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SOME ASPECTS ON PROTEIN MODIFICATION BY ENZYMES

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ABSTRACT

Hydrolysis of protein raw materials by proteases is a process largely used in food industry to either modify the physicochemical properties of proteins (viscosity, emulsifying and stabilizing capabilities) or convert residual protein present in blood and bones of slaughtered animals in marketable products (for instance, decolorized globin, flavor enhancers, animal feed constituents). There are several types of proteases, i.e., endoproteases (pepsin and papain) and exoproteases (carboxypeptidases and aminopeptidases) that act at broad intervals of pH (4.0-10.0) and temperature (50-90^{o}C). In the dairy industry, besides proteases (used in milk clotting), lipases (used in development of aroma and flavor during cheese ripening, such as the organoleptic characteristics of particular cheeses as Camembert, provolone, cheddar, and gouda) and lactase (for dietary products) are also used.

KEYWORDS: Enzymes, proteases, proteins.

INTRODUCTION

Proteins are polymers of amino acids that can be hydrolyzed by proteases.

The modification of proteins has been made in food industry (production of oriental foods, fish paste, sauces, cheese, bread, beer etc.) and chemical industry (leather, detergents, flavor, effluent, and waste treatments).[1] For instance, bakery proteases are used to improve dough handling through increased hydration of proteins and the alteration of elasticity and plasticity of the protein in dough.[2]

The main aim of the enzymatic proteolysis is to increase the commercial value of vegetal and animal protein. Functionally modified proteins – in terms of solubility, stability, flavor enhancement, off-flavor elimination, reduced allergenic action and digestibility – have several applications in the alimentary industry, such as ingredients in thousands of

formulations of food products both for human (flavoring agents, dietetics and health products, infant formulae and clinical nutrition) and animal (flavoring and nutritional agents) uses.

The aim of this work is to present a short discussion about the use of proteases in dairy industry, meat tenderization and in the modification of residual protein resulting from animal slaughtering (blood and bones) and fish canning.

OVERVIEW ON PROTEASES

Proteases, which are found in plants, animals and microorganisms, are classified according to **origin** (papain from *Carica papaya*, ficin from *Ficus sp.*, bromelain from pineapple, for example), **mode of action** – endopeptidases (cleave randomly amide bonds throughout the protein chain) and exopeptidases (cleave amide bonds sequentially from C-terminal (*carboxypeptidase*) or N-terminal (*aminopeptidase*) of a peptide chain), **nature of the active site** (serine protease has a serine at the active site such as trypsin and quimotrypsin; sulphydryl protease has a cysteine at the active site such as ficin, papain, and bromelain; metal protease has a metallic ion (Fe^{+2}, Zn^{+2} or Mg^{+2}) at its active site; acid protease has at least one carboxyl group at the active site, such as pepsin), and value of **optimum pH** (acid protease [4.0 < pH< 6.8], neutral protease [6.9 < pH < 7.3], alkaline protease [7.4 < pH < 8.5] and super-alkaline protease [pH >8.5]).

The criteria for choosing a protease are based on its specificity, necessity or not of cofactor, heat and pH stability, and optimum pH.

Protein hydrolysis can be **partial** (in the case of k-casein precipitation in cheese production), **non-extensive** (when a modification in some functional property is envisaged as viscosity, emulsifying and stabilizing capabilities), and **complete** (such as in the production of soy milk).

The proteolysis can be controlled by either the determination of the total protein content in the raw material or the degree of hydrolysis (DH). Total protein is evaluated by biuret, Lowry, Bradford or Kjeldahl methods, whereas DH is measured by neutralizing free amino acids with sodium hydroxide – the volume of alkali consumed corresponds to the DH. When the protein amino acid composition is known, DH reflects the percent of amide bonds cleaved.[3]

Often a hydrolyzed protein presents a bitter taste due to the presence of medium to short chain hydrophobic peptides (containing high amounts of hydrophobic amino acids). For bitter peptides, the higher the hydrophobicity, the more intense the bitter taste.[3] Moreover, peptides are particularly bitter when hydrophobic amino acids are located near the amino or carboxy terminal or at least one proline is near the center of the peptide chain. Proline alters the conformation of peptide chains by uncoupling bitter reactive sites ("binding site" and "stimulating site") that stimulate the bitter taste receptors on the tongues' papillae. The relation between the position of hydrophobic amino acids and bitterness is pivotal for predicting and controlling the bitter flavor of protein hydrolysate.

To circumvent the bitterness in hydrolyzed proteins, the industry has used methods such as **masking** with other flavorings (monosodium glutamate, citric acid, malic acid, polyphosphate or gelatin), **extraction** with active charcoal, and **limited hydrolysis** using specific proteases (endopeptidases and exopeptidases at balanced amounts in order to reduce the number of hydrophobic amino acids and not modify protein functional properties such as emulsification and foaming).

At first glance, a complete debittering of any hydrolyzed protein can be obtained. However, in practice, the final level of bitterness will be the result of a balance between the cost of debittering, the desired functionality and how much the food flavor is tolerant to the presence of hydrophobic peptides.

Protease in a medium with a low water activity can catalyze the following reaction (transferase activity):

$$\text{Bz-Tyr-NH}_2 + \text{Gly-NH}_2 \leftrightarrows \text{Bz-Tyr-Gly-NH}_2 + \text{NH}_3$$

This approach is used in the synthesis of aspartame (sweetener), in which thermolysin catalyzes the formation of an amide bond between L-aspartic acid and L-phenylalanine.

The partial protein hydrolysis with a protease followed by the concentration of the hydrolysate and renewed incubation with a neutral protease can lead to a gel-like complex called plastein. This gel is recovered by precipitation with ethanol or acetone. Probably, a transpeptidation (similar as the transferase activity) leads to the formation of new insoluble hydrophobic peptides that condense forming a gel.[4]

Dairy Industry

Milk is a nutritive product composed by proteins, fats, lactose, vitamins, salts, and enzymes. Enzymes naturally present in milk – α-amylase, alkaline phosphatase, peroxidase, among others – can affect cheese ripening and heat stability. The presence of alkaline phosphatase in pasteurized milk is an indication that pasteurization was not well carried out.[5]

Several enzymes are used by the dairy industry:

Catalase

Hydrogen peroxide is added to the milk to avoid microbial contamination during the transportation from the farm to the processing plant. Before pasteurization (conducted at 60^{o}C for 30 min, aiming to destroy lipase and phosphatase activities and pathogenic and acidophilus microorganisms), catalase is added in the milk to remove the hydrogen peroxide.

However, in the fabrication of gorgonzola cheese, the presence of hydrogen peroxide – that does not inactivate enzymes and preserve the acidophilus microorganisms – is necessary to develop the characteristic aroma and flavor of this cheese during curing.

Clotting Enzymes

History records indicate that cheese is produced at least since 4,000 B.C. Along the centuries, its production improved empirically, although the technology is well understood and fully controlled nowadays.

Chymosin – acid protease obtained from the stomach of suckling calves, which hydrolyses the amide bond between phenylalanine and methionine from the N-terminal of the k-casein chain – is the most efficient and specific coagulant of milk k-casein. The coagulation process (Figure 1) is influenced by factors such as **chymosin concentration**, which depends on the type of cheese to be produced. For example, in the cheddar production, the concentration is 25 mL of chymosin/100 L of milk, whereas in cottage cheese it is 5 mL of chymosin/100 L of milk; **temperature**; **pH**, which affects the final characteristics of the coagulum, insofar as a pH between 5.8 and 6.5 allows obtaining an elastic, contractile and not granulated k-casein coagulum, meanwhile at a pH < 5.0 the coagulum is granulated and inelastic; **concentration of Ca^{+2}**; **storage of milk at a low temperature** for a long period because the stability of the water/oil emulsion (the real milk nature) can be lost and the coagulation does not occur or it takes a long time (inappropriate for a high-scale cheese production); **fat concentration** in the milk; and **time of coagulation**, which depends on the type of cheese to be produced, generally ranging from 30 to 90 min.

The clotting enzyme power corresponds to the volume of milk (expressed in milliliter) coagulated by 1 mL of enzyme solution for 40 min at 35°C. The substrate used is skim milk powder dissolved in 0.01M calcium chloride solution.

Chymosin availability in the 1970s became a limiting factor for cheese makers because bovine calves were left to grow for meat production, a more lucrative business. Thereby, microbial coagulants obtained from *Cryphonectria parasitica, Bacillus cereus, Mucor pusillus* and *Mucor miehei* were introduced into the market. Chymosin obtained from engineered microorganisms is largely used today in cheese making.[6]

Proteases

The use of proteases aims to affect organoleptic properties (aroma, texture, color and flavor) of ripened cheeses (cheddar, brie and Camembert, for example). The proteolytic activity during the ripening of cheese can be extensive. In hard cheeses, about 30% of the insoluble protein of the curd may be converted into soluble protein, whereas in soft varieties, such as brie or Camembert, over 80% of the insoluble protein can be converted into water-soluble compounds (peptides, amino acids, and ammonia). Apart from the participation of starter cultures, proteolysis and amount of residual enzyme in the curd depend in part on the coagulating enzyme system used in the formation of the curd. The production of cheddar cheese without a starter culture results in a cheese with much lower levels of free amino acids. Production of bitter flavors is generally attributed to the formation of bitter peptides. Bitter flavor results when these peptides are formed faster than they can be broken down by proteolytic enzymes of the starter organisms.[5]

Lipases and esterases

They are enzymes that hydrolyze tri-, di- and monoglycerides present at the oil-water interface in milk to provide dairy products with desired flavors. The reaction seldom completes, as lipases have a high activity on triglycerides, a medium activity on diglycerides, and virtually no activity on monoglycerides.[7] The hydrolysis of a triglyceride by lipase, however, yields free fatty acids in addition to di- and monoglycerides. Animal lipases are lipolytic enzyme preparations derived from animal tissues. The most significant sources of animal lipases are bovine and porcine pancreatic tissues and pregrastic lipases from pregrastic tissues of goat, lamb and calf. Animal lipase powders are produced from edible and government-inspected animal tissues, and standardized before packaging to ensure a consistent strength, uniformity, and purity.

Lactase

Lactose is a disaccharide found in mammal milk. Lactose is a sugar with a low sweetness level, although the individual constituents of lactose (glucose and galactose) have a combined sweetness of about 80% that of sucrose and are three to four times more soluble than lactose. When lactose reaches a 12% concentration (w/w; dry solid basis) in milk or whey, it crystallizes as sharp needles, making the final product improper to use as human food. Lactase splits lactose into glucose and galactose. The main lactase sources are the yeasts *Kluyveromyces fragilis* and *K.lactis* and the fungi *Aspergillus niger* and *A.oryzae*. The pH of maximal activity for yeast lactase ranges from 6.0 to 7.0, whereas for fungal lactase, it ranges from 4.0 to 5.0. Thereby, the yeast and fungal types are suitable for lactose hydrolysis in milk (pH ≅ 6.8) and in whey (pH ≅ 4.5), respectively.

The hydrolysis of lactose in milk and whey enables them to be used in dietary formulations for lactose-intolerant humans. Moreover, hydrolyzed whey is useful in bakery, yoghurt production, confectionary and other industrialized food products (ice cream, condensed milk, frozen yoghurt, spreads, dressings, soft drinks etc.). In bakery goods, 20% of egg protein (albumin) and sucrose can be replaced by whey syrup diluted at 1:1 with water. In yoghurt production, the time of fermentation is shorter because glucose and galactose are metabolized by microorganisms faster than lactose. In confectionary, the hydrolyzed whey syrup is used to replace up to 100% of the sweetened condensed milk in soft caramel formulations and up to 50% in toffee recipes. Ice-cream, condensed milk and frozen yoghurt made with 50%-lactose-hydrolyzed whey syrup present better melt down characteristics, no lactose crystallization, and no sandiness during storage.[5][8][9]

The perspectives of enzymes in dairy industry relies on the production of chymosin through engineered microorganisms, the increase in the availability of thermal sensitive fungi proteases, the use of immobilized chymosin (the 1st step of milk coagulation carried out as a continuous process), and the use of fat capsules (liposome) filled with lipases and esterases, which are added to the milk before coagulation but slowly freed during curd ripening.

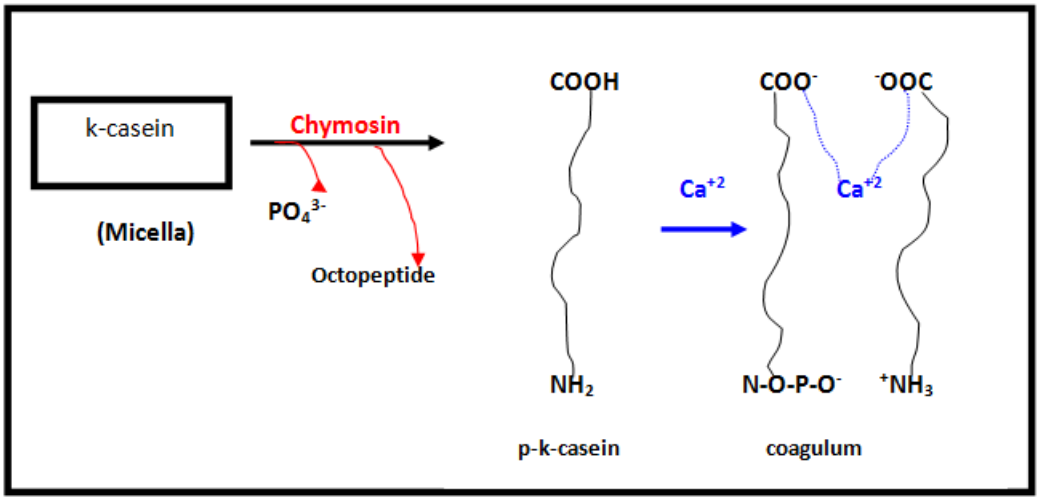

Figure 1: Scheme of milk coagulation. Chymosin hydrolyses k-casein in p-k-casein (PKC), following the combination of PKC with calcium ions.

MISCELLANEOUS USES OF PROTEOLYTIC ENZYMES

Treatment of residual cattle blood with protease

The slaughtering of animals for meat results in the production of residual offal, bones and blood. The blood can be either treated directly with protease or centrifuged for plasma and red cells separation (Figure 2). The plasma is valued as an unmodified protein with emulsifying and gelling properties. The red cell fraction – which contains at least 70% of blood proteins – may be enzymatically treated to break the hemoglobin to decolorize and stabilize the released protein. The decolorized hemoglobin allows using it as a supplementary protein source in food formulations. The use of residual red cells represents a significant economic and environmental benefit.

Figure 2 shows that the first step of blood processing is water addition aiming for both diluting the blood and stimulating hemolysis. The process of hemolysis, which occurs rapidly in pure water, consists in the release of hemoglobin from the stroma of red cells into the surrounding solution. Hemoglobin releasing can be a consequence of lowering the concentrations of salts and other substances due to the entrance of excess water into the cells.

As shown in Figure 2, the resulting products from blood processing are sludge (rich in dark-colored insoluble substances; may be used as animal feed ingredient), decolorized product (mainly globin), and three types of protein hydrolysate, each having a use in animal feed ingredient **(1)**, human food component **(2)** and flavor food enhancer **(3)**.

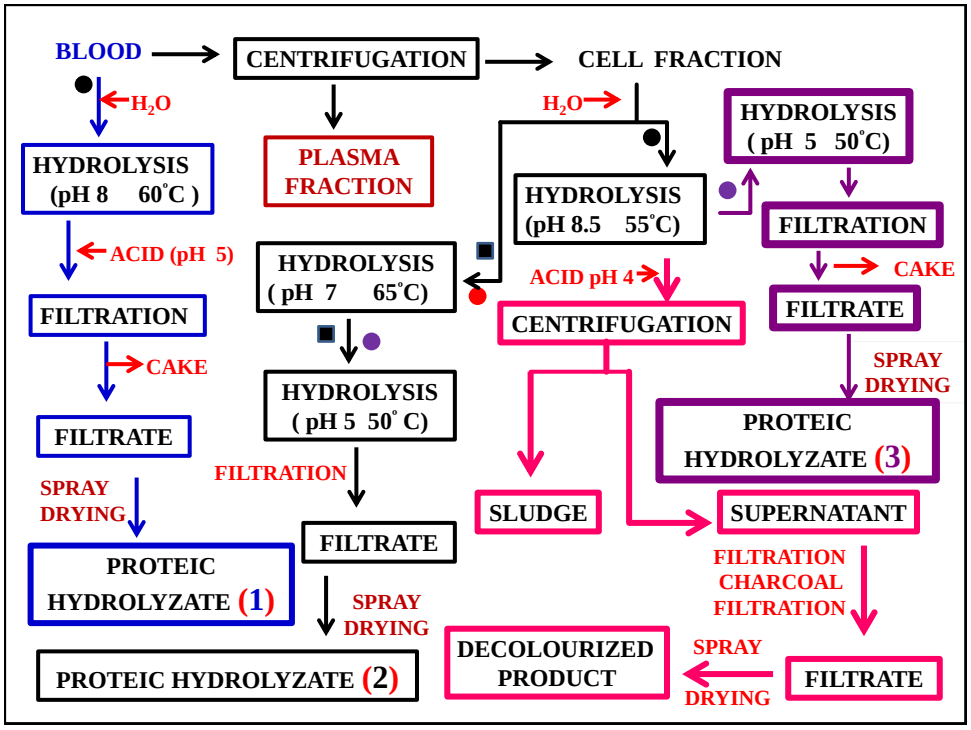

Figure 2: Enzymatic treatment of blood. Symbols: bacterial alkaline protease (●), fungal neutral protease (●), plant protease (■) and fungal acid protease (●).

Recuperation of the residual meat stuck on the bones of slaughtered cattle

The residual meat stuck on the bones represents about 5% of the whole weight of it. The meat can be removed as follows: ground bones are suspended in water containing alkaline protease (0.3% of the whole bones weight) and kept at 60°C for at least 4 hr. After that, the suspension is left decanting and the supernatant is collected and kept at 98°C for 10-20 min. This product is used as supplement in canned meat foods.[3]

Fish protein hydrolysate

The fishery industry handles tons of fishes captured in rivers, lakes, oceans or grown in confinement. This action generates, in turn, tons of residues rich in protein, which can be processed for obtaining derivatives valuable for animal feeding. Moreover, fish with no commercial value captured involuntarily during fishing can also be source of protein derivatives. The fish meat is cut, ground and mixed at 1:1 with water. After maceration, the pH is adjusted to 6.5 and the protease is added on a ratio of 1:150 of whole protein. The mixture is left stirring at 30°C for 1 hr, a period long enough to obtain a degree of hydrolysis of 5%. The pH is adjusted to 3.0 with sulfuric acid followed by the addition of 10% of sodium hexametaphosphate. The phospho-protein precipitate is collected by centrifugation, and washed with water and then with isopropanol. The defatted phospho-protein precipitate is

suspended in water and the mixture is spray-dried. At the end, a fish protein highly concentrated powder is obtained.[3]

Meat Tenderization

After humankind has domesticated several species of mammals millennia ago (cattle, dogs, pigs, sheep, calves etc.), meat soon became a food incorporated into daily cuisine. However, the *rigor mortis* of the muscles following the slaughter of the animal became a problem for chewing. Thereby, the meat industry devoted a considerable time over the years and efforts toward improving meat tenderness without loss of desirable texture.

Tenderness has been achieved through two complementary approaches, i.e., animal breeding (genetic improvement through crossing different lineages of species of bovine cattle, for instance) and aging (the meat is left for 10 to 14 days in aerated chambers kept at 2^{o}C and 84% of humidity).

The natives of Central America developed a tenderization method consisting of immersion of meat cuts in papaya juice or wrapping them in papaya leaves prior to cooking. Based on this technique, the meat industry began to treat meat with proteases as soon as they became available in the market in the 1940s.

The proteolytic enzymes (mainly, papain and bromelain) used commercially for the tenderization of meat are from food sources (papaya and pineapple), and since they have been ingested for centuries without deleterious effects, they have been approved for food use by regulatory officials.[10] There are a few enzymes derived from microbial sources (*Bacillus subtilis* and *Aspergillus* oryzae) that have been approved for meat tenderization.

Commonly, a mixture of papain and bromelain is injected into the jugular vein of the cattle at least 20 min prior to the slaughter, so that the enzymes are distributed all over the body through the bloodstream. The amount of solution introduced varies with age (older animals require almost twice as much solution as a young one), weight, live quality grade, and sex of the animal. The enzyme introduced into the animal in this manner remains dormant until the meat is cooked. Both proteases act directly on the components of muscle tissues. Papain acts on muscle fibers and connective tissues (collagen and elastin), and bromelain has no effect on muscle fibers and acts more intensely on the collagen than on elastin.

The contact enzyme-meat can be carried out through other manners, such as sprinkling the enzyme powder on the meat, dipping the meat in the enzyme solution, spraying an enzyme solution on or into the meat cut by means of an aerosol or injection systems. All such methods have limited applications on a high-scale tenderization process due to the non-uniform distribution of the enzyme in the tissues. The enzyme distribution occurs largely by diffusion, which depends on time, temperature, salt level, and enzyme concentration.

CONCLUSION

Proteases are largely used to hydrolyze proteins (such as collagen in gelatin manufacturing, collagen and elastin in meat tenderization, and k-casein in cheese making) and protein residues from food industry (such as bones and blood of slaughtered animals, and discarded fish). Proteases act at pH and temperature intervals from 4.0 to 10.0 and 50^{o}C to 70^{o}C, respectively. Regarding the pH, the proteases are classified as acid (4.0 < pH < 6.8), neutral (6.9 < pH < 7.3), alkaline (7.4 < pH < 8.5) and super-alkaline (pH > 8.5). The perspective of enzyme development for these uses is based on searching for sources thermally stable and/or specific proteases (carboxypeptidase and aminopeptidase), and immobilization of chymosin (aiming to transform the k-casein into p-k-casein in a continuous process). In dairy industry, lipases (esterases) and lactase are largely used.

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