used to prevent the late blowing. The use of albumine containing lysozyme in concentration of 400-600 μ/ml causes a more or less significative holing (test n. 12 and 13C). Only with test n. 12C it was obtained an almost acceptable quality of cheese, probably depending on the fact that anaerobic sporogenes responsible of the holing were not so active as the germs present in tests n. 12A and 12B. The sensorial test on control cheeses, reported in table n.2, gave the same results. Therefore, by adding egg white or crystallized lysozyme, the cheeses obtained with 500 U of lysozyme/ml had the same behaviour as those obtained with nitrate (test n. 13)

The analysis carried out on cheeses from series n. 12 onwards, on the basis of butyric acid presence, revealed that the addition of lysozyme to cheesemilk not only reduced the holings formation, owed to the blowing germs, but also the butyric acid production when the latter was not sufficiently inhibited.

The not-packed cheeses, waxed after ripening and right away stored, were, from the sensorial point of view, better than the cheeses packed and stored at 8°C. In general, they had an harder consistence and a stronger flavour similar to the GOUDA cheeses.

Exhibit 3

Enzyme Technical Association
Petition for Listing of Egg White Lysozyme on the "National List"

Commercial Confidential Information (CBI) Statement

The information contained in Exhibit 2 of this petition constitutes commercial confidential information concerning an ETA member's proprietary trade secret manufacturing process. As such, **all** of the information contained in Exhibit 2 is protected from public disclosure pursuant to the applicable provisions of the Freedom of Information Act.

A "CBI Deleted" copy is provided with Exhibit 2.

Scheme of cheese ripening and storage

	Results	Evaluation after
Cheese 1	packed and ripened at 15°C	5 weeks
Cheese 2	packed and ripened at 15°C	10 weeks
Cheese 3	packed and ripened at 15°C for 5 weeks and stored at 8°C for 5 weeks	10 weeks
Cheese 4	ripened at 15°C for 5 weeks, waxed and stored at 8°C for 5 weeks	10 weeks

In this way, leaving out the quality comparison between the cheeses obtained with different quantities and type of lysozyme, it was possible to control the following parameters: 1. if the ripening, after 5 and 10 weeks at 15°C, went on normally without any blowing; 2. if quality differences were recognizable between the cheeses ripened after packing and the waxed cheeses.
To be sure that the milk used for cheesemaking was effectively containing the germs responsible of blowing, for each test it was made, still using the same milk, a whole cheese without adding anything that could prevent the late blowing.

All these tests were made with the following types of lysozyme: 1. Egg white separated from fresh egg. 2. Purified and 3-times crystallized lysozyme by Roth Co. 3. Egg albumine (dried and finely pulverized) of Merck Co. The egg albumine had a lysozyme content, in weight, 9,6 times higher than egg white. To complete the test, it was also checked the quality of lysozyme cheeses compared to the quality of nitrate cheeses (test n. 13).

In all tests, the total bacteria count of raw milk resulted of $81x10^4$-$114x10^5$ u.c.f./ml and the pasteurization effect resulted of 91,2-98,5%. This result was sufficient to considerably reduce the number of coliforms. The Weinzirl test on anaerobic sporogenes resulted of 6-10 positive tests on 10 parallel tests.

tests on 10 parallel tests after 7 days at 37°C) and, consequently, a lower sensorial quality of the cheese. The organoleptic test, carried out after 5 and 10 weeks ripening at 15°C on lysozyme cheeses, clearly points out that - countrary to the results of Koterbska (1972) and Aarnes (1974) - a lysozyme concentration of 200 U/ml (about 0,001%) is not sufficient to prevent the late blowing. In three tests out of six (n. 5A-5B-6A) this poor capacity (of lysozyme) was clearly noticed, after 5 weeks ripening, from the point of view of organoleptic quality; in fact, the cheeses showed holes and deformations and also unpleasant taste and flavour. Even with the addition of 300 U of lysozyme/ml of cheesemilk (tests n. 7A-7B) the cheese tended to blow, although the first blowing fault was evident only after 5 weeks ripening, with the holings formation.

Only by adding 400 U of lysozyme/ml of cheesemilk (tests n. 8A-8B) the cheese did not blow. Since all higher concentrations of lysozyme can prevent the late blowing, it can be stated that the suitable minimum concentration is 400 U/ml.

3.3. Influence of the content and type of lysozyme on the cheese quality

After establishing the minimum concentration of lysozyme suitable to prevent the late blowing, it seemed interesting to investigate the possibility to carry out tests on cheese manufacture by using more quantities of milk (vat cheeses) and achieve equivalent results. In the meantime, there was the problem that the quality of lysozyme cheeses was influenced by the quantity and type of added lysozyme.

To this purpose, each test was carried out by means of 2 vats, each containing 100 l of milk of same source and quality, with different quantities of lysozyme,in standard conditions of cheese manufacture. 3 of the 4 whole cheeses obtained were packed, after salting, with paper; the fourth was waxed after 5 weeks ripening. at 15°C. and stored for further 5 weeks at 8°C; afterwards it was subjected to organoleptic test. Of the 3 packed whole cheeses, one was qualitatively exhamined after 5 weeks ripening at 15°C; another after 10 weeks ripening at 15°C; the third after 5 weeks ripening at 15°C and after a storage of furhter 5 weeks at 8°C (See scheme)

Final-version

National Organic Standards Board

Meeting Minutes
November 15 – 17, 2000
USDA/ERS
1800 m Street, NW, South Tower
Waugh Auditorium, 3rd Floor
Washington, DC

Attendance Record:

Members Present: 14

Robert Anderson
Carolyn Brickey
Owusu Bandele
Kim Burton
Rebecca Goldburg
Joan Gussow
Steven Harper
Marvin Hollen
Mark King
William Lockeretz
Betsy Lydon
Stephen Pavich
Eric Sideman
William Welsh

Kyle Moppert, State Representative (LA)
Bob Shine, Certifier Representative (TN)

Members Absent: 0

Other Attendees:

Keith Jones, *Program Manager, National Organic Program (NOP), USDA*;
Michael D. Fernandez, *Assistant to the Administrator, Agricultural Marketing Service (AMS), USDA*
Beth Hayden, *NOP, USDA;*
Mark Keating, *NOP, USDA;*
Richard Mathews, *NOP, USDA;*
Arthur Neal, *NOP, USDA;*
Robert Pooler, *NOP, USDA;*
Toni Strother, *NOP, USDA*;
Kristi Wilson, NOP, USDA; and

Interested persons from the public **(See attachment B)**.

Meeting Purpose:

The principal purposes of this meeting are to provide an opportunity for the NOSB to receive committee reports; receive update from the Aquatic Task Force Working Group; to receive an update from the USDA/NOP, and review materials for possible inclusion on or removal from the National List of Approved and Prohibited Substances. Materials to be reviewed at the meeting are:

time by time, the type of lysozyme, the milk used for the preparation of lysozyme suspension and the ripening cultures in presence of coliforms.

The series of tests n. 4-8 was useful only to establish the lysozyme concentration necessary to prevent the late blowing. Every series was consisting of 3 tests, carried out in different days a week, during which 2 whole cheeses were prepared with different concentrations of lysozyme and a whole cheese was prepared with the same raw milk but without any addition. To be sure that the late blowing was really prevented by the addition of lysozyme, the cheeses were tested both microbiologically and sensorially, not only according to the industrial process, i.e. after a ripening of 5 weeks, but also after a ripening of 10 weeks at 15°C. Since the milks used in different tests change from day to day and are different one from the other, above all as regards the bacteria count, both qualitatively and quantitatively, a verification of the cheeses obtained in different days and with different lysozyme concentrations is, obviously, possible only with a certain reserve.

The results of analysis on milk and cheese are reported in the enclosed table n.1. The total bacteria count of raw milk is between $96x10^4$ and $7x10^5$ germs/ml; the effect of the pasteurization is of 93,35-99,98%; the pasteurization can drastically reduce the number of coliforms. Only in two cases (test n. 5A and 6B), 1 and 0,1 rispectively coliforms/ml of pasteurized milk can be found. These results seem not to influence the number of coliforms in the ripened cheeses.

The test carried out by Weinzirl to determine the number of anaerobic sporogenes bacteria in raw milk, after 3 days incubation, resulted of 2-10 positive tests on 10 parallel tests, and 9-10 positive tests on 10 parallel tests after 7 days incubation for safety's sake. To the high number of positive tests, according to Weinzirl, it also corresponds the fact, not evident in the table, that, except for control cheese of test n. 6C, all control cheeses obtained without any addition of nitrate or lysozyme show, after 5 weeks ripening, a more or less significative late blowing (deformations, holings). The control cheeses of test n. 6C, after 5 weeks and also after 10 weeks ripening, show no visible late blowing, although it can be experienced a quite strong taste (bitter).

While there was no evident correlation between the concentration value of lysozyme and the number of coliforms in ripened cheeses, on the countrary, it seemed that a correlation was existing between the first value (lysozyme concentration) and the Weinzirl test: to a low lysozyme concentration (200-300 U/ml) it was corresponding an high number of anaerobic sporogenes (5-10 positive

periacetic acid, calcium borogluconate, animal enzymes, leather meal and sodium chlorate.

WEDNESDAY, NOVEMBER 15, 2000

CALL TO ORDER – MR. ROBERT ANDERSON, CHAIRPERSON

Mr. Robert Anderson called the meeting to order at 2:20 p.m., he welcomed everyone and thanked them for coming. Mr. Anderson had the National Organic Standards Board (NOSB) members introduce themselves as well as the guests assisting them.

Public Comment Session - Mr. Robert Anderson, Chairperson

Tom Harding, AgriSystems International

He testified in favor of organic certification of sustainably harvested wild caught fish. He told the Board that it must look at the system to determine how these fish are managed. He supported labeling al product in a legal and defined way. He encouraged eco-labeling. He also said that

"access to pasture" must have a clear definition and that the Board must recognize that all operations under all conditions are not appropriate for pasture all the'time. He supported a standards ceiling and recommended that USDA not place a ceiling on standards.

Dennis Blank – He discussed his inability to get free flowing information from USDA or the NOSB. **(See attachment 1)**

Bob Anderson replied that "not one committee on this Board makes a decision away from this table". Mr. Anderson went on to say that never in his six years on the Board, five as Chairperson, did he ever know of any Board member withholding information from the media. Mr. Anderson finally asserted that it is the responsibility of the media is to engage in accurate reporting.**Bruce Krantz, Vice President/General Manager Hynite Corporation** – Mr. Krantz presented comment on the Board's review of Leather Meal. **(See attachment 2)**

Joe Mendelson – Speaking on behalf of the Campaign for Sustainable Agriculture, he referenced a letter attached to a recent survey results. Mr. Mendelson reviewed the content of the letter with the Board, and discussed issues of transparency and development of program manuals. **(See attachment 3)**

Brian Leahy, Executive Director of CCOF – Mr. Leahy requested that the Board reject the petition to approve leathermeal and sodium chlorate, and spoke in support of the label for "made with organic ingredients".

Cissy Bowman - She expressed concern for keeping small farmers on farms and the need for the stakeholders to include these interests. Ms. Bowman encouraged NASOP to be more involved as many new states developing organic programs are not familiar with the stakes involved.

Tom Hutcheson, Organic Trade Association – On behalf of OTA, he welcomed the new NOSB members. Mr. Hutchenson made reference to OTA's historic role in the development of industry and

With the determination of lysozyme content in the milk, it was realized that only a part of the lysozyme added to milk was found. The recovery decreased with the storage time, reaching the 75-85%, according to the quantity added to the milk (the average in the tests n.6-13 was of about 78,8%). Consequently, it can be stated that only a part of the enzyme is linked to the curd.

The recovery of the enzyme in the whey was of about 88-98%. If the lysozyme was added to milk prior to rennet, only a part of it could be found in the relevant whey. In general, this quantity was nearly the 9-10%, so that about the 90% of lysozyme was linked to the curd and this result was considered significative.

3.2. Prevention of the late blowing

Before establishing the lysozyme concentration necessary to prevent the late blowing, it was considered the possibility to produce the EDAM cheese with lysozyme instead of nitrate, according to the production process of Prokopek and Schmanke (1974). To this purpose, a series of tests were carried out in 3 different days a week, by preparing a whole cheese with raw milk added with lysozyme, a control whole cheese with raw milk added with nitrate and a whole cheese made with the same milk but without any addition. These tests, that employed lysozyme quantities of 200-500-1000 U/ml of cheese milk, proved that it is possible to produce the EDAM cheese also with the addition of lysozyme in industrial scale and that without the addition of nitrate or lysozyme the late blowing appears for certain, provided that the content of anaerobic sporogenes is sufficiently high.

Since the manufacture of EDAM cheese, with the addition of nitrate, causes no problems when the operation is made accordingly, no further control tests were deemed necessary. On the other hand, as control test, it was used the cheese obtained without any addition because, for this cheese, only the late blowing test is necessary, provided that, in raw milk, a sufficient number of butyric spores producing the late blowing, is present. Therefore, it is evident that the prevention of the late blowing is owed to the presence of lysozyme.

In every milk, prior and after the pasteurization, it was determined the total bacteria count and the number of coliforms. The cheeses ripened 5-10 weeks were tested from the point of view of the presence of anaerobic sporogenes and analyzed both microbiologically and sensorially. The microbiological test was carried on during the experiments to detect the number of coliforms and anaerobic sporogenes. Furthermore, it was analyzed,

national standards and the offer of the association to continue that role. **(See attachment 4)**

The following people were not present but sent public comment to the Board:

Philip LaRacca, President California Certified Organic Farmers **(See attachment 5)**
Richard C. Nelson, President Nelson & Sons Inc. **(See attachment 6)**
Peter Granger, Washington Fish Growers Association **(See attachment 7)**
Ronald W. Hardy, Professor University of Idaho **(See attachment 8)**
Scott P. Ager, Technical Services Manager CH20, International **(See attachment 9)**

End of Public Comment

The meeting recessed for the day at 3:10 p.m.

THURSDAY, NOVEMBER 16, 2000

The meeting reconvened at 9:15 a.m. Bob Anderson encouraged the Board to stay engaged; stay open and to continue to build on the environment of good working relationships, to strive for more diversity on the Board and in the marketplace. Mr. Anderson passing the gavel to NOSB Chairperson elect Carolyn Brickey. The agenda was reviewed with no changes.

NOSB COMMITTEE ACTION ITEMS – MS. CAROLYN BRICKEY, CHAIRPERSON

Livestock Committee: *Mr. Eric Sideman, Chair*

Mr. Sideman reported that the use of parasiticides in organic livestock production should be the last resort in organic livestock health care, when animals are severely infected. In conventional production, parasiticides are used routinely. He reviewed the history of how the Board approved Ivermectin as one of the three parasiticides submitted for review. The Board chose Ivermectin because it has the widest number of applications. On the other hand, Mr. Sideman pointed out, Ivermectin does pose an important risk that needs to be addressed. Since Ivermectin is also an insecticide it kills dung beetles and other organisms involved in the decomposition of manure. This is a particular concern with slow release formulations of the parasiticide because such products are designed to be active over an extended period and thus a large portion of the manure deposited over the grazing season is resistant to decomposition. Hence the Livestock committee will recommend an annotation to the approval of Ivermectin that will prohibit the slow release formulations.

Emily Brown-Rosen explained the issue of approvals of ingredients for livestock feed. She introduced a proposal from the committee: if materials have been specifically approved for use in organic processing and also are approved either as listed in 21CFR or the American Association of Feed Control Officials (AAFCO)annual publication for use as livestock feed, the material should be allowed for use in organic livestock feed. Betsy Lydon asked if this is a roll back, to review all the approved ingredients in processing and allow them for use as livestock feed ingredients. Mr. Anderson asked if this policy should work in reverse: to approve if not prohibited, by the AAFCO list and CFR 21. Willie Lockeretz asked if there is a realistic difference in environmental concerns in livestock use that would not be present in food processing. Mr. Sideman will prepare a proposed resolution for the Board.

Materials Committee: *Ms. Joan Gussow, Chair*

2043 Mgl by Schleicher and Schüll Co. and by means of water satured n-butanol as solvent and 50% of ethylamine as stationary phase. The fatty acids extract was, immediately after, transformed into the relevant ammonium salt by paper saturation with concentrated ammonia vapours.
The development time was of 16 hours. As indicator, it was used a solution of bromekresole green in absolute alcohol. The fatty acids appear blue on yellow background.

2.4. Preparation and evaluation of the cheeses

The EDAM cheese was produced with pasteurized milk (62-64°C/30 minutes) according to the process established by Prokopek and Schmanke (1974). For the manufacture of each cheese, were used special containers of parallelepiped form, with a capacity of 25 l each, where a total of 100 l of milk was introduced (25 l in each container). The addition of lysozyme in the cheesemaking was made immediately prior to rennet, as concentrated suspension of enzyme in additional pasteurized milk.

In order to evaluate the effects of nitrate and of different lysozyme types on the curdling, it was checked the formation and the consistence of casein curd, by means of a lactodinamograph (Voss and Schmanke, 1973) during the rising of cheese. To estimate the right consistence of curd, it was considered the time necessary to reach a surface of 34 mm. after the breaking of the curd (curdling time).

The evaluation of cheeses ripened 5-10 weeks was made according to Voss (1968) and also to Wasserfall et al. advices (1974) on the basis of the following parameters: external aspect, internal aspect, consistence, flavour and taste.

3. RESULTS OF RESEARCH

3.1. Accuracy in lysozyme determination and recovery of the enzyme in milk and whey

According to the above mentioned method, it was drawn up a standard curve to determine the content of lysozyme.
The correlation between the extinction and the logarithm of the lysozyme concentration resulted in the range of 25-3,125 U/ml with the help of a linear regression analysis, so as to have the samples, after the suitable dilutions, on the curve.
With the help of repeated determinations, different one from the other, it was found, on the same line of the relative error, a value of max. 3,80% for each test. Since every determination of lysozyme content was carried out with an average of values obtained through two different dilutions, each one repeated three times, it was verified that every concentration of lysozyme had a max. error of 2%

Ms. Gussow presented the Materials Database prepared by Organic Materials Review Institute (OMRI). Ms. Brown-Rosen further explained the database. One purpose of the document is to provide a history of materials review for new Board members. Ms. Brown-Rosen asked for suggestions about format or request for additional information. Board members were asked to respond to Joan Gussow or Kim Burton, NOSB Materials Committee, not to Ms. Brown-Rosen or OMRI. Once corrections to format or accuracy are made. The database will come back to the Board for acceptance. Carolyn Brickey noted that the Board would develop a document for historical Board decisions not dealing with materials. This project will begin shortly.

The Materials Committee report was halted for an Environmental Protection Agency (EPA) presentation.

PRESENTATION BY MR. JIM JONES, DIRECTOR, EPA PESTICIDE REGISTRATION DIVISION

Jim Jones explained the status of the inerts review program at EPA. List 3 inerts already in approved organic materials seems like a logical place to begin a review. Owusu Bandele asked if EPA could also review materials for use as fertilizers as well, but EPA does not regulate fertilizers. Keith Jones thought the American Association of Plant Food Control Officials (AAPFCO) might facilitate that. Mr. Jones also discussed a new program that EPA will propose to offer manufacturers who petition EPA the opportunity to obtain a seal that indicates that the product meets OFPA standards for organic use. Manufacturers will need to submit a petition to EPA. This will be a voluntary program at the request of individual pesticide manufacturers. Mr. Sideman raised the question about annotations for organic approval and Jim Jones responded this issue would have to be addressed in the process. Keith Jones asked the Board to think about language for such a label.

Becky Goldberg asked about the public comment period in relation to the March meeting. Jim Jones suggested 90-120 days.

Keith Jones stressed the importance of language on the label. What EPA is doing is allowing additional information to the marketplace, not engaging in oversight of the Board action. The issue is how to communicate annotations. Willie Lockeretz is concerned about use by home gardeners who may misinterpret the EPA label. Keith Jones reminded everyone that NOP does not regulate consumers and home gardeners.

Steve Harper asked how the EPA will deal with materials that the NOSB recommends delisting.

Bob Anderson noted that this is an additional seal that manufacturers would see as a tool, an incentive for organic practice. The EPA organic label would state that organic approval will be allowed according to annotations, according to Jim Jones. Kyle Moppert noted that a violation of label restriction would now be not only an organic violation but also a pesticide violation. Steve Pavich asked how long it would take EPA to come up with this label. Jim Jones indicated that the program could be up and running in about 90 days.

Carolyn Brickey asked for advice about the NOSB petition review process. The Board discussed the opportunities for public comment and whether it would it be available for all material applications. Ms. Brickey mentioned there might be some applications that would definitely not receive NOSB approval and some may not have TAP reviews at all. Kim Burton mentioned the October 1999 time line recommended by the Board and wants to consult with EPA.

2. METHOD

2.1. Determination of lysozyme

The determination of lysozyme uses, as substrate, Micrococcus lysodeikticus cells killed by U.V. rays and, as standard, an egg white lysozyme, three-times crystallized, by Roth Co. of Karlsruhe, with an activity of 20.000 U/mg, according to the method of Smolesis and Hartsell (1948) published by Difco Laboratory (1966): each 5 ml of lysozyme solution is mixed with 5 ml of substrate and its extinction is determined in Elko III, by means of filters S55E, exactly after 20 minutes at room temperature ((21°C).

For the determination of the enzyme in the milk, the whey was quantitatively recovered by acidification with n-HCl at pH 4,6 and subsequent filtration through filter paper n. 595 1/2 by Schleicher and Schüll Co.; afterwards, the pH was increased up to 6,2 with 0,1 n-NaOH. For the determination (of enzyme) in the whey, the final clear solution was centrifuged for 15 min. at about 80.000 G at 2°C and then filtered under vacuum through a cellulose nitrate-filter AE91 by Schleicher and Schüll Co. with 0,8 µm pores.

2.2. Ripening agents and microbiological analysis

As ripening agent, until now, it was used a mixture of microorganisms with a proteolitic activity of nearly 25 µg of released tyrosine/ml of milk in 5 hours at 30°C, and 5% inoculum. Afterwards, the proteolitic activity was determined according to Hull method (1947) with the remarks of Citti et al. (1963)

The microbiological analysis on milk and cheese samples were carried out according to the advices of Wasserfall et al. (1974).

Great care was taken in verifying that the milk used for the cheesemaking and lysozyme suspension was free from any inhibitory substance. For these analysis we would like to thank Mrs. Dr.Suhren of Milk Hygiene Institute.

2.3. Determination of butyric acid

The butyric acid presence in the cheeses was determined by extraction of volatile acidity, according to Ritter method and by separation on paper chromatography. This test was carried out in the decreasing phase by using a paper n.

Materials Committee - Continued: *Ms. Joan Gussow, Chair*

The proposal for materials decisions for Crops, Processing, and Livestock was reviewed. Mr. Anderson suggested that this document go out to the Board with the advanced Board packet.

USDA/NATIONAL ORGANIC PROGRAM UPDATE – *KEITH JONES, PROGRAM MANAGER*

Keith Jones acknowledge the presence of Mr. Michael D. Fernandez, Assistant to the Administrator, Agricultural Marketing Service (AMS), USDA. Mr. Fernandez briefly addressed the Board on behalf of Kathleen Merrigan, Administrator, AMS. Keith Jones discussed the Freedom Of Information Act (FOIA) process.

Keith Jones then reviewed the authorization levels for contracts for service. The Program Manager has authority to execute contracts up to $5,000. Mr. Jones indicated that a contract over $25,000 may not require bidding. Within certain guidelines, contracts can be sole sourced. Mr. Jones addressed in detail the $100,000 contract awarded to OMRI for material technical advisory panel reviews which was originally offered to both OMRI and the Organic Farming Research Foundation (OFRF) at $50,000 respectively. He stated there have been some questions why NOP did not put the materials review contract out for bid. He stated because of the time needed to do a request for proposal it was decided to do a sole source contract under an "urgent and compelling" authorization. Finally, the OMRI contract was submitted to both organizations and OFRF, after review, chose not to execute its purchase order. To continue to obligate the funds, an additional $50,000 was then requested to be awarded to OMRI.

Steve Pavich asked about competition. Mr. Jones said the Department tries to encourage competition for requests for proposals, but indicated that there is little point in requesting proposals if only one person or organization applies. Mr. Jones added that due to the unique and esoteric nature of organic material review, vigorous competition among organizations may not occur.

The discussion returned to FOIA information. Steve Harper asked if some of the information being requested by FOIA could be available on the web. Mr. Jones agreed that some information could be made available, but not unapproved committee minutes and contract details. Until the minutes are approved, they are considered pre-decisional and unavailable to the general public. Willie Lockeretz asked if he should assume that any correspondence between the NOSB and the NOP are subject to FOIA. Mr. Jones answered yes. Any Board business is subject to FOIA.

Keith Jones also explained that NOP is issuing a proposal for a staff person to do administrative assistance for the NOSB. It will be a two-year contract for $20+K and will hopefully be on the street within the week.

Mr. Jones explained that the final rule was undergoing clearance at the Office of Management and Budget and is on target for publication by the year's end. Ms. Brickey asked about advance notice to the Board regarding the release, and Mr. Jones stated the Board would be briefed by a process similar to that used with the March 2000 proposal. Specifically, the rule will be sent to Board members the day before the press conference and will be on the web for pubic viewing the morning of the press conference. Mr. Jones said that Secretary Glickman sees this rule as one of his crowning achievements. Media interest is increasing.

The Final Rule becomes effective 60 days after publication if Congress does not object. Eighteen (18) months after the 60 days, the rule will be fully implemented. Betsy Lydon asked if Congressional

past and will be used in the future.
The trials carried out in the past years did not succeed in finding any substance able to control the late blowing in the cheeses. As reported by Pulay and Krasz (1967) and later by Koterbska et al. (1972), it is possible to prevent the butyric late blowing in the cheeses when the vat milk is added with lysozyme, discovered by Fleming (1922) and afterwards characterized, as egg white at a concentration of 0,1-0,2% (about 80-160 U of lysozyme/ml). These results were ratified by Aarnes (1974) who established that the addition of 1,0-1,2 g. of lysozyme/100 1 of cheesemilk (about 200-240 U of lysozyme/ml) could give good results.

The lysozyme is an enzyme present, in high concentration, in many animals secretions (tears, mucous secretion, egg white, etc.) and is important because it cleaves the glycosidic linkages of the cell wall between N-acetylmuramic acid and N-acetylglucosamine and, consequently, lyses a series of gram+ and gram- bacteria. The gram- bacteria resistance to lysozyme is owed to the presence of CA++ ions, important to stabilize the lipopolysaccharides, so that, through the removal of CA++ ions by EDTA, the activity of lysozyme on murein is restored (Davis et al., 1970).

According to Pulay and Krasz (1967), the enzyme, at a concentration of 1 µg/ml, has no activity on lactic bacteria and coliform bacteria and lyses the cell wall of Cl. butyricum at a concentration of 10 µg/ml in buffer or distilled water at 15°C. According to the results of Rotholz Books (1970), higher concentrations cannot give better results to control or to decrease the growing of clostridia in cheesemilk, whereas the lactic bacteria behaviour on the enzyme is different. According also to Kataoka and Kakae (1972), the enzyme has a different activity on B. subtilis and S. lactis, depending on the enzyme concentration and the growing temperature. According to references (Jolles, 1960) the lysozyme is stable in acidic pH and unstable in alkaline pH. The isoelectric pH is between 10,5 and 11,0. The lysozyme is totally inactivated by pepsin, photoionization, X-rays and surface active agents; it is irreversibly inactivated by iodine. Its molecular weight is between 13.000-18.000.

The results of experiments carried out in Hungary, Poland and Norway to prevent the late blowing in the cheeses clearly point out that the nitrate, usually employed in Middle Europe, can be replaced by lysozyme that is present, in large quantity, in the egg white.
The present research was undertaken upon request of the Ministry of Youth, Family and Health, and by it sponsorized, in order to establish a starting point.

comments, if any, go directly to Keith. Mr. Jones said he would find out about the protocol under The Small Business Regulatory Enforcement Fairness Act of 1996 (SBREFA). Mr. Jones said that no rule of any kind has been rejected under SBREFA.

The top two issues for NOP after final rule roll-out are program manual development and materials (substance) review. NOP will want input from certifying agents about how the rule will work on the ground and will use feedback from certifying agents and the NOSB as a way to prioritize the program manual development.

Bob Anderson asked Mr. Jones about the status of nominations for new Board members. Mr. Jones replied that the nominations are a priority for the Secretary and Administrator Merrigan but that he is not privy to the status of the selection process. Ms. Brickey stressed the need to have new members in place by the next Board meeting which will focus on implementation of the final rule.

Accreditation Committee: *Ms. Betsy Lydon, Chair*

Betsy Lydon asked the Board for confirmation that the Enforcement Task Force is heading in the right direction. She distributed the list of considerations for the memorandum of understanding (MOU)between NOP and the States, the same document as previously seen by the Board. Betsy is asking for any new comments. Two matrices, one for Crops and Handling, one for Livestock were also distributed and she asked to come back with comments by December 1. For the benefit of new members, Diane Goodman explained the relationship of the matrices to the MOU with the States.

MATERIALS PROCESS AND REVIEW - Ms. Joan Gussow, Materials Committee Chair

Periacetic Acid for Crops

Discussion: Steve Pavich explained the committee position on the TAP review and recommended approval with annotations Discussion revolved around the source of acetic acid and the production availability of fermented acetic acid, rather than synthetic acetic acid. The crops committee recommended to allow use for disinfecting equipment, seed and planting stock, and for foliar use for fireblight control. They recommended it be prohibited for soil application, due to concerns that such use is not compatible with a sustainable agricultural system and that alternatives, such as solarization do exist. Foliar use on crops was discussed, and the crops committee found that although the material is broad spectrum in effect, it is of short persistence and breaks down in the environment to water and oxygen. Its potential use as an alternative to antibiotics for control of fireblight was seen as a positive factor.

Several intermediary votes were taken on components of the annotation (vote to allow to disinfect seeds and bulbs; 8-2-4: vote to allow for fireblight control: 10-3-1.) As written, the annotation would not permit soil use.

The TAP review recommended limiting the material to sources derived from naturally fermented acetic acid sources only, however after discussion of the difficulties of identifying and finding sources produced this way, the crops committee agreed to drop this restriction. Steve Harper, processing chairperson, pointed out that the listing should be consistent for all uses (crops, processing, and livestock) and described his research into limited availability of fermented acetic acid sources. When used in processing applications, purity considerations may also limit the source. He also questioned a requirement for a natural source of one component when the material is considered

Institute of Microbiology and State Technological School
for Milk Research - Kiel - Germany

RESEARCH ON CHEESE RIPENING

5. THE USE OF LYSOZYME AS SUBSTITUTE OF NITRATE TO PREVENT THE LATE BLOWING IN CHEESE

By F. Wasserfall, E. Voss and D. Prokopek

1. INTRODUCTION

In the manufacture of cheeses, the nitrate is usually added to the cheesemilk to prevent the late blowing caused by the activity of sporogenic anaerobic bacteria.
The spores of this bacteria play an important role because, being already present in raw milk, they are not destroyed during the thermization (pasteurization), contrary to the most part of vegetative cells, and produce bacteria that affect the good ripening. This fact is considerably dangerous since even few spores present in the cheesemilk may increase so as to start the late blowing (Dorner, 1955; Bollinger, 1952).

The importance of nitrate in preventing the late blowing depends on the fact that the added nitrate is reduced to nitrite by the xanthinoxidase of milk and, consequently, the vegetative forms of the spores, responsible of the late blowing, are inhibited. Among these, the spores of Cl. tyrobutyricum are contemplated. Considering also that the nitrate can react with secondary amines, present in the cheese, producing nitrosamines that are cancerogenic substances, the possibility to avoid the addition of nitrate must be taken into consideration. To answer this question, the following possibilities were exhamined (approval of BAFM, 1968): .
1. The production of a raw milk, if possible free from sporogenic anaerobic bacteria (Wasserfall et al., 1974) and, consequently, the limitation of value of bacteriological content in the silage to hinder the growth of butyric bacteria; 2. The reduction of number of spores by using not legalized hydrogen peroxide or bactofugation; 3. The use of antibiotic nisin or nisin producing bacteria; 4. A modification of the production process through which it could be possible to prevent the increase of said spores.
The above mentioned measures, as ascertained in these tests and in others, up to the present, were not successful in preventing the late blowing and, consequently, the nitrate is still used as in the

a synthetic anyway. The board considered the overall benefits for use as a disinfectant to warrant dropping the restriction on natural sources.

A question was raised about the uses actually requested in the initial petition. The original petition was from the 1995 petition period, and was only a general request for disinfectant purposes in livestock production and handling, although the TAP review covered other uses. The board agreed with NOP staff person Richard Matthews, that as general policy, the NOSB should only be reviewing uses requested by petitioners.

VOTE: Periacetic Acid for Crops

1. Synthetic or Non-Synthetic – The Board voted unanimously that periacetic acid is synthetic.
   14 - yes, 0 – no.
2. Vote to list without annotation: **0 – yes, 14- no**
3. Vote to list with the following annotation: **13 – yes – 0 no, 1 – abstain**

"Allowed to disinfect equipment. Allowed to disinfect seed and asexually propagated planting material (i.e., bulb, corm, tuber) used for planting crops. Allowed for fireblight control only with Experimental Use permit with documentation that alternatives including biocontrols have been tried."

Periacetic Acid for Livestock

Discussion: The board discussed a possible additional allowance for veterinary use, but declined to include that in the annotation, due to lack of established need or direct request from a petitioner.

VOTE: Periacetic Acid for Livestock

1. It was approved as a synthetic: **14-0-0**
2. Approved with the following annotation: **13-0-1**

   "For facility and processing equipment sanitation (barns, milking parlors, processing areas)."

Periacetic Acid for Processing

Discussion: There was discussion about approving materials with an annotation and the implication that certain uses may be prohibited if not specified, which is not entirely correct. Becky Goldberg recommended that prohibitions should be clearly communicated. The TAP review mentioned other uses for peeling and bleaching, these were not recommended by the committee. Also the Board discussed how to communicate changes in annotations that may arise with new petitions for a material already approved. A question was raised as to how to provide information about considered and rejected uses, to discourage people from re-petitioning for uses that have already been rejected.

VOTE: Periacetic Acid for Processing

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56 Late blowing (prevention by the use of Lysozyme)

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1. It was approved as a synthetic: **14-0-0**
2. Vote to list without annotation: **0 – yes, 14- no**
3. Approved with annotation of "Allowed for direct food contact only in wash and/or rinse water. Allowed as a sanitizer on surfaces in contact with organic food." **14-0-0**

Calcium Borogluconate for Livestock

Discussion: The livestock committee recommended allowing and stressed that it is an emergency use treatment that should be needed only on rare occasions. The TAP review suggested language about preventive measures in the annotation, but this was deemed vague and also covered in the proposed rule language that requires preventive practices before medications are used. Dietary adjustment can be made over time to prevent milk fever. The TAP review mentioned use for grass tetany also, but the board declined to allow for that use without more information or a specific request. A suggestion to require a 48-hour withdrawal requirement, as is required by Codex rules for all medications was not supported. Milk is typically not sold for at least 24 hours after parturition, and will contain mostly colostrum for the first 48 hours.

VOTE: Calcium Borogluconate

1. Approved as a synthetic: **14/0/0**
2. Vote to list without annotation: **0 – yes, 14- no**
3. Approved with annotation of "For treatment of milk fever only." **14-0-0**

Sodium Chlorate for Crops - Steve Pavich

Discussion: This material was petitioned for use as a cotton defoliant. Kyle Moppert pointed out that currently most organic cotton is coming from dryland production areas due to reduced insect pressure. Eventually more cotton will be grown in lowland areas or areas on the margin of adaptability and defoliants will be more of an issue in those regions. The board discussed the fact that alternatives seem to be available, and that the existing organic cotton industry is managing without this material.

VOTE: Sodium Chlorate for Crops

1. Vote to consider the material synthetic: **14 – yes, 0 – no**
2. Prohibited Synthetic. **14-0-0**

Leather Meal for Crops -Steve Pavich

Discussion: The crops committee recommended the material be considered synthetic and prohibited. The synthetic determination was made based on the numerous additives introduced through the leather making process. The Board agreed that there are many natural alternatives for fertilizer use that are readily available, and asked what uses the material currently has. The petitioner indicated that leather meal is currently applied to conventional tobacco, citrus, and orchard crops. Willie Lockeretz noted that the record should be clear, that the NOSB rejected the material based on the facts that it is

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RESEARCH ON CHEESEMAKING

5. THE USE OF LYSOZYME AS SUBSTITUTE OF NITRATE TO PREVENT THE LATE BLOWING IN CHEESE

Kieler Milchwirtschaftliche Forschungsberichte 28, 3-16 (1976)

Sonderdruck aus Heft 1 / 28. Band 1976 . Verlag Th. Mann
Hildesheim

synthetic and it has no specific exemption in OFPA.

VOTE: Leather Meal for Crops

1. Vote to consider the material synthetic: **13 – yes; 0 – no. (1 absent)**
2. Vote to add to National List: **0 – yes, 13 – no**

Animal Enzymes for Processing -Steve Harper

There was discussion about which enzymes are currently in use by the industry. The TAP review was presented as a group review of animal enzymes, using animal-derived rennet as the model, and included additional information on six other enzymes. The processing committee considered proposed annotations from the TAP review that restrict incidental additives and preservatives used in enzyme preparations. They noted that powdered forms are preferable, though not always available. Liquid formulations may have sodium benzoate added. The processing committee did not support a requirement for GRAS status. The representative present from the enzyme association explained GRAS as frequently self-imposed, not always FDA- approved with published regulation. FDA has never taken action against enzymes used in the market that claim GRAS and are not regulated by the FDA. It is difficult to determine whether a material is synthetic or natural, depending on the presence of synthetic additives. A request for GRAS status for lysozyme was filed in 1973. The FDA published a *Federal Register* notice in 1998 proposing to affirm it as GRAS. Given the change in policy, the determination is not expected to be granted any time soon. Joan Gussow questioned whether determination of freedom from BSE can readily be determined.

The board decided to list 6 specific animal enzymes as allowed, without annotation. They did not include a listing for lysozyme, which does not have a final GRAS status from FDA. Discussion ended with the fact that the NOSB is voting on the enzymes, not on the additives.

VOTE: Animal Enzymes

1. Vote to consider the material synthetic: **0 – yes; 12 – no. (2 absent)**
2. Vote to list the following materials without annotations and without Lysosyme. **10-yes; 2 – no, 1- abstain (1 absent)**

   Rennet (animal derived); catalase--bovine liver; animal lipase; pancreatin; pepsin; trypsin.

Recessed for the day.

FRIDAY, NOVEMBER 17, 2000

MEETING RESUMED 9:00 a.m. – Carolyn Brickey, Chairperson

Aquatic Task Force Working Group Reports: *Mr. Robert Anderson, Task Force Chair*

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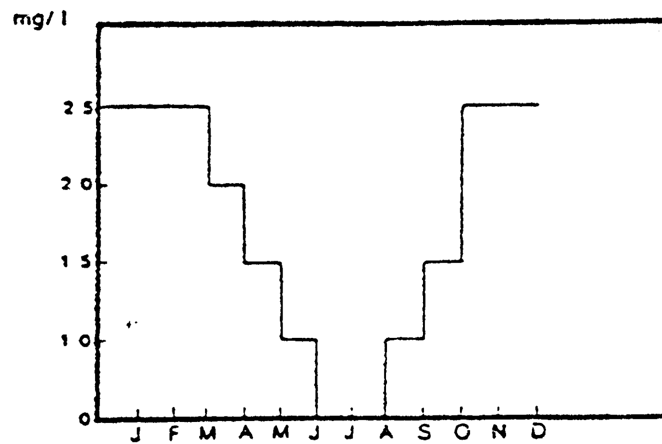
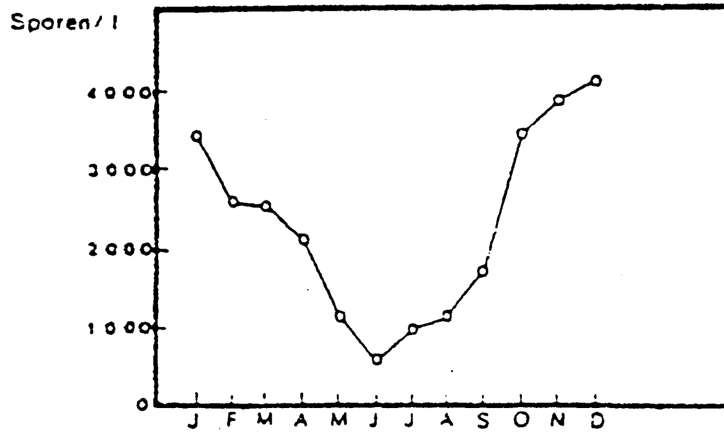
APPENDIX H

Olli Moisio/rt

September 1, 1986

FIGURES: Examples to dose AFILACT, considering the count of spores in cheese milk during the year. (N. Koch, Boehringer Ingelheim KG. in the seminar; Lysozyme in Cheese Manufacture, Kiel January 1986)





Mr. Anderson presented an overview of the intention and structure of the Aquatic Task Force and Working Group. The Task Force is made up of an Aquaculture Working Group and a Wild Aquatic Species Working Group. The chair of each group gave a working report to the NOSB.

Aquaculture Working Group -Ms. Margaret Wittenberg, Chair

Ms. Wittenberg explained that the premise for the working group is to determine the feasibility of establishing organic standards. Margaret addressed the issue of fish meal and fish oils as feedstocks for fish as necessarily organically produced. This led to the question of fish as free-ranging and its comparison to poultry on "free range" which is actually "free range" within a confined area. **(See attachment 10)**

In summary, the majority position of the Working Group is that possibilities exist for organic certification of some aquaculture systems.

Wild Aquatic Species Group -Mr. Miles McEvoy, Chair

Mr. McEvoy summarized the opinion positions of the members of the Working Group regarding the certification of wild fish. **(See attachment 11)**

Summary points:

Wild organic is neither a "no-brainer" nor is it an impossibility. The working group supports labeling to distinguish good stewardship of aquatic species, organic may not be the appropriate label.

Keith Jones made the statement, to provide guidance to the Board, in his own opinion, that there is probably more knowledge about where wild fish go than there is about where cattle graze on rangeland. He praised the progress Miles and Margaret have made in this effort and how they have provided the Board with information they will need to move forward.

Carolyn Brickey and Bob Anderson explained that today's goal was to lay out the parallels and comparisons to help the Board approach this issue, not reach any conclusions.

COMMITTEE ACTION ITEMS – *Carolyn Brickey, Chairperson*

MOTION: Motion by Eric Sideman. The NOSB recommended that the annotation for Ivermectin be amended to prohibit the slow release formulation known as the SR bolus. In addition we request that information continue to be gathered in order to determine if other formulations are a significant risk to decomposition of manure. Marvin Hollen seconded.

REVISED MOTION: The NOSB recommended that the annotation for Ivermectin be amended to prohibit the slow release formulations such as the SR bolus.

The Board discussed whether the annotation would prohibit slow release formulations or to prohibit other formulations. Keith suggests that more data be obtained before the Board makes this decision. Mr. Sideman asked Mr. Jones if this recommendation could make it into the rule. Mr. Jones said that it was not possible now. This raised the question about how changes will be made after the rule is final. Keith responded that the Board will be acting on materials during implementation, so that when the rule is fully implemented, the changes will be reflected. Keith says that any change to the final rule has to go out for public comment, so it is better to raise these issues early in the process. He wants to go through the correction process only once. Kim Burton asked when the National List will be reprinted. **Keith Jones wants to talk about this issue in March. The Board needs to act on materials as quickly as possible to get it incorporated into the rule within 18 months.**

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Olli Moisio/rt

APPENDIX G

September 1, 1986

CHEESE PREFERENCES AND TRENDS IN THE UNITED STATES

Variety of Cheese	Late 1960s % of All Cheese	Early 1980s % of All Cheese
American cheese	45.6	45.2
Cottage cheese	30.9	23.5
Italian varieties	11.2	18.3
Swiss cheese	4.6	4.7
Cream and Neufchâtel	4.3	4.4
Münster cheese	1.0	1.4
Brick	0.8	0.5
Blue cheese	0.7	0.8
All other varieties	0.9	1.2
Total production/year		
Billion pounds	2.8	3.7
Billion kilograms	1.3	1.7

Source of basic information: U.S. Department of Agriculture

The Ivermectin motion passed unanimously (12/0/0). (Mark King and Joan Gussow absent)

MOTION: Motion by Eric Sideman. The NOSB recommends that unless otherwise specified in the annotation any substance on the National List of non-agricultural substances allowed as ingredients in an organic processed food product also be allowed for use in organic animal feed, provided it is regulated in 21 CFR for livestock feed or allowed by FDA with discretion to AAFCO. Betsy Lydon seconded.

Discussion: A number of items on the processing list are used in ingredients for feed additives and have been permitted by the board for human food use, such as citric acid, kelp, kaolin, ascorbic acid, tocopherols, glycerin, lecithin, and potassium carbonate. Their status is not clear under the current livestock regulations, which requires that all synthetics appear on the National list. This motion will save the need to re-petition and re-consider these items individually.

REVISED MOTION: The NOSB recommends that unless otherwise specified in the annotation, any substance on the National List of non-agricultural substances allowed as ingredients in an organic processed food product also be allowed for use in organic animal feed, provided it is approved by FDA in 21 CFR for livestock feed or allowed by FDA discretion as stated by AAFCO. Passed unanimously (11/0/0).

MOTION: Motion by Eric Sideman. Mr. Sideman moved that the minutes from the June 6-7, 2000 be approved as amended. Kim Burton seconded. The motion passed unanimously. (11/0/0).

BREAK FOR LUNCH

Materials Committee Work Plan – *Ms. Kim Burton, Chair*

Kim Burton reviewed the Petition Review Process timeline based on recommendations of the Board that would take 120-150 days from receipt of petition to approval. The Chair suggested that we work backwards, from March approvals back to petition to OMRI. OMRI staff said OMRI needs three months for TAP reviews. If petitions are received by December 1 by NOP they can go to OMRI by December 15. TAP reviews will be open for public comments for 15 days prior to NOSB meetings.

Keith Jones reminded everybody that these contract funds need to be spent which would require the review of 50 materials by October, the end of fiscal year. Keith Jones and Katherine DiMatteo met about three weeks prior to the Board meeting, and he urged OTA to ask the industry to send him a list of material petitions he should be expecting. He has received an indication that about 40 petitions would be sent to NOP. Ms. Brickey asked about a date for OMRI to receive petitions for review in June. Emily Brown Rosen responded with a deadline of February 1, 2001.

Ms. Burton asked about the flow of petitions and requests for comments. Keith wants comments sent to NOP. Dates for petition process and public comment are noted on the chart handed out to the Board.

Livestock Committee Work Plan – *Mr. Eric Siedman, Chair*

Issues identified by Livestock Committee to be addressed in the coming year included:

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APPENDIX F

Olli Moisio/rt

September 1, 1986

TABLE: PRODUCTION OF HARD AND SEMIHARD CHEESES IN CERTAIN COUNTRIES AND ESTIMATES FOR CONSUMER CUTS AND GRATED CHEESE CONSUMED BASED ON FINNISH PERCENTAGES
(Source for production figures: International Dairy Federation, Interim Cheese Market Report, Bulletin No. 175, 1984)

	Produced hard & semihard Cheese '000 tons
Scan	293
Irl	54
UK	224
NL	471
B-LUX	30
FRG	336
F	437
I	500 *
CAN	125 **
USA	1883
TOTAL	4353

* Based on approx. out of total cheese production 625000 tons/year
** Based on approx. out of total cheese production 200000 tons/year

Cost of use — Nitrates to prevent late blowing

Potassium nitrate (KNO_3)

Price in Finland, July 1986 — 5,70 FIM/kg, VAT excluded

Dosage in Finland — 10-15 %/100 l Edam cheese milk

Dosage — 15 g/100 l = 1,62 kg nitrate/1000 kg Edam cheese
(10,8 l milk/1 kg cheese)
1,62 x 5,70 = 9,23 FIM/1000 kg Edam cheese, VAT excluded

Roughly — 0,0Ø1 FIM/kg cheese

Lysozyme, price — 750 FIM/kg

Cost per kg cheese — 750,00 x 0,216 kg/1000 kg cheese = 162,00 FIM/1000 kg cheese

Dosage — 20 ppm in milk = 216 ppm in cheese

Roughly — 0,16 FIM/1000 kg cheese

After Hephner & Assoc. assumed reduction in price (100 %)

= cost of use 0,008 FIM/1000 kg cheese

- Young animal care
- Nutrient management
- Living conditions and stocking rates
- GMO incidentals including compost ingredients, manure from GMO livestock
- Feed additives regarding 100% organic feed, needs to be clarified to determine 5% rule
- Pasture-based living conditions for ruminant animals is still the firm position of the committee but due to the controversial nature of the issue, what pasture-based means needs to be defined.

Emily Brown-Rosen noted the need to address the issue of allowing vitamins as feed additives for livestock, depending on FDA approval by regulation or allowance with discretion for materials listed by AAFCO.

Keith Jones noted that when the final rule comes out, this question may not be an issue, based on any new issues that will be need to be addressed. Many questions and issues may be answered in the Final Rule.

Carolyn Brickey wants to have the work plan for the year posted to the web for industry comment and to provide material for the Board retreat.

Processing Committee Work Plan – *Mr. Steve Harper, Incoming Chair*

The processing committee is waiting for the final rule to determine which parts of the rule will be compatible with processing practices. The committee is also going to be focusing on retailer education and on transitional standards. Steve Harper is waiting for the final rule to formalize the committee work plan.

Accreditation Committee Work Plan – *Mr. Willie Lockeretz, Incoming Chair*

Willie Lockeretz outlined the following issues for attention by the committee this year:

- Continued development on the Enforcement Task Force matrices.
- MOUs with States need to be developed for enforcement
- Peer Review participation with the Department.
- Development of an equitable fee structure especially for small operations.
- Certifier concerns about their role in the politics in accreditation and certification -- more about how they feel rather than about how it will be done.

Tom Hutcheson announced that OCC is hosting an Accreditation Training program for certifiers in February 2, 3, Friday and Saturday. Mark Bradley from the FSIS collaborated with OTA and IOIA in developing the program.

During the final public comment Bob Shine spoke as a small certifier about the issue of the shakeout of small certifiers when the program is implemented and the reaction to the unknown.

Crops Committee Work Plan – *Mr. Owusu Bandele, Incoming Chair*

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APPENDIX E

Olli Moisio/rt

September 1, 1986

TABLE: THE STABILITY OF LYSOZYME AGAINST MECHANICAL STRESS (Wasserfall, 1977. Milchwissenschaft 32 (11)).

Einwirkungsdauer (Minuten)	Prozentuale Aktivität bei Behandlung mit Ultra-Turrax	Ultraschall
0	100	100
1	98,2	58,2
2	99.0	43,6
4	97,8	42,3
8	97,4	38,4

Treatment in 0 °C (Icewater)
Initial lysozyme conc.: approx. 500 E/ml in lysozyme buffer, pH 6,2

Steve Pavich stated the Crops Committee should discuss:

- Compost
- Manure
- GMO
- Transitional Certification

WRAP-UP/NEXT MEETING PLANS – *Ms. Carolyn Brickey, Chairperson*

Ms. Brickey wants this work plan consolidated in a couple of weeks and up on the web.

Travel days for the next NOSB Meeting will be Sunday, March 4, 2001, with a NOSB Retreat on Monday, March 5th, and the NOSB Meeting on Tuesday and Wednesday March 6 and 7. The first day of Expo West is Thursday, March 8th.

Ms. Brickey would like to brief Board members on Conflict of Interest at the retreat.

The June NOSB meeting dates are June 5 – 7, 2001. Suggested locations for the next meeting included La Cross or Madison, WI or the Minnesota area.

The meeting was adjourned at 2:40 p.m.

CAROLYN BRICKEY, Chair

National Organic Standards Board

KEITH JONES, Program Manager

National Organic Program

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APPENDIX D

Olli Moisio/rt

September 1, 1986

TABLE: THE DECREASE IN ACTIVITY OF LYSOZYME IN DIFFERENT PH-LEVELS OF CULTURED MILK
(Wasserfall, 1977. Milchwissenschaft 32 (11))

pH	Einwirkungszeit 1 Std.	2 Std.	6 Std.	24 Std.
4,8[2])	0[3])	0	0	0
5,7	0	0	0	0
6,5	0	0	0	0
7,7	0	0	0	27,0
8,4	0	0	5,6	31,9
9,3	0	7,0	13,3	46,8
10,4	15,9	19,1	22,9	45,2

1) Lysozyme from Roth Co. (Initial conc. 32,1 $^{E}/ml$)

2) pH-value after filling with distilled water and enzyme addition

3) Decrease of activity (%) lounted from the initial activity

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APPENDIX C

Olli Moisio/rt

September 1, 1986

Table 1. Gennemsnit af nogle bedømmelser af Danbo fremstillet af mælk med tilsætning af sporer af Cl. tyrobutyricum.
Average scoring from some gradings of Danbo-cheese manufactured from milk with addition of spores from Clostridium tyrobutyricum.

Tilsætning *Addition*	8 uger *8 weeks* (8 weeks)				16 uger *16 weeks* (16 weeks)			
	Bygning *Texture* (Texture)	Konsistens *Body* (Body)	Lugt og smag *Flavour and taste*	Hovedkarakter *Main score* (Main score)	Bygning *Texture*	Konsistens *Body*	Lugt og smag *Flavour and taste*	Hovedkarakter *Main score* (Main score)
0	5.0 stornullet kruipibet *yeast and united yeast holes*	10.5	8.2 afsmag *off taste*	6.8	4,0 pustet, sprængt *big yeast holes*	9,6 kort, klæg *short, salty*	8,2 afsmag *off taste*	6.3
10 g KNO₃	9.2 kruipibet *united yeast holes*	10.6	9.8 afsmag *off taste*	9.8	9.4 krølpibet *united yeast holes*	11,0	11,0	10,0
1 g lys.	9.8 krølpibet *united yeast holes*	11.5	10.8	10.2	9.7 krulpibet *united yeast holes*	11,0	11,6	10.7
2 g lys.	10.0	10.6	9.8 afsmag *off taste*	10.0	10.2	10,8	11,1	10.6
3 g lys	10.0	9.5 klæg *salty*	9.0 afsmag *off taste*	9.5	8.9 krølpibet *united yeast holes*	10,5	10,5	10.0

Table 2. Gennemsnit af nogle bedømmelser af Danbo fremstillet af almindelig ostemælk.
Average scoring from some gradings of Danbo-cheese manufactured from bulk milk.

Tilsætning *Addition*	8 uger *8 weeks*				16 uger *16 weeks*			
	Bygning *Texture*	Konsistens *Body*	Lugt og smag *Flavour and taste*	Hovedkarakter *Main score*	Bygning *Texture*	Konsistens *Body*	Lugt og smag *Flavour and taste*	Hovedkarakter *Main score*
10 g KNO₃	8,0 krølpibet *united yeast holes*	11,0	11.5	10,0	8,6 krølpibet *united yeast holes*	11,2	11,1	9,8
1 g lys.	10,2	11,6	11,8	11,0	10.7	11,3	11,2	10,7
2 g lys.	10,3	11,2	11,8	10,9	10.4	11,3	11,6	11,0

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subject to the requirements of this AD. For helicopters that have been modified, altered, or repaired so that the performance of the requirements of this AD is affected, the owner/operator must use the authority provided in paragraph (c) to request approval from the FAA. This approval may address either no action, if the current configuration eliminates the unsafe condition, or different actions necessary to address the unsafe condition described in this AD. Such a request should include an assessment of the effect of the changed configuration on the unsafe condition addressed by this AD. In no case does the presence of any modification, alteration, or repair remove any helicopter from the applicability of this AD.

Compliance: Required as indicated, unless accomplished previously.

To detect cracks that could lead to delamination of the tail rotor blade Kevlar tie-bar (Kevlar tie-bar), loss of tail rotor control, and subsequent loss of control of the helicopter, accomplish the following:

(a) Within 10 hours time-in-service (TIS) after the effective date of this AD, and thereafter at intervals not to exceed 250 hours TIS, inspect each Kevlar tie-bar for a crack or delamination in accordance with paragraph B, Operational Procedure, of Eurocopter France Service Bulletin 05.00.34, Revision 3, dated November 14, 1996.

(b) If any delamination or cracking is found during any of the inspections required by paragraph (a) of this AD, remove the blade and replace it with an airworthy blade before further flight.

(c) An alternative method of compliance or adjustment of the compliance time that provides an acceptable level of safety may be used if approved by the Manager, Rotorcraft Standards Staff, Rotorcraft Directorate, FAA. Operators shall submit their requests through an FAA Principal Maintenance Inspector, who may concur or comment and then send it to the Manager, Rotorcraft Standards Staff.

Note 2: Information concerning the existence of approved alternative methods of compliance with this AD, if any, may be obtained from the Rotorcraft Standards Staff.

(d) Special flight permits may be issued in accordance with sections 21.197 and 21.199 of the Federal Aviation Regulations (14 CFR 21.197 and 21.199) to operate the helicopter to a location where the requirements of this AD can be accomplished.

Note 3: The subject of this AD is addressed in Direction Generale De L'Aviation Civile (France) AD 92-185-33(B)R4 dated December 4, 1996.

Issued in Fort Worth, Texas, on February 28, 1998.

Eric Bries,

Acting Manager, Rotorcraft Directorate, Aircraft Certification Service.

[FR Doc. 98-6496 Filed 3-12-98; 8:45 am]

BILLING CODE 4910-13-M

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

21 CFR Part 184

[Docket No. 89G-0393]

Direct Food Substances Affirmed as Generally Recognized as Safe; Egg White Lysozyme

AGENCY: Food and Drug Administration, HHS.

ACTION: Tentative final rule.

SUMMARY: The Food and Drug Administration (FDA) is issuing a tentative final rule to amend its regulations to affirm that egg white lysozyme enzyme preparation, when labeled by the common or usual name "egg white lysozyme" to identify its source, is generally recognized as safe (GRAS) for use in preventing late blowing of cheese caused by the bacterium *Clostridium tyrobutyricum* during cheese production. This action is in response to a petition submitted by Fordras S.A. (formerly SPA-Società Prodotti Antibiotici S.p.A.). FDA has tentatively concluded that this use of the egg white lysozyme enzyme preparation is GRAS only when the ingredient statement for both bulk and packaged food that contains cheese manufactured using egg white lysozyme includes the common or usual name "egg white lysozyme" to identify the source of the protein. To give interested persons an opportunity to comment on this condition of use required for GRAS status, FDA is issuing this tentative final rule.

DATES: Submit written comments by May 27, 1998.

ADDRESSES: Submit written comments to the Dockets Management Branch (HFA-305), Food and Drug Administration, 12420 Parklawn Dr., rm. 1-23, Rockville, MD 20857.

FOR FURTHER INFORMATION CONTACT: Linda S. Kahl, Center for Food Safety and Applied Nutrition (HFS-206), Food and Drug Administration, 200 C St. SW., Washington, DC 20204, 202-418-3101.

SUPPLEMENTARY INFORMATION:

I. Background

In accordance with the procedures described in § 170.35 (21 CFR 170.35), SPA-Societa Prodotti Antibiotici S.p.A., now Fordras S.A., Milan, Italy, submitted a petition (GRASP 9G0355) requesting that egg white lysozyme used to inhibit the bacterium *C. tyrobutyricum* to prevent late blowing of cheese during production be affirmed as GRAS as a direct human food ingredient. FDA published the notice of filing for this petition in the **Federal Register** of October 27, 1989 (54 FR 43861), and gave interested persons until December 26, 1989, to submit written comments.

II. Standards for GRAS Affirmation

Under § 170.30 (21 CFR 170.30), general recognition of safety may be based only on the views of experts qualified by scientific training and experience to evaluate the safety of substances directly or indirectly added to food. The basis of such views may be either: (1) Scientific procedures, or (2) in the case of a substance used in food prior to January 1, 1958, through experience based on common use in food. General recognition of safety based upon scientific procedures requires the same quantity and quality of scientific evidence as is required to obtain approval of a food additive regulation and ordinarily is based upon published studies, which may be corroborated by unpublished studies and other data and information (§ 170.30(b)). General recognition of safety through experience based on common use in food prior to January 1, 1958, may be determined without the quantity or quality of scientific procedures required for approval of a food additive regulation, but ordinarily is based upon generally available data and information concerning the pre-1958 history of use of the substance.

FDA has evaluated Fordras S.A.'s petition on the basis of scientific procedures to whether the petitioned use of egg white lysozyme enzyme preparation to prevent the late blowing of cheese caused by the bacterium *C. tyrobutyricum* during cheese production is GRAS. In evaluating the petition, FDA considered published and unpublished data and information relating to the identity of, characteristic properties of, and estimated dietary exposure to the enzyme component (i.e., lysozyme) of the petitioned enzyme preparation (Refs. 1 through 7). FDA also considered that the source of the petitioned enzyme preparation, egg white, has been safely consumed by humans as a source of food protein throughout recorded history, and, therefore, is GRAS (§ 170.30(d)), and that the methods used for extracting lysozyme from the egg white source do not ordinarily alter the chemical identity and characteristic properties of enzymes (Ref. 8). FDA also considered published scientific review articles (Refs. 1 and 2) and a generally available trade association bulletin (Ref. 7) discussing the use of egg white lysozyme enzyme preparation for its

FINNISH SUGAR CO. LTD. APPENDIX B
Speciality Biochemicals

Olli Moisio/rt September 1, 1986

TABLE: HARD AND SEMIHARD CHEESE VARIETIES, WHERE LYSOZYME COULD BE USED TO PREVENT LATE BLOWING (Note: restrictions by legislation in each country in question!)

Cheese variety	Valio brand name	Country of origin
HARD CHEESE (moisture 34 %)		
Appenzeller		CH
Asiago (old)		US
Parmesan (Grana)	Parmesan	I
Romano		I
Sapsago		US/I
Spalen		I
Caciocavallo		I
Emmental/Swiss	Emmental	CH
Cryere	Cryere	CH
Ricotta (old)		I
SEMIHARD (moisture 34-55 %)		
Edam/Gouda	Edam/Salaneuvos Gouda	NL
Mozzarella	Mozzarella	I
Port Salut *	Luostari	F
Provolone		I
Tilsit	Kesti/Kreivi	FRG
Trappist *		CH/HUN
Svecia		S
Herrgårds ost		S
Danbo	Edam	DK

technical effect of preventing late blowing of cheese contaminated with *C. tyrobutyricum* as well as generally available information documenting that this intended use of the petitioned enzyme preparation has been approved in several European countries (Refs. 9 through 13). Finally, FDA considered generally available and accepted information relating to processing aids used in the manufacture of the enzyme preparation and generally available and accepted specifications for food-grade enzyme preparations (Ref. 14).

III. Safety Evaluation

When present as a contaminant in milk used for cheesemaking, the pasteurization-resistant bacterium *C. tyrobutyricum* ferments lactate to produce carbon dioxide, hydrogen, and volatile organic acids. This fermentation causes a defect in cheese manufacture known as "late blowing," which is typified by abnormal levels of open texture accompanied by undesirable odors and flavors. Late blowing can be a serious economic problem in the manufacture of several varieties of cheese (Refs. 1, 2, and 7).

The contamination by *C. tyrobutyricum* of milk used for cheesemaking, although reducible by good husbandry and hygienic milking practices, is unavoidable. Although treatment with certain chemical agents has been shown to be effective against the problems raised by this contamination, treatment with lysozyme enzyme preparation has been found to be the most effective method of managing the late blowing of cheese contaminated with *C. tyrobutyricum* (Refs. 1 and 2).

A. The Enzyme Component

Enzymes are proteins or conjugated proteins (i.e., a protein that contains a nonamino acid moiety such as a carbohydrate) produced by plants, animals, and microorganisms that function as biochemical catalysts (American Heritage Dictionary of the English Language). Most enzymes are very specific in their ability to catalyze only certain chemical reactions; this high degree of specificity and strong catalytic activity are the most important functional properties of enzymes (Ref. 15).

The Commission on Enzymes of the International Union of Biochemistry has devised a systematic strategy for naming enzymes. This system combines a naming system and a numbering system. For most enzymes, the systematic name is derived from the names of the substrate, product, and type of reaction. The systematic number is based on the class and subclasses to which the enzyme belongs. The systematic name of lysozyme is peptidoglycan *N*-acetylmuramoylhydrolase. Its systematic number is EC No. 3.2.1.17 and its Chemical Abstracts Service Registry Number (CAS Reg. No.) is 9001-63-2.

Lysozyme was first discovered by A. Fleming, who identified lysozyme as an antibacterial enzyme present in nasal mucus membrane (Ref. 3). Subsequently, it was learned that the antibacterial activity of lysozyme occurs because of its ability to catalyze the hydrolysis of the structural polysaccharide peptidoglycan present in cell walls of certain bacteria (Ref. 2). Lysozyme activity has been shown to be present in bacteria, fungi, plants, and almost all animal tissues, with the highest levels found in secretions (including milk, mucus, saliva, and tears) and eggs. Lysozyme is believed to function in all of these organisms and tissues as an endogenous antimicrobial substance (Refs. 1 and 2).

Lysozyme was the first enzyme to have the details of its three-dimensional structure published (Ref. 4), and it has become one of the best characterized of all enzymes, serving as an example for studies of enzyme mechanism and molecular evolution (Refs. 5 and 6). Lysozymes from various organisms are very similar to one another. Egg white lysozyme differs very little in structure, amino acid sequence and composition, catalytic mechanism, and substrate specificity from the enzyme found in human milk, saliva, mucus, and tears (Refs. 3 and 6).

The petitioner provided two published scientific review articles (Refs. 1 and 2) that discuss the use of egg white lysozyme in cheese and other food. The petitioner also provided a generally available trade association bulletin (Ref. 7) that focuses on the use of egg white lysozyme for its technical effect of preventing late blowing in cheese. This bulletin describes the late blowing defect and how it arises, traditional chemical control measures (other than the use of lysozyme) to reduce the problem, and the increasing interest in using lysozyme as a replacement for traditional chemical control measures. In addition, the petitioner provided generally available information documenting that this intended use of the petitioned enzyme preparation has been approved in several countries, including Denmark, France, Germany, Italy, and Spain (Refs. 9 through 13).

FDA considered the estimated dietary exposure to lysozyme for the proposed use in cheese (Refs. 16 and 17). Lysozyme accounts for approximately 3.5 percent of the total protein of domestic hen egg whites (Ref. 7). Whole eggs contain lysozyme at a level of approximately 3,300 parts per million (ppm). The petitioner reported that cheese manufactured using egg white lysozyme enzyme preparation contains a maximum of 400 ppm of lysozyme, or at least 8 times less than eggs on a weight basis. FDA has estimated a long-term mean intake of lysozyme to be 74 milligrams per person per day (mg/p/d) for consumers of eggs and 3.8 mg/p/d for consumers of cheese; the respective 90th percentile intakes are estimated to be 163 mg/p/day and 8.1 mg/p/day. Egg whites from which lysozyme is extracted will be subsequently consumed in other food uses. Thus, there will be no long-term net increase in lysozyme intake by the general population because egg whites without lysozyme will replace egg whites in current use that contain lysozyme (Ref. 16). On a per eating occasion basis, lysozyme intake for cheese consumers may be 16 mg on average, or 22 mg at the 90th percentile level. For comparison, a per eating occasion lysozyme intake for egg consumers may be 264 mg on average, or 416 mg at the 90th percentile level. Thus, lysozyme intake per eating occasion due to cheese consumption may constitute 5 to 6 percent of lysozyme intake due to egg consumption (Ref. 17).

In general, issues relevant to a safety evaluation of proteins such as the enzyme component of an enzyme preparation are potential toxicity and allergenicity (Ref. 18). Proteins derived from egg whites do not raise toxicity concerns because egg whites have been safely consumed by humans as a source of food throughout recorded history without any reports of toxicity. However, proteins derived from egg whites do raise allergenicity concerns because, as with many common foods, there have been reports that consumption of egg whites can cause an allergic reaction in certain individuals, particularly children (Ref. 19). Therefore, FDA considered the question of whether the lysozyme component of egg whites is allergenic.

In evaluating this question, FDA considered a report of an in vitro study of the binding of antibodies to specific egg proteins, where the antibodies were derived from the serum of patients known to be allergic to eggs (Ref. 20). This report suggests that lysozyme was an allergen for some individuals who became sensitive to egg whites. Although this study does not establish that ingestion of egg white lysozyme in cheese will actually cause a clinically

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APPENDIX A

September 1, 1986

TABLE: LYSOZYME EXPERIMENTALS/CHEESE VARIETIES IN CERTAIN EUROPEAN COUNTRIES

Country	Cheese used in trials	Lysozyme dosage U/ml cheese milk
Finland	Edam, Emmental	200-500
Sweden	Edam	?
Norway	Jarlsberg (= Emmental)	
Denmark	Danbo (= Edam)	500
France	Emmental	
West-Germany	Edam, Tilsit, Gouda	500
Italy	* Granapadano, Provolone, Asiago, Montasio	
Hungary	Emmental, Trappist	

At the moment lysozyme is permitted legislatively for cheese in West-Germany (hard and semihard), Belgium, Netherlands, France, Italy and Denmark.

* Granapadano = Grana = Parmesan

significant allergic reaction in such sensitive individuals, FDA is not aware of any data or information that would refute the study's inference that egg white lysozyme may be allergenic. Accordingly, FDA is proposing labeling, as discussed below, to alert the sensitive population to the presence of egg white lysozyme in cheese.

A related question is whether egg white lysozyme, when present in cheese, is capable of inducing an allergenic response in susceptible individuals who have not previously consumed egg whites, e.g., because their customary diet excludes eggs. This question is no different than for any other food containing egg white when consumed by individuals with unknown susceptibility to eggs. The proposed label declaration would provide such individuals with the same protection as that provided by other egg-containing products with ingredient labeling. Thus, individuals who experience an allergic reaction to lysozyme-containing cheese could identify egg white lysozyme as a possible cause of the reaction.

B. Enzyme Source, Manufacturing Methods, and Processing Aids

Commercial preparations of lysozyme are derived from domestic hen egg whites using ion exchange methods and selective precipitation to isolate a highly purified protein fraction that contains mainly lysozyme but also may contain small amounts of other egg white proteins. Consistent with the agency's finding in its GRAS affirmation of microparticulated protein product (55 FR 6384, February 23, 1990), FDA finds that egg whites have been safely consumed by humans throughout recorded history and, therefore, are GRAS (§ 170.30(d)). The agency evaluated the methods used to isolate the enzyme lysozyme from egg whites. These methods are based on generally available and accepted principles of protein purification (Ref. 8). Such methods, if appropriately selected, do not ordinarily alter the chemical identity and characteristic properties of enzymes. Therefore, these methods do not materially change the quality, utility, functionality, or safety of enzymes. Moreover, the retention of the antibacterial activity that is characteristic of egg white lysozyme when egg white-derived lysozyme enzyme preparation is used in cheese evidences that lysozyme in the manufactured enzyme preparation remains unaltered from the lysozyme in egg whites. This is corroborative evidence of the fact that the methods used to isolate lysozyme from egg whites do not materially change the quality, utility, functionality or safety of the enzyme lysozyme.

Enzyme preparations used in food processing are usually not chemically pure but contain, in addition to the enzyme component, materials that derive from the enzyme source. As mentioned above, egg white lysozyme enzyme preparation may contain small amounts of other egg white proteins. A related question is whether such proteins that may be present in the enzyme preparation are allergenic. Even if present, other source-derived proteins would not be a concern because the proposed label declaration for egg white lysozyme would alert individuals who are sensitive to egg whites to the possible presence of other proteins derived from egg whites.

In addition to source-derived materials, enzyme preparations used in food processing usually contain materials that derive from the manufacturing methods used to generate the finished enzyme preparation. The egg white lysozyme enzyme preparation that is the subject of this document complies with the general requirements and additional requirements for enzyme preparations in the Food Chemicals Codex, 4th ed. (Ref. 14). The egg white lysozyme enzyme preparation that is the subject of this document may contain substances that are added to the enzyme preparation, such as preservatives, stabilizers or diluents, and trace amounts of processing aids that are used in its preparation. These substances must be acceptable for general use in foods (Refs. 14 and 15).

C. Labeling as a Condition of Use

Egg whites are known to be an allergenic food source, particularly in children (Ref. 19). There is a literature report (Ref. 20) indicating that lysozyme may in fact have been an allergen for some individuals who became sensitive to egg whites. Although the reported in vitro study does not establish that ingestion of egg white lysozyme in cheese will actually cause a clinically significant allergic reaction in such sensitive individuals, FDA is not aware of any data or information that would refute the study's inference that egg white lysozyme may be allergenic. Therefore, FDA concludes that there is insufficient information in the current record to determine whether the ingestion of egg white lysozyme elicits an allergenic response when consumed by individuals who are sensitive to egg whites. Accordingly, as discussed below, FDA is proposing labeling to alert such individuals to the presence of egg white lysozyme in cheese. Such labeling also would alert the sensitive population to the possible presence of source-derived proteins other than lysozyme in the enzyme preparation.

Under section 409(c)(1) of the Federal Food, Drug, and Cosmetic Act (the act) (21 U.S.C. 348(c)(1)), FDA is authorized, in approving the use of a food additive, to list the conditions under which the additive may be safely used. These conditions may include any labeling requirements that the agency deems necessary to ensure the safe use of the additive. Similarly, under § 184.1(b)(3) (21 CFR 184.1(b)(3)), in affirming a substance as GRAS, FDA is authorized to set forth the particular conditions of use, including labeling, under which there is general recognition among qualified experts that the use of the substance is safe. After careful review of the evidence on the use of egg white lysozyme enzyme preparation in preventing late blowing in cheese, FDA has tentatively concluded that such use is GRAS only when the conditions of its use include a declaration on the label or labeling of the presence of egg white lysozyme in both bulk and packaged food containing such treated cheese. Therefore, this tentative final rule (§ 184.1550(c)(1)) establishes that the declaration of egg white lysozyme enzyme preparation by the common or usual name "egg white lysozyme" is a condition of use required for GRAS status, so that consumers who are allergic to egg white products can be alerted to the presence of the egg white-derived enzyme in treated cheese.

D. Summary and Conclusions

The petitioner provided published data and information relating to the identity of, characteristic properties of, and estimated dietary exposure to the enzyme component (Refs. 1 through 7). The source of the petitioned enzyme preparation, egg white, has been safely consumed by humans as a source of food protein throughout recorded history, and, therefore, is GRAS (§ 170.30(d)). The petitioner provided generally available information showing that the methods used for extracting lysozyme from the egg white source do not ordinarily alter the chemical identity and characteristic properties of enzymes (Ref. 8). Moreover, there is corroborating evidence that the extraction of egg white lysozyme does not change its chemical identity or characteristics because the antibacterial activity of egg white lysozyme is retained. FDA concludes that the methods used to manufacture egg white lysozyme enzyme preparation do not change the safety for food use of the enzyme lysozyme and that toxicological

(10) MARSCHAL R.J., CHAPMAN H.R., GREEN M.L.
The Formation of Curd from Heat-treated Milk XX. Int. Dairy Congr., Paris 1978 Vol. E. 805-806.

(11) GHITTI, MOSCA, LAVEZZARI, BIANCHI-SALVADORI
Produzione di formaggio Grana con l'implego di cloridrato di lisozima. Ind. Latte 1983, 19, 49-61.

(12) CARINI S., MIGNONE, MAZZANTI
Imprego e dosagglo del lisozima in formaggro del tipo Montasro. Ind. Latte 1984, 20, 25-35.

studies are not necessary to establish the safety of lysozyme or other source-derived proteins that may remain in the manufactured enzyme preparation. FDA also concludes that there will be no net increase in dietary exposure of the general population to the commonly consumed enzyme lysozyme due to the proposed use in cheese because lysozyme will simply be transferred from eggs to cheese (Ref. 16).

The petitioner also provided generally available and accepted information relating to processing aids used in the manufacture of the enzyme preparation and generally available and accepted specifications for food grade enzyme preparations (Ref. 14). FDA concludes that substances added to the egg white lysozyme enzyme preparation or potential residues of processing aids used in the manufacturing process do not present a basis for concern about the safety of the egg white lysozyme enzyme preparation.

The petitioner provided published scientific review articles (Refs. 1 and 2) and a generally available trade bulletin (Ref. 7) that discuss the use of the egg white lysozyme enzyme preparation in cheese and other food, including its use for the intended effect of preventing late blowing of cheese contaminated with *C. tyrobutyricum*. The petitioner also provided generally available information documenting that this intended use of lysozyme has been approved in several European countries (Refs. 9 through 13). FDA concludes that generally available and accepted data and information establish that lysozyme will achieve the intended technical effect of preventing late blowing in cheese contaminated with *C. tyrobutyricum*.

Finally, information in the petition and otherwise available to FDA raises the question of whether the lysozyme component of egg whites is allergenic. FDA is proposing labeling to alert individuals who may be sensitive to egg whites to the presence of egg white lysozyme in cheese, including the possible presence of other source-derived proteins that may be present in the enzyme preparation.

IV. Comments

FDA received two comments in response to the filing notice. One comment expressed agreement that lysozyme is GRAS for use in preventing late blowing in cheese and supported the affirmation of GRAS status by the agency.

One comment stated that use of lysozyme as a food preservative may lead to selection of lysozyme-resistant strains of the bacterial food poisoning agents *Listeria monocytogenes* and *C. botulinum*, rendering one of the body's main defense mechanisms useless against resistant strains. The comment likened the potential selection of lysozyme-resistant strains of bacteria to the selection of penicillin-resistant bacteria as a result of its widespread use. The comment pointed out that the body could not readily substitute the lysozyme naturally present in secretions such as tears and saliva for another antimicrobial.

The mechanism of action of lysozyme involves hydrolysis of the structural peptidoglycan present in cell walls of susceptible bacteria. Therefore, development of resistance to lysozyme would require that a bacterium develop a variant of peptidoglycan that is resistant to the action of lysozyme. Development of such a variant peptidoglycan is, in principle, possible. However, as already discussed, lysozyme activity has been shown to be present in bacteria, fungi, plants, and almost all animal tissues. If such relative ubiquity has not resulted in the clinically significant selection of lysozyme-resistant bacteria to date, the use of lysozyme in those cheeses that are susceptible to late blowing is unlikely to favor selection of lysozyme-resistant bacteria and adversely affect the public health. Moreover, FDA is not considering lysozyme for use as a widespread food preservative. Rather, FDA is considering the narrow question of whether the use of lysozyme in preventing late blowing in cheese is generally recognized as safe. FDA disagrees that this limited use in cheese is analogous to the widespread use of antibiotics such as penicillin and the subsequent selection of antibiotic-resistant bacterial strains. Therefore, FDA concludes that the use of lysozyme in preventing late blowing in cheese does not raise concerns about the selection of lysozyme-resistant strains of *L. monocytogenes* or *C. botulinum*.

V. Specifications

The agency finds that, because the potential impurities in the egg white lysozyme preparation that may originate from the source or manufacturing process do not raise any basis for concern about the safe use of the preparation, the general requirements and additional requirements for enzyme preparations in the monograph on Enzyme Preparations in the Food Chemicals Codex, 4th ed. (1996), which are being incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51, are adequate as minimum criteria for food-grade egg white lysozyme enzyme preparation. Lysozyme assay can be performed using a method entitled "Lysozyme hydrochloride, Microbiological Determination," which is included in the petition (Ref. 21) or by using any appropriate validated method.

VI. Conclusions

The agency has evaluated all available information and finds, based upon the published information about the manufacturing methods used in the preparation of egg white lysozyme enzyme preparation, and published data and information about the identity and characteristic properties of egg white lysozyme, that the enzyme component of egg white lysozyme enzyme preparation is unaltered from the lysozyme found in the commonly consumed food, eggs. The agency also finds, based upon generally available and accepted information, that when the preparation is manufactured in accordance with § 184.1550(c), the source, egg whites, and the manufacturing process will not introduce impurities into the preparation that may render its use unsafe. Further, the agency finds, based upon published information, that egg white lysozyme enzyme preparation will achieve its intended technical effect of preventing late blowing in cheese contaminated with *C. tyrobutyricum*. Therefore, the agency tentatively concludes, based upon the evaluation of published data and information, corroborated by unpublished data and information, that the egg white lysozyme enzyme preparation described in the regulation set out below is GRAS for use by the general population in preventing late blowing in cheese.

To give interested persons an opportunity to comment on the proposed label declaration that is a condition of use required for GRAS status, FDA is issuing this tentative final rule under 21 CFR 10.40(f)(6). FDA will review any comments that are relevant to this condition of use and that are received within the 75 day comment period and will respond accordingly to these comments in the **Federal Register**.

VII. Environmental Considerations

The agency has carefully considered the potential environmental effects of this action. FDA has concluded that the action will not have a significant impact on the human environment, and that an environmental impact statement is not required. The agency's finding of no significant impact and the evidence supporting that finding, contained in an environmental assessment, may be seen in the Dockets Management Branch

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(3) WASSERFALL, F., 1978 Grundlagen der Lysozymwirkung bei der Herstellung von Schnittkäse. Teil 2. Milchwissenschaft 33 (2) 91-97.

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(8) KRASZ A. 1982 Methoden zur Bekämpfung der Buttersäuregärung in Käse. Milchwirtschaftliche Benzhle 73/1982. 307-316.

(9) GREEN M.L., MARSCHAL R.J. The Acceleration by Cationic Materials of the Coagulation of Casein Micelles by Rennet. J. Dairy Research 1972, 44, 521-531.

(address above) between 9 a.m. and 4 p.m., Monday through Friday.

VIII. Analysis of Economic Impacts

A. Benefit-Cost Analysis

FDA has examined the impacts of this tentative final rule under Executive Order 12866. Executive Order 12866 directs Federal agencies to assess the costs and benefits of available regulatory alternatives, and, when regulation is necessary, to select regulatory approaches that maximize net benefits (including potential economic, environmental, public health and safety effects; distributive impacts; and equity). According to Executive Order 12866, a regulatory action is "significant" if it meets any one of a number of specified conditions, including having an annual effect on the economy of $100 million, adversely affecting in a material way a sector of the economy, competition, or jobs, or if it raises novel legal or policy issues. FDA finds that this tentative final rule is not a significant regulatory action, as defined by Executive Order 12866. In addition, it has been determined that this final rule is not a major rule for the purpose of congressional review.

The primary benefit of this action is to remove uncertainty about the regulatory status of the petitioned substance. FDA is tentatively affirming the GRAS status of egg white lysozyme in cheese only when the ingredient statement of the bulk and packaged food that contains the cheese includes the common or usual name of the substance, i.e., "egg white lysozyme." The labeling requirement will add a small cost to the future use of the petitioned substance, and therefore, is not a significant action under the Executive Order 12866.

FDA has examined the impacts of this tentative final rule under the Unfunded Mandates Reform Act of 1995 (UMRA) (Pub. L. 104-4). A written statement under section 202(a) of the UMRA is not required for this rule because the rule does not impose a mandate that results in an expenditure of $100 million or more by State, local, and tribal governments in the aggregate, or by the private sector, in any 1 year.

B. Regulatory Flexibility Act

FDA has evaluated this tentative final rule under the Regulatory Flexibility Act. The Regulatory Flexibility Act (5 U.S.C. 601-612) requires Federal agencies to consider alternatives that would minimize the economic impact of their regulations on small entities.

FDA believes that this tentative final rule is not likely to have a significant economic impact on a substantial number of small entities. However, the agency seeks comment on this tentative conclusion. First, FDA is tentatively affirming the GRAS status of egg white lysozyme in cheese only when the ingredient statement of the bulk and packaged food that contains the cheese includes the common or usual name of the substance, i.e., "egg white lysozyme." This labeling requirement will impose only minimal costs to the future use of the petitioned substance. Second, FDA has information that the petitioner does not currently sell egg white lysozyme in the United States (Refs. 22 and 23). Moreover, FDA is not aware of any manufacture or use of cheese containing egg white lysozyme in the United States. If no small entities are currently manufacturing or using cheese containing egg white lysozyme, the proposed labeling requirements would not impose any cost to small entities. However, because FDA does not have any information on whether other entities in the United States are manufacturing or using cheese containing egg white lysozyme, FDA is unable to conclude, in this tentative final rule, that there will be no significant economic impact on a substantial number of small entities. Therefore, the agency seeks comment on the manufacture or use, by any small entity, of cheese containing egg white lysozyme. In its final rule, the agency will, based on any relevant comments received, determine whether there is a significant economic impact on a substantial number of small entities.

IX. References

The following references have been placed on display in the Dockets Management Branch (address above) and may be seen by interested persons between 9 a.m. and 4 p.m., Monday through Friday.

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10. French Republic Ministry of Agriculture Service Note 5236, April 21, 1987.
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16. Memorandum dated March 20, 1990, from Food and Color Additives Review Section, FDA, to Direct Additives Branch, FDA, "Use of Lysozyme to Prevent the 'Late Blowing' of Cheese."
17. Memorandum dated August 5, 1996, from Chemistry Review Branch, FDA, to Biotechnology Policy Branch, FDA.
18. Kessler, D. A., M. R. Taylor, J. H. Maryanski, E. L. Flamm, and L. S. Kahl, "The Safety of Foods Developed by Biotechnology," *Science*, 256:1747-1749 and 1832, 1992.
19. Crespo, J. F., C. Pasqual, A. Ferrer, A. W. Burke, J. M. Diaz Pena, and M. M. Esteban, "Egg White-specific IgE Level as a Tolerance Marker in the Follow-up of Egg Allergy," *Allergy Proceedings*, 15:73-76, 1994.
20. Anet, J., J. F. Back, R. S. Baker, D. Barnett, R. W. Burley, and M. E. H. Howden, "Allergens in the White and Yolk of Hen's Egg; a Study of IgE Binding by Egg Proteins," *International Archives of Allergy and Applied Immunology*, 77:364-371, 1985.
21. Lysozyme Hydrochloride, Microbiological Determination.
22. Letter dated November 25, 1996, from John B. Dubeck, Keller and Heckman, to Linda Kahl, FDA.
23. Letter dated February 28, 1997, from John F. Foley, Keller and Heckman, to Linda Kahl, FDA.

List of Subjects in 21 CFR Part 184

Food ingredients, Incorporation by reference.

Therefore, under the Federal Food, Drug, and Cosmetic Act and under

legislative restriction to use nitrates are obvious preconditions to the growth of lysozyme market in dairy application.

Experiments to find out the dosages of lysozyme needed in different seasons should be carried out in the first place. Valio has stated (Mr. M. Kärki) that they test lysozyme both during the grazing and silage feeding seasons (June-September & November-February), when the count of spores in milk is quite different from each other. The only suggestion for seasonal dosage levels introduced is that of by N. Koch (Boehringer) in the seminar, Lysozyme in cheese manufacture, in Kiel January 1986 (appendix G).

Another application within cheese industry, hinted by C-J Sandström, to inhibit Listeriosis by Listeria monocytogenes would require testing lysozyme's lytic action both on Listeria monocytogenes and on the cheese cultures in question (Brie, Camembert cheese - Penicillium camemberti).

authority delegated to the Commissioner of Food and Drugs and redelegated to the Director, Center for Food Safety and Applied Nutrition, it is proposed that 21 CFR part 184 be amended as follows:

PART 184—DIRECT FOOD SUBSTANCES AFFIRMED AS GENERALLY RECOGNIZED AS SAFE

1. The authority citation for 21 CFR part 184 continues to read as follows:

Authority: 21 U.S.C. 321, 342, 348, 371.

2. Section 184.1550 is added to subpart B to read as follows:

§ 184.1550 Egg white lysozyme.

(a) Egg white lysozyme (CAS Reg. No. 9001-63-2) is the enzyme peptidoglycan *N*-acetylmuramoylhydrolase (EC No. 3.2.1.17) obtained by extraction from egg whites. The enzyme catalyzes the hydrolysis of peptidoglycan in the cell walls of certain bacteria including *Clostridium tyrobutyricum*.

(b) The ingredient meets the general requirements and additional requirements for enzyme preparations in the monograph on Enzyme Preparations in the Food Chemicals Codex, 4th ed. (1996), which is incorporated by reference in accordance with 5 U.S.C. 552(a) and 1 CFR part 51. Copies are available from the National Academy Press, 2101 Constitution Ave. NW., Washington, DC 20418, and may be examined at the Center for Food Safety and Applied Nutrition's Library, 200 C St. SW., rm. 3321, Washington DC, or at the Office of the Federal Register, 800 North Capitol St. NW., suite 700, Washington, DC.

(c)(1) The ingredient is used in cheeses, as defined in § 170.3(n)(5) of this chapter, in accordance with § 184.1(b)(3) at levels not to exceed current good manufacturing practice.

(2) The affirmation of the use of this ingredient as generally recognized as safe (GRAS) as a direct human food ingredient is based upon the following conditions of use:

(i) The ingredient is used as an enzyme as defined in § 170.3(o)(9) of this chapter.

(ii) Current good manufacturing practice utilizes a level of the ingredient sufficient to prevent the late blowing of cheeses caused by the bacterium *Clostridium tyrobutyricum* during cheese production.

(iii) The ingredient statement for both bulk and packaged food that contains cheese manufactured using egg white lysozyme shall include the common or usual name "egg white lysozyme" to identify the source of the protein.

Dated: March 3, 1998.

L. Robert Lake,

Director, Office of Policy, Planning and Strategic Initiatives, Center for Food Safety and Applied Nutrition.

[FR Doc. 98-6571 Filed 3-12-98; 8:45 am]

BILLING CODE 4160-01-F

FEDERAL COMMUNICATIONS COMMISSION

47 CFR Part 73

[MM Docket No. 98-29, RM-9190]

Radio Broadcasting Services; Indian Wells, CA

AGENCY: Federal Communications Commission.

ACTION: Proposed rule.

SUMMARY: This document requests comments on a petition for rule making filed on behalf of Professional Broadcasting, Inc. requesting the allotment of FM Channel 238A to Indian Wells, California, as that community's first local aural transmission service. Coordinates used for this proposal are 33-42-04 and 116-14-47. Indian Wells, California, is located within 320 kilometers (199 miles) of the Mexico border, and therefore, the Commission must obtain concurrence of the Mexican government to this proposal.

DATES: Comments must be filed on or before April 27, 1998, and reply comments on or before May 12, 1998.

ADDRESSES: Secretary, Federal Communications Commission, Washington, DC 20554.

In addition to filing comments with the FCC, interested parties should serve the petitioner's counsel, as follows: John R. Feore, Jr., M. Anne Swanson and Kevin P. Latek, Esqs., Dow, Lohnes and Albertson, 1200 New Hampshire Avenue, NW., Washington, DC 20036-6802.

FOR FURTHER INFORMATION CONTACT: Nancy Joyner, Mass Media Bureau, (202) 418-2180.

SUPPLEMENTARY INFORMATION: This is a synopsis of the Commission's Notice of Proposed Rule Making, MM Docket No. 98-29, adopted February 25, 1998, and released March 6, 1998. The full text of this Commission decision is available for inspection and copying during normal business hours in the FCC's Reference Center (Room 239), 1919 M Street, NW., Washington, DC. The complete text of this decision may also be purchased from the Commission's copy contractor, International Transcription Service, Inc., 1231 20th Street, NW., Washington, DC 20036, (202) 857-3800.

Provisions of the Regulatory Flexibility Act of 1980 do not apply to this proceeding.

Members of the public should note that from the time a Notice of Proposed Rule Making is issued until the matter is no longer subject to Commission consideration or court review, all *ex parte* contacts are prohibited in Commission proceedings, such as this one, which involve channel allotments. See 47 CFR 1.1204(b) for rules governing permissible *ex parte* contacts.

For information regarding proper filing procedures for comments, See 47 CFR 1.415 and 1.420.

List of Subjects in 47 CFR Part 73

Radio broadcasting.

Federal Communications Commission.

John A. Karousos,

Chief, Allocations Branch, Policy and Rules Division, Mass Media Bureau.

[FR Doc. 98-6514 Filed 3-12-98; 8:45 am]

BILLING CODE 6712-01-P

FEDERAL COMMUNICATIONS COMMISSION

47 CFR Part 73

[MM Docket No. 98-28; RM-9234]

Radio Broadcasting Services; Meyersdale, PA

AGENCY: Federal Communications Commission.

ACTION: Proposed rule.

SUMMARY: The Commission requests comments on a petition filed by Douglas M. Dasdorf proposing the allotment of Channel 253A at Meyersdale, Pennsylvania, as the community's second local FM transmission service. Channel 253A can be allotted to Meyersdale in compliance with the Commission's minimum distance separation requirements at city reference coordinates. The coordinates for Channel 253A at Meyersdale are North Latitude 39-48-42 and West Longitude 79-01-36. Since Meyersdale is located within 320 kilometers (200 miles) of the U.S.-Canadian border, concurrence of the Canadian government has been requested.

DATES: Comments must be filed on or before April 27, 1998, and reply comments on or before May 12, 1998.

ADDRESSES: Federal Communications Commission, Washington, DC 20554. In addition to filing comments with the FCC, interested parties should serve the petitioner, his counsel, or consultant, as

Hepner et Associates stated in their report in July 1986 that the market for lysozyme in dairy application is about 30000 kgs/year, and that the market price for lysozyme at the moment is USD 150/kg. This totals a market of FIM 22,0 million per year. This estimate is based on the legislative situation in Europe so e.g. West-Germany and whole of Scandinavia is excluded from this figure. The same report forecasts as a future trend an amount of 500000 tons cheese, which could be prepared with lysozyme in West-Europe. This figure considering IDF-data, may not include Scandinavian amount of about 150000 tons/year. Hepner also states a possible reduction of the price on lysozyme from 150 USD/kg at present to USD 75/kg, before it will conquer the market.

6 Conclusions

The use of lysozyme to inhibit the late blowing phenomena in hard and semihard cheeses has proved to be successful at addition levels of 500 U lysozyme/ml cheese milk (20 g/1000 l).

As lysozyme, according to several research papers, remains untouched in the pH- and temperature/time - ranges used in cheese manufacturing, it is essential to avoid overdosing of lysozyme (about 1000 U/ml), which results in small holed and mild flavoured cheese. Cheese manufacturers should also be properly informed to test their own starter cultures with lysozyme, before production scale cheese manufacturing with it.

The seasonal variations in the count of Clostridia spores in milk are depending very much on the country or even on milk production area in question. Thus the information received e.g. from the possible experiments with Valio are just instructive figures, which serve as quidance for foreign customers in their own needs.

The size of potential market after lysozyme is permitted generally, total 700000 tons cheese per year in West-Europe and 440000 tons cheese in North-America totalling lysozyme 140000 kgs and 88000 kgs respectively. According to today's price level this results in markets of FIM 105.0 million in West-Europe and FIM 66.0 million in North-America. Assuming the probable reduction stated by Hepner and Assoc. we result in markets of FIM 53.0 million and FIM 33.0 million respectively. As the cost of use of lysozyme is very high compared to that of nitrates, this reduction in price and also

U. S. Food and Drug Administration
Center for Food Safety & Applied Nutrition
Office of Premarket Approval

Agency Response Letter
GRAS Notice No. GRN 000064

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

Food and Drug Administration
Washington, DC 20204

April 2, 2001

Mr. Robert H. Sindt
Attorney At Law
1850 M Street, N.W.
Suite 400
Washington, D.C. 20036

Re: GRAS Notice No. GRN 000064

Dear Mr. Sindt:

The Food and Drug Administration (FDA) is responding to the notice, dated December 15, 2000, that you submitted on behalf of Rhodia, Inc. (Rhodia) in accordance with the agency's proposed regulation, proposed 21 CFR 170.36 (62 FR 18938; April 17, 1997; Substances Generally Recognized as Safe (GRAS)). FDA received the notice on December 20, 2000, and designated it as GRAS Notice No. GRN 000064.

The subject of the notice is egg white lysozyme. The notice informs FDA of the view of Rhodia that egg white lysozyme is GRAS, through scientific procedures, for use as an antimicrobial agent in casings for frankfurters and on cooked meat and poultry products. Egg white lysozyme would be used in casings for frankfurters at a concentration of 2.5 milligram (mg) lysozyme per pound (lb) of frankfurter (equivalent to 5.5 mg lysozyme per kilogram (kg) of food) and in cooked meat and poultry products sold as ready-to-eat at a concentration of 2.0 mg of lysozyme per lb of cooked meat or poultry product (equivalent to 4.4 mg of lysozyme per kg of food).

Rhodia's notice describes published information regarding the chemical identity of lysozyme (Chemical Abstract Service Registry Number 9001-63-2). Under a system developed by the Commission on Enzymes of the International Union of Biochemistry, the systematic name of lysozyme is peptidoglycan N-acetylmuramoylhydrolase and its systematic number is EC No. 3.2.1.17. Rhodia describes lysozyme as a natural antimicrobial protein with a molecular weight of approximately 14,300 daltons. The enzyme occurs naturally in many organisms. Hen egg white lysozyme is among the most throughly characterized enzymes, and its three-dimensional structure, mechanism of action, substrate specificity, and other properties have been determined. Lysozyme is inactivated by stomach and intestinal proteolytic enzymes, particularly pepsin.

Dairy Research Institute, Hillerod, states in their report (No. 250), that only one per cent of the lysozyme (Hansozym) they used was lost in whey (5). According to data bases' file search any paper on the lysozyme's binding to milk casein has not been published so far.

Italian paper (11) stated in 1983 that lysozyme is nearly totally retained in curd and is found in the cheese after one year of ripening. Carini et al. (12) found in 1984 that lysozyme lost 30 % of its initial activity during ripening and storage.

5 Markets

In appendix E the production volumes of hard and semihard cheeses in certain OECD-countries are introduced both cost of the use of nitrate and lysozyme.

The countries permitting the use of nitrates (Scandinavia, Ireand, United Kingdom, Netherlands, Belgium and Canada) produce altogether 1197000 tons per year hard and semihard cheeses. When we make an assumption (based on Finnish data) that one half of this amount is such varieties (Edam, Gouda, Tilsit, Grana, Provolone) whereto lysozyme could be used, we result in a figure of about 600000 tons/year. This means 120000 kgs lysozyme worth FIM 90.0 million per year as a total market.

If we count Scandinavia as its own, we find out that about 150000 tons of cheese (0.5 x 293) could be manufactured with lysozyme in Scandinavia. This means 30000 kgs market worth FIM 22.0 million per year.

In the rest of Western Europe (excl. UK) hard and semihard cheese production totals 1828000 tons/year, which results (by factor 0.3) in 550000 tons cheese per year for which lysozyme could be used (= 110000 kg/lysozyme). This corresponds a market of FIM 82.0 million per year.

The markets in the United Kingdom and North-America are more difficult to estimate as the main variety Cheddar doesn't, due to its rapid acidifying, require necessarily lysozyme. According to the data shown in appendix F roughly 18 % (Italian varieties) of the cheese produced could be manufactured with lysozyme. This results in 440000 tons/year, meaning a market of 88000 kgs lysozyme worth of FIM 66.0 million.

Rhodia describes the method of manufacture of egg white lysozyme, which is based on generally available and accepted principles of protein purification. Lysozyme is extracted from fresh egg white, where it is present at approximately 0.3 percent of the egg white, and 3.5 percent of the egg white protein. In the processing, a food-grade inert material (a polymer resin) is mixed with egg white where it specifically binds with the lysozyme. The resin carrying the lysozyme is separated from the egg white, and the lysozyme is removed from the resin by addition of salts and a change in the pH. The enzyme is then concentrated, further purified, and dried. Specifications provided for lysozyme are consistent with the general and additional requirements for enzyme preparations in the Food Chemicals Codex (4th ed., 1996). Rhodia's notice provides intake estimates for the proposed uses of lysozyme based on food consumption data collected by the U.S. Department of Agriculture (1998).

As part of its notice, Rhodia includes the report of a panel of individuals (Rhodia's GRAS panel) who evaluated the data and information that are the basis for Rhodia's GRAS determination. Rhodia considers the members of its GRAS panel to be qualified by scientific training and experience to evaluate the safety of substances added to food. Rhodia's GRAS panel discusses the information and data provided by Rhodia to the panel on the chemical, physical, and antimicrobial properties, manufacture and processing, stability, conditions of anticipated use, and estimates of dietary exposure.

Rhodia's GRAS panel notes that FDA recently evaluated available information on lysozyme and tentatively affirmed that egg white lysozyme, when labeled by the name "egg white lysozyme" to identify its source, is GRAS for use in preventing late blowing of cheese caused by the bacterium *Clostridium tyrobutyricum* during cheese production (the tentative final rule on lysozyme; 63 FR 12421; March 13, 1998). In that tentative final rule, FDA explained that egg whites are known to be an allergenic food source, particularly in children. FDA referenced a literature report indicating that lysozyme may in fact have been an allergen for some individuals who became sensitive to egg whites. Accordingly, FDA proposed that a condition for the safe use of lysozyme would be labeling to alert such individuals to the presence of egg white lysozyme in cheese. Such labeling also would alert the sensitive population to the possible presence of source-derived proteins other than lysozyme in the enzyme preparation.

In its report, Rhodia's GRAS panel discusses, and concurs with, several of the conclusions drawn by FDA in the tentative final rule on lysozyme. Rhodia's GRAS panel concludes that:

- There will be no long term increase in lysozyme intake by the general population because the egg whites from which lysozyme is extracted will be subsequently consumed (without lysozyme) in other food uses.
- The safety of lysozyme as a component of egg white is supported by a long history of safe consumption by humans as a source of food protein throughout recorded history.
- Proteins derived from egg whites do not raise toxicity concerns, and the methods used for extracting lysozyme from the egg white source should not alter either the chemical identity or the characteristic properties of the enzyme.
- FDA's tentative conclusion that egg white lysozyme is GRAS for use in preventing late blowing of cheese caused by the bacterium *Clostridium tyrobutyricum* during cheese production is based, in part, on a presumption that the ingredient would be labeled by the name "egg white lysozyme" to identify its source.

Based on the information provided by Rhodia as well as other information available to FDA, the agency has no questions at this time regarding Rhodia's conclusion that egg white lysozyme is GRAS under the intended conditions of use, provided that the ingredient statement of food products that contain egg white lysozyme contain the name "egg white lysozyme" to identify the source of the protein. The agency has not, however, made its own determination regarding the GRAS status of the subject use of egg white lysozyme. As always, it is the continuing responsibility of Rhodia to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all

After the cheese curd is cut to pieces (cubes) this curd is scalded (warmed up) for 30-60 min to get the whey off the cubes. The temperature used for Edam and Gouda is ranging from 36 °C to 39 °C and for Emmental and Gruyere cheese from 50 °C to 54 °C (max 57 °C). The time of scalding in Emmental cheese can go up to 90 minutes.

Friend et al. (6) found that lysozyme survives heating at 62,5 °C for 30 minutes, but not heating at 75 °C for 15 minutes at the natural pH of milk. This means that lysozymes survives also the scalding of Edam and Gouda cheeses.

The pH of normal milk ranges from 6,5 to 6,8. As the cheese is ready to be pressed (about 20-24 hours) after 3-4 hours from the beginning of cheesemaking the pH of the curd is below 6,5 and after pressing about 5,15-5,25. This means that the pH in milk/curd during cheesemaking doesn't influence on lysozyme activity at all (appendix C) (7). Krasz (8) stated the highest activity of lysozyme at pH 6,15 and practically at the pH range 5,5-6,5 the activity remains stable.

4.2 Mechanical stress

In cheesemaking lysozyme must survive the mechanical stress during cutting scalding and wheying off. Wasserfall (7) showed that lysozyme remained almost untouched the treatment with Ultra-Turrax at 0 °C for 8 minutes (appendix D).

4.3 Binding lysozyme to casein

In cheesemaking the ability of lysozyme to bind to casein is of great importance, because the rate of binding gives the amount of dosage (cost of use) needed to inhibit late blowing. Also binding to casein quarantees the function of lysozyme during cheese ripening and storage.

Wasserfall (7) stated 1977 that 15-17 % of the lysozyme added was recovered in whey and in the water used for curd washing, naturally meaning that 83-85 % of the lysozyme was binded to casein in order to inhibit late blowing. Green et al (9) and Marschal et al. (10) stated in 1977-1978 according to their laboratory experiments with casein micelle suspensions that even as much as 98 % of the lysozyme added adsorbed on casein micelles.

applicable legal and regulatory requirements.

During its evaluation of GRN 000064, FDA consulted with the Labeling and Consumer Protection Staff of the Food Safety and Inspection Service of the United States Department of Agriculture (FSIS). FSIS requests that Rhodia be advised that it may be necessary to modify regulatory standards where a standard of identity prohibits or limits the use of an ingredient. At this time, FSIS is not initiating any substance-specific rulemaking activities to amend food standards until its ongoing standards modernization activities are completed. However, FSIS will be able to address requests for the use of any antimicrobial formulations containing egg white lysozyme on meat and poultry products with prevailing standards of identity provided they are descriptively labeled to clearly distinguish them from the traditional standardized products. It is our understanding that you will seek regulatory guidance from FSIS concerning the use of these antimicrobial formulations in standardized meat and poultry products. You should direct your inquiry to Dr. Robert Post, Director, Labeling and Consumer Protection Staff, Office of Policy, Program Development and Evaluation, Food Safety and Inspection Service, 300 12th Street, SW, Room 602, Washington, DC 20250-3700. The telephone number of his office is (202) 205-0279 and the FAX number is (202)205-3625.

In accordance with proposed 21 CFR 170.36(f), a copy of the text of this letter, as well as a copy of the information in the notice that conforms to the information in proposed 21 CFR 170.36(c)(1), is available for public review and copying on the Office of Premarket Approval's homepage on the Internet (at http://www.cfsan.fda.gov/~lrd/foodadd.html).

Sincerely,
/s/
Alan M. Rulis, Ph.D.
Director
Office of Premarket Approval
Center for Food Safety and Applied Nutrition

cc: Dr. Robert Post, Director
Labeling and Consumer Protection Staff
Office of Policy, Program Development and Evaluation
Food Safety and Inspection Service
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acid bacteria (flavour producers) and this will produce a cheese without holes and of mild flavour. In Finland cheese is called a blind cheese if a cheese type doesn't have holes according its characteristic definitions.

The Dairy Research Institute, Hillerod, Denmark (5), noticed that in addition of 1200 U/ml lysozyme entered to a Edam cheese with small holes or totally 'blind' cheese.

In Edam cheese a blind body means that the growth of flavour/aroma producing bacteria, Str. diacetilactis and Leuconostoc citrovorum, has been insufficient. This phenomena produces also mild or mildless flavour and untypical taste into cheese.

In Emmental and Gruyere cheese the development of flavour and aroma is induced by Lactobacillus helveticus and mainly by propionic acid bacteria. Lb. helveticus has proteolytic activity and degrades casein down to dipeptides and amino acids. Certain amino acids give to cheese its own identical flavour and taste. Lb. helveticus' growth is inhibited by higher lysozyme additions as mentioned earlier in chapter 3.1. Propionic acid bacteria has not been found to be sensitive to lysozyme.

Appendix C, table 1 & 2, shows the Danish cheese grading results in the experiment using lysozyme on Edam cheese (Danbo). In the trials with added Clostridia spores Edam with 1 and 2 grams lysozyme per 100 litres cheese milk was scored of equal quality or better than that manufactured with nitrate (10 g/100 l). In the trials, where normal bulk milk was used for cheese manufacturing, Edam made with lysozyme addition, was graded better than Edam with nitrate in every organoleptical property (texture, body, flavour and taste).

4 Lysozyme in cheese making process

4.1 Temperature and acidity

The best phase to add lysozyme (crystalline lysozyme first dispersed to warm, 29-30 °C, water) is to do it with the starter culture, which is added as soon as the cheese milk is brought to the curdling temperature (29-31 °C) needed for the cheese type.

3.2
Clostridia and coliforms

Wasserfall and Teuber (2) stated in their cheese making experiment in 1978 that lysozyme in concentration of 500 U/ml cheese milk can't only prevent the late blowing provoked by the forms of C. tyrobutyricum sensitive against this enzyme; even spores, the vegetative cells of which are resistant against lysozyme, are hindered to provoke the late blowing in cheese. They concluded that since lysozyme resistance is not lost by one cycle of sporulation of lysozyme-resistant vegetative cells, the above observation on lysozyme resistant spores implies that some steps in the conversion process must be sensitive to lysozyme.

The concentration of lysozyme sufficient to lyse the 99 % of the cells of Cl. tyrobutyricum initially present (initial conc. = 10^5/ml) in Reinforced Clostridial Medium was evaluated as 25 ppm. To achieve a similar percentage of lysis in milk, double concentrations of lysozyme were necessary (50 ppm) (2).

Eschricia coli can cause 'coliform blowing'/small hole formation in the early stage of cheese ripening. The sensitivity of E.coli to lysozyme is debated. Wasserfall (3) states that coliforms are not influenced in the cheese curd and so they must be inactivated by a suitable heating process.

3.3
Organoleptical quality

In the experiments of Federal Dairy Research Center, Kiel, West-Germany (4) Edam cheeese manufactured with lysozyme turned out to be of an equal organoleptical quality (texture, body, holes, flavour and taste) as Edam cheese with nitrate addition. The dosage for lysozyme was 500 U/ml and for nitrate 10 g/100 l cheese milk.

Parallel results have been obtained in experiments in Denmark, Norway, Italy and in Hungary.

The optimum level of lysozyme dosage to cheese milk in the above experiments has proved to be 500 U/ml. If this level seems to be, due to poor raw milk quality, too low, it is in that case better to use a combination of lysozyme and nitrates. As the dosage of lysozyme is about 1000 U/ml, lysozyme will start inhibiting the growth of certain lactic

Material Safety Data Sheet

Date Prepared: 06-Nov-00

Product Name: ***Proprietary Name***

Description: Lysozyme an enzyme extracted from hen egg white		**CAS No:** 9001-63-2
Supplier Address:	*Company Name*	**Phone No.**

Hazardous Ingredients

Ingredient	Percentage	Ingredient	Percentage

Physical Data

Physical State Granulated powder	**Odor and Appearance:** white to off-white powder, no distinct odor		**Odor threshold** not determined (n/d)	**Vapour Pressure** n/d	**Vapour Density** (air =1) n/d
Boiling Point Not applicable (n/a)	**Evaporation Rate** n/d	**Freezing Pt** n/a	**Solubility in H20** soluble	**Specific Gravity** (water=1) n/d	**Melting Point** n/d

Fire and Explosion Data

Flammable No	**under which conditions** Not applicable (n/a)				
Extinguishing Media: Water spray, dry chemical powder, Carbon dioxide, or appropriate foam.					
	Flammable Limits	**Upper** n/a	**Lower** n/a	**Sensitivity to impact** n/d	**Sensitivity to Static** n/d
Flash Point none	**Method** n/a				

Special Fire Fighting Procedures:
Wear self-contained breathing apparatus and protective clothing to prevent contact with skin and eyes.

Unusual Fire and Explosion Hazards:
None.

Today the use of lysozyme in cheese is permitted in Denmark, Belgium, France, West-Germany and Italy.

Appendix B shows the cheese varieties for which lysozyme is applicable. Lysozyme generally is applicable to hard and semi-hard cheeses like Edam, Gouda, Tilsit, Trappist, Emmental and Parmesan types, but not to cheeses with interior mould ripening or with a fast and high acidifying rate like Cheddar-varieties.

3 The influences of lysozyme on cheese

3.1 Lactic acid bacteria of cheese

In an Italian paper (LODI, R. 1983) (1) concerning the activity of lysozyme on different species of lactic organisms (L. bulgaricus, L. helveticus, L. jugurti, L. lactis, L. fermentum, L. plantarum, L. casei, Pediococcus cerevisiae, Str. lactis. Str. thermophilus, Str. faecalis, Str. faecalis var. liquefaciens, Str. faecalis var zymogenes) it was shown that lysozyme does not significantly impair the growth and acidifying, proteolytic and reducing activities of these organisms. Only the strains of Lb. helveticus have been confirmed to be partially sensitive to lysozyme, and especially at levels over 25 ppm (25 g/1000 l cheese milk).

Lb. helveticus is mainly used in starters for Emmental and Cruyere cheese. The most typical organisms used for Edam, Gouda and Tilsit type cheese, namely Str. lactis, Str. cremoris, Str. diacetilactis and Leuconostoc citrovorum, haven't proved to be sensitive for lysozyme at the levels used into cheese milk. At the level over 1000 U/ml cheese milk Streptococcus species have showed decrease in both growth and acidifying properties.

Wasserfall (2) found 500 U/ml as a limit to avoid inhibition on Str. cremoris and Str. diacetilactis. Researchers have not found any lytic action of lysozyme on propironic bacteria, which develope the flavour and holes of Emmental and Gruyere cheese.

In practice, every cheese plant willing to use lysozyme, must test their own starter cultures with lysozyme addition, before production scale cheese manufacturing.

Health Hazard Data

Threshold limit Value (TLV)	LC50	LD50
n/a	n/a	(IPR-MUS) 5800 mg/kg

Route	Effects Immediate and Long Term	First Aid
Inhalation	May be harmful in high dosages and cause allergic reaction in sensitive persons.	Remove to fresh air. Give artificial respiration if not breathing. Get immediate medical attention.
Eyes	May cause irritation.	Immediately flush eyes with lots of running water for 15 minutes, lifting upper and lower eyelids occasionally. Get immediate medical attention.
Skin	May cause allergic reactions in sensitive persons.	Immediately wash skin for at least 15 minutes. Remove contaminated clothing and shoes; wash before reuse. Get medical attention if irritation persists after washing.
Ingestion	May cause allergic reactions in sensitive persons.	Wash out mouth with water provided person is conscious. Call a physician. Get immediate medical attention. Do not give anything by mouth to an unconscious or convulsing person.
Other precautions	This product is not considered to be a carcinogen. The toxological properties have not been thoroughly investigated.	

Reactivity Data

Stable	yes	Unstable (Conditions to Avoid)	Hazardous Decomposition Products	Incompatibility (Materials to Avoid)
Hazardous Polymerization		N/A	N/A	N/A
May Occur:				
Will Not Occur:	xx			

Spill or Leak Procedures

- Wear protective equipment, including: impervious boots, impervious glove, impervious apron and a self-contained breathing apparatus in the pressure demand mode or a supplied-air respirator. If the spill or leak is small, a full face piece air-purifying respirator equipped with particulate filters may be satisfactory.

- Mop up spill area, rinse spill area with large amounts of fresh water. Take care not to allow product to dry prior to clean up. If dried take care when sweeping up residue to avoid raising a dust.

- Comply with all applicable government regulations on spill reporting and handling and disposal of waste.

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LYSOZYME IN CHEESE PRODUCTION

1 Introduction

Several means of preventing the late blowing, caused by anaerobe Clostridia in hard and semi-hard cheese manufacture has been studied so far:

The use of nicin, active against the germination of spores, hasn't shown any interesting results, since it involves deep changes of the microbiological and technological parameters of the cheese.

The use of nitrates or nitrites, although being efficient in preventing the blowing, brings toxicological problems. These additives may result in the formation of nitrosoamines in cheese.

The treatment of milk with IR or UV radiation hasn't shown after all efficient against the late blowing.

Milk bactofugation has also been used for restriction of the number of clostridia spores, but it is an operation requiring considerable capital investment for plants. The decrease in the number of spores, which can be obtained does not solve the problem if the milk is highly contaminated with Clostridia spores.

One method, widely used in Italy, is the addition of formaldehyde to the cheese milk or, for Provolone cheese, in the curd stretching water.

2 Lysozyme and cheese types

The lytic action of lysozyme on the vegetative forms of Clostridium tyrobutyricum and on Clostridia in general has led to its widespread experiment use in cheese production, particularly in the manufacture of medium- and longterm ripening cheeses (hard and semihard cheeses).

In appendix A is shown the cheese varieties of different European countries on which the experiments and use of lysozyme has been carried out. Tests have been done in Scandinavia, France, West-Germany, Austria, Italy and Hungary. In the literature there is no mention about experiments in the UK and the Netherlands.

Special Protection Information

Respiratory Protection:	Not normally required, if dust is a problem wear approved respirator.
Ventilation:	Local mechanical exhaust capable of minimizing emissions at point of use.
Protective Gloves (Type): Chemically resistant	**Eye Protection (Type):** chemical goggles

Other Protective Equipment:
Where necessary use long sleeved shirts, trousers, rubber boots and apron.

Special Precautions

Precautions for Handling and Storage:
Wear appropriate NIOSH/MSHA-approved respirator, chemical-resistant gloves, safety goggles, other protective clothing. Mechanical exhaust required.

Other Precautions:
Avoid contact and inhalation.

The information contained herein is believed to be correct but does not purport to be all inclusive and shall be used only as a guide.

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Scientific Literature Search

Search Parameters: **lysozyme**

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of pure cultures can differ. According to BOTTAZZI (23) 10 % of the "natural" spores of the mixed-spore population of BAB present in milk are resistant to lysozyme. This explains our observation that lysozyme (600 U/ml) is active against spores of certain strains but not against (all) natural spores of BAB. Further research is needed to elucidate the effectiveness of lysozyme against BAB, but it is clear that for Gouda cheese this effect is very limited.

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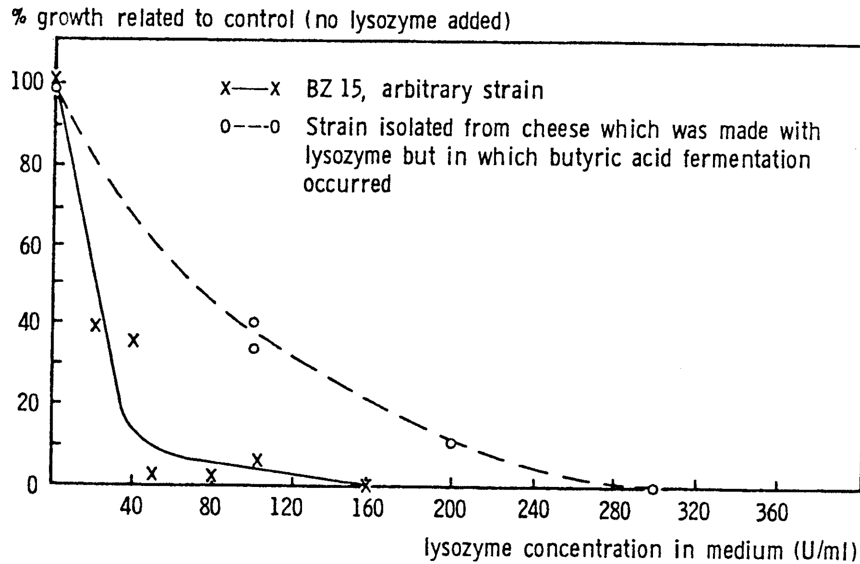

Fig. 4. The resistance to lysozyme of spores of two pure cultures of BAB

Discussion

From the results given in this paper it appears that the usual amount of lysozyme added to milk (about 500 U/ml) can prevent BAF in Gouda cheese made from it only if the number of spores of BAB does not exceed 0.3 spores per ml. This number is normal in milk produced during summertime. In most European countries the number of spores of BAB in milk in winter amounts at least to a level of a few spores/ml. Increasing the amount of the added lysozyme to 6.0 g per 100 l was more effective also in cheese made from milk with 13 spores/ml. However, it is questionable whether it is possible to use 6 g of lysozyme per 100 l of cheese milk in terms of costs and availability. The addition of 6 g of lysozyme/100 l cheese milk did not inhibit the growth and acid production of the mesophilic starter used (BD-starter Bos). The results obtained are not in accordance with those observed by others without due consideration (see references 2, 3, 6 and 7). One of the reasons is that in one experiment described above the cheeses had been stored at 13 °C or even partly at 18 °C. In orientating experiments in which lysozyme was used it appeared that, at ripening at 10 °C, BAF did not occur. Such temperatures or even lower are often used in experiments just referred to, but the ripening temperature for Gouda cheese should be at least 12 °C. Another reason is that many experiments with lysozyme have been carried out with spores of pure cultures of BAB, which may not be resistant to lysozyme. The results in Figures 3 and 4 show that there is a difference in resistance to lysozyme between natural spores and spores of pure cultures of BAB and that the resistance of spores

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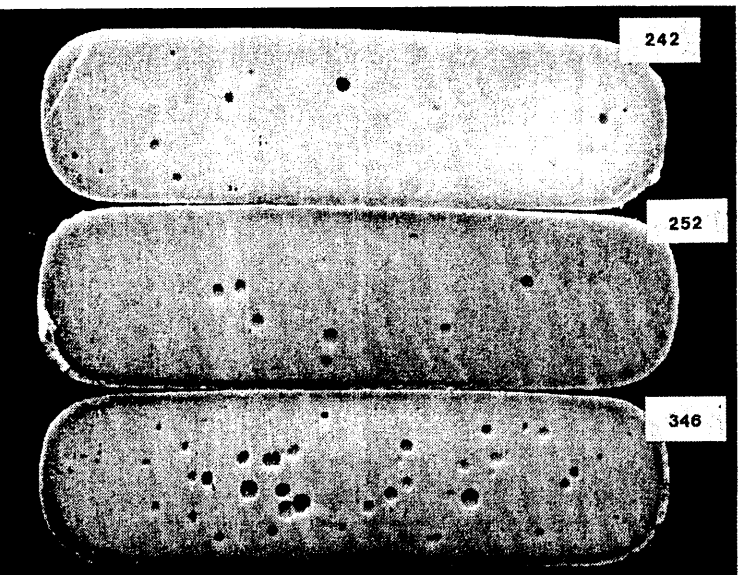
contamination	number of spores of BAB	added per 100 ml	cheese stored at 13 °C	cheese stored at 13 °C and later at 18 °C
BZ 15	5	--	239	--
BZ 15	5	15 g $NaNO_3$	242	244
BZ 15	5	3.0 g lysozyme	252	260
natural	0.8	3.0 g lysozyme	346	377



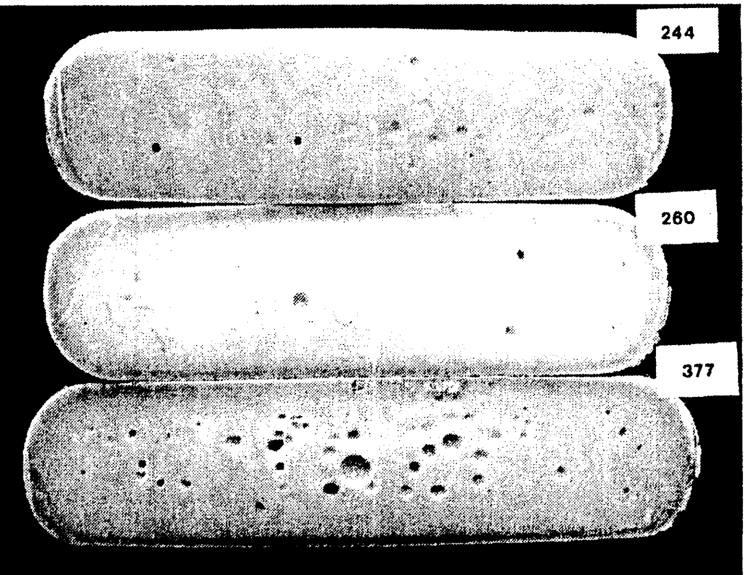


Fig. 3. Cross sections of the cheeses from the third experiment

REFNUM	AUTHOR	YEAR	TITLE	PUBLISHER
571	Adachi, N., J. Azuma, M. Janado, K. Onodera	1973	Solubilization and characterization of ovomucin without chemical modificatio	Agr. Biol. Chem., 37(9), 2175-2180
574	Robinson, D. S., J. B. Monsey	1972	Changes in the composition of ovomucin during liquefaction of thick egg white: the effect of ionic strength and magnesium salts	J. Sci. Fd Agric., 23, 893-904
582	Sleigh, R. W., G. J. H. Melrose, M. B. Smith	1973	Isolation and characterisation of hen egg white ovomucin	Biochimica et Biophysica Acta, 310, 353-360
583	Kato, A., R. Nakamura, Y. Sato	1971	Studies on changes in stored shell eggs: part VII. changes in the physicochemical properties of ovomucin solubilized by treatment with mercaptoethanol during storage	Agr. Biol. Chem., 35(3), 351-356
584	Davis, J. G., C. J. Mapes, J. W. Donovan	1971	Batch purification of ovomucoid and characterization of the purified product	Biochemistry, 10(1), 39-42
585	Thibodeau, S. N., R. D. Palmiter, K. A. Walsh	1978	Precursor of egg white ovomucoid: amino acid sequence of an NH2-terminal extension	The Journal of Biological Chemistry, 253(24), 9018-9023
596	Matsuda, T., K. Watanabe, Y. Sato	1982	Interaction between ovomucoid and lysozyme	Journal of Food Science, 47, 637-641
598	Gorbunoff, M. J.	1979	Purification of ovomucoid by hydroxyapatite chromatography	Journal of Chromotography, 12, 224 228
696	Gooding, K. M., M. N. Schmuck	1983	Purification of trypsin and other basic proteins by high-performance cation-exchange chromatography	Journal of Chromatography, 266, 633-642
817	Mokoto, I., H. Yamada, T. Yasukochi, T. Miki, T. Horiuchi, T. Imoto	1992	Left-sided substrate binding of lysozyme: evidence for the involvement of asparagine-46 in the initial binding of substrate of chicken lysozyme	Biochemistry, 31, 10322-10330
820	Mokoto, I., H. Yamada, T. Yasukochi, R. Kuroki, T. Miki, T. Horiuchi, T. Imoto	1992	Multiple role of hydrophobicity of tryptophan-108 in chicken lysozyme: structural stability, saccharide binding ability, and abnormal pKa of glutamic acid-35	Biochemistry, 31, 5545-5553

In a third experiment again four vats of cheese were made. From one quarter of a portion of naturally contaminated milk cheese was made in the usual way. The milk contained 0.8 spores of BAB/ml. Instead of nitrate 3.0 g lysozyme of preparation 2 per 100 l milk was added (600 U/ml). The remaining quantity of the milk was bactofugated. Thereafter so much of a spore suspension of C. tyrobutyricum BZ 15 was added to the milk that the number of spores of BAB amounted to 5/ml. From one third of this milk, cheese was made without any addition to prevent BAF. To another third 15 g $NaNO_3$/100 l milk was added and to the last part 3.0 g lysozyme of preparation 2 per 100 l was added. The cheeses were stored at 13 °C. After four weeks half of the cheeses from every vat were kept at 18 °C. The cheese was graded after a ripening period of 6 weeks.

Figure 3 shows cross sections of the cheese. Spores of BZ 15, a pure culture of C. tyrobutyricum, caused BAF in cheeses made from milk without any addition to prevent BAF. As was expected, an addition of 15 g $NaNO_3$/100 l prevented BAF by the strain BZ 15, but it was observed too that 600 U lysozyme/ml of cheese milk could prevent BAF by strain BZ 15 (at 5 spores/ml). On the other hand, Figure 3 shows that in cheese made from milk containing 0.8 "natural" spores/ml a clear fermentation of BAF occurred, which was more intensive when the cheese was subsequently stored at 18 °C. Lysozyme is apparently more active against spores of certain pure cultures of BAB than against "natural" BAB-spores. To demonstrate this difference in resistance to lysozyme the strains BZ 15 and Ly 5 were tested for their resistance to lysozyme by the method described in Section 2.7. The results are given in Figure 4. It appears that the strain BZ 15 is more sensitive to lysozyme than the strain Ly 5. The latter strain was isolated from a cheese which had been made with lysozyme but in which BAF occurred.

REFNUM	AUTHOR	YEAR	TITLE	PUBLISHER
1044	Wahlgren, M., T. Arnebrant	1994	Adsorption of lysozyme	COLL
1074	Payne, K. D., S. P. Oliver, P. M. Davidson	1994	Comparison of EDTA and apo-lactoferrin with lysozyme on the growth of foodborne pathogenic and spoilage bacteria	Journal of Food Protection, 57(1), 62-65
1137	Dianoux, A.-C., P. Jolles	1967	Etude d'un lysozyme pauvre en cystine et en tryptophane: le lysozyme de blan d'oeuf d'oie	Biochim. Biophys. Acta, 133, 472-479
1178	Mayes, F. J., M. A. Takeballi	1983	Microbial contamination of the hen's egg: a review	Journal of Food Protection, 46(12), 1092-1098
1179	Salton, M. R. J.	1957	The properties of lysozyme and its action on microorganisms	Properties of Lysozymes, 21, 82-99
1183	Wasserfall, F., M. Teuber	1979	Action of egg white lysozyme on Clostridium tyrobutyricum	Applied and Environmental Microbiology, 38(2), 197-199
1199	Pfeil, W., P. L. Privalov	1976	Thermodynamic investigations of proteins: I. standard functions for proteins with lysozyme as an example	Biophysical Chemistry, 4, 23-32
1206	Wahlgren, M. C., T. Arnebrant, M. A. Paulsson	1993	The adsorption from solutions of beta-lactoglobulin mixed with lactoferrin or lysozyme onto silica and methylated silica surfaces	Journal of Colloid and Interface Science, 158, 46-53
1234	Ibrahim, H. R., H. Hatta, M. Fujiki, M. Kim, T. Yamamoto	1994	Enhanced antimicrobial action of lysozyme against gram-negative and gram-positive bacteria due to modification with perillaldehyde	J. Agric. Food Chem, 42, 1813-1817
1239	Ferrarini, R.	1994	Tecnologie di stabilizzazione integrative a quella microbiologica	
1241	Amati, A., M. Simoni, G. Arfelli, A. Piva	1994	Il lisozime nell'inibizione della fermentazione malolattica: aspetti technologici	

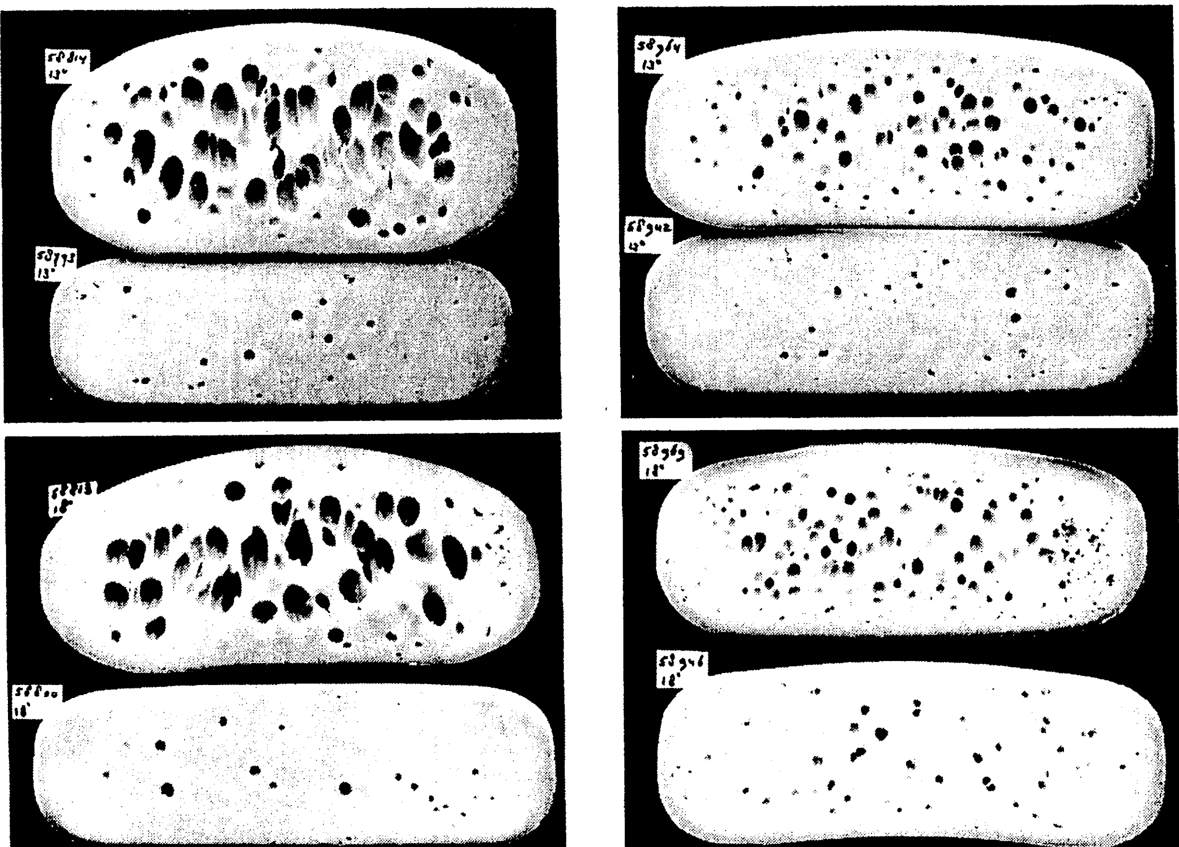


Fig 2. Effect of adding lysozyme to cheese milk on the occurrence of BAF. The spore numbers of the cheese milk were adjusted to 13 spores/ml in the milk of the first three vats and to 1.7 spores/ml in that of the fourth vat. Addition to the four vats was per 100 litres: 15 g $NaNO_3$, 3.0 g, 6.0 g and 3.0 g lysozyme respectively. Cross sections are shown of the cheese after eight weeks; above: cheeses stored at 13 °C; below: cheeses stored for four weeks at 13 °C, thereafter at 18 °C.

58793/58800 milk with 13.0 spores/ml + 15 g $NaNO_3$/100 l
58814/58813 milk with 13.0 spores/ml + 3 g lysozyme/100 l
58942/58946 milk with 13.0 spores/ml + 6 g lysozyme/100 l
58964/58969 milk with 1.7 spores/ml + 3 g lysozyme/100 l

REFNUM	AUTHOR	YEAR	TITLE	PUBLISHER
1297	Braibanti, A., E. Fisicaro	1994	Molecular thermodynamics of the denaturation of lysozyme	Thermochimica Acta, 241, 131-156
1298	Poole, S., S. I. West, C. L. Walters	1984	Protein-protein interactions: their importance in the foaming of heterogeneous protein systems	Journal of Food Agriculture, 35, 701-711
1301	Phillips, L. G., S. T. Yang, W. Schulman, J. E. Kinsella	1989	Effects of lysozyme, clupeine, and sucrose on the foaming properties of whey prtein isolate and beta-lactoglobulin	Journal of Food Science, 54(3), 743-747
1314	Powrie, W. D., S. Nakai	1985	Characteristics of edible fluids of animal origin: eggs	Department of Food Science, University of British Columbia
1316	Pellegrini, A., U. Thomas, R. von Fellenberg, P. Wild	1992	Bactericidal activities of lysozyme and aprotinin against gram-negative and gram-positive bacteria related to their basic character	Journal of Applied Biochemistry, 72, 180-187
1322	Hirs, C. H. W.	0	Chromatography of enzymes on ion exchange resins	
1329	Wang, Y.-B., G.R. Germaine	1993	Effects of pH, potassium, magnesium, and bacterial growth phase on lysozym inhibition of glucose fermentation by Streptococcus mutans 10449	J Dent Res, 72(5), 907-711
1337	Phoebe, C., Jr., K. O'Connor, G. Vella	1994	The purification of human milk whey proteins and peptides by HPLC	Waters Chromatography Division, FASEB/ASBMB Symposium - April, 1991, Poster #5541
1371	Sarkar, D., D. K. Chattoraj	1994	Excess adsorption of lysozyme and water at solid-liquid interfaces	Colloids and Surfaces B: Biointerfaces, 2, 411-417
1372	Pickersgill, R., K. Varvill, S. Jones, B. Perry, B. Fischer, I. Henderson, S. Garrard, I. Sumner, P. Goodenough	1994	Making a small enzyme smaller; removing the conserved loop structure of her lysozyme	FEBS Letters, 347, 199-202
1373	Myrnes, B., A. Johansen	1994	Recovery of lysozyme from scallop waste	Preparative Biochemistry, 24(1), 69-80

Table 2. The effect of lysozyme on the degree of BAF in cheeses made from milk contaminated with different levels of spores of BAB. Lysozyme preparation 2.

Number of spores of BAB/ml cheese milk	Addition per 100 l milk	Degree of BAF after .. weeks							
		cheese stored at 13 °C				cheese stored four weeks at 13 °C and thereafter at 18 °C			
		5	6	7	8	5	6	7	8
13.0	15 g $NaNO_3$	-	-	-	-	-	-	-	±
13.0	3.0 g lysozyme 2)	++	++++	++++	++++	+++	++++	++++	++++
13.0	6.0 g lysozyme 2)	-	-	-	±	-	±	±	+
1.7	3.0 g lysozyme 2)	-	++	++	+++	++	+++	+++	+++

1) see note 2, Table 1

2) 3.0 g lysozyme, corresponding to 600 U/ml
6.0 g lysozyme, corresponding to 1200 U/ml

REFNUM	AUTHOR	YEAR	TITLE	PUBLISHER
1484	Kato, A., H. R. Ibrahim, S. Nakamura, K. Kobayashi	1994	New methods for improving the functionality of egg white proteins	Egg Uses & Processing Technologies, New Developments, CAB International, Sim, J. S., S.
1504	Teotia, J. S., B. F. Miller	1975	Destruction of Salmonellae on poultry meat with lysozyme, EDTA, X-ray, microwave and chlorine	Poultry Science, 54, 1388-1394
1505	Samuelson, K. F., J. H. Rupnow, G. W. Froning	1985	The effect of lysozyme and ethylenediaminetetraacetic acid on salmonella on broiler parts	Poultry Science, 64, 1488-1490
1508	Jenzano, J. W., R. L. Lundblad	1988	Effects of amines and polyamines on turbidimetric and lysoplate assays for lysozyme	Journal of Clinical Microbiology, 26(1), 34-37
1509	Jenzano, J.W. , S.L. Hogan, R. L. Lundblad	1986	Factors influencing measurement of human salivary lysozyme in lysoplate anc turbidimetric assays	Journal of Clinical Microbiology, 24(6), 963-967
1523	Guo, M., G. Narsimhan	1991	Solubility of globular proteins in polysaccharide solutions	Biotechnol. Prog., 7, 54-59
1573	Busse, M., J. Heilmeier, H. Klostermeyer, M. Teuber, U. Krusch, D. Prokopek, N. Koch, S. Carini, E. Neviani, G. Mucchetti	1986	Research on lysozyme for cheesemaking in France	DMZ Deutsche Molkerei-Zeitung
1574	Busse, M., J. Heilmeier, H. Klostermeyer, M. Teuber, U. Krusch, D. Prokopek, N. Koch, S. Carini, E. Neviani, G. Mucchetti	1986	Cheesemaking with lysozyme	DMZ Deutsche Molkerei-Zeitung
1575	Lodi, R., S. Carini	1982	Lysozyme as inhibitor of Clostridial spore germination	Intern. Dairy Congress, 1(1), 507
1576	Verhamme, I., J. Storck, L. Racchelli, A. Lauwers	1988	Lysozyme (N-acetylmuramyl beta(1-4) glycanohydrolase EC 3.2.1.17): Part two: characteristics, purification, and F.I.P. turbidimetric assay of hen egg-white lysozyme	International Pharmacy Journal, 2(5), 168-171
1577	Wasserfall, F., E. Voss, D. Prokopek	1976	Research on cheesemaking; the use of lysozyme as substitute of nitrate to prevent the late blowing in cheese	Kieler Milchwirtschaftliche Forschungsbrichte, 28(1), 3-16

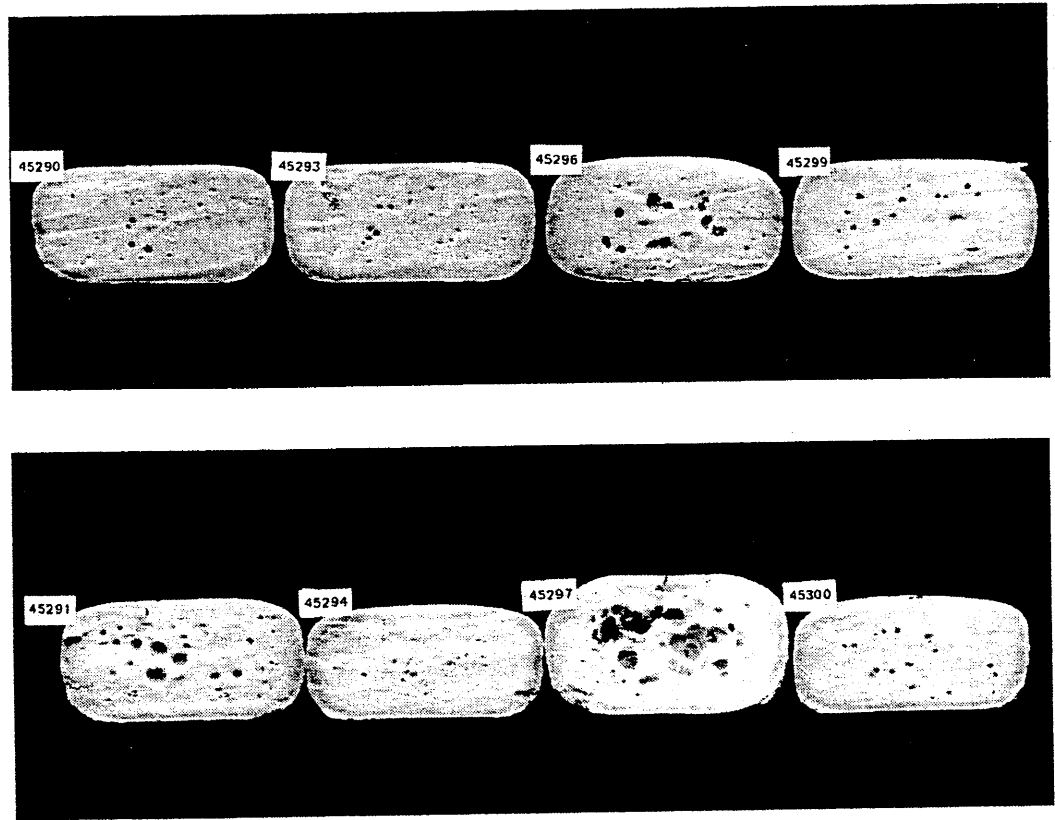


Fig. 1. Effect of adding lysozyme to cheese milk on the occurrence of BAF. The spore numbers of the milk were adjusted to 1.1, 0.27 and 13.0/ml respectively. The control cheese was made from milk with 13.0 spores/ml; 15 g $NaNO_3$/100 l was added instead of 2.5 g lysozyme. Cross sections are shown of the cheese after 24 weeks; above: cheeses stored at 13 °C; below: cheeses stored for four weeks at 13 °C, thereafter at 18 °C.

45290/45291 milk with 1.1 spores/ml
45293/45294 milk with 0.27 spores/ml + 2.5 g lysozyme/100 l
45296/45297 milk with 13.0 spores/ml
45299/45300 milk with 13.0 spores/ml + 15 g $NaNO_3$/100 l

REFNUM	AUTHOR	YEAR	TITLE	PUBLISHER
1676	Ohno, N., D. C. Morrison	1989	Effects of lipopolysaccharide chemotype structure on binding and inactivation of hen egg lysozyme	Eur. J. Biochem., 186, 621-627
1677	Nitta, K., S. Sugai	1989	The evolution of lysozyme and alpha-lactalbumin	Eur. J. Biochem., 182, 111-118
1679	Goldberg, M. E., R. Rudolph, R. Jaenicke	1991	A kinetic study of the competition between renaturation and aggregation during the refolding of denatured-reduced egg white lysozyme	Biochemistry, 30, 2790-2797
1681	Endo, T., T. Ueda, H. Yamada, T. Imoto	1987	pH dependence of individual tryptophan N-1 hydrogen exchange rates in lysozyme and its chemically modified derivatives	Biochemistry, 26, 1838-1845
1687	Eyles, S. J., S. E. Radford, C. V. Robinson, C. M. Dobson	1994	Kinetic consequences of the removal of a disulfide bridge on the folding of he lysozyme	Biochemistry, 33, 13038-13048
1691	Bae, K., H. Oh	1990	Synergistic effect of lysozyme on bactericidal activity of magnolol and honok against a cariogenic bacterium Streptococcus mutans OMZ 176	Arch. Pharm. Res., 13(1), 117-119
1698	Li, Y. M., A. X. Tan, H. Vlassara	1995	Antibacterial activity of lysozyme and lactoferrin is inhibited by binding of advanced glycation-modified proteins to a conserved motif	Nature Medicine, 1(10), 1057-1061
1729	Redfield, C., C. M. Dobson	1988	Sequential 1H NMR assignments and secondary structure of hen egg white lysozyme in solution	Biochemistry, 27, 122-136
1749	Kumagai, I., K.-i. Miura	1989	Enhanced bacteriolytic activity of hen egg-white lysozyme due to conversion trp62 to other aromatic amino acid residues	J. Biochem., 105, 946-948
1753	Smith, L. J., M. J. Sutcliffe, C. Redfield, C. M. Dobson	1993	Structure of hen lysozyme in solution	J. Mol. Biol., 229, 930-944
1764	Barbour, E. K., N. H. Nabbut, W. M. Frerichs, H. M. Al-Nakhli	1984	Inhibition of pathogenic bacteria by camel's milk: relation to whey lysozyme and stage of lactation	Journal of Food Protection, 47(11), 838-840

Table 1. The effect of lysozyme on the degree of BAF in cheeses made from milk contaminated with different levels of spores of BAB. Lysozyme preparation 1.

Number of spores of BAB/ml cheese milk	Addition per 100 l milk	Degree of BAF after .. weeks									
		cheese stored at 13 °C					cheese stored four weeks at 13 °C and thereafter at 18 °C				
		5	6	8	10	24	5	6	8	10	24
1.1	2.5 g lysozyme 1)	-	-	-	+	+	n.d.	n.d.	n.d.	++	+++
0.27	2.5 g lysozyme	-	-	-	-	-	-	-	-	-	±
13.0	2.5 g lysozyme	+	++	++++	++++	++++	+	+++	++++	++++	++++
13.0	15 g $NaNO_3$	-	-	-	-	-	-	-	-	-	-

n.d. = not done

1) 2.5 g lysozyme corresponding to 475 U/ml

2) ± = fermentation doubtful
+ = fermentation slight
++ = fermentation clear
+++ = fermentation strong
++++ = fermentation very strong

REFNUM	AUTHOR	YEAR	TITLE	PUBLISHER
1913	Sashiwa, H., H. Saimoto, Y. Shigemasa, R. Ogawa, S. Tokura	0	Lysozyme susceptibility of partially deacetylated chitin	Int. J. Biol. Macromol., 12, 295-296
1918	Naoe, K., Y. Shintaku, Y. Mawatari, M. Kawagoe, M. Imai	1995	Novel function of guanidine hydrochloride in reverse micellar extraction of lysozyme from chicken egg white	Biotechnology and Bioengineering, 48, 333-340
1924	Bernard, N., C. Jolivalt, J. Schwartzentruber	1996	Protein precipitation by caprylic acid: equilibrium composition data	Biotechnology and Bioengineering, 49, 405-411
1946	Hill, C. P., N. L. Johnston, R. E. Cohen	1993	Crystal structure of a ubiquitin-dependent degradation substrate: a three-disulfide form of lysozyme	Proc. Natl. Acad. Sci. USA, 90, 4136-4140
1947	Ekstrand, B., L. Bjorck	1986	Fast protein liquid chromatography of antibacterial components in milk: Lactoperoxidase, lactoferrin and lysozyme	Journal of Chromatography, 358, 429-433
1952	Amano, K.-i. and E. Ito	1978	The action of lysozyme on partially deacetylated chitin	Eur. J. Biochem, 85; 97-104
1953	Imoto, T., K. Yagishita	1971	A simple activity measurement of lysozyme	Agr. Biol. Chem., 35(7), 1154-1156
1973	Shugar, D.	1952	The measurement of lysozyme activity and the ultra-violet inactivation of lysozyme	Biochimica et Biophysica Acta, 8, 302-309
1975	Knorr, D., K. J. Shetty, L. F. Hood, J. E. Kinsella	1979	An enzymatic method for yeast autolysis	Journal of Food Science, 44, 1362-1365
1976	Digan, M. E., S. V. Lair, R. A. Brierley, R. S. Siegel, M. E. Williams, S. B. Ellis, P. A. Kellaris, S. A. Provow, W. S. Craig, G.	1989	Continuous production of a novel lysozyme via secretion from the yeast, Pichi pastoris	Bio/Technology, 7, 160-164
1977	Ichikawa, K., Y. Shiba, Y. Jigami, N. Serizawa	1993	Secretion and overproduction of carboxypeptidase Y by a Saccharomyces cerevisiae ssl1 mutant strain	Biosci. Biotech. Biochem., 57(10), 1686-1690

concentrate obtained, which contained about thirty times more spores than the milk, was used to adjust the number of spores in the cheese milk to the desired level.

2.7 Estimation of the resistance to lysozyme of spores of pure cultures of spores of Clostridium tyrobutyricum.

Different amounts of a filtered solution of lysozyme were mixed with TGV-agar and plates were made. Equal amounts of a spore suspension in milk, which was pasteurized for 10 min at 80 °C, were plated out on the agar. The plates were incubated and the percentages of spores estimated, which were then germinated and grown out to colonies and compared with those grown on plates without lysozyme.

3. Results and discussion

In the first experiment four vats of cheese were made. The number of spores was adjusted to 1.1, 0.27 and 13.0 spores/ml cheese milk respectively. To the milk 2.5 g lysozyme (preparation 1)/100 l cheese milk (475 U/ml) was added. Control cheeses were made from the milk with 13 spores/ml; 15 g $NaNO_3$ was added per 100 l instead of lysozyme. The results are given in Table 1, and cross sections of the cheeses are shown in Figure 1. It is clear from the results that the added amount of lysozyme could not prevent BAF in the cheese made from milk with 13.0 spores/ml. In the cheeses stored continuously at 13 °C as well as in those stored later on at 18 °C, a very strong BAF was observed. In cheeses made from milk with 1.1 spores/ml a strong fermentation was observed after storage at 13 °C for 4 weeks followed by 20 weeks at 18 °C. When the cheeses were stored continuously at 13 °C, the fermentation was only slight. No BAF was observed in the cheeses made from milk with 0.27 spores/ml.

In a second experiment four vats of cheese were also made. In one part of the milk (three quarters of the total volume) the number of spores was adjusted to 13.0/ml, in the other part (one quarter) to 1.7/ml. The first part was divided into three portions to which per 100 litres 15 g $NaNO_3$, 3.0 g and 6.0 g lysozyme were added respectively. To the milk with 1.7 spores/ml 3.0 g lysozyme was added. Lysozyme preparation No. 2 was used. The main results of this experiment are shown in Table 2 and Figure 2. From these results it is clear that the addition of 3 g lysozyme/100 l cheese milk could not prevent BAF in the cheese made from it. Even in the cheeses made from the milk with 1.7 spores/ml the fermentation was quite serious. Only when 6.0 g lysozyme/100 l was added, the BAF was nearly completely prevented during a ripening time of eight weeks.

REFNUM	AUTHOR	YEAR	TITLE	PUBLISHER
2045	Amati, A., G. Arfelli, M. Simoni, A. Gandini, V. Gerbi, C. Tortia, R. Zironi	0	Lysozyme: a new method to control malolactic fermentation	Istituto di Tecnologie Alimentari - Universita di Udine, 2-18
2054	Cartei, F., G. Cartei, V. Ceschia, S. Pacor, G. Sava	1991	Hematologic effects of oral treatment with lysozyme chloride: A phase-II stud	Current Therapeutic Research, 50(4), 530-537
2064	Verhamme, I., J. Storck, L. Racchelli, A. Lauwers	1988	Lysozyme (N-acetylmuramyl beta(1-4)glycanohydrolase EC3.2.1.17)	International Pharmacy Journal, 2(4), 129-132
2065	Chandan, R. C., R. M. Parry, Jr., K. M. Shahani	1968	Lysozyme, lipase, and ribonuclease in milk of various species	Journal of Dairy Science, 51(4), 606-607
2075	Losnedahl, K. J., H. Wang, M. Aslam, Z. Sixiang, W. L. Hurley	1996	Antimicrobial proteins in milk	Illinois Dairy Report 1996
2145	Bernstein, J. A., A. Kraut, D. I. Bernstein, R. Warrington, T. Bolin, C. P. W. Warren, I. L. Bernstein	1993	Occupational asthma induced by inhaled egg lysozyme	Chest, 103(2), 532-535
2146	Wirth, S. J., G. A. Wolf	1990	Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity	Journal of Microbiological Methods, 12, 197-205
2171	Hancock, R. E. W., P. G. W. Wong	1984	Compounds which increase the permeability of the Pseudomonas aeruginosa outer membrane	Antimicrobial Agents and Chemotherapy, 26(1), 48-52
2178	Becktel, W. J., W. A. Baase	1985	A lysoplate assay for Escherichia coli cell wall-active enzymes	Analytical Biochemistry, 150, 258-263
2185	Thomas, M. J., A. Russo, P. Craswell, M. Ward, I. Steinhardt	1981	Radioimmunoassay for serum and urinary lysozyme	Clin. Chem., 27(7), 1223-1226
2188	Reiter, B.	0	The biological significance of the non-immunoglobulin protective proteins in milk: lysozyme, lacterrin, lactoperoxidase	Chapter 10, 281-337

2.2.1 Afilact from CODIPI, Levallois Perret, France (preparation 1). We found a strength of 19 000 U/mg in this product.

2.2.2 The lysozyme preparation from SPA Società Prodotti Antibiotici SPA Milan, Italy (preparation 2). We found a strength of 20 000 U/mg in this product.

2.3 The number of spores of BAB in cheese milk was estimated according to a modification of the procedure of Van BEYNUM and PETTE (20). An MPN (Most Probable Number)-method was followed, mostly using five tubes in each of four successive 1-ml samples of milk and its decimal dilutions. However, to obtain more accurate estimations of the dilution from which partly positive and partly negative results were to be expected, fifty tubes were examined instead of five. The latter method has been described recently (21).

2.4 In some experiments the milk was "bactofugated" by means of a Westfalia Bacteria Removing Separator, type CNB 130. The removal of spores of BAB amounted to 98 %.

2.5 Pure cultures of Clostridium tyrobutyricum. For the preparation of spore suspensions use was made of the pure strain BZ 15 from the NIZO-collection. The pure strain Ly 5 was also used. This strain was isolated from cheese made from milk to which 3.0 g lysozyme/100 l cheese milk had been added but in which a strong BAF occurred. Pure cultures were obtained by separating visible colonies of BAB which could be easily detected in this cheese after a cross section had been made. After transfer to some 0.2 % solution of trisodium citrate an equal volume of milk was added and the colony was suspended in it. The mixture was heated for 10 minutes at 80 °C in a water bath after which the suspension of spores was smeared on TGV-agar which thereafter was incubated anaerobically. For the composition of TGV-agar see reference (22).

Spore suspensions were made by growing the clostridia for 3 weeks at 37 °C in AC-broth (composition Proteose-peptone No. 3: 2 %; Bacto-beef extract: 0.3 %; Bacto-yeast extract: 0.3 %; Malt extract: 0.3 %; Bacto-dextrose: 0.5 %; ascorbic acid: 0.02 %, all m/v). The broth contained after incubation about 1×10^5 spores/ml. Exact numbers were estimated by plating on TVG-agar.

2.6 Adjustment of the desired numbers of spores of BAB

In some cheese experiments use was made of a suspension with a known number of spores of Clostridium tyrobutyricum BZ 15.

In other experiments the number of clostridial spores in the cheese milk was adjusted as follows: milk was heated by thermization, standardized (fat adjusted), cooled and stored at 5 - 6 °C. The number of spores of BAB was estimated (MPN), which took four days. Then a (calculated) part of the cheese milk was bactofugated. The

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2312	Chiang, B. H., C. K. Su, G. J. Tsai, G. T. Tsao	1993	Egg white lysozyme purification by ultrafiltration and affinity chromatograph	Journal of Food Sci., 58(2), 303-306
2313	Rauch, P., I. Hochel, J. Kas	1990	Sandwich enzyme immunoassay of hen egg lysozyme in foods	Journal of Food Sci., 55(1), 103-105
2314	Howell, N. K., N. A. Yeboah, D. F. V. Lewis	1995	Studies on the electrostatic interactions of lysozyme with alpha-lactalbumin and beta-lactoglobulin	International Journal of Food Sci. and Technology, 30, 813-824

to the cheese milk up to 250 mg/kg had no effect on growth of the starter bacteria, their acid production or proteolysis in cheese. Lactobacillus helveticus, however, was inhibited to some extent and the proteolytic activity diminished. BATTISTOTTI et al. (10) observed some change in organoleptic properties in Grana cheese made from milk to which lysozyme had been added.

Gouda cheese is very susceptible to BAF. In a recent paper (11) we mentioned the critical numbers of spores of butyric acid bacteria (BAB) per ml of cheese milk which would start to cause BAF in Gouda cheese. When no nitrate is added to the cheese milk, this number is about one or two clostridial spores per 200 ml of cheese milk (0.005 - 0.01/ml). When nitrate is added to the cheese milk the critical number is higher (11). Nitrate is an effective means of preventing BAF in Gouda cheese. Low amounts are sufficient to prevent this undesirable fermentation. The contribution of nitrate in cheese to the human daily intake of nitrate is negligible (12). Neither has any indication been found that nitrate in cheese induces the formation of N-nitroso-compounds (13, 14, 15, 16). Nevertheless, some countries require the presence of only low amounts of nitrate in imported cheese.

It was therefore investigated in the Netherlands Institute for Dairy Research (NIZO) at Ede how far lysozyme could prevent BAF in Gouda cheese. The experiments are still going on, but the results obtained so far already justify some important conclusions.

2. Material and methods

2.1 The usual method of making Gouda cheese from pasteurized milk was used. In the experimental dairy Gouda cheeses of 10 to 12 kg were made from 2000 litres of cheese milk. As a control cheese was mostly made from milk to which 15 g $NaNO_3$ per 100 l had been added. In making the experimental cheeses lysozyme was added instead of nitrate. The BD-starter Bos was used in all experiments described. After 5 h, when the pH had reached a value of 5.4, the cheeses were salted as usual in a brine bath (strength 19° Beaumé) at 13 °C. The nitrate content of the brine used for the experimental cheeses was approximately 12 mg/kg, which is very low compared with the usual Gouda cheese brine. The pH reached values after 24 h between 5.10 and 5.20. The cheeses were then stored at 13 °C. After four weeks a part of the cheeses was further stored at 18 °C. During storage the gas formation in the cheeses was checked by sounding the cheeses each week, by taking X-ray photographs (17), by taking photographs of cross sections and by estimation of the redox potential (18).

2.2 The strength of the lysozyme preparations was estimated according to SHUGAR (19) (Boehringer standard; strength 22 000 U/mg).

The following preparations of lysozyme were used:

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THE USE OF LYSOZYME FOR THE PREVENTION OF BUTYRIC ACID FERMENTATION IN GOUDA CHEESE. THE LIMITED EFFECT OF THE ENZYME

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Abstract

The usual amount of lysozyme added to cheese milk (about 2.5 g/~~ml~~ 100 l or 500 U/ml) prevents butyric acid fermentation in Gouda cheese made from it if the number of spores of BAB does not exceed 0.3 spores per ml of cheese milk. The number of spores of butyric acid bacteria in winter milk in most European countries amounts at least to a level of a few spores/ml. This means that, in this concentration, lysozyme is not effective in Gouda cheese made from winter milk. The effectiveness of lysozyme against butyric acid fermentation is therefore very limited. In the discussion an explanation is given why the observation described in this paper is not in accordance with that mentioned by some other investigators.

1. Introduction

PULAY (1) found that lysozyme prevented butyric acid fermentation (BAF) in cheese. WASSERFALL and PROKOPEK (2) studied the use of this enzyme in more detail. Thereafter many data on the effect of lysozyme became available in the literature (3, 4, 5). CARINI and LODI (6) confirmed the anti-blowing effect of lysozyme in Grana and Montasio cheeses made experimentally from milk containing 0.25 - 2.3 clostridial spores per ml cheese milk. Also GHITTI et al. (7) claimed that the addition of 25 mg lysozyme hydrochloride per kg cheese milk prevented BAF in Grana Padano cheese when the clostridial spore counts in milk amounted to 2/ml. LOSI and CHIAVARI (8) reported, however, that the use of lysozyme was effective only when the spore content of the cheese milk was low.

Another question is: does the use of lysozyme affect the development of the starter bacteria (lactic acid bacteria) and the organoleptic properties of the cheese? LODI et al. (9) found that in most cases addition of lysozyme

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satisfactory until further evidence is obtained. Lysozyme appears to be of value in the control of late blowing in countries which prohibit nitrate.

Published information indicates that in other cheese types which are very sensitive to late blowing, such as Gouda, lysozyme used at the current normal addition under normal manufacturing and storage conditions is less effective than the usual amount of nitrate. In this case lysozyme cannot be considered a suitable alternative to nitrate at present.

More information will become available for the various cheese types about the critical number of spores in the raw milk to cause defects when lysozyme is used. Combinations of lysozyme addition and other control measures can then be evaluated further.

REFNUM	AUTHOR	YEAR	TITLE	PUBLISHER
3160	Tsumuraya, K., Y. Hidaka	1980	A sensitive determination procedure for lytic activity of lysozyme in foods	Journal of the Food Hygienic Society of Japan Y-shokuhin Eiseigaku Zasshi, Vol. 21 (2) 144-
3161	Shibata, T., S. Tsuji, K. Kobayashi, Y. Asai, T. Honda, K. Noda, M. Iwaida, E. Mochizuki, O. Sugahara, Y. Ito	1992	Determination of lysozyme in various foodstuffs by high performance liquid chromatography with fluorescence detector	Jpn. J. Toxicol. Environ. Health, 38(5), 437-442
3164	Various	1985	Il lisozima nella caseificazione: suo impiego in Italia e all'estero	Caglio Italiano "Chr. Hansen" s.p.a. Corsico (Milano) - Atti del Simposio
3166	Teuber, M.	1980	Lysozym als Ersatz fuer Nitrat in der Kaeserei	Milchwirtschaftliche Berichte, 63, 129-130
3177	Durance, T. D.	1994	Separation, purification, and thermal stability of lysozyme and avidin from chicken egg white	Egg Uses and Processing Technologies: New Developments, 77-93
3185	Webb, R.	2000	Malo-lactic fermentation - no longer a black hole in the winemaker's universe	The Australian Grapegrower & Winemaker, March 2000, 30-31
3192	Marchal, R., D. Chaboche, L. Marchal-Delahaut, C. Gerland, J. P. Gandon, P. Jeandet	2000	Detection and quantification of lysozyme in Champagne wines	J. Agric. Food Chem., 48(8), 3225-3231
3195	Pellegrini, A., U. Thomas, P. Wild, E. Schraner, R. von Fellenberg	2000	Effect of lysozyme or modified lysozyme fragments on DNA and RNA synthesis and membrane permeability of Escherichia coli	Microbiol. Res., 155, 69-77
3203	Bamberger, U.	2000	Neue Moeglichkeiten-durch Lysozym (New possibilities with lysozyme)	Das Deutsche Weinmagazin, 12, June 10, 32-35
3228	Pellegrino, L., A. Tirelli	2000	A sensitive HPLC method to detect hen's egg white lysozyme in milk and dair products	International Dairy Journal, 10, 435-442
3237	Holen, E., S. Elsayed	1990	Characterization of four major allergens of hen egg-white by IEF/SDS-PAGE combined with electrophoretic transfer and IgE-immunoautoradiography	Int. Arch. Allergy Appl. Immunol., 91(2), 136-141

Typical average composition of the enzyme preparation. The lysozyme hydrochloride is electrophoretically pure. The following commercial specification is relevant as shown:-

Lysozyme Hydrochloride - Food Grade

SPECIFICATIONS

a.	pH	= 3.3 ± 0.3
b.	Identification	= satisfactory
c.	Residue on ignition	= not more than 1.5 per cent
d.	Heavy metals	= not more than 10 ppm
e.	Arsenic	= not more than 1 ppm
f.	Nitrogen content	= 17.3 per cent ± 0.5 per cent
g.	Chloride content	= 3.7 per cent ± 0.5 per cent
h.	Water	= not more than 6 per cent
i.	Assay	= not less than 950 mcg/mg of lysozyme hydrochloride standard calculated on the anhydrous product (Ruyssen and Lauwers)
j.	Microbial limit tests	

Total bacterial count	= not more than 5 x 10^4 col/g
Salmonellae	= absent in 25 g
Staphylococcus aureus, Pseudomonas aeruginosa, E.coli, sulphite-reducing Clostridia	= absent in 1 g
Coliforms	= max 30 col/g

8. TECHNOLOGICAL JUSTIFICATION

In many important cheese producing countries, where the feeding of silage to cows is the normal practice, it is possible to limit the extent of contamination of milk by the adoption of good husbandry practices and effective clean milk production.

Nevertheless, it has been established over many years that additional practices employing mechanical means and more especially chemical control methods must be employed to ensure that brine-salted cheese varieties are protected from late blowing by clostridial infection.

However, there are big differences in the sensitivity to the defect of butyric acid fermentation between the brine-salted cheese varieties. This is due to the composition and desired eye formation of the particular cheese type, and also the size of the cheese, the ripening temperatures and the ripening times applied.

The published information indicates that for some cheese types, lysozyme is a suitable substance for the control of late blowing provided the number of clostridial spores is low. For these cheese types, which may be considered less sensitive to late blowing, the level of lysozyme addition already permitted by regulatory authorities in a number of countries appears to be

REFNUM	AUTHOR	YEAR	TITLE	PUBLISHER
3452	Reiter, B.	1985	The biological significance and exploitation of the non-immunoglobulin protective proteins in milk: lysozyme, lactoferrin, lactoperoxidase, xanthineoxidase	International Dairy Federation Bulletin, No 191 /.1985, 2-35
3454	Kumar, J.K., A.K. Sharma, P.R. Kulkarni	2001	A simple bacterial turbidometric method for detection of S.aureus	International Journal of Food Sciences and Nutrition
3471	Jenish, D'Arcy	2001	Cool wines, hot market: Canada's two biggest wine-makers are on an expansion binge, but their strategies are very different: one is going global while the other targets Canadians.	Maclean's, pp.38
3472	Dunphy, Stephen H.	2001	Washington state wine industry continues to grow, thrive - overview of the state's wine industry, including market size	Seattle Times
3473	Li-Chan, Eunice, S. Nakai		Biochemicals basis for the properties of egg white	Poultry Biology
3476	Hammershoj, Marianne, L. B. Larsen, R. H. Ipsen, K. B. Qvist	2001	Effect of hen egg production and protein composition on textural properties of egg albumen gels	Journal of Texture Studies, 32, 105-129
3493	Murer, G., R. Bene, K. Germann, M. Imre, P. Kotnik, G. Sauseng, M. Trinkel	2001	A new alcohol meter for wine analysis	Anton Paar GmbH
3495	Dutruc-Rosset, Georges	1999	The state of vitiviniculture in the world and the statistical information in 1999	Office International De La Vigne Et Du Vin
3497	Harvey Steiman	2001	Australian abundance: chardonnay joins Shiraz as the best of an ever-rising river of wines from the southern hemisphere	Wine Spectator
3502	O Raeker, M., L. A., Johnson	1995	Cake-baking (high ratio white layer) properties of egg white, bovine blood plasma, and their protein fractions	American Association of Cereal Chemists, Inc.
3503	Johnson, T. M.	1980	Egg albumen proteins interactions in selected food systems	UMI ProQuest Digital Dissertations

6.10 INTERACTION WITH CHEESE INGREDIENTS AND FERMENTATION PRODUCTS

The recovery of lysozyme from vat milk, whey and cheese is practically the total amount added. For the determination in cheese, in all the stages of ripening, it is enough to prepare a simple extraction by water and this confirms that the associations between lysozyme and caseins are very feable (Corradini, 1986).

In the method described by Birkkjaer et al. (1982), the cheese sample and potassium phosphate buffer (used as a cheese dissolution agent in place of citrate which has an inhibiting effect on lysozyme) are heated at 35°C for one hour to release the enzyme.

These workers pointed to the high degree of stability of lysozyme during the maturing of the cheese which allowed the lysozyme activity in the cheese to be used to calculate the amount of lysozyme which was added to the cheesemilk.

7. INFORMATION REQUIRED IN RELATION TO REGULATORY REQUIREMENTS

The following information has been provided by Corradini (1986).

Source of the enzyme preparation. The lysozyme employed in cheesemaking is extracted from hen egg white.

Main activity. For the main activity of lysozyme the information reported in section 6.1 of this paper relates.

Animal toxicity. Many studies confirm that lysozyme has an entirely negligible acute, subacute and chronic toxicity on animals (Barbara and Pellegrini, 1976).

Method of production and controls in production. The following method is reported in the patents GB 1,110,466; Germ. 1617805; Neth. 152,172; USA 3,515,643, Denmark 113,841; Fr. 1,514,474, Jap. 887,334. "Hen egg white is mixed with carboxilic resin buffered at pH 6.0 (about). The lysozyme is adsorbed on the resin from which it is eluted by NaCl solution; after precipitation at pH 10.0, the lysozyme base is dissolved with HCl to pH 3.5 to obtain Lysozyme hydrochloride precipitated by salt". During the process, spectrophotometric and microbiological controls are made, particularly on the eluted solutions and first precipitates.

Food applications. See attached list of related papers.

Activity of commercial preparations. Usually the commercial preparations have an activity in the range 95-100% on anhydrous basis, but it is possible to find commercial preparations with lower activity (about 90%).

Carriers, preservatives etc. Normally lysozyme hydrochloride preparations are without carriers, preservatives or others.

The lower activity preparations have added NaCl.

LYSOZYME PURIFICATION
Citation Information

1. Interference due to non-specific adsorption in ion-exchange chromatography of proteins: the role of initial salt concentration in the separation and analysis of lysozyme.
Ghosh, R.; Cui, Z. F. JOURNAL NAME- Journal of Liquid Chromatography ampersand Related Technologies VOL. 23 NO. 11 2000-06 PP. 1619-1626

2. Synthesis of silica-based weak cation exchanger and separation of lysozyme from egg white.
Li, R.; Chen, G. L.; Lei, J. D.; Li, B. Z.; Li, H. R. JOURNAL NAME- Fenxi Huaxue VOL. 27 NO. 5 1999-05 PP. 517-522

3. Separation of egg white lysozyme by anionic polysaccharides.
Yang, C. C.; Chen, C. C.; Chang, H. M. JOURNAL NAME- Journal of Food Science VOL. 63 NO. 6 Nov-Dec 1998 PP. 962-965

4. Direct lysozyme separation from egg white by dye membrane affinity chromatography.
Grasselli, M.; Camperi, S. A.; Navarro del Canizo, A. A.; Cascone, O. JOURNAL NAME- Journal of the Science of Food and Agriculture VOL. 79 NO. 2 1999-02 PP. 333-339

5. Egg white lysozyme purification with sweet potato ÝIpomoea batatas (L.) Lam¨ leaf preparations.
Hou, W. C.; Lin, Y. H. JOURNAL NAME- Journal of agricultural and food chemistry Nov 1997. v. 45 (11) p. 4487-4489. 1997-11

6. Lysozyme isolation from hen egg-white on fractogel TSK CM-650.
Banka, L.; Petrovic, S.; Becarevic, A. JOURNAL NAME- Lebensmittel - Wissenschaft + Technologie = Food science + technology 1993. v. 26 (1) p. 76-78. 1993

7. Purification of ovalbumin and lysozyme from a commercial product by recycling isotachophoresis.
Caslavska, J.; Gebauer, P.; Thormann, W. JOURNAL NAME- Journal of chromatography Oct 25, 1991. v. 585 (1) p. 145-152. 1991-10-25

8. Purification of lysozyme from hen egg white by affinity chromatography.
Chang, C. T.; Jen, J. C.; Sung, H. Y.; Su, J. C. JOURNAL NAME: Journal of the Chinese Agricultural Chemical Society Sept. 1986. v. 24 (3) p. 272-279. 1986-09

9. Studies on the purification of lysozyme from egg white by ultrafiltration.
Chang, C. T.; Chen, L. H.; Sung, H. Y.; Kao, M. D. JOURNAL NAME- Journal of the Chinese Agricultural Chemical Society Mar 1986. v. 24 (1) p. 86-93. 1986-03

10. Isolation of the lysozyme gene of chicken.
Baldacci, P.; Royal, A.; Cami, B.; Perrin, F.; Krust, A.; Garapin, A.; Kourilsky, P. JOURNAL NAME- Nucleic acids research. p. 2667-2681. June 25, 1979. v. 6 (8) 1979-06-25

6.6 PARTITIONING OF LYSOZYME BETWEEN CHEESE CURD AND WHEY

Wasserfall (1977) reported that a large part of the enzyme was firmly bound to the coagulum and thus was available for prevention of blowing during cheese ripening.

In experiments on Danbo cheese, Birkkjaer *et al.* (1982) measured the levels of lysozyme in the various fractions created during production using *Micrococcus lysodeikticus*. They reported that 99 per cent of the lysozyme in the cheesemilk went with the casein into the cheese and it was not inactivated during cheese ripening. These workers reported that the method which they developed for determining lysozyme in cheese was also suitable for determining the enzyme in cheese milk and whey.

Moskowitz (1986) reported the view of the National Committee of the USA that IDF should prepare an International Standard for the determination of lysozyme in milk, cheese and whey.

6.7 PURITY OF THE LYSOZYME PREPARATION

In France, lysozyme powder must meet the following legal specifications (Garnot 1984):-

The enzyme is extracted from egg white

Arsenic	not more than 1 mg/kg
Lead	not more than 3 mg/kg
Mercury	not more than 0.5 mg/kg
Cadmium	not more than 0.5 mg/kg
Total plate count	not more than 5×10^4/g
Salmonellae	none in 25 g
Pseudomonads	none in 1 g
Coliforms	not more than 30 in 1 g

Commercial preparations may have specifications for yeasts and moulds less than 30 per gram.

6.8 METHOD OF USE OF THE ENZYME

The commercial preparation in the amount required is dissolved in cold sterile water in accordance with the supplier's instructions and added to the cheesemilk prior to the addition of rennet.

6.9 FUNCTION IN THE END PRODUCT - REGULATORY STATUS

Prevention of development of clostridia and butyric acid fermentation. Moskowitz (1986) considers that lysozyme will most likely be considered a food additive.

Regulatory status for intended use still to be defined. van den Berg and Stadhouders (1986) consider that lysozyme is a food additive.

The question of the regulatory status of lysozyme is a matter for the authorities concerned and has still to be decided.

22. Egg white lysozyme purification with sweet potato (Ipomoea batatas (L.) Lam) leaf preparations.
Hou, W-C.; Lin, Y-H. JOURNAL NAME- Journal of Agricultural and Food Chemistry VOL. 45 NO. 11 1997 PP. 4487-4489

23. ISOLATION OF LYSOZYME FROM NURSE SHARK SERUM
HEDAYATI, A.; WARDEN, B. A.; SMITH, S. L. ABBREVIATED JOURNAL TITLE- ABSTR PAP AM CHEM SOC VOL. 203 NO. 1-3 1992 BIOL86 page(s)

24. PURIFICATION OF EQUINE NEUTROPHIL LYSOZYME AND ITS ANTIBACTERIAL ACTIVITY AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA
PELLEGRINI, A.; WAIBLINGER, S.; VON, FELLENBERG R. ABBREVIATED JOURNAL TITLE- VET RES COMMUN VOL. 15 NO. 6 1991 427-435 page(s)

25. MAGNETIC BIOSPECIFIC AFFINITY ADSORBENTS FOR LYSOZYME ISOLATION
SAFARIK, I. ABBREVIATED JOURNAL TITLE- BIOTECHNOL TECH VOL. 5 NO. 2 1991 111-114 page(s)

26. ISOLATION OF A HUMAN LYSOZYME OVERSECRETION YEAST MUTANT WHICH SHOWS A DEFICIENCY IN THE DEGRADATION OF INTRACELLULAR FOREIGN PROTEIN
JIGAMI, Y.; SUZUKI, K.; NAKAHARA, T.; ICHIKAWA, K. ABBREVIATED JOURNAL TITLE- YEAST VOL. 6 NO. SPEC. ISSUE 1990 S412 page(s)

27. STUDIES ON THE LYSOZYME ISOLATION BY ION-EXCHANGE CHROMATOGRAPHY
LEE, S. K.; YOO, I. J.; MIN, B. Y. ABBREVIATED JOURNAL TITLE- KOREAN J ANIM SCI VOL. 31 NO. 12 1989 780-787 page(s)

28. YEAST MUTANTS WITH ENHANCED ABILITY TO SECRETE HUMAN LYSOZYME ISOLATION AND IDENTIFICATION OF A PROTEASE-DEFICIENT MUTANT
SUZUKI, K.; ICHIKAWA, K.; JIGAMI, Y. ABBREVIATED JOURNAL TITLE- MOL GEN GENET VOL. 219 NO. 1-2 1989 58-64 page(s)

29. PURIFICATION OF CAMEL MILK LYSOZYME AND ITS LYTIC EFFECT ON ESCHERICHIA-COLI AND MICROCOCCUS-LYSODEIKTICUS
DUHAIMAN, A. S. ABBREVIATED JOURNAL TITLE- COMP BIOCHEM PHYSIOL B COMP BIOCHEM VOL. 91 NO. 4 1988 793-796 page(s)

30. PROTEIN ANALYSIS OF EARTHWORM COELOMIC FLUID IV. EVIDENCE ACTIVITY INDUCTION AND PURIFICATION OF EISENIA-FOETIDA-ANDREAI LYSOZYME ANNELIDAE
LASSALLE, F.; LASSEGUES, M.; ROCH, P. ABBREVIATED JOURNAL TITLE- COMP BIOCHEM PHYSIOL B COMP BIOCHEM VOL. 91 NO. 1 1988 187-192 page(s)

31. Purification of a lysozyme from Raphanus sativus leaves by affinity chromatography.
Lu ShunDuo; Xu FengCai; Li MingQi JOURNAL NAME- Journal of South China Agricultural University VOL. 16 NO. 4 1995 PP. 78-81

that the level was not more than 300 mg/kg in the cheese (Richardson, 1986).

6. ADDITIONAL INFORMATION CONCERNING LYSOZYME AND ITS USE IN CHEESE MANUFACTURE

6.1 MODE OF ACTION OF LYSOZYME

Lysozyme decomposes the cell wall of clostridia and other Gram-positive bacteria. The enzyme hydrolyses the peptidoglycan constituent of the bacterial cell wall thus cleaving the chemical bonds between the N-acetyl muramic acid and N-acetyl glucosamine sub-units and releasing the protoplasts whcih while retaining many vital functions, including respiration, increase in size and division are not able to have prolonged growth (Ayres, Mundt and Sandine 1980).

6.2 EFFECT ON OTHER SPOILAGE MICRO-ORGANISMS

Lysozyme has no effect on coliforms or other Gram -ve bacteria or yeasts which may cause early blowing in cheese.

6.3 EFFECT ON LACTIC ACID BACTERIA

Jøhnk (1986) reports the practical experience that lysozyme in quantities up to 100 ppm. hardly inhibits the activity of the starter culture. This agrees with the statement by Carini and Lodi (1982), but not these by Bottazzi et al., (1978) and Grazia et al., (1984) which have pointed out that in natural whey cultures used in Italy for cheese manufacture many strains of lactic acid bacteria with a very high sensitivity to lysozyme are present. Teuber (1985) reported that 500 units of lysozyme did not inhibit starter lactic acid bacteria.

It appears therefore that some selection of starter cultures may be necessary for some cheese types.

6.4 STRENGTH OF LYSOZYME PREPARATIONS

Stadhouders et al. (1985) reported using the method of Shugar (1952) to determine the strength of two commercial preparations. The two preparations tested had strengths of 19,000 u/mg and 20,000 u/mg.

By the method of Shugar one unit is that which will cause a decrease in turbidity at 450 nm of 0.001/min at 25°C and pH 7.0 of a cell suspension of Micrococcus lysodeikticus

6.5 HEAT RESISTANCE

A commercial organisation claims that its lysozyme product is heat resistant to 90°C for 10 minutes without showing any noticeable loss of strength. This property may be of value in non-sterilised process cheese.

42. Isolation of lysozyme from equine milk
Yue, Ke; Katsutoshi, Nitta ABBREVIATED JOURNAL TITLE- Huaxue Shijie VOL. 38 NO. 12 1997 PP. 666-667, 672

43. Isolation and purification of lysozyme
Long, Mi; Xie, Xiaojin; Su, Baxian ABBREVIATED JOURNAL TITLE- Zhongguo Yiyao Gongye Zazhi VOL. 28 NO. 10 1997 PP. 470-472

44. Separation and purification of soft-shelled turtle lysozyme
Araki, Tomohiro; Yamamoto, Takaki; Torikata, Takao ABBREVIATED JOURNAL TITLE- Kyushu Tokai Daigaku Nogakubu Kiyo VOL. 17 1998 PP. 35-40

45. Isolation and purification of lysozyme from the Chinese oak silkworm pupae
Lou, Lixin; Yao, Ruhua; Huang, Ziran ABBREVIATED JOURNAL TITLE- Gongye Weishengwu VOL. 27 NO. 1 1997 PP. 21-24

46. A Newly Designed Adsorbent Prepared from Hydroxyapatite Originating from Cattle-Bones for Chromatographic Separation of Albumin and Lysozyme
Akazawa, Toshiyuki; Kobayashi, Masayoshi; Kodaira, Kohei ABBREVIATED JOURNAL TITLE- Bull. Chem. Soc. Jpn. VOL. 70 NO. 9 1997 PP. 2323-2329

47. Liquid-liquid phase separation in supersaturated lysozyme solutions and assocd. preçipitate formation/crystallization
Muschol, Martin; Rosenberger, Franz ABBREVIATED JOURNAL TITLE- J. Chem. Phys. VOL. 107 NO. 6 1997 PP. 1953-1962

48. Characteristics of lysozyme . Part II. Isolation and possibilities of practical applications
Kopec, W.; Trziszka, T. ABBREVIATED JOURNAL TITLE- Przem. Spozyw. VOL. 51 NO. 3 1997 PP. 36-37

49. Study on separation of conalbumin and lysozyme from high concentration fresh egg white at high flow rates by a novel ion-exchanger.
Ming, Fang; Howell, John; Acosta, Fernando; Hubble, John ABBREVIATED JOURNAL TITLE- BIOTECHNOL BIOENG. vol. 42, no. 9, pp. 1086-1090 1993

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51. Thermodynamic study of protein-induced lipid lateral phase separation . Effect of lysozyme on mixed lipid vesicles.
Raudino, A.; Castelli, F. ABBREVIATED JOURNAL TITLE- COLLOID POLYM SCI. vol. 270, no. 11, pp. 1116-1123 1992

5.4 NETHERLANDS

A preliminary report (Stadhouders, de Vries, van den Berg and van der Veer (1985)) has been prepared of full scale investigations in the Netherlands of the use of lysozyme to prevent butyric fermentation in Gouda cheese made from milk containing natural spore contamination. These experiments deal in detail with the critical level of spores in relation to late blowing and on the effects of ripening temperature and lysozyme level. In the meantime it has been proven that cheese made from summer milk with the usual amount of lysozyme is not always safe from butyric acid fermentation. From the results obtained it appears that the usual amount of lysozyme added to cheese milk (500 U/ml) can only prevent butyric acid fermentation in the Gouda cheese made from it, if the number of spores of butyric acid bacteria does not exceed 0.3 spores per ml of cheesemilk. Such a number occurs mostly in the cheese milk produced during summer time. In most European countries the number of spores in the milk produced in winter amounts to at least a few spores/ml. Doubling the amount of the added lysozyme was more effective, also in cheese made from milk with 13 spores/ml. In this case the costs of the treatment are then also doubled. Moreover, recent findings indicate that also in those cases the gas hole formation in the cheese was still somewhat too large. The addition of 6 g of lysozyme/100 l cheese milk did not inhibit the growth and acid production of the mesophilic starter. Up till now, lysozyme is not legally permitted. Stadhouders, Stegink and van den Berg (1986) consider why the effectiveness of lysozyme is very limited against butyric acid fermentation in Gouda cheese.

5.5 FRANCE

In France the use of lysozyme in cheese factories has been temporarily allowed since 1981 (Garnot (1985)). The authorisation was renewed in 1984. At present the use of lysozyme is restricted to hard cooked cheese and hard cheese (such as Saint Paulin) except the cheeses with an 'appelation d'origine' e.g. Beaufort, Comte. The maximum quantity allowed is 30 mg per litre of milk or a residual amount of 400 mg per kg of the final product.

It is estimated (Cretin-Maitenaz (1985)) that around 20 to 25 per cent of the Emmental cheese made in France is produced using lysozyme addition. Bactofugation is frequently given as a preliminary treatment in addition to lysozyme addition.

The Institut Technique du Gruyere (1986) has investigated the effect of lysozyme in different French factories producing Emmental. Lysozyme was added to cheesemilk at 25 mg per litre. If carefully selected strains of lactobacilli were used the effect of the added lysozyme on acidification was very weak. A decrease of 99% in spore level was observed. The quality of the finished cheese was improved:- plus 12.8% in Class A and minus 17.5% in Class D.

5.6 AUSTRALIA

Lysozyme is specifically approved for use by one cheese manufacturer on a 2 year trial basis due to expire in July 1986. Its use/need will be reviewed in Autumn 1986. The maximum level of lysozyme permitted for use in the milk for the manufacture of Swiss, Gouda and Edam cheeses should be such

LYSOZYME CHARACTERIZATION
Citation Information

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Unanue, Emil R.; Suri, Anish; Latek, Robert R.; Kanagawa, Osami; Fremont, Daved JOURNAL NAME- FASEB Journal VOL. 14 NO. 6 April 20, 2000 PP. A1158.

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Wibbenmeyer, J. A.; Xavier, K. A.; Shick, K. A.; Willson, R. C. JOURNAL NAME- Immunotechnology (Amsterdam) VOL. 2 NO. 4 1996 PP. 281

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Bolin, K. A.; Pitkeathly, M.; Miranker, A.; Smith, L. J.; Dobson, C. M. JOURNAL NAME- Journal of Molecular Biology VOL. 261 NO. 3 1996 PP. 443-453

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Elagamy, E. I.; Ruppanner, R.; Ismail, A.; Champagne, C. P.; Assaf, R. JOURNAL NAME- International Dairy Journal VOL. 6 NO. 2 1996 PP. 129-145

6. Cloning and characterization of a bacteriophage lysozyme gene from Haemophilus somnus.
Pontarollo, R. A.; Rioux, C. R.; Potter, A. A. Donachie, W., F. A. Lainson and J. C. Hodgson (Ed.). Haemophilus, Actinobacillus, and Pasteurella; Third International Conference on Haemophilus, Actinobacillus, and Pasteurella (HAP94), Edinburgh, Scotland, UK, 1994. viii+245p. Plenum Press: New York, New York, USA; London, England, UK 1995 PP. 208

7. Characterization of lysozyme crystals with unusually low solvent content.
Nagendra, H. G.; Sudarsanakumar, C.; Vijayan, M. JOURNAL NAME- Acta Crystallographica Section D Biological Crystallography VOL. 51 NO. 3 1995 PP. 390-392

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9. Preparation and preliminary characterization of crystallizing fluorescent derivatives of chicken egg white lysozyme
Sumida, J. P.; Forsythe, E. L.; Pusey, M. L. ABBREVIATED JOURNAL TITLE- J. Cryst. Growth VOL. 232 NO. 1-4 2001 PP. 308-316

2. The lysozyme influences also the lactic acid bacteria development, as is demonstrated by a slower increase of the acidity in cheese whey. Consequently, when lysozyme is employed, fast fermentation with gas production is more possible in Grana cheese produced with raw milk of poor microbiological quality and, particularly, in Provolone cheeses also if produced with pasteurised milk. Often in Grana cheese the fast blowing when lysozyme was employed is due to the development of propionic acid bacteria.

   Moreover, the natural whey culture, as traditionally used in Grana cheesemaking, was sometimes unsatisfactory.

3. Under commercial conditions, the combination of lysozyme with other means or agents (i.e. formaldehyde, bactofugation or other technological improvements) give very good results. Particularly, the combined anti-blowing effects of lysozyme and formaldehyde is higher than either of the single treatments.

4. For the preparation of natural starter culture, it is necessary to have cheese whey with a very low content of lysozyme.

5. For Provolone and other cheeses the available results are not sufficiently clear to forecast the results of lysozyme use under commercial conditions. However, Battistotti et al. (1986) have pointed out that in Provolone cheese the blowing defects are only seldom "late" blowing by butyric acid fermentation, instead the blowing defect in this cheese often comes out in the beginning of the ripening period and is caused by microorganisms against which lysozyme is ineffective.

5.3 DENMARK

Danish experiments (Birkkjaer, Forsingdal, Braun, Madsen and Hansen (1982)) were concerned with use of the enzyme in the manufacture of Danbo, described as a round-holed cheese. Lysozyme was added at 1-2 g/100 l milk and was found to be as effective as potassium nitrate added at 10 g/100 l milk for preventing late blowing of Danbo cheese due to Clostridium tyrobutyricum. Lysozyme had no adverse effects on cheese quality. A combination of lysozyme with KNO_3 for control of late blowing was recommended. The use of lysozyme in cheese production has been legally permitted since early 1984.

Workers at the Danish Government Research Institute, Hillerød, report (Herlev-Jensen, 1986) that strains of Clostridium tyrobutyricum vary in their ability to cause late blowing in Danbo cheese. The same strain may on one occasion produce late blowing while on another it may not. A satisfactory explanation for this variability is not yet available but it is thought that conditions during cheese maturation may have some influence.

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In industrial-scale experiments in Germany (F.R.), Edam and Tilsit type cheese were made in two cheese factories on a 2000 to 5000 l basis. The natural spore content of the milk was around 20 spores per ml. The conclusion was reached that there were no significant differences between lysozyme and nitrate cheeses except in regard to the nitrate content itself. The sensory properties of the cheese were highly acceptable in both instances and represented the usual quality standards of the factory. The lysozyme cheese could be stored for another 6 to 9 months at 12-13°C without blowing problems. Control cheese made without either lysozyme or nitrate was blown after 5 weeks. Similar results were found with Tilsit type cheese. In both cases the experiments were repeated successfully a second time.

From the results obtained it appeared that lysozyme could be used as a substitute for nitrate under the conditions described.

It is emphasised that the action of lysozyme could be aided by controlling the cheese production and ripening conditions (pH value, low ripening temperature, salt concentration).

In the next amendment of the Cheese Laws of Germany (F.R.) the use of lysozyme will be permitted.

van den Berg and Stadhouders (1986) point out that there is a difference in sensitivity between the various strains of Clostridium tyrobutyricum present in the raw milk, when they do not grow in the presence of lysozyme. This helps to explain the different results obtained in cheese experiments carried out with inoculated milk in comparison to those using 'normally contaminated' milk.

5.2 ITALY

Extensive trials have been undertaken in Italy (Emaldi and Corradini, 1985,a) and in August, 1983 the use of lysozyme was legalised for a period of three years in the manufacture of Grana Padano, Provolone, Montasio and Asiago cheese at the maximum amount of 25 mg/l of cheese milk and provided that will not residue more than 300 ppm in the finished cheese.

In September 1986, this authorisation was renewed until 5th September, 1988 (Ministero della Sanita, 1986).

At a meeting held in 1985 under the sponsorship of the Italian Ministries of Agriculture and Forestry and Health the following conclusions were reached (Corradini and Emaldi (1985 b)):-

1. The effectiveness of the lysozyme for the control of butyric fermentations has been proved, but, under commercial conditions, it is possible to have good results in the control of the late blowing defect in Grana Padano cheesemaking only when the Cl. tyrobutyricum spores content is not too high (i.e. the effectiveness is normal when the spores content is in the range of the hundreds per litre of cheesemilk).

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Biological control. Perfiliev and Gudkov (1982) reported that a bacterial preparation - Bioantibut - consisting of a concentrate of lactic acid bacteria exerted an antagonistic action on butyric acid bacteria and when used by the cheesemaking industry in the USSR resulted in the production of cheese with practically no defects caused by butyric acid fermentation. Group B12 wish to have further information on this measure and its ability to prevent late blowing in various cheese varieties.

5. THE USE OF LYSOZYME TO CONTROL BUTYRIC ACID FERMENTATION

Lysozyme (Muramidase) (IUPAC No. 3.2.1.17) is widely distributed in animals and plants. It is present in milk and egg white where it amounts to 0.4 to 0.5 per cent. It was first described in 1922 by Fleming (1922).

Commercial preparations are prepared by the extraction of the enzyme from egg white and may be 98-99 per cent pure lysozyme.

The potential of lysozyme to control butyric acid fermentation in cheese was reported by the Hungarian dairy scientist Pulay (1966) in a contribution to the 17th International Dairy Congress. He was able to demonstrate that late blowing was prevented by the addition of 0.1-2.0 per cent fresh egg white or 0.001 per cent muramidase.

From the late seventies until the present there has been increasing interest in the possibility of using lysozyme as a means of controlling butyric acid fermentation. This interest has been at scientific, technological and commercial levels in several countries.

5.1 FEDERAL REPUBLIC OF GERMANY

The results of laboratory and pilot experiments carried out in Germany (F.R.) have been summarised by Teuber (1985) as follows:-

(a) Lysozyme (from hen egg white) is stable in cheese.

(b) Around 80-90% of the lysozyme becomes bound to the cheese curd.

(c) 500 units of lysozyme per ml of cheesemilk are sufficient to inhibit clostridia without inhibition of starter lactic acid bacteria.

(d) It is important to use crystalline lysozyme dissolved in water. The use of egg white or egg powders leads to an uneven distribution in milk and cheese and does not control late blowing.

(e) The lysozyme must be applied on an activity basis, not a weight basis.

(f) The development of lysozyme-resistant spores was not observed even if lysozyme resistant vegetative cells formed easily.

(g) There were no, or only minor, effects of lysozyme on cheese texture and flavour.

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Good husbandry and hygienic milking practices may lower the spore level by a factor of 10 compared to unclean conditions, however, the spore level may still be dangerously high from the cheesemaker's viewpoint. Many people in the USA believe that the problem of late blowing has been successfully overcome through good sanitation practices (Moskowitz, 1986). van den Berg and Stadhouders, (1986) point out that it cannot be expected that all good husbandry practices and other measures at the farm together, will bring down the contamination in practice to under 5-10 spores per litre. It is necessary therefore for farm level measures to be complemented by control measures at the factory.

4.2 At the Cheese Factory

Segregation of supplies. It is possible under certain conditions to select and bulk the milks which are less likely to be contaminated with clostridial spores for use in cheesemaking. This approach is based on the use of the remainder of the milk for other purposes and on the knowledge that silage feeding is not practiced by all farms. This measure is not suitable for areas where silage is used by all farms or where the milk supplies are going to large specialised cheese factories.

Mechanical methods. Cheese milk can be purified by bactofugation and research has shown that the equipment available to the dairy industry can reduce the spore level in contaminated milk only by about 90% which may not in itself be sufficient to clean normally contaminated milk and avoid the problem of butyric acid fermentation. This technique may be at its limit when the level of contamination is from 2000-3000 spores per litre of milk. In practice, bactofugation reduces the spore level by 65 to 80% and it is very difficult to reach 90% reductions (Institut Technique du Gruyere, 1986). van den Berg and Stadhouders (1986) are of the opinion that even in the summer, bactofugation by itself, cannot achieve the required reduction in spore level.

A process, 'cremage', employs special equipment for separating the milk into two fractions - 70% of the milk which is not contaminated with butyrics, and the remaining 30% of the milk which contains the contaminants. The latter fraction is subjected to bactofugation before being recombined with the larger portion and used for cheesemaking (Kerjean et al., 1985).

Use of Chemicals. Nitrate is an effective means of preventing butyric acid fermentation in Gouda cheese. Small amounts are sufficient to prevent this undesirable fermentation. The contribution of nitrate in cheese to the human daily intake of nitrate is considered to be negligible. Also there is no indication that nitrate in cheese induces the formation of N-nitrosocompounds. Nevertheless some countries require the presence of low amounts of nitrate in cheese when imported.

For many years, Italian law has permitted the use of formaldehyde, at approximately 25 ppm, to prevent late blowing of Grana Padano and Provolone cheese.

LYSOZYME AS ANTIMICROBIAL AGENT IN FOODS
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3.2 Contamination of milk supplies. Studies in the Netherlands (Stadhouders, Hup, Nieuwenhof, 1985) indicated that in summer the contamination is higher than the critical level of 5-10 spores per litre for Dutch cheese. In the Netherlands the contamination in summer amounts to 200-1000 spores per litre and in winter to 2,000-20,000 per litre.

Grass and maize silages are common feeds for milk cows in many countries. The feeding of silage is recognised as a major source of contamination of raw milk with the pasteurisation-resistant spores of Clostridium tyrobutyricum.

In Italy in the Po valley in the areas in which feeding of silage is practised, the milk contamination amounts to 1000-6000 spores per litre in winter and in summer to 300-1500 per litre. (Botazzi et al., 1982).

The most likely source of contamination is faecal material containing such spores. There is a clear correlation between the numbers of spores in silage, cow dung and raw milk.

There is general agreement on the explanation for the abnormal presence of clostridial spores in milk and on the process of contamination:- the spores originate in soil which contains between 500 and 87,000/g of dry matter.

It has been established that hay contains few clostridial spores while maize silage - especially that made from the whole plant, and grass silage, frequently contain dangerously high numbers of spores which lead to dung being produced which if allowed to contaminate the milk is likely to lead to late blowing of cheese.

4. TRADITIONAL CONTROL MEASURES TO REDUCE THE CHANCES OF BUTYRIC ACID FERMENTATION

4.1 At the Farm. In the Netherlands the spore level is taken into account in the payments made to farmers for milk (van den Berg and Stadhouders, 1986).

In certain countries e.g. Switzerland, the Bavarian Alps in Germany (F.R.) and parts of France the prohibition of silage feeding results in milk and cheese which have low numbers of anaerobic lactate-fermenting clostridia.

However the feeding of silage is practised on very many dairy farms throughout the world and the quality of the silage from the point of view of the extent of the presence of clostridial spores depends on many factors e.g. materials used - whether grass or maize, method of production e.g. chemical conservation. A procedure based on the use of formic acid and fermentable carbohydrates has been used in Finland and has been shown to give a silage low in anaerobic spore formers (Teuber, 1985). Where mixing of the fermentation aids with the grass is incorrect the spore level will frequently exceed 1 per ml (van den Berg and Stadhouders, 1986).

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THE USE OF LYSOZYME IN THE PREVENTION OF LATE BLOWING IN CHEESE

1. THE DEFECT

The fault in cheese known as 'late blowing' which is typified by abnormal levels of open texture and accompanying unattractive odours and flavours may be a serious problem in several important varieties of semi-hard and hard cheese including Gouda, Emmental, Grana Padano, Provolone, Danbo and other brine-salted types.

2. THE CAUSE

The cause of 'late blowing' in cheese is the butyric acid fermentation brought about by the germination and growth of clostridial spores, especially those of Clostridium tyrobutyricum, present in the cheese milk. As a result of the butyric fermentation in the cheese, considerable amounts of carbon dioxide and hydrogen are formed and result in blowing of the cheese. At the same time, organic volatile acids are formed during the fermentation and affect the flavour and taste of the cheese.

3. THE SOURCE OF THE CAUSAL ORGANISMS

3.1 Critical numbers of clostridial spores to cause butyric acid fermentation. It has been established that the critical numbers of clostridial spores to cause butyric acid fermentation in 12 kg Gouda cheese are from 5 to 10 per litre (van den Berg et al., 1980). In Dutch investigations an important point with respect to the critical number of spores is the criterion that the presence of somewhat enlarged holes in the cheese of a type like Gouda is a sign of a defective cheese and the result of butyric acid fermentation (van den Berg and Stadhouders, 1986).

Corradini (1986) is of the opinion that 5-10 clostridial spores per litre is too low a level of contamination to cause butyric acid fermentation in Grana cheese.

In the most common German semi-hard cheese of the Gouda, Edam and Tilsit types the presence of about 200 spores per litre of cheesemilk are sufficient to cause late blowing (Teuber, 1985). During the silage feeding season, this level of spores is frequently exceeded in cheese factories in North Germany.

The critical number of spores to bring about butyric acid fermentation which will result in cheese defects probably varies with the type, since between the different brine-salted semi-hard and hard cheeses there are large differences in shape and size of the cheese, pH value, ripening time, ripening temperature and in structure including normal eye formation (van den Berg and Stadhouders, 1986).

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52. Properties and clinical use of quasi-liquid form of lysozyme
Strelets, E. V.; Egorova, E. N.; Chernin, V. V.; Chervinets, V. M.; Bazlov, S. N. ABBREVIATED JOURNAL TITLE- ZHURNAL MIKROBIOLOGII, EPIDEMIOLOGII I IMMUNOBIOLOGII ÝZh. Mikrobiol. Epidem. Immunobiol.¨ no. 3, pp. 14-17 2001-05-00

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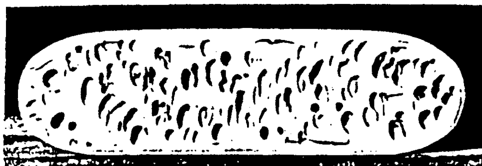

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THE USE OF LYSOZYME IN THE PREVENTION OF LATE BLOWING IN CHEESE

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The report has been prepared by Group B12 (Chairman: Dr R.J.M. Crawford of the U.K.) and approved for publication by Commission B in 1986. In view of the dynamic nature of the subject, Group B12 is keeping an interest in reports being produced by research workers and industry.

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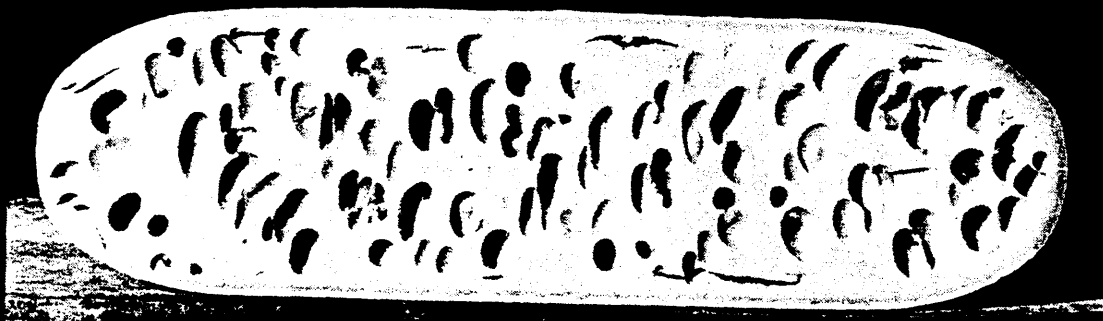
BULLETIN

THE USE OF LYSOZYME IN THE PREVENTION OF LATE BLOWING IN CHEESE



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Lysozyme and Other Proteins in Eggs

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As the vessel that is designed for the embryogenesis of a bird or reptile, it is not surprising that an egg contains numerous antimicrobial proteins. By far the most widely studied of these is the small, but significant, lytic enzyme known as lysozyme. This enzyme has the ability to lyse specific bacteria. It is used in several food and pharmaceutical products. Three other egg proteins (avidin, ovotransferrin and yolk immunoglobulins) have antimicrobial properties of particular interest for application in foods and pharmaceuticals.

Occurrence

Lysozyme was first identified in 1921 in human nasal secretion by Alexander Fleming, who would later discover penicillin. Lysozyme has since been isolated from human tears, saliva and mother's milk, as well as viruses, bacteria, phage, plants, insects, birds, reptiles and other mammalian fluids. Commercially, the most readily available source of lysozyme is the egg white of the domestic chicken (*Gallus gallus*). Lysozyme is probably the most intensively studied of all proteins.

Lysozyme is not, however, the only antimicrobial protein in avian eggs. **Table 1** lists some of the proteins present in avian egg white. Most research to date has focused on the proteins of egg white, perhaps because they can readily be separated by ion-exchange chromatography.

Most yolk proteins, on the other hand, tend to be less water soluble, and because of their close association with lipids, they are somewhat less easily extracted and purified on a large scale. Nevertheless, there are antimicrobial proteins present in the yolk, including immunoglobulins (IgY – the chicken equivalent of IgG) and trace amounts of a biotin-binding protein. It has been proposed that antimicrobials in yolk work primarily to provide the developing chick with passive protection, as is the case with the immunoglobulins.

The four egg proteins that appear at present to have the greatest potential as natural antimicrobials in food and pharmaceutical applications are lysozyme, avidin, ovotransferrin and IgY. The remainder of this article focuses on the properties and applications of these particular egg proteins.

Table 1 Some of the major proteins in egg white and their antimicrobial functions

Protein	*Solids (%)*	*Antimicrobial function*
Ovalbumin	54	Unknown
Ovotransferrin	12	Binds multivalent cations, particularly iron
Ovomucoid	11	Inhibits trypsin and other proteases, antimicrobial properties
Ovoinhibitor	15	Inhibits trypsin, chymotrypsin and other proteases
Ovomucin	3.5	Increases viscosity of egg white preventing bacterial movement
Lysozyme	3.4	Lyses peptidoglycan layer of some Gram-positive organisms
Ovoflavoprotein	0.8	Binds riboflavin (vitamin B_2)
Ovomacroglobulin	0.5	Protease inhibitor
Ficin inhibitor (cystatin)	0.05	Inhibits cysteine proteases
Avidin	0.05	Binds avidin, making it unavailable to organisms

Data from: Ibrahim HR (1997) *Insights into the structure–function relationships of ovalbumin, ovotransferrin, and lysozyme*. pp. 37–56. In: *Hen Eggs Their Basic and Applied Science*. (1997) Yamamoto T, Juneja LR, Hatta H and Kim M (eds) Boca Raton, Florida: CRC Press. Li-Chan ECY, Powrie N and Nakai S (1995) The chemistry of eggs and egg products. In: Stadelman WJ and Cotterill OJ (eds) *Egg Science and Technology*. Pp. 105–175. New York: Food Products Press.

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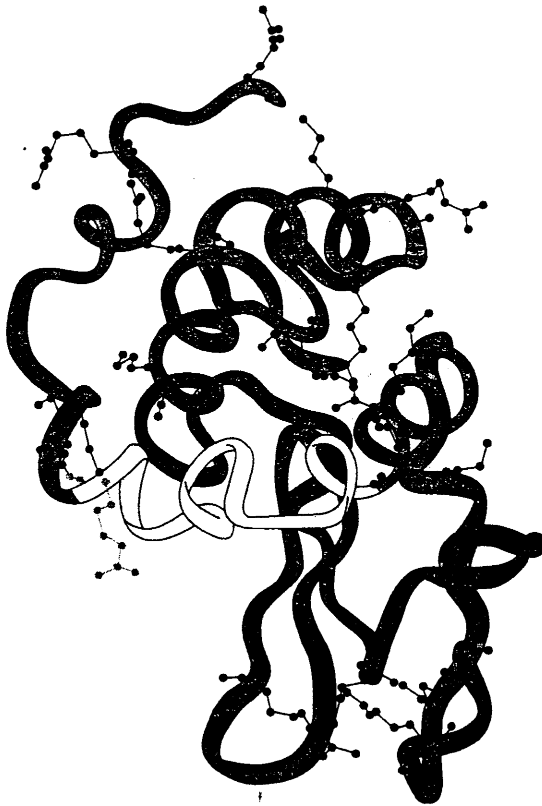

Figure 1 Three-dimensional structure of chicken lysozyme. The active site is highlighted by shading. (Courtesy of Canadian Inovatech Inc., Abbotsford, British Columbia, Canada.)

Structure

It is now recognized that lysozymes from diverse sources fall into several structural classes, with the three most common being type c (chicken), type g (goose) and type v (viral). Chicken lysozyme is composed of 129 amino acid residues with a molecular weight of approximately 14 000.

Figure 1 shows the three-dimensional structure of chicken lysozyme. Lysozyme is used extensively as a model enzyme, partly because it contains all of the twenty common amino acids. An α-helix links two domains of the molecule; one is mainly β-sheet in structure, and the other primarily α-helical. The hydrophobic groups are mainly oriented inward, with most of the hydrophilic residues on the exterior of the molecule.

It is proposed that the enzymatic action of the molecule is dependent on its ability to change the relative position of its two domains by hinge bending. Essentially, the small α-helical connection, or hinge, between the domains can bend sufficiently to cause large conformational changes in the molecule. This permits the enzyme to engage its substrate.

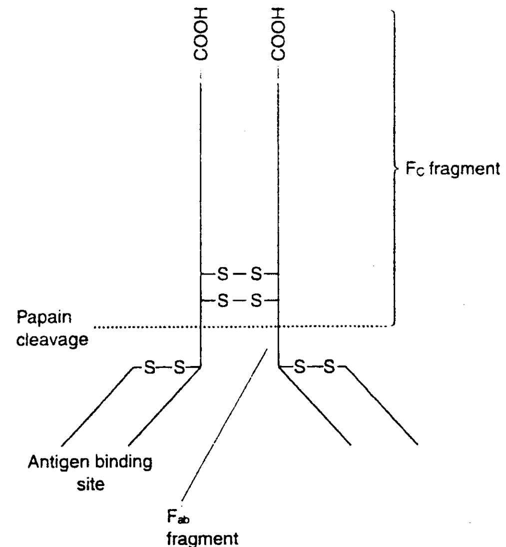

Figure 2 Schematic diagram of a typical immunoglobulin molecule. S-S represents disulphide bonds. F_{ab} is the antigen binding portion of the molecule. F_c is the heavy chain portion of the molecule.

Avidin is a glycoprotein composed of four identical subunits, each with 128 amino acid residues. The molecular weight of the entire molecule is around 67 000. A disulphide bridge links residues four and 83. There are four tryptophan residues per subunit.

Ovotransferrin, also known as conalbumin, is a glycoprotein with a molecular weight of 78 000. It contains two lobes connected by an α-helix. Each lobe is homologous, and can bind an Fe^{+++} ion. The iron-binding site in each lobe is situated between two subdomains. The presence of bicarbonate ion enhances the binding of iron to the molecule.

IgY is similar in structure to mammalian IgG, but with a higher molecular weight (170 000). **Figure 2** shows a schematic representation of the basic structure, with two heavy chains and two light chains, all connected by disulphide linkages, as indicated. The antigenic site is found in the F_{ab} fragment, which can be cleaved from the molecule by means of papain hydrolysis.

Properties

Lysozyme has an extremely high isoelectric point (> 10) and consequently is highly cationic at neutral or acid pH. In solution, lysozyme is relatively stable at pH 3–4 and can withstand near boiling temperatures for a few minutes. As the pH increases, however, its stability decreases. At pH 5.5–6.5 (the pH of many dairy products), lysozyme is stable up to about 65°C. Beyond this point, denaturation accelerates rapidly with temperature. Thus, many precooked food products, in which temperatures of 69–75°C are

their catalytic and proteinase-inhibiting activities respectively, still retained the bactericidal properties. Non-enzymatic killing of two oral streptococci by denaturated human placental lysozyme has been shown by others (Laible & Germain 1985). To our knowledge, however, the same effect with respect to Gram-negative bacteria has not been reported previously. Similarly, the bactericidal properties of the cathepsin G of human neutrophil granules remained unaltered after destruction of its catalytic activity by heating to 90°C (Odeberg & Ohlsson 1975). The antibacterial activity of cationic proteins may be an important factor of non-specific immunity. It has been studied primarily with respect to cationic proteins of neutrophil granules (Gennaro *et al.* 1983; Spitznagel 1984; Ganz *et al.* 1985). Aprotinin and lysozyme are also closely related to host defence affecting Gram-negative and Gram-positive bacteria. There were major differences, however, in the species affected. Lysozyme, for example, did not attack *Staph. aureus* either by muramidase activity or by basic protein mechanism. Lack of cell wall lysis of *Staph. aureus* by lysozyme has been observed previously (Salton & Pavlik 1960). On the other hand, Odeberg & Ohlsson (1975) reported that cathepsin G preferentially kills *Staph. aureus.*

In contrast to previous results (Respaske 1958; Feingold *et al.* 1968; Wilson & Spitznagel 1968; Davis *et al.* 1980), the present study showed that chicken egg white lysozyme inhibited bacterial growth of Gram-negative bacteria without pre-treatment with complement or EDTA.

The cationic property may only be but one important factor for antibacterial activity. The cationic proteinase inhibitors, which we have previously isolated from equine granules (Pellegrini *et al.* 1988), were not bactericidal against the bacterial strains used in this work. Lysine-rich histones were reported to be only slightly bactericidal (Odeberg & Ohlsson 1975), whereas poly-D-lysine and polyarginine had pronounced bacterial killing activity (Laible & German 1985) (the fact that synthetic basic polypeptides were also bactericidal does not *per se* diminish the biological significance of the natural compounds). Hydrophobic interactions were also suggested as possible mechanisms of human defensins (Wilde *et al.* 1989). The HNP-4 defensin, which was 100 times more active against *E. coli* than the other defensins, was also more hydrophobic as judged by its retarded elution from reversed phase chromatography.

We observed an analogous situation, since lysozyme, which was at least 20 times more bactericidal than aprotinin, was eluted at a higher acetonitril concentration than aprotinin in reversed phase chromatography.

It cannot be excluded that additional unknown structural requirements may be necessary for optimal complementarity between cationic proteins and receptors in various bacterial strains. Incubation of *E. coli* with aprotinin or lysozyme led to similar ultrastructural alterations: swelling of the bacterial body and disintegration of cytoplasm. These alterations may have occurred secondary to changes in bacterial wall permeability (Odeberg & Ohlsson 1976). The bacterial wall itself did not disintegrate which was also found in freeze-fracture replicas of *E. coli* incubated with cationic antibacterial proteins of leucocytes (van Houte *et al.* 1977). Thus the effect of lysozyme on *E. coli* could not be primarily related to its muramidase activity. Immunolabelling revealed that both aprotinin and lysozyme were predominantly present within the affected cytoplasm. How cationic proteins gained access to cytoplasm is not understood. Since aggregated and denaturated aprotinin and lysozyme were also detected intracellularly, one might speculate that both proteins were partially degraded by bacterial enzymes without affecting antigenic determinants and functional sites for destruction of bacterial components but enabling them to penetrate the bacterial wall. The basic protein mechanism adds to our understanding of the antibacterial activity and of the biological meaning of lysozyme which is widely distributed in higher vertebrates (Jollès & Jollès 1984). It seems likely that a synergism may exist between the basic protein mechanism and the muramidase activity of lysozyme besides its pure catalytical ability. Aprotinin is one of the most intensely studied proteins concerning its structure and pharmacological use as a proteinase inhibitor (Fritz & Wunderer 1983). The knowledge of its biological function, however, is scarce. Aprotinin was recently found to occur also in ciliar bronchial epithelial cells and in alveolar cells, in addition to mast cells (Businaro *et al.* 1988). Distribution of aprotinin, its antiviral activity assigned to its proteinase inhibition (Zhirnov *et al.* 1982) and its antibacterial activity suggest that the

Fig. 1 Control experiment. Untreated *Escherichia coli* incubated with polyclonal rabbit antilysozyme followed by gold-coupled goat anti-rabbit antibodies. Shrinkage of both cytoplasm and cell wall was due to the fixation procedure. **Fig. 2** *Escherichia coli* exposed to lysozyme for 20 min followed by incubation with rabbit antilysozyme and gold-coupled goat anti-rabbit antibodies. Affected bacteria are swollen and the remaining condensed cytoplasm is labelled by colloidal gold particles. **Fig. 3** *Escherichia coli* exposed to aprotinin for 1 h. The condensed cytoplasm which contains vacuole-like structures is labelled by gold particles after incubation with rabbit antiaprotinin and gold-coupled goat anti-rabbit antibodies. **Fig. 4** *Escherichia coli* exposed to DTT-denaturated lysozyme. The markedly swollen bacterium contains residues of slightly condensed cytoplasm which are labelled by gold particles after treatment with antisera as described in Fig. 2. Bars, 0·5 μm

typically reached during processing, the enzyme may be partially or completely denatured.

Avidin is unique in that its complex with biotin results in an association coefficient (K_a) of about 10^{15}, the strongest such biological association known that does not involve covalent bonding. Avidin is inactivated at 85°C, but the avidin–biotin complex can withstand temperatures greater than 100°C for a brief period.

Ovotransferrin has an isoelectric point of 6.1. Each molecule is capable of binding two atoms of iron. Ovotransferrin will also bind aluminium and copper ions, with the order of strongest to weakest binding being $Fe^{+++} > Al^{+++} > Ca^{++}$.

IgY represents a heterogeneous population of molecules composed of several antibody subclasses, with an isoelectric point ranging from 5.0 to near neutrality. Yolk antibodies are more stable in the neutral pH range up to about 60°C, or above pH 4 up to 40°C. Below pH 4 or above 65°C, the antibody activity is greatly diminished.

Mode of Action

The primary mechanism by which lysozymes lyse microorganisms is by cleaving the bonds between C-4 of *N*-acetylglucosamine and the C-1 of *N*-acetylmuramic acid, the two repeating units of the peptidoglycan layer. It is fairly active against organisms with a relatively accessible peptidoglycan layer (some Gram-positive organisms), but against organisms where this layer is not as accessible (for example, Gram-negative organisms), the enzyme is not able to access its substrate and usually shows little or no antimicrobial effect. Compounds which help to destabilize the outer membrane of Gram-negative organisms (e.g. ethylenediaminetetra-acetate (EDTA)) appear to permit lysozyme to act on some of these otherwise unassailable targets. The lysing action of lysozyme can be quite dramatic when viewed by electron microscopy (**Fig. 3**).

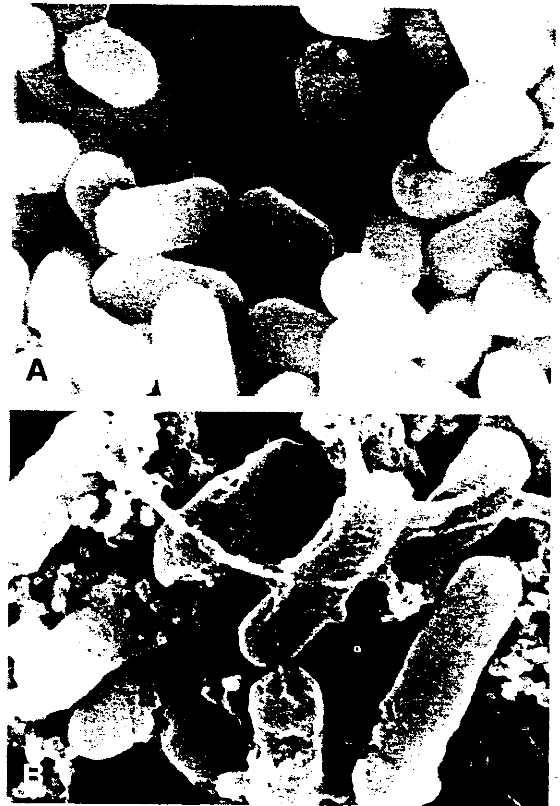

Figure 3 (**A**) Electron micrograph of *L. curvatus* grown in pork juice extract. (**B**) Electron micrograph of *L. curvatus* treated with 500 p.p.m. lysozyme. (Courtesy of Canadian Inovatech Inc., Abbotsford, British Columbia, Canada, and Dr. Frances Nattress, Agriculture & Agri-Food Canada, Lacombe Research Centre, Lacombe, Alberta, Canada.)

The antimicrobial properties of lysozyme are not only limited to its enzymatic action. It is also believed that by adhering to the exterior surface of some microorganisms (especially fungi), it can interfere with cell function and hinder growth and replication. Lysozyme also appears to cause agglutination of bacteria.

The antimicrobial effect of avidin is attributed to its ability to bind strongly with biotin. By depriving microorganisms of this essential nutrient, it has a bacteriostatic effect on biotin-reliant organisms.

For many years, the literature has described a bacteriostatic effect of ovotransferrin. This is attributed to the ability of ovotransferrin to bind iron, and thereby deprive iron-dependent organisms of an essential element. Recent studies have shown that there is also an antimicrobial effect that is relatively independent of the degree of iron saturation of the molecule. It is now believed that a peptide sequence embedded within the primary structure may, in fact, cause direct disruption of cell surfaces. This is based on knowledge of similarities in structure between ovotransferrin and lactoferrin, a milk transferrin containing a bactericidal peptide sequence known as lactoferricin. Recent research has identified a 92 residue sequence in the N-lobe portion of ovotransferrin that displays antimicrobial effect against *Staphylococcus aureus* and *Escherichia coli* K-12. Three disulphide bridges within this sequence contribute to a tertiary structure that is needed for the antimicrobial action.

IgY inhibits bacterial growth through a typical antibody precipitin reaction in which the antibodies coat the outer surface of target organisms and cross-link the cells into an insoluble mass. The optimal pH range for this effect is from pH 5 to 9.

biological meaning of aprotinin may be seen as a defence molecule.

ACKNOWLEDGEMENTS

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WILDE, C.G., GRIFFITH, J.E., MARRA, M.N., SNABLE,

Importance in Avian Eggs

Egg-white proteins protect the egg from invasion by foreign organisms by both physical and chemical defences. By forming a viscous layer between the shell and the yolk, egg white impedes the movement of organisms. Within the egg white, there is a network of fibres believed to be the result of interaction between lysozyme and ovomucin.

The chemistry of the defence in egg white is clearly linked to the antimicrobial proteins discussed earlier. The bactericidal effect of lysozyme is significant, but many organisms that gain access to the interior of avian eggs are Gram negative and therefore unlikely to be susceptible to the lytic action of lysozyme alone. At the basic pH of raw egg white (pH > 9), the iron sequestering ability of ovotransferrin is enhanced and it is also considered to be a major impediment to the growth of many organisms. The proteins presented in Table 1 present a relatively hostile environment to invading microorganisms by making biotin, riboflavin and iron relatively unavailable, inhibiting bacterial proteases and binding bacterial cells together through electrostatic interactions.

Importance in Food and Pharmaceutical Applications

Lysozyme has been used in pharmaceutical and food applications for many years, due to its lytic activity on the cell wall of Gram-positive microorganisms. These organisms are responsible for infections of the human body and for the spoilage of various foods.

Pharmaceutical Applications of Lysozyme

Hen egg white lysozyme is used in 'over-the-counter' drugs in order to increase the natural defences of the body against bacterial infections. Since lysozyme forms part of the human immune system, it has been proposed that supplementation with chicken lysozyme may have benefits. The pharmaceutical use encompasses applications such as otorhinolaryngology (lozenges for the treatment of sore throats and canker sores) and ophthalmology (eye drops and solutions for disinfecting contact lenses). Lysozyme is also added to infant formulae to make them more closely resemble human milk (cow's milk contains very low levels of lysozyme).

A recent publication has renewed interest in the antiviral properties of lysozyme by demonstrating anti-HIV activity of both human and chicken lysozyme in vitro. It is possible that these findings may lead to new means of treatment of this infection.

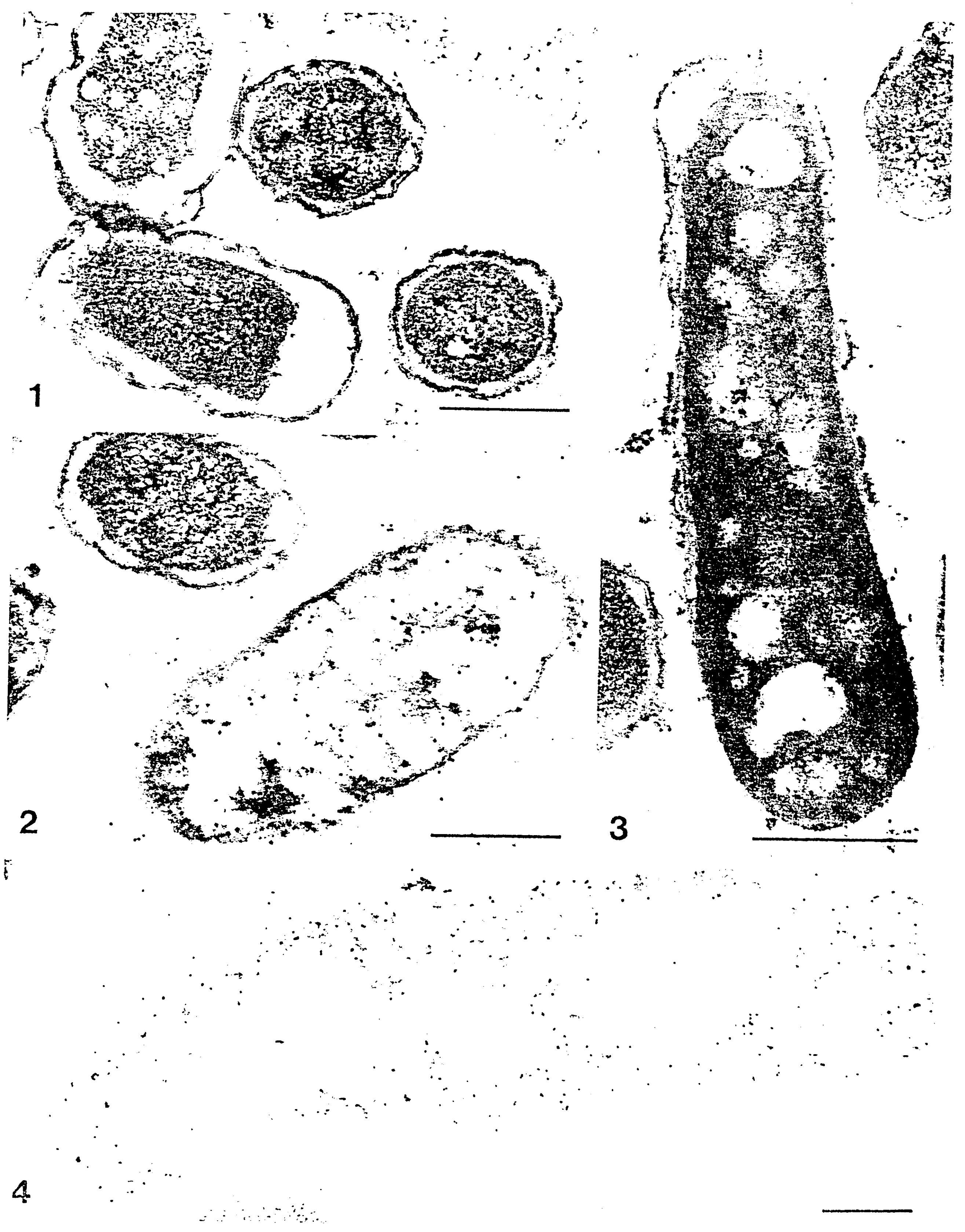
Food Applications of Lysozyme

Much research has been done on the use of lysozyme as a preservative in food products, particularly in the Far East and Japan. Several applications have been described and patented, including the treatment of fresh fruits, vegetables, seafood, meat, tofu, sake and wine.

The most important food application of lysozyme is the prevention of the problem known as 'butyric late blowing', which occurs during the ripening of certain European-type cheeses. This problem is due to contamination of milk by spores of *Clostridium tyrobutyricum*. The origin of this contamination lies in the widespread use of silage as a feed. The spores of *C. tyrobutyricum* are present in the soil and incorporated, together with soil particles, into the corn or hay used to make silage. The spores will proliferate in the silage if a rapid acidification does not take place. When the cows are fed the contaminated silage, the spores are excreted into the manure and, if the milking is not carried out under very strict hygienic conditions (thorough washing of the udder; elimination of the first drops of milk), the spores can subsequently contaminate the milk. It has been demonstrated that a very small amount of manure (less than one gram) is enough to contaminate a tank containing several thousand gallons of milk.

If cheese is made with milk contaminated with spores of *Clostridium tyrobutyricum*, the majority of the spores are retained in the curd. Here, the conditions (absence of oxygen and presence of large amounts of lactic acid) are favourable for the germination and for the development of vegetative forms during the ripening of the cheese. Lactic acid can be metabolized as the primary source of carbon by *C. tyrobutyricum* to produce butyric acid and a combination of two gases: hydrogen and carbon dioxide. The accumulation of butyric acid is responsible for organoleptic defects in the cheese due to the characteristic off-flavour caused by this short-chain fatty acid. The production of large volumes of hydrogen (totally insoluble in the water phase of the cheese curd), and of carbon dioxide (partly soluble), leads to an increase in the internal pressure of the cheese and, subsequently, to the formation of slits and cracks in the cheese during the ripening process. This has a dramatic and detrimental impact on the quality of the cheese and, consequently, on its commercial value. Cheese with a late blowing problem usually has to be downgraded or, in severe cases, cannot be sold at all.

Before the use of lysozyme, cheese makers developed a number of techniques to try to prevent butyric late blowing. There are two commonly implemented techniques: a physical process to eliminate the spores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by centrifugation (known as 'bactofugation'); and the use of chemical inhibitors of *C. tyrobutyricum*, such as nitrates. Neither method can guarantee a complete solution to the problem.

Research begun in the late 1960s and 1970s, followed by cheese trials carried out in Europe in the early 1980s, has demonstrated the efficacy of lysozyme to prevent late blowing in different types of cheese. The principle of lysozyme action is based on its capacity to be retained in the cheese curd, through electrostatic attraction with the casein, and on the stability of its enzymatic activity throughout the ripening process. Lysozyme is active on the vegetative cells of *C. tyrobutyricum*, which appear during the ripening process. The usage level is usually 25 p.p.m. in the cheese milk. At this concentration, most of the lactic cultures used in the production of cheese, although Gram-positive bacteria, are not sensitive to the lytic action of lysozyme.

Lysozyme has been approved by the European Union, and has now been used with success for more than 15 years in several European countries (e.g. France, Italy, Spain, Portugal, Germany, Denmark, The Netherlands). Its use has also been successful in different types of cheeses, such as the hard cheeses (Parmesan, Swiss), the semihard cheeses (Gouda, Manchego), and the soft cheeses (Brie). Lysozyme recently received GRAS (Generally Recognized as Safe) status from the Food and Drug Administration of the United States, and is raising a lot of interest in North America for its application in speciality cheeses.

Because lysozyme is mainly effective against Gram-positive bacteria, recent research has focused on synergistic combinations of lysozyme and other antimicrobial compounds. These combinations often target the Gram-negative organisms against which lysozyme is relatively ineffective alone.

Applications of Avidin

Avidin, in conjunction with biotin, is used in a number of diagnostic and analytical applications, including biotinylated probes for a number of quantitative detection methods, affinity chromatography columns, immunoassays, immunohistochemistry and protein blotting.

Applications of Ovotransferrin

Although patents from the 1970s promoted ovotransferrin as a potential inhibitor of mildew in such Asian dishes as noodles, wonton and fried bean curd, it has not been used extensively for its antimicrobial properties in food applications. More recent patent applications propose the use of ovotransferrin to treat human immunodeficiency virus and to prevent periodontal disease. In Japan, immobilized ovotransferrin has been used to remove iron from drinking water, as well as water for brewing. Also, a company in the Netherlands has recently filed a patent for a nutraceutical drink containing ovotransferrin.

Applications of IgY

A number of recent patents have been issued for the use of IgY (often in the form of a crude extract or even administered directly with the egg yolk). Many include prophylactic or therapeutic use involving passive protection of fish, mammals or humans against pathogenic organisms or viruses. One interesting application involves the use of IgY to target food enzymes that cause deterioration of foods through discoloration, generating off-flavours or odours, or altering important physical properties.

The ability of domestic hens to generate antibodies to a large number of important antigens is likely to lead to the continued growth of applications for IgY.

See also: ***Bacillus***: *Bacillus cereus*. **Cheese**: Microbiology of Cheese-making and Maturation; Microflora of White-brined Cheeses. ***Clostridium***: *Clostridium tyrobutyricum*. **Eggs**: Microbiology of Egg Products. ***Listeria***: *Listeria monocytogenes*. ***Staphylococcus***: *Staphylococcus aureus*. **Starter Cultures**: Cultures Employed in Cheese-making.

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and, after immunization of a rabbit, a monospecific antiserum to lysozyme was obtained. All the antibacterial assays were carried out with lysozyme purified by reversed phase chromatography.

Antibacterial activity of chicken egg white lysozyme and aprotinin

The bactericidal effects of lysozyme and aprotinin on several Gram-positive and Gram-negative bacteria are shown in Table 1. Lysozyme was, on a molar basis, at least 20 times more bactericidal than aprotinin. The bacteriolytic activity of lysozyme towards the same bacteria was also investigated. Bacteriolytic activity was observed only with *B. subtilis* and *Staph. lentus*. Denaturated precipitates of lysozyme and aprotinin produced by reduction and reoxidation at random of the native compounds with dithiothreitol, contained less than 0·2% muramidase and no detectable proteinase-inhibiting activity, respectively, under identical conditions used for the bactericidal assays. Both precipitated compounds, however, retained a significant antibacterial activity as assayed with *E. coli* under the same conditions as described in Table 1. Lysozyme, 12·5 μg per assay, yielded a lg N_0/N_t value of 0·43 and 100 μg aprotinin per assay a lg N_0/N_t value of 1·0. A quantitative comparison of the inhibitory activity, however, is only of limited value because of the different state of aggregation of the native and denaturated compounds.

Electron microscopy

The fixation procedure employed to enhance preservation of antigenicity led to shrinkage of the cell envelope and, in some bacteria, to marked shrinkage of the entire cytoplasm (Fig. 1). Exposure to lysozyme led to swelling of the bacteria. The cytoplasm often appeared to be condensed at the periphery or at one pole or it consisted of only some irregularly shaped little patches (Fig. 2). A clearly visible cell envelope surrounded slightly swollen bacteria whereas in severely swollen bacteria the envelope was distinct only in small areas. The patches and the condensed parts of the cytoplasm were labelled by colloidal gold after incubation of bacteria with antiserum to lysozyme as primary antibody and with gold-coupled goat anti-rabbit serum as secondary antibody (Fig. 2). The morphological changes were time dependent. Suspensions exposed to lysozyme for only 10 min contained only a few cells with swollen cytoplasm whereas after 20 min most of the bacteria were swollen and were twice the size of unaffected cells. Preparations exposed to lysozyme for 1 h contained mainly bacterial ghosts.

Aprotinin had a similar effect on *E. coli* as lysozyme; the time required, however, was much longer. After an incubation period of 1 h, most bacteria still had a condensed cytoplasm but aprotinin could be detected intracellularly (Fig. 3). After exposure for 2 h mainly swollen bacteria and ghosts were observed. Precipitated and resuspended lysozyme devoid of muramidase activity had the same effect as the native compound and was also localized in the cytoplasm (Fig. 4). The same was true for aprotinin.

DISCUSSION

Our results show that aprotinin, a proteinase inhibitor, and chicken egg white lysozyme are capable of killing some Gram-negative and Gram-positive bacteria *in vitro*. The diverse biological and pharmacological functions of the two cationic proteins suggest that their similar bactericidal properties depend on a basic protein mechanism related to their identical IEP of 10·5. Denaturation and precipitation of the two proteins with DTT, reducing intramolecular bonds which subsequently reoxidize randomly, confirm this idea.

The denaturated and precipitated compounds devoid of

Table 1 Antibacterial activity of aprotinin and chicken egg white lysozyme

Bacteria	Strain or type	Activity* 100 μg aprotinin/assay ($1·5 \times 10^{-8}$ mol/assay)	12·5 μg lysozyme/assay ($8·3 \times 10^{-10}$ mol/assay)
Bacillus subtilis	BGA	3·8	3·8
Escherichia coli	ATCC 25922	2·0	1·6
Klebsiella pneumoniae	ATCC 13883	0·7	2·0
Bordetella bronchiseptica	ATB 32 GN (V 1·1)	0·8	1·7
Serratia marcescens	ATCC 8100	0·0	1·6
Staphylococcus lentus	API Staph (V 2·0)	0·7	3·7
aureus	ATCC 25923	0·0	0·0
epidermidis	ATCC 12228	0·2	0·2

* Antibacterial activity is shown as lg N_0/N_t where N_0 refers to the control number of colonies without antibacterial material and N_t refers to the number of colonies containing antibacterial agent after 2 h incubation (Lehrer *et al.* 1983).

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Lactoperoxidase and Lactoferrin

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Introduction

In general, milk is kept on farms for about 2–3 days before being transported to dairy plants, despite the fact that the storage of raw milk for several days at refrigeration temperatures diminishes the quality of dairy products. This deterioration is due to extracellular enzymes synthesized by psychrotrophic bacteria, and a reduction in the shelf life of the products is often observed. Some countries have insufficient cold storage systems for handling milk, leading to the excessive multiplication of bacteria and increases in the acidity of raw milk far beyond the level acceptable for processing. Chemical preservatives, such as H_2O_2 and sodium carbonate, are commonly used in many developing countries with inadequate refrigeration systems to maintain the acidity of milk at low levels, but their undesirable effects on human health are well known. The process of activating the natural antimicrobial systems in milk has been introduced into the dairy industry as an alternative way of preserving milk.

Natural Antimicrobial Systems

The specific immune system in mammals is suppressed during the period following birth, possibly for protection against hypersensitivity and allergic reactions. During this period, the animal's defence system is supported by nonspecific factors in milk.

A great deal of research has been carried out into the antimicrobial factors in milk, and three proteins have been found to be of particular importance: lactoperoxidase (LP), lactoferrin (LF) and lysozyme. There is a similarity between these proteins and leucocytes in terms of their antimicrobial effects. In most cases, the enzymes activate the natural antimicrobial system, resulting in a fatal effect on the target microorganisms. The most important characteristic of natural antimicrobial systems is the simultaneous attack on the oxidative and lytic mechanisms of the microorganism.

The Lactoperoxidase System

The lactoperoxidase system consists of lactoperoxidase, hydrogen peroxide (H_2O_2) and thiocyanate ions (SCN^-).

Components

Lactoperoxidase Lactoperoxidase (EC 1.11.1.7, donor H_2O_2 oxidoreductase) is one of the most abundant enzymes in bovine milk, constituting about 1% of whey proteins. Oxygen metabolism leads to toxic end products, such as oxygen free radicals and H_2O_2, in both prokaryotic and eukaryotic cells unless they are protected by enzymes such as superoxide dismutase, catalase and peroxidases. The primary function of superoxide dismutase is to convert superoxide radicals to H_2O_2. The H_2O_2 is then reduced to H_2O and O_2 by either catalase or peroxidase, the latter involving a variety of electron donors.

The oxidation of molecules by peroxidases leads to the formation of H_2O_2. As early as 1924, it was observed that freshly drawn milk was bactericidal to certain strains; this effect was ascribed to the presence of oxidizing enzymes. A few years later, the involvement of H_2O_2 and SCN^- in the antimicrobial properties of milk was demonstrated. Lactoperoxidase has been found in the milk of all species tested, and in many other types of secretion, e.g. saliva, tears, nasal fluid, uterine luminal fluid and vaginal secretions. Bovine milk contains high levels of LP, which can show activity even at very low concentrations.

Lactoperoxidase is a basic protein with one Fe^{3+}-containing haem group. The amino acid sequence of bovine LP was elucidated in the early 1990s. The molecule consists of 612 amino acid residues constituting a single peptide chain. The haem group is bound to the single peptide chain by a disulphide bond, but LP does not contain free thiol (–SH) groups. The complementary DNA (cDNA) sequences of bovine and human LP show that they are closely related. The predicted molecular weight of bovine LP is 77 500, and its isoelectric point (pI) is 9.8 (**Table 1**).

Some structural similarities between bovine LP, cytochrome *c* peroxidase and horseradish peroxidase have been demonstrated, using nuclear magnetic resonance (NMR). Similar arginine and histidine residues have also been found in these enzymes. Few metal ions are present in bovine LP at significant

Table 1 Specifications of lactoperoxidase and lactoferrin

Properties	*Lactoperoxidase*	*Lactoferrin*
Molecular weight	77 500	78 500
Isoelectric point (pI)	9.8	9.5
pH range	4–7	4–8

incubated with polyclonal rabbit antiaprotinin, diluted 1 : 250 with 0·5% BSA and 1% goat serum in PBS, for 60 min at room temperature. Sections containing *E. coli* exposed to lysozyme were similarly incubated with polyclonal rabbit antilysozyme, diluted 1 : 1000. Subsequently, sections were washed by floating the grids on a drop of 0·5% BSA in PBS (two changes, 10 min each), incubated with a goat antirabbit antibody coupled to colloidal gold, diluted 1 : 100 with 0·5% BSA in PBS. The sections were then washed in 0·05% BSA in PBS (twice for 5 min) and water (four times for 5 min), stained with Mg-uranyl acetate for 4 min and lead citrate for 3 min. For controls, the primary antibodies were replaced by normal rabbit serum diluted 1 : 250, or by 1% goat serum and 0·5% BSA in PBS, and untreated bacteria were incubated as described above.

FPLC reversed phase chromatography on ProRPC column (Hr 5/10) for the analysis of aprotinin

Samples containing 2 mg/ml protein in water were acidified with trifluoracetic acid (final concentration 0·3 v/v) and loaded on a ProRPC column which had been equilibrated with 0·3% trifluoracetic acid in 10% (v/v) acetonitril. Fractions of 0·6 ml were collected and the column was eluted at a flow rate of 18 ml/h. After collecting 25 fractions, two linear gradients from 10 to 27% and from 27 to 60% acetonitril were applied. The fractions were freeze-dried and the contents redissolved in 0·25 ml of water for further analysis and pooling according to the elution diagram.

FPLC reversed phase chromatography on ProRPC column (Hr 16/10) for the analysis and purification of chicken egg white lysozyme

A sample containing 5 mg/ml of chicken egg white lysozyme was acidified with trifluoracetic acid (final concentration 0·3% v/v). The solution (2 ml) was loaded on to the column which was equilibrated with 0·3% trifluoracetic acid in 10% (v/v) acetonitril. Fractions of 3 ml were collected and the column was eluted at a flow rate of 180 ml/h. After collecting eight fractions, the column was first eluted with 25% acetonitril and, after collecting 40 fractions, a linear gradient from 25 to 60% acetonitril was applied. The fractions were freeze-dried and the contents redissolved in 0·5 ml H_2O and the hydrolytic activity of lysozyme toward *M. luteus* was determined.

Reduction and random reoxidation of chicken egg white lysozyme and aprotinin

Five mg of chicken egg white lysozyme were dissolved in 1·0 ml of 0·01 mol/l NaH_2PO_4/Na_2HPO_4 buffer (pH 7·4) containing 0·01 mol/l dithiothreitol (DTT) and incubated at 37°C for 2 h until a precipitate was formed (Laible & Germain 1985). The suspension was centrifuged at 2000 g for 15 min and the supernatant fluid discarded. The precipitate, consisting of randomly reoxidized and aggregated lysozyme, was washed with water 10 times and then freeze-dried. The last wash fluid was tested and found free of antibacterial activity due to traces of DTT or soluble lysozyme. Five mg of aprotinin were dissolved in 5 ml of the phosphate buffer described above, incubated at 37°C for 48 h and the precipitate formed was centrifuged. The sediment was washed with water and then freeze-dried. For use in bactericidal assays weighed amounts of the compounds were suspended in 0·01 mol/l NaH_2PO_4/Na_2HPO_4 buffer. Neither of the denaturated proteins dissolved.

Production of antiserum

Aprotinin purified by reversed phase chromatography was conjugated to rabbit serum albumin by glutaraldehyde as reported by Reichlin (1980), mixed with complete Freund's adjuvant and used to immunize a rabbit.

Antiserum to chicken egg white lysozyme was obtained by immunizing a rabbit with a mixture of purified lysozyme from reversed phase chromatography and complete Freund's adjuvant.

RESULTS

Homogeneity of commercial aprotinin

The elution diagram of commercially available aprotinin from reversed phase chromatography on ProRPC included a main peak and a single small retarded peak eluted with a higher acetonitril concentration consisting of the well-known aprotinin dimer (Trautschold *et al.* 1967). The antibacterial assays were therefore carried out with the commercial aprotinin without further purification.

Homogeneity of commercial chicken egg white lysozyme

The elution diagram of commercially available chicken egg white lysozyme from reversed phase chromatography consisted of two peaks, the first (peak I) eluted with an acetonitril concentration of 25% and the second (peak II) with 35%. The antibacterial activity of both pooled peaks was measured with *E. coli* and *M. luteus*. Peak I did not show any antibacterial activity and was not further investigated. Peak II showed antibacterial activity against both *E. coli* and *M. luteus*, and was identified as lysozyme. It consisted of a single band in SDS polyacrylamide gel electrophoresis

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THE CHEMISTRY OF LYSOZYME AND ITS USE AS A FOOD PRESERVATIVE AND A PHARMACEUTICAL*

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I. CHEMICAL CHARACTERISTICS OF LYSOZYME

Lysozyme was first discovered in 1922 by Alexander Fleming while he was suffering from a cold, when nasal mucus was found to dissolve bacteria on an agar plate. He quickly found that the antibacterial action was due to an enzyme, but that it was only effective against certain bacteria and not those most infectious to man.[1]

Later, in 1963, Jolles and colleagues[2] at the University of Paris and Canfield[3] at the Columbia University College of Physicians and Surgeons discovered the chemical make-up of the protein molecule and mapped the amino acid sequence of lysozyme. The hen egg white lysozyme molecule consists of a single polypeptide chain of 129 amino acids. The lysozyme molecule is cross-linked in four different places by disulfide bridges formed by the combination of sulfur-containing side chains between residues 64-80, 76-94, 6-127, and 30-115. Two of the disulfide bonds must be intact for lysozyme to maintain enzymatic activity. Lysozyme loses its activity if all the –S–S– bonds are reduced. Jolles et al.[2] and Canfield[3] had some disagreements concerning the amino acid sequence of hen egg white lysozyme, and these were later investigated by X-ray crystallography and revisions made where conflicting results had been reported.[4] Figure 1 is a two-dimensional diagram of the amino acid sequence, which summarizes the results of all three research papers. By using X-ray diffraction analysis, secondary and tertiary structures have also been elucidated. Models of these structures can be found in a review written by Philips.[1] The secondary and tertiary structures of lysozyme do not follow basic conformations except for parts of the molecule containing helixes and β-pleated sheet structures.[5] By using optical rotational values, 25% of the lysozyme structure was calculated to be in a helix. The atomic coordinates of the lysozyme structure refined by advancing biochemical procedures have been determined, and structural conformations from binding of the enzyme to metals and other substances have been studied.[6-8]

The hydrophilic and hydrophobic bonds of lysozyme are scattered and not distributed equally along the amino acid chain. For example, residues 38-55 are almost all hydrophilic. The first 40 residues are hydrophilic and hydrophobic and contain amino acids suitable for an α-helix formation. A hydrophobic core is formed with hydrophilic amino acid side chains protruding toward the surface, which is thought to give lysozyme its stability. The opposite

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Both aprotinin and lysozyme were found to penetrate the bacterial wall of Gram-positive and Gram-negative bacteria and disintegrate the cytoplasm, leading to its complete loss. Denaturation of aprotinin and lysozyme did not reduce their bactericidal activity. The results suggest that the bactericidal properties are primarily related to their basic character.

MATERIALS AND METHODS

Reagents were obtained from the following sources: trypsin (from porcine pancreas) and aprotinin from Serva; chicken egg white lysozyme (3 × crystallized) and bovine serum albumin from Sigma; ProRPC column (Hr 5/10 and 16/10) for reversed phase chromatography from Pharmacia; *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 from Roche; *Klebsiella pneumoniae* ATCC 13883, *Serratia marcescens* ATCC 8100, *Staph. epidermidis* ATCC 12228 from Eurodiagnostic, Shoreham-By Sea, Brighton; *Staph. lentus* and *Bordetella bronchiseptica* wild strains were from the Institute of Bacteriology of the Veterinary Hospital, Zürich; *Bacillus subtilis* BGA from Merk, Darmstadt, Germany; *Micrococcus luteus* from Boehringer, Mannheim, Germany; LR white resin from The London Resin Company Ltd, Basingstoke, UK; Vero cells from Flow Laboratories, Rickmansworth, UK; goat antirabbit IgG-colloidal gold (10 nm) from Janssen, Olen, Belgium. All other reagents were of the highest commercially available grade.

Antibacterial assays

Single colonies of bacteria grown on trypticase soy agar plates were inoculated in 50 ml of trypticase soy broth (TSB) and grown overnight at 37°C. One ml of bacterial suspension was diluted 1 : 50 with trypticase soy broth and incubated overnight at 37°C. The culture was then centrifuged at 2000 g for 10 min. The sedimented bacteria were washed twice with 0·01 mol/l NaH_2PO_4/Na_2HPO_4 buffer (pH 7·4) and adjusted to 1×10^6 cfu/ml. Fifty μl of bacterial suspension were added either to 50 μl of purified chicken egg white lysozyme or to 50 μl of aprotinin of appropriate concentrations. One hundred μl of 2% TSB in 0·01 mol/l NaH_2PO_4/Na_2HPO_4 buffer were then added. The mixture was incubated at 37°C for 2 h, serially diluted from 1 : 10 to 1 : 1000 in 0·01 mol/l NaH_2PO_4/Na_2HPO_4 buffer (pH 7·4), and plated on trypticase soy agar. The plates were incubated for between 24 and 48 h at 37°C until distinct colonies could be counted. The assays were conducted in duplicate.

Turbidimetric studies of lysis

Lysozyme hydrolytic activity was determined turbidimetrically by measuring the decrease in absorbance at 546 nm of a suspension of *M. luteus* cells as reported by Weisner (1984). Turbidimetric studies of lysis with the other bacterial species investigated were carried out as follows: 12·5 μl of lysozyme solution (0·05–1·00 mg/ml) in 0·01 mol/l NaH_2PO_4/Na_2HPO_4 buffer (pH 7·4) were added to 750 μl of bacterial suspension (10^8 bacteria/ml in 0·01 mol/l NaH_2PO_4/Na_2HPO_4 buffer (pH 7·4)).

Assay for trypsin inhibitory capacity

The assay for the antitryptic activity of aprotinin was performed with N-benzoyl-L-arginine-*p*-nitroanilide as the substrate according to the method of Geiger & Fritz (1984).

SDS polyacrylamide gel electrophoresis

SDS electrophoresis on polyacrylamide gel (T = 12% C = 7%) was carried out according to Laemmli's (1970) procedure.

Electron microscopy

For ultrastructural studies, 50 μl of *E. coli* suspension (10^8 *E. coli*/ml) were incubated with 50 μl of aprotinin solution (4 mg/ml) and 100 μl of 0·01 mol/l NaH_2PO_4/Na_2HPO_4 buffer (pH 7·4) containing 2% TSB for 2 h at 37°C. Subsequently, the cells were washed with 0·01 mol/l NaH_2PO_4/Na_2HPO_4 buffer (pH 7·4). Five samples were prepared in this way. They were then pooled and fixed with 2% formaldehyde and 0·5% glutaraldehyde in 0·05 mol/l NaH_2PO_4/Na_2HPO_4 (pH 7·4) and centrifuged in microtubes at 3000 g for 25 min at 4°C; 50 μl of lysozyme solution (10 mg/ml) were added to 50 μl of *E. coli* suspension (10^{10} *E. coli*/ml), incubated with 100 μl of 0·01 mol/l NaH_2PO_4/Na_2HPO_4 buffer (pH 7·4) containing 2% TSB for 20 min at 37°C, washed and fixed as *E. coli* incubated with aprotinin. Vero cells were added to stabilize the final pellet. Untreated *E. coli* cells were processed in the same way for control purposes. After fixation, pellets were washed in 0·05 mol/l NaH_2PO_4/Na_2HPO_4 buffer (pH 7·4) for 10 min at 4°C, rapidly dehydrated in a graded series of ethanol starting at 70%, embedded in LR white resin and polymerized for 20 h at 50°C.

Immunogold labelling

Sixty to 80 nm sections collected on bare gold grids (300 mesh) were incubated according to the method described by Geuze *et al.* (1981). After preincubation with 0·5% bovine serum albumin (BSA) and 5% goat serum in phosphate buffered saline (PBS) pH 7·4 (two changes, 10 min each), sections containing *E. coli* exposed to aprotinin were

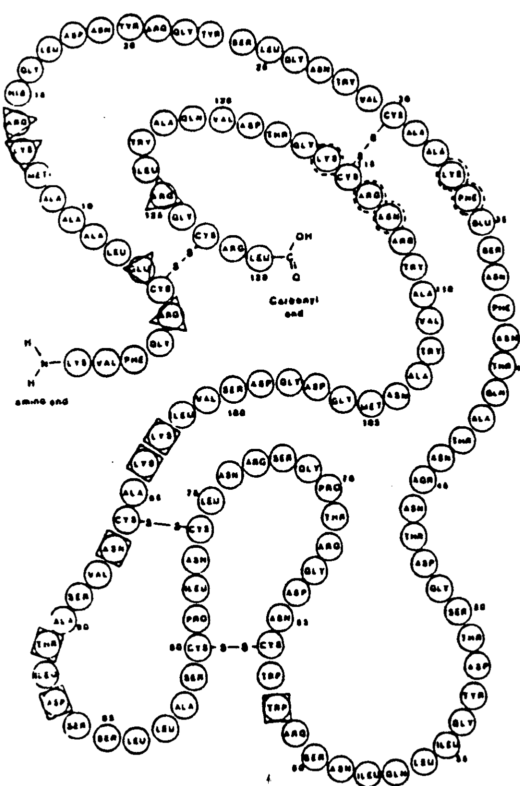

FIGURE 1. The amino acid sequence of lysozyme with the amino acids involved in the three antigenic sites indicated: site 1 (△); site 2 (□); and site 3 (◌).[2-4,96]

side of the molecule contains fewer hydrophobic side chains, and hydrogen bonding is the force of attraction.[5]

Lysozyme is now officially described as *N*-acetylhexosaminodase and is classified as a muramidase.[9] The Commission on Enzymes has assigned the numbers 3.2.1.17 to lysozyme.[10]

The lysozyme molecule can be crystallized out of egg albumen, but may contain two or three components that can be separated by cation-exchange chromatography and moving-boundary electrophoresis. The molecular weight of lysozyme is approximately 14,300 to 14,600 and the isoelectric point is pH 10.7.[11] The acid-base titration curve for lysozyme has been determined and the pK values for the catalytic amino acids found.[12-15] Lysozyme activity rate is highest from pH 3.5 to 7, and the pH range of 5 to 7 is best for lysing of *Micrococcus lysodeikticus*.[16] Lysosyme is stable for several years when stored dry at 5°C.[17]

Lysozyme got its name by being an enzyme that lyses or dissolves bacterial cells. Lysozyme functions by splitting or hydrolyzing the β(1-4) linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine, which are parts of a long, complex sugar molecule found in the outer membrane of many living bacterial cells.[11] Because of the nature of the cell wall, Gram-positive bacteria are most susceptible to lysozyme.

A. Effect of Heat and Chemicals on Lysozyme Activity

Lysozyme is heat stable in acidic solutions, having been reported to withstand 100°C with

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Bactericidal activities of lysozyme and aprotinin against Gram-negative and Gram-positive bacteria related to their basic character

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A. PELLEGRINI, U. THOMAS, R. VON FELLENBERG AND P. WILD. 1992. Bactericidal properties of aprotinin, a proteinase inhibitor and possibly a defence molecule in bovine species, and of chicken egg white lysozyme, known as muramidase, were investigated. Incubation of various bacteria in the presence of either aprotinin or lysozyme showed that both proteins killed Gram-positive as well as Gram-negative bacteria without addition of complement or EDTA. Denaturation of the two proteins by dithiothreitol did not lead to loss of their bactericidal potency. Electron microscopic examination of *Escherichia coli* incubated either with lysozyme or aprotinin revealed that the bacterial cytoplasms gradually disintegrated. Both aprotinin and lysozyme were demonstrated within the affected cytoplasm by immunogold labelling. The results suggest that the bactericidal potency of lysozyme is not only due to muramidase activity but also to its cationic and hydrophobic properties. The bactericidal activity of aprotinin is probably also related to both these properties rather than to its activity as proteinase inhibitor.

INTRODUCTION

Cationic proteins and polypeptides of polymorphonuclear leucocyte granules possess microbicidal properties (Gennaro *et al.* 1983; Spitznagel 1984; Shafer *et al.* 1984; Viljanen *et al.* 1988; Lehrer *et al.* 1989). They are considered to be the non-oxidative defence mechanism of phagocytic cells (Spitznagel 1984). Aprotinin is a proteinase inhibitor with basic character (IEP 10·5) of bovine lung and other organs. Its precise function remains unknown (Fritz & Wunderer 1983). Aprotinin also has antimicrobial properties (Castaldi & Mattiello 1971). The antiviral property of aprotinin is explained by its proteinase inhibition ability (Zhirnov *et al.* 1982). The antibacterial activity, however, is not understood since a wide variety of proteinase inhibitors have no antibacterial properties (Pellegrini *et al.* 1990).

Lysozyme is a muramidase with basic character (IEP 10·5) which is widely distributed in nature. Its antibacterial activity is considered to be strongly related to its catalytic properties that affect some Gram-positive bacteria (Jollès 1964; Nakae & Nikaido 1975; Spitznagel 1984).

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During a previous investigation (Pellegrini *et al.* 1990) we noted that chicken egg white lysozyme, which was present as a contaminant in the commercial preparations of ovoinhibitor and ovomucoid, had bactericidal activity against both Gram-positive and Gram-negative bacteria when tested in a bactericidal assay under the same conditions as the cationic bactericidal proteins of neutrophil granules (Ganz *et al.* 1985). This result was unexpected since it was considered that lysozyme is considered unable to penetrate the outer membrane which protects the peptidoglycan layer of Gram-negative bacteria (Glynn 1968; Nakae & Nikaido 1975; Davis *et al.* 1980; Spitznagel 1984). Gram-negative bacteria are rendered susceptible to the lytic action of lysozyme after the outer membrane has been destroyed by EDTA, antibodies and complement or heat (Warren *et al.* 1955; Repaske 1958; Wilson & Spitznagel 1968; Feingold *et al.* 1968), thereby exposing the peptidoglycan layer to lysozyme.

The purpose of this study was to elucidate the mode of antibacterial activity of aprotinin and lysozyme which have an identical IEP. We were interested to know whether the bactericidal activity of cationic proteins was restricted to granule proteins of neutrophils or whether other cationic proteins with a potential physiological function in antimicrobial defence could act according to the same principle.

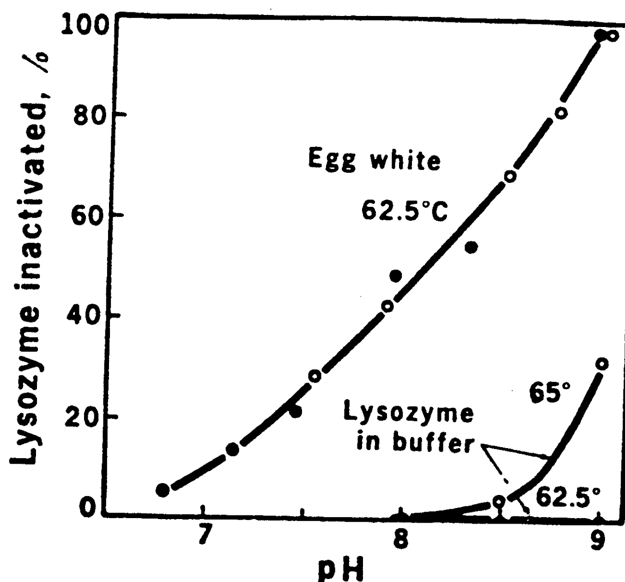

FIGURE 2. Influence of pH on the heat inactivation of lysozyme in egg white containing 30 ppm aluminum and in buffer. The buffer in solution contained about 0.01% lysozyme. All samples were heated for 10 min. Open and closed circles on egg white curve represent two egg white samples.[26]

little loss of activity.[18-21] Matsuoka et al.[22] also found lysozyme to be stable in acidic solution (pH 4.5, 100°C, 3 min; pH 5.29, 100°C, 30 min).

Using loss of activity as a criterion, Beychok and Warner[23] showed that stability in the temperature range of 85 to 95°C was maximum at about pH 5.5. Gorini and Felix[24] reported 25% inactivation of lysozyme at 70°C in borate buffer at pH 7.9 within 30 min. Sandow[25] reported that lysozyme in egg white at pH 8.0 was destroyed in 15 min at 65°C; however, at pH 5 and 60°C, no loss occurred in 60 min. They found lysozyme to be over 50 times more heat stable in phosphate buffer, pH 6.2, than in egg white at 62.5°C. Cunningham and Lineweaver[26] studied the stability of egg white proteins to pasteurizing temperatures above 60°C since egg white functional properties are affected above 57°C when unmodified egg white is heated. Egg white was adjusted to various pH values with 0.1 N HCl, using distilled water to maintain equal dilution. Then 2-mℓ portions were heated 10 min in a water bath at 62.5°C, cooled in an ice water bath, mixed with 8 mℓ of phosphate buffer (pH 6.2), centrifuged, and assayed for lytic activity. Lysozyme inactivation in egg white varied from about 10% at pH 7 to over 95% at pH 9. Neither Al^{3+} nor Fe^{3+} (10^{-3} M concentrations) had any influence on the stability of lysozyme in egg white. There was no inactivation of lysozyme at 62.5°C in phosphate buffer, even at pH 9. At 65°C, inactivation occurred in 10 min at pH 9 (Figure 2). Later, Cunningham and Lineweaver[27] found that loss of lysozyme activity decreased more in egg white at a lower temperature of 62.5°C than in buffer at 65°C because the sulfhydryl group of ovalbumin reduced one or more disulfide bonds in the lysozyme. Ovalbumin is the only egg white protein which contains a sulfhydryl group. Lysozyme inactivation involves a denaturation reaction as well as a sulfhydryl-disulfide interchange. The loss of lysozyme activity on heating lysozyme-ovalbumin solutions is greatly reduced as the NaCl concentration increases. Samples heated to 60°C for 10 min were reduced from nearly complete inactivation to 60% at 0.25% salt, to 42% at 0.5% salt, and to 10% at 1% salt. Therefore, the inactivation reaction occurs more rapidly when

In conclusion, the possibility of using as a food preservative an enzyme that is harmless to humans is attractive, and we believe that the work presented in this study suggests that lysozyme has the potential to serve such a role in specific applications in the food industry.

ACKNOWLEDGMENTS

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lysozyme and ovalbumin are electrostatically combined. None of the other egg white proteins contain sulfhydryl groups or were found to decrease lysozyme activity at temperatures near 60°C. Weaver and Kroger[28] found that activity of 1 mg/mℓ lysozyme in bovine skim milk decreased at temperatures greater than 60°C.

Hayase et al.[29] found lysozyme to polymerize through disulfide linkages, and at 180°C both polymerization and degradation occurred. When the temperature was raised to 200°C, many changes occurred, including cleavage and recombination of peptide bonds. Over 200°C, polymerization and degradation occurred more violently.

Genentech and Genencor,[30] two companies working with genetic engineering, have developed a modified T4 lysozyme in which a new disulfide bond was incorporated into the protein to increase its thermal stability and make it more effective in cheese-making and other food applications. A computerized model of the modified T4 lysozyme is found in the above reference.

Frasco[31] and Frasco et al.[32] studied the mechanism of heat denaturation in lysozyme as it relates to changes in water structure. External nonpolar amino acid residues did not have a significant effect on denaturation, but water freed from external polar amino acid residues played a role in the initial denaturation process. Water migrates to interior peptide-peptide bonds, initiating protein swelling and uncoiling. A mechanism was postulated in which denaturation promotes water structuring by increasing total surface area and polar hydration site availability by reducing protein crystallinity. This encourages shifting of peptide-peptide bonds to peptide-water bonds, causing further protein swelling and uncoiling as water is added to the system. The additional water is laid down in monolayers until a distance is reached where newly added water is no longer under the influence of the protein charge.

Wu[33] and Wu et al.[34] studied the effect of fluctuating temperature on lysozyme activity. Enzymatic reactions were affected differently by fluctuating temperature than simple chemical reactions because activation, deactivation, reactivation, and possibly certain patterns of temperature adaptation unique to enzyme molecules influenced the rate. Overshoot and undershoot phenomena occurred when temperatures were changed and, as a result, nverse compensation was shown. The final product yield after subjecting an enzyme to temperature fluctuation depended on the compensation between the overall magnitude of over- and undershoot from theoretical values. The cycle-down mode and the slower frequency of fluctuation created a greater influence on ratio changes. In the absence of heat inactivation, 20°C for lysozyme, faster rates and higher yields resulted. When heat inactivation was apparent, e.g., 50°C region for lysozyme, the slower frequency and the cycle-down mode resulted in greater inactivation as well as greater activation, but the former effect overshadowed the latter and resulted in a lower yield.

Chang and Carr[35] found lysozyme to be inactive in distilled water. It was long believed that little or no change in globular protein conformation of lysozyme took place upon removal of solvent water. Baker et al.[36] studied hydrogen exchange in lyophilized lysozyme samples, either completely dry or dehydrated at a higher relative humidity, compared with lysozyme in solution. The results indicate that lysozyme does not have the same conformation as it does in aqueous solution.

Many studies of the effect of alcohols on protein conformation have led to the conclusion that the effectiveness of alcohols as protein denaturants increases with increasing hydrocarbon chain length and is explained in terms of a hydrophobic mechanism of alcohol-protein side chain interaction. Tribout and Leonis[37] studied H^+ titration curves of hen egg white lysozyme in the presence of methanol, ethanol, and *n*-propanol in concentrations less than 15% by weight in order to avoid conformational changes. The acidity constants of two groups (whose pH values in water are, respectively, 4.2 and 3.5 corresponding to Asp-101 and Asp-52) are increased in water-alcohol mixtures in comparison to water. This indicates that these alcohols interact within the active site of lysozyme as a function of alcohol concentration and hydrocarbon chain length. No mention was made of lysozyme activity.

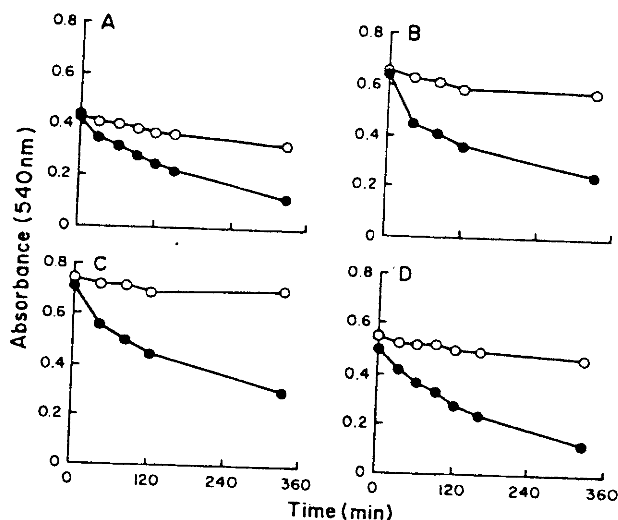

FIG. 4. Lysis of *L. monocytogenes* cells suspended in phosphate buffer and exposed to 10 mg of lysozyme per liter. (A) California; (B) Scott A; (C) V7; (D) $20A_2$ Ohio. Open symbols represent controls without added lysozyme.

TABLE 5. Effect of lysozyme and EDTA on recovery of *L. monocytogenes* on brain heart infusion agar

Treatment	No. of colonies at following dilution:			
	L. monocytogenes Scott A		*L. monocytogenes* Ohio	
	10^{-6}	10^{-7}	10^{-6}	10^{-7}
None	TNTC[a]	250	TNTC	179
Lysozyme, 100 mg/liter	TNTC	232	TNTC	149
EDTA, 1 mM	TNTC	276	TNTC	235
EDTA, 0.5 mM	TNTC	266	TNTC	202
EDTA, 0.05 mM	TNTC	277	TNTC	229
Lysozyme + EDTA, 1 mM	100–150[b]	30–50[b]	0	0
Lysozyme + EDTA, 0.5 mM	TNTC	304	TNTC[b]	50–75[b]
Lysozyme + EDTA, 0.05 mM	TNTC	261	TNTC	184

[a] TNTC, Too numerous to count.
[b] Colonies pinpoint after 3 days of incubation.

luteus did not occur from a culture supernatant not previously dosed with lysozyme.

We found that combining lysozyme with certain chemicals promoted lysis of growing cells of *L. monocytogenes*. The potentiating chemicals were chosen on the basis of previous studies of the activity of lysozyme (17, 18). Addition of DL-lactic acid at 0.85% stopped the growth of the cells and resulted in slow lysis (change of −0.14 optical density unit compared with the control after 24 h) when lysozyme was also present. The most effective potentiator for lysis of *L. monocytogenes* was EDTA (cells lysed −0.46 [measured as optical density at 660 nm] compared with the control). Chemicals assayed as potentiators but which showed no significant differences from the control included potassium sorbate (0.05%), glycine (0.25%), sodium acetate (5 mM), ethanol (0.95%), sodium dodecyl sulfate (0.01%), thioglycolate (5 mM), NaCl (50 mM), dithiothreitol (5 mM), and ascorbic acid (10 mM).

The effectiveness of EDTA and lysozyme against *L. monocytogenes* was also tested for inhibition of colony formation on brain heart infusion agar (Table 5). Although 100 mg of lysozyme per liter or 1 mM EDTA alone did not inhibit two strains of *L. monocytogenes*, the combination caused significant inhibition relative to the controls (Table 5).

DISCUSSION

The present studies indicate that lysozyme effectively lyses and inhibits the growth of several food-borne pathogens and spoilage bacteria. We found that certain strains of *C. botulinum* and four strains of *L. monocytogenes* were lysed by the egg white enzyme. *L. monocytogenes* has recently been associated with human listeriosis transmitted by vegetables, soft cheeses, and pasteurized milk products and is a serious concern as an emerging pathogen in a variety of food products. The results of this study suggest that lysozyme may be effective in foods as a safety factor to assist in the inhibition of *L. monocytogenes*.

The present work also demonstrates that the saccharolytic spoilage thermophile *C. thermosaccharolyticum* is susceptible to the lytic activity of lysozyme. The thermophilic, flat-sour bacterium *B. stearothermophilus* was also highly sensitive, as previously observed (3, 14). Perhaps the structure of the cell walls of certain gram-positive thermophiles contributes to their high sensitivity to lysozyme. Because of their ability to produce extremely heat-resistant spores, *B. stearothermophilus* and *C. thermosaccharolyticum* can present severe spoilage problems in low-acid canned foods. Depending on the product, the commercial sterilization of low-acid canned foods may require considerable quantities of thermal energy to destroy the viability of the thermophilic spores. Since lysozyme has excellent heat resistance at low pHs (1, 8), it may be possible to reduce the thermal energy requirements during canning by including lysozyme in the process. We have obtained preliminary results which indicate that *B. stearothermophilus* spores are readily killed by lysozyme at 70°C (V. L. Hughey, P. Wilger, and E. A. Johnson, unpublished data).

Lysis of several of the pathogenic and spoilage bacteria in the present studies was enhanced and consistently obtained when lysozyme was used in combination with EDTA. This was particularly apparent with certain strains of *C. botulinum*, many of which were completely refractory to lysozyme alone but were inhibited and lysed by lysozyme plus EDTA. The results obtained with *C. botulinum* and *L. monocytogenes* suggest that two factors mainly limit the effectiveness of lysozyme. First, since EDTA is required to obtain consistent lysis, the peptidoglycan substrate may be partially masked by other cell wall components (7, 12, 20). EDTA could allow partial removal of these layers and promote penetration of lysozyme to the peptidoglycan. Second, because cell wall lysis occurred most effectively in cultures slowed in growth rate by lowered temperatures, it is likely that cell wall synthesis in rapidly growing cultures probably exceeds the rate of degradation by lysozyme. The effectiveness of lysozyme at low temperatures is intriguing, because with the increased production of refrigerated foods there is currently concern whether refrigeration is sufficient to restrain growth of low-temperature-growing pathogens such as *C. botulinum* type E (16). The results presented in these studies suggest that the combination of EDTA (or possibly other chelators) and lysozyme may be useful for the prevention of *C. botulinum* growth in foods. The use of lysozyme in prevention of *C. botulinum* food poisoning, however, should be considered with caution. It is also possible that lysozyme might increase the risk of food poisoning by promoting the release of intracellular toxin.

Table 1
STABILITY OF LYSOZYME IN CONCENTRATED SUGAR SOLUTIONS[16]

Temperature (°C)	Time (min)	Remaining lysozyme activity %: pH 6.0, 20% Sugar	pH 6.0, 60% Sugar	pH 3.2, 20% Sugar	pH 3.2, 60% Sugar
	15	89	98	100	100
60	30	85	93	100	100
	60	80	92	100	100
	15	53	100	100	100
80	30	53	100	100	100
	60	35	94	100	100
	15	16.5	32	82	98
100	30	0	15	67	79
	60	0	6.8	25	46

Note: The concentration of lysozyme used was 20 ppm.

Yashitake and Shinichiro[16] found that 10 and 20 μg/mℓ lysozyme maintained 100% activity in mirin liquor (sweetened sake, containing less than 20% alcohol, used for cooking Japanese food) at 37°C for a study period of 20 weeks. Even after 1 hr at 65°C, 100% of the original lysozyme activity was present. From 10 to 50 μg/mℓ of lysozyme maintained 100% of its activity in sake at 37°C for 20 weeks and also for 1 hr at 65°C. In addition, 10 μg/mℓ lysozyme in vinegar had 40% of the original activity after 2 weeks, 5% after 10 weeks, and none at 20 weeks; none of the original activity was found after 1 hr at 65°C; and 20 μg/mℓ lysozyme in vinegar had 100% of its original activity after 2 weeks, 90% after 10 weeks, and none after 20 weeks or for 1 hr at 65°C. Lysozyme was found to rapidly lose its activity in alkaline solutions (above pH 6).

Back et al.,[38] using lysozyme and other proteins, found that sugars and polyols stabilized them against heat. Solutions of proteins were heated at a constant rate, and the temperature of the maximum rate of denaturation (TM) was estimated. The addition of sugar or polyol raised the TM. The magnitude of the stabilizing effect (TM) depended on the nature of the proteins and sugar or polyol. The TM for lysozyme was 18.5°C at pH 3 in the presence of 50% (w/w) sorbitol. It was suggested that stabilization was due to the effects of sugars and polyols on hydrophobic interactions, in which they reduced the tendency for complete transfer of hydrophobic groups from an aqueous to a nonpolar environment.

Yashitake and Shinichiro[16] also found increased heat stability of lysozyme in sucrose. Table 1 shows the stability of 20 ppm lysozyme in sucrose. Activity increased as the pH decreased from 6.0 to 3.2.

Hidaka, as cited in Yashitake and Shinichiro,[16] found that the activity of 20 ppm of lysozyme was stabilized against heat by NaCl, as seen in Table 2. Lysozyme is activated by low concentrations of salt and is inhibited by high concentrations of salt. Kravchenko et al.[39] found that salt was necessary for enzymatic action of lysozyme and that if the ionic strength were higher than 0.05 to 0.1 *M* the lysozyme action on individual substrates increased, whereas the lysis of *M. lysodeikticus* was inhibited. Chang and Carr,[35] using sodium chloride, potassium phosphate, and tris chloride, showed that the activation at low salt concentration was most closely correlated with a nonspecific ionic strength effect and that the inhibition at high salt concentration is most closely correlated with cationic concentration and charge. At a given ionic strength, polyvalent cations are stronger inhibitors

TABLE 3. Lysis[a] of *C. botulinum* in phosphate buffer

Strain[b]	Maximum lysis (%)	Time to maximum lysis (min)	Time to 50% lysis (min)
C. botulinum Hall A	70	150	30
C. sporogenes PA3679	85	40	12
C. sporogenes B1107	85	30	21
C. botulinum 17B	54	140	40
C. botulinum Okra B	65	150	35

[a] Lysis was done at 37°C with cells suspended in 0.067 M phosphate buffer (pH 6.6) containing 100 mg of lysozyme per liter. The decrease in turbidity relative to the control incubation without lysozyme was measured at 540 nm periodically for at least 18 h. The lysis tests were done at least twice on separate days with cultures grown independently.

[b] Isolates *C. botulinum* Alaska E, Iwanai E, Minnesota E, 62A, and 113B and *C. sporogenes* B1106 were repeatedly resistant to lysis.

TABLE 4. Influence of temperature on lysis of *C. botulinum* type E (Iwanai strain)[a]

Temp (°C)	Treatment	Change in A_{540}
4	None (control)	0.0
	Lysozyme	−0.20
	EDTA	0.0
	Lysozyme + EDTA	−0.31
10	Control	+0.19
	Lysozyme	−0.11
	EDTA	+0.18
	Lysozyme + EDTA	−0.36
25	Control	+0.52
	Lysozyme	+0.44
	EDTA	+0.62
	Lysozyme + EDTA	0.0

[a] Cells were grown to mid-exponential phase at 25°C, shifted to the various temperatures, and treated with lysozyme (100 mg/liter) or EDTA (1 mM) or both. The optical densities were monitored for 9 days at 4°C, 6 days at 10°C, and 1.5 days at 25°C.

EDTA with 100 mg of lysozyme per liter in the incubations resulted in greatly improved and repeatable lysis of type E and proteolytic type A and B strains (Fig. 3). Furthermore, the combination of 1 mM EDTA and 20 mg of lysozyme per liter in complex media prevented the growth of proteolytic and nonproteolytic strains, i.e., organisms initially assayed as negative in test 1 (data not shown). Therefore the combination of lysozyme and EDTA was quite effective and consistently inhibited toxigenic *C. botulinum*.

We noticed that the presence of 1 mM EDTA alone slowed the growth of certain *C. botulinum* strains 20 to 30% relative to controls and improved their susceptibility to lysozyme. This information suggests that the growth rate of the cells influences their susceptibility to lysozyme, possibly because the rate of cell wall synthesis exceeds its rate of hydrolysis. To test this, we assayed the effectiveness of lysozyme and EDTA at various temperatures on *C. botulinum* type E, which is capable of growing at temperatures as low as 4°C. An assay of lysozyme at 5.5°C indicated that there was 5,700 U/mg compared with 50,000 U/mg at 25°C. At the lower temperatures the degree of lysis was substantially increased (Table 4), in accordance with the diminishing growth rates, despite the lower activity of lysozyme. It is also possible that EDTA partially disrupts the outer cell wall structure and allows lysozyme to penetrate to the peptidoglycan.

Four strains of *L. monocytogenes* isolated during food poisoning outbreaks were evaluated for their susceptibility to lysozyme. We found that cells of each strain suspended in phosphate buffer were lysed convincingly by lysozyme (Fig. 4). The rate of lysis was relatively slow but steady (Fig. 4), and 50 to 60% reduction in optical density occurred within 2 h, reaching a maximum of 70 to 80% after 6 h. These results were consistent on repeated trials. However, growth of the four strains of *L. monocytogenes* was not inhibited when each was inoculated into media containing lysozyme at 20 or 200 mg/liter (see above), nor did lysis occur when lysozyme was injected into an actively growing culture of *L. monocytogenes*. This ineffectiveness was not due to inactivation of lysozyme by the growing culture, since a sample of the medium withdrawn after 3 days of growth readily lysed *M. luteus* cells suspended in the same medium. Lysis of *M.*

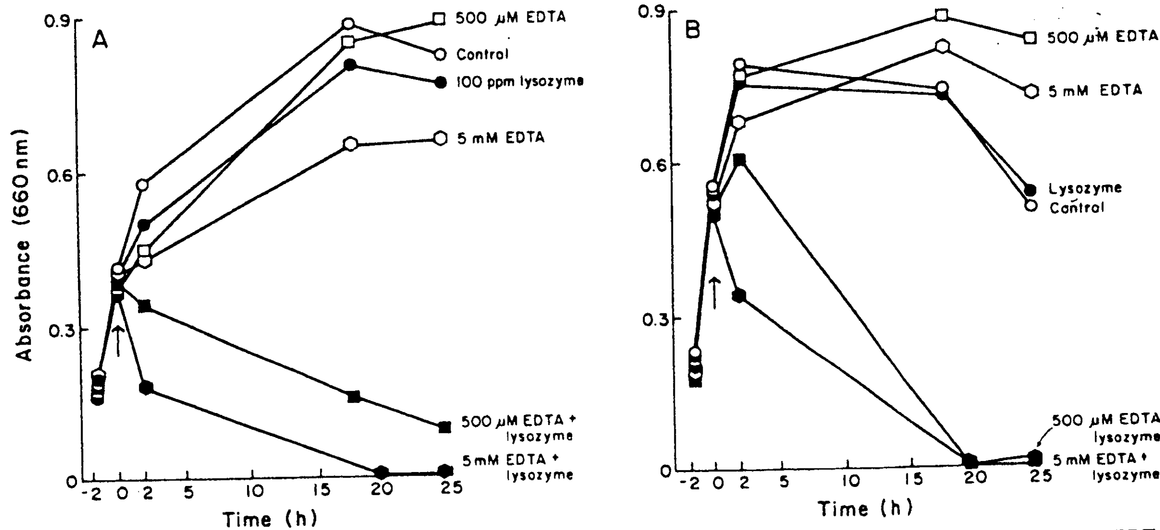

FIG. 3. Influence of EDTA on lysis of growing cells of *C. botulinum* 113B (A) and Hall A (B). Lysozyme (100 mg/liter) and EDTA (1 mM) were added at the arrows.

Table 2
STABILITY OF LYSOZYME IN NaCl SOLUTIONS[16]

Temperature (°C)	Time (min)	Remaining lysozyme activity (%) 0.5% Salt (w/w)	2% Salt (w/w)	5% Salt (w/w)	10% Salt (w/w)
90	30	76.6	87.6	85.6	93.5
	60	61.2	60.8	73.2	70.7
100	30	67.0	79.3	79.3	86.8
	60	39.8	55.6	55.6	58.4

Note: The concentration of lysozyme used was 20 ppm; initial activity was 100%.

Table 3
EFFECT OF POLYSACCHARIDES ON EGG WHITE LYSOZYME ACTIVITY[16]

Polysaccharide	Effect
Carboxy methyl cellulose (carbonic acid radical)	–
Alginate (carbonic acid radical)	–
Pectin (carbonic acid radical)	–
Chondroitin sulfuric acid (sulfuric acid base)	–
Agar (sulfuric acid base)	–
Tannin (sulfuric acid base)	–
Corn starch	+
Tammalindo seed polysaccharide	+
Locust bean gum	+
Low molecular weight pectin	+
Guar gum	+
Tragacinth gum	+

Note: Concentration of lysozyme used was 50 ppm. –, Lysozyme activity decreased greatly; +, lysozyme activity decreased slightly.

than monovalent cations. The effect of cations changes with pH such that the optimum cation concentration decreases with increasing pH. For example, 50 n*M* monovalent cations is optimal at pH 7.0, but at pH 9.0 it is strongly inhibitory. The activity of lysozyme can best be represented by an activity-pH-cation profile rather than by a pH-activity curve. The type of salt used also exerted particular effects. Davies et al.[40] also found lysozyme to remain highly active over the pH range of 5 to 10 if the salt concentration is varied appropriately. However, in contrast to Chang and Carr,[35] Davies et al.[40] found that the inhibition effect of high salt concentration was correlated to ionic strength rather than cationic concentration. Salt does have an effect on the bactericidal effect of lysozymes, and the right concentration must be found for maximum activity.

Hidaka, as cited in Yashitake and Shinichiro,[16] found that polysaccharides with both carboxylic and sulfuric acid bases were inhibitory to lysozyme activity (see Table 3). The inactivation of lysozyme by these compounds is due to precipitation and can be minimized by the addition of 1.0 to 6.0% salt.

B. Effects of Processing on Lysozyme

Damage to proteins from reactions with peroxidizing lipids is an important mechanism

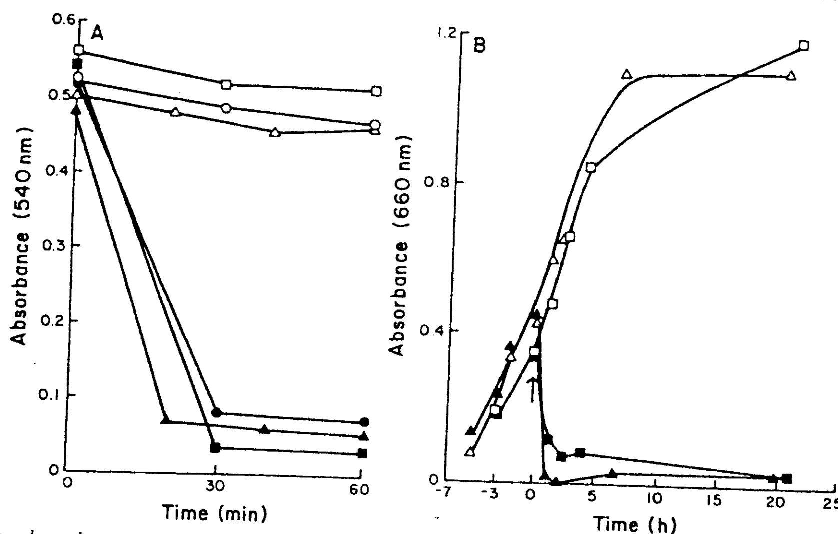

FIG. 1. Lysis of *C. tyrobutyricum* strains by lysozyme. (A) Cells suspended in phosphate buffer and exposed to 10 mg of lysozyme per liter. (B) Growing cells in reinforced clostridial medium exposed to 100 mg of lysozyme per liter at the arrow. Open symbols represent control treatments without lysozyme. Symbols: □ and ■, strain 142; ○ and ●, strain 13; and △ and ▲, strain ATCC 25755.

closely related *C. sporogenes*. *C. botulinum* types A and B and the *C. sporogenes* isolates differed widely in their response to lysozyme in buffer; certain of these strains lysed rapidly (e.g., Hall), whereas others were resistant (e.g., 113B) (Table 3). The differences between certain strains were consistently observed in repeated trials. With other *C. botulinum* strains however, such as Okra B and 17B, as well as *C. sporogenes* PA 3679, repeated trials gave erratic and irreproducible results. In these cases cultures lysed rapidly in some trials but were resistant in others. The type E nonproteolytic cultures were all uniformly resistant to the lytic activity of lysozyme. However, the inclusion of 1 mM

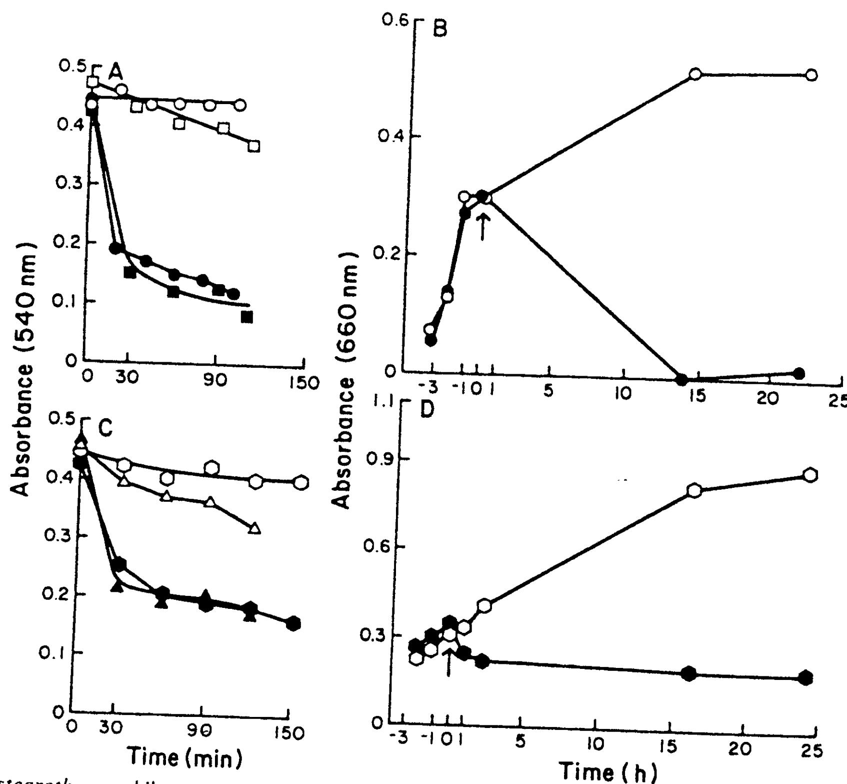

FIG. 2. Lysis of *B. stearothermophilus* and *C. thermosaccharolyticum* by lysozyme. (A) *B. stearothermophilus* cells suspended in phosphate buffer and exposed to 10 mg of lysozyme per liter. (B) Growing *B. stearothermophilus* cells exposed to 100 mg of lysozyme per liter at the arrow. (C and D) Same as A and B, respectively, with *C. thermosaccharolyticum*. Open symbols represent control treatments without lysozyme. Symbols: ○ and ●, *B. stearothermophilus* FS1518; □ and ■, *B. stearothermophilus* NRRL B1172; ⬡ and ⬢, *C. thermosaccharolyticum* HG-8; and △ and ▲, *C. thermosaccharolyticum* TA 5347.

in the processing and storage of foods.[41] Free-radical reactions peroxidize unsaturated fatty acids first into hydroperoxides and then hydroperoxide breakdown products, including malonaldehyde. A common manifestation of damage to proteins exposed to peroxidizing lipids is a decrease in protein solubility. Schaich[42] observed that incubation of lysozyme with peroxidizing methyl linoleate resulted in both decreased solubility of the protein and increased average molecular weight of the soluble fraction. Schaich and Karel[43] later studied production of free radicals in lysozyme because of reactions with peroxidizing methyl linoleate and found that free radical concentration in lysozyme decreased with increasing water activity, probably from radical recombination and cross-linking.

Funes et al.[44] continued the studies of lysozyme with peroxidizing methyl linoleate and found polymerization, loss of biological activity, and other deteriorative changes when lysozyme was exposed to incubation in air with methyl linoleate in a freeze-dried system and when the protein was exposed to the headspace over peroxidizing methyl linoleate or to the vapors of the volatile products of linoleate peroxidation, 2,4-decadienal, *n*-hexanal, and 2-heptenal. Through the use of electron spin resonance, the volatile reaction products generated protein-centered free radicals when lysozyme was exposed to these products. Cross-linking, loss of enzyme activity, and insolubilization increased with increasing water activity. Kanner and Karel[45] found that reactions of lysozyme with peroxidizing linoleate produced lysozyme dimers and higher polymers. The polymerization was due to covalent bonds. The degree of cross-linking, protein insolubilization, and loss of enzyme activity increased with increasing water activity.

Fujimaki et al.[46] used lysozyme and casein to study the effects of roasting at 150 to 300°C on proteins to determine what flavor substances or degradative products would be formed. Lysozyme was used as an example of a pure protein, containing only amino acids. Almost all of the amino acids were decomposed at 250°C. Tryptophan, sulfur-containing amino acids, basic amino acids, and β-hydroxy amino acids are easily decomposed compared with acidic amino acids, proline, aromatic amino acids, except for tryptophan, and amino acids with an alkyl side chain. This could be important in Maillard browning reactions where amino acids react with reducing sugars to form flavor and color components.

Gamma irradiation of egg white caused a decrease in lysozyme activity with increased radiation dose.[47] Kanner and Karel[45] found that gamma irradiation caused polymerization by covalent bonds. Kume et al.[48] found that the inactivation curve of 0.3% lysozyme solution at pH 8.0 was exponential. The dose required to reduce enzymatic activity to 37% of its initial value was 0.35 Mrad. However, lysozyme in egg white was only slightly affected by irradiation, being protected by the high concentration of protein. Mohamed[49] found that 0.85 Mrad of gamma irradiation on 0.5% lysozyme caused no change in density, viscosity, or pH. Growth rates of two strains of *Pseudomonas fluorescens* isolated from milk and eggs were lower when grown on irradiated substrates than on nonirradiated control substrates, but this effect was reversed during 3 weeks of postradiation frozen storage.

Eitenmiller et al.[50] compared effects of gamma irradiation on lysozyme from human milk, bovine milk, and egg white. Activity of the lysozymes decreased exponentially when exposed to increasing doses from 0 to 120 krad. The enzymes were more radiosensitive at 50 than at 500 μg/mℓ. Bovine milk lysozyme was the most sensitive, followed by egg white lysozyme, then human milk lysozyme. Tryptophan was destroyed in egg white and human milk lysozyme. Hydroxyl radicals played a role in the inactivation of all three lysozymes as indicated by the use of hydroxyl radical scavengers, cysteine, and *t*-butanol, which provided radioprotective action.

The type of container used to store lysozyme solutions may affect the activity of lysozyme. Goldblum et al.[51] studied the effect of Pyrex®, polypropylene, and polyethylene containers on immunological components of human milk. Lysozyme concentrations fell 40% after 24 hr in cold storage in Pyrex® and polypropylene containers. Kravchenko et al.[39] found that

TABLE 1. Bacterial species used and aeration conditions for growth

Species	No. of strains tested	Growth medium[a]	Temp (°C) and conditions[b]
Bacillus cereus	6	NB	30; aerated or static where noted
Bacillus stearothermophilus	2	TSB + YE + X	55; static
Campylobacter jejuni	1	BB	37; static
Clostridium botulinum types A and B	5	TSB	37; anaerobic
Clostridium botulinum type E	3	TPGY	30; anaerobic
Clostridium butyricum	1	TSB	37; anaerobic
Clostridium perfringens	2	TSB	37; anaerobic
Clostridium sporogenes	3	TSB	37; anaerobic
Clostridium thermosaccharolyticum	2	Thermo	55; anaerobic
Clostridium tyrobutyricum	3	RCM	37; anaerobic
Escherichia coli O157:H7	1	TSB	37; static
Klebsiella pneumoniae	1	LB	37; aerated
Listeria monocytogenes	4	BHI	37; static
Salmonella typhimurium	1	NB	37; static
Staphylococcus aureus	4	BHI	37; static
Vibrio cholerae	1	NB + NaCl	37; static
Yersinia enterocolitica	1	NB	25; aerated

[a] Abbreviations: BHI, brain heart infusion (Difco Laboratories); BB, brucella broth (Difco); LB, Luria-Bertani medium (per liter: 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl); MRS, lactobacillus MRS Broth (Difco); NB, nutrient broth (Difco); NB + NaCl, nutrient broth (Difco) plus 0.5% NaCl; Thermo, *C. thermosaccharolyticum* medium (per liter: 1.5 g of NaH_2PO_4, 3.0 g of K_2HPO_4, 1.5 g of $(NH_4)_2SO_4$, 1.0 g of $MgCl$, 0.15 g of $CaCl_2$, 10 mg of $FeSO_4$, 5 g of yeast extract, 0.5 g of cysteine, 0.5 ml of 0.2% resazurine, 10 g of xylose); TH, Todd-Hewitt broth (Difco); TSB, Trypticase soy broth (BBL Microbiology Systems); TSB + YE + X, Trypticase soy broth (BBL) plus 0.2% yeast extract (Difco) plus 0.2% xylose.

[b] Aeration conditions were as follows. Strict anaerobes (e.g., clostridia) were grown in anaerobic Hungate tubes under a nitrogen atmosphere. Facultative anaerobes were grown in static tubes (16 by 125 mm) two-thirds filled with medium. Aerated cultures were grown in 5 ml of medium in test tubes on a roller drum.

tibility in phosphate buffer (test 2) and was carried out with EDTA and other potential enhancing chemicals (see Results).

(iv) Test 4. Lysis of cells on agar plates. Lastly we determined the influence of lysozyme and EDTA on the ability of the Scott and Ohio strains of *L. monocytogenes* to form colonies when cells were seeded in brain heart infusion agar. In this procedure, lysozyme or EDTA or both were added to molten agar and the plates were poured and allowed to solidify. *L. monocytogenes* was then plated on the agar surface.

The lysis tests for each organism were done at least twice on separate days with cultures grown independently.

RESULTS

Growth inhibition by lysozyme in complex media. Initially we determined the ability of lysozyme to prevent the growth of bacteria inoculated into complex broth media containing lysozyme. In this assay, 3 bacterial species of 15 examined, *Bacillus stearothermophilus*, *C. thermosaccharolyticum*, and *C. tyrobutyricum*, were found to be completely inhibited (Table 2). Two species, *Campylobacter jejuni* and proteolytic *C. botulinum* type B, were weakly inhibited. *B. cereus* was slightly inhibited under static but not aerated conditions. Several bacteria were not inhibited by lysozyme under optimal conditions for growth (Table 2).

Lysis of nongrowing cell suspensions in buffer and exponentially growing cultures in complex media. The activity of lysozyme against the pathogens and spoilage bacteria was next evaluated by assaying lysis of nongrowing cells by lysozyme (10 or 100 mg/liter) in phosphate buffer and by determining the lysis of young growing cultures upon dosage with lysozyme (100 mg/liter). The latter test was usually done only with bacterial species that exhibited some susceptibility in phosphate buffer and was carried out with EDTA. This chelator was found to be effective in promoting lysis of several of the pathogens and spoilage bacteria (see below) that were not affected by lysozyme alone.

Initially we tested the susceptibility of the cheese spoilage bacterium *C. tyrobutyricum*. Wasserfall and Teuber (23) noted that approximately 90% of a population of *C. tyrobutyricum* cells were inactivated within 2 h by lysozyme. We confirmed the susceptibility of this organism by using two strains isolated from late blowing of cheese in Italy (strains 13 and 142) and with the type strain (ATCC 25755) from the American Type Culture Collection, Rockville, Md. (Fig. 1). Actively growing cultures of *C. tyrobutyricum* in reinforced clostridial medium also lysed when lysozyme (100 mg/liter) was injected into the culture (Fig. 1). The high susceptibility of *C. tyrobutyricum* probably accounts for the effectiveness of lysozyme in preventing late blowing of cheeses (23).

We found in this study that the food spoilage, anaerobic, extreme thermophile *C. thermosaccharolyticum* was highly susceptible to lysozyme (Fig. 2). Cell suspensions in phosphate buffer supplemented with lysozyme (10 mg/liter) cleared to 50 to 60% in 30 min and thereafter lysed slowly for several hours (Fig. 2C). Growing cells were inhibited on introduction of lysozyme (Fig. 2D). We confirmed that the aerobic thermophile *B. stearothermophilus* was also highly sensitive (Fig. 2). The susceptibility of *B. stearothermophilus* to lysozyme has previously been noted by Ashton et al. (3) and Messner et al. (14).

Although several isolates of *C. botulinum* and *C. sporogenes* were not inhibited for growth when inoculated into media containing lysozyme (test 1), several of these strains did lyse when nongrowing cells were suspended in phosphate buffer supplemented with lysozyme (Table 3). We tested eight strains of *C. botulinum* and three strains of the

TABLE 2. Inhibition of growth of various bacteria by lysozyme

Strains[a]	No. inhibited/ no. tested	Growth after 7 days at lysozyme concn (mg/liter) of[b]:	
		20	200
B. cereus	2/5	+ + +(+)[c]	+ + +(+)[c]
B. stearothermophilus	2/2	−	−
C. jejuni	1/1	+	+
C. botulinum (proteolytic) types A and B	1/4	+ +	+ +
C. thermosaccharolyticum	3/3	−	−
C. tyrobutyricum	3/3	−	−
Y. enterocolitica	1/1	+ + +(+ +)[c]	+ + +(+ +)[c]

[a] Bacteria not inhibited included *C. botulinum* (nonproteolytic) types B and E (four of four strains), *C. butyricum* (one of one), *C. perfringens* (two of two), *C. sporogenes* (three of three), *E. coli* O157:H7 (one of one), *K. pneumoniae* (one of one), *L. monocytogenes* (four of four), *S. typhimurium* (one of one), *S. aureus* (four of four), and *V. cholerae* non O:1 (one of one).

[b] All bacterial strains grew to + + + in media without lysozyme.

[c] Static cultures.

lysozyme adhered to glassware, causing poor reproducibility in determinations of lysozyme activity.

C. Sources of Lysozyme

Lysozyme has been found in many substances. The largest concentration is in tears, but for commercial extraction, hen egg albumen has become the major source of this enzyme. Lysozyme concentration in egg albumen is about 0.5% by weight.[52] Arora et al.[53] used five different types of hens and found that fresh egg albumen contained from 2250 to 3270 μg/mℓ. Upon storage, though, the lysozyme content decreased. Delchev and Ionova[54] stored eggs at (1) 0°C and 85% relative humidity (RH) for less than or equal to 5 months and (2) 15 to 20°C for less than or equal to 2 months. Lysozyme contents of egg white decreased gradually during both storage conditions from approximately 3 to approximately 1 mg/mℓ. Kato et al.[55] studied lysozyme content of hen eggs during storage. Centrifugation of thick white gave (1) a liquid and (2) a gel fraction. The ratios of lysozyme concentration and activity were 1.4 to 1.6 before storage and approximately 1.0 after storage for 5 days at 30°C. Lysozyme activity in thick white decreased to approximately 60% after storage for 5 days at 30°C. Loss of lysozyme was reduced at lower temperatures and by storing eggs in a CO_2 atmosphere. Lysozyme plays a role in egg white thinning. Sauter and Petersen[56] found that the loss of egg quality during storage was significantly greater ($p<0.05$) for eggs of low lysozyme content, 43 Haugh units (HU) compared to 34 HU for high lysozyme eggs. Differences in quality losses were particularly noticeable during the first 2 weeks of storage; 5 HU were lost for high-lysozyme eggs and 13 HU for low-lysozyme eggs. Cotterill[57] and Cotterill and Winter[58-60] concluded that lysozyme and ovomucin are electrostatically interacting in thick egg white. The pH of freshly laid egg white is initially 7.6, but increases to near 9.5 during storage due to the loss of CO_2. As the pH increases, it nears the isolectric point of lysozyme and the precipitation interaction time between lysozyme and ovomucin virtually disappears. Therefore it was suggested that the interaction between lysozyme-ovomucin was responsible for maintaining the thick white in a firm gel state, and the absence of reduction of this interaction may be responsible for one stage of egg white thinning. Vadehra et al.[61] found that the cuticle and shell contained little lysozyme, but the outer and inner membranes were rich in lysozyme, containing approximately the same amount per unit of membrane. The amount of activity was dependent on the extraction method, and a saline solution (0.9% NaCl) was found to be essential for lysozyme activity detection.

Fukamizo et al.[62] studied the differences in activity rates of eight avian lysozymes: seven hen-type lysozymes and one goose-type lysozyme with chitopentaose as the substrate (GlcNac)$_5$ at pH 5.0 and 50°C. Except for goose lysozymes, the values of rate constant K_{+1} of cleavage of glycosidic linkages for various lysozymes were different to some extent, whereas the values of K_{-1} and K_{+2} were common to all the lysozymes. Jolles et al.[63] believed that lysozymes belonging to different types — chicken (c) and goose (g) type — and possessing different molecular weights and behaviors might have been derived from a common ancestral protein. Ebner[64] found α-lactalbumin and lysozymes to have 40% of their amino acids in identical positions and suggested a common ancestry.

Lysozymes are also found in animal tissues and serum,[65] as well as in organs, tears, human and cow milk,[66] and cervical mucus.

Pavlovskii et al.[67] determined content and activity of lysozyme in the organs of cattle. Contents (mg/kg) and activities (%) of egg white lysozyme activity using acetone powder of *M. lysodeikticus* cells as substrate (determined by the method of Cherkasov and Kravchenko[68]) were, respectively: spleen, 50 to 160 mg/kg and 70%; thymus, 80 mg/kg and 40%; pancreas, 20 to 35 mg/kg and 20%; liver, trace and not stated; lungs, trace and not stated; and kidneys, trace and not stated. Since the largest amount of lysozyme was found in the spleen of cattle, Pavlovskii and Danilova[69] used affinity chromatography on

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Antimicrobial Activity of Lysozyme against Bacteria Involved in Food Spoilage and Food-Borne Disease

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Egg white lysozyme was demonstrated to have antibacterial activity against organisms of concern in food safety, including *Listeria monocytogenes* and certain strains of *Clostridium botulinum*. We also found that the food spoilage thermophile *Clostridium thermosaccharolyticum* was highly susceptible to lysozyme and confirmed that the spoilage organisms *Bacillus stearothermophilus* and *Clostridium tyrobutyricum* were also extremely sensitive. Several gram-positive and gram-negative pathogens isolated from food poisoning outbreaks, including *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus*, *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Salmonella typhimurium*, and *Yersinia enterocolitica*, were all resistant. The results of this study suggest that lysozyme may have selected applications in food preservation, especially when thermophilic sporeformers are problems, and as a safeguard against food poisoning caused by *C. botulinum* and *L. monocytogenes*.

Lysozyme is an important component in the prevention of bacterial growth in foods of animal origin such as hen eggs (4, 10, 13, 15) and milk (5, 22). The enzyme may also have applications as a preservative in foods that do not naturally possess it. It is attractive as a food preservative because it is specific for bacterial cell walls and harmless to humans. Industrial methods have been developed for its economical recovery from egg whites, and the deproteinized egg whites have been approved for food use in Europe and recently in the United States.

Currently, lysozyme has only limited applications in the food industry. It is added to certain hard cheeses in Europe to prevent gas formation and cracking of the cheese wheels by saccharolytic, butyric-forming clostridia, especially *Clostridium tyrobutyricum* (23). Other potential applications include its use in heat-sterilized products to reduce thermal requirements, its inclusion in immobilized enzyme columns to prevent contamination (9), and its use as a supplement to foods such as poultry, shrimp, sausage, and sake as a preservative (6, 9, 11, 16, 19, 24). In this study we show that lysozyme has antibacterial activity against previously untested food pathogens and spoilage bacteria including thermophilic clostridia, selected strains of *C. botulinum*, and *Listeria monocytogenes*.

MATERIALS AND METHODS

Materials. Egg white lysozyme (2, 8) and dried *Micrococcus luteus* ATCC 4698 cells were provided by Societa Prodotti Antibiotici, Milan, Italy. The preparation of lysozyme supplied by Societa Prodotti Antibiotici contained approximately 50,000 U/mg (21). One unit causes a decrease in turbidity at 540 nm of 0.001 AU/min at 25°C in 0.067 M sodium phosphate (pH 6.6) (21). Its activity remained stable for at least 18 months when stored as a dried powder at 4°C. EDTA (tetrasodium salt) was a product of Sigma Chemical Co., St. Louis, Mo. All other chemical reagents used were commercial products of the highest grade available.

Bacteria and growth conditions. The bacterial species used in this study and media for their cultivation are listed in Table 1. Bacterial strains tested were mainly isolates from outbreaks of food poisoning or spoilage and were obtained mostly from faculty of the Food Research Institute. The individual strains were purified by single-colony isolation before lysozyme treatment.

Activity of lysozyme against pathogens and spoilage bacteria. The antibacterial activity of lysozyme was evaluated by four independent tests.

(i) **Test 1. Growth inhibition of bacteria inoculated to media supplemented with lysozyme.** Filter-sterilized lysozyme was added at 0, 20, or 200 mg/liter to duplicate sets of complex growth media appropriate for each organism (Table 1). The media were inoculated with a 1:1,000 dilution of a young culture (final concentration of cells, ca. 10^5 to 10^6/ml). The tubes were incubated under suitable conditions of temperature and aeration (Table 1) for the organism being studied. After 1, 3, and 7 days, the growth was scored visually as −, +, ++, or +++.

(ii) **Test 2. Lysis of nongrowing bacteria in buffer.** Overnight mid- to late-exponential-phase cultures from 50 to 150 ml of medium were harvested by centrifugation for 20 min at 10,000 × *g*. The pellet was suspended in 5 to 10 ml of 0.067 M sodium phosphate buffer (pH 6.6). Lytic reactions were carried out in Hungate tubes (16 by 120 mm) with butyl rubber stoppers (Bellco Glass, Inc., Vineland, N.J.) in the phosphate buffer. Freshly prepared lysozyme was used to start the reaction; after the addition of the lysozyme (10 or 100 mg/liter), each tube was capped and gently inverted once or twice to mix. The A_{540} was then read in a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.). Initial A_{540} values ranged from 0.5 to 1.4 among the various cultures. The tubes were incubated in a 37°C water bath, and A_{540} were read at 20- to 30-min intervals for a minimum of 2 h depending on the susceptibility of the cultures.

(iii) **Test 3. Lysis of growing cultures after injection of lysozyme.** For a third test of the effectiveness of lysozyme, we determined the rate of lysis upon introduction of lysozyme to growing cultures (A_{660} of 0.2 to 0.5). In this procedure, lysozyme was injected at 100 mg/liter into the cultures, and lysis was determined at 660 nm at appropriate time intervals, but for at least 12 h. This test was usually done only with bacterial species that exhibited some suscep-

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deaminated chitin to isolate and study its properties. It was homogeneous and thermostable in acid and had a maximum activity at pH 6.4 and 0.125 *M* ionic strength. The activity of spleen lysozyme was 70% that of egg albumen lysozyme. Panfil-Kuncewicz and Kisza[70] tested the lysozyme content of cow and human milk and colostrum. The range for cow colostrum was 0.12 to 0.84 μg/mℓ and for cow milk 0.23 to 0.29 μg/mℓ; no definite trends among breeds or time were noted. The range for human colostrum was 37 to 91 μg/mℓ and for human milk 15 to 60 μg/mℓ; again there were no definite trends. It also has been found that human milk lysozyme has approximately the same molecular weight as that derived from egg white, but has twice the specific activity. Chandan et al.[71] reported bovine milk contained 0 to 260 μg of lysozyme activity with an average of 13 μg/100 mℓ of milk. Shahani et al.[72] and Chandan et al.[73] found human milk contained 39 mg/100 mℓ of milk. The conflict in the amount of lysozyme from various sources is due to varying methods of analysis and variations among the same type of samples. Senft et al.[74] tested lysozyme activity in black-pied cow milk at 14-day intervals. Lysozyme concentration in milk increased from 0.16 to 0.32 μg/mℓ during lactation, and there was a significant increase in mean lysozyme concentration between lactations 1 and 2, 3 and 4, and 5 and 6 in cows with healthy udders.

Ereifej and Markakis[75] isolated lysozyme from cauliflower. Testing the lysozyme content of several fruits and vegetables, Chandan and Ereifej[76] found the average lysozyme content (μg/mℓ) of juice to be cauliflower, 27.6; rutabaga, 8.9; papaya, 7.9; cabbage, 2.3; kohlrabi, 3.3; red radish, 4.8; white radish, 4.5; turnip, 1.8; parsnip, 1.6; and broccoli, 8.1. Many of the plants that exhibited lysozyme activity were from the family Brassicaceae. Of the 24 other vegetables and fruits tested, none showed lysozyme activity.

D. Extraction and Quantitation

Alderton et al.[77] developed the classic method of lysozyme extraction. Lysozyme was absorbed on bentonite (a montmorillonite clay), and elution of inactive contaminating proteins was done with successive washings of phosphate buffer (pH 7 to 8) and 5% aqueous pyridine. Elution of lysozyme was accomplished with pyridine-sulfuric acid solution at pH 5. The eluate was dialyzed and freeze dried. A white powder containing 85 to 90% of the lysozyme in egg white was obtained. Alderton and Fevold[78] improved the method and crystallized lysozyme as the salt of several acids and directly from egg white using 5% NaCl. This method has the advantage that other important proteins may be isolated from the same raw material, but has the disadvantage that more than 4 days and several crystallizations are required to achieve yields of 60 to 80% of the total lysozyme in egg white in a pure form.

Matsuoka et al.[79] accelerated crystallization of lysozyme from egg white by addition of nonionic surfactants, poly-(alkylene glycol), or lower alkylene glycol. These researchers used up to 5% NaCl, which they added to egg white homogenate at its isoelectric point of pH 9.5 for 4 days at 4°C. The lysozyme precipitate was dissolved in dilute HCl (pH 4.5) and filtered. To 180 mℓ of filtrate containing 2.0% lysozyme and 3.6% impurity were added 20 mℓ of 20% Nikkol NYS-25poly-(oryethylene)-monosterate made by Nikko Chemical Company, and NaCl up to 5%. The solution was left to stand at 3°C for 6 hr at a pH of 4.5 to give a yield of 95.6% crystalline lysozyme chloride.

Many of the methods developed to isolate lysozyme have used adsorption on chromatography columns. One of the first sorbent materials used was chitin, but new and improved materials have been developed. Cherkasov and Kravchenko[68] found glucochitin, obtained by deamination of chitin (DECH) from crab shells, to be a more specific sorbent for lysozyme than chitin because of the absence of ion-exchange properties. The yield of lysozyme from the egg white of one egg was 100 to 120 mg. Weaver et al.,[80] also using DECH rather than chitin, found that it had a high specificity and capacity for lysozyme (squid DECH more so than crab DECH), along with good stability, and allowed fast flow rates. Using the above

Table 1: **Antibacterial activity of lysozyme against bacterial species**
(Agar well method) Results of Representative trials

Test organism	Concentration of lysozyme					
	5 µg/ml	10 µg/ml	20 µg/ml	100 µg/ml	200 µg/ml	1000 µg/ml
Micrococcus lysodeikticus	20 mm	21 mm	22.5 mm	25 mm	24.5 mm	24 mm
Staphylococcus aureus	–	14 mm	14.5 mm	15.2 mm	14.8 mm	14.6 mm
Bacillus cereus	–	–	–	–	–	–
Escherichia coli	–	–	–	–	–	–
Salmonella typhosa	–	–	–	–	–	–
Shigella dysenterae	–	–	–	–	–	–
Proteus vulgaris	–	–	–	–	–	–
Pseudomonas aeruginosa	–	–	–	–	–	–

– No inhibition.

The quantitative estimation of the action of lysozyme by standard plate count method against *Micrococcus lysodeikticus* indicated that the bacterial count for the control was 34×10^2 and 30×10^8 organisms/ml at 0 and 12 hr respectively (Table 2). The cells of *Micrococcus lysodeikticus* treated with the lysozyme exhibited a decreasing trend i. e. 28×10^7 cells/ml after 12 hr incubation. The lysozyme treatment showed 90.6 % inhibition of the test organisms by standard plate count method.

Table 2: **Effect of lysozyme (10 µg/ml) on the growth of *Staphylococcus aureus* in reconstituted skim milk**

Time (hr)	SPC $\log_{10}$	
	Control	Experimental
0	3.43	3.50
1	3.68	3.64
2	4.39	4.08
4	5.71	5.41
6	6.69	6.29
8	7.61	7.30
12	9.46	9.10

Growth Inhibition

Data regarding the effect of lysozyme (10 µg/ml) on the growth of *Staphylococcus aureus* showed 56 % inhibition in growth (Table 3). Similar observations have been noticed by KARN et al. (9) regarding the inhibitory action of lysozyme on *Staphylococcus aureus*.

Table 3: **Qualitative estimation of antibacterial activity of lysozyme against different species**
Nakamura's Technique

Test organism	Optical density at 540 nm			
	Initial (at 0 hr)	Control without lysozyme	Experimental (with addition of lysozyme (10 µg/ml)	Percent inhibition of lysozyme
Bacillus cereus	0.30	0.20	0.12	77.7
E. coli	0.30	0.20	0.01	56.5
Salmonella typhosa	0.30	0.18	0.08	45.4
Shigella dysenterae	0.30	0.09	0.04	19.2
Proteus vulgaris	0.30	0.23	0.11	63.1
Pseudomonas aeruginosa	0.30	0.22	0.54	60.2

There was 100 % inhibition with *Micrococcus lysodeikticus*

The antibacterial activity of lysozyme on resistant organisms was recorded by modified Nakamura's technique (Table 4). Maximum inhibition by lysozyme was observed with *Bacillus cereus*, which was followed by *Pseudomonas aeruginosa, Proteus vulgaris, E. coli, Salmonella typhosa* and *Shigella dysenteriae* in the decreasing order of inhibition. The present findings are in accordance with the observations of GRULA and HARTSELL (6) in gram-ve bacteria. It may be inferred that low pH potentiates the activity of lysozyme due to the release of the substrate from a bound state within the cell wall.

Table 4: **Quantitative estimation of antibacterial activitiy of lysozyme against different bacterial species**
(Nakamura's technique)

Test organism	Optical density at 540 nm			
	Initial (at 0 hr)	Control without lysozyme	Experimental with addition of lysozyme i. e. 10 µg ml	Percent inhibition due to action of lysozyme
Bacillus cereus	0.30	0.20	0.12	77.7
E. coli	0.30	0.20	0.07	56.5
Salmonella typhosa	0.30	0.18	0.08	45.4
Shigella dysenterae	0.30	0.09	0.04	19.2
Proteus vulgaris	0.30	0.23	0.11	63.1
Pseudomonas aeruginosa	0.30	0.22	0.04	60.2

There was 100 % inhibition with *Micrococcus lysodeikticus*

The effect of alkali presumably results from hydration and dispersion of cell proteins following removal of lysozyme substrate.

Zusammenfassung

In der vorliegenden Arbeit sollte die antibakterielle Wirkung von Lysozym aus Eiklar auf einige pathogene und verderbniserregende Mikroorganismen geprüft werden. Qualitativ erfolgte die Feststellung einer Hemmung mit der Agar-Loch-Technik. Der inhibitorische Einfluß von 10 µg/ml Lysozym wurde über die Ermittlung der koloniebildenden Einheiten quantifiziert und erbrachte für S. aureus eine Hemmung von 56 %. Bei anderen Bakterien (Bacillus cereus, E. coli, Salmonella typhosa, Shigella dysenteriae, Proteus vulgaris und Pseudomonas aeruginosa) resultierten unter Anwendung einer modifizierten Nakamura-Technik Hemmungen von 77,7 %, 56,5 %, 45,4 %, 19,2 %, 63,1 % und 69,2 %.

Summary

The antibacterial activity of egg white lysozyme has been studied against the common pathogens and food spoilage organisms. Agar well method was used for qualitative estimation of inhibitory activity of lysozyme. Quantitative estimation of 10 µg/ml lysozyme by standard plate count showed 56 percent inhibition against S. aureus. In case of other organisms viz., *Bacillus cereus, E. coli, Salmonella typhosa, Shigella dysenteriae, Proteus vulgaris* and *Pseudomonas aeruginosa* by modified Nakamuri's technique showed 77.7 %, 56.5 %, 45.4 %, 19.2 %, 63.1 % and 69.2 % inhibition respectively.

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procedure, researchers obtained a 99% recovery of lysozyme in fresh egg white, human blood, human milk, goat milk, and a commercial hen egg white lysozyme preparation.

Bailon and Nishikawa[81] found lysozyme to bind well to phenyl structures through non-specific hydrophobic adsortion. Lysozyme was purified on agarose derivatives containing a phenylacetyl ligand. The phenylacetylagarose is a general affinity sorbent bearing a ligand of nonbiochemical origin in contrast to those which use nucleotide cofactors. Fernandez-Sousa et al.[82] extracted lysozyme from hen egg white by gel filtration chromatography on agarose columns. This medium was selective for lysozyme because the protein interacted with the agarose matrix and eluted later than the corresponding total volume for the column.

Again improving on the ion-exchange resin technique, Ahvenainen et al.[83] found a simple method that required no pretreatment of egg white other than homogenization. Because of the high viscosity of egg white, it was first adsorbed onto the resin by a batch tank process, followed by washing and elution of the enzyme in a column operation, which achieved an almost 100% recovery of lysozyme. A Japanese procedure patented by Ezaki Glico Eishoku Co.[84] processed egg whites for lysozyme by treating them with specific swelling starches, which adsorbed the lysozyme. A method to concentrate and fractionate egg white by ultrafiltration was developed by Peri and Feresini.[85] Lysozyme retention varied according to membrane size and temperature. A 93% yield was obtained from 0.388 to 0.477 mg/mℓ using the SM 115-33 membrane. Ghielmetti and Trinchera[52] developed a method where egg white was contacted with a weakly acidic ion-exchange resin at pH 6 to 7 and then eluted with salt solution to remove contaminating proteins. The salt concentration was increased and the lysozyme eluted.

Li-Chan and Nakai[86] used cation-exchange chromatography to separate lysozyme from egg white that still retained high functionality. A macroporous weak resin was chosen from various possible strong and weak acid exchangers based on its relatively low cost, high lysozyme recovery, and potential for continuous use. Polyacetylamide gel electrophoresis yielded a lysozyme fraction of high purity, except for possible coelution of avidin, and a protein valued for analytical applications. The lysozyme-free egg white posessed superior whipping, gelling, and emulsifying properties.

Hen egg white is the major source of commercial preparations of lysozyme. Wilkinson and Dorrington[17] described a procedure to remove lysozyme from crude egg white left in broken shells at egg-breaking plants. This protein-rich residual white, unsuitable for human consumption, has been used for stock feed supplementation. Extraction of lysozyme from waste egg white would give the producer a more economical byproduct. The egg white was heat coagulated and centrifuged. The lysozyme stayed in the whey or supernatant where an ion-exchange procedure was used to isolate the enzyme on a cation exchanger using strongly acidic sulfopropyl form on $-C_3H_6SO_3-$ (SP-Protion) exchange medium.

Other procedures for the isolation of lysozyme have been developed for systems other than egg whites. Their purposes were to demonstrate the role of lysozyme as a pharmaceutical and to gain knowledge of lysozyme content of different sources rather than to develop an industrial lysozyme preparation procedure. Germaine and Tellefson[87] developed a method for removing lysozyme from human saliva; Parry et al.[88] isolated lysozyme from human milk; and Pavlovskii and Danilova[69] developed a method for isolating an edible lysozyme preparation from meat industry wastes.

One of the oldest and most widely used procedures for quantitating lysozyme involves the reduction in turbidity of a suspension of dried *M. lysodeikticus* cells in the presence of lysozyme, measured spectophotometrically in an aqueous buffer solution. The activity of the enzyme preparation was recorded as the initial rate of clearing of the turbid suspension, one unit of activity being equal to a decrease in absorbency at 450 nm of 0.001/min at pH 7.0 and 25°C.[40] Parry et al.[89] also used a method that utilized a 50% suspension of UV-killed and lyophilized *M. lysodeikticus* cells in phosphate buffer at pH 6.2, using a 0.3 *M*

20,7 % signifikant höher als der von Nordseekrabben in Aspik mit 15,8 %. Als Ursache dafür kommt der höhere Wassergehalt der dem Lakebad entnommenen „Tiefseekrabben" in Frage. Schon nach 1tägiger Lagerung waren die im wesentlichen auf Wasserübertritt aus den Krabben beruhenden Austauschvorgänge praktisch abgeschlossen. Danach traten signifikante Gewichtsveränderungen nicht mehr auf. Einflüsse der Lagerungstemperatur (4 bzw. 11 °C) ließen sich nicht erkennen. Wie vergleichende Untersuchungen von Krabben mit und ohne Aspik zeigten, übte der Aspik einen konservierenden Effekt aus, so daß die Lagerfähigkeit bis zu 45 Tagen bei 4 °C ohne Qualitätseinbußen gewährleistet war.

Summary

Shrimp in jelly, manufactured under practice conditions, were stored at 4 and 11 °C up to 45 days. The loss in weight of shrimp from Indo-Pacific-Origin was with an average of 20.7 % significantly higher than the loss in weight of shrimp coming from the North Sea with 15.8 %. The higher water content of the shrimp from Indo-Pacific-Origin taken from the brine might be the cause for that. After having stored the shrimp for 1 day, the exchange processe[illegible] already finished. Th[illegible] anymore. Influences [illegible] were not found. A[illegible] without jelly showed [illegible] preserving effect so that a storage was guaranteed up to 45 days at 4 °C.

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Antibacterial activity of lysozyme against some common food poisoning organisms

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Introduction

Lysozyme catalyses the lysis of certain bacteria by hydrolysing the β linkage between muramic acid and glucosamine of glycopolysaccharides of the bacterial cell wall. This antibacterial substance is present in several biological systems, notably in tears, nasal secretions, blood, saline, kidney, spleen, bacteria and milk (3, 4, 7, 8, 14, 15, 17). Although antibacterial activity of egg white lysozyme against Gram+ve bacteria has been studied by several workers (5, 8, 12, 16), the susceptibility of Gram-ve bacteria towards lysozyme has not been thoroughly investigated. The information regarding the antibacterial activity of lysozyme towards potential food poisoning organisms has been rather scanty except few scattered reports by PETERSON and HARTSELL (10) and REPASKE (11). Hence, in the present investigation, an attempt has been made to determine the antimicrobial potentials of lysozyme against some commonly occurring food poisoning including gram-ve ones in milk and milk products.

Materials and methods

Source of Enzyme

Egg white lysozyme was procured from Sigma Chemicals and used without purification for determining its antimicrobial activity.

Culture

The following test organisms used in the study were procured from Post Graduate Institute of Medical Sciences, Chandigarh. *Bacillus cereus, E. coli, Salmonella typhosa Shigella dysenteriae, Staphylococcus aureus, Proteus vulgaris, Pseudomonas aeruginosa. Micrococcus lysodeikticus* was used as a standard culture for determining the inhibiting activity of lysozyme.

Maintenance of different bacterial cultures

All the test organisms were maintained on nutrient agar slants except for *S. aureus*, where BAIRD-PARKER medium was used. These were subcultured regularly at weekly intervals.

Medium for antibacterial activity of lysozyme

Brain heart infusion agar containing 3.7 % Difco dehydrated brain heart infusion agar, was used for the qualitative estimation of antibacterial activity of lysozyme by agar well method.

For quantitative estimation of antibacterial activity of lysozyme, BAIRD-PARKER medium was used for the growth of *Staphylococcus aureus.*

Qualitative estimation of antibacterial activity of lysozyme

1 ml of 50 % cell suspension of the test cultures was inoculated into brain heart infusion medium (10 ml) and poured into sterilized petri plates. Four wells of 0.4 mm diameter were made in each plate and wells were filled with different concentrations of lysozyme. The different concentration used were 5 μg/ml, 10 μg/ml, 20 μg/ml, 100 μg/ml and 200 μg/ml. Then the plates were kept for incubation at 37 °C for 24 hours for the appearance of zones.

Quantitative estimation

Two assay bottles containing 99 ml sterilized skim milk were inoculated with 1 ml of 50 % cell suspension containing approx. 10^3–10^4 cells. In one of the bottles, 1 ml of lysozyme was added to give a final concentration of 10 μg/ml in the medium. The second bottle was kept as control, in which 1 ml of sterilized glass distilled water was added instead of lysozyme solution. Assay bottles were incubated at 37 °C and the counts were recorded at different time intervals i. e. 0, 1, 2, 4, 6, 8 and 12 hrs. The percent growth inhibition was calculated using the formula of BISHOP et al. (2).

% Growth inhibition

$$= \frac{(SPC_{0-12\ hrs\ control}) - (SPC_{0-12\ hrs\ experimental}) \times 100}{(SPC_{0-12\ hr\ Control})}$$

When ($SPC_{0-12\ hr\ Control}$) = (SPC at 12 hr) – (SPC at 0 hr) for the control assay

($SPC_{0-12\ hr\ experimental}$) = (SPC at 12 hr) – (SPC at 0 hr) for the experimental assay (added lysozyme).

Modified Nakamura technique

To the tubes containing 2 ml of 10 μg/ml lysozyme in water (adjusted to pH 3.5), 2 ml of cell suspension were added to give an optical density of 0.30 and incubated for one hr at 45 °C. Sufficient amount of 0.1 M NaOH was then added to adjust the system to pH 10.0–10.5. One control was kept in which lysozyme was absent. Optical density was read immediately and percent lysis was calculated as follows:

$$\%\ lysis = \frac{(O.D_{initial-experimental}) - (O.D_{initial-control})}{(O.D_{initial-experimental})}$$

Results and Discussion

Micrococcus lysodeikticus and *Staphylococcus aureus* were inhibited with different concentrations ranging from 10 μg/ml, 20 μg/ml, 100 μg/ml, 200 μg/ml and 1000 μg/ml of lysozyme (Table 1). On the contrary, other organisms namely *Bacillus cereus, E. coli, Salmonella typhosa, Shigella dysenteriae, Proteus vulgaris* and *Pseudomonas aeruginosa* exhibited resistance to the action of 1000 μg/ml of lysozyme. Similar observations have been recorded by ARAKI et al. (1). A close correlation exists between the content of N-acetylated glucosamine residues in the peptidoglycan component and the restistance of cell wall to lysozyme. From the present study, it may be evident that gram-ve organisms were not inhibited by the action of lysozyme. These observations are in consistent with the reports of SALTON (12, 13) indicating that the presence of lipoprotein layer in gram-ve organisms accounts for the resistance to the action of lysozyme.

NaCl solution to enhance enzyme activity. The materials were added to a cuvette in the following proportions: 1.5 mℓ of the cell suspension, 0.5 mℓ of NaCl solution, and 1.0 mℓ of enzyme solution, making an effective concentration of 0.05 *M* NaCl and 25 mg % cells in the enzyme assay mixture. The mixture was stirred momentarily and the rate of clearance measured spectrophotometrically. The initial transmittance of the assay mixture was approximately 10%. Spectrometer readings were done at 540 nm and no particular time was necessary for the measurements, but the rate of clearing of the suspension by lysozyme had to be linear. Tsumuraya and Hidaka[90] improved on the method by carrying it out turbidimetrically at a higher temperature (60°C) and for a longer time (60 min) than in the conventional method. In order to stimulate the lytic activity of lysozyme, small amounts of nonionic surfactant and NaCl were added to 0.066 *M* phosphate buffer at pH 6.2. This procedure made it possible to determine the lytic activity of hen egg white lysozyme at a concentration of 0 to 15 mg/mℓ of reaction solution.

Weaver et al.[80] assayed lysozyme activity with a modification of the method used by Katz.[91] A suspension of heat-killed *M. lysodeikticus* cells, 30 mg/mℓ, was made in 0.66 *M* phosphate buffer (pH 7.0). The suspension was slurried continuously until the assays were completed. Using a Vortex stirrer, 10 μℓ of lysozyme solution to be assayed was mixed with 3 mℓ of *M. lysodeikticus* suspension in a cuvette and read at 450 nm in a Spectronic® 20 spectrometer set at 0.500 absorbance. Optical density (OD) was taken every 30 sec for 2 min. A decrease in absorbance of 0.001/min was taken as one unit of enzyme activity (U) and the results were expressed as units/mℓ calculated as

$$U/m\ell = \frac{OD/m\ell}{0.001\ OD/min \times 0.01\ m\ell}$$

This method required that samples of high lysozyme content be diluted and *M. lysodeikticus* cell suspensions be prepared fresh daily to obtain accurate results.

D'Orazio et al.[92] used living bacterial cells of *M. lysodeikticus* as substrate for lysozyme activity. The cells were loaded with a marker, trimethylphenylammonium ion, which was released through the action of lysozyme upon the cell wall. The rate of ion release was monitored with a highly selective membrane electrode and related to the concentration of enzyme present.

Watanabe[93] quantitated lysozyme by a modified method of Kakizaki's turbidimetric assay.[94] This method was used to quantitate lysozyme in saliva where lower concentrations are found. A total of 3 mℓ of 0.1% *M. lysodeikticus* in 0.066 *M* phosphate buffer, pH 6.0, and 3 mℓ of sample in 0.066 *M* phosphate buffer, pH 6.0, containing 3% bovine serum albumen (the bovine serum albumen stabilized the centrifuged salvia lysozyme in the homogenate and precipitate) were separately preincubated at 37°C for 5 min, mixed, and incubated again at 37°C for 10 min. The OD was measured at 640 nm. Initial *M. lysodeikticus* sample solution should be approximately 10% transmission.

Galyean and Cotterill[95] developed a method to measure total lysozyme and not only the biologically active enzyme. Pure lysozyme was chromatographed on diethylaminoethyl (DEAE) cellulose, then monitored spectrophotometrically and by biological activity measurement. Some biological activity was lost during chromatography, but standard curves relating enzyme content to chromatographic peak area corrected the loss.

Another procedure for determining lytic activity is the agar plate method where clear zones on agar plates seeded with *M. lysodeikticus* indicate lytic activity. Katz et al.[96] developed an agar plate method to determine lysozyme in mammalian milk: 1.50 g agar (Difco Bacto-agar), 0.2 g NaCl, and 0.002 g *M. lysodeikticus* (heat-killed, dried cells) were dissolved and suspended in 100 mℓ potassium phosphate buffer (pH 7.0) by heating to 90°C with magnetic stirring and without bubbling. The preparation was then cooled to 65 to 70°C

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and poured into or over a suitable glass plate to give a layer 2 mm thick. The agar was cooled and 1-mm holes or wells about 2 cm apart were punched into the agar layer with a transfer pipette. The holes need not penetrate the agar completely. A standard calibration curve was prepared by dissolving increasing amounts of pure hen egg white lysozyme in distilled water; 10-$\mu\ell$ aliquots of lysozyme solution were placed into the wells. The plate was then covered and left standing at room temperature for 3 hr. A clear zone of lysed bacterial cells appeared around those wells containing lysozyme at concentrations greater than 0.0725 $\mu\ell$/10 mℓ. Sensitivity of the method increased with longer standing. The logarithms of the clear zone diameters, including the 1-mm wells, were directly proportional to lysozyme concentration. They were plotted as a straight line on semilogarithmic paper. The logarithms of the diameters of the clarified zones obtained with mammalian milk lysozyme extracts were converted to lysozyme content with the aid of the calibration curve. The results were expressed in units of hen egg white lysozyme. Vakil et al.[97] used a disk-assay technique to quantitate lysozyme; 8 mℓ of melted nutrient agar or any other medium suitable for the growth of a certain test organism, inoculated with 1% of 18- to 24-hr-old nutrient broth culture of an organism under investigation, was poured into a sterile petri dish. Upon solidification of the seeded agar, a sterile filter paper disk of 12.7 mm in diameter was dipped into 0.1% lysozyme solution and placed on the seeded agar surface. The plates were preincubated at 10°C for 2 hr to allow the lysozyme to diffuse into the agar and then incubated at the temperature that was optimum for the test organism. Plates were incubated for 16 to 18 hr and diameters of zone inhibition were measured.

The agar plate method was improved by Gosnell et al.[98] to handle lower concentrations of lysozyme so that 5 μg of egg white lysozyme per 100 mℓ of sample could be determined in 3 hr. The plates were first stained with 0.5 g of buffalo black in 100 mℓ of 7% acetic acid, followed by destaining in 7% acetic acid until satisfactory contrast between the clear zones of lysis and the surrounding seeded agarose was obtained. The researchers found that standard egg white lysozyme solutions remained stable for 7 or more days at 5°C. They noted that the best lyso-plate method included 1.0 g agarose with 0.1 g NaCl in 100 mℓ of a pH 7.0 phosphate buffer seeded with 0.020 g of *M. lysodeikticus* and incubated for 3 hr at 47°C.

A drop method for determination of micrococcal and staphylococcal sensitivity to lysozyme was developed by Safonova et al.,[99] who claimed the method to be simpler and more economical in comparison to serial dilutions in agar and capable of detecting single strains.

E. Enzyme Substrate Reaction of Lysozymes

Lysozymes are effective at lysing bacterial cell walls because they split the linkages in which the carbon 1 in *N*-acetylmuramic acid (NAM) is linked to carbon 4 in *N*-acetylglucosamine (NAG). These residues are linked together in an alternating fashion, with the bond suitable for lysozyme splitting occurring in every fourth residue (Figure 3). In the cell wall, these molecules are linked together by β(1-4)glycosidic linkages in long polysaccharide chains. The polysaccharide chains are cross-linked by short lengths of polypeptide chain and attached to the NAM residues through the lactyl side chain attached to carbon atom 3 in each NAM ring.

The method by which lysozyme lyses its substrate has been defined by Phillips.[1] A lysozyme molecule attaches to the bacterial cell wall by interacting with six exposed amino sugar residues connected by β(1-4) linkages that join the fourth and fifth rings of the polysaccharide chain. In the process, the fourth sugar residue is distorted. Second, glutamate residue 35 of lysozymes transfers its terminal hydrogen atom in the form of a hydrogen ion to the glycosidic oxygen, thus bringing about cleavage of the bond between the oxygen and carbon atom 1 of the fourth sugar residue. This creates a positively charged carbonium ion (C^+) where the oxygen has been severed from atom 1 of the fourth ring. Third, this carbonium

Enterobacter aerogenes 551	580	415	387	230	40	130	290	[illegible]	149	108	70	48
Escherichia coli O157:H7	1160	711	340	200	[illegible]	96	336	221	160	115	74	62
Pseudomonas aeruginosa ATCC 9027	>3170	>3170	>3170	>3170	71.	340	>790	336	218	200	190	120
P. fluorescens 545	>3170	440	190	<4	<4	<4	450	205	31	<2	<2	<2
P. putida 515	>3170	662	231	66	<4	<4	218	150	100	74	62	50
Salmonella typhimurium 617	1340	820	340	180	130	66	325	237	194	149	95	70
Yersinia enterocolitica RS29	262	180	169	91	18	<4	140	120	108	90	<2	<2
Y. ruckeri ATCC 29473	140	95	70	<4	<4	<4	100	75	37	<2	<2	<2

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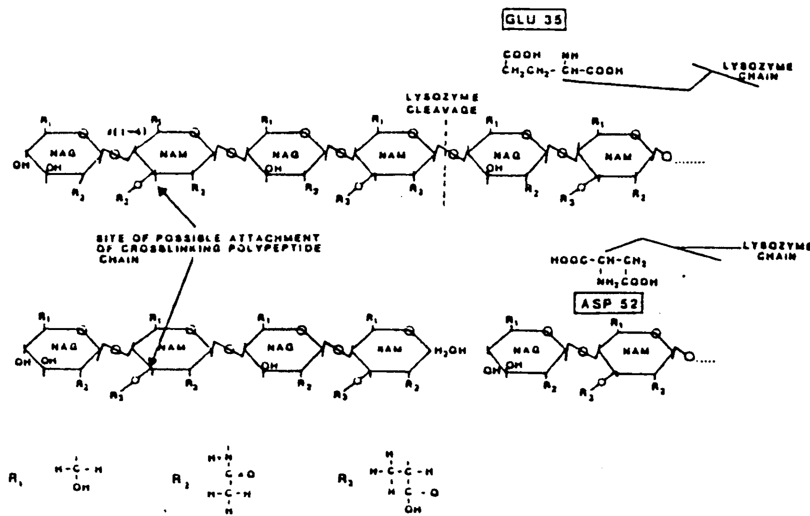

FIGURE 3. The structure shown is of the polysaccharide molecule found in the cell walls of certain bacteria containing the glucose-like amino sugars *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAM) placed alternately in the chain bound by β(1-4) glycosidic linkages. These chains are cross-linked in the bacterial cell wall by short lengths of polypeptide chain connected between NAM residues through the lactyl side chain attached to carbon atom 3 in each NAM ring. Six residues fit into the active site of the lysozyme molecule where glutamic acid (residue 35) and aspartic acid (residue 52) of the lysozyme are involved in the cleavage of the bacterial cell wall polysaccharide.[1,233]

ion is stabilized by its interaction with the negatively charged aspartic acid side chain of lysozyme residue 52 until it can combine with a hydroxyl ion (OH^-) that happens to diffuse into position from the surrounding water, thereby completing the reaction. The surrounding water also supplies a H^+ ion to replace the one lost by residue 35. The lysozyme molecule then falls away, leaving behind a punctured bacterial cell wall.[1]

Three antigenic sites are found on the lysozyme molecule, and their exact boundary, residue, conformational, and directional definitions are described by Atassi and Lee.[100] The amino acids involved in the three sites can be seen in Figure 1. Schindler et al.[101] studied the role of ground-state strain in subsite D in hen egg white and human lysozymes during lysozyme catalysis. Subsite D is described as the fourth subsite from the nonreducing end of at least six GlcNAc (*N*-acetyl-D-glucosamine) moieties in an amino sugar polysaccharide and is the site of bond cleavage. Previously, it was thought that stress in subsite D was responsible for a reduction of the activation energy for bond cleavage in the enzymatic reaction by 5 to 10 kcal/mℓ,[102] but Schindler et al.[101] concluded that stress in subsite D has been overestimated and accounts for only a small part of the observed catalytic effect of the enzyme. Using oligosaccharides containing *N*-acetylmuramic acid, *N*-acethyl-D-glucosamine, or *N*-acetyl-D-xylosamine bound in subsite D, the researchers found no strain involved in the binding of *N*-acetyl-D-glucosamine, but that the lactyl group of NAM (rather than the hydroxymethyl group) is responsible for the apparent stress previously reported for binding at this subsite. The dependence of saccharide binding on pH or on a saturating concentration of Gd(III) and fluorescence difference spectra of the various hen egg white lysozyme saccharide complexes suggest that the conformations of several of the complexes are different from one another and from those proposed previously for a productive complex.

Morozoi and Morozova,[103] and Morozova and Morozoi[104] studied the elastic properties of the hen egg white lysozyme crystal and found that the lysozyme molecule consisted of

TABLE 10.
MINIMUM INHIBITORY CONCENTRATION (μg/mL) AT pH 7 AT DIFFERENT NaCl CONCENTRATIONS OF A COMBINATION OF BUTYLATED HYDROXYANIZOLE (BHA) AND TRIGLYCEROL 1,2 LAURATE (TGL) WITH AND WITHOUT AN EQUAL CONCENTRATION OF EDTA

Organism	L + BHA						L + BHA + EDTA					
NaCl conc. (%)	0.5	1	2	3	4	5	0.5	1	2	3	4	5
Gram-positive												
Bacillus cereus 17	123	66	39	31	13	<4	44	35	32	28	17	6.6
B. licheniformis UG	150	57	39	31	13	<4	54	35	35	26	18	7
B. subtilis 22	139	53	33	26	13	<4	50	37	35	31	17	<2
Lactococcus lactis NCK400	66	53	21	11	<4	<4	44	31	20	13	<2	<2
Listeria monocytogenes ScottA	262	192	123	103	24	22	144	112	90	80	50	30
Staphylococcus aureus ATCC 6538	97	91	71	49	25	20	73	70	69	54	42	28
S. epidermidis ATCC12228	103	91	62	25	<4	<4	61	45	33	30	14	5.7
Gram-negative												
Enterobacter aerogenes 551	580	415	387	230	140	130	290	170	149	108	70	48
Escherichia coli O157:H7	1160	711	340	200	140	96	336	221	160	115	74	62
Pseudomonas aeruginosa ATCC 9027	>3170	>3170	>3170	>3170	711	340	>790	336	218	200	190	120
P. fluorescens 545	>3170	440	190	<4	<4	<4	450	205	31	<2	<2	<2
P. putida 515	>3170	662	231	66	<4	<4	218	150	100	74	62	50
Salmonella typhimurium 617	1340	820	340	180	130	66	325	237	194	149	95	70
Yersinia enterocolitica RS29	262	180	169	91	18	<4	140	120	108	90	<2	<2
Y. ruckeri ATCC 29473	140	95	70	<4	<4	<4	100	75	37	<2	<2	<2

two rigid domains connected by a flexible link. The binding of *N*-acetyl-D-glucosamine in the active-site cleft was accompanied by an increase of about 40% in the interdomain rigidity. Nakanishi and Tsuboi[105] and Nakanishi et al.[106] also studied the fluctuation of the lysozyme structure.

Kato et al.[107] studied the refolding kinetics of hen egg white lysozyme spectrophotometrically and concluded that the kinetics indicated strict biphasic behavior.

F. Lysozyme Complexes

Lysozyme forms complexes with other components and is often rendered inactive. The importance of lysozyme complexes in egg white was discovered when it was noticed that a small amount of yolk within the egg white drastically reduced the volume of the egg white foam.[108] The reason was not apparent until much more work was done with the various egg white proteins. Fleming and Allison[109] reported that a mixture of egg white with an equal amount of yolk resulted in the loss of antibacterial action except against *M. lysodeikticus*. Dehydrated whole egg retained only part of its lysozyme activity.[110] Cunningham and Cotterill[111] showed that egg yolk inactivated purified lysozyme in a pH 6.2 phosphate buffer. Cunningham[112] and Cunningham and Cotterill[111] observed a change in the ion-exchange chromatographic patterns of lysozyme in egg white contaminated with yolk. There were only two lysozyme peaks in yolk-contaminated egg white compared with three peaks in uncontaminated egg white. A total lack of lysozyme in the chromatographic separation of whole egg was reported by Parkinson.[113]

Galyean et al.[114] performed a battery of tests in which they found that 10% yolk totally inhibited lysozyme during the first minute of reaction, but lysis still occurred after longer reaction times. Activity at various pH levels between 5.0 and 9.0 in egg white containing 5% yolk paralleled that of yolk-free egg white. Maximum activity was observed about pH 8. Centrifuging increased the lysozyme activity in supernatant containing yolk at pH values of 5 to 8. Both salt and urea partially reactivated yolk-inhibited lysozyme. Both compounds dissociate electrostatic complexes. Lipovitellin (egg yolk component) inhibited lysozyme activity. These data led researchers to suggest that the mechanism of inhibition involved electrostatic interaction between lysozyme and yolk components.

It is well known that the β(1-4)linked polymer of *N*-acetyl-D-glucosamine is a substrate of lysozyme. The pentamer and higher polymers are readily cleaved by lysozyme, but monomer, dimer, trimer, and tetramer are bound by lysozyme to form complexes with longer lifetimes and are inhibitors for lysozyme.[102,115]

Sodium dodecyl sulfate (SDS), a detergent, forms a stable complex with lysozyme without causing a gross conformational change in the enzyme molecule, but some SDS binds to the active-site cleft and strongly inhibits its activity. Hydrophobic regions and positive charges from the protein side and a hydrophobic tail (possibly more than eight carbons in alkyl chain) and a negative charge from the detergent side were required for formation of the complex.[116] Blake[117] reported that the lysozyme molecule unfolded when SDS was present, yet the four disulfide bonds remained intact.

Other substances with which lysozyme forms complexes include thymus nucleate and yeast nucleate,[118] bovine plasma albumen,[119] ovomucin,[120,121] conalbumin,[122] ovalbumin,[27,123,124] thyroxine or thyroglobulin,[125] imidazole and indole derivatives such as histamine and indole-3-propionic acid,[126] and insulin.[127]

Polyvalent cations in solution and divalent cations such as cobalt (CO^{+2}),[128] manganese,[129] mercury,[130] and copper[131] inhibit the enzymatic activity of hen egg white lysozyme, although only at relatively high concentrations (10^{-3} to 10^{-2} *M*). Teichberg et al.[132] found that copper inhibited noncompetitively, with an inhibition constant Ka = 3.8×10^{-2} M^{-1}. Beddell et al.[133] found that iodine irreversibly inactivated hen egg white lysozyme in small amounts at acid or neutral pH. Metal ions seem to bind carboxyl groups of Glu-35 and Asp-52 in the active site of the enzyme.[129]

TABLE 9.
MINIMUM INHIBITORY CONCENTRATION (μg/mL) AT DIFFERENT pH'S OF A COMBINATION OF BUTYLATED HYDROXYANIZOLE (BHA) AND TRIGLYCEROL 1,2 LAURATE (TGL) WITH AND WITHOUT AN EQUAL CONCENTRATION OF EDTA

Organism	BHA + TGL				BHA + TGL + EDTA			
pH	7	6	5.5	5	7	6	5.5	5
Gram-positive								
Bacillus cereus 17	123	48	15	<4	44	26	14	<2
B. licheniformis UG	150	48	22	<4	54	27	11	<2
B. subtilis 22	139	53	26	<4	50	37	21	<2
Lactococcus lactis NCK400	66	53	33	15	44	45	29	16
Listeria monocytogenes Scott A	262	150	110	39	144	97	65	31
Staphylococcus aureus ATCC 6538	97	62	42	14	73	66	42	24
S. epidermidis ATCC12228	103	97	48	<4	61	58	24	<2
Gram-negative								
Enterobacter aerogenes 551	580	262	245	190	290	251	218	165
Escherichia coli O157:H7	1160	363	300	160	336	218	204	165
Pseudomonas aeruginosa ATCC 9027	>3170	>3170	>3170	1160	>790	500	437	220
P. fluorescens 545	>3170	200	66	<4	450	110	45	<1
P. putida 515	>3170	>3170	200	<4	218	190	60	<1
Salmonella typhimurium 617	1340	475	280	125	325	218	190	165
Yersinia enterocolitica RS29	262	170	125	75	140	115	62	11
Y.ruckeri ATCC 29473	140	75	<4	<4	100	36	<2	<2

CONCLUSION

No one combination of antimicrobial agents tested effectively inhibited both Gram-positive and Gram-negative bacteria. However, their efficacy was greatly enhanced when combined with EDTA at low pH (≤ 5.5) or high (≥4%) NaCl concentrations. The results indicate that lysozyme and monolaurin could be used as one of a number of hurdles to prevent the growth of spoilage and pathogenic bacteria in foods.

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Yajima et al.,[134] while studying the effect of lysozyme on hiochi bacteria in sake, found that lysozyme was inhibited in the presence of either peptone, beef liver extract, or boiled soybean which were usually added to culture media as growth-promoting substances. Lin[135] found that the reaction of hen egg white lysozyme with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide resulted in the modification of 8 of 11 carboxyl groups and destruction of cell wall lytic activity.

II. EFFECT OF LYSOZYME ON SOME BACTERIAL STRAINS

In order to better understand how lysozyme will function as a preservative in foods, it is important to see how it affects certain strains and types of bacteria.

Salton[136] studied the fragments derived from the action of lysozyme on the lysozyme-sensitive bacteria *M. lysodeikticus, Sarcina lutea*, and *Bacillus megaterium*. A review of the studies of lysozyme action on microorganisms was done by Salton in 1957.[137] The present review focuses on more recent experimentation and on lysozyme as a preservative and therefore does not include many of the references cited by Salton.[137]

Salton and Pavilik[138] used various Gram-positive bacteria to study the degree of susceptibility of their cell walls to lysozyme. They studied Gram-positive strains of *Bacillus, Corynebacterium, Lactobacillus, Micrococcus, Sarcina, Sporosarcina, Staphylococcus*, and *Streptococcus*. The isolated walls from all the organisms were sensitive to lysozyme. *Staphylococcus aureus* cell walls were the most resistant, with a percent turbidity reduction in 24 hr at 37°C of 9%. Cell wall isolates from *Bacillus* organisms had turbidity reductions ranging from 51 to 95%; *Corynebacterium*, 92%; *Lactobacillus arabinosus*, 22%; *Micrococcus*, 72 to 98% (*M. lysodeikticus* was 98%); *Sarcina lutea*, 98%; *Sporosarcina ureae*, 98%; *Staphylococcus*, 9 to 91%; and *Streptococcus faecalis*, 73%. The activity of lysozyme was not correlated with total amino sugar contents. Only *L. arabinosus* walls showed an increased sensitivity to lysozyme after removal of O-ester groups or the teichoic acid. Apart from substituent groups, the author suggested that differences in lysozyme sensitivity may be the proportion of *N*-acetylamino sugars linked in a specific manner. It is assumed that if lysozyme is a 1,4 β-*N*-acetylhexosaminidase and that the walls may have 1,4 1,6 and/or 1,3, β glycosidic bonds between the *N*-acetylamino sugars, then the presence of a high proportion of 1,4 bonds between NAM and NAG would make the wall more sensitive to lysozyme than one containing a high proportion of 1,6 or 1,3 linkages.

Peterson and Hartsell[139] tested 135 Gram-negative bacterial species for sensitivity to lysozyme spectrophotometrically and for viability on agar. They concluded that lysozyme will act on Gram-negative cells if certain experimental conditions are provided. These conditions are that the cells be grown for 18 hr at 25°C; that they be exposed as unwashed cells to lysozyme at pH 3.5 in dilutions of hydrochloric acid and incubated at 45°C for 1 hr; and that N/20 sodium hydroxide be added to the suspension to pH 9.8 before the temperature becomes lower than 40°C. The effect of the enzyme would not be observed without these conditions. These data were organized into three groups according to the extent of lysis and decreasing sensitivity to lysozyme: group I, sensitive; group II, relatively sensitive; and group III, insensitive. The order of decreasing lysozyme sensitivity was group I, *Salmonella, Brucella;* group II, *Klebsiella, Shigella, Neisseria, Pseudomonas, Pasteurella, Erwinia, Escherichia;* and group III, *Vibrio, Proteus*. Viability studies on agar plates showed that the lysozyme-insensitive species and certain relatively easily lysed species were bacteriostatic compared with the sensitive cultures; however, lysozyme had an inhibitory action. The killing and the lytic action were of the same relative order.

Vedmina et al.[140] tested the sensitivity of lysozyme against 476 strains of Gram-negative bacteria. They found high resistance of *Vibrio cholerae Eltor* and *Pseudomonas* to lysozyme. Cultures of various sensitivity included *Aeromonas*, enteropathogenic *Eschericia coli*, and NAG-vibrios.

TABLE 8.
MINIMUM INHIBITORY CONCENTRATION (μg/mL) AT pH 7 AT DIFFERENT NaCl CONCENTRATIONS OF A COMBINATION OF BUTYLATED HYDROXYANIZOLE (BHA) AND MONOLAURIN (ML) WITH AND WITHOUT AN EQUAL CONCENTRATION OF EDTA

Organism	BHA + ML						BHA + ML + EDTA					
NaCl conc. (%)	0.5	1	2	3	4	5	0.5	1	2	3	4	5
Gram-positive												
Bacillus cereus 17	14	7.2	5.7	4	3	<2	10	9	6	4.8	2.5	<1
B. licheniformis UG	17	12	7.8	5.3	3.6	<2	10	9.7	6.6	5.6	3.3	2.4
B. subtilis 22	14	6.7	5.3	4	<2	<2	9	8	5.8	4.2	<1	<1
Lactococcus lactis NCK400	7.2	4	4	<2	<2	<2	5.5	5	3.5	2.4	<1	<1
Listeria monocytogenes ScottA	31	16.7	13.2	11.3	9.7	6.7	19	14	10	9	8.5	5
Staphylococcus aureus ATCC 6538	10	5	4.5	3	<2	<2	7	5.5	3.6	3.3	2.4	2.4
S. epidermidis ATCC12228	7	3	<2	<2	<2	<2	5	2.6	1.9	1.5	<1	<1
Gram-negative												
Enterobacter aerogenes 551	660	330	289	170	100	74	252	180	112	93	45	40
Escherichia coli O157:H7	>1580	>1580	237	96	96	33	385	218	130	96	37	24
Pseudomonas aeruginosa ATCC 9027	>1580	>1580	>1580	>1580	>1580	194	>795	450	290	290	165	1.7
P. fluorescens 545	680	420	320	<2	<2	<2	165	96	60	<1	<1	<1
P. putida 515	>1580	620	237	115	<2	<2	385	203	95	84	<1	<1
Salmonella typhimurium 617	>1580	>1580	290	140	80	15	450	243	141	103	34	<1
Yersinia enterocolitica RS29	220	195	140	71	<2	<2	100	97	72	52	18	<1
Y. ruckeri ATCC 29473	130	123	115	<2	<2	<2	80	74	62	<1	<1	<1

Since lysozyme has been isolated from human and bovine milk, researchers have sought to determine how the enzyme affects bacteria in the milk. Vakil et al.[97] used eight Gram-positive strains (including two lactic acid bacteria) and five Gram-negative organisms that were either live resting cells or UV-killed cells and found that all of them were susceptible to bovine milk muramidase and all, except the lactic acid bacteria (*Lactobacillus casei* and *Streptococcus lactis*), were susceptible to human milk muramidase. The Gram-positive organisms were *M. lysodeikticus*, *Streptococcus lactis*, *Lactobacillus casei*, *Staphyloccus aureus*, *Sarcina lutea*, *Streptococcus faecalis*, *Bacillus subtilis*, and *B. cereus;* and the Gram-negative organisms were *E. coli*, *Serratia marcescens*, *Proteus vulgaris*, *Pseudomonas fluorescens*, and *P. aeruginosa*. Disk-assay technique was used to determine lysozyme sensitivity to bovine and human milk and egg white lysozyme in combinations with NaCl, ethylenediaminetetraacetic acid (EDTA), NaCl and EDTA, and by itself. The effect of the additives varied depending on the source of lysozyme and their combinations, which are discussed in a later section. The experimenters concluded that lysozyme played a significant role in the inherent antibacterial activity of milk. Shahani[141] came to a similar conclusion when he found that human and bovine milk lysozyme lysed cells of 13 types of bacteria. Goudswaard et al.[142] found that lysozyme interfered with microbiological screening for penicillin in cow milk because of its lytic effect on *B. stearothermophilus* subsp. *calidolactis*. This lytic effect was demonstrated microscopically.

Korhohen[143] also found that 50 samples of colostrum and milk obtained from five Finnish Ayrshire cows in days 1 to 14 of lactation inhibited the test organism *B. stearothermophilus* subsp. *calidolactis* used in the thermocult test for antibiotics. The presence of lactoferrin and lysozyme was responsible for this inhibition.

Bottazzi et al.[144] studied the effect of lysozyme on thermophilic (heat-stable) lactic acid bacteria. The natural culture of lactic acid bacteria grown in whey was very sensitive to lysozyme. They found that concentrations up to 10 ppm lysozyme were proportional to the lytic effect. More lysozyme was necessary to give an inhibitory effect in milk than in whey for strains of *Lactobacillus helviticus* and *L. fermentum* isolated from natural whey. *L. helviticus* in milk was inhibited by greater than 50 ppm lysozyme, and the authors believed that the difference was due to the absorption of lysozyme onto casein. Some strains of *L. fermentum* could grow on MRS in the presence of 30 ppm lysozyme. Lysozyme should not be added directly to the milk during manufacturing or processing of Italian long-ripening cheeses because of the high level needed, but rather should be added after cutting the curd when only 5 to 10 ppm would be needed. Lysozyme should not be added during the phase of acidification of the cheese to avoid selective action of lactic microflora from the natural whey culture, giving an irregular fermentation.[144]

Trentini and Murray[145] observed the lysis of *Caryophanon latum* by phase-contrast microscopy. Pretreatment of cells with pronase, a lipase-phospholipase C mixture, EDTA, glutaraldehyde, or heat did not alter the pattern of lysis, nor did it render the remaining peptidoglycan more susceptible to attack.

Lysozymes have been shown to be more detrimental to spoilage and pathogenic bacteria in milk than to the lactic acid-producing bacteria. In fact, lysozyme in small concentrations was found to activate the growth of two *Streptococcus cremoris* strains that are used in cheese processing.[146] Because of this, lysozyme has been suggested as a preservative in dairy-fermented products such as cheese. Studying the factors involved, Panfil-Kuncewicz and Kisza[147] added 100 mg/ℓ egg white lysozyme to raw milk and determined the percent of acid-forming bacteria in total bacterial counts. Initially, 56% of the total bacteria were acid formers, 45% after incubation for 2 hr at 30°C without lysozyme and 66% after similar incubation with lysozyme. Samples of sterile skim milk were incubated for less than or equal to 24 hr with lactic acid-producing bacteria. Initial titratable acidities and increases in acidities after incubation were for control samples and samples with lysozyme (100 mg/

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TABLE 7.
MINIMUM INHIBITORY CONCENTRATION (μg/mL) AT DIFFERENT pH'S OF A COMBINATION OF BUTYLATED HYDROXYANIZOLE (BHA) AND MONOLAURIN (ML) WITH AND WITHOUT AN EQUAL CONCENTRATION OF EDTA

Organism	BHA + ML				BHA + ML + EDTA			
pH	7	6	5.5	5	7	6	5.5	5
Gram-positive								
Bacillus cereus 17	14	5.4	3.6	<2	10	5.3	3.4	<1
B. licheniformis UG	17	5	3	<2	10	3.3	2.2	<1
B. subtilis 22	14	5.7	3.7	<2	9	5.3	3.8	<1
Lactococcus lactis NCK400	7.2	4.5	3	<2	5.5	4.5	3.5	1.4
Listeria monocytogenes Scott A	31	10	7.2	5.3	19	12	8	3
Staphylococcus aureus ATCC 6538	10	4.5	3.5	2.6	7	3.4	2.3	1.5
S. epidermidis ATCC12228	7	4.5	3.3	<2	5	3.8	3.6	<1
Gram-negative								
Enterobacter aerogenes 551	660	437	290	207	252	155	145	110
Escherichia coli O157:H7	>1580	>1580	900	195	385	235	165	65
Pseudomonas aeruginosa ATCC 9027	>1580	>1580	>1580	>1580	>795	>795	>795	180
P. fluorescens 545	680	580	290	<2	165	104	85	<1
P. putida 515	>1580	>1580	890	<2	385	290	85	<1
Salmonella typhimurium 617	330	280	200	96	207	124	100	80
Yersinia enterocolitica RS29	220	180	150	100	100	75	65	12
Y. ruckeri ATCC 29473	130	115	21	<2	80	45	25	<1

Antimicrobial Activity of Triglycerol 1,2 Laurate in Combination with Butylated Hydroxyanizole

Inhibition by combinations of TGL and BHA (Table 9) could be explained solely on the basis of BHA concentrations (Razavi-Rohani and Griffiths 1994; Razavi-Rohani and Griffiths 1996). The presence of EDTA increased the inhibitory effect of TGL + BHA against all the bacteria tested (Table 9).

Again, pH had a pronounced effect on inhibition by TGL + BHA (Table 9). Although EDTA enhanced the inhibitory effect of the combination at neutral pH, as the pH decreased EDTA became less effective; indicating that the inhibition was mostly due to the change in pH.

For all the organisms tested, the antimicrobial activity of TGL + BHA increased with increasing NaCl concentration especially when EDTA was present (Table 10). This was particularly true for Gram-negative bacteria.

ℓ, respectively: (1) butter starter using four strains of *S. diacetilactis* and *S. lactis* at 28°C— 7.6 and 7.5, and 31.6 and 30.3; (2) cheese starters using *S. lactis*, *S. diacetilactis*, and two strains of *S. cremoris* at 28°C — 7.8 and 7.7, and 31.9 and 31.8; (3) using three strains of *L. bulgaricus* at 37°C — 8.6 and 8.7, and 50.8 and 46.0. The results indicated some inhibition of starter cultures by lysozyme, but very little compared with the lysis of the spoilage organism *E. coli*. *E. coli* strain T-28 was added at 70,000 cells per milliliter to milk. The mean counts after incubation for 2 hr at 37°C without lysozyme and with 150 mg/ℓ plus NaCl (which was added to stimulate lyses) were 162,000 and 88,000 cells per milliliter, respectively.

Carroll,[148] on the other hand, suggested that lysozyme may have limited importance as a protective agent against coliform bacteria. In a study of lysis by bovine sera of washed coliform bacteria in various buffer systems, no correlation was found between killing by serum and sensitivity to lysis by lysozyme. EDTA markedly potentiated serum lysis in the presence of added lysozyme. Lysozyme itself was not lytic, but Carroll later absorbed serum with bentonite or bacteria and found that lysozyme was involved in lysis but not in the killing of coliforms by bovine serum.[149]

Silberstein and Inouye[150] used lysozyme in combination with three detergents — disodium EDTA, Brij-58, or Triton® X-100 — to lyse *E. coli* to remove DNA strains from the bacterial membrane strands.

Mineyeva et al.[151] found that three periods (a lag period and periods of intensive and decelerated spheroplast formation) were detected when lysozyme acted upon the cells of *Anacystis nidulans*. This was due to pecularities in the cell wall structure of the cyanobacterium and the heterogeneity of the culture.

Chassy and Giuffrida[152] found that some lactic acid producers were more suceptible to lysis by lysozyme when the growth medium was supplemented with chemicals. Streptococci, lactobacilli, *Actinomycetes*, propionibacteria, and pediococci cells were more susceptible to lysis by lysozyme when the growth medium was supplemented with L-threonine and/or L-lysine. When grown on glucose-containing media, cells were more easily lysed than those grown on other substrates. Polyethylene glycol was used as an osmotic stabilizer, but was also found to enhance the extent of lysis. These researchers also found tnat dilute tris (hydroxymethyl) aminomethane buffer was superior to the buffer systems most commonly used in lysozyme-based lysis techniques. Stationary-phase cells of *L. casei* and *S. mutans* were more easily lysed than log-phase cells. Wasserfall[153] found contradictory results compared with other researchers. *E. coli* was not inhibited in cheese curd by lysozyme, so heat treatment remained essential for its destruction. Strains of *S. lactis*, *S. cremoris*, *S. diacetilactis*, and butter starter cultures in the resting cell state were used to determine the effect of lysozyme on them. The main conclusions were that prolonged contact with the phosphate buffer alone caused inactivation of resting cells and that lysozyme enhanced this effect. Streptococci were inhibited to differing extents, with *S. diacetilactis* being least resistant and *S. lactis* being more resistant. Butter starter cultures were restricted in acid-forming capacity.

Many types of spoilage organisms shorten the shelf life of foods. Lysozyme can lengthen the shelf life of foods by inhibiting these organisms. Gardner and Nikoopour[154] studied the growth of *P. fluorescens* on 1% lysozyme substrate. A prolonged lag phase, accompanied by a 96% reduction in the initial bacterial population, was observed followed by a sharp increase in bacterial numbers during the second week. The results suggested an alteration in the growth characteristics of the test organism or a substrate-induced selection of organisms within the test strain, which were resistant to lysozyme activity. Fluorescence production, negligible during the first few days, increased rapidly during the second week of incubation. The fluorescent pigment was bright blue in contrast to the bright green observed on conalbumin substrates.

positive and Gram-negative) with the exception of two pseudomonads, *P. aeruginosa* and *P. putida* (Razavi-Rohani and Griffiths 1996). When these two inhibitors were combined in equal amounts the effect on Gram-positive bacteria was additive but the effect on the Gram-negative bacteria was no greater than that observed with BHA alone (Table 7). This confirms the finding of Kabara (1979) that phenolic antioxidants have synergistic activity with ML when used against *S. aureus*.

The inhibitory activity of ML against all the organisms tested was increased by EDTA and similar, but less dramatic, results were observed for BHA (Razavi-Rohani and Griffiths 1994; Razavi-Rohani and Griffiths 1996). When these two inhibitors (ML + BHA) and EDTA were added in equal amounts, the effect on all bacteria was also amplified, but the effect of BHA with EDTA on Gram-negative bacteria was greater than that of BHA + ML + EDTA (Table 7). Kabara (1979, 1980) stated that BHA and EDTA enhanced the preservative qualities of monolaurin. In the present study, this was only confirmed for Gram-positive organisms. In other work, Notermans and Dufrenne (1981) found that the inhibitory effect of ML + BHA + EDTA on toxin production by *Cl. botulinum* was not more than that produced by ML alone. These differences in efficacy may be due to species and strain variations or may reflect differences in methodology used to determine MIC.

When ML and BHA were combined, they were more effective against Gram-positive bacteria at low pH (Table 7) and their effects were synergistic (Razavi-Rohani and Griffiths 1994; Razavi-Rohani and Griffiths 1996). This pH effect was enhanced by EDTA (Table 7). With Gram-negative bacteria, lowering the pH did not make ML + BHA more inhibitory than BHA alone, either in the presence or absence of EDTA (Razavi-Rohani and Griffiths 1996). As the pH decreased, the inhibitory effect of the combination (ML + BHA) increased and was made more effective by addition of EDTA (Table 7).

The inhibition of Gram-positive bacteria by the combination (ML + BHA) increased with increasing NaCl concentrations in the medium (Table 8). The combination of ML + BHA was more effective than either ML or BHA alone (Razavi-Rohani and Griffiths 1994; Razavi-Rohani and Griffiths 1996). Also, the synergistic action of EDTA was not marked (Table 8). Different results were obtained with the Gram-negative species. Whereas addition of NaCl increased the bactericidal effects of ML + BHA, the efficacy of the combination was greater than ML alone (Razavi-Rohani and Griffiths 1994) but not as great as that observed for BHA (Razavi-Rohani and Griffiths 1996). As with other systems, EDTA enhanced the inhibition of Gram-negative bacteria by ML + BHA (Table 8).

Safonova et al.[155] reported that the minimum inhibitory concentration of lysozyme for *Micrococcus lutens* ranged from <0.0003 to 1 mg/mℓ. *M. varians* was stable to lysozyme. The minimum inhibitory concentration for all the strains was 8 mg/mℓ.

Using direct microscopic counts, Hall et al.[156] found lower total counts than expected in some egg products after bacterial decomposition. After testing, they found this to be due to lysis by lysozyme in the eggs. It was suggested that the bactericidal effectiveness of lysozyme in reducing bacterial populations should not be overlooked in producing low-bacterial count, pasteurized products. Ashton et al.[157] confirmed these results when he found commercial crystalline egg albumen to contain lysozyme. They cautioned that destruction of the thermophilic aerobes by lysozyme should be considered when performing counts on egg products. The addition of lysozyme to pasteurized milk has been shown to reduce substantially the direct microscopic count.[158]

The use of lysozyme against some pathogens has also been studied. Teotia and Miller[159] contaminated turkey drumsticks and whole carcasses with *Salmonella senftenberg*. A 0.1% solution of lysozyme completely eliminated *S. senftenberg* 775W at 22°C within 3 hr after contamination. *Clostridium botulinum* type E spores were found to have greater heat resistance after being exposed to lysozyme. The D80°C value (time for 90% destruction at 80°C) for untreated spores was 1.51 min, and 20.84 min for the treated spores. These D values were higher than previously recorded and should be taken into consideration in the preservation of food by thermal processing.[160] Alderton et al.[161] investigated the effect of lysozyme on the recovery of heated Ca-formed *C. botulinum* 62A and untreated *C. botulinum* 1304E spores. Survivor curves were obtained when the *C. botulinum* 62A spores were heated in strained asparagus puree. The D235°F values were 3.0 and 1.10 min, with and without lysozyme, respectively. When the heating temperature was raised to 245°F, the difference in D values with and without lysozyme seemed to increase. The D245°F was 0.2 min without lysozyme and 0.8 min with lysozyme. Lysozyme in the recovery medium enhanced and speeded outgrowth of heated *C. botulinum* spores. The spore recovery of type E spores was more dramatic than for the A spores as measured by colony formation. The measured heat resistance of type E spores was about 1800-fold and type A spores up to 3-fold.

Poncelet and Catteau[162] found that 5 μg/mℓ lysozyme increased the number of *C. perfringens* spores identified in foods when it was added to 1.5% VF sulfite agar and the food was dissolved in 180 mℓ of a solution containing 0.1% tryptone plus 0.85% NaCl. Ionesco[163] found that lysozyme increased the rate of germination of *C. difficile* from lower than 10^{-5} to 5×10^{-3} spores per milliliter. All spores are initiated by lysozyme when previously treated by sodium thioglycolate. These spores were lysozyme dependent for germination.

Mustafa[164] found that some strains of clostridia increased in turbidity when exposed to lysozyme. *C. multifermentuns*, *C. lentoputrescens*, and *C. tertium* released reducing sugars when exposed to lysozyme. Distorted cells in Gram-stained smears of lysozyme-treated cells were observed with suspensions of *C. hemolyticum*, *C. butyricum*, *C. multifermentans*, and *C. lentoputrescens*.

Ng and Garibaldi[165] found that incubating and shaking *S. aureus* in liquid whole egg caused a decline in viability because of lysozyme. The action of lysozyme was not attributed to its bacterial lytic property, but to the basicity of the lysozyme molecule. The natural pH of the egg rises from 7.2 to between 8.0 and 8.2 during shaking as a result of CO_2 loss. It was postulated that the rise in pH rendered the bacterial cells more negatively charged so that in the presence of positively charged molecules of lysozyme a complex was formed, agglutinating the cells.

Itakura[166] found that lysozyme was only partially effective against *S. aureus* alone, but in combination with a small amount of amoxicillin, lysozyme was very effective at lysing the cell walls. Safonova et al.[167] found that lysozyme changed the OD of suspensions of *S. aureus*, *S. epidermidis*, and *S. saprophyticus*, although the effect was different from that of

TABLE 6.
MINIMUM INHIBITORY CONCENTRATION (µg/mL) AT pH 7 AT DIFFERENT NaCl CONCENTRATIONS OF A COMBINATION OF LYSOZYME AND BUTYLATED HYDROXYANIZOLE (BHA) WITH AND WITHOUT AN EQUAL CONCENTRATION OF EDTA

Organism	L + BHA						L + BHA + EDTA					
NaCl conc. (%)	0.5	1	2	3	4	5	0.5	1	2	3	4	5
Gram-positive												
Bacillus cereus 17	62	42	30	18	9	<4	33	27	21	19	16	4.5
B. licheniformis UG	62	31	22	13	6	<4	33	27	18	16	16	6
B. subtilis 22	71	40	30	20	8	<4	42	35	24	22	22	<2
Lactococcus lactis NCK400	50	21	14	<4	<4	<4	40	31	18	8	<2	<2
Listeria monocytogenes ScottA	97	85	62	36	27	9	80	80	48	43	43	22
Staphylococcus aureus ATCC 6538	50	42	36	19	12	10	42	28	28	20	16	14
S. epidermidis ATCC12228	70	57	52	38	30	<4	45	24	21	21	18	11
Gram-negative												
Enterobacter aerogenes 551	230	200	160	116	76	62	150	123	100	62	58	33
Escherichia coli O157:H7	320	231	160	130	71	45	207	195	123	70	54	40
Pseudomonas aeruginosa ATCC 9027	>3170	>3170	>3170	760	570	71	500	290	140	130	100	84
P. fluorescens 545	505	380	120	<4	<4	<4	130	90	50	15	<2	<2
P. putida 515	>3170	580	158	100	<4	<4	170	130	80	43	21	<2
Salmonella typhimurium 617	330	300	160	116	66	38	207	182	123	70	66	40
Yersinia enterocolitica RS29	150	132	103	62	<4	<4	90	84	79	54	28	<2
Y. ruckeri ATCC 29473	91	84	62	<4	<4	<4	74	70	66	<2	<2	<2

M. luteus. The OD characteristics of *S. saprophyticus* were characterized by some properties which provided a supposition that the cell wall structure of the organism is different from the other two strains, which had similar cell wall characteristics.

Safonova et al.[168] studied the nature of the OD of suspensions of *S. aureus* and *M. luteus* cells at various concentrations of egg white lysozyme and two products of its chemical transformation. A decrease in OD of the suspensions at relatively low concentrations of lysozyme, i.e., 0.032 to 0.064 mg/mℓ, was due to lysis of both microbial species. The increase in OD at 0.5 to 2.0 mg/mℓ of lysozyme indicated the formation of insoluble aggregates of an electrostatic character. Lysozyme was the positively charged component and the microbial cell material was the negatively charged component. Lysozyme aggregates with *M. luteus* were composed of lysis acid products, and with *S. aureus* the aggregates were composed of lysis products and the intact live cells.

Wooley and Blue[169] studied the in vitro effect of EDTA, tris(hydroxymethylamino-methane)HCL (tris) buffer, and lysozyme solution on 16 pathogenic bacteria of medical or veterinary importance. Marked decreases in bacterial count occurred with *Pseudomonas aeruginosa*, *E. coli*, *Moraxella oslensis*, and *Campylobacter fetus*; smaller decreases occurred with *Salmonella typhimurim*, *Shigella boydii*, *Aeromonas hydrophi*, *Proteus mirabilis*, *Listeria monocytogenes*, and *Erysipelothrix insidiosa*. The test solution had no effect on *Klebsiella ozaenae*, *Brucella canis*, *Corynebacterium pyogenes*, *C. renale*, *Streptococcus equi*, and *Staphylococcus aureus*.

III. LYSOZYME AS A PRESERVATIVE IN FOOD

A. Miscellaneous Foods

There has been considerable interest in lysozyme as a food preservative, mostly in Japan, where the majority of the work has been performed using lysozyme in a food system. Akashi[146] found that egg white suspensions had a lytic action on *E. coli* suspensions. Food products such as sausages, fish cakes, and bacon could be preserved with lysozyme.

The Japanese have many patents on the use of lysozyme as a preservative in foods. A patent was issued for the preservation of fresh vegetables, fish, meat, and fruit by coating the surface with lysozyme.[170] Taiyo Food Co.[171] patented a process to preserve tofu bean curd by adding lysozyme to soya milk during processing. Kanebo Ltd.[172] rendered food packaging films antiseptic by coating them with lysozyme obtained from egg whites. Dried milk compositions for pediatric use were preserved by incorporation of lysozyme derived from egg whites along with ovalbumin and ovomucin.[173] Francis[174] and Carlsson et al.[175] found that antibodies, lactoferrin, and lysozyme in human breast milk acted complementarily to prevent gastroenteritis and helped prevent allergies, therefore, lysozyme has been added to infant formulas. Nishihava and Isoda[176] added lysozyme to infant's dry milk powder to simulate human milk, which contains lysozyme, to see if the number of *Bifidus bacillus* in the intestine would increase. *B. bacillus* is said to contribute to a healthy flora and aid in digestion. Lysozyme added to the milk powder did increase the *B. bacillus* of the intestine of the infants used as subjects.

Oysters, shrimp, and other seafoods were claimed to be preserved in refrigerated storage by treatment with aqueous solutions of lysozyme and NaCl in a Japanese patent by Eisai Company.[177] The same company patented a process to preserve fresh marine products by soaking them in aqueous solutions containing a lysozyme salt, amino acids, and NaCl.[178] Eisai Company[179] also claimed that lysozyme was very valuable in the preservation of wine and sake and patented a process in which wines were stabilized by addition of lysozyme or its salts, together with *p*-hydroxybenzoic esters. Eisai obtained a Japanese patent whereby specific lysozyme materials and β-glycopyranose aerodehydrogenase were added to sake to improve storage stability.[180]

TABLE 5.
MINIMUM INHIBITORY CONCENTRATION (μg/mL) AT DIFFERENT pH'S OF A COMBINATION OF LYSOZYME AND BUTYLATED HYDROXYANIZOLE (BHA) WITH AND WITHOUT AN EQUAL CONCENTRATION OF EDTA

Organism	L + BHA				L + BHA + EDTA			
pH	7	6	5.5	5	7	6	5.5	5
Gram-positive								
Bacillus cereus 17	62	36	20	<4	33	28	12	<2
B. licheniformis UG	62	36	12	<4	33	18	9	<2
B. subtilis 22	71	57	6	<4	42	31	15	<2
Lactococcus lactis NCK400	50	45	36	12	40	35	31	14
Listeria monocytogenes Scott A	97	90	50	41	80	74	54	18
Staphylococcus aureus ATCC 6538	50	40	40	22	42	40	37	16
S. epidermidis ATCC12228	70	66	30	<4	45	35	14	<2
Gram-negative								
Enterobacter aerogenes 551	230	170	160	140	150	140	123	100
Escherichia coli O157:H7	320	200	200	160	207	149	115	45
Pseudomonas aeruginosa ATCC 9027	>3170	>3170	>3170	660	500	410	355	180
P. fluorescens 545	505	110	<4	<4	130	70	<2	<2
P. putida 515	>3170	>3170	<4	<4	170	150	8	<2
Salmonella typhimurium 617	330	280	200	96	207	124	100	80
Yersinia enterocolitica RS29	150	123	103	48	90	66	11	9
Y. ruckeri ATCC 29473	91	49	41	<4	74	35	13	<2

Antimicrobial Activity of Monolaurin in Combination with Butylated Hydroxyanizole

Monolaurin is extremely inhibitory towards most Gram-positive bacteria but is not active against Gram-negative genera (Kabara 1979, 1984; Razavi-Rohani and Griffiths 1994). A similar specificity is found for BHA (Chang and Branen 1975; Shih and Harris 1977; Branen *et al.* 1980; Raccach 1984), but certain Gram-negative bacteria are susceptible to inhibition by the antioxidant (Robach *et al.* 1977; Davidson and Branen 1980; Gailani and Fung 1984). ML inhibited all the Gram-positive bacteria used in this study and MIC's were in the range 8 to 96 μg/mL (Razavi-Rohani and Griffiths 1994). The same study revealed that none of the Gram-negative bacteria tested were affected by ML concentrations up to 1580 μg/mL. It was also found that BHA concentrations between 46 and 66 μg/mL inhibited all the strains of bacteria tested (both Gram-

Yashitake and Shinichiro[16] used information from Ezai concerning lysozyme preparations made by them with the trade name Amicannon®. The use of lysozyme as a preservative in the following foods was suggested by them in combination with other additives:

- Kimchi pickles are a fermented Korean food made from Chinese cabbage, hot peppers, vinegar, and garlic. Japanese potato salad is prepared from mashed potatoes, mayonnaise, and thinly sliced vegetables. Egg white lysozyme and amino acids were found to be the best combination for preserving both of these products.
- Sushi (rice seasoned with sugar and vinegar and rolled in layered seaweed with either pickled turnip, radish, grated carrot, meat, or other filler in the center) can be preserved by dissolving lysozyme in the vinegar used to make it.
- Chinese noodles could be prepared with organic acids, hydrogen peroxide, or propylene glycol as a preservative. The use of lysozyme and amino acids would enhance preservation, especially in combination with propylene glycol.
- Creamed custard is heated in processing but because of the high sugar content lysozyme activity is not lost. Amino acids and lysozyme in combination with alochol make a good preservative of this food.

A few scientific papers have been published in relation to the patent subject matter, but most research seems to involve industrial secrets. Yajima et al.[181] found that lysozyme was a strong inhibitor of Hiochi bacteria (sake-putrefying lactic acid bacteria) in sake. Sake contains less than 20% alcohol and has a pH of 4.0 to 4.5. The minimum concentration of lysozyme needed to inhibit *Lactobacillus heterohiochii* was 10 ppm; *L. homohiochii*, 20 ppm; *L. fermenti*, 1.25 ppm; and *L. acidophilus*, greater than 100 ppm. The minimum concentration of salicylic acid needed to cause inhibition of these bacteria was 500 ppm under the same conditions. Enzymatic activity was not lost during pasteurization and more than 85% of the initial enzymatic activity of lysozyme remained after 1 year of preservation at room temperature. Persimmon tannin caused lysozyme to precipitate in the sake, but activity was hardly affected by an enzyme used for removal of dregs. A total of 10% of lysozyme at a concentration of 10 ppm was absorbed in 100 ppm activated carbon. No objectionable flavor was detected in the sake treated with lysozyme.

Yajima et al.,[134] in another study on the effect of lysozyme on Hiochi bacteria in sake, found that *L. acidophilus* (H-7) was resistant to lysozyme — unlike *L. heterohiochii* (H-1), *L. homohiochii* (H-42), and *L. fermentum* (H-34) — but found them all to be inhibited in sake containing more than 15% ethanol. They reported that the growth inhibitory action of lysozyme on Hiochi bacteria was more potent in sake pretreated with activated carbon than in sake without pretreatment. Butyl *p*-hydroxy-benzoate enhanced the inhibitory effect of lysozyme, but it caused an unfavorable change in the taste of sake. The minimum inhibitory concentration of lysozyme in sake of approximately 20% alcohol content was 5 ppm. A commercial sample of sake with added lysozyme maintained the enzyme activity even in the presence of residual kakishibu (tannin prepared from Japanese persimmon). Eisai Company[182] prevented degradation of sake by *Lactobacillus* organisms by adding lysozyme or its salts, together with *p*-hydroxy-benzoic esters. Hara et al.[183] found that the minimum inhibitory concentration of lysozyme against Hiochi bacteria was increased considerably by larger inocula and only slightly by young subcultures. Uchida et al.[184] used lysozyme to inhibit Hiochi bacteria in Mirin liquor, a sweetened sake used for cooking. *L. heterochiochii*, *L. fermenti*, *L. plantarum*, and *L. casei*, all of which spoil the wine, were isolated from Mirin liquor and used as test organisms. The growth of these organisms was completely inhibited by 20 ppm lysozyme. After 1 year of storage at room temperature, more than 95% of the original activity of lysozyme remained. No loss of lysozyme activity was observed after pasteurization at 65°C for 5 hr. The clarification treatment of Mirin liquor with wheat

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L) AT DIFFERENT pH'S OF A
OL 1,2 LAURATE (TGL) WITH
RATION OF EDTA

	L + TGL + EDTA			
5	7	6	5.5	5
7	115	48	16	<2
<4	95	30	10	<2
<4	160	58	24	<2
60	80	90	75	30
80	250	195	110	50
10	220	150	90	45
<4	130	100	100	<2
70	>1580	>1580	>1580	>1580
70	30	>1580	>1580	250
70	>1580	>1580	>1580	310
70	170	115	<2	<2
4	210	170	6	<2
70	>1580	>1580	>1580	500
70	>1580	>1580	221	18
4	195	90	<2	<2

but two pseudomonads when
, the antimicrobial effect of
that found when either of the
inhibition was due to BHA.
the inhibitory effect of the
(Table 5). Improvements to
binations were also observed
acreased. This was especially
(Table 6).

TABLE 4.
MINIMUM INHIBITORY CONCENTRATION (μg/mL) AT pH 7 AT DIFFERENT NaCl CONCENTRATIONS OF A COMBINATION OF LYSOZYME AND TRIGLYCEROL 1,2 LAURATE (TGL) WITH AND WITHOUT AN EQUAL CONCENTRATION OF EDTA

Organism	L + TGL						L + TGL + EDTA					
NaCl conc. (%)	0.5	1	2	3	4	5	0.5	1	2	3	4	5
Gram-positive												
Bacillus cereus 17	>3170	1300	1000	820	660	400	115	100	95	74	70	54
B. licheniformis UG	>3170	940	760	440	190	150	95	90	90	66	66	54
B. subtilis 22	>3170	1650	1500	1200	170	<4	160	150	125	70	65	<2
Lactococcus lactis NCK400	205	150	96	66	<4	<4	80	80	45	40	<2	<2
Listeria monocytogenes ScottA	>3170	>3170	>3170	>3170	>3170	>3170	250	237	220	220	194	194
Staphylococcus aureus ATCC 6538	660	480	380	340	340	130	220	200	115	150	160	170
S. epidermidis ATCC12228	580	169	200	150	116	570	130	100	70	70	70	62
Gram-negative												
Enterobacter aerogenes 551	>3170	>3170	>3170	>3170	>3170	>3170	>1580	>1580	250	250	200	85
Escherichia coli O157:H7	>3170	>3170	>3170	>3170	>3170	>3170	>1580	>1580	>1580	990	95	84
Pseudomonas aeruginosa ATCC 9027	>3170	>3170	>3170	>3170	>3170	>3170	>1580	>1580	>1580	500	330	290
P. fluorescens 545	>3170	>3170	>3170	<4	<4	<4	170	110	50	<2	<2	<2
P. putida 515	>3170	>3170	>3170	>3170	<4	<4	210	170	140	70	<2	<2
Salmonella typhimurium 617	>3170	>3170	>3170	>3170	>3170	>3170	>1580	>1580	>1580	>1580	>1580	180
Yersinia enterocolitica RS29	>3170	>3170	>3170	>3170	445	<4	>1580	360	280	245	220	<2
Y.ruckeri ATCC 29473	>3170	>3170	>3170	>3170	<4	<4	195	115	102	<2	<2	<2

gluten and persimmon tannin gave a 2.5% loss of lysozyme activity. The combined treatment of Mirin liquor with glutentannin and 400 ppm of activated carbon reduced the lysozyme activity by 15%. No flavor defects were detected by the presence of lysozyme at 20 ppm.

Nakagawa and Maeshigi[185] added a commercial lysozyme-glycine mixture at 1.5% to potato salad and extended the shelf life at 35 to 37°C from 23 to 43 hr. The addition of a commercial hydrolyzed egg white preparation at 5% to cooked burdock with soy sauce extended the shelf life from 13 to 18 hr at 35 to 37°C. No synergistic effects were noted for the lysozyme mixture and the egg white preparation on extending the shelf life of a sauce for fried fish. Reed,[186] in reviewing lysozyme use in foods, mentioned lysozyme being used to preserve caviar, but no references were found.

Dubois-Prevost[187] reviewed the use of lysozyme for infant feeding by way of addition to formula milk to yield a product that more closely resembled human milk. Sawada et al.[188] related the lysozyme treatment of milk to its ability to support *L. bifidus* growth. They claimed that cow milk may be sterilized by treating with 0.05 to 0.1 mg lysozyme per milliliter of milk maintained at 37°C for 3 hr, then heated to 80°C for 30 min to inactivate the lysozyme. This resulted in a product that favored the propagation of *L. bifidus* in the infant and resulted in a milk product that "cannot be differentiated from human milk in regard to *L. bifidus* content".

B. Cheese

The food in which the effect of added lysozyme has been studied most extensively is cheese. Bakri and Wolfe[189] showed that lysozyme caused destabilization of casein micelles in a manner similar to the action of rennin. Kisza et al.[190] reported that added lysozyme hastened the digestion of milk proteins by pepsin. Panfil-Kuncewicz and Kisza[191] studied the proteolytic susceptibility of cow milk to lysozyme and found that reconstituted dried skim milk with $CaCl_2$ increased the time before onset of coagulation, but coagulation time was reduced with increasing amounts of lysozyme. Reconstituted 100 or 25% dried milk modified with lysozyme also showed the same time reductions but, in addition, showed a reduction of coagulum rigidity. Lysozyme added to milk accelerated release of soluble nitrogen compounds by pepsin, and this effect increased with increasing lysozyme concentration. Vakil et al.[97] studied the susceptibility of several microorganisms to milk lysozyme and concluded that the enzyme played a significant role in the inherent antibacterial activity of milk.

The effect of lysozyme on some cheese cultures was discussed in the previous section. Butyric acid bacterial contamination in Edam cheese caused late blowing of cheese, making it unsuitable for human consumption. To overcome the problem, producers must use milk free of butyric acid bacteria or decrease the number of butyric acid spores in the cheese milk (such as by bactofugation), change the cheesemaking procedure (such as salting the cheese early), use bacteria that produce nisin or other substances inhibitory to butyric acid bacteria, or add inhibitory substances such as nitrate, H_2O_2, or lysozyme.[192] There are many areas in the world where grasses are heavily contaminated with butyric acid bacteria, such as in the Zubawy district of Poland; therefore,[193] milk free of butyric acid bacteria is impossible. Methods such as bactofugation are costly and time consuming. The growth of the milk starter culture is a highly regulated, very important operation in cheesemaking. The addition of another culture to compete with the starter culture is not desirable. The nisin or other inhibitory substance produced by bacteria may also be inhibitory to the starter culture. Nitrate addition to form nitrite, which inhibits butyric acid bacterial growth, has been a popular method widely used by the industry. Nielsen[192] discussed some of these options in an effort to find a replacement for nitrite including the use of lysozyme and early salting of the cheese where $\leq$2250 g salt per 100 ℓ of cheese milk at an early stage in samso cheese manufacturing inhibited butyric acid bacteria. Aarnes[194] discussed the mode of action

ɔod. The use of combination
ɔitors may maximize the
Identification of optimal
ɔwer concentrations of these

tive against Gram-positive
/ity against Gram-negative
monoglyceride, monolaurin
ting emulsifying properties,
ɔara 1979; Shibasaki 1979,

many natural systems. This
y than Gram-negatives that
layer (Davidson and Branen
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ore resistant Gram-negative
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al compound to be used,
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ating agents, pH and NaCl

IODS

Canadian Lysozyme Inc.,
te (TGL) was provided by
ıydroxyanizole (BHA; 2,(3)-
raacetic acid (EDTA) and

sodium citrate (SC) were all from Sigma Chemical Co. (St. Louis, MO). Monolaurin (ML) was obtained from Lauricidin Inc. (Galena, IL).

When inhibitors were used in combination they were mixed to obtain equal mass. The stock solutions were prepared in water, except ML which was dissolved in ethanol. The solutions were sterilized by filtration through a 0.22μ disposable sterile filter (Millipore Inc., Bedford, MA).

Organisms

The test organisms were *Bacillus cereus* 17, *Bacillus licheniformis* UG, *Bacillus subtilis* 22, *Lactococcus lactis* NCK 400, *Listeria monocytogenes* Scott A, *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Enterobacter aerogenes* 551, *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas fluorescens* 545, *Pseudomonas putida* 515, *Salmonella typhimurium* 617, *Yersinia enterocolitica* RS29 and *Yersinia ruckeri* ATCC 29473.

The inhibitory activity of the chemicals was determined against organisms grown on Brain Heart Infusion (BHI) broth (Difco, Detroit, MI) containing 1.2% agar (Difco), adjusted to pH 5, 5.5, 6 and 7 with 1M HCl or 1M NaOH prior to autoclaving. Autoclaving had no effect on final pH. The NaCl concentration in the medium was varied between 0.5 to 7%.

Determination of Minimum Inhibitory Concentration

The minimum inhibitory concentrations (MIC) of the inhibitors were determined using a Spiral Plater Model D (Spiral System Instruments Inc., Bethesda, MD) according to the Preliminary User Guide of Spiral System Instruments (Anon 1985). In order to compare results with standard MIC values, the results obtained were increased two-fold (Anon 1985).

Petri plates (15 cm diameter) were filled with BHI agar (55 mL) to produce an agar depth of 3.3 mm then kept at room temperature until they were used. The inhibitor solution was deposited on the surface of the agar with the Spiral Plater (Hill and Schalkowsky 1990). This dispensed an accurate volume of solution at an ever decreasing rate radially from the center to the periphery of the plate. The plates were left for 5 to 7 h to allow a concentration gradient of the inhibitor to form throughout the agar (Schalkowsky 1986; Hill and Schalkowsky 1990; Molitoris *et al.* 1990).

Overnight BHI broth cultures of the test organisms grown at 37C were diluted 10-fold in BHI broth tempered at 37C and incubated for a further 1 to 2 h at that temperature. This ensured that the bacteria were in the exponential growth phase. The turbidity of each culture was adjusted with tempered BHI broth to 0.5 on the MacFarland scale (corresponding to a population of approximately 1×10^8 cfu/mL). A gradient plate, obtained as described above,

and application of some substances used to counter butyric acid fermentation as well as substances preventing mold growth such as sorbic acid, benzoic acid, and pimaricin. Wasserfall[195] reviewed the use of all these options to improve the quality of cheese and concluded that, so far, the only suitable measures were the improvement of milk quality and the use of lysozyme. Practical difficulties and cost seem to exclude other options.

Wasserfall et al.[196] made Edam cheese incorporating lysozyme in the form of concentrated suspensions just before the addition of rennet. The minimum amount of lysozyme required to prevent late blowing of cheese made from low-quality milk was about 500 U/mℓ, corresponding to approximately 0.6% egg white. Purified lysozyme and egg white were both effective inhibitors, but dried ovalbumin failed to completely inhibit the defect. No significant differences were found organoleptically between cheese made with lysozyme or with nitrate, nor did lysozyme cause any effect on the process of cheesemaking. Koterska et al.[193] used only 0.2% egg white on four different batches of Edam cheese containing progressively increasing amounts of butyric acid spores and found that even this amount improved the cheese organoleptically and microbiologically.

One of the butyric acid bacteria that causes Italian cheeses and Edam- or Gouda-type cheese to undergo late blowing or late gassing is *Clostridium tyrobutyricum*. Wasserfall and Teuber[197] used egg white lysozyme at a concentration of 500 U/mℓ to kill 99% of 5×10^5 resting vegetative cells of *C. tyrobutyricum* within 24 hr of incubation at 25°C. Spores were resistant to lysozyme and proliferating vegetative cells were severely inhibited. Although lysozyme-resistant cells developed in growting cultures in the presence of lysozyme, the overall outgrowth of spores to vegetative cells was delayed by 1 day in the presence of 500 U of lysozyme per milliliter. It was suggested that this inhibition by lysozyme of the outgrowth of spore cells into vegetative cells of the lactate-fermenting *C. tyrobutyricum* was the basis for the observation that lysozyme can substitute for nitrate in preventing the late gas defect. Wasserfall and Prokopek[198] prepared Edam cheese without lysozyme and with 500 U/mℓ of lysozyme from 25 ℓ low-count milk containing approximately 1000 *C. tyrobutyricum* spores per liter. After 5 weeks of ripening, cheese made with lysozyme had resisted late blowing, while the other was no longer suitable for consumption. The investigators found this to be true even in the presence of lysozyme-resistant vegetative cells. Ferrari and Dell'Acqua[199] patented a process in the U.K. whereby lysozymes or their nontoxic salts were added to butter or cheese to prevent the development of undesirable microorganisms. One of the suggested applications involved the addition of 50 ppm lysozyme to renneted milk in granan cheesemaking. The inventors found that after 24 months of ripening the physical and organoleptic properties were excellent, whereas control cheese was blown because of *C. tyrobutyricum*. These same findings were common to other Italian cheese varieties.

While studying the recovery and stability of lysozyme, Wasserfall[200] recovered 97 to 100% of lysozyme added to acid whey, but only 75 to 85% of lysozyme added to milk was recovered in whey after acidification. The recovery of lysozyme added to rennet whey was 88 to 93%, but only 8.7 to 11% of lysozyme added to cheese milk was recovered in whey after renneting. His conclusion was that a large part of the enzyme added to milk was firmly bound to the coagulum and therefore was not available for prevention of late blowing during cheese ripening. He also found that storage stability of lysozyme preparations descreased with increasing temperature and alkalinity.

C. Meat

The influence of lysozyme in meat products has also been investigated. Akashi[201-203] completed a series of studies on the preservative effect of lysozyme added to cooked sausage, salami sausage, and Vienna sausage. In the cooked sausage study, samples of ground beef were treated with 3% NaCl, 12.5 ppm $NaNO_2$, and 50 or 200 ppm lysozyme; 200 ppm

was then inoculated with culture using a cotton-tipped swab and streaking from the periphery to within 10 mm of the center of the plate (Hill and Schalkowsky 1990; James 1990; James *et al.* 1991). Control plates containing no inhibitor were streaked to verify that any inhibitory action observed was not due to the medium.

After the inoculum was sufficiently absorbed into the agar, the plates were inverted and incubated for 24 h at 37C or 25C, depending on the optimum growth temperature of the bacterium. The end point of growth for each organism was determined by measuring the distance from the center of the plate to the point at which observable growth began. The MIC was calculated by using the deposition factor for the Model D Plater (Anon 1985).

The experiments were carried out at least in triplicate and the results presented are averages.

RESULTS AND DISCUSSION

Antimicrobial Activity of Lysozyme in Combination with Monolaurin

Lysozyme alone had no inhibitory effect on any of the bacteria tested except for *L. lactis* NCK 400 (Razavi-Rohani and Griffiths 1996). ML, when present at concentrations between 8 and 96 μg/mL, inhibited only Gram-positive bacteria (Razavi-Rohani and Griffiths 1994). All Gram-positive bacteria were inhibited by combinations of ML and lysozyme when both were present at concentrations between 6 μg/mL (for *Staph. epidermidis* ATCC 12228) and 90 μg/mL (for *L. monocytogenes*) (Table 1). These MIC's were only slightly lower than observed with ML alone which indicated that the inhibition was predominantly due to the presence of the monoglyceride. The combination of lysozyme and ML tested was not effective against the Gram-negative bacteria.

EDTA, when added to this combination at the same concentration as the individual components, made 3 species of Gram-negative bacteria more susceptible to inhibition at neutral pH (Table 1). Previous work had shown that ML in the presence of EDTA resulted in inhibition of all of the Gram-negative test organisms (Razavi-Rohani and Griffiths 1994) and when the chelating agent was used with lysozyme, *P. fluorescens*, *P. putida*, *Y. enterocolitica* and *Y. ruckeri* were the only Gram-negative bacteria tested to be inhibited (Razavi-Rohani and Griffiths 1996).

The inhibition of Gram-positive bacteria by the combination of lysozyme and ML was markedly affected by pH (Table 1). In the case of Gram-negative bacteria, lowering the pH was not effective except for *P. putida* 515 which was inhibited at ML and lysozyme concentrations of less than 2 μg/mL. *P. fluorescens* 545 was inhibited at combined ML and lysozyme concentrations of 29 μg/mL at pH 6 and concentrations of less than 2 μg/mL were required to

lysozyme alone; or (as control) 3% NaCl and 12.5 ppm $NaNO_2$. The treated sausage was kept at 75°C for 1 hr and stored at 10°C for 4 weeks. It was found that the meat was more effectively preserved by lysozyme in combination with NaCl and $NaNO_2$ than by either lysozyme or salt alone for heated samples. Only Gram-negative rods were isolated from all heat-treated samples. Lysozyme was unaffected by the heat treatment. The best preservation was obtained for unheated samples by lysozyme alone, rather than NaCl-$NaNO_2$ control. *Micrococcus* and *Streptococcus* appeared on samples treated with lysozyme alone in addition to Gram-negative rods. A Gram-positive rod appeared in the control sample in addition to the types already mentioned.

Salami sausages containing 0.015% of 2[2-furyl-3-(5-nitro-2 furylacrylamide)] (AF2), 0.1% sorbic acid, and 0.01% spices, including pepper, nutmeg, coriander, onion, ginger, cinnamon, and garlic, were cut in half and coated on the inside and outside surface areas by Akashi[202] with four different preparations: (O) 0.5 mℓ salad oil; (L) 3 mℓ of 0.05% lysozyme in M/10 phosphate buffer (pH 6.5); (LO) 0.05 g of lysozyme added to 0.5 mℓ of salad oil; and (LOS) 0.05 g of lysozyme dissolved in 3 mℓ of M/10 phosphate buffer (pH 6.5) mixed with 0.5 mℓ of salad oil and 0.1 g of sorbitan monooleate, an emulsifier. The sausages were stored at 37°C (80% humidity) for 7 days or at 20°C + 1°C for 57 days. Sausages were examined for viable bacteria count, slime formation, thiobarbaturic acid (TBA) value, volatile base nitrogen (VBN), and red color intensity. The (LO) treatment was the best preservative followed by (LOS), (L), (O), and the control.

Akashi[203] subjected Vienna sausages to nine different treatments using lysozyme. Vienna sausage is a semidry meat product that is processed by stuffing ground, cured meat into natural desalted sheep casings, followed by cooking at 75°C for 15 min and drying at 80°C for 10 min. During storage, the sausage is subject to color fading and sliminess due to microbial growth. In Japan, where the study was undertaken, casings are often dipped into a chemical preservative before use, but the residual-absorbed additive plus the amount added to the cured meat could exceed prescribed legal limits, so lysozyme was studied as a possible alternative.

The treatments included combinations of lysozyme in the meat preparation, dipping the casings in a 0.05% M/10 lysozyme phosphate buffer solution before stuffing the cured meat, and dipping the finished Vienna sausage in the 0.05% lysozyme phosphate buffer (pH 6.5) after cooking. He also used chemical preservatives, nitro-furylacrylamide (0.03%) and sorbic acid (0.01%). Vinyl bags, each containing 30 sausages, were stored for 5 days at 30°C. Color fading, sliminess, and viable cell count were used as parameters for evaluating keeping quality. Species of bacteria found initially on sausages after lysozyme treatment were *Streptococcus*, *Pseudomonas*, *Achromobacter*, and *Flavobacterium*. Toward the middle to last stages of the storage time, these bacteria decreased and the flora were predominately made up to *Micrococcus*, *Lactobacillus*, *Leuconostoc*, and a lactic group of *Streptococcus*. Vienna sausages were best preserved by a combination of dipping the casings in 0.05% lysozyme plus phosphate buffer (6.5), adding 0.5% lysozyme to the cured meat, and dipping the sausage in 0.05% lysozyme in phosphate buffer (pH 6.5) after cooking. In practice, adding 0.05% lysozyme to cured meat would be expensive, so a combination of dipping casings in 0.05% lysozyme plus phosphate buffer (pH 6.5) at 4°C for a holding time of 24 hr and dipping sausage in 0.05% lysozyme was recommended.

Akashi and Oono[204] studied the preservative effect of egg white lysozyme on nonpackaged Kamaboko. Kamaboko was processed from frozen lizard-fish meat to which 3% NaCl was added. The meat was kneaded and the following experimental preparations were made and added to the meat: (1) 0.6 ppm/kg AF2; (2) 0.1% sorbic acid; and (3) 0.05% egg white lysozyme. The fish cakes were also dipped in 1% gelatin-0.05% lysozyme solutions as another experiment. The fish cakes were stored at 10°C for 14 days. Viable bacteria count, slime changes, VNB, binding capacity, and brown color changes were monitored. Kamaboko

ped swab and streaking from plate (Hill and Schalkowsky lates containing no inhibitor observed was not due to the

into the agar, the plates were depending on the optimum point of growth for each e from the center of the plate The MIC was calculated by r (Anon 1985).
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a of the bacteria tested riffiths 1996). ML, when bited only Gram-positive Gram-positive bacteria were when both were present at midis ATCC 12228) and 90 C's were only slightly lower d that the inhibition was ceride. The combination of the Gram-negative bacteria. e same concentration as the m-negative bacteria more evious work had shown that of all of the Gram-negative nd when the chelating agent a, *Y. enterocolitica* and *Y.* ed to be inhibited (Razavi-

e combination of lysozyme the case of Gram-negative or *P. putida* 515 which was f less than 2 μg/mL. *P.* lysozyme concentrations of 2 μg/mL were required to

inhibit *Y. ruckeri* at pH 5.5. Addition of EDTA had little or no effect on the inhibition of Gram-positive bacteria by ML and lysozyme regardless of pH. However, in the case of most Gram-negative bacteria, EDTA amplified the effect at low pH. The exceptions were *S. typhimurium* 617 and *E. aerogenes* 551 which were unaffected even at pH 5 (Table 1).

TABLE 1.
MINIMUM INHIBITORY CONCENTRATION (μg/mL) AT DIFFERENT pH'S OF A COMBINATION OF LYSOZYME AND MONOLAURIN (ML) WITH AND WITHOUT AN EQUAL CONCENTRATION OF EDTA

Organism	L + ML				L + ML + EDTA			
pH	7	6	5.5	5	7	6	5.5	5
Gram-positive								
Bacillus cereus 17	38	10	4.5	<2	50	7	4.5	<2
B. licheniformis UG	46	9	6	<2	62	6	3.3	<2
B. subtilis 22	46	9	6	<2	85	6	4.5	<2
Lactococcus lactis NCK400	7	5.3	4.5	<2	7	4.5	3.9	<2
Listeria monocytogenes Scott A	90	27	9	2.7	230	13	7	3.5
Staphylococcus aureus ATCC 6538	10	5	4	<2	10	5	4	<2
S. epidermidis ATCC12228	6	5.5	4.5	<2	4.5	4.2	4.2	<2
Gram-negative								
Enterobacter aerogenes 551	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580
Escherichia coli O157:H7	>1580	>1580	>1580	>1580	>1580	380	290	100
Pseudomonas aeruginosa ATCC 9027	>1580	>1580	>1580	>1580	>1580	>1580	>1580	290
P. fluorescens 545	>1580	29	<2	<2	115	70	<2	<2
P. putida 515	>1580	>1580	>1580	<2	185	170	90	<2
Salmonella typhimurium 617	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580
Yersinia enterocolitica RS29	>1580	>1580	>1580	>1580	>1580	>1580	90	<2
Y. ruckeri ATCC 29473	>1580	>1580	<2	<2	150	90	<2	<2

The inhibitory effect of this combination (ML + lysozyme) increased slightly with increasing NaCl concentration (Table 2), except for the strains of *E. aerogenes*, *E. coli*, *P. aeruginosa* or *S. typhimurium* tested, even when the NaCl concentration reached 5%. The addition of EDTA to this combination was not effective on Gram-positive bacteria but, at NaCl concentrations of $\geq$ 4% all Gram-negative bacteria tested became more susceptible to inhibition by ML and lysozyme (Table 2).

Table 4
SYNERGISTIC EFFECT OF LYSOZYME AND GLYCINE AGAINST THREE BACTERIA[14]

Lysozyme (ppm)	Remaining bacteria (%)					
	0% Glycine	0.2% Glycine	0.4% Glycine	0.6% Glycine	0.8% Glycine	1.0% Glycine
Bacillus subtilis						
0	100	77	47	35	27	14
10	65	39	33	12	5	5
20	23	16	7	3	2	0
40	9	6	1	0	0	0
80	2	2	0	0	0	0
Escherichia coli S-8						
0	100	92	78	24	18	15
10	95	97	42	21	18	11
20	96	91	37	9	8	7
40	96	72	24	2	0	0
80	92	69	20	0	0	0
Staphylococcus aureus 209-P						
0	100	97	95	97	84	89
10	92	102	91	88	85	77
20	91	92	56	19	17	13
40	93	86	44	8	5	0
80	87	86	36	4	0	0

preserved with lysozyme in the meat performed better than that with AF2 or sorbic acid in the meat, but dipping the fish cakes in the gelatin lysozyme solution increased the preservative effect with AF2 and sorbic acid in the meat over the Kamaboko with lysozyme in the meat. Egg white lysozyme exhibited similar binding capacity and brown color changes as AF2 or sorbic acid.

Akashi[205] reviewed the uses of lysozyme as a food preservative and pointed out some of the possible problems, including allergic and antigenic aspects of ingested lysozyme.

D. Yeast Autolysis

Lysozyme has also been used in the production of yeast autolysate.[206] Intact yeast cells were treated with lytic enzymes including zymolase and lysozyme, which increased the release of nitrogen and proteins during incubation. Pancreatin or pronase also added during incubation of cells with lytic enzymes caused concurrent hydrolysis of the yeast proteins. The precipitable yeast protein at pH 4.5 decreased from 73 to 21% within 60 min.

E. Additives Used with Lysozyme

To enhance the effectiveness of lysozyme as a food preservative, experiments have been done using lysozyme in combination with other substances to increase the bactericidal effect. Some of the substances used successfully have been phytic acid (chelating agent), butyl *p*-hydroxybenzoate (POBB)[134,207] *p*-hydroxy-benzoic esters,[182] β-glycopyranose aerodehydrogenase,[180] amino acids,[208] hydrogen peroxide, and organic acids such as ascorbic acid.[209] The amino acid that has been most effective is glycine (Table 4), which is used commercially in combination with lysozyme in Japan.[16,207,208]

Chassy and Giuffrida[152] used lysozyme in a tris(hydroxymethyl) aminomethane buffer instead of the common buffer systems used with lysozyme and found it to be superior as discussed previously. Polyethylene glycol added to the lysozyme buffer solution as an osmotic stabilizer was also found to enhance the extent of lysis. Supplementation of the growth

TABLE 2.
MINIMUM INHIBITORY CONCENTRATION (μg/mL) AT pH 7 AT DIFFERENT NaCl CONCENTRATIONS OF A COMBINATION OF LYSOZYME AND MONOLAURIN (ML) WITH AND WITHOUT AN EQUAL CONCENTRATION OF EDTA

Organism	L + ML						L + ML + EDTA					
NaCl conc.(%)	0.5	1	2	3	4	5	0.5	1	2	3	4	5
Gram-positive												
Bacillus cereus 17	38	23	12	10	6	3	50	33	18	15	10	3
B. licheniformis UG	46	18	11	11	4	<2	62	40	9	6	6	<2
B. subtilis 22	46	27	13	11	6	<2	85	52	24	24	10	<2
Lactococcus lactis NCK400	7	6	4.5	4.5	4.5	<2	7	5	4	3	<2	<2
Listeria monocytogenes ScottA	90	43	44	16	10	9	72	30	21	15	9	7
Staphylococcus aureus ATCC 6538	10	7	7	4.5	4.5	3.5	14	7	5.7	4.2	4	3.5
S. epidermidis ATCC12228	6	3.5	3	2.5	<2	<2	4.5	4.2	3.4	3.1	2.9	<2
Gram-negative												
Enterobacter aerogenes 551	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	330	170	90
Escherichia coli O157:H7	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	330	66	48
Pseudomonas aeruginosa ATCC 9027	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	330	220
P. fluorescens 545	>1580	90	<2	<2	<2	<2	115	79	<2	<2	<2	<2
P. putida 515	>1580	>1580	>1580	>1580	>1580	<2	170	123	123	123	100	<2
Salmonella typhimurium 617	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	194	74
Yersinia enterocolitica RS29	>1580	>1580	>1580	>1580	>1580	<2	>1580	360	330	280	120	<2
Y.ruckeri ATCC 29473	>1580	>1580	>1580	<2	<2	<2	150	123	115	<2	<2	<2

medium with L-threonine and/or L-lysine frequently produced cells that were more susceptible to lysis by lysozyme. Glucose-containing media produced cells that were more easily lysed than those from cultures grown on other substrates.

Salt was used by Parry et al.[89] to enhance lysozyme activity in a technique designed to spectrophotometrically determine the rate of clearing of a suspension of lyophilized *M. lysodeikticus* cells. They found that the addition of salt to the assay system with egg white lysozyme did not stimulate the rate of lysis but improved the linearity of the reaction rate along with the reproducibility. They also reported that NaCl was essential for bovine milk lysozyme activity and as an activator for human milk lysozyme.

Weaver and Kroger[28] used potassium dichromate in human milk as a preservative and found no adverse effect on measurement of lysozyme activity. Vakil et al.[97] studied lysozyme from bovine and human milk and egg white in the pure form with NaCl, EDTA, and NaCl plus EDTA on various organisms, both UV-killed and living. They found that NaCl accelerated the lysis of both live and UV-killed cells of *M. lysodeikticus* and *Sarcina lutea* by the two milk lysozymes, but egg white lysozyme was only accelerated by NaCl when using live cells. The lysis of other bacteria sensitive to the lysozymes was impaired by NaCl. EDTA had little or no effect upon the enzymatic activity of the milk lysozymes but accelerated lysis of live cells of *M. lysodeikticus* by egg white lysozyme. In general, EDTA augmented the lysis of both live and UV-killed cells of *B. subtilis* and *P. aeruginosa* by bovine milk lysozyme. However, the lysis of other sensitive bacteria, under similar conditions of assay, was depressed by EDTA. When using actively growing bacteria, the additions of either NaCl or EDTA in general improved the lysis of the sensitive organisms by the milk lysozymes and egg white lysozyme. NaCl and EDTA were essential in lysing *E. coli* and *B. cereus*. This study indicated that differences exist not only in the lytic activity of the lysozyme from different sources, but also in the additive effect of combinations on different bacteria.

IV. PHARMACEUTICAL USES OF LYSOZYME

Lysozyme is well known as an effective immunological agent and has been called an "endogenous antibiotic". Because of this characteristic, it is used in human therapy for the treatment of viral and bacterial infections. Lysozyme has an analgesic effect when administered to patients suffering from cancer and has been used as a potentiating agent in antibiotic therapy. It has been used in the prophylaxis and therapy of leukopenia induced by antiblastic and ionizing radiation.[52] EDTA-tris-lysozyme solutions are effective in the treatment of coliform infections of the bladder in human patients[210] and of experimentally induced pseudomonas cystitis[211] and otitis externa in dogs.[212] The Japanese use lysozyme in cold medicines sold over the counter.[213]

Lysozyme concentration has been used as an indicator of health and infection in animals and humans. Kageoka et al.[214] used peroxidase and lysozyme activities of leukemic cells as an indicator of the disease. Kavanova et al.[215] found blood serum concentration of healthy humans to be 7.75 ± 0.28 mg/ℓ, whereas that of patients with varicose ulcers, erysipelas, and particularly microbial eczema showed a statistically significant increase. The increase associated with psoriasis was not statistically significant.

Falchuk et al.[216] found that the serum lysozyme levels of patients with Crohn's disease (also known as ileitis or regional enteritis) were substantially elevated compared with normal levels and levels of patients with other inflammatory bowel diseases. A total of 19 normal patients contained 8.8 ± 0.3 μg/mℓ serum lysozyme, 13 ulcerative colitis patients had 9.3 ± 0.6 μg/mℓ, 6 bacterial or nonbacterial enteritis patients had 8.9 ± 0.7 μg/mℓ, and 25 Crohn's disease patients had 26.3 ± 1.4 μg/mℓ serum lysozyme. Weaver and Kroger[28] found no correlation between somatic cell counts of bovine milk and lysozyme concentration. Meyer et al.[217] infected individual quarters of cow udders with *S. aureus* and found that the

Pseudomonas aeruginosa ATCC 9027	>1580	>1580	>1580	>158	580	>1580	>1580	>1580	>1580	>1580	330	220
P. fluorescens 545	>1580	90	<2	<2	[illegible]	<2	115	79	<2	<2	<2	<2
P. putida 515	>1580	>1580	>1580	>1580	>1580	<2	170	123	123	123	100	<2
Salmonella typhimurium 617	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	>1580	194	74
Yersinia enterocolitica RS29	>1580	>1580	>1580	>1580	>1580	<2	>1580	360	330	280	120	<2
Y.ruckeri ATCC 29473	>1580	>1580	>1580	<2	<2	<2	150	123	115	<2	<2	<2

The antibacterial effect of ML + lysozyme was no greater than that observed with ML alone at all the conditions of pH, NaCl and EDTA concentrations used (Razavi-Rohani and Griffiths 1994). Thus, it can be concluded that lysozyme had no synergistic effect when added to ML.

Antimicrobial Activity of Lysozyme in Combination with Triglycerol 1,2 Laurate

Except for the strains of *L. lactis*, *S. aureus* and *S. epidermidis* that were inhibited by between 205 and 660 μg/mL of TGL + lysozyme, all the other organisms tested were not inhibited by concentrations of these agents up to 3170 μg/mL (Table 3). When compared with their inhibitory action alone (Razavi-Rohani and Griffiths 1996), combining the two agents had no significant effect. The presence of EDTA did increase the antimicrobial effect of the combination (lysozyme + TGL) against all of the Gram-positive and three of the Gram-negative bacteria used in this study (Table 3).

The inhibitory action of this combination against all the Gram-positive bacteria, except *L. lactis* NCK 400, was increased by lowering the pH of the growth medium (Table 3). This effect of pH was enhanced even further in the presence of EDTA (Table 3). However, for Gram-negative bacteria, the synergistic effect of acidified medium was only observed for *P. putida* 515 and *Y. ruckeri* ATCC 29473 at pH 5. When EDTA was added all the Gram-negative strains were inhibited at pH 5 except for *E. aerogenes* 551 which was not inhibited even at concentrations of lysozyme and TGL up to 1580 μg/mL (Table 3).

When the NaCl concentration in the medium was increased, the antimicrobial activity of TGL + lysozyme against the majority of Gram-positive bacteria tested was enhanced (Table 4). A similar effect was observed for four of the eight Gram-negative strains tested. With the addition of EDTA to the medium containing NaCl, the inhibitory activity of TGL + lysozyme was increased for all the bacteria studied (Table 4). However, it was noted that the inhibitory effect of the combination (TGL + lysozyme) was no greater than that observed with TGL alone (Razavi-Rohani and Griffiths 1994).

Antimicrobial Activity of Lysozyme in Combination with Butylated Hydroxyanizole

With the exception of two of the pseudomonads that were not inhibited, even at lysozyme and BHA concentrations of 3170 μg/mL, all the other bacterial strains tested were inhibited, with MIC's ranging between 50 and 505 μg/mL for each of lysozyme and BHA (Table 5). Lysozyme alone was not effective against the same bacteria (with the exception of *L. lactis* NCK 400) when added at concentrations up to 3170 μg/mL (Razavi-Rohani and Griffiths 1995). In the

lysozyme concentration of the milk rose rapidly within 10 hr. Lysozyme concentrations reached a maximum 34 to 48 hr after initial treatment regardless of initial lysozyme concentration. *S. aureus* has been found to produce lysozyme,[218-221] and it was determined that production of lysozyme could be a better index of pathogenic staphylococci than the coagulase measurement, especially in cases of animal origin strains. Seleim et al.[221] tested 88 strains of animal origin and 40 strains of human origin. Of 103 strains from pathogenic cases of human and animal origin, 89 (86.4%) were lysozyme producers and 86 (83.5%) were coagulase positive. Out of 75 strains isolated from pathogenic cases of animal origin, 75 (100%) were lysozyme producers and 71 (94.6%) were coagulase positive. On the other hand, out of 28 strains isolated from pathogenic human cases, 14 (50%) were lysozyme producers and 15 (53.6%) were coagulase positive.

Hawiger[220] found staphylococcal lysozyme splits *N*-acetylamino sugars from susceptible substrate and was similar to egg white lysozyme in its optimal temperature for reaction, optimal pH, activation by NaCl and Ca^{2+} ions, and inactivation by Cu^{2+} ions and sodium dodecyl sulfur. It differs from egg white lysozyme in its temperature susceptibility range (staphylococcal lysozyme is inactivated at 56°C). It acts on whole cells and cell walls of *M. lysodeikticus*, murein from *S. aureus* 524, and cell walls of *S. epidermidis* Zak, the latter not being susceptible to egg white lysozyme. The mechanism of action was analogous to egg white lysozyme, but the specificity may differ. The role of staphylococcal lysozyme is unclear.

Lysozyme has also been used as a marker in the therapy of acute and chronic urinary tract infections. The determination of lysozyme in cerebrospinal fluid and blood serum is helpful in differentiating between bacterial and aseptic meningitides or infections. Elevated fecal lysozyme excretion in adolescents is an indicator for a chronic inflammatory bowel disease. Monitoring fecal lysozyme excretion can be used as a marker for a relapse and to monitor therapeutic efficiency in patients with inflammatory bowel disease. A consistent high level of fecal lysozyme excretion in adults over the age of 40 is an indicator for possible colorectal tumors and warrants more thorough investigation.[222]

A. Oral Cavity

Watanabe[93] studied lysozyme of saliva from clinically healthy subjects and patients suffering from periodontal disease. The lysozyme content in the homogenate and precipitate from the diseased group was statistically higher than that from the normal group ($p<0.05$). The activity and content of lysozyme in the precipitate were found to be 70 to 80% of that in the homogenate. The addition of mucin to whole saliva slightly increased the activity of lysozyme in the supernatant. The addition of plaque bacteria to whole saliva reduced the activity of lysozyme in the homogenate by approximately 20%. The addition of human serum to whole saliva increased the activity of lysozyme in the supernatant fivefold.

Richter and Slama[223] assessed lysozyme values in saliva of children after tonsillectomy, after adenotomy, and in a control group without surgical operation. No fluctuations were found in the control group. Lysozyme levels before operations were elevated, but declined to normal values 1 month after the operations.

Zajaczkowska-Bialowas et al.[224] studied the relationship between salivary lysozyme activity and dental caries, and Murai et al.[225] studied the effect of a lysozyme-containing chewing gum on chronic marginal periodontitis. Two experimental groups, each composed of 35 individuals, ages 19 to 53, were used. One group was given gum (3.2 g) with lysozyme (30 mg) and the other group, gum without lysozyme. They were instructed to chew the gum for 5 min after breakfast, lunch, and dinner for 3 weeks. The authors reported favorable results with the lysozyme-containing chewing gum. Statistically significant differences were found between the two experimental groups concerning bleeding, swelling, reddening, and pus discharge. A statistical difference was not found in pocket depth and dental plaque. No

TABLE 3.
MINIMUM INHIBITORY CONCENTRATION (μg/mL) AT DIFFERENT pH'S OF A COMBINATION OF LYSOZYME AND TRIGLYCEROL 1,2 LAURATE (TGL) WITH AND WITHOUT AN EQUAL CONCENTRATION OF EDTA

Organism	L + TGL				L + TGL + EDTA			
pH	7	6	5.5	5	7	6	5.5	5
Gram-positive								
Bacillus cereus 17	>3170	580	125	7	115	48	16	<2
B. licheniformis UG	>3170	440	150	<4	95	30	10	<2
B. subtilis 22	>3170	870	217	<4	160	58	24	<2
Lactococcus lactis NCK400	205	300	300	260	80	90	75	30
Listeria monocytogenes Scott A	>3170	>3170	>3170	380	250	195	110	50
Staphylococcus aureus ATCC 6538	660	205	245	110	220	150	90	45
S. epidermidis ATCC12228	580	450	450	<4	130	100	100	<2
Gram-negative								
Enterobacter aerogenes 551	>3170	>3170	>3170	>3170	>1580	>1580	>1580	>1580
Escherichia coli O157:H7	>3170	>3170	>3170	>3170	>1580	>1580	>1580	250
Pseudomonas aeruginosa ATCC 9027	>3170	>3170	>3170	>3170	>1580	>1580	>1580	310
P. fluorescens 545	>3170	>3170	>3170	>3170	170	115	<2	<2
P. putida 515	>3170	>3170	>3170	<4	210	170	6	<2
Salmonella typhimurium 617	>3170	>3170	>3170	>3170	>1580	>1580	>1580	500
Yersinia enterocolitica RS29	>3170	>3170	>3170	>3170	>1580	>1580	221	18
Y.ruckeri ATCC 29473	>3170	>3170	>3170	<4	195	90	<2	<2

same study it was observed that BHA inhibited all but two pseudomonads when present at concentrations of ≤ 664 μg/mL. Thus, the antimicrobial effect of lysozyme + BHA in combination was greater than that found when either of the two compounds was present alone, but most of the inhibition was due to BHA. In the presence of the chelating agent, EDTA, the inhibitory effect of the combination (lysozyme + BHA) was heightened (Table 5). Improvements to antimicrobial efficiency of lysozyme + BHA combinations were also observed when the medium acidity and NaCl concentration increased. This was especially true when EDTA was also present in the medium (Table 6).

adverse effects were observed in the administration of lysozyme-containing chewing gum. A British patent 948377 dealing with lysozyme for prevention of tooth decay was mentioned by Yashitake and Shinichiro.[16]

Sabau et al.[226] found that a combination of lysozyme and fluorine on experimentally induced dental caries in white rats on the cariogenic Larson diet exhibited a carioprotective effect. The experimental group of 19 rats treated with lysozyme and fluorine on the cariogenic Larson diet had 4.42 $\pm$ 0.38 cavities per rat, a group of 18 rats on the Larson diet treated with lysozyme had 5.39 $\pm$ 0.23 cavities per rat, and the control experimental group of 18 rats on the Larson diet had 8.27 $\pm$ 0.39 cavities per rat.

Blumberger and Glatzel[227] studied the effect of spices on lysozyme activity of saliva. Chili peppers, curry, ginger, paprika, pimento, pepper, mustard, lemon juice, and sugar were used along with some bitter flavors including aloes, gentian, hops, oranges, rhubarb, and vermouth. Intensity of reactions was to a large extent individually determined. The administration of spices increased the amount of saliva secreted per unit of time, with a maximum of three to seven times the resting value caused by mustard, pepper, and lemon juice. Bitter flavors slightly increased the saliva production, but lysozyme activity per milliliter stayed the same or slightly decreased. Lysozyme activity per minute was less than in the resting state following bitter flavor components. The authors hoped to prove that the defensive power of saliva caused by lysozyme could be intensified by the right choice of spices.

Chassy and Giuffrida[152] lysed oral streptococci with lysozyme suspended in dilute tris(hydroxymethyl) aminomethane-hydrochloride buffer containing polyethylene glycol. Zajaczkowska-Bialowas and Kunicka[228] found that during administration of certain sulfamides and antibiotics the amount of salivary lysozyme activity decreased, but increased after regression of the inflammatory process and discontinuation of drugs. Data on the role of oral lysozyme and other oral enzymes in the antimicrobial defense of microorganisms are reviewed by Barabash and Levitsky.[229]

Miller and Lacey[230] found that 80 to 90% of all clinical isolates of *S. aureus* produced β-lactamase, which rendered them penicillin resistant and also less susceptible to the killing effect of lysozyme. They found that this was particularly true at lysozyme concentrations of 100 to 500 μg/mℓ. Both β-lactamase-negative derivatives and those producing β-lactamase were more susceptible to lysozyme at pH 5. Differences were less marked, but usually apparent with larger inocula, with cells in stationary phase, and in the presence of 1 mol ferric chloride.

S. aureus is present in the anterior nares, although the organism may be carried at other sites such as the perineum. Probably by originating in tears, lysozyme occurs in nasal secretion at concentrations about 100 μg/mℓ, which may be relevant to the selection of β-lactamase producing *S. aureus* in the anterior nares.

B. Dermatology

Lysozyme is an active part of the defense system in the skin. Ogawa[231] found that human skin contained 60 to 120 μg of lysozyme per wet weight gram, a value almost equal to that of saliva. Lysozyme activity in the epidermal portion was approximately three times greater than the dermal portion of the skin.

Kavanova et al.[215] first demonstrated lysozyme activity in skin homogenate, and partially isolated the enzyme by means of gel filtration on Sephadex®. The mean lysozyme value in skin homogenate amounted to 47.1 μg/g tissue. The concentration of lysozyme on skin surfaces of various parts of the body were also determined. The forearm contained 4.03 $\times$ 10^{-3} μg/cm^2, the forehead contained 2 times as much, and the palm of the hand had 7 times as much lysozyme. Concentration in a microbial eczema lesion was 22.55 $\times$ 10^3 μg, compared with a site without lesions containing 6.60 $\times$ 10^{-3} μg.

compounds so as to reduce the amounts added to food. The use of combination preservative systems rather than single inhibitors may maximize the multifunctional properties of these chemicals. Identification of optimal preservative systems should then allow the use of lower concentrations of these agents in food (Kabara 1982; Raccach 1984).

Fatty acids and their derivatives are effective against Gram-positive bacteria, molds and yeasts but have little activity against Gram-negative organisms (Kabara 1979, 1984). Of these lipids, the monoglyceride, monolaurin (glyceryl monolaurate; ML), in addition to exhibiting emulsifying properties, possesses the highest antimicrobial activity (Kabara 1979; Shibasaki 1979, 1982).

Lysozyme is an antimicrobial agent found in many natural systems. This enzyme lyses Gram-positive bacteria more readily than Gram-negatives that contain a protective lipoprotein-lipopolysaccharide layer (Davidson and Branen 1993). Because it lyses some bacteria and is generally regarded as safe for humans, lysozyme has potential for the preservation of food.

Phenolic antioxidants like butylated hydroxyanizole (BHA) also exert an antimicrobial effect. They are active against a wide variety of organisms but their use is limited by toxicological considerations. Their antibacterial effects have been described by many workers (Chang and Branen 1975; Robach *et al.* 1977; Stern *et al.* 1979; Kabara 1980, 1981, 1982; Raccach 1984).

In foods EDTA is used primarily as an antioxidant. It exerts little antimicrobial effect by itself but can potentiate preservative action (Kenward *et al.* 1979; Razavi-Rohani and Griffiths 1994). EDTA is believed to facilitate the action of antimicrobial agents, especially on the more resistant Gram-negative bacteria, by binding divalent cations in bacterial cell walls (Kato and Shibasaki 1976; Kabara 1981).

As well as the nature of the antimicrobial compound to be used, environmental factors such as pH, salt concentration and temperature need to be addressed when formulating a preservative system (Kabara 1981). In this study, we have examined the antibacterial activity of different combinations of food-grade additives and ascertained the effect of chelating agents, pH and NaCl concentration on their efficacy.

MATERIALS AND METHODS

Chemicals

Lysozyme chloride was obtained from Canadian Lysozyme Inc., Abbotsford, B.C., Canada; triglycerol 1,2 laurate (TGL) was provided by Atkemix Inc. (Brantford, ON., Canada); butylated hydroxyanizole (BHA; 2,(3)-tert-butyl-4-hydroxyanizole), ethylenediamine tetraacetic acid (EDTA) and

C. Lysozyme and Certain Antibiotics

Itakura[166] studied the effect of lysozyme on *S. aureus* in combination with amoxicillin, a synthesized penicillin, and found a synergistic effect. Little lysis took place with lysozyme alone, but with a small amount of amoxicillin, lysis occurred in proportion to the amount of lysozyme added. Electron microscope studies indicated bacterial solubility of cell membranes. Application of these results would be in treating dermatological diseases since large amounts of lysozyme are found in the upper layers of skin.

When cells of *Rhizobium japonicum* were treated or pretreated with ampicillin or penicillin and subsequently treated with lysozyme, the rate of lysis was enhanced and marked damage occurred to the cells. Ampicillin and penicillin are thought to cause disorganization of the cell wall and render the mucopeptide substrate accessible to the action of lysozyme.

Rozgonyi and Redai[232] found that the natural meticillin resistance of staphylococci increased slightly in the presence of lysozyme. Under the same conditions, strains sensitive to the antibiotic or adapted to meticillin resistance showed an increased sensitivity.

Barach and Adams[233] found that lysozyme was inhibited by the antibiotics polymyxin, neomycin, or kanamycin. Kornfeld[234] found that lysozyme enhanced detection of zones of inhibition in a paper-disk or cylinder-plate method for detection of penicillin or agar seeded with *S. lutea*. Lower quantities of antibiotic could be detected by the increased size of the inhibition zone.

Fernandez-Sousa et al.[82] found that the lytic activity of hen egg white lysozyme towards bacterial cells of *M. lysodeikticus* was pH dependent and was inhibited by several aminoglycosidic antibiotics whose structure is related to the saccharidic substrates of the enzyme. The concentration of antibiotics necessary to yield 50% inhibition at pH 4.0 and 6.2 was in decreasing order: neomucin B < gentamicin C_{1a} < kanamycin A < dihydrostreptomycin, according to the number of positive charges they bear. The extent of inhibition was greater at pH 4.0 than 6.2, except for dihydrostreptomycin.

D. Optometry

Ueda et al.[235] found that the lysozyme concentration in tears of 111 normal subjects was 4697 ± 1544 μg/mℓ (mean ± SD), and the lysozyme concentration of subjects with keratoconjunctivites sicca (KCS) was significantly lower. The lysozyme concentration of tears in 168 subjects with Sjogren's syndrome was 1626 ± 1063 μg/mℓ which was significantly lower than the tears of normal subjects. Harada[236] studied the bactericidal substances in human tears (lysozyme, immunoglobulins, and β-lysin) and found that lysozyme, which is excreted from the main lacrimal gland, was the most effective against *B. subtilis*. The concentration of lysozyme was 6.9 ± 2.2 μg/mℓ (mean ± SD) in tears of adults (25 to 36 years old), 6.6 ± 2.5 μg/mℓ in children (7 to 12 years old), and was decreased to 6.3 ± 0.9 μg/mℓ in tears of older people (55 to 70 years old). This was suggested as part of the reason why older people contract ocular diseases more frequently.

E. Lysozyme Gene Isolation and Cloning

As the study of lysozyme and its uses continues, the isolation of the lysozyme gene would be an important event. Baldacci et al.[237] isolated the gene structure. Electron microscopic studies showed that the plasmid carrying a double-stranded lysozyme cDNA was split by at least three introns. The length of the gene was about 3.9 kb, 6 times longer than lysozyme in RNA. Nguyen-Huu and Stratmann[238] found that the chicken lysozyme gene contained several intervening sequences. Sippel et al.[239] cloned the chicken lysozyme sequence in vitro.

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INHIBITION OF SPOILAGE AND PATHOGENIC BACTERIA ASSOCIATED WITH FOODS BY COMBINATIONS OF ANTIMICROBIAL AGENTS

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ABSTRACT

The antibacterial activity of combinations of lysozyme, monolaurin (ML), triglycerol 1,2 laurate (TGL) and butylated hydroxyanizole (BHA) against 7 Gram-positive and 8 Gram-negative bacteria was studied at different pH, NaCl and EDTA concentrations by the spiral gradient end point test. The inhibitory effect of lysozyme in combination with ML was slightly greater for Gram-positive than for Gram-negative bacteria, but their combined effect was not markedly more inhibitory than ML alone. Lysozyme and TGL together were only inhibitory at low pH and high NaCl concentrations in the presence of EDTA. There was an increase in inhibition when lysozyme and BHA were combined. For Gram-positive bacteria, inhibition by ML and BHA together was more marked than when either was present singly. However, ML decreased the antibacterial activity of BHA against Gram-negative bacteria. Similarly, TGL was antagonistic to BHA action against both Gram-positive and Gram-negative bacteria. In general, the inhibition produced by all combinations was greater as the pH decreased and the NaCl concentration increased, especially in the presence of EDTA.

INTRODUCTION

Consumer dislike for food additives and the limited efficacy of some food preservatives has prompted investigations into more effective use of these

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V. SUMMARY

Lysozyme (or muramidase) is an enzyme that consists of 129 amino acids, cross-linked by 4 disulfide bridges. Lysozyme functions in lysing bacteria by splitting the β(1-4) linkages between NAM and NAG, which are the components making up bacterial cell walls. Lysozyme is found in many places including animal tissues and serums, as well as in organs, tears, human and cow milk, and cervical mucus. The major commercial source for lysozyme is hen egg white.

Lysozyme has been extracted from eggs by a salting out process and by chromatographic methods. Lysozyme is quantitated by a procedure where the reduction in turbidity of a suspension of *M. lysodeikticus* is measured in an aqueous buffer solution, or by the agar plate method where clear zones on agar plates seeded with *M. lysodeikticus* are an indication of lytic activity.

Stability of lysozyme in various chemical solutions and temperatures was reviewed. Lysozyme has been found to be heat stable in acidic solutions but to be inactivated quickly in alkaline conditions. The effects of fluctuating temperatures on lysozyme were studied and over- and undershoot of theoretical values were found. Alcohols of 15 and 20% did not denature lysozyme. Sugars, polyols, and NaCl stabilized lysozyme against heat. Polysaccharides with both carboxylic and sulfuric acid bases were inhibitory to lysozyme.

Reactions with peroxidizing methyl linoleate caused polymerization, loss of activity, and other deteriorative changes; 0.35 Mrad of irradiation was required to reduce the enzymatic activity of a 0.3% lysozyme solution at pH 8 to 37%.

Lysozyme complexes with certain components, which renders the enzyme inactive. Some of the components with which lysozyme has been demonstrated to complex include egg yolk and some egg-white components, monomers, dimers, trimers, and tetramers, imidazole and indole derivatives, polyvalent cations, and divalent cations.

The effect of lysozyme on different strains of dairy starter cultures has been studied because lysozyme has been found to prevent late blowing of cheese. Lysozyme is more inhibitory to the deleterious organisms than to the dairy starter cultures. Some researchers have reported that lysozyme will cause lysis of *E. coli*. It appears that the amount of lysozyme necessary to inhibit growth of the organism in various food products needs to be researched further. Lysozyme effectively inhibited the growth of *P. fluorescens* and *S. senftenberg* on poultry. *C. botulinum* spores had greater heat resistance after being exposed to lysozyme.

The Japanese have been the largest users of lysozyme in practical applications. They have patented processes using lysozyme as a preservative on fresh fruits and vegetables, tofu bean curd, seafoods and meats, and wine and sake. Lysozyme was also used as a supplement to infant-feeding formulas to make them more closely resemble human milk. The shelf life of cooked burdock with soy sauce and of potato salad was extended with a lysozyme-glycine mixture and a commercial hydrolyzed egg white preparation. The product on which most of the research using lysozyme as a preservative has been done is cheese. Lysozyme prevents late blowing of semihard cheeses such as Edam and Gouda and some Italian varieties by inhibiting the growth of butyric acid bacteria, particularly *C. tyrobutyricum*.

Pharmaceutical use of lysozyme has included treatment of viral and bacterial infections. EDTA-tris-lysozyme solutions have been effective in treatment of coliform infections of the bladder of experimentally induced pseudomonas cystitis. The level of serum lysozyme in humans and animals has been used as a marker of infections. Lysozyme has been shown to increase the effectiveness of some antibiotics.

The use of lysozyme as a preservative is a very interesting area of research and has a lot of potential in the food industry, especially within the U.S. The use of lysozyme in different foods, with various additives, and methods of application need to be explored.

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cate that lethal damage to microbial DNA from irradiation results in the loss of ability to reproduce. Damage to other sensitive and critical molecules (e.g., membranes) may also have an effect (ICMSF, 1980).

Several factors can affect the sensitivity of irradiation. Removal of oxygen can increase the resistance of microorganisms to the lethal effect of irradiation. Removal of water also increases the resistance of microorganisms. Finally, the suspending medium or type of food can greatly affect irradiation.

Only if consumer perception is changed to accept irradiated foods will we see more use of irradiation as our food supply grows.

Packaging

Packaging aids in preserving the quality of foods by protecting against damage during storage, transport, and retail handling. Protection can be classified as chemical, where the package may prevent the passage of water vapor, oxygen, or other gases, or may act selectively and only let certain gases pass; physical, where the package may protect against light, dust and dirt, weight loss, and mechanical damage; or biological, where the package may prevent entry of microorganisms or insects, or may affect the mode or rate of spoilage or the survival and growth of pathogens in foods.

The factor most important to the microbiology of packaged foods is the relative permeability of the packaging material to oxygen, carbon dioxide, and water vapor, particularly if the air spaces within the package are evacuated or filled with preservative gases at time of closure (Cavelt, 1968). Films permeable to water vapor and gases more permeable to oxygen than to CO_2, or only loosely applied, may protect against contaminating organisms but do not affect the growth of organisms which may already be on the food.

Another type of packaging we see quite often and are bound to see more of is aseptic packaging. Aseptic filling is the process of filling a sterile food into a sterile container under aseptic conditions. There are three types of aseptically filled packages; tin cans, sterilized by steam and filled and sealed in a sterile chamber; flexible containers, decontaminated by ultraviolet light, alcohol, peroxide, or a combination of these, filled by injection, and sealed in a sterile atmosphere (Shaw, 1977); and extruded polyethylene or polystyrene containers, sterilized in the heat of the extrusion or forming process and filled and sealed in a sterile room (Berry, 1975).

Interaction of Factors

Any of the various factors reviewed above (temperature, water activity, pH, etc.) would probably need to be used at an extreme concentration or level in a food to be an effective antimicrobial agent when used alone. Thus, temperatures would either be freezing or boiling. The a_W of the food would need to be quite low (<0.85 for bacteria). The pH of the food would either be above 9 or below 2 to restrict microbial growth. Conditions such as these would restrict the development or marketing of a variety of foods. Such conditions also fail to allow the optimum quality of the food to be produced or delivered to the consumer. Generally, when factors such as these are at their optimum for the development of food quality, they also allow the development of microbiological spoilage flora.

Recent efforts have recognized the importance of interaction of various antimicrobial factors to produce microbiologically safe food. The significance of this interaction lies in the fact that had these antimicrobials been used separately, stability from spoilage would not have occurred. Yet, it is in the combination or interaction of these factors that such stability can be achieved. The effects of these interactions may be additive or synergistic.

Studies on the effectiveness of the interaction of various factors have increased in number and diversity in the recent literature. Combinations of a_W, pH, salt, sorbate, and other chemicals have been investigated for antimicrobial effectiveness against *C. botulinum* (Baird-Parker and Freame, 1967; Garcia and Genigeorgis, 1987; Garcia et al., 1987; Hauschild and Hilsheimer, 1979; Notermans et al., 1985; Sperber, 1982), *Yersinia enterocolitica* (Stern et al., 1980), *Listeria monocytogenes* (Conner et al., 1986; George et al., 1988; Shahamat et al., 1980), *Staphylococcus aureus* (Lahellec et al., 1981; Raccach, 1981; Stern et al., 1979), *Clostridium sporogenes* (Robach, 1980), mycotoxin production (Bullerman, 1985), osmophilic yeast (Restaino et al., 1982), and *Salmonella* spp. (Rubin et al., 1982; Sofos, 1986). The use of culture selection on product attributes as well as antimicrobial capabilities in some fermented products has also been investigated (Friend et al., 1983; Raccach, 1981; Rubin et al., 1982).

With the greater emphasis on the development and marketing of refrigerated foods by the food industry, use of antimicrobial agents is growing in importance. Temperature abuse of refrigerated products during distribution or retailing or in the hands of the consumer would increase the potential for a health-hazard situation. The National Food Processors Association is advocating the use of one or more antimicrobial factors (also called hurdles or barriers) in refrigerated foods, in addition to refrigeration to provide product safety (NFPA, 1988a; b).

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Organic Acids

Organic acids are considered natural antimicrobials which occur widely in nature. Today, food manufacturers make use of several organic acids to aid in the preservation of processed food products. The organic acids and their derivatives that are typically used as antimicrobial agents include acetic acid, benzoic acid, propionic acid, and sorbic acid.

Acetic acid occurs naturally in vinegar at a level of 4%. Acetic acid and its salts—sodium acetate, calcium acetate, calcium diacetate, and sodium diacetate—assert their antimicrobial activity up to pH 4.5. Acetic acid is commonly used in pickled meat and fish and in condiments such as catsup, mayonnaise, and pickles (Chichester and Tanner, 1972; ICMSF, 1980).

Benzoic acid occurs naturally in cranberries, prunes, cinnamon, and cloves (ICMSF, 1980). Benzoic acid is primarily used as an antimycotic agent at levels of 0.05–0.1% of the undissociated acid. It is most effective in controlling yeasts and molds and least effective in controlling bacteria (Chichester and Tanner, 1972). The sodium salt of the acid, being more soluble than the acid, is generally used as the antimicrobial in foods.

Propionic acid and its salts have been used as antimicrobials for centuries. They are effective mold inhibitors, yet at similar levels show no effectiveness against yeasts. They have little inhibitory effect against bacteria, with the exception of their ability to inhibit the organism which causes rope in bread (Chichester and Tanner, 1972). Propionate has been estimated to account for approximately 75% of all chemical preservatives consumed today, primarily because of its extensive use in bread and bakery products. It is also one of the least expensive preservatives available.

Sorbic acid is the only unsaturated organic acid permitted as a food preservative (ICMSF, 1980). It is an effective antimicrobial at pH values up to 6.0 in food products. It is currently used as a preservative in fruit juices and soft drinks. The free acid and its potassium salts, collectively referred to as sorbates, are typically used in food systems. Sorbates are generally more effective against yeasts and molds than against bacteria (Lück, 1972; 1976).

Curing Salts and Related Materials

Curing was originally developed to aid in the preservation of food by the addition of sodium chloride. Sodium nitrate, however, a natural impurity of sodium chloride, was eventually shown to be the responsible factor in the development of the pink-to-red pigment in meat, the cured meat color. Since then, nitrite, rather than nitrate, has been shown to be the important compound in color formation. Binkerd and Kolari (1975) reviewed the history of nitrite and nitrate as curing agents for meats.

Curing has evolved over the years to include additional processes, as well as other additives. Among the processes which may be considered as curing aids are fermentation, smoking, drying, and heating. Among the additives used in curing, which are known collectively as adjuncts, are ascorbates, phosphates, glucono-delta-lactone, and sugars. Adjuncts are utilized to maintain desirable changes, but may also enhance safety.

Most cured products must still be kept refrigerated, despite the addition of adjuncts or further processing, to remain safe and wholesome. In recent years, packaging improvements have contributed to furthering the shelf life of refrigerated cured products. Cerveny (1980) described many changes in the production and marketing of cured meats in the U.S. in the 1900s.

The preservation and safety of a cured meat product is the result of the interaction of several factors in a system designed to yield a safe, wholesome product. The main components of the system are the curing salts; the microbial content of the raw product; the type, temperature, and duration of the processing; and the types and numbers of microorganisms that can survive the curing process (ICMSF, 1980).

Antibiotics

Antibiotics, produced either naturally by microorganisms or synthetically, can inhibit microbial growth when used at high dilution. Small residues carried over from treated animals are generally considered unacceptable in our food supply. The only antibiotic approved for use in our food supply is nisin (CFR, 1988a), a polypeptide produced from *Streptococcus lactis* Lancefield Group N microorganisms. Similar nisin-like substances are commonly produced by lactic streptococci (Hurst, 1967).

The effectiveness of nisin when used as an antimicrobial in foods decreases with increase in size of the microbial inoculum (Scott and Taylor, 1981; Rayma et al., 1981). The stability of nisin solutions also decreases as the pH increases (Ramseier, 1960; Rayman et al., 1981; Scott and Taylor, 1981). In addition, nisin is not inhibitory against yeasts, fungi, and Gram-negative organisms. The activity against Gram-positive organisms can also vary (Mattick and Hirsch, 1947; Gowans et al., 1952).

The application of nisin in food preservation has been extensively studied and reviewed (Jarvis and Morisetti, 1969; Marth, 1966; Hall, 1966; Hawley, 1957). In general, it has been found that successful application of nisin as a preservative requires that the food be acidic to ensure stability of the antimicrobial during processing and storage, and that the spoilage organisms to be controlled be Gram-positive and/or nisin-sensitive.

Nisin has been proved to be nontoxic and was given international acceptance in 1969 (WHO, 1969). It was recently approved for use as an optional antimicrobial agent for use in the inhibition of growth of *C. botulinum* spores, as well as toxin formation, in certain pasteurized cheese spreads. In conjunction with that ruling, FDA has proposed to amend the standards of identity for pasteurized cheese spread to permit the use of nisin as an antimicrobial agent (CFR, 1988b).

Irradiation

Irradiation, use of ionizing radiation, is typically characterized by high energy content, great penetrating power, and lethality resulting from action at the cellular level (ICMSF, 1980). Practical application of ionizing radiation to destroy microorganisms on commercial products has really developed within the past two decades. Early work clearly established the potential usefulness of irradiation as a means of food preservation. During the early 1960s, some governments permitted the use of ionizing radiation for processing a limited range of foods. Subsequently, permission to use ionizing radiation in the preservation of foods was withdrawn in the U.S. because experimental evidence suggested that irradiation could induce potentially mutagenic, teratogenic, or carcinogenic compounds in some foods. Studies investigating the claim of such hazardous compounds have shown that the process is totally safe and suggest that irradiation may again be approved as a food processing method (FAO/IAEA/WHO Expert Committee, 1977). It is primarily the misinformation associated with irradiation that causes public concern and nonacceptance.

Ionizing radiation and the effects on microorganisms have been extensively studied (ICMSF, 1980). Ionizing radiation has been shown to have several advantages over other methods for destroying bacteria in foods: it is highly lethal, and the dose can be adjusted to yield a pasteurizing or a sterilizing effect; at lower levels (<0.5 Mrad) it produces virtually no organoleptically detectable change in the product; even at higher doses (>1 Mrad), total chemical change in the food is small; there are no residues of nonfood material; negligible heat is produced, so that raw products retain their raw food characteristics and foods may be processed in the frozen state; and penetration of radiation is instantaneous, uniform, and deep, permitting precise process control (in contrast to heat).

Irradiation has also shown some disadvantages over other methods for destroying microorganisms in foods: enzymes are usually not inactivated at bactericidal dose levels and may remain active in food during storage, and chemical changes, though small in total amount, may cause unacceptable organoleptic changes in sensitive foods or foods given high dose levels (ICMSF, 1980).

The death of microorganisms is a consequence of the ionizing action of high-energy radiation. Most studies indi-

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factor in developing safe, shelf-stable food products. Several publications have addressed this topic in depth (Birnbaum, 1981; Jermini and Schmidt-Lorenz, 1987; Troller, 1986).

pH

Microorganisms have minimum, optimum, and maximum pH values for growth (ICMSF, 1980). Molds, yeasts, and bacteria can be categorized by their ability to grow in various pH ranges. The pH of the food plays a significant role in determining the types of microorganisms capable of growing and causing spoilage, a desired fermentation, or a potential health hazard. In general, molds can grow at a lower pH than yeasts, and yeasts are more tolerant of low pH than are bacteria. Bacterial spore germination can be inhibited by low pH (acid conditions). The pH of the food is instrumental in determining the thermal processing conditions required to provide commercial sterility. Foods are categorized by the Food and Drug Administration (CFR, 1987) as acid (natural pH < 4.6), acidified (acid addition provides pH < 4.6), or low acid (pH > 4.6).

In the unprocessed state, most foods such as meat, fish, and vegetables are slightly acidic (pH 5.5–6.8), most fruits are moderately acidic (pH 3.0–4.6), and some foods, such as egg whites, are alkaline (pH > 7.0). Lowering the pH of the foods, either naturally or artificially, has been used for centuries to enhance microbiological stability and preserve foods. Microbiological fermentations of milk, cucumbers, and meats are examples where the pH has been lowered by naturally produced acids to provide more safe and stable products such as cheese, yogurt, pickles, and sausages.

The pH of a food product contributes to its stability from microbiological pathogens. Because of their low pH, acid and acidified foods, while still subject to spoilage, will not support the growth of bacterial pathogens. However, low-acid foods of pH >4.6 are not microbiologically stable and may support microbial pathogen growth. While pH cannot solely be used as a preservative (Vasavada, 1988), it may be used in combination with other factors to contribute to the stability of the product. Pasteurized processed cheese spread is an example of a product where pH is used in conjunction with other factors to provide stability against *Clostridium botulinum* in a low-acid product (Tanaka et al., 1986).

Gases and Modified and Controlled Atmospheres

For many years, various naturally occurring and manufactured gases and vapors have been known to inhibit or to kill microorganisms. Several of these have been studied to determine their potential usefulness in increasing the storage life of foods. The antimicrobial activities of a few of these can be discussed in detail: carbon monoxide, carbon dioxide, ethylene oxide, propylene oxide, sulfur dioxide, and ozone (Davidson et al., 1983; Gammon and Kereluk, 1973). Nitrogen and oxygen, though used frequently in the packaging and storage of foods, do not directly inhibit the growth of microorganisms. Nitrogen flushing, however, can be used to inhibit mold growth.

Recent research has shown carbon monoxide to have potential use with foods (Davidson et al., 1983). CO at 1% has been shown to effectively inhibit psychrotrophs (Clark et al., 1976). CO has also been shown to inhibit yeasts and molds which tend to cause postharvest decay in fruits and vegetables (Aharoni and Stadelbacher, 1973; Barkai-Golan and Aharoni, 1976). The potential toxicity of this compound to workers requires special handling procedures.

The antimicrobial effectiveness of a gas may be dependent on a number of factors associated with the food, its processing, and the microorganisms. Carbon dioxide, for example, has been shown to inhibit or kill various microorganisms depending on the concentration of the gas used, the temperature of incubation, the age of the cells used, and the water activity of the microbial medium. While the degree of inhibition varies with the organism and the type of food involved, a 10% level of CO_2 usually gives about 50% inhibition on the basis of total counts after a given incubation time (Ledward et al., 1971; Clark and Lentz, 1969). CO_2 has been used frequently to control microorganisms in vacuum-packaged meats, for controlled-atmosphere storage, and with carbonated beverages. Studies also have been reported in which CO_2 stimulates the outgrowth of bacterial spores (Enfors and Molin, 1978; Holland et al., 1970). To use CO_2 as an antimicrobial agent, or preservative, sufficient scientific evidence demonstrating its efficacy with a specific product should be obtained.

Sulfur dioxide is broadly effective against yeasts, molds, and bacteria (Dziezak, 1986). It is used extensively for controlling the growth of undesirable microorganisms in soft fruits, fruit juices, wines, sausages, fresh shrimp, and pickles. It tends to react with many organic compounds in aqueous solution to form sulfite compounds, which are inhibitory to some bacteria. The antimicrobial activity of SO_2 is related largely to the unbound nonionized molecular form (Hailler, 1911). Therefore, treatments to kill or inhibit microorganisms are most effective at pH <4.0, where this form prevails. SO_2 tends to penetrate the microbial cell more readily than the ionic species. SO_2 itself is very reactive and probably interacts with many cell components; however, the precise cause of cell inhibition or death is unknown. Sulfite compounds have received much attention in recent years because of individual hypersensitivities to the compounds in food (Taylor et al., 1986).

Gaseous ethylene oxide has been widely used to reduce microbial contamination and to kill insects in various dried foods (Griffith and Hall, 1938; 1940). Among the foods treated have been gums, spices, dried fruits, corn, wheat, barley, dried egg, and gelatin (ICMSF, 1980). Concern over toxicity of residues of ethylene oxide has more strictly regulated the use of this gas.

Propylene oxide has been less studied than ethylene oxide. However, it appears that the general effects of concentration, relative humidity, and temperature are similar (Bruch and Koesterer, 1961; Phillips, 1968; Skinner and Hugo, 1976). Bacteria are more resistant than yeasts and molds. Bacterial spores are more resistant than the vegetative cells. In the United States, propylene oxide has been used as a fumigant for the control of microorganisms and insects in bulk quantities of goods such as cocoa, gums, processed spices, starch, and processed nutmeats (ICMSF, 1980).

Ozone has been shown to elicit different sensitivities in different organisms, similar to the other gases (Broadwater et al., 1973). In terms of inhibition, bacteria are more susceptible to ozone than yeasts and molds (Ingram and Barnes, 1954). Bacterial spores are 10–15 times more resistant than vegetative cells. Ozone appears to attack many vital constituents of microbial cells, but the precise cause of cell death is unknown. Commercially, ozone has been used primarily for the sterilization of water (Guinvarch, 1959; Bean, 1959). It has also been used as a maturing agent in ciders and wine and to sterilize the interior of soft drink bottles prior to filling (Torricelli, 1959).

Modified- or controlled-atmosphere packaging of foods refers to the incorporation of specific gases into the package. The difference between the two rests with the control of the gas mixture throughout the shelf life of the product. "Modified" simply indicates an initial change in the internal atmosphere with no subsequent control. Vacuum packaging, which also fits in this realm, is packaging without gas. The potential dangers with each type of atmospheric modification are virtually the same (reviewed by Eyles and Warth, 1981; Hintlian and Hotchkiss, 1986).

The hazards from *Clostridium botulinum* growth with the modified- or controlled atmosphere packaging or vacuum packaging of various foods have also been extensively studied (Kautter et al., 1981; Post et al., 1985; Stier et al., 1981). Modifying, controlling, or removing the gases from within food packages may control the spoilage flora to a limited extent, but such practice presents a significant danger of *C. botulinum* growth in non-shelf-stable, high-moisture foods. In such packaged foods, the spoilage flora, which warn the consumer of potential hazards via foul odors, are suppressed. Therefore, growth and toxin production by *C. botulinum* can occur prior to any obvious sign of spoilage. Thus, such packaging cannot be relied upon to maintain the microbiological safety of foods. Any interactive effect with other preservatives would need to have sufficient scientific data to establish its efficacy.

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Present and Future Use of Traditional Antimicrobials

A variety of antimicrobial agents and processes have been and will continue to be used, alone and in combination, to preserve foods

M.K. Wagner and L.J. Moberg

☐ THROUGHOUT HISTORY, the problem of food spoilage has plagued man. Early attempts to preserve food focused on the most readily available substances or processes, such as using sugars, salts, spices, and wood smoke. Today, however, preservation has utilized such factors as temperature, water activity, pH, gases, organic acids, salts, antibiotics, irradiation, packaging, and various combinations of these factors. No matter which factors are selected, use of the proper antimicrobial is dependent on the chemical properties of the antimicrobial; the properties and composition of the food product; the type of preservation system, other than chemical, used in the food; the type, characteristics, and number of microorganisms; the safety of the antimicrobial; and the cost effectiveness of the antimicrobial (Branen, 1983).

An advanced and more technical application of preservatives and preservation processes in today's food supply has allowed for several new and innovative products to be introduced, many displaying a longer shelf life (Vasavada, 198). This review will examine the activity and application of several antimicrobials or antimicrobial processes utilized in today's food supply, and will speculate on future uses of these traditional preservation systems.

Temperature

Through the centuries, man has learned to utilize temperature as a means for preserving his food supply. Temperature plays a critical role in retarding as well as eliminating the growth of spoilage and pathogenic microorganisms (ICMSF, 1980). Chilling of the foods is an effective way to retard spoilage. Freezing of the foods allows for their preservation for longer periods of time. Mild heating can be used to dry and therefore preserve foods. Heating at higher temperature can be used to kill microorganisms and eliminate spoilage altogether, if the foods are suitably packaged.

In addition to using the hot and cold temperature extremes for food preservation, use of ambient temperature also proves beneficial. Foods can be quickly fermented with appropriate microorganisms at these ambient temperatures. While the organoleptic character of the food will be changed, its edibility and even its stability will also be enhanced. A wide variety of fermented foods and beverages has evolved, with lactic and alcoholic fermentations, or combinations of the two, as the major fermentations employed in today's food industry (Gibbs, 1987).

Microorganisms can be grouped into different classes (psychrophile, mesophile, thermophile) according to their optimum growth temperature (ICMSF, 1980). In general, as the temperature is lowered from the optimum, microbial growth slows and eventually stops. When the temperature finally reaches the freezing point, growth generally ceases, but some bacteria survive virtually unharmed. Other bacteria are susceptible to damage during freezing and will die. In general, most spores survive freezing unchanged, while most vegetative cells sustain varying levels of damage from one or more steps of the freezing process (Grecz and Arvay, 1982). This damage may result only in sublethal injury, and may not significantly interfere with their eventual repair and potential growth in nonfrozen food. Thus, freezing is not a means to eliminate spoilage and pathogenic bacteria (Obafemi and Davies, 1986). However, this injury may make them more susceptible to the action of other antimicrobial agents. Unfortunately, freezing temperatures that often cause the maximum damage to bacteria also cause the maximum damage to foods.

As the temperature increases above the optimum growth temperature for the microorganisms, injury and death will eventually occur. Mild exposure to these temperatures induces stress and may cause sublethal injury to cells. As with freezing, this stress or injury may increase their sensitivity to antimicrobial agents.

Temperature, but only at its extremes, can be used to preserve food or protect it from spoilage. Freezing will prevent microbial growth, and thus spoilage, as long as the food is maintained frozen (Speck and Ray, 1977). Thermal processing of food under appropriate conditions will destroy microbial spores and cells and provide a shelf-stable product. Refrigeration serves only as a temporary means to retard spoilage and pathogenic bacteria. However, several new pathogens that grow at refrigeration temperatures have recently been identified; therefore, refrigeration cannot be solely relied upon to provide safety to high-moisture, non-shelf-stable products. Nevertheless, as discussed later, refrigeration may be used interactively with another preservative to provide the desired safety.

Water Activity

The growth and metabolism of microorganisms invariably depend on the presence of water in an available form (ICMSF, 1980). The water activity (a_w) of a food provides an objective measure of the amount of water available for microbiological usage. Generally, bacteria require an a_w above 0.91, yeasts above 0.88, and molds above 0.75. Knowledge of the a_w limits for a microorganism can be useful in designing microbial stability into a food product.

Components of a food affect its water activity. The a_w in a food can easily be reduced by increasing the concentration of solutes in the aqueous phase of the food. This can be accomplished by either adding solutes or removing water. In curing, salting, syruping, or sugaring, solutes are contributing to the lower a_w. In dehydration, water removal is contributing to the lower amounts of available water. Food dehydration can be accomplished either by freezing with accompanying removal of water (freeze drying), or by heating to evaporate existing water. In general, the gentler the drying process, the greater the chance that contaminating microorganisms will survive. Freeze drying has little effect on organism viability, whereas the more severe spray drying or roller drying using high temperatures to drive off the water may destroy the majority of vegetative cells present.

Water activity, alone or in combination with other preservatives or factors, can be influential in controlling the growth of spoilage and pathogenic microorganisms. A knowledge of the minimum a_w at which an organism can grow is a major

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V. SUMMARY

Lysozyme (or muramidase) is an enzyme that consists of 129 amino acids, cross-linked by 4 disulfide bridges. Lysozyme functions in lysing bacteria by splitting the β(1-4) linkages between NAM and NAG, which are the components making up bacterial cell walls. Lysozyme is found in many places including animal tissues and serums, as well as in organs, tears, human and cow milk, and cervical mucus. The major commercial source for lysozyme is hen egg white.

Lysozyme has been extracted from eggs by a salting out process and by chromatographic methods. Lysozyme is quantitated by a procedure where the reduction in turbidity of a suspension of *M. lysodeikticus* is measured in an aqueous buffer solution, or by the agar plate method where clear zones on agar plates seeded with *M. lysodeikticus* are an indication of lytic activity.

Stability of lysozyme in various chemical solutions and temperatures was reviewed. Lysozyme has been found to be heat stable in acidic solutions but to be inactivated quickly in alkaline conditions. The effects of fluctuating temperatures on lysozyme were studied and over- and undershoot of theoretical values were found. Alcohols of 15 and 20% did not denature lysozyme. Sugars, polyols, and NaCl stabilized lysozyme against heat. Polysaccharides with both carboxylic and sulfuric acid bases were inhibitory to lysozyme.

Reactions with peroxidizing methyl linoleate caused polymerization, loss of activity, and other deteriorative changes; 0.35 Mrad of irradiation was required to reduce the enzymatic activity of a 0.3% lysozyme solution at pH 8 to 37%.

Lysozyme complexes with certain components, which renders the enzyme inactive. Some of the components with which lysozyme has been demonstrated to complex include egg yolk and some egg-white components, monomers, dimers, trimers, and tetramers, imidazole and indole derivatives, polyvalent cations, and divalent cations.

The effect of lysozyme on different strains of dairy starter cultures has been studied because lysozyme has been found to prevent late blowing of cheese. Lysozyme is more inhibitory to the deleterious organisms than to the dairy starter cultures. Some researchers have reported that lysozyme will cause lysis of *E. coli*. It appears that the amount of lysozyme necessary to inhibit growth of the organism in various food products needs to be researched further. Lysozyme effectively inhibited the growth of *P. fluorescens* and *S. senftenberg* on poultry. *C. botulinum* spores had greater heat resistance after being exposed to lysozyme.

The Japanese have been the largest users of lysozyme in practical applications. They have patented processes using lysozyme as a preservative on fresh fruits and vegetables, tofu bean curd, seafoods and meats, and wine and sake. Lysozyme was also used as a supplement to infant-feeding formulas to make them more closely resemble human milk. The shelf life of cooked burdock with soy sauce and of potato salad was extended with a lysozyme-glycine mixture and a commercial hydrolyzed egg white preparation. The product on which most of the research using lysozyme as a preservative has been done is cheese. Lysozyme prevents late blowing of semihard cheeses such as Edam and Gouda and some Italian varieties by inhibiting the growth of butyric acid bacteria, particularly *C. tyrobutyricum*.

Pharmaceutical use of lysozyme has included treatment of viral and bacterial infections. EDTA-tris-lysozyme solutions have been effective in treatment of coliform infections of the bladder of experimentally induced pseudomonas cystitis. The level of serum lysozyme in humans and animals has been used as a marker of infections. Lysozyme has been shown to increase the effectiveness of some antibiotics.

The use of lysozyme as a preservative is a very interesting area of research and has a lot of potential in the food industry, especially within the U.S. The use of lysozyme in different foods, with various additives, and methods of application need to be explored.

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Table 5. Percent Activity of Lysozyme[1] in Antimicrobials, an Antioxidant, and Carbohydrates[2].

ANTIMICROBIALS	PERCENT ACTIVITY[3]	STANDARD DEVIATION
1% potassium sorbate	95.2	±0
1% sorbic acid	95.2	±0
ANTIOXIDANT		
5% n-propyl gallate	85.7	±0
CARBOHYDRATES		
5% flour	91.3	±0
5% corn starch	96.0	±0

[1]5mg/ml of lysozyme were added to each sample.
[2]Lysoplate method (Gosnell et al., 1975) was used.
[3]There were 6 replications per value.

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Table 4. Percent Activity of Lysozyme[1] in Spices[2].

SPICE	PERCENT ACTIVITY[3]	STANDARD DEVIATION
1% black pepper	105.2	±0
1% white pepper	101.3	±0
2% mustard	101.3	
30% soy sauce	100.0	±0
5% chili powder	96.0	±0
1% paprika	93.5	±0
3% sage	82.7	±0.04
5% ginger	88.0	±0
4% cinnamon	84.0	±0
3% Wrights® liquid smoke	97.1	±0
CURING INGREDIENTS		
5% Quick Cure®	109.1	±0
1% sodium tripolyphosphate	105.2	±0

[1]5mg/ml lysozyme added to each spice.
[2]Lysoplate method (Gosnell et al., 1975) was used.
[3]There were 6 replications per value.

EXHIBIT II

Table 3. Percent Activity of Lysozyme[1] in Gums[2].

GUM	PERCENT ACTIVITY[3]	STANDARD DEVIATION
guar	100.0	±0
arabic	100.0	±0
CMC	97.4	±0
colloid 488T (alginate)	95.2	±0
locust bean	95.2	±0
tragacanth	95.2	±0
760 MB colloid (carrageenan)	66.7	±0

[1]5mg/ml of lysozyme was added to each gum.
[2]Lysoplate method (Gosnell et al., 1975) was used.
[3]There were 6 replications per value.

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Table 2. Percent Activity of Lysozyme[1] in Sweeteners[2].

SWEETENER	PERCENT ACTIVITY[3]
40% glucose	109.7[a]
20% corn SW80® (high fructose corn syrup)	105.1[a,b]
40% corn SW 80® (high fructose corn syrup)	103.8[a,b,c]
20% glucose	103.5[a,b,c]
10% glucose	103.4[a,b,c]
40% sucrose	99.0[b,c,d]
20% sucrose	98.4[c,d]
20% sorbitol	97.8[c,d,e]
10% sucrose	96.4[d,e]
2.5% Kitchen Klatter® (calcium saccharin)	91.6[e,f]
10% lactose	91.1[e,f]
30% lactose	90.4[e,f]
20% Kitchen Klatter® (calcium saccharin)	89.7[f]
10% fructose	86.3[f]
40% fructose	86.4[f]
.01g/ml Equal® (aspartame)	84.8[f]

[1]200μg/ml of lysozyme were used in each solution.
[2]These values were determined spectrophotometrically (Parry et al., 1969)
[3]There were 8 replications per value.
Values with the same letter are not significantly different.

33. Ministero della Sanita' Italiano: Decreto 1 agosto 1983 (Gazzetta Ufficiale della Repubblica Italiana No. 228 del 20-08-1983)
34. Ministero della Sanita' Italiano: Decreto 18 settembre 1986 (Gazzetta Ufficiale della Repubblica Itàliana No. 231 del 4-10-1986)
35. Ministerio de Sanidad y Consumo - Direccion General de Salud Publica - Registro General Sanitario No. Expediente 31.831 - Madrid 20 de Junio de 1983
36. Funfte Verorderung zur Anderung der Kaseverordnung vom 14 November 1985

1-7-87

Table 1. Percent Activity of Lysozyme[1] in Amino Acid Solutions[2].

AMINO ACID	PERCENT ACTIVITY[3]
5% histidine (w/v)	114.0[a]
5% lysine	112.0[a,b]
5% glycine	107.0[b,c]
5% L-cysteine	106.0[b,c]
5% L-threonine	104.0[c,d]
5% lysine HCL	104.0[c,d]
5% serine	103.0[c,d,e]
5% L-arginine	102.0[c,d,e,f]
5% L-proline	98.0[d,e,f,g]
5% DL aspartic acid	98.0[d,e,f,g]
1% inosine	97.0[d,e,f,g]
1% leucine	96.0[e,f,g,h]
1% glutamic	96.0[e,f,g,h,i]
5% valine	96.0[e,f,g,h,i]
5% L-asparagine	95.0[f,g,h,i,j]
5% L-argenine	92.0[g,h,i,j]
1% tyrosine	91.0[g,h,i,j]
5% DL phenylalanine	91.0[g,h,i,j]
1% cystine	89.0[h,i,j,k]
5% DL alanine	89.0[i,j,k]
5% hydroxyl-L-proline	88.0[j,k]
1% tryptophan	83.0[k]

[1]200μg/ml of lysozyme were used in each solution.
[2]Values were determined spectrophotometrically (Parry et al., 1969)
[3]There were 8 replications per value.
Values with the same letter are not significantly different.

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Egg-white lysozyme as a food preservative: an overview

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The use of lysozyme as a food preservative and the factors affecting lysozyme activity (temperature, chemicals, processing and complexes) is reviewed. Lysozyme inhibits the growth of deleterious organisms thus prolonging shelf life. Chemicals used to improve the preservative effect of lysozyme and those that inhibit the enzyme are discussed along with the stability of lysozyme in various chemical environments. Lysozyme has been used to preserve fresh fruits and vegetables, tofu bean curd, seafoods, meats and sausages, potato salad, cooked burdock with soy sauce, and varieties of semi-hard cheeses such as Edam, Gouda and some Italian cheeses. Lysozyme added to infant feeding formulae makes them more closely resemble human milk.

Keywords: Egg-white; food products; lysozyme; preservative

Introduction

One of the egg-white proteins, lysozyme (or muramidase), is an enzyme that consists of 129 amino acids, cross-linked by four disulphide bridges. Lysozyme functions as a food preservative by destroying certain bacteria. This it does by splitting $\beta(1-4)$ linkages between N-acetyl-muramic and N-acetyl-glucosamine, the components making up bacterial cell walls. The most important commercial source of lysozyme is egg-white of the domestic fowl.

The purpose of this overview is to discuss those factors that affect the activity of lysozyme (temperature, chemicals, processing and complexes), how lysozyme acts on bacteria, and the use of lysozyme as a food preservative.

Activity of lysozyme

Effect of heat and chemicals on lysozyme activity

Lysozyme is heat stable in acidic solutions, having been reported to

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Table 1 Stability of lysozyme in concentrated sugar solutions

		Remaining lysozyme activity (%)			
		pH 6.0		pH 3.2	
Temperature (°C)	Time (min)	20% sugar	60% sugar	20% sugar	60% sugar
60	15	89	98	100	100
	30	85	93	100	100
	60	80	92	100	100
80	15	53	100	100	100
	30	53	100	100	100
	60	35	94	100	100
100	15	16.5	32	82	98
	30	0	15	67	79
	60	0	6.8	25	46

The concentration of lysozyme used was 20 p.p.m.
Adapted from Yashitake and Shinichiro.

effect (increase in T_M) depended on the nature of the proteins and sugar or polyol. The T_M for lysozyme was 18.5°C at pH 3 in the presence of 50% (w/w) sorbitol. The researchers suggested that stabilization was due to the effects of sugars and polyols on hydrophobic interactions, which reduced the tendency for complete transfer of hydrophobic groups from an aqueous to a non-polar environment.

Yashitake and Shinichiro (1977) also found increased heat stability of 20 p.p.m. lysozyme in sucrose (*Table 1*). The percentage remaining activity increased at 60% sugar and as pH decreased from 6.0 to 3.2.

Hidaka, as cited in Yashitake and Shinichiro (1977), found that the activity of 20 p.p.m. lysozyme was stabilized against heat by NaCl (*Table 2*). Lysozyme is activated by low concentrations of salt and inhibited by high concentrations. Kravchenko *et al.* (1967) found that salt was necessary for enzymatic action of lysozyme, and that if the ionic strength was higher than 0.05–0.1 M, the lysozyme action on individual substrates increased, whereas the lysis of *Micrococcus lysodeikticus* was inhibited. Chang and Carr (1971), using sodium

Table 2 Stability of lysozyme in NaCl solutions

		Remaining lysozyme activity (%)			
Temperature (0°C)	Time (min)	0.5% salt (w/w)	2% salt	5% salt	10% salt
90	30	76.6	87.6	85.6	93.5
	60	61.2	60.8	73.2	70.7
100	30	67.0	79.3	79.3	86.8
	60	39.8	55.6	55.6	58.4

The concentration of lysozyme used was 20 p.p.m.; activity was 100% at beginning.
Adapted from Yashitake and Shinichiro.

Table 3 Effect of polysaccharides on egg-white lysozyme activity

Polysaccharide	Effect
Carboxy methyl cellulose (carbonic acid radical)	–
Alginate (carbonic acid radical)	–
Pectin (carbonic acid radical)	–
Chondroitin sulphuric acid (sulphuric acid base)	–
Agar (sulphuric acid base)	–
Tannin (sulphuric acid base)	–
Corn starch	+
Tammalindo seed polysaccharide	+
Locust bean gum	+
Low molecular weight pectin	+
Guar gum	+
Tragacinth gum	+

–, lysozyme activity decreased greatly; +, lysozyme activity decreased slightly. Concentration of lysozyme used was 50 p.p.m.
Adapted from Yashitake and Shinichiro.

chloride, potassium phosphate and Tris chloride, showed that the activation at low salt concentration was most closely correlated with a non-specific ionic strength effect and that the inhibition at high salt concentration was most closely correlated with cationic concentration and charge. At a given ionic strength, polyvalent cations are stronger inhibitors than monovalent cations. The effect of cations changes with pH, such that the optimum cation concentration decreases with increasing pH. For example, 50 nM monovalent cations are optimal at pH 7.0, but at pH 9.0 they are strongly inhibitory. The activity of lysozyme can best be represented by an activity–pH–cation profile rather than by a pH–activity curve. The type of salt used also exerted particular effects. Davies *et al.* (1969) found lysozyme to remain highly active over the pH range of 5–10 if the salt concentration was varied appropriately. However, in contrast to Chang and Carr (1971), the latter authors found that the inhibition effect of high salt concentration was correlated to ionic strength rather than cationic concentration. Salt does have an effect on the bactericidal action of lysozyme and the right concentration must be found for maximum activity.

Yashitake and Shinichiro (1977) found that polysaccharides with carboxylic acid and sulphonic acid groups were inhibitory to lysozyme activity (*Table 3*). The inactivation of lysozyme by these compounds was due to precipitation and could be minimized by the addition of 1.0–6.0% salt.

Effects of processing on lysozyme

Damage to proteins from reactions with peroxidizing lipids is an important mechanism in processing and storage of foods (Awad *et al.*, 1968). Free radical reactions peroxidize unsaturated fatty acids first into hydroperoxides; the hydroperoxides are then broken down to products including malonaldehyde. A common manifestation of damaged proteins exposed to peroxidizing lipids is a decrease in protein solubility. Schaich (1974) observed that incubation of lysozyme with peroxidizing methyl linoleate resulted in both decreased solubility of the protein and increased average molecular weight of the soluble fraction. Schaich and Karel (1975) later studied production of free radicals in lysozyme that had reacted with peroxidizing methyl linoleate. They found that free radical concentration in lysozyme decreased with increasing water activity,

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and copper (Feeney *et al.*, 1956), inhibit the enzymatic activity of hen egg-white lysozyme, although only at relatively high concentrations (10^{-3} to 10^{-2} M). Teichberg *et al.* (1974) found that copper inhibited non-competitively with an inhibition constant Ka = $3.8 \times 10^{2}\,M^{-1}$. Beddell *et al.* (1975) found that iodine irreversibly inactivated hen egg-white lysozyme in small amounts at acid or neutral pH. Metal ions seem to bind carboxyl groups of Glu-35 and Asp-52 in the active site of the enzyme, according to McDonald and Phillips (1969).

Yajima *et al.* (1971), while studying the effect of lysozyme on hiochi bacteria in sake, found that lysozyme was inhibited in the presence of either peptone, beef liver extract or boiled soybean, ingredients often added to culture media as growth promoting substances. Lin (1970) found that the reaction of hen egg-white lysozyme with 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide resulted in the modification of eight of 11 carboxyl groups and the destruction of cell wall lytic activity.

Effect of lysozyme on some bacterial strains

To comprehend how lysozyme functions as a preservative in foods, it is important to understand the effect of lysozyme on certain strains and types of bacteria. Salton (1956) studied the fragments derived from the action of lysozyme on the lysozyme-sensitive bacteria, *Micrococcus lysodeikticus*, *Sarcina lutea* and *Bacillus megaterium*. A review of the studies of lysozyme action on microorganisms was undertaken by Salton (1957).

Salton and Pavilik (1960) used various Gram-positive bacteria to study the degree of susceptibility of their cell walls. They studied strains of *Bacillus*, *Corynebacterium*, *Lactobacillus*, *Micrococcus*, *Sarcina*, *Sporosarcina*, *Staphylococcus* and Gram-negative *Streptococcus*. Isolated walls from all the organisms were sensitive to lysozyme. *Staphylococcus aureus* cell walls were the most resistant, with a percentage turbidity reduction in 24 h at 37°C of 9%. Cell wall isolates from *Bacillus* organisms had turbidity reductions ranging from 51% to 95%, *Corynebacterium*, 92%; *Lactobacillus arabinosus*, 22%; *Micrococcus*, 72–98% (*M. lysodeikticus* was 98%); *Sarcina lutea*, 98%; *Sporosarcina ureae*, 98%; *Staphylococcus*, 9–91%; and *Streptococcus faecalis*, 73%. The activity of lysozyme was not correlated with total amino sugar contents.

Peterson and Hartsell (1955) tested 135 bacterial species for sensitivity to lysozyme by spectrophotometrically checking their dissolution after incubation at 45°C for 1 h. These data were organized into group I (sensitive), group II (relatively sensitive) and group III (insensitive). The order of decreasing lysozyme sensitivity was: group I – *Salmonella*, *Brucella*; group II – *Klebsiella*, *Shigella*, *Neisseria*, *Pseudomonas*, *Pasteurella*, *Erwinia*, *Escherichia*; group III – *Vibrio*, *Proteus*. Viability studies on agar plates showed that the lysozyme-insensitive species and certain species relatively easily lysed were not killed to the extent of the sensitive cultures; however, lysozyme had an inhibitory action. The killing and the lytic action were of the same relative order.

Vedmina *et al.* (1979) tested the sensitivity of lysozyme against 476 strains of Gram-negative bacteria. They found high resistance to lysozyme in *Eltor vibrio cholerae* and *Pseudomonas*. Cultures of various sensitivity included *Aeromonas*, enteropathogenic *Escherichia coli* and NAG-vibrios.

Since lysozyme has been isolated from human and bovine milk, researchers have sought to determine how the enzyme affects bacteria in the milk. Vakil *et al.* (1969) used eight Gram-positive strains (including two lactic acid bacteria)

and five Gram-negative organisms that were either live resting cells or UV-killed cells. They found that all of them were susceptible to bovine milk muramidase and all except the lactic acid bacteria (*Lactobacillus casei* and *Streptococcus lactis*) were susceptible to human milk muramidase. The Gram-positive organisms were *Micrococcus lysodeikticus*, *Streptococcus lactis*, *Lactobacillus casei*, *Staphylococcus aureus*, *Sarcina lutea*, *Streptococcus faecalis*, *Bacillus subtilis* and *Bacillus cereus*, and the Gram-negative organisms were *Escherichia coli*, *Serratia marcescens*, *Proteus vulgaris*, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa*. The disc assay technique was used to determine lysozyme sensitivity to bovine milk, human milk and egg-white lysozyme alone or in combinations with NaCl, ethylene diamine tetraacetic acid (EDTA), or NaCl plus EDTA. The effect of the additives varied depending on the source of lysozyme and their combinations, which will be discussed in a later section. The experimenters concluded that lysozyme played a significant role in the inherent antibacterial activity of milk. Shahani (1970) came to a similar conclusion when he found that human and bovine milk lysozyme lysed cells of 13 types of bacteria. Goudswaard *et al.* (1978) found that lysozyme interfered with microbiological screening for penicillin in cow milk because of its lytic effect on *Bacillus stearothermophilus* subsp. *calidolactis*. This lytic effect was demonstrated microscopically.

Korkohen (1978) also found that 50 samples of colostrum and milk obtained from five Finnish Ayrshire cows in days 1–14 of lactation inhibited the test organism *B. stearothermophilus* subsp. *calidolactis* used in the thermocult test for antibiotics. The presence of lactoferrin and lysozyme was responsible for this inhibition.

Bottazzi *et al.* (1978) studied the effect of lysozyme on thermophilic lactic acid bacteria. The natural culture of lactic acid bacteria grown in whey was very sensitive to lysozyme. They found that concentrations up to 10 p.p.m. lysozyme were proportional to the lytic effect. More lysozyme was necessary to give an inhibitory effect in milk than in whey for strains of *Lactobacillus helviticus* and *L. fermentum* isolated from natural whey. *L. helviticus* in milk was inhibited by greater than 50 p.p.m. lysozyme, and the authors believed that the difference was due to the absorption of lysozyme onto casein. Some strains of *L. fermentum* could grow on MRS in the presence of 30 p.p.m. lysozyme. Lysozyme should not be added directly to the milk during manufacturing or processing of Italian long-ripening cheeses because of the high level needed, but rather after cutting the curd when only 5–10 p.p.m. would be needed. Lysozyme also should not be added during acidification of cheese, to avoid selective action of lactic microflora from the natural whey culture giving an irregular fermentation.

Trentini and Murray (1975) observed the lysis of *Caryophanon latum* by phase contrast microscopy. Pretreatment of cells with pronase, a lipase–phospholipase C mixture, EDTA, glutaraldehyde or heat did not alter the pattern of lysis or render the remaining peptidoglycan more susceptible to attack.

Lysozyme has been shown to be more detrimental to spoilage and to pathogenic bacteria in milk than to lactic acid-producing bacteria. In fact, lysozyme in small concentrations was found to activate the growth of two *Streptococcus cremoris* strains that are used in cheese processing (Akashi, 1972a). Because of this, lysozyme has been suggested as a preservative in dairy fermented products such as cheese. Studying the factors involved, Panfil-Kuncewicz and Kisza (1976) added egg-white lysozyme to raw milk and determined the percentage of acid-forming bacteria. Initially, 56% of the total

synergistically with lysozyme.

D_{235} values were 3.0 min and 1.10 min, with and without lysozyme, respectively. When the temperature was raised to 245°C, the difference between *D* values seemed to increase: 0.2 min without lysozyme and 0.8 min with lysozyme. Lysozyme in the recovery medium enhanced and speeded outgrowth of heated *C. botulinum* spores.

Mustafa (1963) noted that some strains of clostridia increased in turbidity when exposed to lysozyme. *C. multifermentans*, *C. lentoputrescens* and *C. tertium* released reducing sugars when exposed to lysozyme. Distorted cells in Gram-stained smears of lysozyme-treated cells were observed with suspensions of *C. hemolyticum*, *C. butyricum*, *C. multifermentans* and *C. lentoputrescens*.

Ng and Garibaldi (1975) found that incubating and shaking *Staphylococcus aureus* in liquid whole egg caused a decline in viability because of lysozyme. The action of lysozyme was not attributed to its bacterial lytic property, but to the basicity of the lysozyme molecule. The natural pH of the egg rises from 7.2 to between 8.0 and 8.2 during shaking, as a result of carbon dioxide loss. The authors postulated that the rise in pH rendered the bacterial cells more negatively charged so that in the presence of positively charged molecules of lysozyme a complex was formed that agglutinated the cells.

Itakura (1978) observed that lysozyme alone was only partially effective against *S. aureus* but, in combination with a small amount of amoxicillin, lysozyme was very effective at lysing cell walls. Safonova *et al.* (1979) found that lysozyme changed the optical density of suspensions of *S. aureus*, *S. epidermidis* and *S. saprophyticus*, though the effect was different from that of *Micrococcus luteus*. The optical density of *S. saprophyticus* was characterized by some properties indicating that the cell wall structure of the organism differs from those of the other two strains, which were similar.

Safonova *et al.* (1981) studied the nature of the optical density of suspensions of *S. aureus* and *M. luteus* cells at various concentrations of egg-white lysozyme and two products of its chemical transformation. A decrease in optical density of the suspensions at relatively low concentrations of lysozyme, i.e. 0.032–0.064 mg/ml, was due to lysis of both microbial species. The increase in optical density at 0.5–2.0 mg/ml of lysozyme indicated the formation of insoluble aggregates of an electrostatic character. Lysozyme was the positively-charged component and the microbial cell material was the negatively-charged component. Lysozyme aggregates with *M. luteus* were composed of lysis acid products and aggregates with *S. aureus* were composed of lysis products and the intact live cells.

Wooley and Blue (1975) studied the *in vitro* effect of EDTA, Tris/HCl buffer and lysozyme solution on 16 pathogenic bacteria of medical or veterinary importance. Marked decreases in bacterial count occurred with *Pseudomonas aeruginosa*, *Escherichia coli*, *Moraxella oslensis* and *Campylobacter fetus*, and smaller decreases with *Salmonella typhimurium*, *Shigella boydii*, *Aeromonas hydrophi*, *Proteus mirabilis*, *Listeria monocytogenes* and *Erysipelothrix insidiosa*. The test solution had no effect on *Klebsiella ozaenae*, *Brucella canis*, *Corynebacterium pyogenes*, *Corynebacterium renale*, *Streptococcus equi* and *Staphylococcus aureus*.

Lysozyme as a preservative in food

Considerable interest has been stimulated in using lysozyme as a food preservative, mostly in Japan, where the majority of the work has been performed using lysozyme in food systems. Akashi (1972a) found that

egg-white suspensions had a lytic action on *Escherichia coli* suspensions. Food products such as sausages, fish cakes and bacon could be preserved with lysozyme.

The Japanese have many patents on using lysozyme as a preservative in foods. A patent was issued for the preservation of fresh vegetables, fish, meat and fruit by coating the surface with lysozyme (Kanebo Ltd., 1973, Japanese patent 4831-905). Taiyo Food Co. Ltd. (1972) patented a process to preserve tofu bean curd by adding lysozyme to soya milk during processing (Japanese patent 46-336/72). Dried milk compositions for paediatric use were preserved by incorporating egg-white lysozyme with ovalbumin and ovomucin (Morinaga Milk Industry Co. Ltd., 1970, Japanese patent 16-780/70). Francis (1980) and Carlsson *et al.* (1979) found that antibodies, lactoferrin and lysozyme in human breast milk acted complementarily to prevent gastroenteritis and helped prevent allergies; therefore, lysozyme has been added to infant formulae. When Nishihava and Isoda (1967) added lysozyme to infants' dry milk powder, to simulate human milk which contains lysozyme, the number of *Bifidus bacillus* in the intestines increased. *B. bacillus* is said to contribute to a healthy flora and aid in digestion.

Oysters, shrimps and other seafood were claimed to be preserved in refrigerated storage by treatment with aqueous solutions of lysozyme and NaCl in Japanese patent 5710/72 by Eisai Co. Ltd. in 1972. The same company patented a process (Japanese patent 19576/71) to preserve fresh marine products by soaking them in aqueous solutions containing a lysozyme salt, amino acids and NaCl in 1971. They also claimed that lysozyme was very valuable in the preservation of wine and sake, and patented a process in 1971 (Japanese patent 3-115/71) by which wines were stabilized by addition of lysozyme or its salts, together with *p*-hydroxybenzoic esters. Eisai (1980) also obtained a Japanese patent for adding specific lysozyme materials and ß-glycopyranose dehydrogenase to sake to improve storage stability (Japanese examined patent 5535105).

Yashitake and Shinichiro (1977) used information from Eisai concerning lysozyme preparations made by them with the trade name Amicannon. The use of lysozyme as a preservative in the following foods was suggested by them in combination with other additives.

1 Kimchi is a fermented Korean food made from Chinese cabbage, hot peppers, vinegar, and garlic. Japanese potato salad is prepared from mashed potatoes, mayonnaise, and thinly sliced vegetables. Egg-white lysozyme and amino acids were found to be the best combination for preserving both these products.
2 Sushi (rice seasoned with sugar and vinegar rolled in laver seaweed, with either a pickled turnip, radish, grated carrot, meat, or other filler in the centre) can be preserved by dissolving lysozyme in the vinegar.
3 Chinese noodles could be prepared with organic acids, hydrogen peroxide or propylene glycol as a preservative. The use of lysozyme and amino acids would enhance preservation, especially in combination with propylene glycol.
4 Creamed custard is heated in processing, but because of the high sugar content lysozyme activity is not lost. According to Yashitake and Shinichiro (1977), amino acids and lysozyme in combination with alcohol make a good preservative of this food.

starch decreased lysozyme activity only slightly.

CONCLUSIONS

The basic amino acids increased the activity of lysozyme over 100% against lyophilized cells of M. lysodeikticus, but whether this effect was due to ionic strength was not determined. The sugars, glucose and high fructose corn syrup (because of its glucose content) also enhanced lysozyme activity. Aspartame decreased lysozyme activity to 84.8%, which was more than other sweeteners. The only gum that was found to be inhibitory to lysozyme was carrageenan, which contains sulfated polymers. Ingredients used in curing and spices used in further processed meats enhanced lysozyme activity, whereas sage, ginger, and cinnamon in high concentrations were slightly inhibitory. Antimicrobials did not seem to affect lysozyme activity, but the antioxidant, n-propyl gallate, decreased lysozyme activity slightly.

In order for lysozyme to be effective as a food preservative, the types of food and food ingredients that are compatible or enhance its enzymatic activity must be determined. The results of this study suggest that lysozyme could be an effective preservative of foods with a high natural sugar content but not of artificially sweetened products. The spices and curing ingredients of further processed meats may enhance lysozyme activity and longevity in the product. The addition of the antimicrobials tested would not decrease lysozyme activity significantly and possibly could

type cheese to undergo late blowing or late gassing is *Clostridium tyrobutyricum*. Wasserfall and Teuber (1979) used egg-white lysozyme at a concentration of 500 units/ml to kill 99% of 5×10^5 resting vegetative cells of *C. tyrobutyricum* within 24 h of incubation at 25°C. Spores were resistant to lysozyme, however proliferating vegetative cells were severely inhibited. Though lysozyme-resistant cells developed in growing cultures in the presence of lysozyme, the overall outgrowth of spores to vegetative cells was delayed one day in the presence of 500 units of lysozyme/ml. The researchers suggested that this inhibition of the lactate-fermenting *C. tyrobutyricum* was the basis for the observation that lysozyme can substitute for nitrate in preventing the late gas defect. Wasserfall *et al.* (1976) prepared Edam cheese without lysozyme and with 500 units/ml of lysozyme from 25 litres of low-count milk containing approximately 1000 *C. tyrobutyricum* spores/l. After five weeks of ripening, cheese made with lysozyme had resisted late blowing, while the other was no longer suitable for consumption. The investigators found this true even in the presence of lysozyme-resistant vegetative cells. Ferrari and Dell Acqua (1979) patented a process in the UK (patent application 2014032A) in which lysozymes or their non-toxic salts were added to butter or cheese to prevent the development of undesirable microorganisms. One of the suggested applications involved the addition of 50 p.p.m. lysozyme to renneted milk in grana cheese making. The inventors found that after 24 months of ripening the physical and organoleptic properties were excellent, whereas control cheese was blown because of *C. tyrobutyricum*. These same findings were common to other Italian cheese varieties.

While studying the recovery and stability of lysozyme, Wasserfall (1977) recovered 97–100% of lysozyme added to acid whey, but only 75–85% of lysozyme added to milk was recovered in whey after acidification. The recovery of lysozyme added to rennet whey was 88–93%, but only 8.7–11% of lysozyme added to cheese milk was recovered in whey after renneting. His conclusion was that a large part of the enzyme added to milk was firmly bound to the coagulum and was therefore not available for prevention of late blowing during cheese ripening. He also found that storage stability of lysozyme preparations decreased with increased temperature and alkalinity

Meat

The influence of lysozyme in meat products has also been investigated. Akashi (1969, 1970, 1971) completed a series of studies on the preservative effect of lysozyme added to cooked sausage, salami sausage and Vienna sausage. In the cooked sausage study, samples of ground beef were treated with: 3% NaCl, 12.5 p.p.m. $NaNO_2$, and 50 or 200 p.p.m. lysozyme; 200 p.p.m. lysozyme alone; or (as control) with 3% NaCl and 12.5 p.p.m. $NaNO_2$. The treated sausage was kept at 75°C for 1 h and stored at 10°C for four weeks. Akashi found that the meat was more effectively preserved by lysozyme in combination with NaCl and $NaNO_2$ than by either lysozyme or salt alone. Omitting the effect of heat, lysozyme retarded microbial growth to a greater extent than did the standard preservative mixture of NaCl and $NaNO_2$.

Salami sausages containing 0.015% of 2[2-furyl-3-(nitro-2 furyl acrylamide], 0.1% sorbic acid, and 0.01% spices, including pepper, nutmeg, coriander, onion, ginger, cinnamon and garlic, were coated on the inside and outside surfaces with four different preparations: (O) 0.5 ml salad oil; (L) 3 ml of 0.05% lysozyme in M/10 phosphate buffer (pH 6.5); (LO) 0.05 g of lysozyme added to

0.5 ml of salad oil; and (LOS) 0.05 g of lysozyme dissolved in 3 ml of M/10 phosphate buffer (pH 6.5) mixed with 0.5 ml of salad oil and 0.1 g of sorbitan monooleate, an emulsifier (Akashi, 1970). The sausages were stored at 37°C (80% humidity) for seven days or at 20°C + 1°C for 57 days. Sausages were examined for viable bacteria count, slime formation, TBA (thiobarbaturic acid) value, VBN (volatile base nitrogen), and red colour intensity. The LO treatment was the best preservative followed by LOS, L, O and the control.

Vienna sausage is a semi-dry meat product stuffed in natural sheep casings. The casings were shipped to Japan from Australia dried and salted. Casings were desalted in water at room temperature for 24 h before they were stuffed with Vienna sausage mix. Akashi (1971) subjected Vienna sausages to nine different lysozyme treatments, including combinations of lysozyme in the meat preparation, dipping the casings in a 0.05% M/10 lysozyme phosphate buffer solution before stuffing the cured meat, and dipping the finished Vienna sausage in the 0.05% lysozyme phosphate buffer (pH 6.5) after cooking. He also used nitro-furylacrylamide (0.03%) and sorbic acid (0.01%). Colour fading, sliminess, and viable cell count were used as parameters to evaluate keeping quality. Species of bacteria found initially on sausages after lysozyme treatment were *Streptococcus, Pseudomonas, Achromobacter* and *Flavobacterium*. Toward the middle to last stages of storage time the number of these bacteria decreased, and the flora was predominantly made up of *Micrococcus, Lactobacillus, Leuconostoc* and a lactic group of *Streptococcus*. Vienna sausages were best preserved by a combination of dipping the casings in 0.05% lysozyme plus phosphate buffer (pH 6.5), adding 0.055% lysozyme to the cured meat, and dipping the sausage in 0.05% lysozyme in phosphate buffer (pH 6.5) after cooking.

Akashi and Oono (1972) studied the preservative effect of egg-white lysozyme on non-packaged Kamaboko. Kamaboko was processed from frozen lizard-fish meat to which 3% NaCl was added. The meat was kneaded, and the following experimental preparations were made and added to the meat: (1) 0.6 p.p.m./kg AF2 (2-[2 furyl-3-(5-nitro-2 furyl) acrylamide]); (2) 0.1% sorbic acid; and (3) 0.05% egg-white lysozyme. The fish cakes were also dipped in 1% gelatin–0.05% lysozyme solutions as another experiment. The fish cakes were stored at 10°C for 14 days. Viable bacteria count, slime changes, VNB, binding capacity and brown colour changes were monitored. Kamaboko preserved with lysozyme in the meat performed better than mixtures with AF2 or sorbic acid in the meat, but dipping fish cakes with AF2 and sorbic acid in the meat in the gelatin lysozyme solution increased the preservative effect. Egg-white lysozyme exhibited binding capacity and brown colour changes similar to those of AF2 or sorbic acid.

Akashi (1972b) reviewed the use of lysozyme as a food preservative and pointed out some of the possible problems, including allergic and antigenic aspects of ingested lysozyme.

Yeast autolysis

Lysozyme has also been used in the production of yeast autolysate (Knorr *et al.*, 1979). Intact yeast cells were treated with lytic enzymes including zymolase and lysozyme, which increased the release of nitrogen and proteins during incubation. Pancreatin or pronase also added during incubation of cells with lytic enzymes caused concurrent hydrolysis of the yeast proteins. The precipitable yeast protein at pH 4.5 decreased from 73% to 21% within 60 min.

glucose in high fructose corn syrup seemed to be the enhancing factor because fructose alone depressed lysozyme activity (86% activity). Table sugar (sucrose) did not lower lysozyme activity. Calcium saccharin (91.6%) and aspartame (84.8%) slightly decreased lysozyme activity.

The gums, guar and arabic (Table 3), did not decrease lysozyme activity at all; CMC decreased it only slightly (97.4%); and alginate, locust bean, and tragacanth lowered lysozyme's activity to 95.2%. 760 MB colloid, a carrageenan greatly decreased lysozyme activity (66.7%). Although commercial carrageenan is a mixture of various types, it is approximately 60% kappa and 40% lambda carrageenans, which are both sulfated polymers. Yashitake and Shinichiro (1977) also found that sulfuric acid-based gums decreased lysozyme activity greatly, and guar, locust bean gum, and tragacanth only decreased lysozyme activity slightly.

The effects of spices and cure ingredients on lysozyme are shown in Table 4. Ingredients that would commonly be used in further processed meats, such as the mustard, pepper, paprika, smoke, and curing ingredients, either enhanced lysozyme activity or were only slightly inhibitory. Sage, ginger, and cinnamon decreased lysozyme activity, but the concentrations of these spices used would not normally be found in foods.

Antimicrobials in a 1% solution (higher concentration than would be allowed in food products) only slightly decreased lysozyme activity (95.2% activity) (Table 5). The antioxidant, n-propyl gallate, decreased lysozyme activity to 85.7%, but flour and corn

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a high fructose corn syrup (corn SW 80®, containing 80% fructose) from ADM Corn Sweeteners, Clinton, IA.

The spices were purchased at a grocery store. The sodium tripolyphosphate, Granular 00-22, was made by Calgon Corp. Quick Cure® (Custom Food Products, Inc, Chicago, Ill.) is composed of salt, sugar, 1% sodium nitrate, maple sugar, and not more than 1% propylene glycol added as an anti-caking agent. The corn starch was from Busch Industrial Products Corp., St. Louis, Mo., and the isolated soy (Ardex DNV, dispersible and 94% protein) was made by ADM, Decauter, IL. The antimicrobials, potassium sorbate (Sorbistat-K®) and sorbic acid (Sorbistat®), were obtained from Pfizer, New York, N.Y. n-Propyl gallate (No. P-3130) was purchased from Sigma Chemical Co.

RESULTS and DISCUSSION

The combinations of amino acids with lysozyme are illustrated in Table 1. The highest activity (114%) was with 5% histidine. The 112% activity with 5% lysine was not significantly different. Other amino acids that produced more than 100% activity included 5% glycine, 5% L-cysteine, 5% L-threonine, 5% lysine HCL, 5% serine, and 5% L-arginine.

The amino acids that increased lysozyme activity against M. lysodeikticus were basic. Their influence may simply be due to pH and ionic charge, as reported by Chang and Carr (1971).

The sweeteners, glucose and high fructose corn syrup, increased lysozymes' activity above 100% activity (Table 2). The

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The Properties of Lysozyme and Its Action ON MICROORGANISMS

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INTRODUCTION

The bactericidal and bacteriolytic properties of various body fluids, tissues, secretions and microbial culture filtrates have long been known. The possibility that bacteriolytic substances may possess properties in common with enzymes was considered by Nicolle (1) in the course of a study of lytic factors produced by *Bacillus subtilis*. In 1909, Laschtschenko (2) investigated the bactericidal action of egg white from the hen, and he concluded that germicidal activity was due to an enzyme. The 'plasmolytic' action of the egg white on *B. subtilis* was followed microscopically and the sensitivity of other *Bacillus* spp. and *Proteus* spp. was determined. Fleming (3) found that a powerful bacteriolytic substance capable of rapidly lysing thick suspensions of certain bacteria, was widely distributed in nature. As this substance had properties akin to those of "ferments," Fleming (3) called it a "lysozyme." It was detected in tears, nasal mucus, saliva, blood serum and plasma, in many other tissues and secretions of human and animal origin, and it was present in egg white and turnips (3). Of all the materials examined, egg white was the richest source of lysozyme (3). Fleming (3) also isolated a gram positive coccus which he called *Micrococcus lysodeikticus* ('lysis indicator') and this organism proved to be particularly susceptible to the lytic action of lysozyme. Thus Fleming's investigations provided a name that has been universally adopted for the bacteriolytic substance, a great deal of information about its distribution, and a suitable test organism that still appears to be the most sensitive indicator of "lysozyme."

Until Meyer *et al.* (4) discovered that lysozyme digestion of bacterial substrates was accompanied by a liberation of reducing groups, the enzyme could only be defined by its ability to lyse certain bacteria. Early investigations based solely on lytic properties indicated the widespread distribution of lysozyme. The possibility that bacteria themselves, as well as animals and plants, may contain such an enzyme was raised by Fleming and Allison (5). They discovered that a substance lytic for *Micrococcus lysodeikticus* was liberated when cell suspensions of the same organism were dissolved by lysozyme from tears.

Although Laschtschenko (2) described the bactericidal enzyme in egg white as a proteolytic enzyme and Hallauer (6) reported that lysozyme action was accompanied by a liberation of amino nitrogen, there has been abundant confirmation of the release of reducing substances reported by Meyer *et al.* (4). If lysis and liberation of reducing groups are accepted as the primary manifestations of lysozyme action, it can be said with certainty that lysozyme occurs in egg white (4, 7), saliva, tears, pig duodenal secretion (7), polymorphonuclear leucocytes (7, 8), and ficus and papaya extracts (9). In addition to reducing groups, N-acetylhexosamine is detectable after digestion of bacterial substrates with lysozyme from egg white (7, 10); lysozyme from most of the sources mentioned above has also brought about liberation of acetylhexosamine (7).

Recent investigations of the bacteriolytic enzymes from actinomycetes studied by Welsch (11) and isolated by Ghuysen (12) have shown that they digest bacterial cell-wall substrates from many of the organisms used as lysozyme indicators (13). Unlike lysozyme, these enzymes liberate amino acids and not reducing substances (13). This emphasizes the need for caution in applying the term "lysozyme" to all enzymes capable of lysing bacteria sensitive to egg white lysozyme. From the beginning of this century there have been many reports of bacteriolytic fluids containing what we now know to be enzymes. The similarity of the leukins (Pettersson, 14) to lysozyme has been pointed out and discussed by a number of investigators (8, 15, 16), and the resemblance of plakin to lysozyme is striking (17, 18). It will be of great interest to see how closely the activities of these various enzymes coincide with those of the well characterized lysozymes of plant and animal origin.

Lysozyme has been the subject of several earlier reviews (16, 19–21). In recent years a great deal of information on the structure and properties of lysozyme as a protein (22) has been gained; this enzyme has also found increasing usefulness in studies of bacterial structure, and a more detailed picture of its enzymic properties is beginning to emerge (23). The vast majority of investigations on the action of lysozyme on microorganisms have been performed with egg white lysozyme, so this review will be concerned mainly with that enzyme and unless otherwise specified "lysozyme" will refer to the enzyme from egg white.

PROPERTIES OF CRYSTALLINE LYSOZYMES

Meyer *et al.* (24) demonstrated that purified egg white lysozyme was a basic protein. Abraham and Robinson (25) achieved a high degree of purification of lysozyme, and indeed obtained crystalline preparations; the yields of the crystalline form were insufficient for physico-chemical characterization. Alderton, Ward and Fevold (26) worked out suitable methods for the purification of lysozyme and succeeded in isolating it in a crystalline form in sufficient quantities to demonstrate its homogeneity, and chemical and physical properties. Although lysozyme prepared by adsorption on bentonite was homogeneous as determined by the criteria of electrophoresis, sedimentation and diffusion (26), heterogeneities of crystalline preparations have been detected by immunological (27) and chromatographic (28) methods of analysis. Tallan and Stein (28) have shown that three chromatographically distinct, active components may occur in crystalline lysozyme. The most nearly homogeneous preparation, judged on chromatographic behavior, was the isoelectric precipitate, recrystallized once (28). Lysozyme prepared in the form of the carbonate and stored in the dry state showed a progressive change in the proportions of the three chromatographic components.

Investigations with crystalline lysozyme have confirmed its basic nature; it has an isoelectric point at pH 10.5–11 and is a protein of comparatively low mol wt, 14,700 (22). Two striking features of its amino acid composition are the absence of sulfhydryl groups and the high content of arginine. Lysine is the N-terminal amino acid (29), and the C-terminal amino acid is leucine (30).

Jollès and Fromageot (31) isolated two proteins from rabbit spleen, both of which were found to have a lytic action on *Micrococcus lysodeikticus*. One of the proteins was obtained as a crystalline substance, and qualitatively, its lytic action was exerted on the same microorganisms that were attacked by egg white lysozyme (32). This lytic protein had a mol wt of 14,267 and an amino acid composition similar to that of egg white lysozyme (32). Its resemblance to egg white lysozyme became even more striking when Jollès and Fromageot (33) found that it possessed the same N-terminal and C-terminal amino acids as lysozyme, *i.e.*, lysine and leucine respectively.

calculated.

The ingredients tested included 22 amino acids, 7 gums that were hydrated and diluted to a pipetteable solution, 8 sweeteners in various concentrations, 13 spices and curing ingredients, 5% flour solution, 5% corn starch solution, antimicrobial solutions of 1% potassium sorbate and 1% sorbic acid, and the antioxidant, propyl gallate, in a 5% solution. Other antioxidants would not disperse in a liquid nonlipid solution. Dry materials were weighed on a weight/volume basis and liquid samples on a volume/volume basis.

Some of the amino acids were donated by Ajinomoto U.S.A., Inc., Teaneck, N.J. including inosine, L-threonine, L-serine, L-lysozyme monohydrochloride, L-leucine, L-valine, and L-proline.

The gums were donated by Tic Gums, Inc, New York, and included colloid #488T (the sodium salt of alginate isolated from kelp plant, _Laminaria digitata_, used as a thickener); colloid 760MB (a carrageenan standardized to yield a specific viscosity in milk systems); guar; arabic; locust bean gum; and tragacanth. Sodium carboxymethylcellulose (CMC) was obtained from Dupont (P-75-L), Wilmington, Delaware.

The sweeteners included the noncaloric sugar substitutes Kitchen Klatter® (water, calcium saccharin 2.25%, propylene glycol USP 1.5%, benzoic acid 0.1%, methyl paraben 0.05%); and Equal® (dextrose with dried corn syrup, aspartame (Nutra Sweet brand), silicon dioxide, cellulose, tribasic calcium phosphate, and cellulose derivatives); glucose; sucrose; lactose; fructose; and

TABLE 1

Some features of the composition and physico-chemical properties of lysozymes

Amino Acid	Egg White	Rat Spleen "Protéine Lysante II"	Papaya Latex
	Number of amino acid residues		
Aspartic acid	20	20	22
Arginine	11	6	13
Glutamic acid	4	11	11
Histidine	1	1	3
Lysine	6	6	10
Proline	2	5	18
Phenylalanine	3	3	12
Tryptophane	8	2	7
N-terminal amino acid	Lysine	Lysine	Glycine
Molecular weight	14,700	14,267	24,745
Iso-electric point	pH 10.5-11		pH 10.5

Data summarized from references 22, 29, 32-34.

The only other lysozyme isolated in a sufficiently purified state for physico-chemical characterization is that of papaya latex (34). Smith *et al.* (34) obtained the papaya lysozyme as a crystalline mercury derivative. A basic isoelectric point (pH 10.5), a relatively low mol wt of 25,000 and a high content of arginine and basic amino acids were some of the properties papaya lysozyme possessed in common with egg white lysozyme and the lytic protein from rabbit spleen (34). Unlike the lysozymes of animal origin, the papaya lysozyme contained glycine as its N-terminal amino acid (34).

Table 1 summarizes the more outstanding similarities and differences of amino acid composition and other general properties of the three lysozymes obtained as crystalline proteins or as crystalline derivatives.

NON-ENZYMIC REACTIONS OF LYSOZYME

Agglutination of Bacterial Cells

In their studies of the lytic and flocculating action of egg white, Friedberger and Hoder (35) noted that organisms not lysed by high dilutions of egg white were flocculated. Both lysozyme activity and flocculating capacity of the egg white were optimal at the same sodium chloride concentrations, were adsorbed on to charcoal and kaolin, and were inactivated under the same conditions of heating. They concluded that the lytic action and the ability to agglutinate resistant bacteria were two manifestations of the same substance. Flocculation of living and heat killed cell suspensions of both sensitive and resistant bacteria have been reported by a number of investigators (16, 36-38). Thompson (16) observed occasional agglutination of the very susceptible micrococci and *Sarcina* spp. before lysis commenced. There is little doubt that the flocculation of bacterial suspensions by lysozyme is due to basic properties, and in this respect it behaves very similarly to other, non-enzymic, basic substances, *e.g.*, protamines, basic polypeptides and cationic detergents (39). Lysozyme, in common with other basic compounds, would be attracted by negatively charged groups on the bacterial surface, the neutralization of which would account for the observed agglutination.

Formation of Complexes with Acidic Substances

Many substances, including simple synthetic surface active compounds and naturally occurring acidic polymers of bacterial origin, can combine with lysozyme. Glassman and Molnar (40) found that "Lorol"[1] (Na salt of straight chain alcohol sulfate; C_{12} the principal homologue) formed insoluble complexes with lysozyme, complete precipitation occurring at a mass ratio of lorol to lysozyme of 0.3. However, for complete inhibition of lytic activity, amounts in excess of those required for the stoichiometric complexes were necessary. Inhibition of lysis of *Micrococcus lysodeikticus* by ribonucleic acid, heparin and gum arabic was reported by Nihoul *et al.* (41). Simmons (42) showed that inhibitory effects of substances of biological origin could be the result of the formation of insoluble complexes; a mucoid lysozyme salt precipitated from solution on the addition of an acidic submaxillary mucoid to lysozyme solutions. The inhibitory action of heparin has been confirmed by others (43, 44). Hyaluronate, type II pneumococcal polysaccharide, glutamyl polypeptide from *Bacillus anthracis*, Vi antigen, ribonucleic acid and deoxyribonucleic acid inhibited the lytic action of lysozyme, and on the addition of lysozyme to solutions of these acidic polymers, fine precipitates were formed (45). Skarnes and Watson (45) concluded that the inhibition resulted from the formation of an acid-base complex, rather than a coating of the bacterial substrate by the inhibitory substances. The effect of electrolytes and ionic strength on the reversal of this inhibition supported this conclusion (45). Caputo (46) studied the reaction of solutions of crystalline lysozyme and crystalline pepsin at different levels of pH. The formation of protein-protein complexes at pH levels where the proteins carried opposite charges was demonstrated; the complex gave a single electrophoretic boundary and sedimented as a single component (46). As with other lysozyme complexes, the complex with pepsin could be dissociated with sodium chloride and its solubility was sensitive to changes in ionic strength (46).

[1] Manufactured by E. I. du Pont de Nemours & Co., Wilmington, Delaware.

Another manifestation of the ability of lysozyme to form complexes with bacterial cells was the increase in turbidity observed when high concentrations of the enzyme were added to suspensions of heat killed microorganisms (38). Heated cells of both resistant and sensitive organisms showed such increases on the addition of lysozyme and identical effects were given when the basic polypeptide, salmine, was used instead of lysozyme (38). The reduction or absence of lysis on the addition of high lysozyme concentrations to sensitive bacteria has been noted by Lwoff *et al.* (47) with *Bacillus megaterium* and more recently by Wilcox and Daniel (48) with *Micrococcus lysodeikticus*. The decreased lysozyme activity was attributed to the formation of a stable enzyme substrate complex. The sodium ion concentration had a marked influence on the phenomenon; the inhibition at high lysozyme concentrations was reduced by increasing the sodium ion concentration (48). Thus the enzyme substrate complex behaved in a very similar fashion to complexes between lysozyme and acidic substances not attacked by this enzyme.

SUSCEPTIBILITY OF BACTERIA TO THE LYTIC ACTION OF LYSOZYME

There have been many investigations of the susceptibility of microorganisms to lysozyme and the ability of the enzyme to lyse bacterial cells has been assessed qualitatively by noting whether clearing has been partial or complete, or quantitatively by measuring the change in the turbidity of the suspensions. Apart from the influence of pH, temperature and ionic environment on the activity of lysozyme there are many other factors that can modify the extent of reduction of turbidity. The frequency and accessibility of the linkages in the bacterial substrate attacked by lysozyme will have an important effect on the response to the lytic action of the enzyme. Partial clearing of a bacterial suspension could be due to small amounts of substrate in individual cells or to mixed populations of lysozyme sensitive and resistant cells.

In addition to variations in the lysis attributable to the quantity of substrate in the cells, factors affecting the physico-chemical properties of cellular protoplasm and protoplasmic membranes will profoundly influence the final degree of clearing achieved. Maximum clearing would be expected where the intracellular proteins are in a readily soluble state. Treatments resulting in the denaturation or coagulation of bacterial proteins, or in the fixation of cellular structures such as the protoplasmic membrane will reduce the apparent lytic activity of the lysozyme. Since purified lysozyme is devoid of proteolytic activity (4) this reduced lysis is due to the insolubility of intracellular proteins and does not constitute proof that the substrate has been modified so that it can no longer be attacked by lysozyme. Both untreated bacteria and cells pretreated in a variety of ways have been used for the determination of sensitivity to the lytic action of lysozyme, and the various factors just mentioned have markedly influenced the extent of lysis.

Sensitivity of Untreated Bacteria

The early investigations on the bactericidal and bacteriolytic properties of tears and egg white established the sensitivity of organisms belonging to the following genera: *Bacillus*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Proteus* and *Brucella* (2, 3). Although some organisms were killed by lysozyme, visible clearing of the suspensions could not be detected (3). Under conditions giving rapid lysis of thick suspensions of *Micrococcus lysodeikticus*, Fleming (3) was unable to detect lysis of organisms of the coli-typhoid group. Since these early studies, a large number of organisms have been tested for their sensitivity to the lytic action of lysozyme from various sources and many of the results have

A lysozyme standard was prepared daily in phosphate buffer, pH 6.2. The percent activity of lysozyme in the test sample was calculated by the ratio of its value to the standard value.

200μg/ml of lysozyme was used in the standard and all test solutions for spectrophotometric determinations. The test for activity was performed at a wavelength of 540nm, 1 hour after lysozyme addition to the test medium. The initial rate of reaction of lysozyme was calculated (change in absorbance/min.) as <u>M. lysodeikticus</u> cells were lysed and the solution cleared. Eight determinations were done for each sample. Analysis of variance was calculated (Snedecor and Cochran, 1976). The samples that were not suitable for spectrophotometric determinations were tested by the lysoplate method. An agar preparation, containing 1.0g agarose with 0.1g NaCl in 100ml of pH 7.0 phosphate buffer seeded with 0.02g of <u>M. lysodeikticus</u>, was heated to 65 - 70°C, while being stirred. The agar was poured into a 2 qt. oblong Pyrex glass baking dish. After solidification, holes or wells were bored into the agar with the small end of a transfer pipette, and 5μg of solution were deposited into each hole with a microtiter pipette. Standards were prepared with 3, 5, and 7 mg/ml of lysozyme and run simultaneously with the samples. The plates were incubated for 3 hrs at room temperature, and diameters of the lysed areas were measured using a pair of dividers and a ruler. Six determinations were done for each standard and sample. The calculated linear gradient of the standard solutions was used to determine the concentration of the samples. Means and standard deviations were

been reviewed by Thompson (16, 49). It is inevitable that studies of the sensitivities would reveal some conflicting conclusions and many of these would in all probability be due to strain differences, differences of growth conditions and media, and variations in the test conditions and source of the material to be tested for "lysozyme" activity. However, despite the fact that there has been no standardized method for testing the susceptibility of bacteria to "lysozyme," there is a body of agreement supporting several broad conclusions. Some of the most easily lysed bacteria belong to these genera: *Micrococcus*, *Sarcina*, *Staphylococcus*, *Bacillus*. Untreated, living gram negative bacteria, when tested under conditions giving rapid lysis of indicator organisms, have been generally found to be more resistant. As discussed below, gram negative bacteria receiving certain pre-treatments can display sensitivity to the lytic action of lysozyme. It is abundantly clear, however, that the lysozyme substrate is not confined to gram positive bacteria and the sensitivity of certain gram negative bacteria is comparable to that of many gram positive organisms (3, 16, 50). There are also wide variations in the extent of lysis shown by organisms belonging to the same genus (16, 21, 23), and the fact that it is possible to obtain less sensitive strains of *M. lysodeikticus* by growth in the presence of lysozyme (6, 51, 52) indicates that not all cells display the same susceptibility.

Repaske (53) recently made the interesting observation that the following gram negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa* and *Azotobacter vinelandii*, were lysed without any pretreatment if exposed to lysozyme at pH 7.6 in the presence of Versene[3] (ethylenediamine-tetraacetic acid). Lysozyme alone was without effect on all three organisms, but Versene alone had an appreciable lytic action on *P. aeruginosa* only.

Sensitivity of Killed Bacteria

Fleming and Allison (5) demonstrated that heat-killed cells of *Micrococcus lysodeikticus* were still attacked by lysozyme; clearing was not complete but the bacterial protoplasm could then be digested with trypsin. The reduced lysis observed with heat-killed cells of micrococci exposed to lysozyme from various sources has been confirmed by many others (6, 35, 38, 54, 55). In

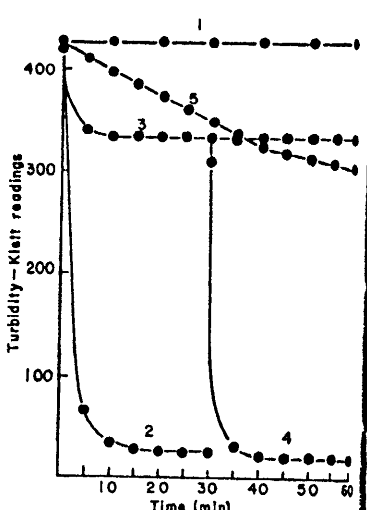

Figure 1. Lysis of *Micrococcus lysodeikticus* in 0.1 M phosphate buffer at 37 C (38). Curve 1, untreated and heated cells suspended in buffer alone; curve 2, lysis of unheated cells with crystalline lysozyme, 5 μg/ml, pH 6.2; curve 3, heated cells incubated with crystalline lysozyme, 5 μg/ml, pH 6.2; curve 4, heated cells incubated with lysozyme as in curve 3 for 30 min, followed by sedimentation of the cells, resuspension in buffer at pH 7.6 and incubation with crude trypsin, 1 mg/ml; curve 5, lysis of heated cells with crude trypsin, 1 mg/ml, pH 7.6.

a study of bacterial lysis in terms of cellular structure, Salton (38) showed that the decrease in turbidity of heated cells of *M. lysodeikticus* treated with lysozyme corresponded to the dissolution of the cell walls and the residual turbidity was due to the lysozyme-insoluble coagulated protoplast. Figure 1 illustrates the turbidimetric changes observed with lysozyme and trypsin-treated suspensions of heated and untreated *M. lysodeikticus*. Examination of preparations of both *M. lysodeikticus* and *Bacillus megaterium* in the electron microscope showed the structural changes caused by treatment with lysozyme. A comparison of the appearance of heated cells of *B. megaterium* before and after incubation with lysozyme showed that lysozyme removed the cell

[3] Supplied by Bersworth Chemical Company, Framingham, Mass.

wall, leaving the coagulated protoplasm in the form of rod shaped bodies (shown in Plate I). Earlier experiments performed by Welshimer and Robinow (56) indicated that lysozyme treatment of both heated and formalin fixed cells of *B. megaterium* produced many "small bacilli" that correspond to the coagulated "protoplasts" illustrated in Plate I. Thus, any agent capable of 'fixing' the bacterial protoplast membrane or denaturing the protoplasmic contents of the cell, will decrease the extent of lysis occurring with lysozyme. Epstein and Chain (7) found that even *M. lysodeikticus* will show incomplete or no lysis if its proteins are denatured by heat, organic solvents or iodine. Pretreatment of *B. megaterium* with polymyxin, prior to removal of the wall with lysozyme, has been shown by Newton (57) to give "fixed" protoplasts very similar to those obtained by heat or formalin fixatives.

To avoid some of the day to day variations in the preparation of suspensions of *Micrococcus lysodeikticus* for assays of the lytic activity of lysozyme, Smolelis and Hartsell (58) investigated the effects of pretreating the cells by quick freezing, ultraviolet killing, acetone drying and killing with phenol, Roccal and Emulsept.[4] Killing the bacteria with phenol and detergents reduced their sensitivity to lysis. For quantitative assay, cells that were killed by ultraviolet light and stored at 4 C in the frozen-dried state gave the most reproducible results (58).

Lysozyme action on a number of gram negative bacteria could be demonstrated if the pH of the cell-lysozyme mixture was not above pH 3.5 and if incubation was performed at 45 C (59). Using essentially the same conditions, Peterson and Hartsell (60) found many gram negative bacteria susceptible to the lytic action of lysozyme; since the cells suspended at pH 3.5 had been killed by acid, the effect of lysozyme on viability under these conditions could not be studied (60). The decreasing order of sensitivity to lysozyme was: group I. *Salmonella*; *Brucella*; group II. *Klebsiella*, *Shigella*, *Neisseria*, *Pseudomonas*, *Pasteurella*, *Erwinia*, *Escherichia*; group III. *Vibrio*, *Proteus*. The Nakamura (61) technique was an essential step for the demonstration of a lytic effect by lysozyme (60). Warren, Gray and Bartell (62) were able to lyse *P. aeruginosa*

[4] Roccal supplied by Winthrop-Stearns, Company; New York, N. Y. Emulsept by Emulsol Corporation, Chicago, Ill.

with lysozyme, provided the cells were heated or treated with acetone. Lysis occurred over a wide range of pH, with an optimum between pH 7-8. Apart from pretreatment with acetone or heating, no special conditions were necessary to demonstrate a lytic effect on 10 different species of gram negative bacteria (62). As with many of the techniques required to demonstrate the susceptibility of gram negative bacteria to lysozyme (53, 60) the treatment with acetone alone gives rise to appreciable lysis in the absence of lysozyme (62).

Factors Affecting the Lytic Activity of Lysozyme

Early investigations (3, 5) had shown that the lytic activity of lysozyme in tears and other sources could be influenced by temperature, pH, and salt concentration. Increased rates of lysis have been noted with temperature increases up to 60 C (5, 16, 58). The pH for maximum lysis of *Micrococcus lysodeikticus* by lysozyme is between pH 6.0-7.0 (16, 58, 63). Little lysis occurs in the absence of sodium chloride, and at high concentrations the lysis is inhibited (5, 16, 58). Smolelis and Hartsell (58) have found that for maximum lytic activity in the presence of potassium salts, the ionic strength must be about 0.1. When tested in solutions of magnesium or calcium salts, the extent of lysis was less than that obtained with potassium and sodium salts (58). The inhibitory action of heavy metal cations has been reported by several investigators (63, 64).

Apart from the physical environment in which the lytic tests are carried out, cultural conditions are also reported to influence the sensitivity of the bacterial cells to lysis by lysozyme (16, 65, 66). Lysis of intact bacteria is a complex process involving a breakdown of the surface structures of the cell. Thus, any factor affecting the stability of the cell surface structure (cell wall and protoplasmic membrane), or any substance blocking the interaction of lysozyme with its substrate (*e.g.*, acidic polymers, capsular substances), will alter the response to the lytic activity of the enzyme. Whether the influence of growth conditions upon the sensitivity of an organism to lysis can be explained in the above terms or whether it is due to more fundamental changes in the production of the lysozyme substrate during growth, cannot be said at this stage. The experiments of Weibull (67) at least

closely correlated with a nonspecific ionic strength effect and that inhibition at high salt concentration was most closely correlated with cationic concentration and charge. Back et al. (1979) tested lysozyme with other proteins and found that both sugars and polyols stabilized them against heat. Polysaccharides with both carboxylic and sulfuric acid bases were reported to decrease lysozyme activity (Yashitake and Shinichiro, 1977).

The use of lysozyme is a more specific, targeted approach to food preservation than conventional methods. Understanding the effects of food ingredients on lysozyme activity is important,if the enzyme is to be used effectively. Many of the published reports on the effects of food ingredients have been commercial propaganda and only included a small number of ingredients.

The purpose of this study was to investigate the effects of various food ingredients on lysozyme activity, especially compounds that might work to enhance activity, and to determine in what foods lysozyme would be most effective as a preservative.

MATERIALS AND METHODS

Grade I lysozyme (Sigma no. L-6876) from chicken egg white, 54,000 units per mg protein, and lyophilized <u>Micrococcus lysodeikticus</u> cells (Sigma no. 4698), ATCC 4698 were used.

Lysozyme activity was tested either by monitoring the clearing of a suspension of <u>M. lysodeikticus</u> because of lysis with a spectrophotometer (Parry et al., 1969) or by measuring the zone of lysis in <u>M. lysodeikticus</u>-seeded agar (Gosnell et al., 1975), depending on the turbidity and dispersibility of the test medium.

make it clear that if the environment used for lysis is osmotically suitable, the partial lysis is due to the formation of stable protoplasts and not to inhibition of the lysozyme. Similar results have been obtained recently with *Micrococcus lysodeikticus* and *Sarcina lutea* (68). These studies again emphasize the important influence the intracellular osmotic pressure and the osmotic pressure of the external medium may have in the over-all lysis of bacteria by lysozyme.

THE SITE OF LYSOZYME ACTION

Few have ever doubted but that lysozyme brings about the lysis of certain sensitive bacteria by a profound alteration in the properties of the surface structure of the cell. There has, however, been some doubt as to whether the protoplasmic membrane or the cell wall, or both structures, are attacked by lysozyme. Early observations of the microscopic sequence of changes effected by lysozyme from several sources have been in complete agreement that a marked swelling of the cells occurs before lysis and that this is due to an alteration of the cell wall (3, 6, 63, 69). Meyer, *et al.* (4) believed that lysis resulted from hydrolysis of a mucoid substance in the bacterial membrane. Boasson (63) concluded that the cell wall of *Micrococcus lysodeikticus* was not destroyed by the action of lysozyme, but that it became more permeable to the cellular contents. Epstein and Chain (7) suggested that the polysaccharide attacked by lysozyme was an essential element for the maintenance of the morphological structure of bacteria that were susceptible to lysis. That the lysozyme substrate was not the sole component of the walls of some bacteria was evident from the results of experiments with a strain of *Bacillus subtilis* (7); this would explain why certain organisms were killed but not lysed by the enzyme. The cellular component digested by lysozyme was found in the insoluble fraction after mechanical disintegration of *Micrococcus lysodeikticus*, and Epstein and Chain (7) concluded that it was in a more highly polymerized form than that isolated from the cells by chemical methods of extraction. Welshimer and Robinow (56) believed the cell wall was the first structure attacked by lysozyme, but were uncertain as to whether it was completely digested, or whether there was any lysozyme action on the cytoplasmic membrane.

Electron microscopy was used in studying the bacteriolytic effects of lysozyme from tears (70); the swelling and liberation of cell contents were observed, but the cell membrane was intact after lysis. Kern *et al.* (71) also observed in the electron microscope the changes occurring during incubation of *Micrococcus lysodeikticus* and *Staphylococcus aureus* with lysozyme. With the sensitive *M. lysodeikticus*, they noted the following sequence of events: swelling of the cells, cell wall rupture and dispersal of contents, dissolution of the cell contents and membrane leaving only two small electron-dense bodies from each lysed cell (71).

Most of the evidence strongly suggested that the bacterial cell wall was the site attacked by lysozyme, but confirmation of this had to await the development of suitable techniques for the isolation of cell walls. Isolation of the wall as a single morphological entity of the bacterial cell was achieved by mechanical disintegration. Such preparations of cell walls were finally freed of other structural elements and cytoplasmic constituents by centrifugation and suitable washing procedures (72); the cell walls so isolated gave stable, milky white suspensions. The direct action of lysozyme on the walls of *Micrococcus lysodeikticus* was demonstrated by Salton (73). Complete dissolution of the walls occurred, and on examination in the electron microscope there was no evidence for the existence of a lysozyme resistant framework forming part of the cell wall of *M. lysodeikticus* (73). From the appearance of cell walls examined at the stage of 'half-lysis' it was clear that much of the rigidity of the wall had been lost (73); this would account for the swelling of the cells observed by previous workers (16, 63, 70, 71).

The elegant technique of phase contrast microscopy combined with immunological reactions, developed by Tomcsik (74), was used by Tomcsik and Guex-Holzer (75) in a study of the action of lysozyme on a *Bacillus* sp. and it enabled them to conclude that lysozyme acted specifically on the cell wall. By microscopic examination of stained, crushed cell preparations of *Bacillus megaterium*, before and after incubation with lysozyme, Welshimer (76) was able to demonstrate the dissolution of the walls and cell wall fragments.

Tomcsik's investigations on the structure of the capsule of *B. megaterium* M showed that the polysaccharide capsule is serologically indistinguishable from the cell wall substance and that it is also degraded by lysozyme (77, 78). These results show that at least in some bacteria the lysozyme substrate may be present also in the capsular layer, and it is presumably at a much lower level of polymerization than the substrate in the wall. The discovery that incubation of *Vibrio cholerae* with lysozyme enhanced its sensitivity to an otherwise feebly lytic phage also suggests the presence of surface components susceptible to digestion with lysozyme (79).

ACTION OF LYSOZYME ON AND THE CHEMICAL CONSTITUTION OF ISOLATED SOLUBLE AND CELL WALL SUBSTRATES

Soluble Substrate Isolated from Whole Bacteria

Before the recent discovery that the substrate of lysozyme could be isolated simply in the form of a cell wall preparation, attempts were directed at purification of the cellular component by various extraction procedures. Hallauer (6) and Meyer *et al.* (4) were the first to extract the substrate in a soluble form. Alkaline digestion of whole bacteria and precipitation of the substrate from the crude extract was used by Meyer *et al.* (4). The materials isolated in this way were mucopolysaccharides; those from *Sarcina* sp. contained 6.73% N and 19.5% reducing sugar. Lysozyme liberated reducing substances from these substrate fractions (4).

Epstein and Chain (7) isolated the substrate from *Micrococcus lysodeikticus* by dissolving the bacteria in hot formamide or by dissolution in alkaline hypochlorite at 0 C. The product extracted by the formamide method gave the greatest increase in reducing value (15% expressed as glucose) on incubation with lysozyme. In agreement with Meyer *et al.* (4) these investigators (7) concluded that the action of lysozyme was hydrolytic. The dialysable fraction of the lysozyme digested substrate contained free reducing substances (a total of 41% after acid hydrolysis), gave a strong reaction for N-acetylhexosamine and contained 6.4% N. Evidence for the liberation of a substance giving the reactions of a ketohexose was presented (7). A number of other organisms were tested for the presence of lysozyme substrate by extracting under conditions used for *M. lysodeikticus* and determining the amounts of N-acetylhexosamine liberated after incubation with the enzyme. Substrate was found in all organisms sensitive to lysozyme (*Bacillus subtilis*, *Brucella abortus*, *Sarcina* sp. and two organisms isolated from air). Only traces of the substrate were found in *Staphylococcus albus*, *Bacterium coli* and *Pseudomonas pyocyanea* (7).

Meyer and Hahnel (10) isolated the soluble substrate from *Micrococcus lysodeikticus* by treating the cells with 0.5 N sodium hydroxide in an atmosphere of nitrogen. The mucopolysaccharide contained 5.5–6.5% N and 23–30% hexosamine. They confirmed the liberation of acetylhexosamine and demonstrated a reduction in viscosity accompanying the action of lysozyme on the soluble substrate. The acetylhexosamine reaction accounted for about half the reducing value (10%) obtained after digestion.

Polysaccharide fractions were isolated by Webb (37) from *Staphylococcus citreus* by extraction with distilled water at 100 C for 5 hr and from *Clostridium welchii* by treatment with 0.05 N sodium hydroxide at 60 C for 18 hr. The purified fractions on incubation with lysozyme gave maximum reducing values of 1.42–1.75% for *S. citreus* and 1.16–2.17% for *C. welchii*. No other properties of these polysaccharides were given.

Susceptibility of Isolated Cell Walls to Lysozyme

One of the most sensitive methods for detecting the presence of the lysozyme substrate is the determination of changes in turbidity on incubation of isolated cell walls with the enzyme. As already pointed out, such a method avoids many of the complications encountered with intact bacteria (23). Cell walls may be isolated by mechanical disintegration of bacteria, and other structural elements such as flagella, cytoplasmic granules and soluble constituents may be removed by differential centrifugation and washing cell wall deposits in the centrifuge (72). An electron micrograph of cell walls of *Bacillus megaterium* prepared in this way is illustrated in Plate II, and the dissolution of the walls of this organism and those of *Bacillus subtilis* and *Bacillus cereus* by lysozyme is shown in figure 2.

Dissolution of isolated bacterial cell walls by lysozyme has now been established for a number of bacteria; some walls such as those of *Micrococcus lysodeikticus*, *Sarcina lutea* and *Bacillus megaterium* are completely digested by lysozyme (23). As shown in figure 2, only partial dissolution of the walls of some bacteria may occur and the residual turbidity appears to be caused by

INTRODUCTION

Lysozyme has long been used as a bacteriostatic or bactericidal agent, starting with the preliminary work of Fleming in 1922 (Phillips, 1966). More recently, the use of lysozyme as a preservative in foods was reviewed (Proctor and Cunningham, 1988). The Japanese have patented many applications of lysozyme for preserving foods. One patent was issued for the preservation of fresh vegetables, fish, meat, and fruit by coating the surface with lysozyme (Kanebo Ltd, 1973). Many of the patents and commercially prepared reports have found lysozyme to be a more effective preservative in combination with other substances. Some substances that enhance lysozyme activity are phytic acid (chelating agent), butyl p-hydroxybenzoate (POBB) (Yajima et al., 1971; Yashima et al., 1972), p-hydroxy-benzoic esters (Eisai Company, 1972), β-glycopyranose aerodehydrogenase (Eisai, 1980), hydrogen peroxide, and organic acids such as ascorbic acid (Miller, 1969). The Japanese have combined lysozyme with the amino acid, glycine (Yashitake and Shinichiro, 1977) and reported that they worked synergistically against Bacillus subtilis, Escherichia coli S-8, and Staphylococcus aureus 209-P.

Lysozyme activity has been tested in the presence of salt. Chang and Carr (1971), using NaCl, potassium phosphate, and tris chloride, showed that activation at low salt concentration was most

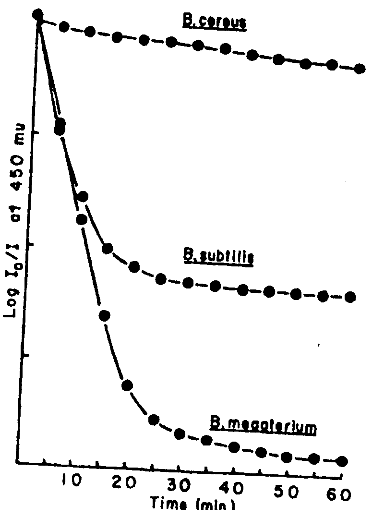

Figure 2. Dissolution of cell walls of *Bacillus cereus*, *Bacillus subtilis* and *Bacillus megaterium* by lysozyme (23). Incubation with 50 μg lysozyme/ml, in 0.1 M phosphate buffer, pH 6.0, at 37 C.

the thin, residual wall structures resisting the action of lysozyme (23). Recent investigations by Grula and Hartsell (80) have demonstrated the dissolution of the walls of *Neisseria flava* by lysozyme at pH 7 and only partial reduction in turbidity of walls from other species. Romano and Nickerson (81) were able to digest the isolated cell walls of *Streptomyces fradiae* with lysozyme. Only partial clearing of cell wall suspensions of *Staphylococcus aureus* occurred on incubation with lysozyme; this was contrasted with the complete digestion of the walls by the purified enzymes from actinomycetin (13). Lysozyme had no action on the cell walls of *Clostridium welchii*, *Streptococcus* spp., *Escherichia coli*, *Rhodospirillum rubrum*, or *Pseudomonas aeruginosa* when the sensitivity was determined turbidimetrically under conditions giving a rapid and complete lysis of the isolated walls of *M. lysodeikticus* (82).

Cell Wall Composition and Sensitivity to Lysozyme

The qualitative reactions of several fractions isolated from *M. lysodeikticus* led Hallauer (6) to the conclusion that the substrate attacked by lysozyme was of mucoid nature (a "glykoproteid"), and subsequent studies with both soluble substrate and the cell walls have been in complete agreement (4, 7, 10, 83). The composition of the cell walls of several organisms attacked by lysozyme is given in table 2. The walls of *M. lysodeikticus*, *Sarcina lutea* and *Bacillus megaterium* are composed of about 50% polysaccharide as estimated by reducing values after acid hydrolysis, and approximately 50% peptide material (82, 83). The nitrogen contents of walls and soluble substrates are very similar and in both instances exceed the values expected on the basis of hexosamine being the only nitrogenous constituent. The presence of significant amounts of amino acids in the lysozyme substrates was established during the investigations of cell wall composition (83). Both amino acids and hexosamine are major constituents of the lysozyme sensitive wall of *Streptomyces fradiae* (81).

The cell walls of both lysozyme sensitive and resistant bacteria belonging to the gram positive group have been characterized as muco complexes; they contain both peptide and polysaccharide components (82, 83). One of the most striking features of the chemical composition of the cell walls of gram positive bacteria was the relative simplicity of amino acid constitution (82, 83). The restricted amino acid composition of the walls of a large number of gram positive bacteria including both lysozyme sensitive and resistant organisms has been confirmed in the extensive survey by Cummins and Harris (84). The hexosamine contents of the cell walls of gram positive bacteria appear to be generally higher than those of gram negative organisms

TABLE 2

The composition of cell walls of several lysozyme sensitive bacteria (23, 82, 83)

Bacteria	Percentage Dry Weight of Cell Walls			
	N	P	Reducing substances*	Amino sugar*
Micrococcus lysodeikticus	8.7	0.09	45	16
Sarcina lutea	7.6	0.22	46	16
Bacillus megaterium	5.3	0.42	48	18

* Determined after hydrolysis with 2 N HCl, 2 hr at 100 C.

TABLE 3

Principal amino acid and amino sugar constituents of bacterial cell walls (13, 82, 83)

Bacteria	Alanine	Glutamic Acid	Glycine	Lysine	Diamino-pimelic Acid	Glucos-amine	Unknown Amino Sugar*
Lysozyme sensitive							
Micrococcus lysodeikticus	+++†	++	++	++	−‡	++	+
Sarcina lutea	+++	++	++	++	−	++	+
Sporosarcina ureae	+++	+++	++	++	−	++	+
Bacillus megaterium	+++	+++	−	−	+++	++	+
Bacillus subtilis	+++	+++	−	−	+++	++	+
Lysozyme resistant							
Bacillus cereus§	+++	+++	−	−	+++	++	+
Staphylococcus aureus§	+++	+++	+++	++	−	+	+
Streptococcus faecalis	+++	+++	+	++	−	++	+¶
Clostridium welchii	+++	+++	++	−	+++	++	+

* Probable structure suggested by Strange (91) is 3-o-α-carboxethylhexosamine.
† "+" indicates the relative size of the ninhydrin positive spots corresponding to the various amino acids and amino sugars on paper chromatograms of cell wall hydrolysates.
‡ "−" means no detectable amino acid.
§ Slow and incomplete digestion of walls by lysozyme.
¶ Unknown amino sugar and galactosamine reported by Cummins and Harris (84).

(82, 83). Following the discovery of an unknown amino sugar in spore peptides (85), this amino sugar was found to be a characteristic component of the walls of all the gram positive bacteria so far investigated (84, 86). The major amino acid, amino sugar and sugar constituents identified in the walls of a number of lysozyme sensitive bacteria are compared with those of cell walls of several lysozyme resistant organisms in table 3.

A consideration of the results presented in table 3 and those of Cummins and Harris (84) clearly shows that lysozyme sensitivity cannot be 'diagnosed' by qualitative inspection of cell wall composition; there is no single amino acid, amino sugar or indeed sugar component that appears to confer lysozyme sensitivity on a particular organism. There have been some superficial similarities suggesting that lysozyme sensitivity was greater in those walls possessing amino sugars and glucose as the only reducing substances (23), but the absence of glucose in the walls of *Sporosarcina ureae* questions the validity of such a correlation. Nor is the susceptibility governed simply by the total amino sugar content; the cell walls of *Streptococcus faecalis* have a high amino sugar content but they share lysozyme resistance with the walls of gram negative bacteria containing only small amounts of hexosamine (82).

The discovery that the principal low mol wt fragment liberated from cell walls by lysozyme, is an acetyl-amino sugar complex of glucosamine and the unidentified amino sugar (87) provides a clue as to the characteristics of cell wall composition that may govern the response to the action of lysozyme. Although the cell walls of the gram positive bacteria possess both glucosamine and the unknown amino sugar, it may well be that in the walls of some bacteria these amino sugars are more frequently linked together in the form of a disaccharide and it could be the number of such units in the wall that would govern the sensitivity to digestion with lysozyme.

In contrast to the gram positive bacteria, the walls of the gram negative organisms possess an amino acid composition that more closely resembles that of most proteins (82). Small amounts of 'hexosamine' are present in the walls of gram negative bacteria (82) and recent investigations have shown that both glucosamine and the unknown amino sugar may also be cell wall constituents of certain gram negative species (88). Thus some gram negative bacteria contain both amino sugars that occur in the form of an amino sugar complex in certain lysozyme sensitive, gram positive bacteria. Considering the degree of sensitivity of gram negative bacteria such as *Brucella abortus* (3, 7) and *Achromobacter fischeri* (50) and the presence of a considerable

ABSTRACT

The effect of various food and food ingredients on lysozyme activity was studied using a spectrophotometric and a lysoplate technique in which the lysis of lyophilized M. lysodeikticus cells is monitored. Basic amino acids, glucose, curing ingredients, and some spices (mustard, and pepper) were found to enhance lysozyme activity. The noncaloric sweetener, aspartame, and the gum, carrageenan, decreased lysozyme activity to 84.8% and 66.7% respectively. Most other ingredients tested decreased lysozyme activity only slightly.

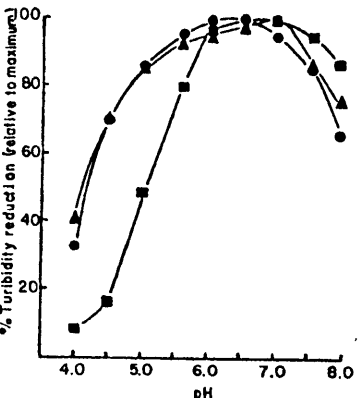

Figure 3. Influence of pH on lysozyme activity as measured by the turbidity reduction of cell-wall suspensions (87). ●——● *Micrococcus lysodeikticus;* ▲——▲ *Sarcina lutea;* and ■——■ *Bacillus megaterium* cell wall suspensions. *M. lysodeikticus* incubated with 1 μg lysozyme/ml, 60 min; *S. lutea* 2.5 μg lysozyme/ml, 90 min; *B. megaterium* 50 μg lysozyme/ml, 60 min. 0.1 M acetate buffer (pH 4–5.5), 0.1 M phosphate buffer (pH 5.5–8). Temperature of incubation, 37 C.

amount of substrate in cells of the former species (7), it would be surprising if lysozyme action on the substrates occurring in gram negative organisms is not accompanied by the liberation of a similar amino sugar complex.

THE MECHANISM OF LYSOZYME ACTION ON BACTERIAL CELL WALLS

Turbidity Reduction and Liberation of Reducing Substances

With the isolated cell walls as substrates for lysozyme, two manifestations of the enzyme's activity (dissolution of the walls and liberation of reducing groups) may be followed. The optimum pH for the dissolution of the walls was determined turbidimetrically, and figure 3 summarizes the results for *Micrococcus lysodeikticus*, *Sarcina lutea* and *Bacillus megaterium*. It is evident that lysozyme has a broad optimum between pH 6–7, and the optimum at pH 6.5 for *Micrococcus lysodeikticus* agrees well with that found for lysis of whole cells (58, 63). When lysozyme activity was followed by estimating the amounts of reducing substances liberated, the composition of the buffer was found to have a marked influence on the pH activity curves (87); discontinuities occurred on changing from acetate to phosphate buffer at pH 5.5. In an earlier study of the action of lysozyme on suspensions of *S. lutea*, Dickman and Proctor (89) demonstrated that buffer composition may strongly influence lysozyme activity. The discontinuities in the pH curves for the liberation of reducing substances by lysozyme from the isolated cell wall substrates could be eliminated by using buffer of constant ionic strength. Thus the most probable explanation of some of the discontinuous pH activity curves reported earlier (10) may be variations in the ionic strength of the buffers used to cover a wide range of pH. Lysozyme activity, at least with whole cells, is maximal at ionic strengths between 0.1–0.2 (58, 89) and the results with isolated walls of *M. lysodeikticus* indicate a plateau at $\mu = 0.1$ (13).

When the progress of the digestion of cell walls of *Micrococcus lysodeikticus*, *Sarcina lutea* and *Bacillus megaterium* by lysozyme was followed by simultaneous determination of turbidity reduction and liberation of reducing substances, the two reactions were closely parallel (87).

Physico-chemical Properties of Cell Wall Digests

Apart from the liberation of reducing substances, including N-acetylhexosamine, (7, 10) and the reduction in viscosity of the soluble substrate (10), the products of lysozyme action have received little attention.

Recent investigations have shown that incubation with lysozyme results in the complete dissolution of the walls of *M. lysodeikticus*, *Sarcina lutea* and *Bacillus megaterium*; the digests contain a complex mixture of fragments of different mol wt (82, 87). Ultracentrifugal analysis of lysozyme digested walls has shown that the major components are of the order of 10,000–20,000 mol wt. After dissolution of the walls by lysozyme some of the products are sufficiently small to pass through dialysis tubing, and it was of interest to determine the relative proportions of dialyzable and non-dialyzable components in the digested walls. About 50% of the original weight of cell wall was rendered dialyzable after complete digestion of *M. lysodeikticus* walls with lysozyme. Electrophoresis of the non-dialyzable fractions from *M. lysodeikticus*, *S. lutea* and *B. megaterium* cell wall digests showed the presence of a number of different electrophoretic components, some of which possess high negative mobilities (87). The results of an examination of the physico-chemical properties of digests of the walls of the three organisms used in this investigation (87) are summarized in table 4.

TABLE 4

Physico-chemical properties of lysozyme digested cell walls (87)

Cell Wall Digest	Ultracentrifugal Analysis S_{20} (Svedbergs)	Per Cent Non-dialyzable Material	Number of Electrophoretic Components*
Micrococcus lysodeikticus	1.15	50	3
Sarcina lutea	1.27	70	3
Bacillus megaterium	1.50	60	2

* Minimum number of components in the non-dialyzable fractions.

Richmond (90) has shown that a lytic enzyme produced by *Bacillus subtilis* has very similar properties to egg white lysozyme when tested on isolated cell wall substrates. The similarity to lysozyme was even more striking on examination of the physico-chemical properties of the walls of *Micrococcus lysodeikticus* digested by the bacterial enzyme; the dialysis characteristics and the electrophoretic patterns were very similar indeed to results obtained with walls digested by egg white lysozyme.

The Nature of Some of the Fragments Resulting from Lysozyme Action

The liberation of reducing groups and substances giving the reactions of N-acetylamino sugars has been confirmed with the isolated walls as substrates for lysozyme (87). Reducing values of 10–12% after lysozyme action on the cell walls of *Micrococcus lysodeikticus* (87) agree well with the amounts liberated from the soluble substrates of the same organism (7, 10).

Paper chromatography of lysozyme digested walls of *Micrococcus lysodeikticus*, *Sarcina lutea* and *Bacillus megaterium* revealed the presence of a component common to all three preparations and this substance was found to pass into the dialyzable fraction on dialysis. The component liberated by lysozyme behaved similarly to N-acetylglucosamine; it reacted with aniline phthalate and the N-acetylamino sugar spray reagents when separated on paper chromatograms. Unlike N-acetylglucosamine it behaved as an acidic substance on electrophoresis on paper. Hydrolysis of this "small fragment," followed by chromatographic examination on paper showed that it was composed of glucosamine and the unidentified amino sugar found in spore peptides and bacterial cell walls. Thus the principal small substance liberated by lysozyme is not free N-acetylamino sugar (7, 10) but an amino sugar complex of glucosamine and an amino sugar possessing a carboxyl group (87, 91); the evidence also suggests that both amino sugars are present as the acetyl compounds (87), so that the complex is probably in the form of a disaccharide. The "unidentified amino sugar" having the properties of an acidic hexosamine has been provisionally characterized by Strange (91) as 3-*o*-α-carboxyethyl hexosamine.

No cell wall amino acids are liberated as a result of dissolution of the walls with lysozyme (87). This has been further confirmed by studying the products of the reaction of untreated cell walls and digested walls with 1-fluoro 2,4 dinitrobenzene (FDNB) (92). In all three cell wall preparations (*Micrococcus lysodeikticus*, *Sarcina lutea* and *Bacillus megaterium*), the N-terminal amino acid has been identified as alanine. The detection and estimation of the mono-dinitrophenol (DNP) derivative of lysine has shown that a high proportion of the lysine residues in the walls of *M. lysodeikticus* have free amino groups. About one third of the diaminopimelic acid residues in *B. megaterium* walls have amino groups free. The formation of mono-DNP diaminopimelic acid established that only one of the two amino groups of this substance was available for reaction with FDNB. A small increase in the alanine residues with free amino groups occurs after digestion of *M. lysodeikticus* walls with lysozyme, and it is probable that this increase results from an unmasking and a more complete reaction with FDNB rather than an enzymic reaction liberating the amino groups (92). No other end group amino acids appear after digestion with lysozyme, and the increase in alanine is very small in comparison to the liberation of reducing groups (92). A small amount of a dinitrophenol derivative of what appears to be the unknown amino sugar was obtained from FDNB treated digests.

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Activity of Egg White Lysozyme in Various Food Systems.

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The interesting observation that cell walls with their free amino groups blocked by reaction with FDNB are still digested by lysozyme clearly shows that the state of the amino groups has little influence on the reaction of the enzyme with its substrate (92). This was perhaps not surprising, as the main action of lysozyme involves the splitting of amino sugar linkages, and it is probable that most of the amino sugar residues participating in the enzyme reaction are the acetylamino sugars.

A preliminary examination of the properties of the fast-moving electrophoretic component in the non-dialyzable fraction of *Micrococcus lysodeikticus* cell wall digests has shown that, qualitatively, it contains the same amino acids and amino sugar constituents that are present in the intact cell wall. Other fractions contain different proportions of both polysaccharide and peptide residues (87). The evidence so far available suggests that lysozyme does not split the cell wall into simple peptide and polysaccharide fragments, but rather into fragments containing different proportions of amino acids, amino sugars and in some instances sugars [in those walls containing glucose as the other reducing sugar (83)]. It is thus probable that the cell walls of these lysozyme sensitive bacteria have a highly branched structure in which peptides and peptide-amino sugar complexes may be glycosidically linked through terminal amino sugars to other amino sugar and sugar residues of polysaccharide components. There is now abundant evidence for the existence of hexosamine containing peptides of bacterial origin, as shown by the work of Strange and Powell (85); and these peptides are closely related to similar complexes of bacterial cell walls. It seems likely that the types of cell wall fragments that arise from digestion with lysozyme could do so by a splitting of an amino sugar complex from amino sugar-containing peptides and peptide-amino sugar-polysaccharide complexes.

PROTOPLAST FORMATION AND STRUCTURAL ANALYSIS OF BACTERIA WITH LYSOZYME

Because of their circumscribed sphere of action on particular substances, enzymes have been particularly useful in analysing cellular structures and the relationships between the various structures and the biochemical functions of the cell. Lysozyme has already given much information, and it is now being employed more extensively in the isolation and study of various morphological entities of the bacterial cell.

When *Bacillus megaterium* cells were suspended in a medium of suitable osmotic pressure and the dissolution of the cell walls by lysozyme was allowed to proceed, the structural integrity of the cell was retained in the form of a spherical bacterial protoplast (Weibull, 67). The complete digestion of the wall and exposure of an entirely different surface at the periphery of the protoplast has been demonstrated by a variety of methods including electron microscopy (67), immunological reactions (78), and reaction with a specific bacteriophage (67). This information, together with the knowledge that the isolated cell walls of this organism are completely digested by lysozyme into relatively low molecular weight compounds (23, 87), provides a sound structural basis for defining the bacterial protoplast as an osmotically stabilized cell from which the cell wall has been completely and selectively removed by some suitable method such as enzymic digestion. The osmotic stabilization refers more especially to a functional protoplast rather than a cytologically demonstrable protoplast that may have been "fixed" by appropriate techniques. In using lysozyme for the isolation of bacterial protoplasts, it seems important that the complete dissolution of the wall should be demonstrated. As pointed out by Epstein and Chain (7) and confirmed later (23), some bacterial cell walls may be composed of two structural entities. The use of the term protoplast seems unjustified if some residual cell wall structure remains after treatment with lysozyme, even if the transformation from a rod shaped cell to a spherical form has occurred. Protoplasts of very similar appearance in the electron microscope to those isolated from *B. megaterium* (67) have been obtained by lysozyme treatment of *Bacillus subtilis* by Wiame *et al.* (93). Mitchell and Moyle (68) have recently obtained stable protoplasts from two other organisms, *Micrococcus lysodeikticus* and *Sarcina lutea*. The walls of these two organisms are completely digested by lysozyme (23, 73).

Although osmotically sensitive "protoplasts" have been recently obtained from *Escherichia coli* by treatment with lysozyme, the "protoplasts" may still be infected with T_2 bacteriophage after the transformation from rods to spherical protoplasts has taken place (94). The work of Weidel *et al.* (95) has established the presence of T_2 phage receptor in isolated cell wall preparations of *E. coli*. By dissociation in 90% phenol a layer of the wall showing the typical antiviral specificity of the receptor for T_3 as well as T_4 and T_7 phages was uncovered. The localization of the T_2 phage receptor in the cell wall, together with the demonstration that "protoplasts" may be infected with T_2 bacteriophage, indicate that part of the wall of *E. coli* remains after formation of "protoplasts" by treatment with lysozyme.

CONCLUSIONS

The enzymic properties of lysozyme can be defined in terms of the following three experimental determinations: 1. turbidity reduction of isolated cell wall structures or lysis where the wall is *in situ* as with intact bacterial cells; 2. liberation of reducing groups; 3. liberation of an acetylamino sugar complex of glucosamine and the acidic hexosamine (91). It seems desirable that the term *lysozyme* should be reserved for those enzymes possessing all three properties; the demonstration that an enzyme displays all three reactions constitutes excellent evidence for regarding it as a lysozyme. If lysis or dissolution of isolated walls (reaction 1) were the sole property used in classifying an enzyme as a "lysozyme," enzymes such as those from actinomycetin (11, 12) would be wrongly placed in the lysozyme group. However, a clear indication of the group to which an enzyme belongs may be gained by determining whether reducing groups are liberated (as by lysozyme) or whether amino acids [the main products of digestion of walls by purified enzymes from actinomycetin (13)] are split off. Actinomycetes have been regarded as producing "lysozyme" (96), and it will be of considerable interest to find out whether they produce enzymes liberating reducing groups and an amino sugar complex of the type released by lysozyme as well as the peptidase type. The enzyme purified by McCarty (97) and shown to act on isolated walls of hemolytic streptococci does so with a liberation of substances giving the reactions of acetylamino sugars (98); this enzyme did not lyse *Micrococcus lysodeikticus* and may represent an enzyme differing from the above types (lysozyme and peptidase from *Streptomyces* sp.).

Enzymes possessing the three properties listed in the preceding paragraph have been isolated in a crystalline form from hen egg white, rabbit spleen and papaya latex. Of these only egg white lysozyme has been fully characterized in terms of the three reactions. In addition to carrying out certain enzymic reactions in common, these enzymes also show a number of striking similarities in protein structure. All three are basic proteins of relatively low mol wt, and their amino acid constitution is characterized by a high content of arginine and an apparent absence of sulfhydryl groups. The presence of a common peptide with an N-terminal lysine and C-terminal leucine has been established for the lysozyme from egg white and the lysozyme from rabbit spleen. The lysozyme of plant origin, on the other hand, had a N-terminal glycine. Thus in addition to the enzymic properties, the basic nature of the protein and type of N-terminal amino acid may provide further evidence of similarity to other lysozymes. Indeed, Smith *et al.* (34) did not suspect that they had isolated a lysozyme until they discovered the remarkable similarity in amino acid composition and other chemical and physical properties between their protein and lysozymes of egg white and spleen!

The presence in many bacterial cell walls of the two amino sugars liberated by lysozyme from certain walls in the form of a complex suggests that lysozyme sensitivity should be more widespread than that observed. However, the mode of linkage of the two amino sugars in the bacterial cell wall muco complexes is unknown and the type of linkages broken by lysozyme have not yet been established [Meyer *et al.* (4) believed they were definitely not α-glycosidic bonds].

Because of their basic properties, lysozymes can form complexes with negatively charged substances. For this and a number of other reasons, a negative test for lytic action on an intact bacterial cell is insufficient reason for concluding that lysozyme substrate is absent.

The action on isolated walls provides a more sensitive method, but again a negative test under limited experimental conditions would not provide proof of the absence of substrate. The important effects of ionic strength on the enzyme reaction and the possibility of the presence of

hyaluronic acid and hyaluronidase was probably due to the short digestion period used.

Although chitinase caused a decrease in turbidity of the hyaluronic acid preparation and the development of a positive Elson-Morgan reaction, it did not change the viscosity of the solution. It seems probable that the positive Elson-Morgan reaction obtained with hyaluronic acid and chitinase resulted from chitinase action on an impurity in the substrate material.

Lysozyme did not produce any change in the solution of hyaluronic acid. However, both lysozyme and chitinase hydrolyzed an appreciable amount of chitin and BLS. It was not possible to make a quantitative comparison of the activities of the two enzymes on chitin since the chitinase used was not a purified preparation.

Since chitosan was not attacked by any of the enzymes, it seems probable that chitinase acts only on substrates in which the nitrogen is acetylated.

The ability of lysozyme to attack chitin indicates that this enzyme possesses β-glucosaminidase activity, since chitin is a straight-chain polymer of N-acetylglucosamine linked by β-glucosaminidic linkages. Accordingly, the capacity of lysozyme to hydrolyze the simple α and β linkages of synthetic phenyl-N-acetylglucosaminide was tested.

The action of lysozyme on α- and β-phenyl-N-acetylglucosaminide

Lysozyme was tested for its hydrolytic activity on the synthetic glycosides α- and β-phenyl-N-acetylglucosaminide. The enzyme–substrate mixtures were incubated at 37° for 24 and 48 hours. Control tests were made with hyaluronidase, emulsin, and chitinase. The results presented in Table I show that, whereas chitinase and emulsin hydrolyzed the synthetic β-glycoside, lysozyme and hyaluronidase did not. None of the enzymes hydrolyzed the α-glycoside.

TABLE I

THE CAPACITY OF LYSOZYME, EMULSIN, HYALURONIDASE, AND CHITINASE TO HYDROLYZE THE SYNTHETIC GLYCOSIDES α- AND β-PHENYL-N-ACETYLGLUCOSAMINIDE

Enzyme	Concentration of enzyme/ml	6 glycoside			7-glycoside (1.0 mg/ml)	
		μg/ml	pH	Phenol liberated μg/ml	pH	Phenol liberated μg/ml
L	10 μg	100	6.65	0		
L	500 μg	1000	6.5	0	6.5	0
E	10 μg	100	4.5	0		
E	20 μg	200	4.5	0		
E	500 μg				5.2	145
H	15 TRU	950	4.5	0		
H	10 TRU					
H	5 TRU				5.2	0
C					5.5	0
					7.0	240

Symbols: L = lysozyme, E = emulsin, H = hyaluronidase, C = chitinase, TRU = Turbidity reducing units of activity for hyaluronic acid. 0 = no phenol liberated.

DISCUSSION

The observation that crystalline egg-white lysozyme hydrolyzes chitin is evidence that it possesses β-glucosaminidase activity. Its lack of action on the simple synthetic

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β-phenyl-glycoside may be explained in two ways. The first, but least probable, explanation is that lysozyme cleaves only the end reducing acetylglucosamine groups of polysaccharide chains or their branches. If this were true it would readily account for the capacity of lysozyme to split acetylglucosamine from chitin and BLS, but not form the synthetic β-phenyl-glycoside. However, the viscosimetric data obtained by MEYER AND HAHNEL[2] indicate that lysozyme cleaves its bacterial substrate at random within the molecule. Hence, the second and more probable explanation is that lysozyme is a polysaccharidase with a restricted capacity for attacking low molecular weight substrates or phenyl derivatives.

The possibility that the action of lysozyme on chitin could have been due to the contaminating β-glucosaminidase in the crystalline lysozyme preparation does not appear likely, since detectable activity of lysozyme on chitin was observed to occur even with concentrations of lysozyme as low as 10 ug/ml. The alternative possibility that the chitin preparation may have contained a contaminating substrate susceptible to attack by lysozyme is also improbable since it has been recently shown that over 50% of the chitin substrate can be digested with lysozyme[16].

The hydrolysis of a component in the hyaluronic acid preparation by chitinase was evidenced by a decrease in the turbidity of the solution and the development of a strong Elson-Morgan reaction. However, the substrate component concerned in these changes was probably not hyaluronic acid since there was no concurrent viscosity change.

SUMMARY

Substances of natural and synthetic origin were used to determine the chemical linkages attacked by crystalline egg-white lysozyme. Whereas, lysozyme was observed to possess β-glucosaminidase activity, as indicated by its capacity to hydrolyze chitin, a known β-glucosaminide bonded by 1,4 linkages, it failed to hydrolyze either the α or the β form of synthetic phenyl-N-acetylglucosaminide.

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other components blocking the interaction of substrate and enzyme may require further exploration in determining the presence or absence of the lysozyme substrate.

Lysozyme has become an extremely useful enzyme for the isolation of bacterial structures and intracellular components, and for the specific dissolution of the cell wall in the preparation of bacterial protoplasts. If the latter term is to carry valid structural implications, then the ability of lysozyme completely to dissolve the wall under the conditions used for protoplast formation should be established.

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Chitosan, a deacetylated, partially depolymerized product was prepared by heating chitin at 100° for 48 h in a solution containing 40% NaOH after the method of DARMON AND RUDALL[7]. The colloidal form of chitin used was prepared by the gradual addition of dilute ethanol to a solution of chitin in concentrated HCl.

The hyaluronic acid preparation (HA) was made from freshly-collected human umbilical cords by a procedure based on the method of SCHMIDTH AND FABER[8] and SEASTONE[9]. The cords were washed free of blood, passed through a meat grinder and homogenized with one volume of water in a Waring Blendor. The homogenized material was centrifuged, the supernatant fluid collected, and the homogenization of the sediments repeated in a similar manner several times. The proteins were denatured by repeated mixing with a chloroform pentanol mixture, the protein layer being discarded after centrifugation. The supernatant fluid was acidified with glacial acetic acid to pH 4.0 and poured into two volumes of chilled 95% ethanol. The crude fibrous precipitate was collected by centrifugation and dissolved in a solution of 5% sodium acetate. The remaining protein was removed by repeated treatment with a chloroform-pentanol mixture and adsorption on zinc hydroxide[2]. The deproteinized solution was acidified with acetic acid to pH 4.0 and the material precipitated with ethanol as above. The resulting pale gray precipitate was dissolved in water and reprecipitated with ethanol several times. The preparation was finally washed with ether, dried *in vacuo* and tested with hyaluronidase in the usual manner. When the material was incubated at pH 5.2 with 5 turbidity reducing units (TRU)/ml of hyaluronidase, using toluol or thiomersalate as preservative, a marked reduction in turbidity and viscosity occurred. The results of these tests are included in Figs. 1 and 2.

β-phenyl-N-acetylglucosaminide was synthesized by the method of ROSEMAN AND DORFMAN[10].

The preparations of crystalline egg-white lysozyme, α-phenyl-N-acetyl-glucosaminide, hyaluronidase and emulsin were obtained from various sources*,**,***,§. The chitinase was prepared from cultures of a *Streptomyces* sp. by the method of REYNOLDS[6].

With the exception of chitin, which was in the form of a suspension in Sorensen's $M/100$ phosphate buffer the other substrates and enzymes were dissolved separately in $M/100$ phosphate buffer. The various digestion mixtures were prepared in duplicate in screw-capped tubes when small volumes were used, and in stoppered 50 ml Erlenmeyer flasks when larger volumes were used. The digestion mixtures were composed of one volume of enzyme solution and one volume of substrate solution. Control samples containing buffer and each reactant solution were included.

Two drops of toluol or 0.01% thiomersalate were added to each vessel as preservative and the various samples of control and digestion mixtures were agitated in a water bath at 37°. The extent of substrate digestion was determined at intervals using various methods. In certain instances the protein was removed from aliquots of the samples with phosphotungstic acid and the extent of digestion determined by the method of ELSON AND MORGAN[11].

Although there is uncertainty about what was being measured in the present work by the Elson-Morgan reaction it was presumably N-acetylhexosamine end groups rather than hexosamine or N-acetylhexosamine. It is probable that not all of the amino-sugar liberated from bacterial substrate by lysozyme is N-acetylglucosamine. It has been recently reported by STRANGE AND DARK[12] that a considerable amount of an unidentified amino sugar is present in the cell walls of bacteria, including *M. lysodeikticus*, which gives the Elson-Morgan reaction. Despite the uncertainty about what was being measured by the Elson-Morgan reaction, the values obtained with the various substrates in the present work were calculated as N-acetyl-glucosamine.

In those instances in which turbidity change was followed, the samples were placed directly in colorimeter tubes.

In the case of the synthetic glycosides, the phenol liberated was determined colorimetrically after deproteination by the method of FOLIN AND CIOCALTEU[13].

In those instances in which change in viscosity was followed the viscosimetric measurements were made using a vertically supported U-tube viscometer into which 6.0 ml of solution were pipetted. The U-tube was immersed in a water bath at 37°. Flow time was determined at known intervals and compared to that of pure water.

A Model 9 Coleman nephelometer was used for measurements of optical density and an Evelyn colorimeter for colorimetric measurements.

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* The crystalline egg-white lysozyme was generously supplied by Armour and Co., Chicago, Ill.

** The α-phenyl-N-acetylglucosaminide was generously supplied by Dr. S. ROSEMAN, University of Chicago, Chicago, Ill.

*** The hyaluronidase was generously supplied by the Schering Corp., Bloomfield, N.J.

§ The emulsin was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

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RESULTS

Enzyme-substrate cross-reactions

Since B.L.S.[1], HA[11], and chitin[15] are polysaccharides containing N-acetylglucosamine, it appeared possible that their respective "specific" enzymes, lysozyme, hyaluronidase, and chitinase might show cross reactivity for them. Accordingly, several experiments were performed to test this hypothesis. Although the concentrations of the enzymes and substrates were varied in different experiments, the pattern of results was similar. The data are presented in Figs. 1–4.

Hyaluronidase did not lead to a positive Elson-Morgan reaction with any of the substrates even though it readily depolymerized hyaluronic acid as indicated by a marked reduction in viscosity. Failure to obtain the Elson-Morgan reaction with

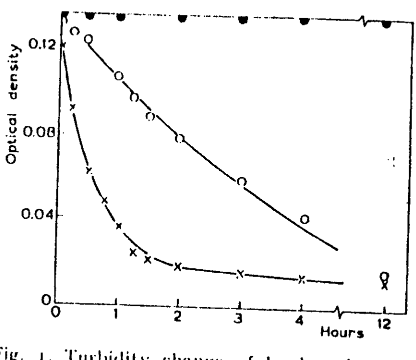

Fig. 1. Turbidity change of hyaluronic acid caused by various enzymes at 37°. The pH of the reaction mixtures was 5.5 for hyaluronidase and 7.0 for lysozyme and chitinase. Symbols: ● lysozyme; ○ = chitinase; × = hyaluronidase.

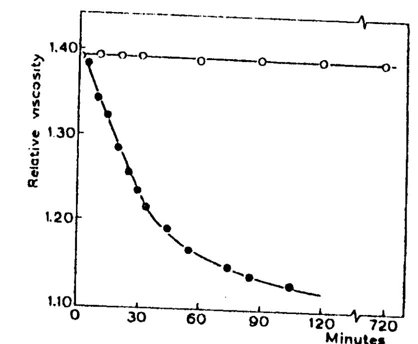

Fig. 2. Viscosity change of hyaluronic acid caused by various enzymes. Immersed U-tube viscometer immersed in water bath at 37°. The pH of reaction mixtures was 5.5 for hyaluronidase and 7.0 for lysozyme and chitinase. Symbols: ○ = lysozyme and chitinase; ● = hyaluronidase.

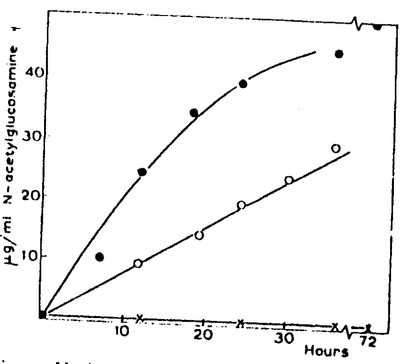

Fig. 3. Hydrolysis of various polysaccharides by lysozyme. Incubations were conducted at 37°, pH 7.0. The concentration of lysozyme was 100 μg/ml. The concentration of substrates was 0.5 mg/ml. Symbols: ● = Bacterial lysozyme substrate; ○ = chitin; × = chitosan and hyaluronic acid.

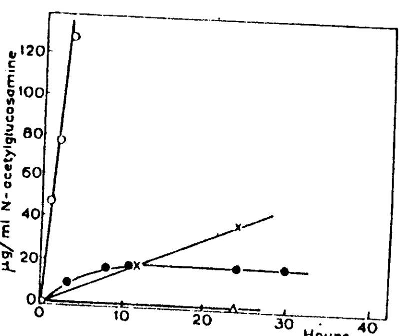

Fig. 4. Hydrolysis of various polysaccharides by chitinase. Incubations were conducted at 37°, pH 7.0. The concentration of substrates was 0.5 mg/ml. Symbols: ○ = chitin; × = hyaluronic acid; ● = bacterial lysozyme substrate; △ = chitosan.

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En conclusion, l'étude *in vitro* des cellule[s] la RNase peut pénétrer dans certaines cell[ules] métabolisme de l'ARN, ni celui des protéine[s] tumeur de Landschütz nous indique, d'autre part, qu'au cours de la première phase de son action, tout au moins, la RNase accélère l'incorporation des bases pyrimidiques dans l'ARN sans altérer le métabolisme des bases puriques ni celui des protéines.

RÉSUMÉ

La pénétration de la ribonucléase dans les cellules d'ascites de la tumeur de Landschütz traitées *in vitro* s'accompagne d'une élévation importante du métabolisme des bases pyrimidiques de l'ARN; le métabolisme des bases puriques de l'ARN et celui des protéines ne sont pas modifiés par l'enzyme.

Dans les cellules de la moelle osseuse, traitées *in vitro*, la ribonucléase ne modifie pas le métabolisme de l'ARN ni celui des protéines.

Les faits observés sont mis en relation d'une part avec la variation de la teneur en ribonucléase des deux types de cellules et d'autre part, avec les propriétés de leur métabolisme nucléinique.

SUMMARY

Penetration of ribonuclease into cells of Landschütz ascites tumour, treated *in vitro*, is accompanied by an important elevation in the metabolism of the pyrimidine bases of RNA; the metabolism of the purine bases of RNA, and that of proteins, is not modified by this enzyme.

Ribonuclease does not modify the metabolism of RNA or of proteins in the bone marrow cells, treated *in vitro*.

The observed facts are brought into relation with the variation in ribonuclease content of the two types of cells, and, on the other hand, with the properties of their nucleinic metabolism.

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THE β-GLUCOSAMINIDASE ACTIVITY OF EGG-WHITE LYSOZYME*

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It is generally accepted that the lysis of susceptible species of bacteria by lysozyme is secondary to the initial action of the enzyme on a mucopolysaccharide substrate(s) in the cell wall.

EPSTEIN AND CHAIN[1] showed that the action of lysozyme on certain susceptible species of bacteria results in rapid and complete lysis of living cells but not of cells killed by heat. Despite this lack of lysis of heat-killed cells, they were attacked by lysozyme and reducing sugars liberated. EPSTEIN AND CHAIN also reported that when lysozyme was allowed to act on mucopolysaccharide-containing extracts of susceptible bacteria, the reducing sugar, N-acetylglucosamine and an unidentified ketohexose were liberated. MEYER AND HAHNEL[2] presented evidence indicating that lysozyme contributes only to a step preparatory to the lysis of bacterial cells. SALTON[3] has shown that the action of lysozyme on the isolated cell walls of lysozyme-sensitive bacteria results in a complex mixture of fragments ranging in size from mol. wt. 20,000 to small fragments composed largely of an amino sugar complex presumed to be a disaccharide of glucosamine and an unidentified amino sugar.

Although the investigations of MEYER and co-workers[4] have shown that lysozyme does not exert α-glucosidase, protease, kinase, amylase, lipase, or phosphatase activity, the chemical linkage(s) which lysozyme is capable of cleaving have not been established.

The purpose of the present investigation was to determine the linkage(s) which may be attacked by lysozyme by the use of various natural and synthetic substrates.

MATERIALS AND METHODS

Unless otherwise specified, the following materials and methods were employed throughout the investigation.

The bacterial lysozyme substrate preparation (BLS) was made from a lysozyme-susceptible strain of *Sarcina lutea* by the method of MEYER AND HAHNEL[2] using wet-packed cells instead of acetone-dried organisms. The suitability of the BLS preparation was tested by reacting it with lysozyme under toluene at 37° for 4 to 8 days at pH 6.5–7.0[5]. The digestion of the substrate was rapid as indicated by the development of a strong Elson-Morgan reaction within the first hours of incubation. In one experiment, the amount of liberated material, calculated as N-acetylglucosamine, released following prolonged treatment was equal to 37% of the total weight of the substrate[5].

The chitin preparation was highly purified***. Its chemical analysis has been reported by REYNOLDS[6].

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