STABILITY OF PAPAIN IN AQUEOUS ORGANIC SOLVENT BY REVERSE PHASE LIQUID CHROMATOGRAPHY

MUHAMMAD YUNUS BIN ZAINAL ABIDIN

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ABSTRACT

Papain is one of the proteolytic enzymes found in the latex of the leaves and of the green fruit of the papaya tree (*Carica papaya L.*). It's known to be highly used in the several industry like food, medical, brewing and many more. Hence, the objective of this research is to study the stability of papain in aqueous organic solvent and secondly to purify the papain using the Amberlite™ XAD7HP as the reverse phase liquid chromatography adsorbent. The research is based on experimental lab research. The effects of different concentrations of the water-miscible organic solvents acetonitrile and ethanol on the stability of papain in aqueous solution were studied. Batch adsorption using Amberlite™ XAD7HP was carried out to purify papain from papaya juice. The purified papain was analysed using the Bradford assay, the proteolytic papain activity assay and SDS PAGE. As for conclusion the of the study. The result obtained proved that papain has exhibited higher stability in aqueous acetonitrile than in ethanol under small margin. Decreases in the activity of the enzyme were observed at organic solvent concentrations above 60%. Papain with high purity was achieved by adsorption using the Amberlite™ XAD7HP.

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ABSTRAK

Papain adalah salah satu enzim proteolitik yang ditemui dalam getah daun dan buah-buahan hijau pokok betik (*Carica papaya L.).* Ia dikenali kerana sangat digunakan dalam beberapa industri seperti makanan, perubatan, minuman dan banyak lagi. Oleh itu, objektif kajian ini adalah untuk mengkaji kestabilan papain di akueus pelarut organik dan kedua untuk membersihkan papain menggunakan Amberlite™ XAD7HP sebagai fasa terbalik kromatografi cecair adsorben. Penyelidikan adalah berdasarkan kepada penyelidikan makmal eksperimen. Kesan yang berbeza kepekatan pelarut asetonitril air larut organik dan etanol pada kestabilan papain dalam larutan akueus telah dikaji. Batch penjerapan menggunakan Amberlite™ XAD7HP telah dijalankan untuk membersihkan papain dari jus betik. Papain disucikan telah dianalisis menggunakan cerakin Bradford, papain proteolitik aktiviti cerakin dan SDS PAGE. Sebagai kesimpulan kajian. Hasil kajian yang diperoleh membuktikan bahawa papain telah menunjukkan kestabilan yang lebih tinggi dalam asetonitril akueus daripada dalam etanol bawah margin kecil. Penurunan dalam aktiviti enzim yang dapat diperhatikan pada kepekatan pelarut organik melebihi 60%. Papain dengan kesucian yang tinggi telah dicapai oleh penjerapan menggunakan Amberlite™ XAD7HP.

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LIST OF ABBREVIATIONS

ACN	Aqueous acetonitrile
BAEE	Benzoyl-Arginine Ethyl Ester
CO_2	Carbon Dioxide
CD	Circular dichroism
EDTA	Ethylenediaminetetraacetic acid
NaOH	Sodium hydroxide
rpm	Revolutions per minute
SDS-PAGEi	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
THF	Tetrahydrofuran
Z-gly-pNP	N-Carbobenzoxy-glycine p-nitrophenyl ester

LIST OF SYMBOL

cm	Centimetre
hr	Hour
L	Litre
m	Metre
mg	Milligram
ml	Millilitre
min	Minute
Kg	Kilogram
s	Second
V	Volume
Umoles	U moles of tyrosine enzyme
%	Percentage
℃	Celsius Degree
µ	Specific growth rate

CHAPTER 1

INTRODUCTION

1.1 Background of proposed study

Stability of papain in aqueous organic solvent by reverse phase liquid chromatography was a research that focused on finding the highest activity of enzyme in different organic solvent by reverse phase liquid chromatography. As early in 20th century research have been done to discover the potential of papain. Papain, also known as papaya proteinase I, is a highly stable cysteine protease enzyme present in papaya (*Carica papaya*) and mountain papaya. It belongs to the papain superfamily, as a proteolytic enzyme, papain is crucial in many biological processes in all living organisms (Tsuge *et al.*, 1999). Recent years show it have a well known application in the line of medical application, food industry, forage

industry, leather industry, textile industry, cosmetic industry, brewing industry, etc. In a medical application, studied done by Huet (2006) proves that. As a protein digesting, papain is used in combating *dyspepsia* and other digestive disorders and disturbances of the gastrointestinal tract. Thus avoid the risk of having digestive disorder. The importance of papain can also be seen in human body requirement. In tough cuts of meat, muscle fibers are tightly bundled and held together by proteinaceous material. The papain helps break this down, as well as tenderizing the meat. Hence a better separation of molecules based on their polarity using reverse phase liquid chromatography (RPLC) was conducted. Principally, RPLC was a separation of molecules based upon their interaction with a hydrophobic matrix which is largely based on their polarity. The papain that is bound to the hydrophobic matrix in aqueous buffer and was eluted from the matrix using a gradient of organic solvent. RPLC has been chosen as an analysis tool in this research because of its wide application in the industry which is allows precise control of the variables like organic solvent type as well as truly proven cost effective.

1.2 Problem Statements.

One of the common diseases faced by Malaysian citizen is Prostate Inflammation (PI). The new statistic show by the Malaysia Ministry of Health shows that PI diseases affect about 10 percent of the male population at least once in their lifetime. The main causes of these diseases are infection by the bacteria in their prostate gland.

According to Habermacher (2006) there are four stages of prostatitis inflammatory which are acute bacterial prostatitis, chronic bacterial prostatitis, chronic prostatitis and lastly asymplomatic inflammatory prostatitis. The highest cases was recorded was chronic prostatitis which is about 90 percent compared to the other 3 cases. Thus, a high purity of papain supplement is in great demand to treat the PI disease.

Pharmaceuticals industry is not the only one that is benefit from papain enzyme existence. Some other industries like breweries, food, leather, detergents, meat and fish processing are also using it. Good quality papain has very good domestic as well as export demand, The US market has been estimated at up to double the EU market or roughly 300 to 400 Mts per year. This shows that there is a demand in papain will continue to be steady as year's progress.

There are many studies show an alternative small scale papain isolation through purification technology which involved the use of various expensive and difficult manual chromatographic columns with different mechanisms including ion exchange, covalent, or affinity chromatography (Nitsawang *et al*., 2006) . However,

no literature has found to report the purification of papain from papaya fruit using reverse phase liquid chromatography (RPC). Therefore, the development of a direct and simplified purification of papain using batch adsorption of RPC was studied

1.3 Research objectives.

The main objective of this research is:

1. To test the activity of papain in aqueous organic solvent using acetonitrile and ethanol

2. To purify papain from papaya juice using reverse phase liquid chromatography adsorbent.

1.4 Scope of Research Work.

The downstream process of purifying papain can be considered complex molecule because it only active at certain condition as well as having a denaturation that will affect the process. There is many protocols and parameter that must be considered such as time of incubation, concentration of solvent, and many more.

In achieving the aim of purification process by research. The stability of the papain in various condition such as concentration of solvent, incubation of time and many more must be investigate, Once the highest activity condition of enzyme are known, Purification is done in order to remove the unwanted molecule and have a high quality enzyme for chemical process. Lastly analysis of sample will be done to see the result of the purification.

The first part of research are focusing on finding the stability of papain by comparing between two organic solvent that is acetonitrile and ethanol at a different volume ratio of concentration ranging from 0% to 100%. From the result a graph will be plotted to show a trend of enzyme activiy.

After the entire stability test done, the purification part it will be done by using the batch adsorption of Amberlite^{TM} XAD7HP. The purpose of purification is to get higher purity of papain to sell to the industry. This was followed up by the analysis of the sample by the bradford assay and the SDS-PAGE. Both are done in order to determine the concentration of protein in the solution and compare the purity based on the parameter used for the first part.

1.5 Significance of Propose Study

Papain is one of the enzymes that have the widest application in its family. Such example is in medical supplement where diseases like inflammatory of prostatitis is being treated with papain as it core medicine supplement. However, producing the papain supplement, the enzyme first must be extract from *carica papaya* at a very highest stability of enzyme. Thus it comes for the rationalize of this research to find the highest stability of enzyme condition for further purification of papain.

Other than that, ethanol alongside with acetonitrile is also being used in this research as a solvent in order compare the highest stability of papain with this solvent. Recent years show that a decreasing demand of acrylonitrile due to financial crisis, have affect the worldwide shortage of acrylonitrile (Brettschneider *et al.*, 2010). Thus, these studies will determine whether ethanol will provide sufficient backup for acrylonitrile in RPLC method.

CHAPTER 2

LITERATURE REVIEW

2.1 Papain

Papain is the scientific name for protease enzyme isolated from papaya enzyme. From papaya it can be obtained by cutting the skin of the unripe papaya followed up with drying the latex which flow through the cut. The active of papain can be predicted through the colour of the papayas, the greener the papaya, more active is the papain.

As an early 1950s. The action of papain was first investigated by Roy in an article published in the *Calcutta Medical Journal* entitled "The Solvent Action of Papaya Juice on Nitrogeno us Articles of Food". However in the late twentieth

century only a serious research done by (Storer, 1994) who partially named papain and purified the product from the sap of papaya (Menard, 1998). As a day goes on more research come to follow with Tsuge (2009) asserts that papain can be classified as importance in many biological processes in all living organisms as it was one of the proteolytic enzyme. Some areas of medicine and food field, papain extensive proteolytic activity is applied towards proteins, amino acid esters, short chain peptides and amide links (Uhlig, 1998).

The structure of papain also truly gives its functionality that help to understand how this proteolytic enzyme works and how it's useful for a variety of purpose. Besides that, Papain is unusually defiant to high concentrations of denaturing agents, such as, 8M urea or organic solvent like 70% EtOH. Optimum pH for activity of papain is in the range of 3.0-9.0 which varies with different substrate (Edwin *et al.*, 2000).

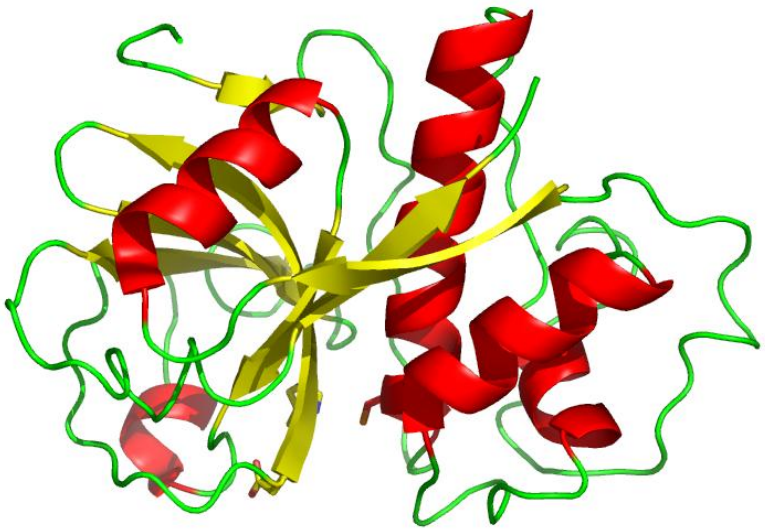

Figure 2.1 Structure of papain (Source: Storer, 1994)

2.2 Stability of Papain

Papain can be considered as a highly stable enzyme. In a report Choudhury (2009) states that a crystalline suspension of papain is stable at 5°C for 6-12 months. With the stabilizing agents are EDTA. cysteine and dimercaptopropanol. The basic reaction condition was reported around pH 6-7. Even under a wide range of condition at elevated temperature, papain a cycteine hydrolase has been found out very stable and active (Cohen *et al*., 1986).

Besides that in a study about the effects of water-miscible organic solvents in aqueous solution on the activity and conformational stability of papain by Szabo (2006). The alterations in the secondary and tertiary structures of the enzyme were by means of fluorescence spectroscopic and far- and near-UV circular dichroism (CD) measurements found out that papain exhibited high stability in aqueous acetonitrile (ACN), ethanol and 1,4-dioxane. Decreases in the activity of the enzyme were observed at organic solvent concentrations above 60%. Tetrahydrofuran (THF) caused a dramatic reduction in activity even at low concentrations (5–10%). The solvent-induced structural changes were followed by means of circular dichroism (CD) and intrinsic fluorescence spectroscopy measurements. The decreases in enzyme activity at 90% THF or 90% 1,4-dioxane were accompanied by the loss of the tertiary structure. However, at 90% ethanol and 90% ACN, papain exhibited an increased amount of the helical conformation, with little change in the tertiary structure.

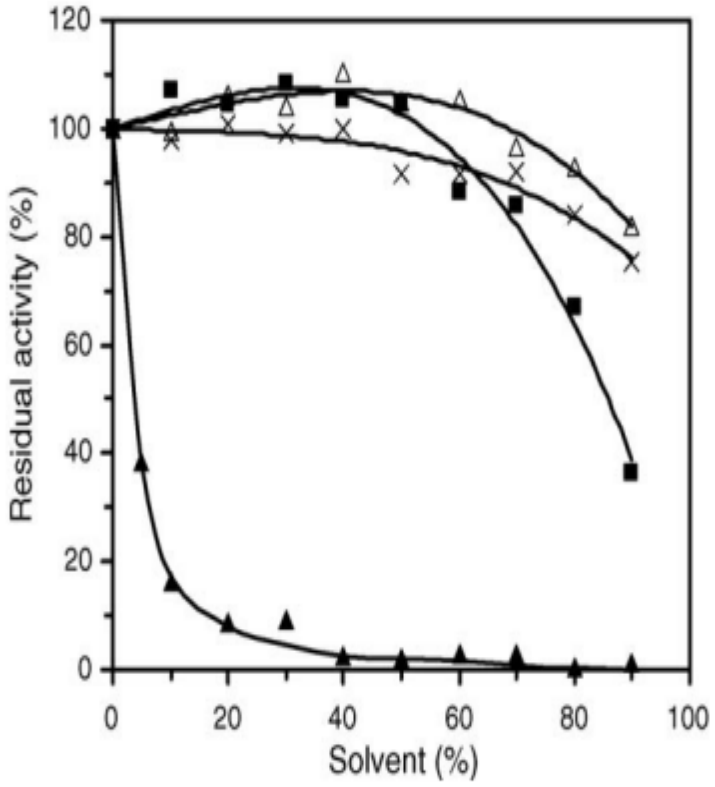

Figure 2.2 Effects of different concentrations of acetonitrile(☐ 1,4-dioxane tetrahydrofuran△) and ethanol (×) on papain activity in aqueous solution. Experiments were carried out at 25 ◦C with incubation for 20 min, at an enzyme concentration of 0.1 mg/ml. (Source: Szabo *et al*., 2006)

2.3 Purification of Papain

Purification can be definite as extraction of a single enzyme protein from. cells, tissue, etc. which may contain more than 1000 different proteins and lots of other biomolecules. Papain can be extracted from fresh latex of *carica papaya*. However in conducting the purification there is some structure and boundaries that must be keep intact. Such as pH, temperature, salt concentration etc. Some common type of purification of papain known was crystallization, precipitation and for physical method there is also gel filtration and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page). In his research, Rubens (2000) stated that the papain thus obtained is practically pure measured using Z-gly-pNP and BAEE as substrates. Papain crystallized by this method, without the use of high concentrations of salts or thiol-containing substances such as cysteine and dithiothreitol, is obtained in the form of a complex with natural inhibitors existent in latexwhich can be removed by dialysis. Hence, SDS-page is one type of analysis of purification that can be applied in order to separate proteins accordingly by size and get high purity of papain.

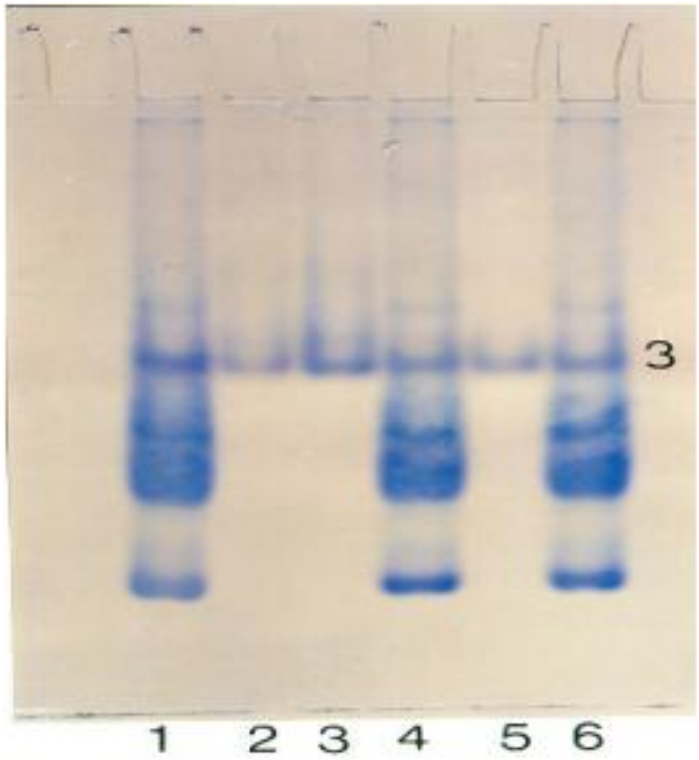

Figure 2.3: Nondenaturing polyacrylamide gel electrophoresis using 12% acrylamide gel in the b-alanine acetic acid buffer at pH 4.3. Staining was by Brilliant Blue G-colloidal. In the columns 1, 4 and 6, were added aliquot of the fraction 2 (crude latex). In the column 2 and 3, aliquot of fraction 4.In the column 5, aliquot of fraction 5 (recrystalization). The purity of the band 3 was confirmed electrophoresis and immunologic assay (Source: Basílio, 1993).

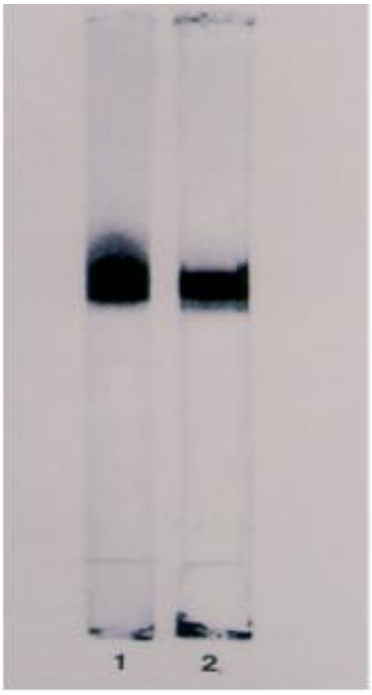

Figure 2.4: Nondenaturing polyacrylamide gel electrophoresis using 12% acrylamide gel and ß-alanine buffer, pH 4.3. Staining was by Coomassie Blue R-250. Gel 1 Mixture of peak 2 with classic papain. Gel 2 mixed of peak 2 with the papain extracted by this method. (Source: Monti *et al*,.2000)

2.4 Aqueous Organic Solvents

In some cases the enzymes might become inactive this is because proteins are not that stable. This is due to the hydrophobic effect, one of the dominant contributors to protein folding and stability, would not stabilize the native structure of enzymes in nonpolar organic solvents, Thus in order to get higher activity aqueous organic solvent is need but must be mix with water cause to much of it might cause denature of protein (Ghosh, 2006), but the question is how much water is necessary. The enzyme can't "see" more than a monolayer or so of water around it. The data suggests that the nature of the organic solvent is very important. Most hydrophobic solvents are best in terms of their ability to maintain active enzymes. Chymotrypsin retains 10^4 more activity in octane than pyridine, which is more hydrophilic than octane. The more polar the solvent, the more it can strip bound water away from the protein. If you add 1.5% water to acetone, the bound water increases from 1.2 to 2.4%, and the activity of chymotrypsin increases 1000 fold (Klijn, J.2005). Whereas, in a research done by Stevenson (1991), Papain is active in solvents ranging in polarity from acetonitrile to tetrachloromethane. With the optimal activity in each solvent varied only about three to four fold, but the amount of added water required to achieve it varied from 4% (v/v) in acetonitrile to 0.05% (v/v) in tetrachloromethane. Later it also believes that, papain can catalyze reactions under a variety of conditions in organic solvents but its substrate specificity is little changed from that in aqueous media Stevenson (1991). Thus it can be concluded that the presence of aqueous of organic solvent in enzyme might just help in getting enzyme of highest activity.