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Understanding the Molecular Mechanisms of Proteases in Bioprocessing: A Review on the Future of the Food Industry

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Research Article

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ABSTRACT

Proteases are enzymes that break down proteins through peptide bond catalysis. A protease is a single polypeptide chain of some 250 amino acids and is devoid of sulfhydryl groups. The COOH-terminal tryptic peptide of the protease molecule contains some 43 residues. Because of this unique structure and mechanism, they play a crucial role in the bioprocessing industry, especially in food processing applications. Herein, the sources of proteases are discussed as plant-derived, animals-derived, and microbes-derived in the food processing industry, and each has certain unique characteristics: Plant-derived proteases exhibit alkaline pH optima, temperature stability, and lower allergenic potential, making them suitable for a range of food processing needs. Animal-derived proteases contribute to sensory effects in foods and exhibit versatility in functioning at extreme pH conditions and high or low temperatures. Microbes-derived proteases offer a wide pH range, thermostability, specificity and making them more valuable tools in food processing. Overall, the activity of proteases is influenced by several factors, including temperature, pH, substrate concentration, and the presence of inhibitors. Optimal protease activity is typically observed at alkaline pH and a temperature of around 37°C. The enzyme activity increases with increasing substrate concentration until saturation is reached. The presence of inhibitors can affect protease activity, necessitating their removal for accurate measurement. Current research has shifted toward the development of engineered enzymes with enhanced properties for food processing. These engineered proteases can exhibit improved stability and activity under specific conditions, leading to enhanced efficiency and specificity in protein degradation. Such advancements hold great potential for enhancing food production processes.

Keywords: Proteases; Food industry; Enzymes; Sources; Mechanisms; Food processing

INTRODUCTION

Proteases are a class of enzymes that catalyse the hydrolysis of peptide bonds within proteins. Peptides are remarkable catalysts of nature that play crucial roles in accelerating biochemical processes by breaking down proteins into smaller fragments. Enzymes importance spans an array of sectors from food, beverages to baking, detergent, medical, and textiles. In the food industry, proteases emerge as key orchestrators, shaping the transformation of raw materials into an array of consumable delights. Their enzymatic actions span across an array of activities including tenderization of meat, curding of milk of cheese production, enhancement of baking and brewing processes, and extraction of bioactive compounds from agricultural sources [1]. Proteases are sourced from all living organisms (animals, fungi, viruses, plants, bacteria, and humans) because of their diverse importance. Protease-producing microorganisms are found in various environments, including soil, wastewater, sludge, and undersea fumaroles. Harnessing the catalytic process of proteases in industries has the unique opportunity to not only optimize their operations but also contribute significantly to a more sustainable and harmonious coexistence with the planet owing to the fact that they are the most diverse enzymes used in bioprocessing. Proteases, on the basis of pH, are divided into acidic, neutral and alkaline. Among them, alkaline proteases are the most important for industrial purposes mainly due to their capability to withstand higher pH conditions.

The basic mechanisms of enzyme workings require that, for catalysis to occur quickly, lowering the activation energy is a necessity for binding to the substrate. To increase specificity, enzymes exhibit high substrate specificity so that binding occurs accurately in the active site, leading to an enzyme-substrate complex, while the pH depends on the types of enzymes involved. Protease displayed around 75% effectiveness within the pH spectrum of 7.0 to 9.0, showing full efficiency at pH 8.0. However, at pH 3.0 and 10.0, the protease effectiveness diminished significantly to 30% and 22%, respectively [2]. Overall, enzymes work better and faster at low temperatures between 32°C to 35°C. As the food industry seeks innovative ways to enhance both efficiency and product quality, an understanding of molecular mechanisms is paramount. This review seeks to add to the already established knowledge by simplifying the molecular mechanisms underlying the actions of proteases, their applications in the food industry as well as future trends and research directions.

LITERATURE REVIEW

Structure and mechanisms of action

The protease is a single polypeptide chain of some 250 amino acids and is devoid of sulfhydryl groups. The COOH-terminal tryptic peptide of the protease molecule contains some 43 residues. By catalysing the peptide bond of amino acid residues, proteases can break down long chains into shorter and smaller fragments. This happens because the peptide bond is found between the amino acids and helps to hold them together. During this action, those that detach from the chain are called exopeptidases while those that remain and invade the internal bond proteins are called endopeptidases [3]. Endopeptidases include chymotrypsin, pepsin, papain, trypsin, etc. The breaking of peptide bonds results in two nucleophilic processes.

Glutamine, aspartic, and metalloproteases facilitated breakdown of peptide bonds by water molecules leading to a process called hydrolysis. Glutamine proteases play a very vital role in intracellular protein degradation and processing. The well-known group of glutamine proteases is the cysteine cathepsins, which are involved in lysosomal protein degradation and various physiological processes. The catalytic triad such as serine, threonine, and cysteine proteases utilization of nucleophilic residue to form a covalent bond between the protease and the substrate protein. Following this, an acyl-enzyme intermediate is generated, and this intermediate is subsequently hydrolysed by an activated water molecule [4]. This two-step process finalizes catalysis, releasing the remaining portion of the product and restoring the enzyme to its original state (free enzyme). There are various classes of proteases, each with unique structural features and mechanisms of action. Some common classes of proteases include:

Serine proteases: These enzymes contain a serine residue within their active site, which acts as a nucleophile in the cleavage reaction. Examples include trypsin, chymotrypsin, and elastase.

Cysteine proteases: These proteases use a cysteine residue as a nucleophile to cleave peptide bonds. Papain and caspases are examples of cysteine proteases.

Aspartic proteases: These enzymes utilize aspartic acid residues within their active sites to catalyse peptide bond hydrolysis. Pepsin and cathepsin D are examples of aspartic proteases.

Metallo proteases: Metal ions, often zinc, are involved in the catalytic mechanism of metalloproteases. Matrix metalloproteinases and aminopeptidases are examples of this class.

Threonine proteases: These proteases have a threonine residue in their active site, which acts as the nucleophile in the cleavage process. Examples include the proteasome and HIV protease.

Amino acid sequence: The primary structure of a protease enzyme is composed of a specific sequence of amino acids. This sequence determines the enzymes catalytic activity and substrate specificity.

Active site: Protease enzymes contain an active site, which is a region within the protein where the catalytic reaction occurs. This active site typically consists of specific amino acid residues that interact with the substrate and facilitate the cleavage of peptide bonds.

Cofactors and coenzymes: Some protease enzymes require cofactors or coenzymes to function effectively. These small molecules may assist in substrate binding, catalytic activity, or overall enzyme stability.

Domain structure: Many protease enzymes have distinct structural domains that contribute to their overall structure and function. Domains may have specific roles in substrate binding, regulation, or interaction with other molecules.

Zymogen form: Some protease enzymes are initially synthesized as inactive zymogens (proenzymes) and require proteolytic cleavage to become active. This activation step helps prevent unwanted proteolysis within the producing organism.

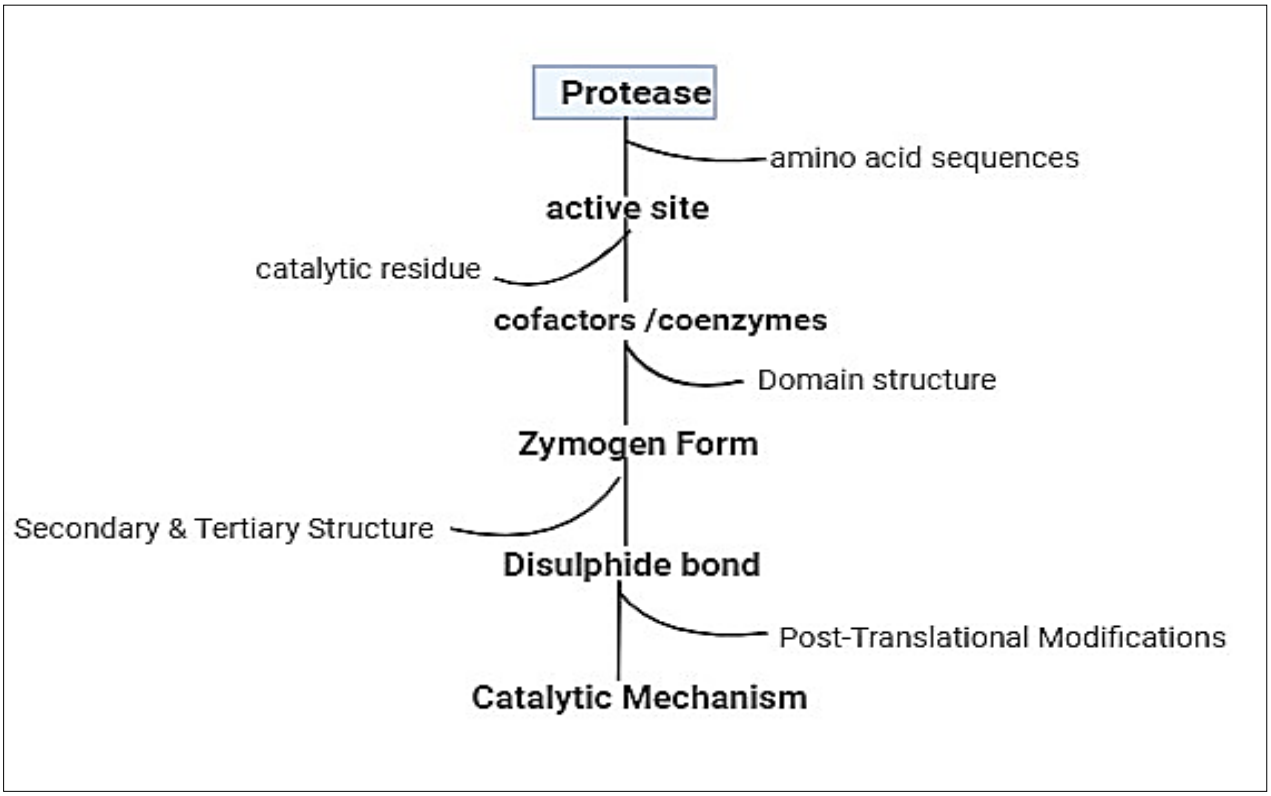
Secondary and tertiary structures: Protease enzymes have complex three-dimensional structures resulting from interactions between amino acid residues.

Disulphide bonds: In some cases, disulphide bonds between cysteine residues contribute to the enzymes stability and proper folding.

Post-translational modifications: Protease enzymes may undergo various post-translational modifications, such as glycosylation, phosphorylation, or lipidation.

Catalytic mechanism: The protease enzymes catalytic mechanism involves interactions between the active site residues, substrate, and water molecules. Different classes of proteases employ distinct mechanisms, such as serine proteases, cysteine proteases, aspartic proteases, and metalloproteases (Figure 1).

Figure 1. Shows the schematic diagram of protease structure.



Proteases in food production

Protease enzymes are used in food production to increase the stability of dough fermentation and moisture content, improve flavour enhancement, texture, colour, nutrient release, tenderization and extend the shelf life of foods etc., Here, we will discuss proteases used in the food industry that are plants-derived, animals-derived and microbes-derived [5].

Plants derived proteases

Plant-derived proteases have several unique characteristics compared to proteases derived from animals and microbes. These include optimum pH, Temperature stability, allergenicity, and substrate specificity.

pH: Plant-derived proteases often have a pH optimum in the alkaline range, typically around pH 8-10. This is in contrast to animal-derived proteases, which usually have a pH optimum in the acidic to neutral range, and microbial

proteases, which can have a wide range of pH optima depending on the source. The alkaline range is attributed to their adaptation to the plant cell environment which enables them to carry out their proteolytic activities.

Temperature stability: plant-derived proteases have thermal stability so they tend to be more stable at higher temperatures which makes them preferable during food processing that requires high temperatures. Their thermal stability is influenced by several factors such as,

Adaptation to environmental conditions: Most plants are exposed to various environmental stresses, which include high temperatures, contributing to their heat-resistant and stable to function effectively in their natural habitat.

***Structural features*:** Includes increased disulphide bonds, higher content of hydrophobic amino acids, and improved protein folding are capable of enhancing their resistance to denaturation at high temperatures.

Enzyme activation: Most proteases require high temperatures for their activation.

Substrate specificity

They often exhibit different substrate specificities compared to animal and microbial proteases. They may have a preference for specific peptide bonds or amino acid sequences, which can influence their effectiveness in different applications.

Allergenicity

Plant-derived proteases are generally considered to have lower allergenic potential compared to animal-derived proteases. This is an important consideration in industries such as food and pharmaceuticals, where allergenic reactions can be a concern. Examples of such enzymes include amylases, cellulases, xylanases, pectinases, bromelain and papain.

Animals-derived proteases

This group of enzymes holds immense prominence compared to those of plants and microbes. While there can be some overlap, certain features are more prominent in one group than the other. They can contribute to complex sensory effects in foods due to their precise and specific cleavage of proteins resulting in unique flavours, textures, and aromas [6]. In the cases of proper functioning, some animal's derived proteases are known to function at high temperatures (thermophilic), which can be of need during applications that involve cooking, baking, or other high-temperature treatments.

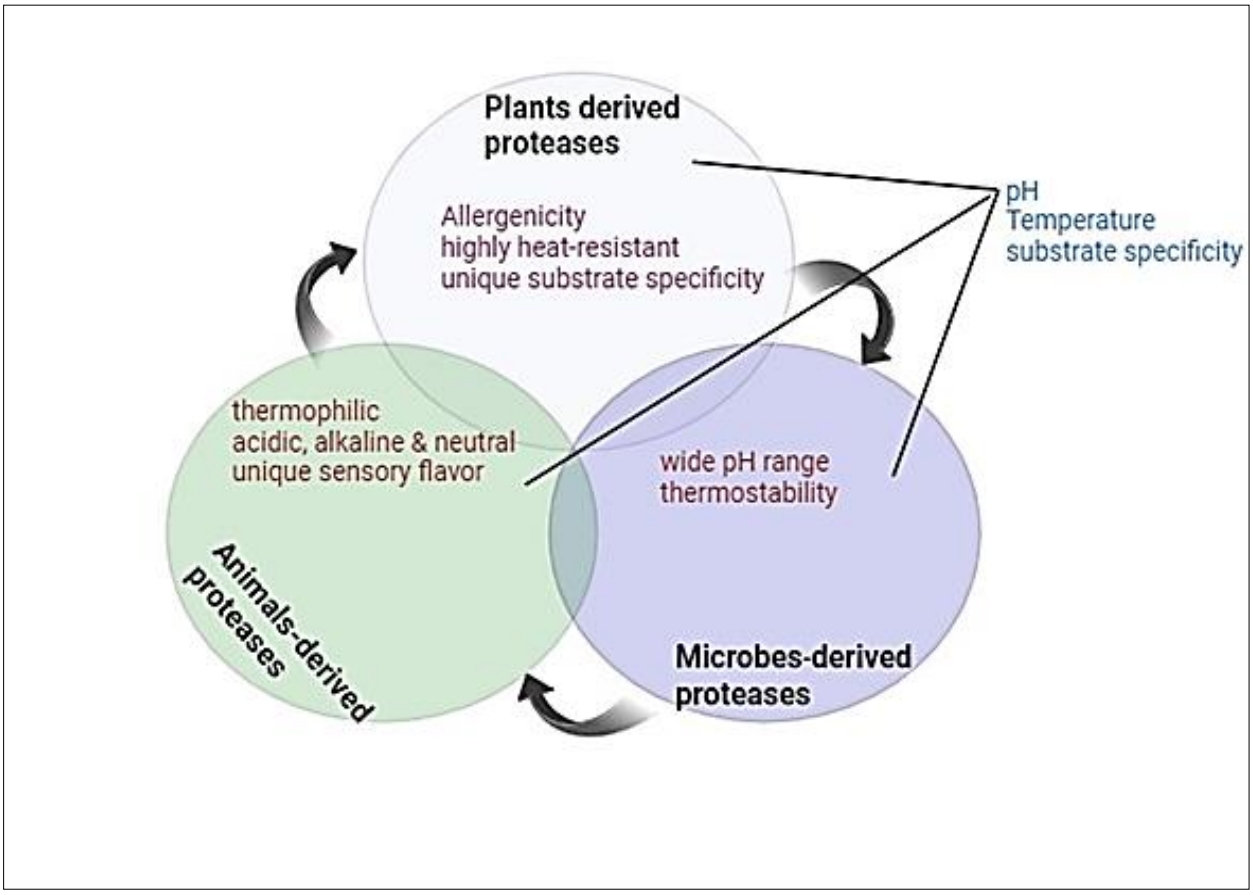
pH: In contrast to plants, some animal-derived proteases can maintain their activity under extreme pH conditions, both acidic and alkaline as well as in neutral conditions. This adaptability can be useful in food processing methods that involve pH changes during different stages of production.

Substrate specificity: This have evolved to be target-specific and can effectively cleave complex protein structures, leading to the generation of peptides with desired bioactivities. For example, proteases in the gastric juices of animals are specialized for digesting dietary proteins. This specificity can lead to unique sensory and flavour outcomes in food products. Examples are ficin, chymosin (rennin), trypsin, and thrombin.

Microbes derived proteases

This are widely used in various food processing applications, including meat tenderization, flavour enhancement, coagulation of milk, and protein hydrolysis. They also offer several unique characteristics. They exhibit a wide pH range, allowing them to function in different food processing conditions. They also possess thermos-stability, making them suitable for high-temperature processes [7]. Microbes-derived proteases are known for their specificity, enabling precise control over protein hydrolysis. Additionally, they can be produced on a large scale using fermentation techniques, making them economically viable for industrial applications. Genetic modification techniques can also be applied to enhance their properties or develop tailor-made proteases for specific food processing needs. Examples of such proteases include neutrase, savinase, alcalase, and flavourzyme sourced from *Bacillus amyloliquefaciens*, *Bacillus lentus*, and *Aspergillus oryzae* respectively. They are used in the production of protein hydrolysates, flavour enhancers, and meat tenderizers (Figure 2).

Figure 2. Illustrates the plant, animal, and microbes-derived proteases as well as their similarities like pH, temperature, and substrate specificity.



Applications of protease in food processing

Proteases find crucial roles in food processing as shown in the table below, their activities have been harnessed in different sectors of the food industry (Table 1).

Table 1. This table contains examples of some frequently used proteases in the food industry, organisms from which they are sourced and their mode of action using three sectors of the food industry.

Process	Enzyme	Source	Action
Tenderization	Papain	*Carica papaya*	Degrades myofibrillar and connective proteins
	Bromelain	Pineapple	Degrades myofibrillar proteins and breakdown under natured collagen
	Ficin	Figs	Degrades myofibrillar proteins and breakdown undenatured collagen
	Cathepsins B and Cathepsins D	Lysozyme	myofibrillar breakdown, weaken intramuscular connective tissue
	Actinidin	Kiwi fruit	Targets myofibrillar proteins in meat
	Ginger rhizome protease	Ginger rhizome protease	Peptide bond breakdown
Dairy industry	Chymosin	Animals (calf stomach) and microbial vegetable sources	Cleaves κ-casein, leading to curd formation
	Aminopeptidase	*Lactobacillus*	Secretes of single Amino acid residue
	Acid proteinase	*Aspergillus*	Milk coagulation
	Pepsin	Porcine stomach lining	Cleaves κ-casein, leading to curd formation
Baking and brewing industry	Papain	Papaya fruit	Hydrolysis of gluten proteins
	Amylase Proteases	Microorganisms (bacteria, fungi)	Breakdown of starch into sugars
	Flavourzyme	Microorganisms (bacteria, fungi)	Hydrolysis of proteins into peptides
	Neutrase	Microorganisms (bacteria)	Hydrolysis of proteins into smaller peptides
	Protamex	Microorganisms (bacteria)	Cleaves protein polyphenol complexes

DISCUSSION

Effects of temperature, pH, substrate and inhibitors in proteases activity

In reference to an experiment, the protease activity of the purified enzyme was found to be maximum at 37°C. This indicates that the enzyme exhibits optimal activity at this temperature. Measuring activity was measured at different pH values (4, 5, 6, 7, and 8) and it was found that the enzyme exhibited maximum activity at alkaline pH. The enzyme activity assay was conducted with different concentrations of β-casein as the substrate. It was

observed that the protease activity increased with increasing substrate concentration up to a certain point, after which it reached saturation. Furthermore, the use of inhibitors to remove protease inhibitors and EDTA before the protease assay indicates that inhibitors can affect protease activity and their removal is necessary for accurate measurement.

Similarly, the optimum pH for protease activity was found to be between 8.0 and 9.0. The enzyme showed a 25% increase in activity compared to pH 7.0. However, the hydrolytic activity decreased sharply at acidic pH values below 7.0, with over 75% of the original activity being lost [8]. The enzyme was more stable in the pH range of 7.0 to 10.0 when incubated for 1 hour, while the optimum temperature for protease activity was observed at 40°C, with substantial activity between 30°C and 50°C. At higher temperatures of 50°C and 60°C, the activity decreased to 97% and 85%, respectively. The enzyme was stable at temperatures between 40°C and 60°C, retaining more than 75% of the initial activity after 1 hour of incubation. In determining the protease activities using sulphanilamide azocasein substrate, one unit of proteolytic enzyme activity was defined as the amount of azocasein hydrolyzed during 1 hour of incubation at 37°C. Furthermore, the presence of these Zn^{2+}, Co^{2+} and Fe^{3+} ions reduced the hydrolytic activity of the proteases as an inhibitory effect.

Future trends in protease research

Proteases have diverse importance in other areas like cancer therapy and disease treatment, compared to their diversification in bioprocessing industries [9]. In the food industry, genetic engineering techniques have been employed greatly to engineer proteases with additional potential to improve the efficiency and specificity of protein degradation, leading to enhanced food processing and production. They can be designed to have specific properties that are desirable for food processing. For instance, to have enhanced stability and activity under specific processing conditions, such as high temperatures or low pH [10]. Common types of such enzymes are described below

- Savinase can be isolated from *Bacillus* and it is engineered to enhance flavor and hydrolysis.
- Flavorzyme possesses enhanced flavor, hydrolysis, and vigorous production of bioactive peptides.
- Neutrase is enhanced for flavor development and engineered to have a high degree of specificity for peptide bonds, allowing for controlled protein degradation and the production of peptides with specific functionalities.
- Alcalase is engineered to enhance its production of protein hydrolysates and peptides.

Research is likely to focus on developing proteases that can effectively target byproducts and waste generated during food production. Engineered proteases could help extract valuable components from waste materials, reducing environmental impact and enhancing the utilization of resources.

CONCLUSION

Away from food processing, the food sector is undergoing significant progress due to technological shifts that align with changing consumer preferences and eco-friendly concerns. Health-focused customers are increasingly interested in functional foods and nutraceuticals. Digital platforms and online sales are reshaping food distribution, and enhancing customer satisfaction. Food safety and transparency are being met through traceable supply chains and digital food management systems. Tailored nutrition is becoming more accessible through personalized diets and AI-guided suggestions. Robots and automation are simplifying restaurant operations and food production. 3D food printing is revolutionizing meal personalization and sustainability, while efforts are underway to minimize food

waste. These trends collectively reform the food industry, adding to the already established protocols when using proteases to foster more sustainable, effective, and consumer-centric approaches.

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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