An Overview of Papain Enzyme Characteristics, Applications and Production

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Abstract

Enzymatic processing, a longstanding practice in biotechnology, leverages enzymes as eco-friendly catalysts for biochemical reactions. Papain is one of the most significant cysteine proteases derived from the latex of the unripe papaya plant. Papain has gained significant attention for its diverse applications in the food, pharmaceutical, and cosmetics industries. The industrial-scale production of papain is essential to satisfy the growing global demand for this enzyme. This paper highlights the versatility and economic relevance of papain, providing insights into its characteristics, production methods, and expanding market trends in various industries while highlighting the synergy between natural resources and biotechnological advancement. For this purpose, we performed a comprehensive literature analysis of the papain enzyme, spanning from January 1961 to May 2024, with Google Scholar as our main source of information.

Keywords: Papain, Papaya, Cosmetics, Protease, Protein

Introduction

The fruit of the papaya trees yields the enzyme papain. The papaya (*Carica papaya*) belongs to the Caricaceae family, and its origin can be traced to the Caribbean coast of Central America. The plant family is native to tropical and subtropical regions, encompassing 31 recognized species in America. The most prevalent among these is cultivated primarily for economic purpose (Nordin, 2010).

It is postulated that early civilizations cultivated papaya around 1600 AD, at which time several species were cultivated in Malaysia (Nordin, 2010). In the sixteenth century, this fruit was cultivated in India. The fruit has a soft and weak texture, with a stem that is usually unbranched and soft. It has large clusters and long stem leaves, and it quickly grows, with some specimens reaching lengths of up to 20 meters (Tigist et al., 2016). The papaya plant exhibits a morphology similar to palm leaves, with large, uniaxial palms. Young plants exhibit

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a single stem during the initial one to two years of growth. However, large side branches may also be observed in areas with high fertility. The papaya leaf exhibits a deep edge, formed by a smooth and hollow petiole, which is supported. The mature papaya fruit bears a resemblance to that of the cantaloupe in terms of its appearance (Nordin, 2010). The unripe green papaya and the papaya tree contain several valuable enzymes, including papain, chemopain, caricaine, and glycerol endopeptide (Paul et al., 2013). The papaya plant is rich in valuable substances, including vitamins A, B, C, and E, antioxidants, magnesium, potassium, pantothenic acid, folate, and fiber beta-carotene. This compound has been shown to prevent damage caused by free radicals, which can lead to cancer. Additionally, it has been demonstrated to prevent heart disease associated with diabetes and to reduce blood cholesterol levels. Additionally, the leaves of this tree exhibit a multitude of beneficial properties. For instance, they contain a considerable quantity of alkaloids, amino acids, carbohydrates, steroids, glycosides, proteins, phenols, and flavonoids (Ayodipupo Babalola et al., 2024; Oliver-Simancas et al., 2024; Tigist et al., 2016).

The application of enzymatic processing has a rich and effective history within the realm of biotechnology. Enzymes, which are typically proteins generated by various living organisms, function as catalysts for biochemical reactions in living systems. Additionally, they serve as efficient catalysts under laboratory conditions and in industrial settings.(Jegannathan and Nielsen, 2013). Enzymes are regarded as a valuable alternative to chemical processes that contribute to environmental pollution. Every enzyme catalyzes a particular chemical reaction, some of which are impossible to accomplish by any other method. Currently, approximately 5, 500 enzymes are known, which are named based on the reaction they catalyze. The toxicity of the enzyme is usually decreased when it is released into the environment after being utilized in industrial production. Compared to conventional industrial procedures, these characteristics enable manufacturers to make items of equivalent or even higher quality while producing less hazardous waste (Jegannathan and Nielsen, 2013; Kant Bhatia et al., 2021). Enzymes are widely recognized for their ability to accelerate chemical reactions. They typically function by lowering the activation energy required for a reaction, thereby facilitating a faster process without modifying the enzymes' structure. Additionally, the enzymes remain unchanged during the course of the reaction (Dianaty and Nordin, 2010; Tigist et al., 2016). Advances in genetic engineering have enhanced and expanded the applications of enzyme technology, contributing to its development.(Beilen and Li, 2002). Enzymes are utilized in several industrial processes such as that produce pulp, leather, textiles, detergents, foods, beverages, animal feed, pharmaceuticals, desirable chemicals, cosmetics, and biodiesel require the use of enzymes. Enzymes are referred to as digestive supplements in the realm of food science and are utilized in the nutrition of athletes. Furthermore, it has been demonstrated that enzymes offer a range

of advantages in enhancing human well-being, with applications, the treatment of digestive disorders and weight management. Moreover, given the growth of the enzyme sector, it is conceivable that enzymes may become a viable investment (Nordin, 2010)

Figure 1 provides an overview of various industrial applications of enzymes: superoxide dismutase, protease, lipase, glutathione peroxidase, thiol peroxidase, glucose oxidase, and laccase peroxidase (Debata et al., 2021; Naveed et al., 2021; P. Choudhury and Bhunia, 2015; Janetzki et al., 2023; Jeon et al., 2021; Musameh et al., 2006; Choi et al., 2015; dos Santos Bazanella et al., 2013; Gaur et al., 2018; Shin et al., 2019; Jeon et al., 2021). Each of these enzymes has a distinct application within the industry (Choi et al., 2015; Kant Bhatia et al., 2021). Proteases are among the most valuable enzymes and account for around 60% of the entire enzyme market. Proteases, especially papain, are known as hydrolases and have the ability to hydrolyze peptide bonds. The spectrum of applications for proteases is extensive, with these enzymes present in all animal and plant tissues or microorganisms. These enzymes perform a variety of physiological functions, and thus, the most suitable protease can be selected for a specific process based on the type of hydrolysis or their ability to tolerate specific environmental conditions, such as temperature and pH (Beilen and Li, 2002; Ozhelvaci and Steczkiewicz, 2023; Tacias-Pascacio et al., 2021).

The global market of Papain

A heightened social awareness has a beneficial impact on the utilization of natural extracts. The demand for papain has increased in recent years due to changes in the biocatalysts industry and the growing market for natural extracts and plant products. The primary market for papain is divided into several regions. The global market for papain is distributed across the following regions (Figure 2): North America, Europe, Asia Pacific, Latin America, the Middle East, and Africa. North America is currently the largest consumer of papain, followed by Europe. In the Asia-Pacific region, the greatest increase in demand is observed in conjunction with an increase in meat and beer consumption. In the regions of Latin America, the Middle East, and Africa, wage increases have been observed, yet the demand for enzymes is not expected to reach the same levels as in other parts of the world. In 2019, North America accounted for 34% of the global papain market. The food and beverage industry represents the largest consumer of papain enzyme, with a growth rate of 4.4 % (Lapuente Salinas, 2021).

In this review, we have conducted an extensive literature analysis of the papain enzyme, covering the period from 1961 to May 2024, utilizing Google Scholar as our primary resource. The objective of this review is to explore the characteristics, applications, and purification methods of both native and recombinant papain enzyme production.

Characteristics and structure of papain enzyme

As previously stated, the papain enzyme is a proteolytic enzyme commonly derived from the juice of papaya plants. The greener

and more unripe the fruit is, the more active the papain enzyme is. The enzyme's primary amino acids are arginine, lysine, and phenylalanine. The unique architecture of this enzyme has allowed researchers to determine its function and explore its possible applications. The structure of papain is available in the Protein Data Bank (PDB) under the identifier 1CVZ (Figure 3). Upon examination of the figure, it can be determined that this enzyme has a molecular weight of 23,406 Daltons and comprises 212 amino acids, with three disulfide bonds. The catalytic residues are Gln 19, Cys25, His158, and His159 (Amri and Mamboya, 2012; Tsuge et al., 1999). Papain is initially expressed propapain, which necessitates various chemical alterations to transform it into its active form. Subsequently, the enzyme is cleaved into smaller components, referred to as subunits, which are subsequently assembled to create the active enzyme.(Paul et al., 2013). The enzyme possesses a free sulfhydryl group within the enzyme active site. This group is largely concealed within the crystalline structure of the enzyme, but it can be observed in stoichiometric quantities following the enzyme's activation with mercaptan or sodium borohydride. Oxidation using performic acid is necessary to convert the sulfur group into a stable cysteine residue. Alternatively, mercaptoethanol can be used in urea and alkylation of active sulfide groups with iodoacetate (Anfinsen and Haber, 1961). However, in recent studies to identify the sequence containing the group and determine the position of disulfide bridges from papain deactivated with carbon-14 iodoacetate, the catalytic active sulfhydrylation method has been employed. In this study, the sulfhydryl group in the active site of the enzyme was converted to carboxymethyl with an efficiency of approximately 53%. However,

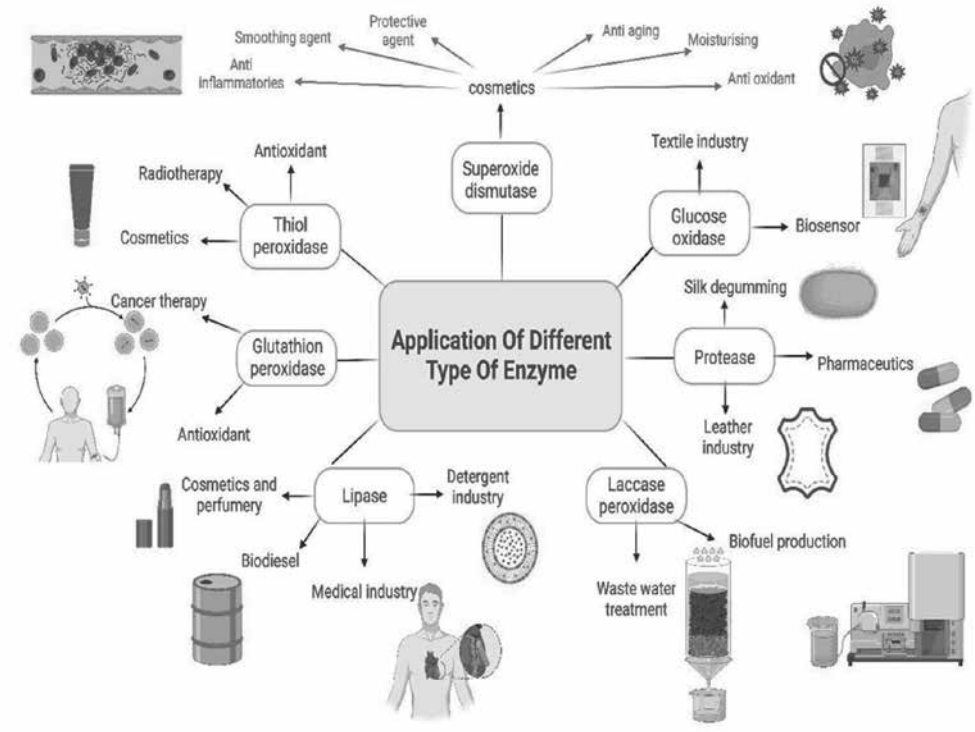

Fig. 1. The application of different types of enzymes (created by authors)

the presence of disulfide bridges has been shown to impose constraints on the structural properties of papain. Consequently, to overcome these limitations, the overall structure of the enzyme must be folded in a manner that allows for the accommodation of these disulfide bridges (Light et al., 1960; Ozhelvaci and Steczkiewicz, 2023).

The mechanism of action

The mechanism of action of the papain enzyme indicates that the enzyme breaks peptide bonds through a proton transfer from Cys25 by His159. The sulfhydryl group on Cys25 frequently forms a covalent bond with various enzyme substrates. His159 provides support for Cys25, while His159 maintains Arg175 in a stable state. His159 and Cys25 are involved in the catalytic mechanism

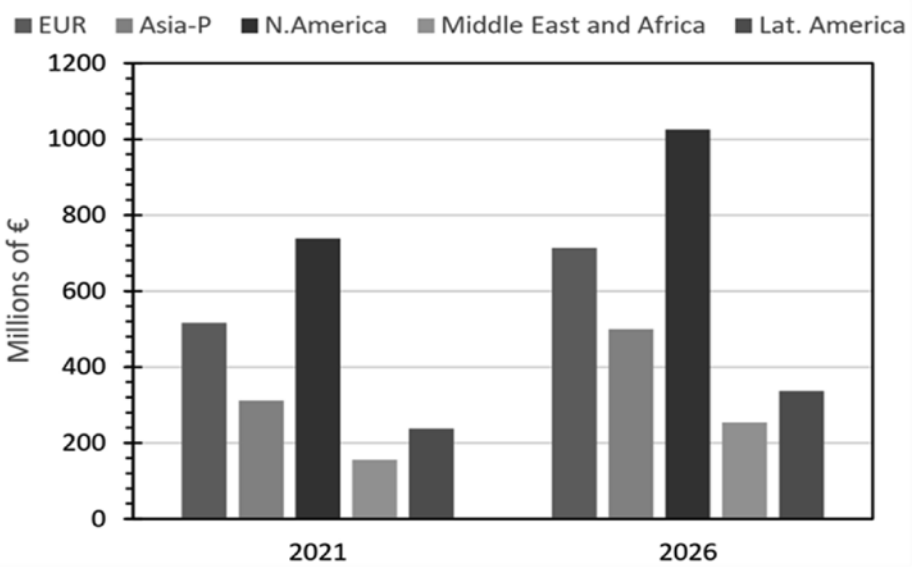

Fig. 2. The papain enzyme market worldwide across five regions, along with the consumption levels within the food industry for the year 2019, and a comparative analysis with projections for 2026

Fig. 3. 3D structure of papain enzyme in PDB under the code 1CVZ

of the active site. Cys25 functions as a nucleophile, initiating the attack on the carbonyl carbon of the peptide structure. This reaction results in the release of the amino peptide group and the formation of a covalent bond between the enzyme and the peptide. Subsequently, the enzyme is deacetylated by a water molecule, resulting in the release of the terminal carboxypeptide (Joshi, 2015).

Papain enzyme activity

To determine the activity of the papain can use different methods such as nitrophenyl ester (Paul et al., 2013), but the specific method for the assessment of enzyme activity is the utilization of Benzoyl-L-Arginine-Ethyl-Ester (BAEE). The speed of reaction is measured by UV absorption at 285 nm. As a result, one active enzyme unit for papain is defined as the activity resulting from the hydrolysis of 1 micromole per minute at 37 °C. Enzyme activity can also be measured simply by using chromatography and adding acetic acid (Nordin, 2010; Rinaldi et al., 2021).

Stabilization of papain

Most enzymes lose their biological activity at room temperature within one or more months. For this reason, enzymes must be stable after purification and purification. In the cosmetics industry, for instance, the products contain various surfactants and oils that can cause enzyme dysfunction. Enzymes need to be combined in various configurations to guarantee the stability of the products. Dextran, polyethylene glycol (PEG), and glucan can be utilized for this purpose. In this method, these materials are linked with the enzyme papain, thereby enhances its stability. The outcomes of the experiment were unexpectedly impressive, revealing that the enzyme activity achieved an efficiency ranging from 85 % to 95 %. Each of three substances contributed to an improvement in enzyme stability, observed at both ambient and elevated temperatures. However, glucan exhibited the most favorable results, maintaining enzyme activity at approximately 95% on average (even after one month of enzyme storage) at room temperature and 45 °C. The preservation of papain enzyme using PEG and dextran demonstrated 27 % and 42 % enzyme activity after one month, respectively (Sim et al., 2000). Research indicates that papain extracted from the sap of the papaya plant exhibit a semi-complete structure under low pH condition. The papain solution remains stable in the presence of several denaturing agents, and its complain activity can be well observed after recrystallization in 70 % methanol and 8 M urea (Nordin, 2010). Significant research has been conducted to immobilize papain enzyme, and several notable studies have been conducted in this area. One such study by Hastuti et al. employed alginate hydrogels for the purpose of enzyme immobilization (Hastuti et al., 2024).

Applications of papain enzyme

The papain enzyme has a wide range of applications in various industries, making its production in large quantities a matter of significant importance for those industries (Table 1). For instance, this enzyme is employed in the food industry to enhance the tenderness of meat during the production and processing stages. Additionally, it can be

employed in the pharmaceutical industry for the design and production of new drugs. The enzyme is employed in the fisheries industry to degrade peptide residues. Moreover, it is utilized in the cosmetics industry to synthesize natural-based cosmetics (Channamade et al., 2021; Tacias-Pascacio, Morellon-Sterling, et al., 2021). Additionally, it has applications in the production of nanocomposites in dentistry(Sharafeddin et al., 2024). In addition to the aforementioned applications, this enzyme is employed as a cleansing agent in the textile and leather industries (Syahdan et al., 2024), where it is utilized to remove excess hair from the skin of animals (Ahmed et al., 2024). Due to its antibacterial properties, this enzyme is also employed in medical applications (Yulirohyami et al., 2024). Furthermore, this enzyme is employed in the production and extraction of natural oils, including coconut oil (Jakfar et al., 2023).

Cosmetics industry applications

The cosmetics industry is consistently striving to innovate and offer consumers a diverse range of products. One notable trend is the growing preference for natural cosmetics, which are perceived as being safer for the environment. However, the use of chemical substances in cosmetic products has also been identified as a concern. These substances, such as iodopropenyl butyl carbamate, have been linked to allergic reactions. Butyl paraben, resorcinol, and ethylhexyl methoxycinnamate have been identified as endocrine disruptors, which may result in consumer exposure daily (Garcia and Gonçalves, 2021).

Increasing the amount of skin collagen

Type 1 collagen is a fundamental component of the extracellular matrix and is recognized as a pivotal element in maintaining the structural integrity of the second layer of the skin. Therefore, the aging of the skin can be attributed to a reduction in the quantity of type 1 collagen present in the dermis (Jung et al., 2007). The samples treated with papain exhibited a slightly higher amount of collagen, which may be attributed to the effect of papain on collagen proteolysis. This indicates that papain may enhance the firmness and elasticity of the skin (Banchhor and Saraf, 2008).

Hydrolyzing agent

Natural human fibroblasts undergo a limited number of divisions on the skin surface and gradually reach an irreversible growth arrest state. This process is known as replicative aging. While old fibroblasts are alive and fully functional, they exhibit numerous morphological and biochemical alterations in comparison to their younger counterparts. The use of papain enzyme, which hydrolyzes proteins and damaged connective tissue, has been reported to dissolve approximately 15 % of damaged connective tissue, resulting in a clear and bright appearance of the skin (Banchhor and Saraf, 2008).

Skin hydration

The capacity of the skin to retain water also increases with the increase in papain concentration. This may be due to an increase in pH levels. An increase in pH levels has been demonstrated to enhance the skin's water-holding capacity (MILES CL , 1970). Additionally, the water supply to the stratum corneum of the skin, along with the

Table 1. The application of papain enzyme

Application	Function	Reference
Cosmetics	Enhance collagen	(Banchhor and Saraf, 2008)
	Hydrolyze agent	(Banchhor and Saraf, 2008)
	Skin hydration	(Banchhor and Saraf, 2008)
Anti-tumor activity	Modify leukocytes	(Fazolin et al., 2020)
	Reduce swelling	(Banchhor and Saraf, 2008)
	Radioprotective	(Fazolin et al., 2020)
Anti-inflammatory	Increase absorbance of vitamins C and E.	(Shouket et al., 2020)
	Break the Fab fragment of the antibody to reduce pain and inflammation	(Shouket et al., 2020)
Ms patients	Destroy immune clot	(Krishnamoorthy et al., 2009)
	Increase Fab and useful in immunohistochemical	(Banchhor and Saraf, 2008)
Textile and leather	Removal of hair, and dust from wool before dyeing	(Lapuente Salinas, 2021; Shouket et al., 2020; Syahdan et al., 2024)
Fishery industry	Produce active compound	(Lapuente Salinas, 2021)
	Health promoter for human	(Tacias-Pascacio, Castañeda-Valbuena, et al., 2021)
Antimicrobial activity	Against a wide range of gram-positive and negative bacteria	(dos Anjos et al., 2016; Eshamah et al., 2014)
	Antifungal and anti-biofilm activity	(Mohamed et al., 2018)
Wound healing	with alginate maintains the optimal level of moisture	(Moreira Filho et al., 2020)
	Remove necrotic tissue.	(Dhivya et al., 2018)
	With bacteria cellulose	(Jurkevicz et al., 2024)
Dentistry	Production of nanocomposite	(Sharafeddin et al., 2024)
Food industry	Meat tenderness	(Barekat and Soltanizadeh, 2017)
	Degrade myofibrillar	(Barekat and Soltanizadeh, 2017; Shouket et al., 2020)
	Increase melting and elasticity of Nabulsi cheese	(Amri and Mamboya, 2012)
	Use to prepare sweet	(Amri and Mamboya, 2012)
	Chewing gums	(Shouket et al., 2020)
	Coconut oil	(Jakfar et al., 2023)

retention of intracellular water, is improved. Consequently, the utilization of papain enzyme in cosmetics lead to enhanced skin hydration and a more vibrant appearance (Banchhor and Saraf, 2008).

Antitumor activity

The enzyme papain has been demonstrated to enhance the immune system's function in the context of cancer treatment. This is achieved through its ability to control and modify leukocytes in response to the immune reaction. Additionally, papain has been demonstrated to reduce swelling and redness in the prostate, and it has functional properties against fungi. However, it is now widely accepted that this enzyme is an effective treatment for cancer. For instance, this enzyme is employed as a dietary supplement in the treatment of skin cancer. When combined with other enzymes, such as trypsin and chymotrypsin, papain has demonstrated significant radioprotective and antitumor effects in mice (Banchhor and Saraf, 2008; Fazolin et al., 2020). The Fab fragment, prepared using papain proteolysis, provides a basis for further genetic manipulation of the binding site, with the objective of improving tumor targeting. Papain has gained value as a supplement in the treatment of skin cancer and even to reduce the effects of radiation therapy and chemotherapy. It enhances the skin's ability to absorb nutrients, including vitamins C and E, and essential fatty acids, by increasing their bioavailability (Banchhor and Saraf, 2008).

Anti-inflammatory activities

A further function identified for this enzyme is related to its ability reducing pain and anti-inflammation for allergies, headache, and toothache, all without causing side effects for the user. Detrich (2012) employed this enzyme in the treatment of sports injuries, which resulted in the treatment of sports injuries, leading to a recovery period ranging from 3 to 8 days (Shouket et al., 2020).

Improving MS patients

Papain has been demonstrated to break up and destroy immune system clots that circulate in the blood (Krishnamoorthy et al., 2009). These clots are responsible for slowing the progressive myelin degeneration that is observed in multiple sclerosis (MS). This phenomenon improves blood circulation, promotes tissue repair, and aids in the transport of vital nutrients to the affected regions, while also expediting the clearance of waste products and bolstering the immune response. Stabilized papain serves as an optimal choice for generating Fab and Fc fragments of various IgG receptors. By examining the interactions between Fc receptors and immunoglobulins, researchers can investigate the phenomenon of body cell surface detection. The application of Fab fragments is beneficial in immunohistochemical studies (Banchhor and Saraf, 2008; Lehmann, 1992).

Textile and leather industries

Papain plays an essential role in numerous processes within the textile and leather industries. In 2013, Bushra and Tjul demonstrated that papain is employed in the removal of dust from wool and silk prior to dyeing. The leather industry encompasses several distinct processes, including soaking, dehairing, tapping, and tanning. Previously, chemical substances such as sodium sulfide

were utilized to purify leather, which has resulted in significant environmental and wastewater concerns. An alternative method is the use of enzymes such as papain, which have become commonplace. Currently, papain is employed in a variety of industrial applications, including the leather and fur industries, hair removal, leather tanning, and dyeing. Additionally, this enzyme is one of the most effective proteolytics in woolen fabrics and is highly beneficial for enhancing the visual appeal of this fabric (Lapuente Salinas, 2021; Shouket et al., 2020).

Fisheries industry

The papain enzyme is utilized extensively in the fisheries sector, where it plays a crucial role in the production of active biopeptides. In addition to their nutritional value as a source of amino acids, peptides have been shown to have beneficial effects on health. They have the capacity to engage directly with human metabolic pathways, functioning as health enhancers and contributing to the mitigation of the aging process. The remaining proteins of the fisheries and fishing industries, as well as the use of papain in their hydrolysis processes, serve as exemplary cases of efforts in this field. In addition, the incorporation of papain in this industry yields hydrolyzed products with a less bitter taste, a characteristic that expands their applicability within the food industry (Lapuente Salinas, 2021; Tacias-Pascacio, Castañeda-Valbuena, et al., 2021).

Antimicrobial property

One of the bacterial species that has been studied for its susceptibility to the antimicrobial properties of papain is several species of Alicyclobacillus. This is a gram-positive, thermophilic, acidophilic, and non-pathogenic bacterial species that is capable of forming spores. These bacteria are commonly found in soil and are often associated with the deterioration of acidic products, such as juices and beverages. Additionally, the antimicrobial activity of the extract was observed against Staphylococcus aureus and Listeria monocytogenes. Notably, the hydrolysis of papain occurs within approximately 120 minutes, resulting in a mixture that inhibits the growth of most Gram-positive and negative bacteria. Nevertheless, other studies have identified antibacterial activity against *Bacillus subtilis*, *Enterobacter cloacae*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhi*, *Staphylococcus aureus*, and *Proteus vulgaris* (dos Anjos et al., 2016; Eshamah et al., 2014). The research conducted by dos Anjos et al., (2016) involved the measurement of the minimum inhibitory concentration (MIC) and the minimum concentration to kill bacteria (MBC) of papain. The minimum inhibitory concentration (MIC) for various strain of Alicyclobacillus was determined to range from 0.98 to 3.91 μg/ml. Regarding the minimum concentration required to eradicate bacteria among different strains, the range is from 3.91 to 15.60 μg/ml. Furthermore, another antimicrobial property against *Klebsiella pneumoniae* bacteria has been identified. *K. pneumoniae* is a gram-negative, non-motile, enclosed, lactose-fermenting, facultative anaerobes bacterium belonging to the Enterobacteriaceae family represents the second most prevalent element of the intestinal aerobic bacterial microbial flora

and is the most common cause of hospital and community-acquired infections. They exhibit a remarkable ability to withstand various environmental conditions, including the presence of antimicrobial agents. To evaluate the efficacy of papain enzyme against this bacterium, its activity was assessed in terms of biofilm removal at varying concentrations. The most significant reduction occurred at a concentration of 100 mg/ml, where various strains of *Klebsiella pneumoniae* exhibited varying levels of inhibition. At this concentration, the capacity to inhibit biofilm formation ranged from 21.51 to 58.85 %, while the ability to eliminate and eradicate Kenny biofilm ranged between 45.9 to 55.07 % (dos Anjos et al., 2016; Mohamed et al., 2018; Tacias-Pascacio, Castañeda-Valbuena, et al., 2021; Yulirohyami et al., 2024).

Wound healing

Active dressings are defined as dressings that are designed to transfer molecules that facilitate wound healing or are composed of materials that possess endogenous therapeutic activities. These materials include natural polymers such as chitosan, collagen, bacterial cellulose, fibroin, and alginate. One advantage of using these active compounds on polymer matrices is that the drugs can be released in a controlled manner. This alginate, in conjunction with papain, demonstrates the capacity to facilitate the healing process by removing excess alginate and maintaining the optimal moisture balance. This makes it an attractive candidate for use as a wound dressing. Currently, this enzyme is available on the market as an ointment and gel specifically designed for wound healing. Its potential application in the treatment of wounds and burns has also been identified. This efficacy is attributed to its bactericidal and anti-inflammatory properties, which facilitate the healing process. It also removes necrotic tissue and dead cell parts, which accelerates the healing process (Moreira Filho et al., 2020; Tacias-Pascacio, Castañeda-Valbuena, et al., 2021). Additionally, the combination of papain and alginate represents an effective approach for the removal of necrotic or dead tissue at the wound area. In other words, alginate serves as a surface for the immobilized papain enzyme. Once it is stabilized, papain retains its enzymatic activity, safeguarding by the alginate. This process enhances blood circulation to the injury, thereby accelerating the healing process. Subsequently, the wound then undergoes the healing process (Dhivya et al., 2018; Moreira Filho et al., 2020). In 2024, Jurkevicz and colleagues developed an effective platform for the application of papain enzyme as a wound dressing by extracting cellulose from the bacteria responsible for its production. This innovation proved to be highly beneficial in alleviating pain and inflammation at the wound site (Jurkevicz et al., 2024).

Food industry

Papain is employed in a multitude of applications within the food industry. One of the most important characteristics of meat is tenderness, which strongly affects its consumer acceptability. The proteolytic enzymes, including papain, as well as the loss of connective tissue compounds, especially collagen, are responsible for the tenderness and softness of meat, which in

turn increases customer satisfaction. This enzyme can degrade myofibrillar proteins and connective tissue. Research conducted by Wang and Maynard demonstrated that immersing freeze-dried pork pieces in a papain solution significantly enhances the tenderness and flexibility of the muscle(Barekat and Soltanizadeh, 2017). Papain is a valuable component of the food industry, serving as a softener comparable to collagenase. Additionally, this enzyme can be employed as a clarifying agent in the food industry, enhancing the melting and elasticity of Nabulsi cheese, which has a fibrous structure, utilized in the preparation of kenafa and various confections (Amri and Mamboya, 2012). Papain has been employed at various concentrations (0.25, 0.50, 0.75, and 0.1 µg/ml) to soften chicken legs (Syahdan et al., 2024), with a concentration of 0.25 µg/ml demonstrating tender meat compared to the control group. Additionally, papain can be employed as a softener in chewing gums and production of herbal oil (Jakfar et al., 2023; Shouket et al., 2020).

Precautions

Overuse of the usual dose and allergic reactions

Exceeding a daily intake of 400 mg of the substance is not recommended, as it may lead to irritation of the gastric and pharyngeal regions. To reduce the risk of adverse effects, it is advised that the dosage be distributed across multiple meals. It is contraindicated for children, pregnant women, and lactating mothers to use this product (Lapuente Salinas, 2021; Shouket et al., 2020). Additionally, there is evidence of allergic reactions in humans and animals when consuming papain. Allergic reactions have been documented in the use of skin creams, eye lenses, and shampoos containing this enzyme. However, allergic reactions can also be caused by the direct consumption of papain as a food supplement. The allergic reaction in the majority of individuals manifests as hives, although a small proportion of individuals also experience respiratory allergies. In any case, these reactions manifest in a small percentage of the consumer population. Consequently, it is imperative for manufacturing companies to include contraindications on the packages (Lapuente, 2021).

Papain and other drugs and diseases

It should be noted that this enzyme should not be used in individuals who are taking anticoagulants, particularly Coumadin and Plavix, as it has the potential to slow blood clotting. Furthermore, consumption of papain is contraindicated in individuals with diabetes, as it has been demonstrated to lower blood sugar levels and precipitate hypoglycemia. Individuals undergoing surgical procedures are advised to cease papain consumption two weeks prior to the procedure to prevent excessive bleeding (Lapuente Salinas, 2021).

Extraction and Purification of Papain Enzyme

Papain extraction

Numerous techniques have been proposed for the extraction of papain enzyme from the fruit of the papaya plant. In general, mature green papaya fruits are collected early in the morning, after which the white juice is extracted by cutting the skin. The procedure of crushing and pounding the

fruit commences in the early morning and concludes in the mid-morning, a time characterized by elevated humidity levels, a period when humidity is high, as the flow of plant sap diminishes in condition of low humidity). It is recommended that the fruits be harvested between four and seven days after they have reached maturity. A thin slice is sufficient to extract the juice. Subsequently, the plant sap should be stored in containers made of stainless steel and in the shade, as this environment minimizes the enzymatic reactions that result in the loss of valuable enzymes in plant sap. It is essential to emphasize that a 2 mm stainless steel blade should be utilized for cutting, as this facilitates the blending of the plant juice with the starch-rich fruit juice, ultimately producing a juice of comparable quality to that of the fruit juice. Prolonged exposure can adversely affect the plant. Before storage, plant juice should be preserved in a sodium hydroxide solution at a temperature of minus -20 °C to prevent oxidation (Dubois et al., 1988; Paul et al., 2013). Additionally, it is crucial to complete the collection of plant sap within a maximum of 1-2 minutes, as extending this duration may lead to contamination and a decline in enzyme activity. However, more recent techniques have been developed to extract papain from plant sap. One such method is two-phase extraction in an aqueous medium, which results in a higher degree of papain purity in a significantly shorter time than the previous method. This method asserts that the extracted papain is free from contamination and protease activity. Even in the present era, methodologies have been proposed for the extraction of papain enzyme from plant sap without the use of salt or thiol, and instead through the utilization of natural inhibitors that are present in the sap of the plant. The enzyme is then extracted. Papain can also be extracted from the fruit's skin. In this method, the fruit skin is first cut into small pieces and then dried at a temperature of 55 ºC. The chopped skins are then dissolved and immersed in a large mixer with distilled water. The resulting solution is filtered and centrifuged, resulting in the purification of the papain enzyme in a pure form (Abdeldaiem et al., 2019; Jain and Student, 2020).

Purification of papain

One of the major challenges currently facing scientists is the purification of papain enzyme. This work necessitates the use of highly precise techniques and well-equipped devices. Purification serves to reduce contamination and adjust the concentration of the various substances that comprise the enzyme, thereby increasing the specific activity of the enzyme or protein. Once the crude enzyme extract has been prepared, a range of purification methods are employed (Table 2). The resulting extract is then subjected to fractionation, which involves the separation of proteins into distinct fractions. In the initial stages of this process, the researchers employ a complex function of pH, temperature, salt concentration, and other factors to determine the difference in protein solubility (Jain and Student, 2020).

Purification by sedimentation method

This method is a straightforward approach to protein purification. Ammonium sulfate salt is typically employed in the precipitation

method due to its high solubility in water and its capacity to interact with a multitude of water molecules. Nitsawang et al. (2006) conducted a study in which the method involved in two steps. In the initial stage, 45% saturated ammonium sulfate was employed, while in the subsequent stage, sodium chloride was utilized. The results of this method demonstrated that unrefined papain exhibited a maximum activity of 39%, which increased to approximately 86-90% following purification. However, in addition to the two salts identified, chemical reagents can also be employed for precipitation. Nitsawag et al. (2007), demonstrated that ethanol alcohol can be employed for this precipitation process (Jain and Student, 2020; Nitsawang et al., 2006).

Purification by reverse micellar method

Purification by reverse micellar method allows for the purification of all proteins or enzymes. It facilitates the dissolution of the protein in the aqueous medium while the remainder remains in the organic phase. The reason for this is that micelles are thermally stable and nanosurfactant in size, encapsulating a small amount of water in aqueous solutions. This method was first described by Juang and Mathew (2005). The extraction of raw papain was conducted using a sample and a similar amount of aqueous and organic solution (Lee and Chong, 2011). Following this, the mixture was then centrifuged and extracted. The upper phase of the solution was subjected to re-extraction, during which the new aqueous phase included 10 % isopropyl and tri-n-octyl dimethylammonium surfactant (Jain and Student, 2020).

Purification by two-phase method in water

Biphasic extraction in water is employed to purify protease from crude extract, as it is a selective method in downstream processes. Nitsawang et al. (2006) demonstrated that this method is a superior alternative to salt purification for papain. Liet et al. (2010) purified papain by creating a system in tubes comprising an enzyme solution, polyethylene glycol, salt solution, and deionized water. The raw plant sap was purified. In 2013, modifications were made to this method, and the enzyme solution, comprising tetrabutylphosphonium bromide and potassium phosphate, was added to the column. Additionally, A 0.05 M NaCl solution was incorporated to rectify the ionic strength, and the concentration, temperature, and pH were set to 150 mM, 30 °C, and 7.5, respectively (Benavides et al., 2008; Jain and Student, 2020).

Purification by three-phase method

This method represents a straightforward and expeditious approach to protein purification and concentration. In 2010, Chaiwut, Pintathong, and Rawdkuen employed this method to purify papain. In the initial stage of the procedure, ammonium sulfate and butanol were employed. Subsequently, the lower phase was subjected to treatment with a butanol and ammonium sulfate solution, resulting in a concentration of approximately 55% (w/v) (Dennison and Lovrien, 1997; Jain and Student, 2020).

Purification by drying method

The drying method represents the primary determinant of the final quality of papain. Subsequently, the vegetable sap is spread in stainless trays and dried at 55 °C for

approximately four hours. It is then ground with potassium meta bisulfate and in a mill and roller until it becomes powder. According to the Science Company, the drying process continues until the product is separated into sheets with a porous structure (Baines and Brocklehurstt, 1979).

Sun drying is the least effective method for producing a high-quality papain enzyme. It reduces enzyme activity to a significant extent and causes the enzyme to turn brown. However, sun drying is still a common method for purifying papain enzyme in many countries. The sap is then poured into large trays and left to dry in the sun. The activity and quality of raw papain that has been dried in the sun increases with the use of preservatives. The application of preservatives has been demonstrated to enhance the visual appeal, olfactory profile, chromaticity, and enzymatic activity of the papain enzyme compared to a control sample. However, the treatment with sodium meta bisulfite at a concentration of 0.1% has been found to yield the most favorable outcomes. The addition of 1.0 % benzoic acid to sun-dried raw papain resulted in an improvement in both appearance and color, compared to sodium benzoate. However, sodium benzoate was found to enhance enzyme activity. Another potential solution is drying in an oven. For instance, in Sri Lanka, clay ovens with a height of approximately one meter are utilized. The duration of the drying process varies, with an average of four to five hours at a temperature of 35 to 40 ºC. Subsequently, the dried product should be stored in closed and light-proof containers and a cool environment. Another drying method is spray drying (Lester et al., 2004), which is not feasible on a small scale. Papain that has been dried in this manner exhibits greater enzyme activity than other types of papain and is completely soluble in water. It is of the utmost importance to exercise caution when handling this type of papain, as inhalation of the enzyme can lead to the development of allergies and potentially fatal lung diseases. Consequently, papain that has undergone this process is encased in a gelatin coating and then subjected to laboratory testing to ascertain its proteolytic activity (Paul et al., 2013).

Purification by Sephadex G-75 method

Monti and Carmelita posit that plant sap is immediately utilized for papain purification, or it is stored at -8 °C in a nitrogen atmosphere following extraction. Subsequently, to prepare an EDTA extract with a pH of 7, the plant juice is added to the preparation, which is then carried out with continuous shaking under nitrogen. Subsequently, the suspension is subjected to centrifugation for a period of 30 minutes, after which its optical absorption is measured at a wavelength of 280 nm. Thereafter, its molecular weight is determined (Paul et al., 2013).

Recombinant papain enzyme Recombinant proteins have been widely used for decades in various fields, such as determining enzyme activity or investigating new drug targets (Jame-Chenarboo et al., 2022; Nekouei et al., 2018). One of the most effective methods for producing papain enzymes is genetic engineering and biotechnology. A summary of some of these types of recombinant enzymes can be found in Table 3 below.

Table 2. Different type of papain purification

Method	Substance	Reference
Sedimentation	Ammonium sulfate	(Jain and Student, 2020; Nitsawang et al., 2006)
Reverse micellar	Isopropyl Tri-n-octyl dimethylammonium	(Jain and Student, 2020; Lee and Chong, 2011)
Two-phase in water	Polyethylene glycol Deionized water Tetrabutylphosphonium bromide Potassium phosphate	(Benavides et al., 2008; Jain and Student, 2020)
Three-phase	Ammonium sulfate Butanol Potassium meta bisulfate	(Dennison and Lovrien, 1997; Jain and Student, 2020)
Drying	Benzoic acid Sodium benzoate Sodium meta bisulfite	(Baines and Brocklehurstt, 1979; Lester et al., 2004; Paul et al., 2013)
Sephadex G-75	EDTA Nitrogen atmosphere	(Paul et al., 2013)

Table 3. Reported studies on recombinant papain enzyme

Article	Year	Reference
Cloning and sequencing of papain-encoding cDNA	1986	(Cohen et al., 1986)
Active papain from insoluble recombinant propapain	1992	(Taylor et al., 1992)
papain-like cysteine protease from the inclusion body	2004	(Brömme et al., 2004)
Production of recombinant propapain with high yield	2009	(D. Choudhury et al., 2009)
papain-like cysteine protease from inclusion bodies of *E. coli*	2015	(Ling et al., 2015)
Codon-Optimized *Carica papaya* Papain Sequence in the Methylotrophic Yeast Pichia pastoris	2015	(Werner et al., 2015)
Design of Recombinant Papain-Like Cysteine Protease	2017	(Gorokhovets et al., 2017)
Extraction, sequence alignment of recombinant papain	2024	(Asian and Biotechnol, 2024)

Conclusion

Over the past few decades, enzyme-based processes have been progressively replacing traditional chemical processes in a multitude of fields, particularly in the chemical and pharmaceutical industries. The advent of novel enzyme engineering technologies, coupled with mounting economic pressure and growing public concern about environmental pollution, will accelerate the replacement of traditional chemical processes with enzyme-based alternatives. Consequently, researchers will have the opportunity to discover new applications and technologies in the field of enzymes.

One of the most significant categories of enzymes is that of proteases. These enzymes possess several distinctive characteristics, including rapid action, activity in mild environmental conditions, high specificity, and biodegradability. Considering the challenges currently facing the enzyme production industry, proteases offer a significant opportunity for industrial-scale solutions to the challenges that lie ahead. One of the proteases is the papain enzyme. This enzyme is distinguished by a unique structural configuration that confers a distinctive functional profile. This enzyme is naturally derived from the papaya plant. The plant in question is indigenous to tropical climates and offers significant economic benefits to farmers. The enzyme's efficacy is contingent upon its continued activity. In order to achieve this goal, it is necessary to purify the enzyme. Various techniques have been proposed for this purpose. All of these methods enhance the selectivity of the enzyme to perform different processes. In general, this enzyme is employed in a wide range of fields. Papain plays a pivotal role in numerous industries, including food, pharmaceuticals, cosmetics, detergents, textiles, and leather.

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