Enzymes
Handling/Processing

Identification of Petitioned Substance

This Technical Report addresses enzymes used in used in food processing (handling), which are traditionally derived from various biological sources that include microorganisms (i.e., fungi and bacteria), plants, and animals. Approximately 19 enzyme types are used in organic food processing, from at least 72 different sources (e.g., strains of bacteria) (ETA, 2004). In this Technical Report, information is provided about animal, microbial, and plant-derived enzymes generally, and more detailed information is presented for at least one model enzyme in each group.

Enzymes Derived from Animal Sources:
Commonly used animal-derived enzymes include animal lipase, bovine liver catalase, egg white lysozyme, pancreatin, pepsin, rennet, and trypsin. The model enzyme is rennet. Additional details are also provided for egg white lysozyme.

Chemical Name:
Rennet (animal-derived)

Other Names:
Bovine rennet
Rennin
Chymosin
Prorennin
Rennase

Trade Name:
Rennet

CAS Number:
9001-98-3

Other Codes:
Enzyme Commission number: 3.4.23.4

Chemical Name:
Peptidoglycan N-acetylmuramoylhydrolase

Other Name:
Muramidase

Trade Name:
Egg white lysozyme

CAS Number:
9001-63-2

Other Codes:
Enzyme Commission number: 3.2.1.17

Enzymes Derived from Plant Sources:
Commonly used plant-derived enzymes include bromelain, papain, chinitase, plant-derived phytases, and ficin. The model enzyme is bromelain.

Chemical Name:
Bromelain

Other Names:
Ananus comosus (pineapple)
Ananus bracteatus
Bromelin
Pineapple enzyme

Trade Name:
Bromelain

CAS Numbers:
9001-00-7 (fruit bromelain); 37189-34-7 (stem bromelain):

Other Codes:
Enzyme Commission number: 3.4.22.32 (stem bromelain); 3.4.22.33 (fruit bromelain)

Enzymes Derived from Microbial Sources:
The model enzyme is acidic pectinase from the fungus *Aspergillus niger.*

Chemical Names of components:
poly(1,4-α-D-galacturonide)glycanohydrolase; pectin pectylhydrolase; poly(1,4-α-D-galacturonide)lyase; pectin lyase; L-Arabinofuranoside arabinofuranohydrolase; 1,5-L-Arabinan arabinofuranohydrolase; Exo-polygalacturonase; Endo-1,4-*β*-galactanase; Pectin acetylesterase; Exopolygalacturonase lyase

Other Names:
Pectase
Polygalacturonase

Trade Names:
Pectinex 3XL(R)
Ultrazym

CAS Number:
9032-75-1

Other Codes:
Enzyme Commission numbers: 3.2.1.15; 3.1.1.11; 4.2.2.2; 4.2.2.10; 3.2.1.55; 3.2.1.99; 3.2.1.67; 3.2.1.89; 3.1.1.6; 4.2.2.9

Characterization of Petitioned Substance

Composition of the Substance:

Enzymes are a specific type of proteins that catalyze chemical reactions (Kirk and Othmer, 1947). Biologically-active proteins are considered the active components of enzymes. Proteins have highly complex structures and may be conjugated with metals (e.g. iron, manganese, cobalt, etc.), carbohydrates, or lipids. The naming convention for enzymes includes a description of the substance's function and has the word ending in '-ase.'

Animal-derived rennet is a commercial extract containing the active enzyme rennin, also known as chymosin. The product, generally referred to as 'rennet,' is the aqueous extract prepared from dried, cleaned, frozen, or salted fourth stomachs of calves, goat kids, or lambs. The structure of rennin consists of a single polypeptide with an internal disulfide bridge (The Food Chemicals Codex, 1996; USDA, 2000).

Egg white lysozyme is an antimicrobial protein (i.e., a protein with the ability to inhibit or kill microorganisms) comprised of 129 amino acid residues. Although lysozyme can be found in many organisms (including plant tissues), it is found in large quantities in chicken egg white (FDA, 2000).

Bromelain refers to a group of sulfhydryl-containing, proteolytic enzymes extracted from pineapple. The final product, extracted from the stem, core, juice, or peel of the pineapple, is processed into a yellow to grey powder (Thomas Research Inc., 1998).

The enzyme pectinase refers to a composition of multiple enzymes with the principal enzymes being pectin methylesterase, pectin lyase, and polygalacturonase. Pectinase is produced by the controlled fermentation of nonpathogenic and nontoxicogenic strains of bacteria and fungi that is then isolated from the growth medium (FAO, 2000). The information presented in this Technical Report focuses on an acidic pectinase isolated from *Aspergillus niger* and will, for simplicity, be referred to as 'pectinase' in this report.

Properties of the Substance:

Enzyme preparations may consist of parts of cells, whole cells, or cell-free extracts from a particular source. Enzymes may be in liquid, semi-liquid, or dry form. Enzymes are generally considered to be soluble in water and insoluble in alcohol, ether, and chloroform. Liquid enzyme preparations are typically in aqueous solution and have similar physical/chemical properties as water. In general, the boiling point of these materials is slightly above 100°C (212°F). Liquid enzyme preparations usually range in color from tan to dark brown. Dry enzyme preparations are typically fine powders and are tan or off-white in color (The Food Chemicals Codex, 1996).

Animal-derived rennet products are clear amber to dark brown liquid preparations or white to tan powders (Scholar Chemistry, 2009; USDA, 2000). Rennin is the milk-coagulating enzyme of the mucosa of the fourth 'true' stomach (abomasum) of young calves (Frankhauser, 2009). Egg white lysozyme is generally distributed as an odorless, white powder with a sweet taste. Egg white lysozyme readily dissolves in water, but does not dissolve well in organic solvents (Kewpie Corporation, 2010).

Bromelain is a water soluble, light grey to yellow amorphous powder with a molecular weight of 33 kDa. Bromelain remains active as an enzyme between a pH of 4.5 and 9.8. The effective temperature range for bromelain is 40–65ºC (Gautam et al., 2010; FAO, undated).

Pectinase is readily soluble in water and practically insoluble in ethanol and ether. The substance is generally distributed as off-white to tan amorphous powders or tan to dark brown liquids. The unformulated product is usually diluted and standardized with food-grade glycerol (also called glycerin), water, and potassium chloride to obtain commercial products (FAO, 2000).

Specific Uses of the Substance:

Enzymes are natural protein molecules that act as highly efficient catalysts in biochemical reactions. They are used to carry out naturally occurring biological processes that are useful in the processing of food products or ingredients. For example, enzymes are commonly used in the production of sweeteners, chocolate syrups, bakery products, alcoholic beverages, precooked cereals, infant foods, fish meal, cheese and dairy products, egg products, fruit juice, soft drinks, vegetable oil and puree, candy, spice and flavor extracts, and liquid coffee, and are also used for dough conditioning, chill proofing of beer, flavor development, and meat tenderizing. Enzymes can also be used to help reduce production costs, reduce the length of time required for aging foods such as cheese, clarify or stabilize food products, and control the content of alcohol and sugar in certain foods (Enzyme Technical Association, 2001).

Microbial enzymes used in food processing and are typically sold as enzyme preparations, which are mixtures with the desired enzyme activity that contain preservatives (such as boric acid and natamycin), stabilizers (such as salts and aminoacetic acid), and other metabolites of the production strain (Pariza and Johnson, 2001).

Rennet is a fluid that contains the protease, rennin, which occurs in the gastric juices of human infants and is also contained in the stomach of calves and other ruminants (FDA, 2006; Mosby's Medical Dictionary, 2009). Rennet hydrolyzes polypeptides (e.g., casein) yielding peptides of lower molecular weight. When added to milk, it cleaves a single bond in k-casein leaving an insoluble fraction para-k-casein. This process causes milk to curdle and clot, making animal-derived rennet useful in cheese production (FAO, 1992).

Egg white lysozyme controls the proliferation of bacteria during fermentation or food processing and has been shown to possess antimicrobial properties especially in relation to *Clostridium tyrobutyricum* (Kewpie Corporation, 2010; FDA, 2000). Therefore, it is used to improve the shelf life of chilled foods and confectionary products and has been used to preserve fresh fruits and vegetables, tofu bean curd, seafood, meats and sausages, potato salad, cooked burdock with soy sauce, and varieties of semi-hard cheeses such as Edam, Gouda, and some Italian cheeses (Cunningham et al., 1991). Egg white lysozyme is also incorporated into casings for frankfurters and in cooked meat and poultry products that are sold as ready-to-eat (FDA, 2000). Unlike other model enzymes, egg white lysozyme does not exhibit lytic activity against the lactic acid bacteria that are critical for cheese fermentation.

Plant enzymes are commonly used in food processing and pharmaceutical and healthcare products. Bromelain has primarily been used in meat tenderizing products (Haslaniza et al., 2010); it is the main ingredient in powdered meat tenderizers sold at grocery stores. Bromelain is also added to baked goods to degrade gluten, making dough easier to process. Protein supplements are often produced using bromelain because it can hydrolyze the protein in soybeans to create soluble protein that is more easily absorbed by the intestine (Guangxi, 2011; Marinova et al., 2008). This enzyme is also added to improve the taste and quality of goods such as crackers and bread; and it is used to clarify apple juice, produce soft sweets, clot milk for cheese production, and flavor food (Guangxi, 2011).

Pectinase is used in the manufacture of fruit juice and wine, primarily to reduce viscosity, improve filtration and clarity of products, and prevent particle sedimentation and pectin gel formation (FAO, 2000). Specifically, pectinase is used to de-polymerize and esterify plant pectins in fruits such as apples, lemons, cranberries, oranges, cherries, grapes, and tomatoes. It is also added to sparkling clear juices in order to increase the juice yield during pressing of the fruit and straining of the juice and to remove suspended matter to create sparkling clear juices that are free of haze. The application of pectinase also enables the entire fruit to be liquefied, which improves saccharification and thus sweetness, reduces waste and energy use per unit of juice produced, and improves aroma and color (Kashyap et al., 2001).

The juices produced with the addition of pectinase include:

- Sparkling clear juices (apple, pear, grape); and
- Juices with clouds (citrus, prune, tomato, nectars).

Acidic pectic enzymes used in the fruit juice and wine making industries often come from fungal sources, especially *Aspergillus niger* (Kashyap et al., 2001). Pectinases derived from bacterial and fungal species are used in the textile industry to aid in the retting and degumming of fiber crops, including hemp, flax, jute, ramie, kenaff, and coir from coconut husks. Retting is a fermentation process in which certain bacteria and fungi (including *Aspergillus spp.*) decompose the pectin of the bark of a plant and release the fiber (Kashyap et al., 2001).

Other uses of pectinase include (Kashyap et al., 2001):

- Treatment of pectic wastewater;
- Oil extraction from rape seed, coconut germ, sunflower seed, kernel, olives, etc.;
- Paper making; and
- Coffee and tea fermentation.

Approved Legal Uses of the Substance:

The U.S. Department of Agriculture (USDA) permits the use of enzymes in organic food processing (handling) as specified in 7 CFR § 205.605:

> "The following nonagricultural substances may be used as ingredients in or on processed products labeled as 'organic' or 'made with organic (specified ingredients or food group(s))' only in accordance with any restrictions specified in this section.
>
> (a) Nonsynthetics allowed:
>
> Animal enzymes—(Rennet—animals derived; Catalase—bovine liver; Animal lipase; Pancreatin; Pepsin; and Trypsin).
>
> Egg white lysozyme (CAS # 9001–63–2).
>
> Enzymes—must be derived from edible, nontoxic plants, nonpathogenic fungi, or nonpathogenic bacteria."

Animal-derived rennet and bovine rennet are generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA) (21 CFR 184.1685). The Select Committee on GRAS Substances determined that, because rennin would be rapidly inactivated by digestion, the substance is unlikely to exert significant proteolytic activity on the mucosa of the alimentary tract. No adverse effects have been reported in infants fed milk coagulated with rennin preparations. Teratogenicity tests on rennet by the chick embryo method have yielded negative results. FDA concluded that no evidence in the available information on rennet demonstrates or suggests reasonable grounds to suspect that rennet is a hazard to the public when used at current levels and in the manner now practiced or at levels that might reasonably be expected in the future (FDA, 2006).

The requirements provided by the FDA for specific standardized food products containing animal-derived rennet are provided in 21 CFR 131 and 133. The requirements provide descriptions of each dairy product (e.g., sour cream contains no less than 14.4% milk fat an acidity of at least 0.5%), allowed optional ingredients (e.g., vitamins, salt, and rennet), and labeling requirements including the name(s) allowed on product labels. Products detailed in 21 CFR 131 and 133 that allow animal-derived rennet as an optional ingredient include sour cream; a number of soft, semi-soft, semi-hard, and hard cheeses; cottage and cream cheeses; and low fat cheeses made from skim milk.

Egg white lysozyme was included as part of the tentative final[1] rule (21 CFR 184) on direct food substances affirmed as GRAS in 1998. In 2000, a GRAS petition was submitted to FDA for egg white lysozyme. FDA follow up was identified; however, it is unknown if a conclusion was made on the GRAS status of egg white lysozyme (FDA, 2000).

Bromelain is GRAS when used with good manufacturing practice. It is also in compliance with the requirements of the Food Chemicals Codex, 3rd edition (FDA, 1995a).

Pectinase is an allowed food additive under the Food, Drug, and Cosmetic Act. Pectinase has been self-declared GRAS by the Enzyme Technical Association. Based on information available to FDA, the agency had no questions regarding the conclusion drawn by the Enzyme Technical Association that pectinase preparations from *Aspergillus niger* are GRAS under the intended conditions of use. FDA has not, however, made its own determination regarding the GRAS status of pectinase enzyme preparations and affirms that it is "the continuing responsibility of each manufacturer to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements" (FDA, 2002).

<u>Action of the Substance</u>:

Enzymes are natural protein molecules that act as highly efficient catalysts in biochemical reactions. A catalyst is a substance that accelerates or initiates a chemical reaction without itself being consumed in the process. Therefore, enzymes help a chemical reaction take place efficiently and quickly by increasing the reaction rate of a biochemical process (Enzyme Technical Association, 2001).

Rennet is a coagulant used to curdle milk to be made into cheese or sour cream. The milk-clotting effect of rennin, the active enzyme in rennet, is due to a specific and limited hydrolysis of the k-casein surrounding the protein micelles (an aggregate of surfactant molecules dispersed in a liquid colloid) in milk. As a result, the micelles lose their electrostatic charge and are able to aggregate with the help of calcium and phosphorus ions to form a network that traps the fat micelles. A gel structure or curd is formed (Kirk and Othmer, 1947; USDA, 2000).

Egg white lysozyme acts as an antimicrobial agent by inhibiting the growth of deleterious organisms, thus prolonging shelf life of a variety of food products. The antimicrobial properties of egg white lysozyme are associated with its ability to catalyze the hydrolysis (i.e., the splitting apart of molecules with water) of structural polysaccharide peptidoglycan molecules present in the cell walls of certain bacteria. The cell walls of many gram-positive bacteria (e.g., *Clostridium tyrobutyicum*, which is commonly found in cheese) contain polysaccharide peptidoglycan and are thus broken down by egg white lysozyme. However, egg white lysozyme does not affect the beneficial lactic acid bacteria used in cheese fermentation (FDA, 2000).

[1] A tentative final rule is a rule that has been proposed by FDA but that has not subsequently been finalized. Therefore, the rule surrounding the GRAS status for egg white lysozyme has not yet been finalized although it was affirmed as GRAS in the issuance of the tentative final rule.

The main component of bromelain is a sulfhydryl proteolytics fraction, which is responsible for breaking down large protein molecules. For example, bromelain tenderizes meat by breaking the cross-links (connections) between its muscle fibers. Bromelain is also used to break down the gluten in bread dough; it has been popular in this industry due to how quickly it works, even in less-than-ideal conditions (e.g., high or low temperatures) (Polaina and McCabe, 2007). Bromelain can also be used in milk clotting because it degrades the casein (protein) in milk, causing it to gel. Bromelain's enzymatic activity (how efficiently the enzyme works) is measured using several systems including rorer units (r.u.), gelatin dissolving units (g.d.u), or milk clotting units (m.c.u.) (Thomas Research Inc., 1998).

The primary two constituents in pectinase are pectin methylesterase and polygalacturonase. Pectin methylesterase demethylates pectin and polygalacturonase hydrolyzes the a-1,4-galacturonide bonds in pectin. Pectic enzymes are available both in liquid or solid forms and in various strengths as measured by level of enzyme activity. For pectin, this is measured by the ability of the enzyme to hydrolyze the glycosidic bond between the biopolymer pectin of repeating chains of the sugar galactose or galacturonic acid (USDA, 2003).

Combinations of the Substance:

Enzymes are often packaged with various carriers that do not have catalytic activity and they may or may not be synthetically derived (USDA, 2003). Synthetic preservatives are usually added during processing and may be added during the final preparation to prevent microbial growth, stabilize the preparation, and maintain the desired enzymatic activity (Pariza and Johnson, 2001). Enzyme preparations may also include antioxidants, carriers, stabilizers, humectants, and diluents and other food-grade substances consistent with current good manufacturing practice (USDA, 2000). A complete list of food additives is available at http://www.fda.gov/Food/FoodIngredientsPackaging/FoodAdditives/FoodAdditiveListings/ucm091048.htm**.** In addition, enzymes are often used in combination with other enzymes (USDA, 2003).

Substances used in commercial rennet preparations include salt (sodium chloride), propylene glycol, sodium benzoate, boric acid, and sodium propionate (USDA, 2000).

No specific combination products were identified for egg white lysozyme or bromelain. Lysozyme is directly added to foods as a hydrochloride salt (FDA, 2000).

Pectinase is often used with cellulases, hemicellulases, amylases, and proteases. Several of these additional classes of enzymes are also produced by *Aspergillas niger* (White and White, 1997). Specifically, for apple juice, which in its natural state is unfiltered and unclarified and contains a high percentage of pulp, a combination of pectinase and cellulases is added to increase overall juice yield. A combination of amylase and pectinase can be used to break down starches in fruit that cause haziness in the juice (Kashyap et al., 2001). Pectinase is usually diluted and standardized with food-grade glycerin, water, and potassium chloride to obtain commercial products (FAO, 2000).

Status

Historic Use:

Enzymes have been utilized in food production since ancient times. They are commonly used in the baking, dairy, and brewing industries (FDA, 1995b). Today, a large amount of commercially prepared foods contain at least one ingredient that has been made using enzymes. Over time, progress in the field of biotechnology has made it possible to isolate and characterize the specific enzymes responsible for the processes that produce a variety of foods. In early food production, the enzymes in yeasts and bacteria were used to make cheese, vinegar, wine, and beer. The use of enzymes helps improve quality in food processing by making the chemical reactions more predictable and controlled (Enzyme Technical Association, 2001).

Over time, specialized strains of enzymes have been developed to improve the flavor and quality of foods. Enzymes can also be used in food processing to help reduce production costs, reduce the length of time required for aging foods such as cheese, clarify or stabilize food products, and control the content of alcohol and sugar in certain foods (Enzyme Technical Association, 2001).

Rennet: Animal-derived rennet has been used in cheese making since ancient times for the coagulation of milk. This form of rennet is used in traditional cheesemaking operations and is made from a collection of enzymes from the fourth stomach of ruminant animals (kid, calf, or lamb) (Frankhauser, 2009).

Many enzymes are now considered to be genetically modified and are used in food production because they are less expensive to produce. First introduced in 1990, fermentation produced chymosin rennet (FPC) is a widely used substitute for animal-derived rennet. FPC is produced by taking the rennin-producing gene out of the animal cell's DNA and inserting this gene into the DNA of a yeast, mold, or bacteria host. Following insertion of the gene, the production of chymosin enzyme is initiated within the host and chymosin is cultivated and fermented. According to the culture companies, FPC is used in the production of nearly 70 percent of cheese in North America (Madison Market, 2011; Yacoubou, 2008). Genetically modified rennet (fermentation produced chymosin) is prohibited for use in organic agriculture under 7 CFR 205.105(e) and 7 CFR 205.301(f).

Microbial rennet refers to a coagulating agent produced by a specific type of fungus, yeast, or mold that is grown and fermented in a laboratory. This form of rennet is generally considered to be 'vegetarian-friendly' because the enzyme produced is not derived from an animal. Although microbial rennet is generally favored by vegetarians, commercial cheesemakers agree that products made with this type of rennet typically result in a flavor of bitterness, which is especially noted when cheese is aged. True microbial rennet is cheaper than animal-derived rennet but not as inexpensive as fermentation produced chymosin (FPC) and has become difficult to find because its use has been replaced by FPC rennet (Madison Market, 2011; Yacoubou, 2008).

True vegetable rennet (versus the term 'vegetarian rennet' which is used interchangeably with microbial rennet) comes from plants which produce enzymes that have coagulating properties. Examples include nettles, cardoon thistle, or fig tree bark. Some disadvantages to using vegetable rennet are that they often produce an undesirable and bitter effect on cheese flavor and are a little more unpredictable when used in some cheese (Fletcher, 2011).

Egg white lysozyme: Lysozyme was first discovered in 1922 after being identified as an antibacterial enzyme present in the nasal mucus membranes of humans. Subsequently, in addition to human lysozyme, several classes of lysozymes have been identified in nature such as type C (chicken; domestic laying hens), type G (goose), type V (bacteriophages), and type H (plants). Because lysozyme is abundant in the albumen of domestic hen eggs, egg albumen has been the traditional source of lysozyme for use in foods, and egg white is considered a GRAS substance. Lysozyme is directly added to foods as a hydrochloride salt to specifically inactivate or inhibit spoilage and growth of pathogenic bacteria (FDA, 2000). Egg white lysozyme does not exhibit lytic activity (or causing cell destruction) against the beneficial lactic acid bacteria used in cheese fermentation (FDA, 2000).

Bromelain: Bromelain was first isolated from pineapple in the late 1800s (Gautam et al., 2010). It is unclear when the enzyme was first used in the food processing industry.

Pectinase: Pectinases were some of the first enzymes to be used in homes, and commercial application was first observed in the 1930s for the preparation of fruit juices and wines. Acidic pectic enzymes used in fruit juice and wine making industries often come from fungal sources, specifically *Aspergillus niger*. The juices produced by these commercial industries include (Kashyap et al., 2001):

- Sparkling clear juices (apple, pear, and grape); and
- Juices with clouds (citrus, prune, tomato, nectars).

OFPA, USDA Final Rule:

The use of nonsynthetic animal-derived rennet and egg white lysozyme are permitted in organic food processing accordance with 7 CFR 205.605(a). These enzymes are permitted for use as an ingredient in or on processed products labeled as "organic" or "made with organic (specified ingredients or food group[s])" as specified in 7 CFR 205.605(a).

The use of enzymes that are derived from edible, nontoxic plants, nonpathogenic fungi, or nonpathogenic bacteria as ingredients in or on processed products labeled as "organic" or "made with organic (specified ingredients or food groups))" is permitted in accordance with 7 CFR 205.605(a). This includes pectinase preparations from *Aspergillus niger* and plant-derived bromelain and papain enzymes.

International:

The Canadian General Standards Board permits the use of egg white lysozyme and animal-derived rennet in organic food processing. Animal-derived rennet is described as a nonorganic ingredient that is not classified as a food additive. Animal-derived enzymes must be guaranteed free of specified risk materials including the brain, skull, trigeminal ganglia (nerves attached to the brain), tonsils, eyes, spinal cord, and dorsal root ganglia (nerves attached to the spinal cord) of ruminants aged 30 months or older and the distal ileum (portion of the small intestine) of ruminants of all ages. Animal-derived enzymes, including rennet, should be from an organic source unless no such source is commercially available (Canadian General Standards Board, 2009).

The Canadian General Standards Board also permits the use of any preparations of enzymes normally used in food processing derived from edible, nontoxic plants, nonpathogenic fungi, or nonpathogenic bacteria (Canadian General Standards Board, 2009). Therefore, the Canadian organic standards allow the use of pectinase and bromelain in organic food processing.

The European Economic Community (EEC) Council Regulation (EC) No 889/2008, Article 27, 1(b) indicates that the use of "enzymes normally used in food processing" is permitted in organic food processing practices (EC No 889/2008). This would include animal-derived rennet, egg white lysozyme, pectinase preparations from *Aspergillus niger*, and plant-based enzymes such as bromelain.

The Codex Alimentarius Commission organic food guidelines allow preparations of micro-organisms and enzymes, specifically, "any preparations of micro-organisms and enzymes normally used in food processing, with the exception of micro-organisms genetically engineered/modified or enzymes derived from genetic engineering" (Codex Alimentarius Commission, 1999; USDA, 2000).

The most recent edition of the International Federation of Organic Agriculture Movements (IFOAM) Basic Standards considers enzymes acceptable for use in organic food processing provided they are based on the established Procedure to Evaluate Additives and Processing Aids for Organic Food Products (IFOAM, 2005; USDA, 2003). These standards are generally parallel to the OFPA criteria.

Evaluation Questions for Substances to be used in Organic Handling

Evaluation Question #1: Describe the most prevalent processes used to manufacture or formulate the petitioned substance. Further, describe any chemical change that may occur during manufacture or formulation of the petitioned substance when this substance is extracted from naturally occurring plant, animal, or mineral sources (7 U.S.C. § 6502 (21)).

Rennet: Although animal-derived rennet has been used since ancient times for the production of cheese, there are now more modern methods for production.[2] Traditionally, the fourth stomach (i.e., the abomasum) of four-week-old goat kids or calves are dried, cleaned, and then sliced into pieces, called abomasums strips, before being stored in either whey or saltwater. Vinegar or wine can be added to the solution to lower the pH. This salt solution of rennet and abomasum strips is mixed well and allowed to settle for two to three days at room temperature. The mixture is then filtered through a coarse sieve and a fine mesh (muslin) cloth. Filtering through muslin cloth should be repeated a few times to obtain the desired clear filtrate. A small amount of boric acid (added to the filtrate) acts as a preservative. The filtered solution can then be used to coagulate milk. Approximately one gram of this solution can coagulate two to four liters of milk (O'Connor, 1993).

When manufactured for industrial use, the mucosa of the abomasum of young calves is minced and the pH is adjusted to between 2 and 3 by adding hydrochloric acid. The mixture is incubated at 42°C (110°F) to convert the zymogen ("enzyme maker") prorennin to rennin. The pH of the mixture is next adjusted to 5.5 with the addition of sodium phosphate. In the presence of phosphate, the mixture becomes fluid and is dried in a vacuum and powdered. The product contains some fat, which is removed from the dried powder by solvent extraction. The solvent is usually acetone or alcohol, residues of which are easily removed from the preparation (Kirk and Othmer, 1947; USDA, 2000).

In the United States, microbial rennet and fermentation-produced chymosin[3] (FPC) are now more widely used in cheese making than animal-derived rennet. Approximately 70 percent of all cheese is produced with FPC, while approximately 25 percent is made with microbial coagulants, and the remaining 5 percent is made from calf rennet (Yacoubou, 2008). Microbial rennet describes a coagulating agent produced by a specific type of mold, fungus, or yeast organism, grown and fermented in a lab. Some consider this vegetarian-friendly, as the enzyme produced is not derived from an animal. While cheaper than animal rennet, true microbial rennet is now hard to find because it has been replaced by FPC rennet (Fletcher, 2011).

FPC is made by taking the rennin-producing gene out of the animal cell's DNA and then inserting into the bacteria, yeast, or mold host cell's DNA. Once inserted, the newly placed gene initiates the production of the chymosin enzyme within the host, which is then cultivated and fermented and produces chymosin. Production of FPC is more economical and does not produce a flavor of bitterness when used in cheesemaking. FPC rennet is derived from a genetically modified organism (GMO). Generally, ingredient labels do not distinguish between this type of microbial rennet (FPC) and the original non-GMO type (true microbial rennet) (Yacoubou, 2008).

Therefore, most conventional (i.e. non-organic) cheese in North America is made from vegetarian-friendly but animal-origin, GMO-derived FPC rennet (Fletcher, 2011).

Egg white lysozyme: To manufacture egg white lysozyme, the lysozyme is extracted from fresh egg white by mixing in an inert polymer resin that binds to the lysozyme. The resin carrying the lysozyme is separated from the egg white. The lysozyme is then removed from the resin through the addition of salts.

[2] Rennet produced from genetically modified microorganisms is now readily available and is often used in industrial cheese making because it is less expensive than animal-derived rennet (O'Connor, 1993). The use of genetically-modified microbial rennet is not permitted by NOP for use in organic foodhandling/processing.

[3] Chymosin is considered an alternative name for 'rennet'

The lysozyme is then concentrated, purified, and dried. Although the resulting purified protein, on a dry, basis is almost 100 percent lysozyme, small amounts of other egg white proteins may be present (FDA, 2000).

Bromelain: Bromelain is extracted from the pineapple's fruit, stem, peel, or juice, although studies have suggested that stem bromelain has a higher enzymatic activity than other parts of the fruit (Guatam et al., 2010). Bromelain usually functions without the addition of activators like calcium chloride. The preliminary extraction involves crushing pineapples in roll presses and further crushing to extract stem and fruit juice. Bromelain is then further isolated, separated, and purified using chromatography, ultrafiltration, precipitation (with substances such as ethanol and ammonium sulfate), freeze-drying (lypophilization), and other procedures. For example, an extraction process may include breaking down the pineapple stem using a blender, filtering the pineapple product, precipitating the filtrate twice with acetone, then freeze-drying the product to obtain the crude bromelain powder (Ngampanya and Phongtongpasuk, 2006). Recent studies have also shown that a process in which liquid pineapple juice is mixed with a surfactant (i.e., detergent) can be used to extract and purify bromelain without negatively affecting the structure or solubility of the protein, a problem with some purification methods (Fileti et al., 2009).

Pectinase: Pectinase is produced by the controlled fermentation of nonpathogenic and nontoxicogenic strains of *Aspergillus niger* that are isolated from growth medium (FAO, 2000). Microbial strains that are known to produce pectinase are grown in culture medium. Pectinase preparations from *Aspergillus niger* are considered nonsynthetic as pectinase is an enzyme released from the natural fermentation process of certain bacterial and fungal species (FAO, 2000). Isolation of the enzymes from their intracellular sources generally begins with separation from the media, usually by physical means such as centrifuging and sorting by specific gravity. The cell walls of the organisms are then destroyed through a mechanical process of homogenization, similar to that used on milk. The substrate often contains various grains and synthetic nutrients (USDA, 2003). Pandey (2006) indicated that production of extracellular pectinase can be induced if the culture medium contains pectic material, such as citrus peel or beet pulp. Extracellular production, where the fermentation organism excretes the enzymes in a form that can be safely isolated, does not necessarily involve breaking the cell walls of an organism to recover the enzyme. However, techniques such as ion exchange may be used to remove impurities in extracellular production (USDA, 2003).

<u>Evaluation Question #2:</u> Is the substance synthetic? Discuss whether the petitioned substance is formulated or manufactured by a chemical process, or created by naturally occurring biological processes (7 U.S.C. § 6502 (21)).

All enzymes included on the National List have been previously classified as nonsynthetic under 7 CFR 205.605(a).

No synthetic versions of animal-derived enzymes were identified. Animal-derived enzymes are nonsynthetic materials that are often treated with solvents or stabilized with synthetic antioxidants or preservatives (USDA, 2000). Animal-derived rennet and egg white lysozyme are considered nonsynthetic on the National List. Synthetic additives, including those solvents discussed in Evaluation Question #1, may be used to produce enzyme products. However, the use of synthetic solvents in the extraction and preparation the enzymes does not necessarily render the enzymes synthetic. Enzymes remain classified as nonsynthetic if they do not undergo a chemical change during extraction and formulation.

Bromelain refers to a naturally occurring (nonsynthetic) group of enzymes found in the pineapple. Various chemical processes, such as precipitation with synthetic products such as ammonium sulfate, are often used to extract and purify the bromelain from the pineapple.

Pectinase preparations from *Aspergillus niger* are considered nonsynthetic as pectinase is an enzyme released from the natural fermentation process of certain bacterial and fungal species (FAO, 2000). Extraction of the enzymes from intracellular sources or by extracellular production is performed using physical and/or chemical processes as discussed in Evaluation Question #1. Because extraction is pH dependent, the pH may be adjusted through the use of various strong acids (e.g., sulfuric acid) or bases such as sodium hydroxide. Specific enzymes are then precipitated or absorbed by the use of a variety of chemical constituents and/or ion exchange columns. Purification and standardization may also be required and these processes generally involve the use of synthetic substances (USDA, 2003).

Evaluation Question #3: Provide a list of non-synthetic or natural source(s) of the petitioned substance (7 CFR § 205.600 (b) (1)).

Rennet is commercially available as a nonsynthetic animal-derived enzyme. Animal-derived enzymes are naturally occurring proteins that are ubiquitous in living organisms. They are derived from animals that have been used as sources of food and have been safely consumed as part of the human diet throughout history. Specifically, animal-derived rennet is obtained from the fourth stomach of young goats and calves (FDA, 1995b; USDA, 2000).

Genetically-modified versions of rennet (e. g. fermentation produced chymosin) are available for commercial use and are generally considered to be nonsynthetic. True vegetable rennet and microbial rennet are also considered to be nonsynthetic. Fermentation produced chymosin, microbial rennet, and vegetable rennet are produced by basic biological processes including fermentation and are not considered to be formulated products (Madison Market, 2011; Yacoubou, 2008). Genetically modified rennet (fermentation produced chymosin) is prohibited for use in organic agriculture under 7 CFR 205.105(e) and 7 CFR 205.301(f), which prohibit the use of methods involving recombinant DNA technology as well as the labeling of foods as organic that are produced using recombinant DNA technology (an excluded method).

Lysozymes are present in bacteria, fungi, plants, and animal tissues; high concentrations are found in milk, saliva, mucus, and tears. Egg white lysozyme is found specifically in the egg whites of domestic laying hens (FDA, 2000).

Although bromelain can only be obtained from pineapple, other plant-derived enzymes with similar activities, such as papain, are available for commercial use.

A commonly utilized natural source of pectinase is the mold *Aspergillus niger*. Pectinase can also be derived from *Rhizopus oryzae* and *Aspergillus aculeatus* (Pariza and Johnson, 2001).

Evaluation Question #4: Specify whether the petitioned substance is categorized as generally recognized as safe (GRAS) when used according to FDA's good manufacturing practices (7 CFR § 205.600 (b)(5)). If not categorized as GRAS, describe the regulatory status. What is the technical function of the substance?

Table 1 provides a partial list of key enzyme preparations that have been affirmed as GRAS by FDA for specified or unspecified food uses (21 CFR Part 184). Conditions for their use are prescribed in the referent regulations and are predicated on the use of nontoxicogenic strains of the respective organisms and on the use of current good manufacturing practice (21 CFR 184.1(b)) (FDA, 2009).

Animal-derived rennet is considered GRAS by FDA according to 21 CFR 184.1685 (FDA, 2006). Animal-derived rennet acts as a coagulating agent used to curdle milk for making cheese or sour cream (FDA, 2006).

Egg white lysozyme was included as part of the tentative final rule (21 CFR 184) on direct food substances affirmed as GRAS in 1998. In 2000, a GRAS petition was submitted to FDA for egg white lysozyme. FDA follow up was identified; however, it is unknown if a conclusion was made on the GRAS status of egg white lysozyme (FDA, 2000).

Table 1. Partial List of Enzymes Preparations Affirmed as GRAS in 21 CFR 184

Section Number	Description of Enzyme Preparation
184.1012	Alpha-amylase enzyme preparation from *Bacillus stearothermophilus;* used to hydrolyze edible starch to produce maltodextrin and nutritive carbohydrate sweeteners
184.1024	Bromelain derived from pineapples, *Ananas comosus* and *Ananas bracteatus;* used to hydrolyze proteins and polypeptides
184.1027	Mixed carbohydrase and protease enzyme product derived from *Bacillus licheniformis* for use in hydrolyzing proteins and carbohydrates in the preparation of alcoholic beverages, candy, nutritive sweeteners, and protein hydrolysates
184.1034	Catalase from bovine liver used to decompose hydrogen peroxide
184.1316	Ficin (peptide hydrolase) from the genus *Ficus* to hydrolyze proteins and polypeptides
184.1372	Insoluble glucose isomerase enzyme preparations derived from recognized species of precisely classified, nonpathogenic, and nontoxicogenic microorganisms, including *Streptomyces rubiginosus*, *Actinoplane missouriensis*, *Streptomyces olivaceus*, *Streptomyces olivochromogenes*, and *Bacillus coagulans* grown in a pure culture fermentation that produces no antibiotic
184.1387	Lactase enzyme preparation from *Candida pseudotropicalis* for use in hydrolyzing lactose to glucose and galactose
184.1388	Lactase enzyme preparation from *Kluyveromyces lactis* (previously called *Saccharomyces lactis*) for use in hydrolyzing lactose in milk
184.1415	Animal lipase (triacylglycerol hydrolase) derived from the edible forestomach of calves, goat kids, or lambs; used to hydrolyze fatty acid glycerides
184.1420	Lipase enzyme preparation from *Rhizopus niveus;* used in the interesterification of fats and oils
184.1443	Malt (α-amylase and β-amylase) from barley to hydrolyze starch
184.1583	Pancreatin (peptide hydrolase) from porcine or bovine pancreatic tissue; used to hydrolyze proteins or polypeptides
184.1585	Papain derived from papaya, *Carica papaya L.*
184.1595	Pepsin (peptide hydrolase) from hog stomach; used to hydrolyze proteins
184.1685	Rennet (animal derived) and chymosin preparation from *Escherichia coli* K-12, *Kluyveromyces marxianus* var. *lactis* or *Aspergillus niger* var. *awamori* to coagulate milk in cheeses and other dairy products
184.1914	Trypsin (peptide hydrolase) from porcine or bovine pancreas; used to hydrolyze proteins
184.1924	Urease enzyme preparation from *Lactobacillus fermentum* for use in the production of wine
184.1985	Aminopeptidase enzyme preparation from *Lactococcus lactis*used as an optional ingredient for flavor development in the manufacture of cheddar cheese

As mentioned previously and as shown in Table 1, bromelain has been affirmed GRAS by FDA (FDA, 1995a).

Pectinase is considered a food additive under the Food, Drug, and Cosmetic Act. Pectinase has been self-declared GRAS by the Enzyme Technical Association. Based on information available to FDA, the agency had no questions regarding the conclusion drawn by the Enzyme Technical Association that pectinase preparations from *Aspergillus niger* are GRAS under the intended conditions of use. FDA has not, however, made its own determination regarding the GRAS status of pectinase enzyme preparations and states that it is "the continuing responsibility of each manufacturer to ensure that food ingredients that the firm markets are safe, and are otherwise in compliance with all applicable legal and regulatory requirements" (FDA, 2002).

Table 4 provides a compilation of microbially-derived enzymes that the FDA recognized as GRAS in opinion letters issued in the early 1960s. The opinions are predicated on the use of nonpathogenic and nontoxicogenic strains of the respective organisms and on the use of current good manufacturing practice (FDA, 2009).

Table 2. Partial List of Microbially-Derived Enzymes that FDA Recognized as GRAS in Opinion Letters

Description of Enzyme Preparation
Carbohydrase, cellulase, glucose oxidase-catalase, pectinase, and lipase from *Aspergillus niger*
Carbohydrase and protease from *Aspergillus oryzae*
Carbohydrase and protease from *Bacillus subtilis*
Invertase from edible baker's yeast or brewer's yeast (*Saccharomyces cerevisiae*)

<u>Evaluation Question #5:</u> Describe whether the primary function/purpose of the petitioned substance is a preservative. If so, provide a detailed description of its mechanism as a preservative (7 CFR § 205.600 (b)(4)).

Generally, enzymes would not be considered preservative materials. The products of enzyme activity could conceivably act as preservatives, but these would result from the breakdown of the food material itself, not from an outside source. Food qualities are changed by enzymatic activity, but this change should not necessarily be construed as a means of recreating qualities of the original product lost in processing. While enzymes can be used to transform food into a more stable product, these processed foods are generally identified as different from their raw ingredients (USDA, 2003).

Liquid preparations of enzymes may be prone to spoilage by microbial contamination, and preservatives are almost always added during processing and after final preparation (Pariza and Foster, 1983; USDA, 2000).

Animal-derived rennet does not act as a preservative. Its primary action is to coagulate milk to form cheese. Preservatives may be added during processing and after the final preparation of cheese products (USDA, 2000).

Egg white lysozyme does act as a preservative because it inhibits the growth of deleterious organisms, prolonging the shelf life of food products. 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. Egg white lysozyme is an important preservative in cheese manufacturing and minimizes the process called 'late blowing,' which is caused by the fermentation of butyric acid. Spores of *Clostridium tyrobutyricum* are capable of surviving the normal heat treatment of milk during the production of cheese and later propagate to cause late blowing. During the ripening of salt brined, semi-hard and hard cheeses, Clostridia ferments lactate into butyric acid and large quantities of gas (CO_2 and H_2). The formation of gas produces undesirable effects in texture (cracks and irregular eyes) and the acids cause unacceptable tastes and smells (CSK Food Enrichment, 2011).

There are no indications that bromelain has any preservative properties (USDA, 1996).

The use of pectinase neither increases nor decreases the shelf life of a raw product. In a natural situation, various enzymes are produced by the plant or other organisms to accelerate decay, decompose cell walls, increase sugar content, and release the nutrients contained in the fruit and other plant organs during the senescence process (USDA, 2003).

<u>Evaluation Question #6:</u> Describe whether the petitioned substance will be used primarily to recreate or improve flavors, colors, textures, or nutritive values lost in processing (except when required by law) and how the substance recreates or improves any of these food/feed characteristics (7 CFR § 205.600 (b)(4)).

Animal-derived rennet is not used for any of the purposes listed in Evaluation Question #6

Egg white lysozyme is used to prevent *Clostridia* bacteria from undergoing the process of fermentation during cheese manufacturing, which turns lactate into butyric acid and large quantities of gas (carbon dioxide (CO_2) and hydrogen (H_2)). The formation of gas produces undesirable effects in texture, which includes cracks and irregular eyes, and the acids cause unacceptable tastes (CSK Food Enrichment, 2011).

Bromelain (and other plant-based enzymes such as papain) are very popular mainly because they do not cause a bitter taste when hydrolyzing large molecules. Bromelain is also added to some foods to improve flavor or texture, mainly for the purpose of meat tenderization (Polaina and McCabe, 2007).

The use of pectinase enhances processing by enabling the entire fruit to be liquefied. This has the effect of improving saccharification and thus sweetness, improving aroma and color, enhancing clarity, removing haze, preventing gel formation, and increasing fruit juice yield (Kashyap et al., 2001). Juices extracted from ripe fruit contain a significant amount of pectin. Pectin imparts a cloudy appearance to the juice and results in an appearance and texture that many consumers do not find appealing. Although pectinases naturally occur in most fruits used to make juice, manufacturers often add pectinase to produce clear juice in a shorter amount of time (ETA, 2001).

<u>Evaluation Question #7</u>: Describe any effect or potential effect on the nutritional quality of the food or feed when the petitioned substance is used (7 CFR § 205.600 (b)(3)).

Enzymatic activity on foods is specific and usually results in a significant change in the characteristics of the substrate. Most studies show that nutritional quality as measured by vitamin and mineral content as well as other parameters is maintained. In some cases, because of the enzyme's role in the removal of the non-nutritional part of the food and in making the nutrients of the food more digestible, enzymes can measurably improve the nutritional quality of food (USDA, 2003).

If rennet is not used, milk will not curdle and cheese and other food products reliant on this process cannot be produced (Kirk and Othmer, 1947).

Egg white lysozyme is commonly used in food processing to decrease the loss of nutritional quality caused by thermal processing. The enzyme acts as an antimicrobial agent and is considered to be thermally stable. The use of egg white lysozyme may reduce the amount of thermal processing (including pasteurization and heat sterilization) needed during food manufacture, which also minimizes the loss of nutritional quality (Rahman, 2007). Egg white lysozyme does not exhibit lytic activity against the beneficial lactic acid bacteria used in cheese fermentation (FDA, 2000).

Bromelain and other plant-based enzymes break down proteins in foods, making them easier to digest. No information regarding the impact of bromelain on the nutritional quality of foods was available, but bromelain is not anticipated to affect nutritional properties.

It is unlikely that the use of pectinase will have effects on the nutritional quality of food.

<u>Evaluation Question #8:</u> List any reported residues of heavy metals or other contaminants in excess of FDA tolerances that are present or have been reported in the petitioned substance (7 CFR § 205.600 (b)(5)).

The Food Chemicals Codex is a compendium of internationally recognized standards for the purity and identity of food ingredients that is compiled by the Committee of the Food Chemicals Codex. This committee is charged by the FDA to provide information on the purity of food ingredients and specifications for food additives, GRAS substances, and any other ingredients to allow for a safer, more uniform use of food chemicals than the Food, Drug, and Cosmetic Act regulations could allow when used alone (IOM, 2003). The Food Chemicals Codex, places the following limits on residues in enzymes used in food production and processing:

- Arsenic (As): not more than 3 ppm
- Coliforms: not more than 30 per gram
- Heavy metals as lead: not more than 0.004 percent
- Lead (Pb): not more than 10 ppm
- *Salmonella* spp: negative by test

The Food Chemicals Codex also states that "although tolerances have not been established for mycotoxins, appropriate measures should be taken to ensure that the products do not contain such contaminants" (USDA, 2003).

<u>Evaluation Question #9:</u> Discuss and summarize findings on whether the manufacture and use of the petitioned substance may be harmful to the environment or biodiversity (7 U.S.C. § 6517 (c) (1) (A) (i) and 7 U.S.C. § 6517 (c) (2) (A) (i)).

Enzymes are catalysts and are used in small amounts to achieve the desired effect. For example, the maximum amount of animal-derived rennet used to clot milk is 0.036 percent (Pariza and Foster, 1983; USDA, 2000). Enzymes are biochemically active proteins. Heat, light, and air can cause irreversible degradation of enzyme activity. Thus, enzyme preparations should be protected from heat (normally they are kept under refrigeration) and stored in the dark. Like all proteins, enzymes are biodegradable (USDA, 2000).

Enzyme manufacturing must occur in sanitary conditions and under good manufacturing practices in order to be suitable for use in human food processing. Normal food factory waste treatment in industrialized nations reduces biological oxygen demand and thus practically eliminates the risk of environmental contamination. Release of enzymes into the environment is generally not a concern. They are active at very low concentrations, and each enzyme's action is specific to a very narrow range of substrate(s). They can be relatively stable molecules, but as mentioned above, are generally degradable by heat or other environmental factors. Enzymes in the environment may accelerate the rate that pollutants are metabolized. This may be detrimental, beneficial, or have no net effect depending on the substrate and metabolites present. The escape of enzyme-producing organisms into the environment is not considered an environmental concern (Kirk and Othmer, 1947).

Extraction of bromelain is considered a sustainable process because it allows for the use of parts of the pineapple (e.g., stem, peel, and leaves) that would otherwise be thrown away (Gimeno et al., 2010).

<u>Evaluation Question #10:</u> Describe and summarize any reported effects upon human health from use of the petitioned substance (7 U.S.C. § 6517 (c) (1) (A) (i), 7 U.S.C. § 6517 (c) (2) (A) (i)) and 7 U.S.C. § 6518 (m) (4)).

It is possible that enzymes could pose a threat to human health and safety. Enzymes can remain active after they are digested and, as proteins, cause allergic reactions in sensitive individuals (Tucker and Woods, 1995). Enzyme preparations can produce sensitivity reactions upon inhalation or skin contact. There have been reports of production plant workers experiencing allergic reactions and primary irritation following skin contact with enzymes or inhalation of dust from concentrated enzymes. FDA reports that it is not aware of any allergic reactions associated with the ingestion of food containing enzymes commonly used in food processing (FDA, 1995a).

Animal-derived enzymes, including rennet, are naturally occurring proteins that are ubiquitous in living organisms. They are derived from animals that have been used as sources of food and have been safely consumed as part of the human diet throughout human history. Animal-derived enzymes are used extensively as medical adjuvants. Pancreatin, a mixture of lipase, proteases, and amylase, is used as a supplement by patients with cystic fibrosis to improve the digestibility of food. One enzyme component of pancreatin, trypsin, is also used alone to improve protein digestibility (FDA, 1995b).

General lysozyme activity has been found in fungi, bacteria, plants, and almost all animal tissues. Specifically, high concentrations of lysozymes are found in human milk, saliva, and tears. It is unlikely that the use of egg white lysozyme would cause harm to human health. The FDA has determined that there is insufficient current information to establish whether the ingestion of egg white lysozyme elicits an allergic response when consumed by sensitive individuals (FDA, 2000).

Bromelain, when administered in therapeutic doses, can sometimes result in an allergic reaction. One 10-year-old patient being treated for sinusitis with bromelain had a mild allergic reaction; this patient had a pineapple allergy (Braun et al., 2005). However, the reaction was self-limiting and bromelain treatment was continued. Researchers have also documented cases of occupational bromelain-induced asthma, noting that while bromelain is a strong sensitizer, sensitization usually occurs due to inhalation and not ingestion; therefore, sensitization would only be expected in occupational settings (Gailhofer et al., 1988). As a medical supplement, bromelain has been used as a digestive aid and has also been used for its wound healing, anti-inflammatory, anti-coagulating, and ani-tumor effects (Thomas Research Inc., 1998). Studies of oral administration of bromelain show low toxicity; the LD_{50} (lethal dose killing 50% of animals) was reported to be greater than 10 g/kg. Dogs administered up to 750 mg/kg-day bromelain for six months showed no adverse effects. Rats dosed with 1.5 g/kg-day did not experience carcinogenic or teratogenic (i.e., developmental) effects (Thomas Research Inc., 2010). Clinical studies on humans have shown a low potential for side effects, unless an individual has hypertension; one report suggested that pre-existing hypertension may be exacerbated by therapeutic doses of bromelain (Thomas Research Inc., 2010).

The possible presence of mycotoxins from either the source organism or a competing organism that invades the fermentation media is a primary concern with the use of enzymes from fungal sources. Many of these organisms are capable of producing antibiotics. While good manufacturing practices require that nonpathogenic strains be used, quality control and Hazard Analysis Critical Control Point (HACCP) need to be sufficient to ensure that both the strains and the media avoid contamination with pathogens and toxins. For example, *Aspergillus niger* is capable of producing low levels of toxins, but most strains are considered nontoxic because the levels of toxins are so low (Pariza and Foster, 1983).

<u>Evaluation Information #11:</u> Provide a list of organic agricultural products that could be alternatives for the petitioned substance (7 CFR § 205.600 (b)(1)).

Enzymes are natural catalysts that accelerate or initiate a chemical reaction. They are unique in their ability to break down protein in food. As a result, enzymes can only be substituted with another enzyme with the same function, e.g. from other sources (e.g., replacing a fungal enzyme with a plant-derived enzyme which can act on the same substrate). In addition, many enzymes have specific functions that are unique (or not common in other enzyme types). For example, rennet clots milk to make cheese, but egg white lysozyme cannot be used to make cheese (only preserve it) because it does not have adequate enzymatic activity against lactic acid bacteria.

Cheese is the food created by the clotting action of rennet on the milk of cows, sheep, and goats. Plant-based enzymes have been used in cheese making, with ficin (from figs) used most extensively (CRC, 1980); however, it is unclear if the clotting action of plant-based enzymes is as effective as rennet. Another alternative to animal-derived rennet for producing cheeses with the appropriate characteristics is genetically engineered chymosin, which the National Organic Standards Board (NOSB) previously reviewed in 1996 and determined to be incompatible with organic food handling (USDA, 2000).

There were no alternatives to bromelain identified besides other plant-, bacteria-, or animal-derived enzymes. However, it should be noted that consumers and processing facilities can tenderize meat with physical methods (e.g., using a hand-held meat tenderizer) rather than applying bromelain meat tenderizing products.

Additional Questions Specific to Enzymes

The following additional questions were posed by the NOSB Handling Committee to aid the National List review for enzymes use in organic handling/processing. In posing these questions, the NOSB Handling Committee stated that, "The technical report should specifically address any new food uses, manufacturing methods, or sources of enzymes since the previous technical reports, and should thoroughly evaluate alternative organic inputs which could provide similar functionality in foods."

Additional Question #1: Are animal enzymes from organic sources available?

As described in the 2000 TAP Review for animal-derived enzymes, animal-derived enzymes could be produced from organic livestock (USDA, 2000). However, no information has been identified to confirm the use of organic livestock as a source of animal-derived rennet.

While this report provides information specific to animal-derived rennet, it is possible to produce rennet from non-animal sources, including plants and microorganisms. In 2008, it was estimated that only five percent of cheese in the United States was produced using animal-derived rennet (Yacoubou, 2008). Genetically modified rennet (e.g. fermentation produced rennet) is prohibited for use in organic agriculture and is not permitted for use in products labeled as organic. Vegetable rennet could be classified as organic because true vegetable rennet can be produced from plants including nettles, cardoon thistle, or fig tree bark (Fletcher, 2011). If these plants were grown in a manner that adhered to the principles of organic agriculture, the enzymes derived from them could be considered organic.

It is unclear if egg white lysozyme is obtained commercially from eggs labeled as organic. However, due to the high cost of organic eggs, this process is unlikely since it would significantly increase the cost of production (USDA, 2009).

Additional Question #2: Are other enzymes from organic sources available?

Although organic plants could be used to make organic plant-derived enzymes, no sources of organic bromelain, ficin, papain, or other plant enzymes were identified.

Additional Question #3: What are the specific organic food uses for animal enzymes?

Egg white lysozyme may be used to preserve fresh, organic fruits and vegetables, organic tofu bean curd, and ready-to-eat products like cooked meat and poultry. Egg white lysozyme may also be used in the casings for organic frankfurters. Animal-derived rennet could be used to make organic cheese and sour cream; however, vegetarians would likely prefer organic cheese made with microbial or other non-animal sources. Because of the lower cost and consumer demand for cheeses produced without animal-derived products, only five percent of cheeses produced in the United States are created using animal-derived rennet (Yacoubou, 2008). Cheeses containing animal-derived rennet can be labeled as organic. Blue cheese products are frequently still made using animal-derived enzymes (Organic Valley, 2011).

Additional Question #4: What are the specific organic food uses for enzymes from edible, nontoxic plants, nonpathogenic fungi, or nonpathogenic bacteria?

Bromelain may be used in organic crackers, breads (and other baked goods), and soy-based protein supplements, as described in the "Specific Uses of the Substance" section. Bromelain and other plant-based enzymes like ficin may be used in the production of organic cheese from organic milk.

Microbial enzymes are often used to make organic cheeses as a form of "vegetarian rennet." Microbial and fungal enzymes are used widely in food processing; thus, they are likely in a variety of organic food products including breakfast cereals, candy, corn syrup, beer, milk, fruit juice (made using pectinase), and mayonnaise (Underkofler et al., 1957).

Additional Question #5: Are any synthetic solvents used in the extraction (or other manufacturing) process of enzymes?

Synthetic solvents are used in the manufacturing of enzymes. Solvents such as acetone and alcohol are often used in the manufacturing of animal-derived rennet and plant-based enzymes such as bromelain.

References:

Braun, J.M., Schneider, B., Beuth, H.J., 2005. Therapeutic use, efficiency, and safety of the proteolytic pineapple enzyme bromelain-POS in children with acute sinusitis in Germany. In Vivo, 19(2):417-421. Retrieved May 23, 2011 from http://www.ncbi.nlm.nih.gov/pubmed/15796206 [abstract]

Canadian General Standards Board, 2009. Retrieved April 11, 2011 from http://www.tpsgc-pwgsc.gc.ca/ongc-cgsb/internet/bio-org/index-eng.html

Chemical Rubber Company (CRC), 1980. Handbook of Food Additives. Boca Raton: CRC Press. Retrieved July 8, 2011 from http://books.google.com/books?id=2w__X6I0_5wC&dq=ficin+in+cheese+making&source=gbs_navlinks_s

Codex Alimentarius Comission, 1999. Section 3.4 of the Codex Guidelines, GL 32-1999, Retrieved July 28, 2011 from vwww.codexalimentarius.net/download/standards/360/cxg_032e.pdf

CSK Food Enrichment, 2011. Lysozyme. Retrieved June 1, 2011 from http://www.cskfood.com/ingredients/307.html

Cunningham, F. E., Proctor, V. A., Goetsch, S. J., 1991. Egg-white lysozyme as a food preservative: an overview, World's Poultry Science Journal, (47): 141-163. Retrieved June 1, 2011 from http://journals.cambridge.org/action/displayAbstract?fromPage=online&aid=5140424

Enzyme Technical Association (ETA), 2001. Enzymes: A Primer on Use and Benefits Today and Tomorrow. Retrieved March 24, 2011 from http://www.enzymetechnicalassoc.org/benefits_paper.pdf

Enzyme Technical Association (ETA), 2004. Enzyme preparations used in food preparation. Retrieved July 8, 2011 from http://www.enzymetechnicalassoc.org/fcclist.pdf.

The European Economic Community (EEC), Council Regulation (EC) No 889/2008. Retrieved April 11, 2011 from http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:250:0001:0084:EN:PDF

FAO, 1992. Bromelain. Retrieved May 25, 2011 from www.fao.org/ag/agn/jecfa-additives/specs/Monograph1/Additive-060.pdf

FAO, 1992. Rennet. Retrieved April 14, 2011 from http://www.fao.org/ag/agn/jecfa-additives/specs/Monograph1/Additive-373.pdf

FAO, 2000. Pectinase from *Aspergillus niger*, Var. Retrieved April 14, 2011 from http://www.fao.org/ag/agn/jecfa-additives/specs/Monograph1/Additive-305.pdf

FDA, 1995a. Subchapter B: Food for human consumption. Part B: Direct Food substances affirmed as GRAS. Section 184.1024: Bromelain. Retrieved May 24, 2011 from http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?FR=184.1024

FDA, 1995b. Enzyme preparations from animal and plant sources; affirmation of GRAS status as direct food additives. Retrieved April 12, 2011 from http://gpo.gov/fdsys/pkg/FR-1995-06-26/html/95-15239.htm

FDA, 2000. Lysozyme GRAS Notice. Retrieved May 31, 2011 from http://www.accessdata.fda.gov/scripts/fcn/gras_notices/grn0064.pdf

FDA, 2002. Agency Response Letter GRAS Notice No. GRN 000089. Retrieved April 15, 2011 from http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/GRASListings/ucm154613.htm

FDA, 2006. Database of Select Committee on GRAS Substances (SCOGS) Reviews: Rennet. Retrieved April 8, 2011 from http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=scogsListing&id=271

FDA, 2009. Partial List of Enzyme Preparations That are Used in Foods. Retrieved April 18, 2011 from http://www.fda.gov/Food/FoodIngredientsPackaging/ucm084292.htm

Fileti, A.M.F., Fischer, G.A., Santana, J.C.C., Tambourgi, E.B., 2009. Batch and continuous extraction of bromelain enzyme by reverse micelles. Brazilian Archives of Biology and Technology, 52 (5):1225-1234. Retrieved May 23, 2011 from http://www.scielo.br/pdf/babt/v52n5/v52n5a21.pdf

Fletcher, J., 2011. Cheese Focus: The Critical Ingredient, Specialty Food Magazine. Retrieved July 28, 2011 from http://www.specialtyfood.com/news-trends/featured-articles/retail-operations/cheese-focus-critical-ingredient/

The Food Chemicals Codex, 1996. Fourth edition.

Frankhauser, D., 2009. Rennet for Making Cheese, University of Cincinnati Clermont College. Retrieved April 8, 2011 from http://biology.clc.uc.edu/fankhauser/cheese/rennet/rennet.html

Gailhofer, G., Wilders-Truschnig, M., Smolle J., Ludvan, M., 1988. Asthma caused by bromelain: an occupational allergy. Clin Allergy, 18(5):445-450. Retrieved May 23, 2011 from http://www.ncbi.nlm.nih.gov/pubmed/3233722 [abstract]

Gautam, S.S., Mishra, S.K., Dash, V., Goyal, A.K., Rath, G., 2010. Comparative study of the extraction, purification, and estimation of bromelain from stem and fruit of pineapple plant. Thai J. Pharm. Sci. 34:67-76. Retrieved May 23, 2011 from http://www.pharm.chula.ac.th/tjps/ContentVol34No2/V34-2Art3%20pp67-76.pdf

Gimeno, C., Seguí, L., Fito, P., 2010. Valorisation of industrial pineapple residues: Bromelain separation followed by bioethanol fermentation. International Conference of Food Innovation 2010. Retrieved May 24, 2011 from http://www.foodinnova.com/foodInnova/docu2/339.pdf

Guangxi Nanning Javely Biological Products, Co., Ltd., 2011. Bromelain. Retrieved May 25, 2011 from http://papain.diytrade.com/sdp/204028/4/cp-1018388/0.html

Haslaniza, H., Maskat, M.Y., Wan Aida, .W.M., Mamot, S., 2010. The effects of enzyme concentration, temperature, and incubation time on the nitrogen content and degree of hydrolysis of protein precipitate from cockle (Anadara granosa) meat wash water. International Food Research Journal, 17:147-152.

IFOAM, 2005. Basic Standards for Organic Production and Processing. Retrieved July 28, 2011 from http://www.ifoam.org/about_ifoam/standards/norms/norm_documents_library/norms_documents_library.html

Institute of Medicine (IOM), 2003. Food Chemicals Codex. Prepared by the Committee on Food Chemicals Codex with support from the U.S. Food and Drug Administration. Washington, DC: National Academies Press; 998 pps. Retrieved July 8, 2011 from http://books.google.com/books?id=k7Mk7-SUIKkC&printsec=copyright#v=onepage&q&f=false

Kirk, R. and Othmer, D., 1947. Encyclopedia of Chemical Technology, Volume 5. The Interscience Encyclopedia, Inc., New York.

Kashyap, D.R., Vohra, P.K., Chopra, S., Tewari, R., 2001. Applications of pectinases in the commercial sector: a review, Bioresource Technology, 77: 215-227. Retrieved April 18, 2011 from http://infolib.hua.edu.vn/Fulltext/ChuyenDe2009/CD211/46.pdf

Kewpie Corporation, 2010. Food Additive: Egg White Lysozyme. Retrieved May 31, 2011 from http://www.kewpie.co.jp/english/fc/imgs/02/ewl.pdf

Madison Market, 2011. Rennet. Retrieved July 11, 2011 from http://www.madisonmarket.com/index.php?page=rennet

Marinova, M., Cuc, N.T.K., Tchorbanov, B., 2008. Enzymatic hydrolysis of soy protein isolate by food grade proteinases and aminopeptidases of plant origin. Food Biotechnology 22(3):835-838.

Mosby's Medical Dictionary, 2009. Elsevier, 8th edition. Retrieved April 8, 2011 from http://medical-dictionary.thefreedictionary.com/rennin

Ngampanya, B., and Phongtongpasuk, S., 2006. Effects of Sucrose on crude bromelain production of in vitro culture of pineapple (*Annas comusus* par. “Pattavia”). Kasetart J (Nat. Sci), 40: 129-134. Retrieved May 23 from http://kasetsartjournal.ku.ac.th/kuj_files/2008/A080403102631.pdf.

O’Connor, C., 1993. Traditional cheese making manual, ILCA (International Livestock Centre for Africa), Addis Ababa, Ethiopia. Retrieved April 12, 2011 from http://www.fao.org/sd/erp/toolkit/BOOKS/Cheese.pdf

Organic Valley, 2011. Products- Cheese. Retrieved July 11, 2011 from http://www.organicvalley.coop/products/cheese/crumbles/

Pandey, A., 2006. Enzyme Technology. Retrieved April 15, 2011 from http://books.google.com/books?id=x13NiCGHMt8C&pg=PA283&lpg=PA283&dq=taking+pectinase+from+growth+media&source=bl&ots=mNKdaNb_ff&sig=ggh9nrg2670mz2C3yh0Kq7percc&hl=en&ei=HqioTfCZMebV0QHotdz5CA&sa=X&oi=book_result&ct=result&resnum=3&ved=0CCIQ6AEwAjgK#v=onepage&q&f=false

Pariza, M.W. and Foster, E.M., 1983. Determining the safety of enzymes used in food processing, J. of Food Protection 36:453-468.

Pariza, M.W. and Johnson, E., 2001. Evaluating the Safety of Microbial Enzyme Preparations Used in Food Processing: Update for a New Century, Regulatory Toxicology and Pharmacology, 33:173-186.

Polaina, J., McCabe, A.P., 2007. Industrial Enzymes: Structure, Function, and Applications. New York: Springer; 654 pp.

Rahman, S., 2007. Handbook of Food Preservation. Retrieved June 1, 2011 from http://books.google.com/books?id=sKgtq62GB_gC&pg=PA293&lpg=PA293&dq=lysozyme+impact+on+nutritional+quality&source=bl&ots=DFHb6MMBzV&sig=6atcAfEg7V3jTp4J-kHRezcNfMM&hl=en&ei=gnnmTZG4LaTh0QGjnZX2Cg&sa=X&oi=book_result&ct=result&resnum=1&ved=0CBoQ6AEwADgU#v=onepage&q=lysozyme%20impact%20on%20nutritional%20quality&f=false

Scholar Chemistry, 2009. Material Safety Data Sheet: Rennin, CAS # 9001-98-3. Retrieved April 8, 2011 from http://www.sargentwelch.com/pdf/msds/Rennin_604.00.pdf

Thomas Research Inc., 1998. Monograph: Bromelain. Alternative Medicine Review, 3(4):302-305. Retrieved May 18, 2011 from http://www.thorne.com/altmedrev/.fulltext/3/4/302.pdf

Thomas Research Inc., 2010. Monograph: Bromelain. Alternative Medicine Review, 15(4):361-368. Retrieved May 24, 2011 from http://altmedrev.com/sobi2.html?sobi2Task=dd_download&fid=492

Tucker, G.A. and Woods, L.F.J., 1995. Enzymes in Food Processing, London: Blackie Academic and Professional. Retrieved April 14, 2011 from http://books.google.com/books?id=-4ZnMbryTqkC&printsec=frontcover&dq=Tucker+and+Woods.+1995.+Enzymes+in+food+processing&source=bl&ots=yVGw479lCc&sig=N95ceZwLZAQ2HC78oOv26qeYcXU&hl=en&ei=lxCnTeLJMse1tgeX-8mFAQ&sa=X&oi=book_result&ct=result&resnum=1&ved=0CBgQ6AEwAA#v=onepage&q&f=false

Underkofler, L.A.; Barton, R.R., and Rennert, S.S., 1957. Microbiological process report. Production of microbial enzymes and their applications. Presented at the Symposium of the Society for Industrial Microbiology, Storrs, Connecticut, August, 1956. Retrieved July 8, 2011 from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1057391/pdf/applmicro00317-0062.pdf

USDA, 1996. TAP Review: Enzymes: plant. NOSB/National List Comment Form: Processing.

USDA, 2000. TAP Review: Animal Derived Enzymes (Compiled by OMRI).

USDA, 2003. TAP Review: Enzymes, Plant, and Fungal. NOSB/National List Comment Form: Processing.

United States Department of Agriculture (USDA), 2009. Organic prices. Data sets. Retrieved July 12, 2011 from http://www.ers.usda.gov/Data/OrganicPrices/

White, J.S. and D.C White, 1997. Source Book of Enzymes. Boca Raton: CRC Press. Retrieved April 11, 2011 from http://books.google.com/books?id=VN1P6oQkLcIC&printsec=frontcover&dq=White+and+White,+1997.+Source+Book+of+Enzymes&hl=en&ei=CyKjTbnuBpLpgQetlbzkBQ&sa=X&oi=book_result&ct=result&resnum=1&ved=0CEQQ6AEwAA#v=onepage&q=White%20and%20White%2C%201997.%20Source%20Book%20of%20Enzymes&f=false

Yacoubou, J., 2008. An Update on Rennet, Vegetarian Journal Issue 3. Retrieved July 11, 2011 from http://www.vrg.org/journal/vj2008issue3/update_renet.htm