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JUDUL : EXTRACTION OF PAPAIN ENZYME FROM PAPAYA LEAVES USING ENZYME ASSISTED METHOD

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EXTRACTION OF PAPAIN ENZYME FROM PAPAYA LEAVES USING ENZYME ASSISTED METHOD

ROSWANI BINTI SHAIMI

A thesis submitted in fulfillment
of the requirements for the award of the Degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
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DECEMBER 2010

I declare that this thesis entitled “Extraction of Papain Enzyme from Papaya Leaves using Enzyme Assisted method” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.”

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Name : Roswani Shaimi

Date : 3 December 2010

To my beloved parents, brothers and sisters

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In the name of Allah S.W.T. the most gracious and most merciful, Lord of the universe, with His permission, Alhamdulillah the project has been completed. Praise to Prophet Muhammad S.A.W., His companions and to those on the path as what He preached upon, might Allah Almighty keep us His blessing and tenders.

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ABSTRACT

Papain is highly appreciated in medical field that prevents several chronic diseases such as cardiovascular disease, cancer and diabetes. Therefore, the objective of the research is to extract papain enzyme from papaya leaves using enzyme assisted method and determine papain activity. The Cellullase assisted extraction process was optimized by varying different parameters such as pH of extraction process, Cellulase concentration, Solid to Liquid ratio, incubation time and incubation temperature. Each 5 g of papaya leaves were ground and mixed with acetate buffer at a different Solid to Liquid ratio (1:5 - 1:25 g/ml) and adjusted with different pH (3-8). Cellulase was quantified and dispersed in acetate buffer (Na_2HPO_4—citric acid) in different concentration (0.5 - 2.5 mg/ml).The enzymatic pretreatment was carried out and continued for enzyme assisted extraction process for various temperature and time (50-70^oC and 1-5 h respectively). The optimal extraction conditions that satisfied the above constraints were found to be at pH 7, 1.5 mg/ml of Cellulase, solid to liquid of 1:10 (g/ml), an extraction time of 4 h and at an extraction temperature of 65^oC. Under the optimum conditions, the extraction yield of papain was successfully achieved as much as 3.8018 µmole with 2.0910 Units/ml in Cellulase treated sample. Therefore, the potential for the papain enzyme in medical field can be fulfilled using enzyme assisted extraction.

ABSTRAK

Papain sangat dihargai dalam bidang perubatan yang mencegah beberapa penyakit kronik seperti penyakit jantung, penyakit kanser dan diabetes. Oleh kerana itu, tujuan dari penelitian ini adalah untuk mengekstrak enzim papain dari daun betik menggunakan kaedah pendekatan enzim dan menentukan aktiviti enzim papain. Proses pengekstrakan melalui kaedah pendekatan Cellullase telah dioptimumkan dengan mempelbagaikan parameter yang berbeza seperti pH untuk proses pengekstrakan, kepekatan selulase, nisbah pepejal kepada cecair, masa inkubasi dan suhu inkubasi. Setiap 5 g daun betik dikisar dan dicampur dengan buffer asetik pada pelbagai nisbah pepejal kepada cecair(1:5 - 1:25 g/ml) dan disesuaikan dengan pH yang berbeza (3-8). Selulase telah disukat dan dimasukkan dalam buffer asetat (Na_2HPO_4-sitrat asid) dengan kepekatan berbeza (0.5-2.5 mg /ml). Rawatan awal enzimatik dilakukan dan dilanjutkan untuk proses pengekstrakan melalui penambahan enzim dengan pelbagai suhu dan masa (50-70^oC dan 1-5 jam untuk masing-masing). Selanjutnya proses diteruskan dengan penapisan dan sentrifugasi. Parameter optimum diperolehi seperti berikut: pH untuk proses pengekstrakan adalah 7, 1.5 mg / ml untuk Selulase, 1:10 untuk nisbah pepejal kepada cecair, inkubasi selama 4 jam pada 65^0C. Dalam keadaan yang optimum, hasil pengekstrakan papain telah Berjaya diperolehi sebanyak 3.8018 μmole dengan 2.0910 Unit / ml pada sampel Selulase di rawat. Oleh kerana itu, potensi enzim papain dalam bidang perubatan dapat dipenuhi dengan menggunakan kaedah pendekatan enzim untuk pengekstrakan.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS/ABBREVIATIONS

A	-	Absorbance
Asn	-	asparagines
C	-	Celcius
cm	-	centimeter
Cys	-	Cysteine
F-C	-	Folin & Ciocalteu's Phenol Reagent
g	-	gram
g	-	gravitational acceleration
h	-	hour
His	-	histidine
M	-	Molarity
m	-	mili
m	-	meter
mg	-	milligram
min	-	minute
ml	-	mililiter
mm	-	millimeter
N	-	normality
nm	-	nanometers
rpm	-	revolutions per minute
Trp	-	Tryptophan
µ	-	micro
(v/v)	-	volume per volume
%	-	Percent
^{o}	-	degree

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Papaya Tree

Papaya (*Carica papaya* L.) belongs to the family of Caricaceae. It grows in Australia, Hawaii, Philippines, Sri Lanka, South Africa, India, Bangladesh, Malaysia and also other countries in tropical America. Papaya also known as tepayas by Kadazan Dusun community in East Malaysia, betik in Peninsular Malaysia, lechosa in Venezuela, pawpaw in Sri Lanka and papali in India (Rahman *et al*., 2008).

Papaya is a fast growing plant, rarely branching, semi-woody and short juvenile phase which is 3 to 8 months. When it starts flowering, it will continue to flower and produce fruit. Papaya is cultivated in tropical and subtropical regions. The papaya plants grow until 8–10 m in height with few branches and large leaves that bears yellow egg-shaped.Fruits 5–6 cm in diameter and 6–14 cm long with many seeds inside the fruit (Guillermo, Mario & Peter, 2009).

Many scientifics investigated the biological activities of various parts of *Carica papaya* L. such as fruits, shoots, leaves, rinds, seeds, roots or latex. There are many uses for the whole part of papaya especially in medical properties The papaya fruit contain some immune-stimulating and anti-oxidants agents while the unripe fruits and roots are used for abortifacient activity and also has shown bacteriostatic activity against the

human enteric pathogens. Besides that, the seeds used as a potential-testicular anti – fertility drug while the pulp is used for treating wounds and burns. For the latex and seeds, they are used in the care of gastrointestinal nematode infections and they also have shown anthelmintic activity .Then, for the leaves, they are used to relieve the symptoms of asthma and as vermifuge in treatment of gastric problems, fever and amoebic dysentery (Antonella *et al*., 2007).

1.1.1 Papaya leaves

Various parts of papaya include fruits, shoots, leaves, rinds, seeds, roots or latex have been traditionally used as ethnomedicine for a number of disorders, including cancer. There have been anecdotes of patients with advanced cancers achieving remission following consumption of tea extract made from papaya leaves **(**Morimoto *et al*., 2010).Papaya leaves constitute the most important part of the plant and play a major role in the anabolic activities by means of the so called "green pigment" or "chlorophyll", which they possess in abundance. Photosynthesis occurs within the chloroplast-containing mesophyll layer.

Papaya leaf juice is consumed for anti-cancer activity by people living with some anecdotes. Papaya leaf extracts have also been used for a long time as an aboriginal remedy for various disorders, including cancer and infectious diseases (Morimoto *et al*., 2010).The leaves are also used for relieving the symptoms of asthma and as a vermifuge, in the treatment of gastric problems, fever and amoebic dysentery. Methanolic leaf extract also demonstrated vasodilatatory and anti-oxidant effects, both implicated in the reduction of cardiovascular risks. Papaya leaves are also used in tropical alimentation cooked as a vegetable and in preparation of teas and infusions (Antonella *et al*., 2007).

The leaves of papaya contain many active components that can increase the total antioxidant power in blood. They also can reduce lipid peroxidation level, such as papain, chymopapain, cystatin, α-tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates(Morimoto *et al*., 2010).The content of papain, chymopapain, glycyl endopeptidase and caricain vary in fruit, leaves and roots (Rahman *et al*., 2008).

1.1.2 Papain

C. papaya plant is laticiferous because they contain specialized cells known as laticifers. Lactifiers secrete latex and are dispersed throughout most plant tissues. The papaya latex is well known for being a rich source of the four cysteine endopeptidases namely papain, chymopapain, glycyl endopeptidase and caricain. The content of papain, chymopapain, glycyl endopeptidase and caricain vary in fruit, leaves and roots. Commercially, papaya latex is harvested from fully-grown but unripe fruit. Ripe papaya contains less latex compared to green papaya possibly due to cessation of function or breakdown with age of the latexproducing cells (Rahman *et al*., 2008). When unripe, papaya contains the enzyme papain (EC 3.4.22.2), a cysteine protease. It is also cultivated for the proteolytic enzyme ‘papain’. Papain is a proteolytic enzyme from the latex in the leaf, the stem and the papaya's unripe fruits and possesses a stereospecific esterase activity on appropriate synthetic compounds (Wang, Chen &Wu, 1982).

The 3D structure of papain is well characterized. The enzyme consists of a single polypeptide chain made up of 212 amino acid residues and has a molecular weight of 23,400 Dalton. An interesting feature of papain molecular structure is that it is divided in the form of two distinct domains that are separated by a deep cleft which are L domain and R domain, forming a cleft with the active site (Prakash, Kumar & Sathish, 2009). L domain, which is mainly α-helical, is compromised of residues 10–111 and 208–212 while the R domain contains residues 1–9 and 112–207 and the key feature of the R

domain is its antiparallel β-sheet structure. In the cleft formed between these two domains are the active site residues, Cys-25, His-159 and Asn-178 (Prakash, Kumar & Sathish, 2007).

Latex from fruit is the most common part of the papaya plant being analyzed by scientists for its papain activity. Cysteine proteinases are used widely for protein digestion in the food and pharmaceutical industries. Latex of Carica papaya L., contains a mixture of cysteine endopeptidases such as papain (EC 3.4.22.2) ,chymopapains A and B (3.4.22.6) ,papaya endopeptidase III, papaya endopeptidase IV and endopeptidase U (caricain)(Salas *et al*., 2008).

1.1.3 Application of papain

There are many applications of the papain enzyme that extracted from papaya. The enzyme is used widely as meat tenderizer, and has also several other applications such as for defibrinating wounds, treatment of edemas and shrink proofing of wool (Rajni, Sarote, & Pawinee, 2006). Besides, the papain which is a sulfhydryl protease is one of the most commonly used enzymes in various industries including food, tanning and pharmaceutical industries (Prakash, Kumar & Sathish, 2009). The other uses of papain are cell isolation, breweries, food and pharmaceutical as digestive enzyme, leather, cosmetic and textile industries (Abraham & Sangeetha, 2006). A study show that the papain has been used in meat tenderizers and in face and hair care products. It is also increasingly being used in pharmaceutical preparations and in such diverse manufacturing applications as leather, wool, rayon and beer (Kamalkumar *et al*., 2007). Recently it is proven that the papain has multiple applications in the food industry such as a clarifier in beers, a meat tenderizer and in preparation of protein hydrolysates and the pharmaceutical industry like in treatments for osteoporosis, arthritis, vascular diseases and cancer (Santiago *et al*., 2009).

1.2 Problem Statement

The extraction of papain enzyme is commonly carried out by collecting the latex from green papaya by making incisions in the fruit surface .This damages the fruit and causes it to not meet the specification for commercial values. The decrease in commercial value using the unripe fruit can be reduced by another extracting method which is by using the leaves.

For extraction of papain enzyme, water or organic solvent such as methanol, ethanol and acetonitrile are commonly used as an extraction media. However, the use of aqueous organic solvent as extraction media can change the structural activity of papain due to the decreasing of hydrolytic activity and the number of active sites of papain. Therefore, in the extraction of papain enzyme from papaya leaves, water is applied as the extraction media. This is because water can maintain the structural stability of papain besides it is also very good extractive properties for polar substances compare to the organic solvent. The extraction of papain using water as extraction medium also avoid from toxicity due to the using of papain as the food application.

1.3 Objective

The objective of this research is to extract the papain enzyme from papaya leaves using enzyme assisted method and determine the papain activity.

1.4 Scope

In the extraction of papain enzyme from papaya leaves, it is focused on five scopes to obtain the optimum condition for the extraction.

The first scope of the research is to determine the optimum pH for extraction process which is divided into six parts: pH 3, pH 4, pH 5, pH 6, pH 7and pH 8.It is done to check whether the papain is acidic, neutral or basic enzyme.

The second scope is to determine the optimum concentration of enzyme (Cellulase) in extraction process. Five different concentrations are chosen which are 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml and 2.5 mg/ml.

The third scope is to determine the optimum solid to liquid ratio for extraction process and it is divided into five parts: 1:5g/ml, 1:10g/ml, 1:15g/ml, 1:20g/ml and 1:25g/ml.

The fourth scope is to determine the optimum incubation time for extraction process and it is divided into five parts: 1h, 2h, 3h, 4h and 5 h.

The last scope is to determine the optimum incubation temperature for extraction process. Five different temperatures are chosen which are $50^{o}C$, $55^{o}C$, $60^{o}C$, $65^{o}C$ and $70^{o}C$

1.5 Rationale and Significance

Extraction of papain is not only in the fruit but also in different plants tissue such as roots, stem, petiole and leaves. The extraction of papain enzyme from leaves is introduced in the research. Using this part of the papaya tree (leaves), it does not compete with another part of papaya tree especially fruit in producing end products.

In addition, leaves tissue yield the largest amount of papain enzyme compared to the other part of papaya tree which are stem, petiole and roots (Santiago *et al*., 2009). Then using the leaves byproducts also can manage disposal of tree byproducts for the benefits uses. These byproducts are generally disposed of in open areas. High transport costs limit any secondary uses and in most cases this waste is left to root, producing phytopathogens that cause ecological problems and pose a risk to human health (Santiago *et al*., 2009).

Besides that, using leaves can create waste to wealth application because of the papain enzyme contained in the leaf tissues can be used in industrial fields like pharmaceutical, brewery, meat, dairy, textile, photographic, optical, tanning, cosmetic, detergents, food and leather industry.

CHAPTER 2

LITERATURE REVIEW

2.1 Papaya Leaves

There are several constituents of papaya leaves including the fermenting agent myrosin, alkaloids, rutin, resin, tannins, carpaine, dehydrocarpaines, pseudocarpaine, flavonols, benzylglucosinolate, linalool, malic acid, methyl salicylate, another enzyme, chymopapain (latex and exudate), calcium, iron, magnesium, manganese, phosphorus, potassium, zinc, beta-carotene, B-vitamins and vitamins A, C and E. Papaya leave is an excellent treatment for digestive disorders and extremely useful for any disturbances of the gastrointestinal tract. Papain, the powerful enzyme in papaya, helps to dissolve and digest protein, thus easing stomach ailments and indigestion. Papaya leaves' enzyme, papain, not only digests protein, but it extends its activity to digesting carbohydrate.

The leaves of papaya contain active components that can increase the total antioxidant power especially in blood. It has been shown that the papaya leaves can reduce lipid peroxidation level such as papain, chymopapain, cystatin, α-tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates. Papaya leaf juice was consumed for anti-cancer activity with some anecdotes. Papaya leaf extracts are also used for a long time as an aboriginal remedy for various disorders, including cancer and infectious diseases (Morimoto *et al*., 2010).

Analyses show that the contents of papain, chymopapain, glycyl endopeptidase and caricain vary in fruit, leaves and roots (Rahman *et al*., 2008). Previously, the result shows that the leaf and fruit tissue had the highest protein contents of papaya harvest by-products (stems, unripe fruit, petioles and leaves). Leaf tissue also produced the highest total enzymatic extracts yield which probably corresponds to papain from Carica papaya L harvest by-product (Santiago *et al*., 2009).

2.2 Papain

Papain is a proteolytic enzyme preparation derived from fruits and other parts of papaya such as stem, petioles and leaves. It contained lactose or dextrin. The enzyme activity of papain is not less than 300,000 units per gram. Papain occurs as white to light yellow-brown powders. It is odorless or has slight characteristic odors. Papain is a proteolytic enzyme from plants and possesses a stereospecific esterase activity on appropriate synthetic compounds (Wang, Chen &Wu, 1982). Papain is applied as an enzyme in protein chemistry for the synthesis of many biologically active compounds. It is comprised of a single polypeptide chain which consists of 212 amino acid residues containing a total of 11 primary amino groups which in 10 Lys residues and 1 amino terminal. The enzyme folded into two domains, L domain and R domain forming a cleft with the active site. The first domain contains α-helix, while the second domain has a large content of β-sheet and a lesser amount of α-helix (Simon *et al*., 2009).

Papain is one of the sulfhydryl protease of carica papaya fruit. It is highly stable enzyme based on its interesting molecular structure and its many industrial applications. Its molecular structure consists of two distinct domains, with the active site in the groove between the domains. The first domain (residues 1–110) contains mainly α-helix, while the second domain (residues 111–212) has a large content of antiparallel β-sheet and a lesser amount of α-helix. There are five tryptophan (Trp) residues that are located in the two distinct domains. Three of the five tryptophan (Trp) residues are located in the first

domain (Trp7 in the β-sheet, Trp26 and Trp69 in the α-helical segments), and the other two tryptophan (Trp177 and Trp181) in the coil region of the second domain. One of the seven cysteine residues, which is Cys25, provides the free thiol group of the active site .The others six of cyteine residues which are Cys22–Cys63, Cys56–Cys95 and Cys153–Cys200 form disulfide bridges .Water molecules present in ice-like networks play an important role in the stability of the enzyme, especially at the domain–domain interface (Simon *et al*., 2006).

Papain was a highly active endolytic cysteine protease from Carica papaya .It is stable in harsh conditions and active at low and high temperatures. It also is less expensive than microbial enzymes besides has wide range of specificity and good thermal stability amongst other proteases. Because of such characteristics, the papain has high potential used in detergents. Papain molecules had a molecular weight of 23,000 Da and an isoelectric point of 9.5.Papain molecules consisted of a single peptide chain of 211 amino acid residues folded into two parts that form a cleft and having 11 lysine residues. Papain cleaves peptide bonds involving basic amino acids and it also has an esterase activity. It is used in breweries, food and pharmaceutical, leather, cosmetic and textile industries. The catalytic site of the enzyme contains a catalytic triad Asn-His+-Cys−, which exists as zwitterions. Papain can be chemically modified by different dicarboxylic anhydrides of citraconic, phthalic, maleic and succinic acids as Lysine residues are not a part of active site in papain. These anhydrides react with the ε-amino group of lysine residues and change its charges from positive to negative, leading to a shift in pH optima of the enzyme from 7 to 9(Abraham & Sangeetha, 2006).

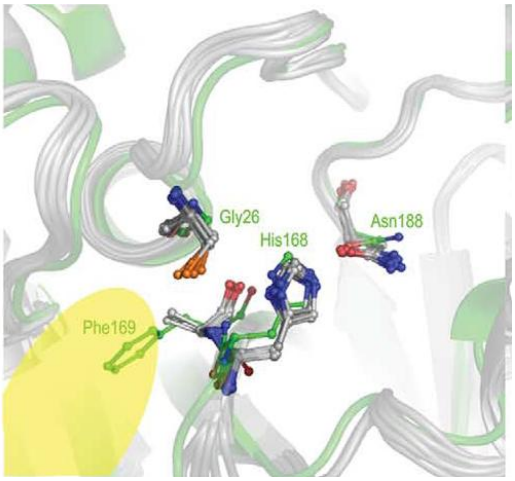

Figure 2.1: Active site residues, Cys-25, His-159 and Asn-178 of the papain between L and R domains (Gong *et al*., 2006)

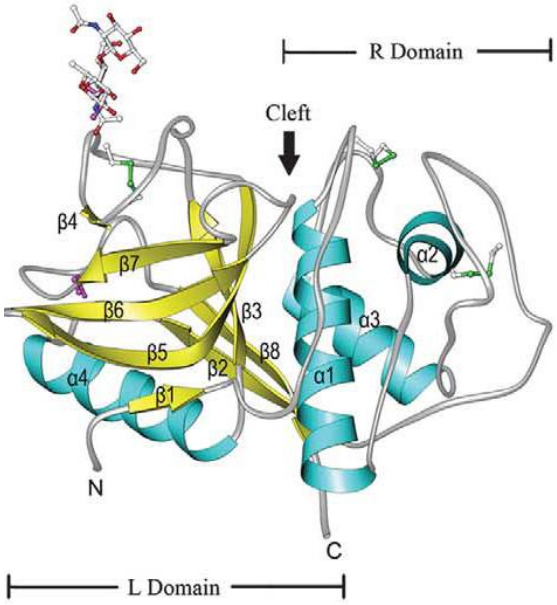

Figure 2.2: Active site in the cleft between these L and R domains (Gong *et al*., 2006)

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2.3 Hydrolysis pretreatment

The plant cells are surrounded by a complex cell wall matrix composed of carbohydrate molecules (cellulose, hemicelluloses, and pectic polysaccharides) as well as proteins. In order to achieve higher extraction, the activity for disrupting cell wall structure must be done to obtain the target product as intracellular product. Before the hydrolysis treatment, the samples must be treated into smaller particle such as grinding or crushing. During the hydrolysis treatment on the degraded surface, the middle lamellae is degraded causing cell tissue slowly and gradually lose cellular and sub-cellular organization as the walls and cytoplasm become disrupted (Silva *et al*., 2009).

There are many methods for hydrolysis treatment that have been published either in physical methods or chemical and physicochemical methods. The physical methods include disruption the cell in bead mill, using a rotor-stator mill, French press and ultrasonic vibration. For chemical and physicochemical method, it includes disruption the cell by using detergents, enzyme, solvents and osmotic shock. From the previous study, the enzyme-assisted extraction is a method applied to the study secondary metabolites releasing from biogenic materials. This kind of hydrolysis treatment has advantages of environmental friendship, high efficiency and easy operation process. It also has been represented as an alternative way for natural product extraction. Hydrolytic enzymes including cellulase, beta-glucosidase and pectinase, which are commonly used in extraction can interact on cell wall, break down its structural integrity so as to increase the releasing of intracellular products (Fu *et al*., 2009).

2.3.1. Enzymatic Assisted Extraction

Analysis shows that the cell wall degrading enzymes can improve the extraction of phenols from fruit skins. The enzyme assisted release of phenols from the cell wall matrix occurs via enzyme catalyzed hydrolytic degradation of the cell wall

polysaccharides that are presumed to retain the phenolics in the polysaccharide-lignin network by hydrogen or hydrophobic bonding. The use of cell wall degrading enzymes also increased the mass transfer of total phenols, with proteases having a particular increasing effect on the yield of chlorogenic acid. This study showed that phenols can be selectively extracted by varying the extraction conditions and by adding cell wall degradation enzymes. Another mechanism may be the direct enzyme catalyzed breakage of the ether and/or ester linkages between the phenols and the plant cell wall polymers. Fungal pectinases are the most widely used cell wall degrading enzymes in the fruit industry. In apple juice processing, the pectinases improve the press capacity and efficiency via viscosity lowering of the mash and are used for the juice clarification as well (Meyer, Pinelo & Zornoza, 2008).

In order to choose the best enzyme to increase the yields of taxanes notably, the activity of individual and complex enzymes was compared in this section. Enzyme-assisted extraction of paclitaxel and other taxanes, namely 7-xyl-10-DAT, 10-DAT, cephalomannine and 7-epi-10-DAT from needles of T. chinensis was carried out in present study. The effect of three hydrolytic enzymes which were Cellulase, Beta-glucosidase and Pectinase was compared. Cellulase catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose polymers. Pectinase has the ability to disintegrate pectic compounds and pectin while Beta-glucosidase breaks the beta-1,4 glucosidic linkages in glucosides. Although Beta-glucosidase was proved to be most effective for extracting taxanes from needles of T. chinensis, the cost is too high to afford in industry. Cellulose was chosen for the treatment of the needles by considering the economic effect (Fu *et al*., 2009)

2.3.2. Ultrasonic Assisted Extraction

Ultrasonic-assisted extraction is one of the important techniques for extracting the compounds from the vegetal materials and it is quite adaptable on a small or large

scale. The ultrasonic device is cheaper compared with other extraction techniques such as microwave-assisted extraction. Its operation is also much easier. The general ultrasonic devices are ultrasonic cleaning bath and ultrasonic probe system. When sonicating liquids at high intensities, the sound waves that propagate into the liquid media results in alternating high-pressure (compression) and low-pressure (rarefaction) cycles, with rates depending on the frequency. During the low-pressure cycle, high-intensity ultrasonic waves create small vacuum bubbles or voids in the liquid. When the bubbles attain a volume at which they can no longer absorb energy, they collapse violently during a high-pressure cycle. This phenomenon is termed cavitations. When these bubbles reach resonance size, they collapse releasing mechanical energy in the form shock waves. The shock waves disrupts cell in the suspension.

From the previous research, an ultrasonic probe system was chosen as the ultrasonic device to extract the intracellular product. In this study, the feasibility of the extraction of epimedin C from fresh leaves of Epimedium using ultrasonic probe system was demonstrated. The high extraction yield of epimedin C was obtained under an optimum extraction condition. The high yield was obtained form the extraction temperature of 50 °C, methanol concentration 60% (v/v), ratio of liquor to solid 30 ml $g^{-}1$, and ultrasonication time for 15 min. Ultrasound could result in the disruptions of leaf tissues and cell walls, which enhanced the mass transfer of the solvents into the leaf materials and the soluble constituents into the solvents (Wang *et al*., 2009)

Previously, the study introduced ultrasonication in the traditional enzymatic release of protein- and phosphate bound thiamin and riboflavin in the determination of vitamin B1 and B2 in foods. Unfortunately, the ultrasonication process did not show any effect on the efficacy of the enzymes but enabled the enzymatic treatment to be performed within 1 h, as a replacement for 4–18 h incubation for vitamin B1 and 18 h incubation for vitamin B2 in the standardized methods (Jakobsen, 2008).

2.4 Method of Extraction

Many extraction methods including microwave-assisted extraction, Soxhlet extraction, Percolation, bubble column extraction (BCE), Supercritical fluid extraction (SFE), reverse micellar extraction and Heat-refluxing extraction had been reported.

2.4.1 Heat-refluxing extraction

A conventional method of heating-refluxing extraction using ethanol–water (80:20, v/v) was performed .According to the preliminary investigation, target compositions were extracted by adding 20 g of pigeonpea leaves into 400 ml of solvent in a round bottom flask. The extraction was employed to optimum condition of 65 ^{0}C for 2 h under magnetic stirring at 500–700 rpm. The extracting solution was filtered by membrane filtration and analyzed by HPLC (Fu *et al*., 2009)

2.4.2 Soxhlet extraction, Percolation, bubble column extraction (BCE)

In the extraction of solanesol from tobacco, the extraction methods using Soxhlet extraction, Percolation and bubble column was compared. A bubble column reactor is basically a cylindrical vessel with a gas distributor at the bottom. The gas is spurge in the form of bubbles into either a liquid phase or a liquid–solid suspension. In this device, bubbles were introduced into liquid–solid system (made of the extraction solvent and material) to increase the turbulence in the medium and transfer coefficient (Zu, Zhao & Li, 2009).

For this experiment, percolation was conducted using a glass column. Material was packed into the column and the solvent was added continuously to percolate through the material packing and collected. The flow rate was set at and lastly the infiltrated

solution was filtered through a 0.45 µm mmembrane filter before chromatographic analysis (Zu, Zhao & Li, 2009).

A conventional Soxhlet extraction was used. The materials were ground and packed into a filter paper. This cartridge was placed inside the Soxhlet extractor, which was placed on top of a round-bottomed flask filled with the solvent. The system was boiled using a bath boiler until the extraction was completed. After filtration, the extract was fixed volume in 10 ml volumetric flasks prior to chromatographic analysis (Zu, Zhao & Li, 2009).

Result shows that the yields of solanesol by the above three extraction methods (percolation, Soxhlet and BCE) are similar, but the extraction time varies with the different extraction methods. Percolation, Soxhlet extraction and BCE need 24 h, 6 h and 54min respectively to complete the extraction. BCE reduces the extraction time, proving it is a fast and efficient method for the extraction of solanesol (Zu, Zhao & Li, 2009).

2.4.3 Supercritical fluid extraction (SFE)

From the previous study of extraction of rose geranium oil, the supercritical fluid extraction (SFE) using supercritical CO_2 was used to produce a natural extract from Portuguese-grown geranium as a high quality material for application in perfumery. As SFE uses gentle operating conditions (low temperature, close to ambient), the SFE extract has been recognized as having a superior quality in terms of odor and taste, with fresher characteristics and resembling more to its natural source, especially when compared with distilled oils. Unfortunately, SFE is a very expensive technology due to the high investment costs and safety precautions of working at high pressures. SFE is profitable only when applied to high-added value, to obtain ultra-pure products or if impose by regulatory restrictions on residues .Pressure and temperature are the most

important operating parameters that affect the supercritical fluid selectivity, yield and extraction rate, especially in the vicinity of the critical point. Other operational parameters are the pre-treatment of plant material, solvent flow rate, extraction time and design of extractor (relation height/width). The physical properties of the solute, namely vapour pressure, polarity and molecular weight, affect its solubility in the supercritical fluid (Mata, Gomes & Rodrigues, 2007).

2.4.4 Reverse micellar extraction

Extensive studies have been done on reverse micellar extraction of proteins using an anionic surfactant, bis (2- ethylhexyl)sulfosuccinate (AOT) .The distribution of proteins between the micellar phase and an aqueous phase is largely determined by the environments of bulk aqueous phase, i.e., pH, ionic strength and type of salt. Parameters related to the organic phase also affect the partition of protein, such as the concentration and type of surfactant, presence of co-surfactant and type of solvent. By controlling these parameters, the extracted fraction can be varied via variations of protein–micelle electrostatic, hydrophobic and steric interactions.

In order to employ reverse micelles for protein separations, the micelles should exhibit two characteristic features. Firstly, they should be capable of solubilizing proteins selectively. This protein uptake into the reverse micelles is referred to as forward extraction process. Second, it should be possible to release the protein from the reverse micelles so that a quantitative recovery of purified protein can be achieved. This is referred to as the back extraction process.From the result about 60–65% of papain was forward extracted (1st step) without much difficult (Juang & Mathew, 2005).

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2.5 Thermal Stabilization of Papain

A major limitation to industrial use of the enzyme is their relative instability under operational conditions, which involve exposure to extremes of temperature, pressure, pH, denaturant and organic solvents. The native structure of any protein is only marginally stable and will always fluctuate non-native states, the rate of which increases under extreme environmental conditions. One of the major means of enzyme inactivation is thermal denaturation. There are several advantages in conducting an enzyme reaction at elevated temperature such as increase solubility of the substrate, decrease microbial contamination, decrease viscosity and also increase reaction rate. The search for development of thermostable enzymes for use in the food and pharmaceutical industries is receiving considerable research interest. There are two options to increase the stability of a protein. The first one is to modify the solution condition and the second option is to modify the protein itself. The second one option in protein modification is not recommended because of the cost factor

In the previous research, the solution condition was modified to increase the stability of protein. The various substances that can act at high concentrations strongly affected stability of the folded protein. These substances are known as cosolvents and many of them are referred to stabilize the native structure of protein. Cosolvents like sugars and polyols have been known to have a profound effect on stability, structure and function of a protein. From the result, it showed that, cosolvents used namely sorbitol glycerol, sucrose and xylose can increase the thermal stability in a concentration dependent manner. These data suggest that, though all the cosolvents used in the above study tend to stabilize the protein against thermal denaturation. (Prakash,Kumar & Sathish, 2007).

2.5 Stability of Papain in Organic Solvents

Water-miscible organic solvents can interact with enzyme and the water molecules associated with the protein structure but it can reduces the activity and/or stability of the enzymes. Using of aqueous organic solvent as extraction media can change the structural activity of papain due to the decreasing of hydrolytic activity and the number of active sites of papain. In the previous research, the stability of papain in aqueous organic solvents such ethanol, 1,4-dioxane, tetrahydrofuran (THF) and acetonitrile (ACN) were studied. For the result, the effects of the organic solvents on the activity of papain in aqueous solution were different. THF proved to be the most destabilizing solvent for papain while ACN, ethanol and 1, 4-dioxane exhibited high stability in aqueous. Activity of the enzyme was decreased with organic solvent at concentrations above 60% (Simon *et al*., 2006).

In the study on the effects of chemical modification of papain by mono- or dicarboxylic acid anhydrides on the catalytic activity and conformational stability in aqueous organic media, ethanol (EtOH), acetonitrile (ACN) and tetrahydrofuran (THF) were used .Chemical modification of papain with monocarboxylic acid anhydrides (acetic or propionic) resulted in higher catalytic activity and conformational stability than that with dicarboxylic acid anhydrides (citraconic, maleic or succinic) (Simon *et al*., 2009).

2.6 Analysis Method

There were many analysis methods done for the previous research.For the analysis done of papain assay, the N-Benzoyl-r_-arginine ethyl ester(BAEE) used as substrate.The reaction was carried out at 37 ^{0}C and mixture contained BAEE, mercaptoethanol, ethylenediaxninetetraacetatic acid (EDTA), sodium chloride and native papain. Sodium hydroxide solution was added and the reaction was terminated by

the addition of 30% acetic acid. The reaction mixture was filtered and the filtrate was analysed by high-performance liquid chromatography (HPLC).The amount of the product was linearly related to the peak height and the rate of the enzyme reaction was accurately determined from the peak height of benzoylarginine (Wang, Chen & Wu ,1982).

The other analysis of papain assay ,N-acyl-D,L-amino acid methyl ester was used as a substrate .The papain assay was carried out at pH 6.5 with mixture contain of methanol, phosphate buffer and N-acyl- D,L-amino acid methyl ester. The mixture incubated at 37°C for 5 min with stirring. The reaction was terminated by addition of 30% acetic acid. The reaction mixtures were filtered and the filtrates were analyzed by HPLC .The rate of enzyme reaction was determined from the peak height of the N-acyl amino acid product (Wang, Chen & Wu,1982).

Another enzyme assay was performed by using N-a-benzoyl-Larginine-*p*-nitroanilide hydrochloride (L-BAPA) as a substrate. The analysis was carried out at 37^{o}C. Each sample was added to the mixture contained citric acid, disodium phosphate buffer ,cysteine, HCl and Na_2EDTA .The mixture was added to the substrate solution, N-a-benzoyl-Larginine-*p*-nitroanilide hydrochloride (L-BAPA) which was prepared by adding the dimethyl sulfoxide(DMSO).this solution was diluted in citrate-phosphate buffer and stopped by adding of 60% (v:v) acetic acid. Released 4-nitroaniline was determined by measurement of the absorbance at 410 nm. One unit of activity corresponds to the release of 1 nmol of 4-nitroaniline (0.8800 $M.cm^{-1}$) per s from L-BAPA at pH 6.4 and 37°C (Dekeyser *et al*., 1997).

CHAPTER 3

METHODOLOGY

Papain is an intracellular product. To extract papain enzyme from papaya leaves, firstly, the cell wall must be degrade or disrupt to release the intracellular into the solvent. In this research, water was used as extraction media to extract intracellular from the cell. Mechanical action such as grinding was applied to reduce the particle size for increasing the superficial area, thus increase mass transfer of the target product. Then, the enzyme-assisted extraction was employed in this research to weaken the cell wall rendering the intracellular materials more accessible. Cellulase was used in study due to its performance to destroy .Cellulase can destroy the structures of plant cells and results in higher extraction yields (Fu et al., 2009). Next, the papain was extracted in hot water extraction by using shaking water bath. Further process was solid-liquid separation where the semi solid samples were separated using filtration and centrifugation method. Lastly, the enzymatic extract were undergone protein determination using colorimetric method by UV-Vis spectrophotometer at wavelength 660 nm.

3.1. Materials and Reagents

Papaya leaves were collected from Orchard Gambang, Kuantan. Leaves in good shape, soft texture and green or fresh leaves were used in the assays. Disodium hydrogen phosphate(Na_2HPO_4),citric acid anhydrous, potassium phosphate dibasic(K_2HPO_4) ,HCI, casein, trichloroacetic acid(TCA), Folin & Ciocalteu Reagent, sodium carbonate, L-cysteine, sodium acetate, calcium acetate, sodium hydroxide (NaOH) and cellulase produced from *Aspergillus Niger* were purchased from Sigma Chemical Company .

3.2 Apparatus

3.2.1 Shaking Water bath

Water bath is a device used for regulating the temperature of substances subjected to heat. Water baths are used to heat those substances, which can't be heated directly on Bunsen burner or hot plate or any other media. However only those materials whose has less than water boiling point can be heated with water bath. Shaking Water Bath is one type of water bath. Shaking water baths are used when the lab operation requires precise temperature control and a smooth reciprocal shaking motion. Some of their applications include warming reagents, Coliform determinations, sample thawing, bacteriological examinations and microbiological assays. The shaking water bath was applied in this research. It was used in enzymatic pretreatment, enzyme extraction and enzyme analysis process.

Figure 3.1: BS-21 Shaking water bath

3.2.2 Centrifuge

A centrifuge is equipment, generally driven by an electric motor, which puts an object in rotation around a fixed axis then applying a force perpendicular to the axis. The centrifuge works using the sedimentation principle, where the centripetal acceleration causes more dense substances to separate out along the radial direction (the bottom of the tube). By the same token, lighter objects will tend to move to the top. A centrifuge uses centrifugal force (g-force) to isolate suspended particles from their surrounding medium. Applications for centrifugation are many and may include sedimentation of cells and viruses, separation of subcellular organelles, and isolation of macromolecules such as DNA, RNA, proteins, or lipids. In this research, the centrifuge was used for separation process. The supernatant that obtained in this process was used for papain determination.

Figure 3.2: Eppendorf 5810 R Centrifuge

3.2.3 UV-Vis Spectrophotometer

Spectrophotometer is equipment that used to determine the extent of absorption of various wavelengths of *visible* light by a given solution is commonly known as *colorimetry*. This method is used to determine concentrations of various chemicals which can give colours either directly or after addition of some other chemicals. All spectrophotometer instruments designed to measure the absorption of radiant energy have the basic components such as a Light, a filter or monochromator, cuvette for the sample and the blank, a radiation detector (phototube) to convert the radiant energy received to a measurable signal; and a readout device that displays the signal from the detector. In the research, UV-Vis spectrophotometer was applied in papain assay procedure to determine the absorbance of L-cysteine (papain) at wavelength 660 nm.

Figure 3.3: Hitachi U-1800 UV-Vis Spectrophotometer

3.2.4 pH meter

A pH meter is an electronic instrument used to measure the pH (acidity or alkalinity) of a liquid (though special probes are sometimes used to measure the pH of semi-solid substances). A typical pH meter consists of a special measuring probe (a glass electrode) connected to an electronic meter that measures and displays the pH reading. The pH probe measures pH as the activity of hydrogen ions surrounding a thin-walled glass bulb at its tip. The probe produces a small voltage (about 0.06 volt per pH unit) that is measured and displayed as pH units by the meter .In this research, pH meter was used to measure the pH of the solution for the extraction of papain enzyme. The pH was measured between pH 3 to 8.

Figure 3.4: Bench models pH meter

3.3 Methods

3.3.1 Sample preparation

Papaya leaves that obtained from Orchard Gambang, Kuantan initially washed before other treatment applied. After washed, papaya leaves were cut into smaller pieces (2x2 mm) and weighed about 5 g for each experiment. The surfaces of papaya leaves were make sure entirely dry during weighing process.

Figure 3.5: Orchard Gambang, Kuantan

Figure 3.6: Preparation of papaya leaves

3.3.2 Grinding

Each 5 g of papaya leaves were ground for 1 minute and mixed with acetate buffer (0.2 M Na_2HPO_4—0.1 M citric acid) at a different solid to liquid ratio (1:5 - 1:25 mg/ml) and adjusted with different pH (3-8).The 2x2 mm of particle size was required in the extraction of papain enzyme. Intraparticle diffusion resistance is small for small particle because of the smaller diffusion path. Therefore, the extraction yield will increase with the decrease of particle size. Reduction of particle size would increase the superficial area available for mass transfer and, then increase extraction yield.

3.3.3 Enzymatic pretreatment

Cellulase was quantified and dispersed in acetate buffer (0.2 M Na_2HPO_4—0.1 M citric acid) containing ground papaya leaves in different concentration (0.5-2.5 mg/ml). The slurries with the cellulase were incubated at 50^{o}C and agitated in a water bath at a constant speed of 80 rpm. Reactions were carried out for a maximum period of 2 h. Cellulase would interact on cell wall, break down its structural integrity so as to increase the releasing of components in the papaya leaves. The mechanism for enzyme-assisted extraction can weaken or break down the cell wall rendering the intracellular materials more accessible for extraction.

Figure 3.7: Cellulase for Enzymatic pretreatment process

3.3.4. Enzyme extraction

The hot water extraction method was applied for the extraction of papain enzyme from papaya leaves. Subsequent to the enzymatic pretreatment, the slurries were further undergone extraction process. Extraction time of papain was varied for 1,2,3,4, and 5 h to determine the time of extraction required for maximum yield of papain from papaya leaves. The mixture was kept in a thermostatically controlled stainless steel water bath at the required temperature. The temperatures of the water bath during extraction were maintained at 50, 55, 60, 65 and 70 ^{0}C and heating was done in close conditions. At the end of reaction, the mixture was heated at 75^{o}C for 10 min to denature the Cellulase activity.

3.3.5. Solid-liquid separation

The semi solid samples initially filtered to remove the leaf pulp and retain its aqueous solution. The semi solid sample was filtered by filter cloth and retained solution was further used for centrifugation .Centrifugation was carried out at 3250g for 10

minutes at 20^{o}C. The supernatants called enzymatic extract were used for protein determination.

3.3.6. Enzyme analysis

Papain activity in the papaya leaves was determined using colorimetric method. The substrate of a 0.65% casein solution was added in 50 mM potassium phosphate buffer.1 ml protease solution that was prepared in sodium acetate buffer and calcium acetate buffer was added to this 5 ml substrate solution. This mixture was incubated at 37^{o}C for 10 min, and the reaction stopped by adding 5 ml of 110 mM trichloroacetic acid (TCA). It was incubated again at 37^{o}C for 30 min. 2 ml sample was taken of it and added to a solution of 5 ml of 500 mM Na_2CO_3 and 1 ml Folin & Ciocalteu that was prepared at 1:3 ratios. This mixture was left to react for 30 min at 37^{o}C. Absorbance of the releasing of Cysteine was read at 660 nm. Further dilution was done if the solution was too concentrated. In this experiment, all samples were diluted in a 10 dilution factor. A standard curve of 1.1 mM Cysteine was prepared to determine the concentration of L-cysteine released.

3.3.7. Enzyme quantification

The enzyme concentration was determined using L-cysteine as a standard. L-cysteine is one of the active site residue in papain enzyme(Prakash *et al*., 2007).The papain enzyme possesses seven cysteine residues but only one of them ,Cys25 provides the free thiol group of the active site.(Simon *et al*., 2009).

3.3.8 Experimental design

Table 3.1: Experimental procedure for PH effect on extraction of papain

PH	Enzyme concentration	Solid to liquid ratio	Incubation time	Incubation temperature
3	1 mg/ml	1:10	4 h	65^{o}C
4	1 mg/ml	1:10	4 h	65^{o}C
5	1 mg/ml	1:10	4 h	65^{o}C
6	1 mg/ml	1:10	4 h	65^{o}C
7	1mg/ml	1:10	4 h	65^{o}C
8	1 mg/ml	1:10	4 h	65^{o}C

The example of experimental design was showed in the Table 3.1 for the pH effect on extraction of papain. Experimental procedure for the other effects which were enzyme concentration, solid to liquid ratio, incubation time and also incubation temperature were done in the similar design. In study of pH (3-8) effect, the pH that showed the highest concentration of L-cysteine was used for determining the second effect on the extraction of papain which was enzyme concentration (0.5-2.5 mg/ml).Then, the enzyme concentration that show the highest concentration of L-cysteine was used for determining the third effect on the papain extraction which was solid to liquid ratio (1:5-1:25 g/ml). Procedure was repeated for the incubation time and incubation temperature in the same design .The optimized condition in the extraction of papain enzyme was obtained.

CHAPTER 4

RESULT AND DISCUSSION

A systematic investigation of the parameters such as pH, enzymes concentration, solid to liquid ratio, incubation time and incubation temperature was carried out in a laboratory scale to determine conditions which enable the production of papain enzyme from papaya leaves using enzyme assisted method. Only one process parameter was varied at a time. The extraction of papain enzyme was done through 3 replicates. The effects of each process parameter on the extract yield of papain enzyme were discussed individually in the following sections

L-cysteine was declared as the papain enzyme in this research. Folin & Cioalteus Phenol reacted with free cysteine to produce a blue colored chromophore (part of a molecule responsible for its color), which was quantified and measured as an absorbance value on the spectrophotometer. The more cysteine that was released from casein, the more chromophores was generated. Absorbance values generated were compared to L-cysteine standard curve, which was generated by reacting known quantities of cysteine with the F-C reagent to correlate changes in absorbance with the amount of cysteine in micromoles. From the standard curve, the concentration of the L-cysteine was determined.

From the concentration of L-cysteine determined, the activity of the papain was also performed in terms of Units, which was the amount in micromoles of cysteine equivalents released from casein per minute.

$$units / ml(enzyme) = \frac{L - cysteine(\mu mole) * (11)}{(1) * (10) * (2)}$$

11= Total volume (in milliliters) of assay

10=Time of assay (in minutes) as per the Unit definition

1=Volume of Enzyme (in milliliters) of enzyme used

2=Volume (in milliliters) used in Colorimetric Determination

4.1 Effect of pH

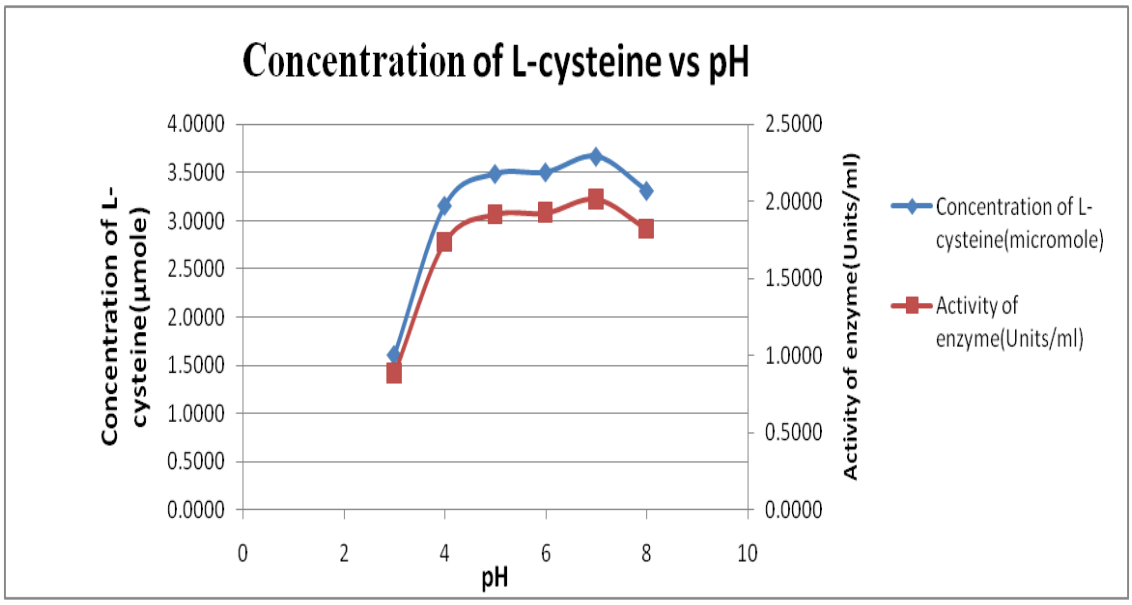

Figure 4.1: Effect of pH on papain extraction

Figure 4.1 showed the effect of different pH on papain extraction. pH was varied for 3, 4, 5, 6, 7 and 8. pH that was studied including acidic condition(pH 3 to pH 6), neutral condition(pH 7) and basic condition(pH 8) in determining the suitable condition for extraction of papain enzyme. From the result, condition in neutral which was pH 7 showed the highest concentration of papain enzyme followed by pH 6 (3.5018 µmole), pH 5 (3.4846 µmole), pH 8(3.3089 µmole), and pH 4 (3.1546 µmole). The lowest concentration of papain enzyme was in acidic condition which was pH 3.The highest concentration of papain in pH 7 was 3.6647 µmole while the lowest concentration in pH 3 was 1.6030 µmole.

From the Figure 4.1 also, the activity of the papain enzyme was determined. The activity of papain enzyme showed that, the more concentration of cysteine released from casein, the stronger the activity of papain enzyme. The activity of the papain enzyme was directly proportional to the concentration of the papain generated. Papain activity

for the pH 7was 2.0156 Units/ml followed by pH 6 (1.9260 Units/ml), pH 5 (1.9165 Units/ml), pH 8(1.8199 Units/ml), and pH 4 (1.7350 Units/ml). The lowest papain activity was 0.8817 Units/ml in pH 3.

pH is a measure of how acidic or basic a solution is; that is, how many H+ or OH- ions there are. These ions are charged, and charged molecules tend to pull on other molecules. Therefore, if too many ions are present, the enzyme may be denatured .This is because the enzyme is twisted and pulled so out of shape that it can no longer function. In this research, the optimal pH for the papain activity was in neutral condition. Aqueous phase pH influenced the available charges on papain and altered the electrostatic interaction between the papain, which was one of the major driving forces for extraction of papain (Rastogi & Nandini, 2009). Transfer of papain into aqueous phase took place by selecting the suitable pH. The pH of the system influenced the ionizable groups of a papain and alters the papain surface charges (Rastogi, Babu & Raghavarao, 2008). In general, the pH had influence on papain partitioning, either by changing the charge of the solute or by altering the ratio of the charged species present. When papain was in solution at the pH of their isoelectric point (pI), they will had no net molecular charge. Electrostatic repulsion between papain molecules will then be at a minimum and interactions via hydrophobic groups on the surface of the papain was more likely to occur (Rawdkuen, Chaiwut & Benjakul, 2010). At high pH (8.0), the yield and partition coefficient were lowered compared to neutral pH (7.0). This was probably due to inappropriate state of the charge in enzyme active site at the alkaline condition.

The increasing of papain content in aqueous phase pH from 5.0 to 7.0 may be attributed to the increase in charge density on papain resulting in increased electrostatic interaction. Further, increase in aqueous phase pH will result in obvious in negative charge on the target papain, which led to strong electrostatic interaction (Rastogi & Nandini, 2009), thereby increase in extraction of target papain but in the extraction efficiency of papain at pH 8.0, it was not found to be significantly. In pH 3 and pH 4, the effects of pH on papain extraction yield attributed to the action of H + on papaya leaves.

The acidic condition altered papaya leaves structures and H+ might opened up parts of the leaves structure which were usually inaccessible but in the strongly acidic condition, the structural of enzyme were altered and it can no longer function (Liang & Xu, 2001). It led to the decreasing of the concentration of papain in pH 3 and pH 4.

This result was agreed by Rastogi and Nandini, 2009 that stated “lipase was extracted from the organic phase to a fresh aqueous phase in 0.05 M potassium phosphate buffer (pH 7.0).

4.2 Effect of enzyme concentration

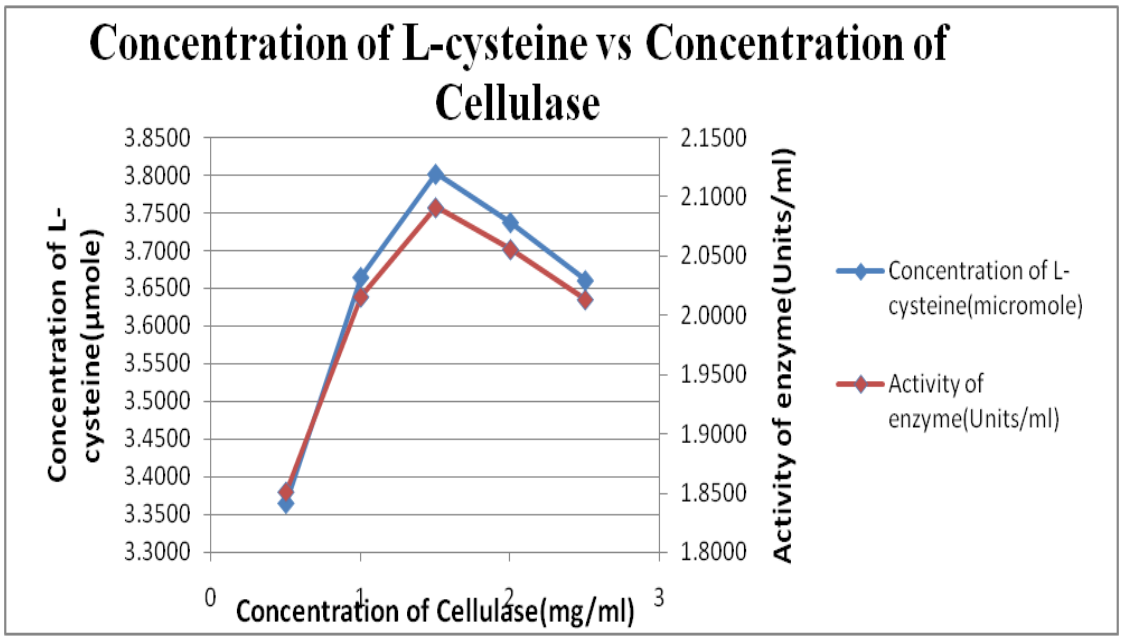

Figure 4.2: Effect of enzyme concentration on papain extraction

As shown in Figure 4.2, the extraction of papain enzyme was influenced by different concentration of enzyme. The result showed, the highest concentration of papain was 3.4561 mol/l in 1.5 mg/ml of enzyme followed by 2 mg/ml (3.7375 µmole), 1 mg/ml (3.6647µmole) and 2.5 mg/ml (3.6604µmole). The lowest concentration of papain was 3.3646 µmole in 0.5 mg/ml of enzyme.

The activity of the papain enzyme also was determined. From Figure 4.2, the activity of papain enzyme showed that, the higher concentration of cysteine released from casein, the stronger the activity of papain enzyme. The activity of the papain enzyme was directly proportional to the concentration of the papain generated. Papain activity in 1.5 mg/ml was 2.0910 Units/ml followed by 2.0556 Units/ml in 2 mg/ml, 2.0156 Units/ml in 1 mg/ml and 2.0132 Units/ml in 2.5 mg/ml. The lowest papain activity was 1.8505 Units/ml in 0.5 mg/ml of enzyme.

With an increase of cellulase concentration up to 1.5 mg/ml, the extraction yields of papain increased. As the enzyme concentration increases, the hydrolysis rate was increase because substrate molecules can easily contact with enzyme molecules, so more reactions will take place. Concentration of 1.5 mg/ml indicated that this concentration provided sufficient amounts of cellulase for the hydrolysis of cell wall. Thus, 1.5 mg/ml was selected as suitable concentration for the consumption of cellulase.

The enzyme concentration was decrease in 0.5 to 1.0 mg/ml of enzyme compared to the enzyme concentration in 1.5 mg/ml. This was because the hydrolysis of cell wall goes down. The rate of the reaction decreases because there were no more enzyme molecules available to catalyze the reaction of hydrolysis. Substrate inhibition occurred when excessive amounts of substrate were present. This was due to the fact that there were so many substrate molecules competing for the active sites on the papain enzyme surfaces that they blocked the sites and prevent any other substrate molecules from occupying them. This causes the reaction rate (hydrolysis process) to drop. At 2.0 to 2.5 mg/ml of enzyme, the very high enzyme concentration made the substrate concentration become rate-limiting, so the rate stopped increasing. The rate of the reaction of hydrolysis decreased because there was no more substrate to be catalyzed.

Cell walls of plants have important functions in the different tissues that they are part of. Throughout the enzymatic degradation of the cell wall of plants small structure changes in the polysaccharide that they are made of can alter considerably the properties of the wall. Plant cell wall consists of a rigid skeleton of cellulose embedded in a gel-like matrix composed of pectic compounds, hemi-cellulose and glycoprotein. Cellulase catalyzes the breakdown of cellulose into glucose, cellobiose and higher glucose polymers (Fu *et al*., 2009) .The extraction yields of papain in treated samples increased notably with treatment by enzyme preparations. Cellulase hydrolyzed cellulose in papaya leaves, released papain from cell walls and increased the extraction efficiency. Cellulase could improve extraction yield mainly because it can penetrate the matrix material, rupturing the cell walls, resulting in papain being more easily released from the

matrix into the extraction medium. Also, Cellulase can enhance the extracting power of the solvent by driving solvent into the matrix to extract the targeted components.

The result was supported by Fu *et al*., 2009 that said "the optimum extraction of paclitaxel was occurred at 1.5 mg/ml of cellulase. The cellular target of paclitaxel's action is tubulin, a protein capable of undergoing polymerization to produce microtubules."

4.3 Effect of solid to liquid ratio

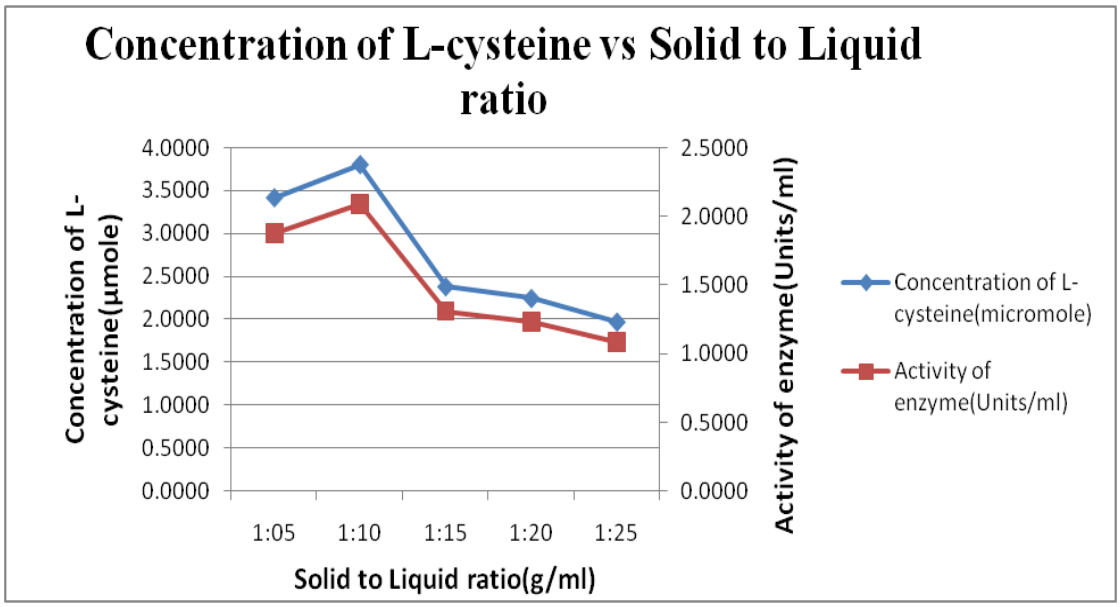

Figure 4.3: Effect of Solid to Liquid ratio on papain extraction

In investigating the influence of ratio of solid to liquid ratio on yields of papain enzyme, several tests were performed at different ratios. The effect of solid to liquid from 1:5 to 1:25 g/ml was shown in Figure 4.3. Seen from Figure 4.3, with increase in solid to liquid ratio, the extraction rate of papain also increased until the optimum condition achieved. The results show that, if the yield was expressed by the ratio of papain extracted, the smaller the solid to liquid ratio was, the better the yield was.

Figure 4.3 showed the highest concentration of papain was 3.8018 µmole in 1:10 ratio(g/ml) followed by 3.4161 µmole in 1:5 ratio(g/ml) , 2.3788 µmole in 1:15 ratio(g/ml) and 2.2417µmole in 1:20 ratio(g/ml). The lowest concentration of papain was 1.9673 µmole in 1:25 ratio (g/ml).

From the figure 4.3 also, the activity of the papain enzyme was determined. The activity of papain enzyme showed that, when the concentration of cysteine released increased from casein, the activity of papain enzyme rise up. The activity of the papain enzyme was directly proportional to the concentration of the papain generated. Papain activity for the 1:10 ratio was 2.0910 Units/ml followed by 1:5 ratio (1.8788 Units/ml), 1:15 ratio (1.3083 Units/ml) and 1:20 ratio (1.2329 Units/ml). The lowest papain activity was 1.0820 Units/ml in1:25 ratio (g/ml).

The result showed the extraction yields of papain increased with higher of solid to liquid ratio until it achieved optimum ratio for the papain extraction. When the solid to liquid ratio increased from 1:5 to 1:10 (g/ml), the yield of papain increased .It was obvious that the solid to liquid ratio was useful for improving extraction yields. The dissolution of papain enzyme into the solvent was a physical process. Water content in the applied solvent played an important role in the extraction. Swelling of the plant material by water enhanced the extraction efficiency .When the amount of solvent increase, the chance of papain enzyme come into contact with the solvent goes up, which lead to the higher extraction rate.

Increasing solid to liquid ratio was good for reactive medium diffusion making the reaction more sufficient but when solid to liquid come to 1:15, increasing the ratio was not helpful to extracting rate. In the tested range of 1:15–1:25 (g/ml), there was no significant difference, which was probably due to the larger volume of solvent causing excessive swelling of the materials (Fu *et al*., 2010) .The decrease in the extraction yield as the solid to solvent ratio was increased from 1:15 to 1:25 was due to the fact that the concentration of solvent was not high enough to allow for extract transfer from the material matrix. It was found that a larger solvent volume did not lead to a higher papain yield from papaya leaves (Ye *et al*., 2011).

Using a large amount of solvent was not considered cost-effective due to the high operating cost of energy consumption (Phisalaphong & Boonkird, 2008).

Consequently, the ratio of the extraction of papain enzyme was selected at 1:10 g/ml. The value of 1:10 g/ml was considered as the optimal ratio of solid to liquid for the extraction of papain which also can reduce processing costs. Therefore, the solid to liquid ratio of 1:10(g/ml) was sufficient to reach the high extraction yield.

This result agreed by Liu, Guan & Sun, 2008 that stated “10 g of defatted oat bran sample and 100 ml of deionized water was used in determine the optimized of the enzymatic pretreatment in oat bran protein extraction by particle swarm optimization algorithms for response surface modeling.”

4.4 Effect of Incubation time

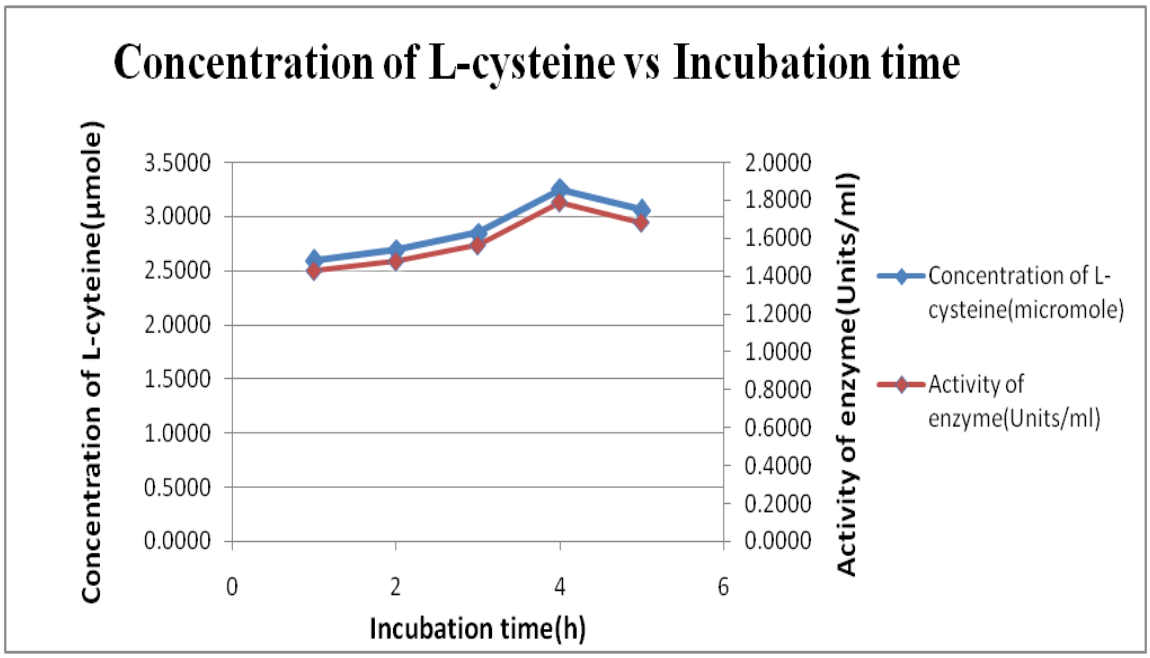

Figure 4.4: Effect of incubation time on papain extraction

Next, the effect of incubation time on the extraction yields of papain was examined. It was necessary to select a proper time to guarantee completion of the extraction. Studies were carried out at different times which were 1, 2, 3, 4 and 5 hour. From figure 4.4, an increase in the extraction yields was observed with the extending of time from 1 h to 4 hr .The highest concentration of papain was found in the 4 h incubation time which was 3.2532 µmole followed by 5 h (3.0603µmole) , 3 h(2.8417µmole),2h (2.6874µmole) of incubation time. The lowest concentration of papain was in the 1 h of incubation time which was 2.5974 µmole.

From the figure 4.4 also, the activity of the papain enzyme was determined. The activity of papain enzyme showed that, the higher concentration of cysteine released from casein, the stronger the activity of papain enzyme. The activity of the papain enzyme was directly proportional to the concentration of the papain generated. Papain

activity for the 4 h was 1.7893 Units/ml followed by 5h, 3h ,2 h and 1 h which were 1.6832 Units/ml, 1.5629 Units/ml, 1.4781 Units/ml and 1.4286 Units/ml respectively.

During the initial 1 h to 4 h, the extraction of papain was considerably enhanced rapidly and reached its maximum at 4 h. After 4 h, the extraction yields decreased about 6.3%. Extraction yield decreased with the extension of incubation time due to the degradation of papain structure. Prolonging the contact time increased the extraction of papain until it reached the optimum time. The longer of extraction time, more solvent could penetrate the cells and at the same time increased the surface contact (Fryer *et al*., 2010), therefore a greater quantity of papain could be extracted.

The extraction time of papain in 4 hours indicated that the time was long enough for the extracting process. Using a longer time for extracting process was not considered cost-effective due to the high operating cost of energy consumption. Consequently, the extraction time of papain was selected in 4 hours.

This result supported by Flavio &Tatiana, 2005 that said “the results showed that best conditions for extracting pectic enzymes were 1.0 M NaCl, for 4 h for both enzymes, whereas pH 7.5 and 4.0 were the appropriate parameters for extracting pectinesterase (PE) and polygalacturonase (PG) , respectively.”

4.5 Effect of Incubation temperature

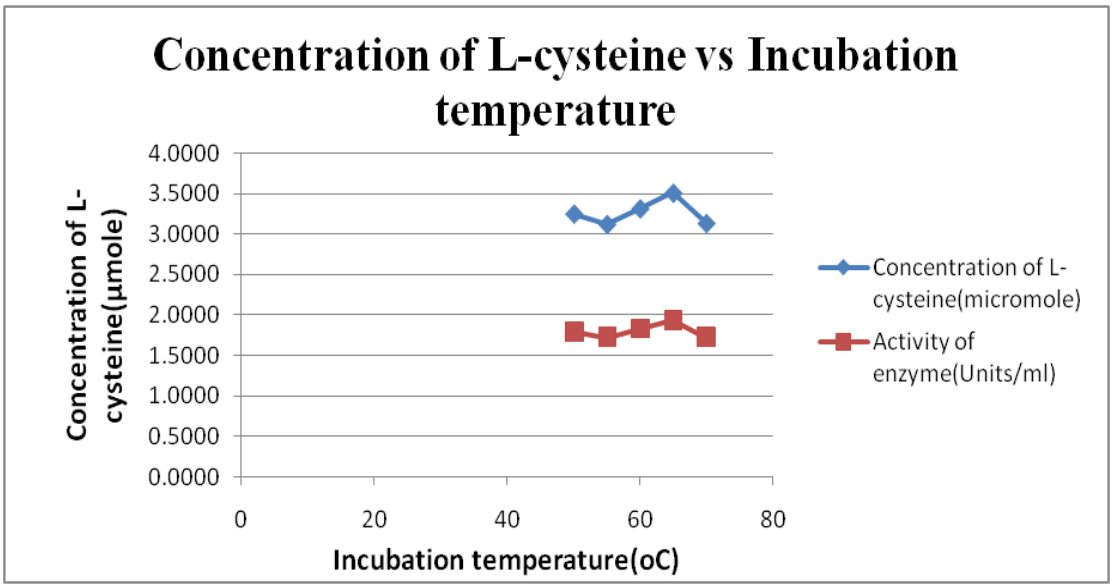

Figure 4.5: Effect of incubation temperature on papain extraction

High papain extraction may not be achieved in a short time at low temperatures whilst high temperatures have disadvantages such as higher energy. So, in our present study, a medium temperature range from 50 to 70 °C was selected and its effect on papain enzyme extraction was studied.

As it can be seen from Figure 4.5 , the highest extraction yields of papain appeared at temperatures of 65^0C that contribute 3.5104µmole followed by temperature at 60^0C,50^0C and 70^0C which were 3.3175 µmole , 3.2532 µmole and 3.1375 µmole respectively. The lowest extraction yield was at temperature 55 ^{0}C which was 3.1246 µmole.

From the figure 4.5 also, the activity of the papain enzyme was determined. The activity of papain enzyme showed that, when the concentration of cysteine released increased from casein, the activity of papain enzyme was higher. The activity of the

papain enzyme was directly proportional to the concentration of the papain generated. Papain activity at 65^{o}C was 1.9307 Units/ml followed by 1.8246 Units/ml at 60^{o}C, 1.7893 Units/ml at 50^{o}C and 1.7256 Units/ml at 70^{o}C. The lowest papain activity was 1.7185 Units/ml at 55^{o}C.

Heat treatment is routinely performed to accelerate the mechanism of the diffusional process when extracting from plants (Fauduet *et al*., 2008). Extraction temperature is a factor that must be studied to increase the extraction yield of papain. The extraction yield considerably increased from 50^{0}C to 65^{0}C although the extraction yield at 55^{o}C showed an inconsistent reading. It was might caused by mistake in the papain analysis step. The amount of casein added that act a substrate was not enough for the digestion of papain. .

Extraction of papain was increase with the higher temperature (50^{o}C to 65^{o}C) but when extraction temperature goes by certain threshold (70 °C), the extraction yield started to decrease. Higher temperature caused the decrease of intermolecular interactions within the solvent, gave rise to higher molecular motion, and increased the solubility. The increasing temperature may also improve the release of targeted papain from the cytoplasm of plant materials, and as a result, the availability of papain increased. At higher temperature, the solvent viscosity decreased and the diffusivity increased, resulted for the increasing of extraction efficiency (Fu *et al*., 2010). Higher incubation temperature may improve the recovery, because incubation in hot water can extract some pectic polysaccharides from cell wall and weaken the cell wall integrity (Hossain, Li & Smith, 2006). Possibly, as a result, the solvent containing water can easily get in contact with the papain materials, and the recovery is improved. Increased temperature also promoted solvent extraction by enhancing both diffusion coefficients and the solubility of papain content. Increasing temperature favored the release of papain content with the breakdown of cellular constituents of plant cells which leads to increase cell membrane permeability (Tan *et al*., 2010). Hence, the efficiency of extraction increased.

Extraction yield of papain increased with the increasing temperature, but increasing the temperature at 70^{o}C decreased the yield of the papain .It showed that increasing the extraction temperature could changes in papain structure and the enzyme denatured.

In order to avoid decomposition and scorching under high temperature, 65^{o}C was selected as the optimum temperature for the extraction of papain enzyme.

This result agreed by Fu *et al*., 2010 that stated "maximum extraction yields of pinostrobin (PI) in pigeonpea leaves were at temperature 65^{o}C. PI has showed many bioactivities such as inhibiting the human placental aromatase, DNA topoisomerase I, decreasing the proliferation of MCF-7 cells, inducing mammalian phase 2 detoxication enzymes, antioxidant enzymes, and anti-Helicobacter pylori activities."

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The extraction of papain enzyme from papaya leaves were successfully achieved by enzyme assisted method. Optimized extraction yields of papain were achieved by operating at suitable pH, suitable concentration for the consumption of cellulase, solid to liquid ratio which was efficient and feasible, sufficient extraction time to overcome diffusion limitations and at a moderate extraction temperature. The optimal extraction conditions that satisfied the above constraints were found to be at pH 7, 1.5 mg/ml of cellulase, solid to liquid of 1:10 (g/ml), an extraction time of 4 h and at an extraction temperature of 65^{o}C. Passing through the treatment by cellulase, extraction yield of papain enzyme were found to be 3.8018 μmole with 2.0910 Units/ml which give confidence that the optimized process can be carried out without loss of efficiency at an industrial scale. Enzyme assisted extraction provide a feasible way for the extraction of papain enzyme from papaya leaves due to the advantage of environment-friendliness, lower cost, easy operation and higher efficiency, and it is promising for industry application broadly.

5.2 Recommendations

In the extraction of papain enzyme, the downstream processing is the most important process to obtain the purified enzyme. Previously, purification of papain by two-step salt using ammonium sulfate and also sodium chloride was done. However, the purified enzyme still remains contaminated with other proteases. Therefore, an alternative purification of papain is suggested by extraction in aqueous two-phase. Aqueous two-phase systems (ATPS) are made up of two polymers or one polymer and a salt in water. For extraction in aqueous two-phase system, defined amounts of solid polyethylene glycol (PEG)–phosphate and ammonium sulfate $(NH_4)_2SO_4$ is used.

Papaya leaves is the rich source of the cysteine endopeptidases, including papain, glycyl endopeptidase, chymopapain and caricain. Therefore in determine the presence of purified papain, it is recommended to apply ion-exchange chromatography on fast protein liquid chromatography (FPLC) and also cathodic gel electrophoresis. In FPLC, the percentages of peak areas of papain and other proteins is obtained from an automatic integrator. The purity of papain is specified as the percentage peak area of papain with respect to the total peak area. For the cathodic gel electrophoresis method, the protein samples migrate towards the cathode during electrophoresis. The relative concentration of the papain is quantified by measuring the absorbance of the protein bands at 595 nm using densitometer.

The extraction of papain enzyme for this research was carried out in laboratory scale using enzyme assisted method. For the suggestion, the supercritical fluid extraction can be applied to enhance the extraction yield and obtain the pure products. Supercritical fluid extraction is becoming increasingly popular, particularly those that employ environmentally benign supercritical carbon dioxide (SC-CO2). This interest is due to the time savings involved when substituting SC-CO2 for a liquid solvent and the reduction and/or elimination of organic solvents. It is profitable when applied to scale up process, to obtain ultra-pure products.

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APPENDIX A

PREPARATION OF REAGENT

PRINCIPLE:

$$\text{Casein} + H_2O \xrightarrow{\text{Protease}} \text{Amino Acids}$$

(L-cysteine is Amino acids)

CONDITIONS: T = 37°C, pH = 7.5, A 660nm

METHOD: Colorimetric

READENTS:

1. A: 50 mM Potassium Phosphate Buffer, pH 7.5
2. B:0.65 % Casein
3. C:110 mM Trichloroacetic Acid,6.1 N
4. D:0.5 mM Folin & Ciocalteu's Phenol Reagent(F-C)
5. E: 500 mM Sodium Carbonate (Na_2CO_3)
6. F: 10 mM Sodium Acetate Buffer with 5 mM Calcium Acetate, pH 7.5 at 37 °C (Enzyme Diluent)
7. G: 1.1 mM L-Tyrosine Standard (Standard Solution)
8. H: Protease Enzyme Solution

REAGENT PREPARATION:

1. A: 50 mM Potassium Phosphate Buffer, pH 7.5
   Prepared using 11.4 mg/ml of potassium phosphate dibasic, trihydrate in deionized water and pH was adjusted with 1M HCI.

2. B:0.65 % (w/v) Casein
   Prepared by mixing 6.5 mg/ml of casein in 50 mM potassium phosphate buffer. Gradually increase the solution temperature with gentle stirring to 80-85^{o}C for about 10 minutes until a homogenous dispersion was achieved. It is very important not to boil the solution. The pH was then adjusted if necessary with 1M NaOH and 1 M HCI.

3. C:110 mM Trichloroacetic Acid(TCA),6.1 N
   Prepared by diluting a 6.1N stock 1:55 with deionized water. Trichloroacetic acid is a strong acid and should be handled with care.

4. D:0.5 mM Folin & Ciocalteu's Phenol Reagent(F-C)
   Prepared by diluting 1:3 with deionized water. The solution reacted with cysteine to generate a measurable color change that directly related to the activity of protease. Folin's Phenol Reagent is an acid and should be handled with care.

5. E: 500 mM Sodium Carbonate (Na_2CO_3)
   Prepared using 53 mg/ml of anhydrous sodium carbonate in deionized water.

6. F: 10 mM Sodium Acetate Buffer with 5 mM Calcium Acetate, pH 7.5 at 37 °C (Enzyme Diluent)
   This solution used to dilute enzyme solutions.

7. G: 1.1 mM L-Cysteine Standard (Standard Solution)

   Prepared using 0.13 mg/ml L-cysteine in deionized water and heated gently until the cysteine dissolves. As with casein, do not boil this solution. L-cysteine standard was allowed to cool to room temperature. This solution used to make standard curve.

8. H: Protease Enzyme Solution

   Immediately before use, 0.5 ml protease was dissolve in 0.5 ml enzyme diluent solution.

APPENDIX B

ENZYMATIC ASSAY OF PROTEASE: CASEIN AS A SUBSTRATE

Pipette the following reagents into suitable vials (in milliliters):

1. Reagent B(Casein) 5 ml

Equilibrate in 37^{o}C for about 5 minutes .Then add

2. Reagent H(enzyme solution) 1 ml

Mix by swirling and incubate at 37 °C for exactly 10 minutes. Then add:

3. Reagent C(TCA) 5 ml

Incubate the solution at 37^{o}C for 30 minutes. Then add

The following reagents were pipette into test tube

Solution from previous sample 2 ml

4. Reagent E(Na_2CO_3) 5 ml

5. Reagent D(F-C) 1 ml

The solution was mixed by swirling and incubated at 37^{o}C for 30 minutes. Then, they were cooled at room temperature. After that the absorbance was measured by a spectrophotometer using a wavelength of 660 nm.

APPENDIX C

CONSTRUCTION OF STANDARD CURVE

A standard curve was prepared by pipetting the following reagents into suitable vials (in milliliters):

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Blank
Reagent G	0.05	0.10	0.20	0.30	0.40	0.50	0.00
Deionized Water	1.95	1.90	1.80	1.70	1.60	1.50	2.00
Reagent E (Na_2CO_3)	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Reagent D (F-C)	1.00	1.00	1.00	1.00	1.00	1.00	1.00

The solution was mixed by swirling and incubated at 37 °C for 30 minutes. Then, they were allowed to cool to room temperature. The absorbance was read at 660nm for each of the vials in suitable cuvettes.

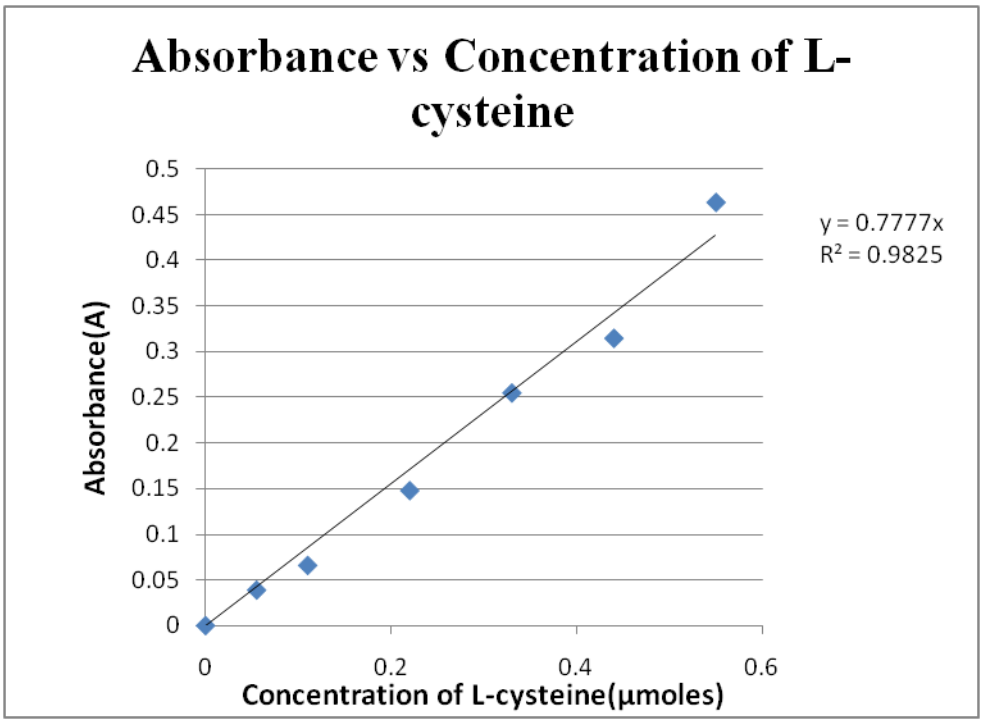
APPENDIX D

MEASURING ABSORBANCE AND CALCULATING ENZYME ACTIVITY

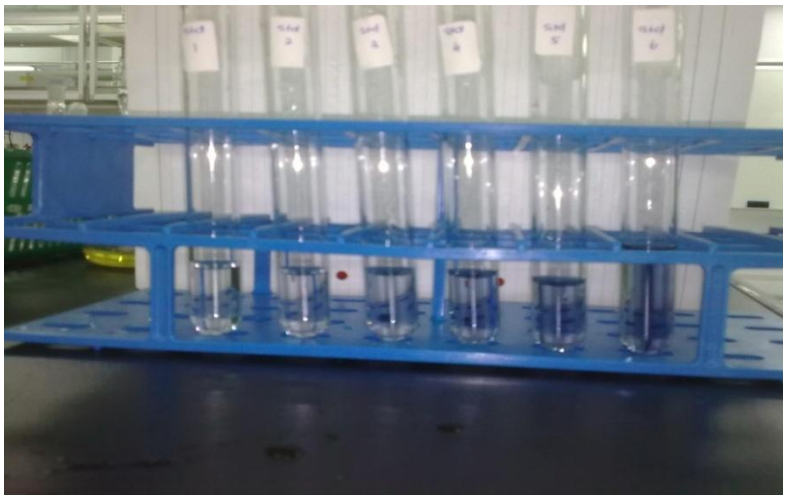
The standard curve was created using a graphing program to plot the change in absorbance of the standard on the Y axis versus the amount in micromoles for each of 5 standards on the X axis

Concentration of L-Cysteine(µmole)	Absorbance(A)
0	0
0.055	0.039
0.11	0.066
0.22	0.148
0.33	0.255
0.44	0.315
0.55	0.464

Absorbance reading for L-cysteine standard curve



L-cysteine standard curve



The color development of standard curve

A line of best fit and corresponding slope equation was generated. The slope equation was y=0.7777 x. The result in the micromoles of L-cysteine liberated during this particular proteolytic reaction could be obtained by inserting the absorbance value for one of the test sample into the slope equation.

To get the activity of enzyme activity in Units/ml,the following calculation was performed

$$Units/ml(enzyme) = \frac{\mu mole(L-cysteine)*(11)}{(1)*(10)*(2)}$$

11 = Total volume (in milliliters) of assay

10 = Time of assay (in minutes) as per the Unit Definition

1 = Volume of enzyme (in milliliter) of enzyme used

2 = Volume (in milliliters) used in Colorimetric Determination

APPENDIX E

BUFFER PREPARATION

The Acetate Buffer (disodium hydrogen phopphate, Na_2HPO_4, and Citric Acid anhydrous) was used in the research

- Solution A (0.2 M Na_2HPO_4): 0.028 g/ml of Na_2HPO_4 was prepared with deionized water
- Solution B (0.1M Citric Acid): 0.019g/ml of citric acid was prepared with deionized water

Referring to table, the volume of solution A and solution B were mixed together to get for desired pH

0.2 M Na_2HPO_4/ml	0.1 M Citric Acid/ml	pH
20.55	79.45	3.0
38.55	61.45	4.0
51.50	48.50	5.0
63.15	36.85	6.0
82.35	17.65	7.0
97.25	2.75	8.0

Acetate Buffer solution

THE EXTRACTION OF PAPAIN ENZYME FROM PAPAYA LEAVES USING ENZYME ASSISTED METHOD

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