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Cysteine Proteases from *Carica papaya*: An important enzyme group of many industrial applications

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Abstract:*Cysteine proteases (Thiol Proteases) are specific group of proteases which carry nucleophilic cysteine thiol in their structure. They are usually obtained from different tropical and subtropical fruits such as papaya, kiwifruit and pineapple. This group of enzyme gain more interest nowadays based on their wide range of applications in textile, food, and medical industries. This review summarizes the recent development of the production of these enzymes using papaya (Carica papaya) as main source of this group of important biocatalysts. Itcovers also the new trends applied for extraction, purification, and modification of this enzyme group. In addition, the new extended applications of cysteine proteases were also discussed as well.*

Keywords: *Cysteine Protease, Papaya, Carica papaya, enzyme extraction, purification*

I. Introduction

Enzymes are key elements in our life through governing many metabolic pathways in all living organisms. In addition, nowadays, they play great roles in many agricultural and industrial applications. Based on their wide range of biocatalytic properties they are widely used in biorefinery, feed, and food industries, and pharmaceutical industries [1-7]. In addition, they also important biocatalysis for the production of bioactive molecules for the development of new types of more efficient antibiotics [8-10]. Proteases are among the widely used enzymes worldwide in different applications based on their wide range of catalytic properties and serve in many industries [11-12]. Of these, cysteine proteases which are also known as thiol proteases are enzymes that primarily function in degrading and breaking down protein structures. Cysteine protease is one of the well-studied proteolytic enzymes in plants with action similar to that of pepsin in gastric juice. They possess immense biological value and could be found in virtually every bacteria, eukaryotes and viruses. Currently, the MEROPS protease classification system has listed out fourteen cysteine proteases super-families with several others yet to be assigned. Of these different super-families of the enzyme, the one with plant origin especially extracts of the tropical plant *Carica papaya* containing latex consisting of a cysteine endopeptidase mixture such as papain, papaya endopeptidases III, papaya endopeptidase IV, caricain, chymopapain A and B, and papaya proteinase IV which constitute more than 80 % of the whole enzyme fractions are considered to be of highest commercial significance due to their various applications in different industrial processes [13-15]. Compared with other enzymes, Papain has been the most extensively studied and more easily purified despite it being a minor constituent totaling approximately 8 % among the papaya endopeptidase [16]. From the aforementioned list of high potential enzymes extracted from *Carica papaya*, Papain is the current main source of empirical data for the cysteine proteases super-family with the most extensive characterisation in regards of its kinetic data as well as the structural viewpoint of the enzyme. Papain and related cysteine proteases are widely distributed in the plant kingdom and some are also found in the bacteria which are known to be virulent and primarily acting as defense factors for the hosts and pathogens [17]. Papain is the eponym of the C1 family of proteases which belongs to clan A of the cysteine protease. This enzyme contains a large number of endopeptidase and fewer exopeptidase. Interestingly, Papain has been recorded as the first protease where its crystalline structure was determined by X-ray methods and then refined to its three-dimensional structure which was determined at a resolution of 1.65 A resolution in 1984 and regarded as one of the archetypes in this family of enzymes. [16,18]. Structurally, a papain molecule undergoes folding producing two compact interacting domains. Papain cysteine protease is the most numerous family of the cysteine protease class with the size of 23.4 kDa with an isoelectric point of 9.5 consisting of 212 amino acid with four disulfide bridge and catalytically important residues in the following positions: Gln19, Cys25, His158, and His159 [19-21]. All members of the papain family contain cysteine and histidine at their active site, forming a catalytic dyad. The mechanistic geometry of its active site was reviewed for the first time in 1982 [16]. The catalytic residues Cys25 and His159 are found to be located at

the opposite end of a cleft delimiting the surface between these two domains. The former is part of an α-helix structure of one domain whilst the latter is contained within a β-sheet of the other domain's surface.

Carica Papaya The Main Source Of Cysteine Protease

Carica papaya is a botanical name of papaya, a type of tropical fruit tree from the species in the genus carica of the plant caricaceae. The papaya is a tree-like, large plant, with a single stem growing from 5 to 10 (16 to 33 feet) tall, with large leaves around 50 to 70 cm in diameter and spirally arranged concisely to the top of the trunk. The tree is usually unbranched with seven, deeply palmately lobes on the leaves [22, 23]. Young leaves of papaya have high value of phytochemicals which decreases as the leaves matured. The study on the papaya leaf extract on the anticancer properties has been reported as it can inhibit the activity of tumour cell lines. The leaf extracts were shown to significantly inhibit the proliferative responses of solid tumour cell lines derived from cervical carcinoma, breast adenocarcinoma, hepatocellular carcinoma, lung adenocarcinoma, pancreatic epitheloid carcinoma, and mesothelioma [24]. The matured flowers that appear on the axils of the leaves will grow into large fruits that are considered ripe when the skin has attained amber to orange blue and the fruits feel soft [25,22]. Papaya fruits can form in various shapes whereby fruits from female tress are more spherical compared with the hermaphroditic tress depending upon modifying factors that affect the flower morphology during ontogeny. Fruits can be in shape of pyriform (pear shaped), spherical, or cylindrical with the pyriform hermaphrodite fruit as the most common in trade [26]. The fruit is highly appreciated worldwide for its flavour, nutritional qualities and digestive properties. Analysis on the nutritional content of 100 g of papaya fruits shows that it contains 88.06 g water, 0.47 g protein, 0.26 g fat, 10.82 g total carbohydrate, 1.7 g fiber and contain highest carotenoids, potassium, fibre, and ascorbic acid content per serving among other fruits [27]. Papaya is not only used as a food and in cooking aid but the other parts such as stem and bark may also be used in rope production. Furthermore, the whole plant parts including fruits, roots, bark, peel, seed, and pulp are also known to have benefit in medicinal properties due to its high content of vitamin A, B, C, minerals, potassium, magnesium, folate, fibre, and proteolytic enzymes [22]. Green papaya fruit, leaves and bark's latex are rich in enzyme known as papain, a protease which is usually extracted, dried and used as chewing gum for digestive problems, toothpaste, and meat or other proteins tenderizer. Moreover, this enzyme is also strongly believed to have antifungal, antiviral, and antibacterial properties as well. According to Lu,[23] these proteolytic enzymes can be found in younger plants than the older ones. The papaya fruits will take around 22-24 weeks to develop and in countries with colder climate, the development of fruits may take 2-3 weeks longer than normal. The total fruit starch will start to decline during the first 11 weeks and sugars, however, will start to accumulate during the last 4-6 weeks of fruit development. The fruit is climacteric and yellow skin was developed from the stigma end and endocarp outward will form and develop the internal flesh colouring and softening [26]. Fruit softening is a major factor that determines the quality and marketing of the papaya. The ripening of the fruits involves a complex series of physiological and biochemical modifications, changes in cell wall structure, flavour, aroma, and pigment biosynthesis. The criterion to assure the ripening, shelf life, and adequate sugar accumulation inside the fruits is depend on the yellow colour that appear in the fruits skin [28, 26]Table 1 summarises the different stages of papaya maturity

Table 1: Stages of papaya maturity according to the skin colour when stored at room temperature (20-24 °C) [24]

Maturity stages	Score range
0	Fruit completely developed. 100 % green skin colour.
1	Represent papaya with yellow colour cover area of between 1 % until 25 % of the skin fruit surface.
2	Represent papaya with ¼ mature. Fruit skin with up to 50 % until 75 % yellow covered area.
3	Represent papaya with ¾ mature. The fruit surface covered with yellow area with up to 50 % until 100 %.
5	Mature. Fruit covered with 76 % yellow colour or totally yellow colour and only area near the stem is green.

Extraction Of Papain From Papaya Latex

Plants usually develop their own defence mechanism strategies to protect themselves from predators and pathogens as they exposed to a variety of environmental stresses. Papaya, which is a latex containing plant, used this as its strategy to defend against pathogens. Latex released out by mechanical wounding will rapidly coagulate effectively sealing the wounded area to protect the fruit from the entry of pathogens which could later damage the phloem cells within [29].Papaya latex is a milky-like thixotropic fluid (exhibiting a stable form at rest but becoming less viscous when stressed) that contains about 15 % of dry matter in which forty present of the dry matter is constituted by enzymes mainly cysteine endopeptidase where altogether they account for more than 80 % of the whole enzyme fraction. Papaya endopeptidase stored in latex as an inactive preform. These enzymes will change rapidly into active mature enzymes after the release of latex from the plants [16]. The latex usually coagulates almost immediately upon release, unless it is brought to a high pH upon collection [30].

Latex is obtained by tapping; a process where the skin of the unripe papaya is cut and latex which flows from the cut collected and dried. According to Amri*et al.* [20], the greener the fruit, the more active is the papain. The collection of latex starts with the single wounding of unripe fruits at the plant with a steel razor blade. The samples collected in Eppendorf tube and dropped into a dry-ice bath followed by storage at – 70℃ until used. The sample duplicates were obtained from the different fruits. The higher yield of papain produced per fruit was recorded at 8.17 g and the highest papain produced per plant was recorded at 686.29 g in a period of 6 months. Commercial available crude latex was usually obtained from the sun-drying process of the whole papaya latex but it is hampered with high possibility for contamination. Spray dried is thus preferable in which the preparations have been freed from any insoluble material and sterilized through membrane filtrations [16]. Previous research done by Moutime*et al.* [15] shows that, cysteine proteinase from *C. Papaya* become activated during clot formation cause by latex bleeding from injuring leaves or fruits of *C. papaya*. The bleeding of latex will lead to the coagulation process where plants defence themselves against possible pathogen attack. Latex produced by *C. papaya* is slightly different from other plant's latex such as latex that produced by rubber tree in which the coagulation involve the aggregation of rubber particles whereas in *C. papaya* the latex produced mainly made up of protein.

Table 2: Latex collection stages from *Carica papaya*

Method used	Latex collection	Storage	References
Latex is collected early morning (7.00 am-8.00 am), as the flow of latex is slow during the day. Latex dried at room temperature before triturated using mortar and pestle and sieved through a mesh size 170.	1-2mm deep vertical incisions on the skin of unripe but matured fruits.	Stored at 4 - 8˚C until used	[45]
Crude extract preparations; Latex is mixed with cold distilled water with ratio 1:1 and gently stir for 1 h. Mixture is centrifuged at 9000g for 20 min and filtered before undergoes freeze-drying.	Four to six longitudinal incisions were made on the green papaya fruit using a stainless steel and latex was collected using a receiving container.	Latex stored below 10 ˚C and used within 3 h.	[14]
The samples obtained from different fruits (100-200 µl sample^{-1}) were collected in eppendorf tubes containing 150 µl water, or 5 mM MMS (Methyl methane thiosulfanate) and drop into the dry-ice bath.	Wounding of unripe fruit at the plant with steel razor blade.	Samples stored at – 70˚C until used	[15]

1. Papain Purification

The broad usage of product contained papain especially in the pharmaceutical area which required highly purified protein made the purification of papain becomes more important in the industry. Purification of papain from papaya latex has traditionally been achieved by precipitation methods [31]. Salt precipitation method is the conventional way that was previously applied in papain purification. Low purity of papain with totaling up to 39 % and contamination with other proteins has become major constraints when dealing with large scale production and purification of papain. [32,33].Purification with various chromatographic techniques including ion exchange, covalent or affinity chromatography has become an alternative strategy to overcome the drawbacks in traditional method. Aqueous two-phase system (ATPS) made up of two polymers or one polymer and a salt in water have shown interesting potential for downstream processing of proteins. Research done by Nitsawang*et al.*[32] shows the purification process from *C. Papaya* latex by using ATPS composed of PEG and ammonium sulfate obtained highly pure papain in much shorter processing time. The maximum concentrations of the phase components that could be used for purification of papain from papaya latex comprising of 12 % (w/w) PEG 6000-15 % ammonium sulfate and the system that exceed this value resulted in highly viscous mixture and triggered the precipitation of protein from the latex.

2. Modification on Papain Extraction and Purification Methods

2.1. Modification on Salt Purification Method

Recent advances in extraction technology have also pave ways in the development and optimisation of different papain extraction methodologies. In regards to the conventional salt precipitation method, Braia*et al.* attempted in using different salt complexes to better exploit the unique relationship between enzymes and polyelectrolytes [34]. Here, a poly (vinyl sulfonate) complex (PVS) was used in replacement of the traditional ammonium sulphate to induce precipitation of papain from papaya latex. The experimental setup involves the formation of papain-poly (vinyl sulfonate) complexes which could be precipitated at pH lower than 6 with PVS/Papain stoichiometric ratio of 1:279. It was also reported that the ionic strength of the salt plays a key role within the effective formation of the complex and that the presence of polymer aids in increase enzymatic

activity whilst preventing auto degradation [34]. Interestingly, this modification demonstrated significant increase of papain recovery in comparison to conventional salt purification (89% to 39%). Nevertheless, the process is still susceptible to the weakness of salt purification whereby contaminants with similar properties to papain are found in abundance within the agglomerated complex. Hence, this modified precipitation method is suggested as an appropriate first step within a larger papain purification strategy.

2.2. Modification on Aqueous Two-Phase System (ATPS)

Regarding the aqueous two phase system (ATPS) proposed for papain purification, it was reported that there is significant difficulty in separating the extracted papain from the extraction media namely polyethylene glycol (PEG). Thus, to address this particular issue, Mingliang*et al*. proposed a combination of the aforementioned ATPS system with in situ immobilisation of papain [35]. Here, they experimented on four different immobilisation techniques consisting of the use of aminated supports, chitosan beads, activated supports and the preparation of cross-linked papain aggregates (CLEA). Based on their findings, it was found that using supports for papain immobilisation from the PEG phase allowed for yield of more than 85% to be achieved albeit at a substantial reduction of papain activity (between 28.2% to 52.5%). This was attributed to the inherent disadvantage of carrier-bound enzymes namely the dilution of catalytic activity due to the presence of large proportion of non-catalytic mass. On the other hand, the formation of CLEA was shown to possess great promise with a reported 120% enzymatic activity and near complete immobilisation of papain within the PEG phase. The hyper activation of the enzyme is inferred to have been caused by the conformational changes of the protein as it is brought into an aggregated state [36].

2.3. Application of Three Phase Partitioning (TPP)

Another relatively novel technique of papain extraction was proposed by Chaiwut*et al*. through the application of three phase partitioning (TPP) [37].This process is similar to that of ATPS in which ammonium sulphate and PEG are used at certain saturation to induce protein precipitation. However, the difference is that in TPP t-butanol is added to produce a three-phase layer system in which small molecular weight compound such as lipids and phenolics could be removed [38]. Due to this increase in extraction efficiency, Chaiwut*et al*. explored its application of extracting papain directly from papaya peels instead of the latex. Throughout the experimental phase, it is reported that the bulk of protease are partitioned within the aqueous phase with papain found in the interphase. By running two TPP cycles, it was observed that 89.4% of papain recovery was obtained in the interphase which is comparable to the ATPS method without the need for papaya latex as the initial starting papain source. Furthermore, increased purification fold of 18 was achieved following the second TPP depicting high degree of purity with the use of this extraction method.

3. Strategies in Enhancing Enzyme (Papain) Stability

The drawbacks when dealing with the enzymes are the behaviour of the enzymes which are often not sufficiently stable in the desired media. Even a slight conformational change can give an enormous decline in the activity of the enzymes. Most of the enzymes will lose its function or some may denatured if exposed under a range of conditions such as extremes of temperature, or pH value, physical forces and aqueous-organic or gas liquid interfaces. Hence, improvements in enzyme stability by using several strategies such immobilization, chemical modification, protein engineering, and medium engineering can enable further practical applications of the enzymes. Immobilized method are more preferred compared with their free forms as it generally more stable and easier to handle without any contaminations of the reaction products with the enzyme [39].

4. Application of Cysteine Protease

Moving on to the applications of papain-like cysteine proteases, it has been mentioned that there are myriads of industrial sectors in which they are utilised. This enzyme was first used for industrial application in 1911 for beer chill-proofing [40]. Nowadays, due to its biological importance, properties and function of papain, it has been applied in many areas such as, cosmetic, leather, textiles, detergents, food industry, peptides production, synthesis of molecules, and pharmaceuticals industry [33]. There is strong evidence that latex produced by certain plants contains several compounds that give protection from the environmental damage and*Carica papaya* plays an important part of an induced defense mechanism [15]. Proteases are protein digesting biocatalysts long time used in food industry. In the food and beverage industries, the enzyme is used for processes including beer clarification, tenderising meat as well as the preservation of certain spices. The powder of crude papain has the largest application as food supplements even though many of the studies reported about the crystallization of papain and chemo-papain. This is due to the positive effect of the crude powder on the degradation of casein and whey proteins from cow's milk in the stomach of the infants[27].Apart from the aforementioned applications, papain-like cysteine proteases have also been shown to have values for several medical and para-medical applications. For instance, they have been identified as one of the key

proteolytic activities contributor in disorders relating to degenerative and invasive immune system[41]. As an example, the enzyme cathepsin K is shown as the major influence towards bone degradation in osteoclasts and as such its selective inhibition is hypothesised to be advantageous in treating osteoporosis and several forms of arthritis[36]. Moreover, papain-like cysteine proteases are also seen with vital roles in the growth, cell differentiation and signalling as well as host invasion of pathogenic parasitic organisms. The latex of *C. papain* was also believed to act as pesticide as the larvae of silkworm died when fed with the leaves that contains the latex but not when the latex was washed away from these leaves or selectively inactivated by N-[N-(L-3-trans-carboxyoxirane-2-carbonyls)-L-leucyl]-agmatine. In addition, they also usually function as the virulence factor whereby the host immune system is proteotically degraded by said enzymes[43].

Table 3: Applications and benefits of papain

Industries	Application/Benefit	Mechanism	References
Medicinal	Healing of burn wound	It will show faster wound contraction (process of shrinkage of area of wound) and shortening the epithelialisation time.	[44,45]
	Dressing for wound debridement.		[45]
	Medication for kidney stones, hypertension, urinary tract disorders, abdominal pain during menstruation, analgesic, dysentery, diarrhoea, and fever.		[46]
	Enhance the production of hydrogen and degradation of glucose, proteins, and lipids.	Hydrolyse large proteins into smaller peptides and amino acids.	[47]
Food	Meat tenderiser, beer chill-proofing,		[16,34,28]
	Cheese production, extraction of flavour and colour compounds from plants.		[35,34]
Textile	Detergents (laundry, dishwashing) and blood stains remover		[21,42,43]

II. Conclusion

Throughout this short review, it is seen that cysteine proteases is a group of the many useful enzymes abundant in different part of papaya plant*Carica papaya*. Type and concentration of the enzyme is highly dependent on the plant part and fruit is usually considered as the much rich part of this type of enzyme. The selection of enzyme collection method from fruit latex and furthermore the storage conditions are critical factors in this process. In addition, the extraction method and purification play also critical role in designing cost effective process to develop proper industrial platform for this enzyme production which has growing applications in different field as shown in this work

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