Chapter-4

Isolation of papain from ripe papaya peel using aqueous two-phase extraction and compare with conventional technique

Chapter 4

Isolation of papain from ripe papaya peels using aqueous two-phase extraction and compare with conventional method

4.1 Introduction

Due to their ubiquitous nature, proteolytic enzymes (EC 3.4) are found in many organisms, including animals, plants and microorganisms **[1]**. In spite of the wide substrate specificity of enzymes from plants, they are also able to function in a wide range of temperatures and pH conditions as well as in the presence of additives and other organic compounds **[36]**. As a result, this kind of enzyme is extensively utilized in various industrial practices, such as in food, cleansing agent, waste, pharmaceuticals, and leather **[32]**.

A tropical fruit of great economic importance, papaya (*Carica papaya L.*) is widely grown throughout the year and recognised as one of the most significant commercial fruits among the *Caricaceae* family **[30,40]**. Due to non-utilization of papaya peel for commercial purposes, it is disposed of as waste and contributes to environmental pollution across the globe. Nevertheless, papaya peel has the ability to be used as it is a beneficial resource for the manufacture of natural enzymes such as papain (EC 3.4.22.2) **[28]**.

The enzyme papain is a member of the cysteine protease family and generally appears as a minor component in papaya fruit latex **[23]**. The enzyme has been extensively used in the food industries for meat tenderization and for various medicinal usages **[28]**. As a result, cysteine protease must be of high purity in order to fulfil the varied commercial and scientific requirements. In the traditional method, the purification process involves many steps, discontinuous operations, and a high labour intensity, all of which increases the cost and cause yield losses **[43]**. For the purifying of protein products, industries are seeking fast and economical downstream processes, including those that can produce high yield and high purity **[15]**. The aqueous two-phase system (ATPS) is a method capable of meeting these criteria. The best reason for this is that it

operates under mild conditions, is easy to scale up, has a rapid process, uses low-cost materials, and minimizes protein denaturation **[3,10]**.

The use of ATPS, which comprise either two polymers or one polymer and a salt solution mixed with water, has demonstrated promise in the downstream processing of proteins. These systems can consolidate the clarification, concentration, and purification of the desired product into a single unit operation **[16,35]**. High output separation and purification of enzymes have been successfully achieved through the use of ATPS **[18,21,37]**. A number of processing parameters can be adjusted to have the desired partition of proteins while using such systems **[2,25]**. When compared with polymer–polymer–water systems or the polymer–salt–water systems prove to be more beneficial in regards to their low cost and viscosity. The polyethylene glycol (PEG)–phosphate is the most widely used system among the latter systems **[19,37]**; however other multivalent anion salts have also been useful **[38]**.

The aim of this study was to examine the potential of using anaqueous two-phase system for seperation of cysteine protease from papaya peel. Moreover, the influence factors such as concentration of PEG, concentration and type of salt, and the analyte concentration on the partition behaviour of cysteine protease were also studied.

4.2 Material and methods

4.2.1 Raw materials

Fresh ripe papaya fruit of 14°Brix was procured from a local market in Tezpur, Assam, India. Analytical-grade materials were utilized throughout the experiment. Bovine serum albumin (BSA), polyethylene glycol (PEG) 6000 (g/mol), L-tyrosine, Coomassie brilliant blue G-250, ethanol and casein were all provided by Zenith India Pvt. Ltd., India. Trichloroacetic acid (TCA), aluminium sulphate, and sodium sulphate were procured from Merck, India.

4.2.2 Extraction procedure of papain from papaya peel

Fresh papaya was cleaned with distilled water and peeled to obtain papaya peel. Using a laboratory grinder (Philips HL 1632, India), the peel was grinded into paste with

phosphate buffer (1:1) at pH 9. The final paste was passed through a muslin cloth before being centrifuged (7000 rpm for 15 min at 4 °C) to get the crude enzyme extract.

4.2.2.1 Experiment design and Preparation of PEG/Salt ATPS

All the experiments for partitioning were done according to full-factorial design. As per the preliminary experiments, a suitable amount of PEG (6000 g/mol) with various concentrations (10%, 12%, 14%, and 16%), ammonium sulphate (10%, 12%, 14%, 16%, and 18%, w/w), sodium sulphate (10%, 12%, 14%, 16%, and 18%, w/w), and 20% (w/w) crude feedstock were chosen for this study. As per the different combinations, polymer and salts were weighed to prepare phase systems in 50 mL graduated centrifuge. The required quantity of distilled water was then added to the mixture, bringing the final volume to 50 mL. The content was thoroughly homogenized for equilibration, and phase separation was then accomplished by centrifuging at 7000 rpm for 10 min. Next, the proteolytic activity and protein concentrations of both the top and bottom phases were assessed.

4.2.3 Extraction of papain enzyme using ethanol

A modified version of the 70% ethanol extraction method was used to prepare papain extract from ripe papaya peel **[33]**. The extraction process was conducted by mixing 10 g of papaya peel with 100 mL of 70% ethanol in a 250 mL beaker, and the mixture was blended. The extraction was carried out at room temperature for 72 hours, with regular stirring every 4 hours. Afterward, the extract was filtered using Whatmann No. 1 filter paper. The resulting solution was subjected to evaporation in a water bath set at 50°C, leading to the formation of a greasy substance. This extract was then transferred to sterile screw-capped bottles, properly labeled, and stored under refrigerated conditions until further use. Then, cysteine protease activity and protein concentrations were estimated.

4.2.4 Analytical Tests

4.2.4.1 Proteolytic activity assays

The protease activity of the enzyme was evaluated by performing the casein digestion method. A mixture of 0.1 mL of the enzyme solution, 0.8 mL of pH 9.0

phosphate buffer, and 0.2 mL of 0.03M cysteine-0.006M EDTA was prepared for the proteolytic reaction. The mixture was then incubated for 10 minutes at 37°C before adding 1 mL of 1.5% casein solution to initiate the reaction.Exactly 5 minutes later, 3 mL of a 5% TCA solution was added to terminate the reaction. The supernatant was produced by centrifugation at 7000 rpm for 20 min (Eppendorf, 5804R, Germany), and it was then filtered through filter paper. The absorbance at 275 nm was measured at UV-Vis spectrophotometer (Agilent Technologies, USA, Cary 60 UV Vis). The protease activity of the samples was measured in Units (U) which is the micromoles (µM) of tyrosine equivalents released from casein per minute, determined from the standard curve of tyrosine.

4.2.4.2 Protein content assay

Protein content was determined using the Coomassie Brilliant Blue G-250 method **[8]**. A total of 100 mg of Coomassie Brilliant Blue G 250 was dissolved in 50 mL of 95% ethanol and 100 mL of 85% phosphoric acid to make the Bradford reagent, and the final volume was adjusted to 1.0 L with distilled water. To estimate protein, 100 µL of enzyme-containing sample was mixed with 2.0 mL of Bradford reagent, and the volume was made up to 3.0 mL using 0.1 M phosphate buffer. The absorbance of protein was determined at 595 nm using a UV-Vis spectrophotometer (Agilent Technologies, USA, Cary 60 UV Vis). A standard curve of bovine serum albumin (10–100 g/mL) was used to determine the protein concentration.

4.2.5 Determination of Partition Coefficient, Purification Factor and Yield

The partition coefficient (K_e) was calculated by dividing the protease activity in the top phase (A_T) by the protease activity in the bottom phase (A_B) (as shown in Equation 1).

$$K_e = \frac{A_T}{A_B} \qquad (1)$$

To determine the specific activity of the enzyme, the total activity of protease (U) was divided by the total protein content (mg) using Equation 2.

$$Specific\ Activity = \frac{Total\ protease\ acitivity}{Total\ protein\ content} \qquad (2)$$

The purification factor (PF) of cysteine protease was determined by dividing the specific activity of the enzyme in the top phase by the specific activity of the enzyme in the bottom phase, as shown in Equation 3.

$$Purification\ Factor\ (PF) = \frac{Specific\ acitvity\ of\ top\ phase}{Specific\ activity\ of\ bottom\ phase} \quad (3)$$

The equation 4 was utilized to calculate the yield of cysteine protease in the top phase (Y):

$$Y\ (\%) = \frac{100}{(1 + \frac{1}{[VR].[K_e]})} \quad (4)$$

where K_e represents the partition coefficient of cysteine protease and VR represents the volume ratio of the top phase to the bottom phase.

4.2.6 Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was utilized to determine the distribution of MW of the extracted materials following the Laemmli method **[22]**. A buffer solution comprising 0.5 M Tris-HCl (pH 6.8), 4% SDS, and 20% glycerol was combined with the sample in a 1:1 ratio. For the reducing condition, 10% β-mercaptoethanol was added to the sample buffer. Subsequently, 20 µg of protein samples were loaded onto polyacrylamide gels, consisting of 12% running gels and 4% stacking gels. Electrophoresis was carried out at a constant current of 15 mA/gel. Following separation, the protein bands were stained using a 0.02% Coomassie Brilliant Blue R-250 solution, and subsequently de-stained using a mixture of acetic acid and methanol.

4.2.7 GEL permeation chromatography

The molecular weight (MW) of the extracted papain from best selected method was assessed using gel permeation chromatography (GPC) employing an HSP gel RT 5.0 THF 3 µm column. Tetrahydrofuran was used as the mobile phase, flowing at a rate of 0.5 ml/minute, while the temperature was maintained at 25°C. The GPC system utilized for the analysis was sourced from Waters Corporation, USA, following the methodology outlined by Huang et al. **[17].** The extracted papain was dissolved in tetrahydrofuran at a

concentration of 1 mg/ml, and a 20 µl volume of this solution was injected in triplicate. The relative MW was calculated by comparing the results to a calibration curve prepared using serial concentrations of polystyrene with a MW range of 162-22,000 Da.

4.2.8 Statistical analysis

The obtained triplicated results of the samples were analyzed through analysis of variance (ANOVA) test using IBM SPSS 24.0 software followed by Duncan test considering with a 5% significance level ($p \leq 0.05$).

4.3 Result and Discussion

4.3.1 Aqueous two-phase extraction

The behavior of biomolecules in ATPS during partitioning is a critical parameter for the characterization, purification, and separation of these molecules. The partitioning of the enzyme is influenced by a large number of interdependent characteristics, such as the kind of phase-forming components, the pH of the system, the MW of the phase-forming polymers, and the phase composition **[14]**.

Based on the isoelectric point of the papain enzyme, the pH range was chosen. According to reports, papain's isoelectric point (pI) is 8.75 **[31]**. If the pH measured is higher than its pI for that particular enzyme, the enzyme gets negatively charged and repels from the bottom salt-rich phase to the top polymer-rich phase **[16]**. The partition coefficient rises and its interaction with PEG gets greater. Proteins with positive charges tend to distribute to the salt-dense bottom phase, whereas those with negative charges have a preference for the upper phase enriched with polymers **[6]**. However, some findings indicated that when the protein charge switched from positive to neutral, there was a significant rise in K_e **[24]**.

Tables 4.1 and 4.2 display the partition coefficients for enzyme (K_e), protein (K_p), purification fold (PF), and yield (Y%) of papain enzyme, which highlights the ideal phase system for enzyme recovery. The system's volume ratio (VR) varied from 0.2 to 1.7. Papain preferred to partition to the top phase, as shown by the K_e values that were obtained from all systems being higher than 1.

4.3.1.1 Effect of salt concentration on extraction

One of the important components of the ATPS approach is the selection of phase-forming salt. Two PEG/salt systems were investigated in this study to find out how phase-forming salts affected the partition of papain. Due to the ability to encourage hydrophobic interactions between proteins, sulphate salts (ammonium sulphate and sodium sulphate) were employed **[12]**. Sulphate salts were also selectively removed from protein surfaces, which may be exploited to achieve the highest degree of selectivity while proteins were purified using hydrophobic interaction chromatography **[4,5]**. This system was chosen for further study because papain partitions more well in the PEG/sulphate system.

A two-phase system made of PEG and ammonium sulphate was investigated for partitioning papain because ammonium sulphate is frequently used to separate proteases. The extraction setup was maintained at a steady pH of 9.0 and a temperature of 35°C, and the optimal condition for the selective separation of proteins with protease activity was attained by utilizing 10% PEG 6000 and 18% $(NH_4)_2SO_4$. This resulted in a partition coefficient (Ke) of 1.43, a purification factor of 4.08, and a yield of 26.40% for papain, as outlined in Table 1.The next most effective condition was found to be 12% PEG with 18% $(NH_4)_2SO_4$. Table 1 illustrates that because ATPS didn't reach its saturation point in 10% PEG 6000 + 12% $(NH_4)_2SO_4$ and 10% PEG 6000 + 14% $(NH_4)_2SO_4$, ATPS partition didn't take place.

According to Salabat's phase diagram for the PEG/$(NH_4)_2SO_4$ system, a system containing 12% (w/w) PEG 6000 and 15% (w/w) $(NH_4)_2SO_4$ resulted in two distinct phases. This was found to be the highest concentration of the phase-forming components that could be used for purifying papain from papaya peel. Adding $(NH_4)_2SO_4$ salt, that creates water structures, can lower the concentration of "free water" in the upper phase, which in turn lowers the volume of the upper phase **[42]**.

Here, the impact of Na_2SO_4 concentration on papain extraction efficiency was also studied. The partition coefficient (1.20) of the PEG 6000/Na_2SO_4 ATPS phase formation was highest at 10% PEG + 18% Na_2SO_4 system, followed by 10% PEG + 16% Na_2SO_4 (Table 4.2). With a partition coefficient (K_e) of 1.20, the protease enzyme extraction was more noticeable in top phase. Additionally, positively charged proteins

typically partitioned predominantly to the bottom phase in PEG/salt systems, whereas negatively charged proteins preferred the top phase. Papain may be an anion, which would cause the enzyme to partition to the upper phase. Additionally, Tables 4.1 and 4.2 showed that the salting out effect caused the partitioning coefficient (K_e) with increase in salt concentration.

Sulfate salts have the capacity to induce protein-protein hydrophobic interactions **[12]**. The PEG/$(NH_4)_2SO_4$ system had the highest purification fold and extracted the highest proportion of papain to the top phase, indicating that hydrophobicity had an impact on the enzyme's extraction in ATPS (Table 4.2). The intensity of the hydrophobic contact between the protein and PEG molecules would rise as the hydrophobicity difference between the two phases increased, and facilitating extraction. The PEG/$(NH_4)_2SO_4$ system may be a superior approach for partitioning papain. The use of PEG/sulfate aqueous two-phase systems has thus enabled the partitioning and purification of numerous enzymes and/or proteins **[20,29,41]**.

Table 4.1: Effect of PEG 6000 and ammonium sulphate salt on partitioning of papain enzyme

Phase Composition	VR	K_p	K_e	Specific Activity		PF	Yield (%)
				Polymer	Salt		
10%PEG6000/12%$(NH_4)_2SO_4$	0.42	0.421 ± 0.033^{bc}	1.036 ± 0.015^{de}	3.450 ± 0.110^{e}	1.558 ± 0.091^{cd}	2.21	30.31
10%PEG6000/14%$(NH_4)_2SO_4$	0.35	0.468 ± 0.019^{bc}	1.071 ± 0.035^{d}	3.584 ± 0.159^{e}	1.688 ± 0.058^{b}	2.12	27.26
10%PEG6000/16%$(NH_4)_2SO_4$	0.28	0.505 ± 0.030^{a}	1.177 ± 0.086^{bc}	4.066 ± 0.048^{cd}	1.456 ± 0.086^{ef}	2.79	24.78
10%PEG6000/18%$(NH_4)_2SO_4$	0.25	0.527 ± 0.042^{a}	1.434 ± 0.006^{a}	4.916 ± 0.159^{b}	1.203 ± 0.087^{ab}	4.08	26.38
12%PEG6000/10%$(NH_4)_2SO_4$	-	-	-			-	-
12%PEG6000/14%$(NH_4)_2SO_4$	0.51	0.333 ± 0.052^{ef}	1.045 ± 0.003^{de}	3.498 ± 0.304^{e}	1.705 ± 0.200^{b}	2.05	34.76
12%PEG6000/16%$(NH_4)_2SO_4$	0.38	0.338 ± 0.041^{e}	1.216 ± 0.021^{b}	4.642 ± 0.621^{bc}	1.628 ± 0.066^{b}	3.62	31.60
12%PEG6000/18%$(NH_4)_2SO_4$	0.28	0.511 ± 0.067^{a}	1.374 ± 0.059^{a}	5.907 ± 0.689^{a}	1.111 ± 0.069^{g}	4.17	27.78
14%PEG6000/10%$(NH_4)_2SO_4$	-	-	-			-	-
14%PEG6000/12%$(NH_4)_2SO_4$	0.78	0.294 ± 0.040^{g}	1.052 ± 0.029^{d}	6.030 ± 0.692^{a}	1.668 ± 0.093^{bc}	3.61	45.07
14%PEG6000/16%$(NH_4)_2SO_4$	0.47	0.355 ± 0.063^{de}	1.043 ± 0.037^{de}	4.495 ± 0.193^{bc}	1.525 ± 0.196^{cd}	2.94	32.89
14%PEG6000/18%$(NH_4)_2SO_4$	0.38	0.488 ± 0.047^{ab}	1.241 ± 0.067^{b}	4.541 ± 0.277^{bc}	1.785 ± 0.168^{ab}	2.54	32.04
16%PEG6000/10%$(NH_4)_2SO_4$	1.77	0.410 ± 0.018^{c}	1.023 ± 0.025^{e}	3.452 ± 0.132^{e}	1.384 ± 0.032^{ef}	2.49	64.42
16%PEG6000/12%$(NH_4)_2SO_4$	1	0.516 ± 0.036^{a}	1.075 ± 0.101^{d}	3.901 ± 0.345^{de}	1.873 ± 0.112^{a}	2.08	51.80
16%PEG6000/14%$(NH_4)_2SO_4$	0.61	0.404 ± 0.016^{cd}	1.179 ± 0.040^{bc}	4.389 ± 0.208^{bcd}	1.503 ± 0.050^{cd}	2.92	41.83
16%PEG6000/18%$(NH_4)_2SO_4$	0.47	0.400 ± 0.008^{cd}	1.177 ± 0.058^{bc}	3.918 ± 0.248^{de}	1.331 ± 0.23^{f}	2.94	35.61

where, VR = volume ratio; K_p = partition coefficient of protein; K_e = partition coefficient of cysteine protease; PF = Purification factor. Values are reported as mean ± standard deviation of three replications. Means followed by the different superscript small letters within a column are significantly different (p>0.05).

Table 4.2: Effect of PEG 6000 and sodium sulphate salt on partitioning of papain enzyme

Phase Composition	VR	K_p	K_e	Specific Activity		PF	Yield (%)
				Polymer	Salt		
10%PEG6000/12%Na_2SO_4	0.35	0.544 ± 0.003^{de}	1.081 ± 0.014^{bc}	6.845 ± 0.167^{bc}	5.171 ± 0.144^{a}	1.32	24.45
10%PEG6000/14%Na_2SO_4	0.31	0.692 ± 0.039^{cd}	1.064 ± 0.009^{c}	5.832 ± 0.176^{cd}	3.516 ± 2.606^{cd}	1.65	22.56
10%PEG6000/16%Na_2SO_4	0.25	0.817 ± 0.014^{b}	1.132 ± 0.033^{b}	7.808 ± 0.300^{b}	4.772 ± 0.231^{ab}	1.63	18.08
10%PEG6000/18%Na_2SO_4	0.21	0.921 ± 0.427^{a}	1.204 ± 0.038^{a}	8.338 ± 0.197^{b}	2.772 ± 0.100^{cd}	3.01	14.85
12%PEG6000/10%Na_2SO_4	0.56	0.675 ± 0.046^{cd}	1.081 ± 0.020^{bc}	11.331 ± 2.589^{a}	4.946 ± 0.449^{ab}	2.29	34.12
12%PEG6000/14%Na_2SO_4	0.28	0.490 ± 0.125^{ef}	1.065 ± 0.009^{c}	5.977 ± 0.895^{cd}	4.411 ± 0.387^{bc}	1.35	20.81
12%PEG6000/16%Na_2SO_4	0.25	0.576 ± 0.085^{d}	1.037 ± 0.026^{de}	4.866 ± 0.374^{de}	2.687 ± 0.226^{e}	1.81	19.42
12%PEG6000/18%Na_2SO_4	0.21	0.804 ± 0.187^{b}	1.042 ± 0.032^{d}	3.942 ± 0.199^{f}	2.550 ± 0.094^{ef}	1.54	16.77
14%PEG6000/10%Na_2SO_4	0.66	0.506 ± 0.054^{e}	1.054 ± 0.036^{cd}	6.828 ± 0.302^{bc}	3.273 ± 0.315^{cd}	2.08	38.5
14%PEG6000/12%Na_2SO_4	0.51	0.577 ± 0.023^{d}	1.066 ± 0.019^{c}	4.720 ± 0.174^{de}	2.555 ± 0.141^{ef}	1.84	32.36
14%PEG6000/16%Na_2SO_4	0.42	0.341 ± 0.066^{f}	1.048 ± 0.025^{d}	4.671 ± 0.276^{de}	2.458 ± 0.037^{f}	1.9	28.61
14%PEG6000/18%Na_2SO_4	0.35	0.552 ± 0.029^{de}	1.126 ± 0.043^{b}	8.233 ± 1.083^{b}	2.452 ± 0.127^{f}	3.35	23.71
16%PEG6000/10%Na_2SO_4	0.78	0.465 ± 0.068^{ef}	0.983 ± 0.036^{e}	6.195 ± 1.420^{cd}	2.821 ± 0.279^{e}	2.19	44.24
16%PEG6000/12%Na_2SO_4	0.56	0.672 ± 0.096^{cd}	1.025 ± 0.027^{de}	5.186 ± 0.371^{de}	3.374 ± 0.211^{cd}	1.53	35.33
16%PEG6000/14%Na_2SO_4	0.51	0.853 ± 0.153^{ab}	1.081 ± 0.051^{bc}	4.614 ± 0.584^{de}	3.589 ± 0.256^{cd}	1.28	32.05
16%PEG6000/18%Na_2SO_4	0.42	0.764 ± 0.062^{c}	1.035 ± 0.025^{de}	4.387 ± 0.287e	3.229 ± 0.100^{cd}	1.35	28.86

where, VR = volume ratio; K_p = partition coefficient of protein; K_e = partition coefficient of cysteine protease; PF = Purification factor. Values are reported as mean ± standard deviation of three replications. Means followed by the different superscript small letters within a column are significantly different (p>0.05)

4.3.1.2 Effect of polymer concentration on extraction

The partitioning of papain was significantly affected by the PEG 6000 concentration (10%, 12%, 14%, and 16%, w/w), as demonstrated in Tables 4.1 and 4.2. It was evident from Tables 4.1 and 4.2 that when the quantity of salt increased, papain's enzyme activity increased in the polymer phase. Because volume exclusion has a stronger impact than salting out, papain concentration has increased. The steric exclusion effect of polymer and the hydrophobic bond between polymer and proteins both play a significant role in determining the maximal protein concentration in the top phase. Among all ATPS tested, the treatment with 10% PEG 6000 and 18% $(NH_4)_2SO_4$ was the most effective at partitioning papain into the top PEG-rich phase and unwanted proteins and contaminates into the bottom salt phase, followed by 12% PEG 6000 and 18% $(NH_4)_2SO_4$. Under these circumstances, 26.38% of the enzyme was obtained in the top phase, giving an approximate PF of 4.08. When compared to other proteins, papain partitioning to the bottom phase was substantially reduced, which led to an increase in PF. However, it has been shown that systems with a substantial amount of polymer or high molecular weight polymers and high salt concentrations led to the partitioning of biomolecules at the interphase because of the combined effects of salting out and volume exclusion **[27]**.

Two significant aspects that have a direct influence on the extraction efficacy of the biomolecules in ATPS were hydrophobic interactions and hydrogen bonding. According to the results, papain extraction with PEG/$(NH_4)_2SO_4$ ATPS may rely mostly on hydrophobic contact between papain and the polymer.

According to Tapadar et al. **[34]**, the optimal condition for partitioning xylanase activity was achieved using a combination of 12% PEG-6000 and 15% $(NH_4)_2SO_4$ in an extraction system at 40°C and pH 6. Under these conditions, the maximum purification fold of 4.94 was obtained with a yield of 69.68% for xylanase. Similar to this, de HC Maciel et al. **[11]** stated that when a large PEG molar mass (6000g/mol) was utilized, the enzyme partitioning to the top phase was seen. Additionally, Fakhari et al. **[13]** demonstrated the activation of PEG 6000 with epichlorohydrin, which was covalently linked to aminodiacetic acid to partition A.Niger.

Because the partition effect was more prominent with PEG molecular weights between 4000 and 6000 g/mol than with lower molecular weights of PEG **[26]**. However,

all of the studies in the current work used just PEG-6000 molecular weight, making it difficult to comment on the influence of MW on papain's partitioning behavior. It should be noted that the complex relationship between the size of the biomolecule, its surface characteristics, its net charge, the system pH, the system temperature, and the MW of the polymer determines how the biomolecule partitions in ATPS **[7]**. The tendency for partitioning into one phase also tends to rise when the size of the biomolecule increases, while the partition coefficient typically decreases as the PEG chain length increases. The net charge of the protein has an impact on the partitioning behavior as well. Because of this, the enzyme might be accommodated in the top phase when utilizing PEG with a high MW (6000 g/mol).

4.3.2 Ethanol extraction

The papain enzyme was extracted from ripe papaya peel using 70% ethanol. The protease activity of the crude papain enzyme was determined using the casein digestion method, and it was found to be 0.315 U. The protein content of the extract was measured to be 83.14 µg/mL. In a study by Chaiwut et al. **[9]**, proteases were obtained from papaya peel extract and papaya latex using different extractants, including methanol (75%), ethanol (70%), and 2-propanol (67%). The highest proteolytic yield (57.6%) was achieved using ethanol (70%) as the extractant compared to other precipitation agents. The specific activity of the unripe latex extract was found to be higher than that of other extracts. The specific activity of the papaya peel extract was determined to be 3.79 U/mg. These results were then compared to the optimized ATPS method using 10% PEG 6000 + 18% $(NH_4)_2SO_4$. From Table 4.3, it was observed that the papain extracted by ATPS exhibited superior results compared to the enzyme extracted using ethanol.

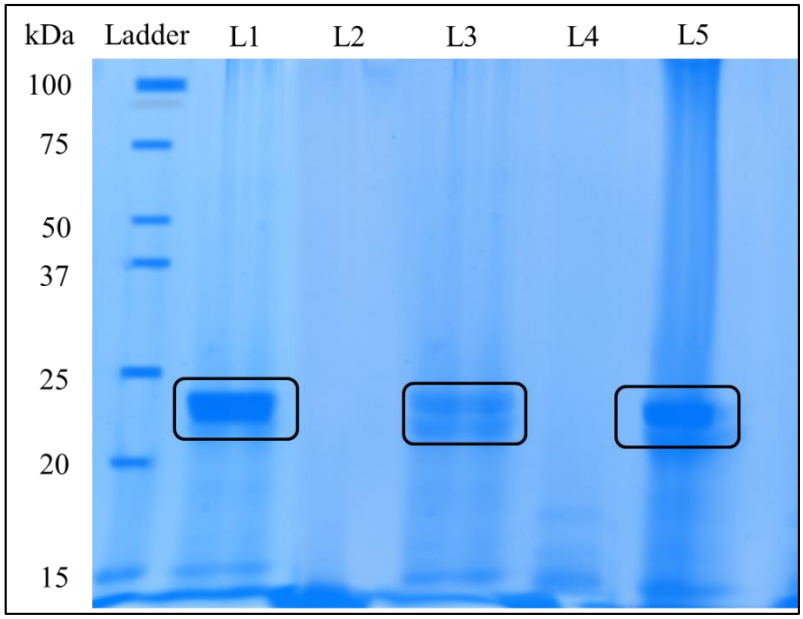
Table 4.3: Properties of extracted papain enzyme

	Protease Activity (U)	**Protein Content (µg/mL)**	**Specific Activity (U/mg)**
Ethanol	0.315 ± 0.027^{b}	83.14 ± 1.75^{b}	3.79 ± 0.097^{b}
ATPS	0.476 ± 0.042^{a}	97.13 ± 2.43^{a}	4.91 ± 0.148^{a}

values are presented as mean ± standard deviations. Means in a same column with different superscripts indicate significant difference ($p < 0.05$).

4.3.3 SDS-PAGE

Electrophoretic patterns of the extracted papain, partitioned from the 10% PEG 6000 + 18% $(NH_4)_2SO_4$ ATPS fraction, the 10% PEG 6000 + 18% Na_2SO_4 ATPS fraction, and the ethanol extract, were displayed in Figure 4.1. The migration of proteins from the crude protein sample into the top and bottom phases of the ATPS and ethanol extract was clearly distinguishable. The molecular weights of the isolated enzymes ranged from 20 to 25 kDa, facilitating comparison with protein markers (Ladder). In the 10% PEG 6000 + 18% $(NH_4)_2SO_4$ ATPS, a prominent band corresponding to the papain enzyme was observed in the top phase (L1), while no band was present in the bottom phase (L2) as shown in Figure 4.1. Comparable results were obtained with the 10% PEG 6000 + 18% Na_2SO_4 ATPS, although with lower concentrations compared to the $(NH_4)_2SO_4$ ATPS. In the composition of the bottom phase, no noticeable zones of a specific enzyme were observed, most likely due to the relatively low papain content, resulting in undetectable proteolytic activity. The different amounts of interfering proteins and other enzymes in each phase likely contributed to the variation in protein bands between the polymer and salt phases. In the case of ethanol extraction, a band appeared between 20-25 kDa, indicating the presence of papain, but it also exhibited impurities (L5). However, it is crucial to accurately quantify which protein band corresponds to papain. Notably, previous studies by Vernet et al. **[39]** reported that a SDS-PAGE examination revealed the purified papain isolated from papaya to have a molecular weight of approximately 23.4 kDa, which aligns with the obtained result. From the SDS-PAGE analysis, it is evident that the polymer phase of the 10% PEG 6000 + 18% $(NH_4)_2SO_4$ ATPS fraction yielded the most favorable result.

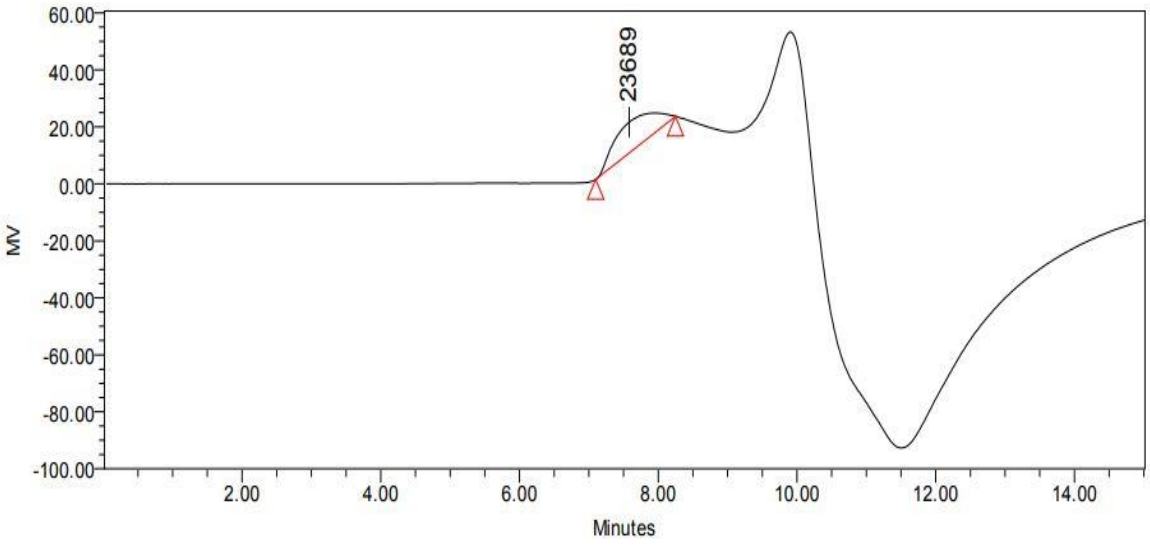


where, L1 is 10% PEG 6000, L2 is 18% $(NH_4)_2SO_4$, L3 is 10% PEG 6000, L4 is 18% Na_2SO_4 and L5 is ethanol extract

Figure 4.1: SDS-PAGE patterns of cysteine protease from papaya peel partitioned

4.3.4 Gel permeation chromatography

The results indicated that the polymer phase obtained from the 10% PEG 6000 + 18% $(NH_4)_2SO_4$ aqueous two-phase system (ATPS) fraction demonstrated the best overall performance. To further confirm the extraction and purification of papain, gel permeation chromatography analysis was conducted on the enzyme extracted from the polymer phase. The results, as depicted in Figure 4.2, revealed a molecular weight of 23.6 kDa for the extracted enzyme. This finding aligns with the work of Vernet et al. **[39]**, who reported that purified papain isolated from papaya typically has a molecular weight of approximately 23.4 kDa. Therefore, the obtained result further supports the successful extraction and purification of papain from papaya peel using the ATPS method and can be used in various food applications.



GPC Results

	Retention Time (min)	% Area	Mn	Mw	MP	Mz	Mz+1	Poly dispersity
1	7.583	100.00	20254	22442	23689	24648	26705	1.108034

Figure 4.2: GPC analysis of extracted papain

4.4 References

1. Aehle, W. *Enzymes in Industry: Production and Application*; Wiley-VCH, New York, USA, 2004.
2. Albertsson P.Å. *Partition of Cell Particles and Macromolecules*. 3rd ed., Wiley, New York, USA, 1986.
3. Andersson, E., & Hahn-Hägerdal, B. Bioconversions in aqueous two-phase systems. *Enzyme and Microbial Technology*, 12(4), 242-254, 1990.
4. Arakawa, T., & Narhi, L. O. Solvent modulation in hydrophobic interaction chromatography. *Biotechnology and Applied Biochemistry*, 13(2), 151–172, 1991.
5. Arakawa, T., & Timasheff, S. N. Mechanism of protein salting in and salting out by divalent cation salts: balance between hydration and salt binding. *Biochemistry*, 23(25), 5912–5923, 1984.
6. Asenjo, J. A., & Andrews, B. A. Aqueous two-phase systems for protein separation: a perspective. *Journal of Chromatography A*, 1218(49), 8826–8835, 2011.
7. Baskir, J. N., Hatton, T. A., & Suter, U. W. Protein partitioning in two-phase aqueous polymer systems. *Biotechnology and Bioengineering*, 34(4), 541–558, 1989.
8. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72(1-2), 248–254, 1976.
9. Chaiwut, P., Nitsawang, S., Shank, L., & Kanasawud, P. A comparative study on properties and proteolytic components of papaya peel and latex proteases. *Chiang Mai J. Sci*, 34(1), 109–118, 2007.
10. Chen, J. P., & Lee, M. S. Enhanced production of Serratia marcescens chitinase in PEG/dextran aqueous two-phase systems. *Enzyme and Microbial Technology*, 17(11), 1021–1027, 1995.
11. de HC Maciel, M., Ottoni, C. A., Herculano, P. N., Porto, T. S., Porto, A. L., Santos, C., ... & Souza-Motta, C. Purification of polygalacturonases produced by Aspergillus niger using an aqueous two-phase system. *Fluid Phase Equilibria*, 371, 125–130, 2014.
12. Eiteman, M. A., & Gainer, J. L. (1991). Partition of isomeric dipeptides in poly (ethylene glycol)/magnesium sulfate aqueous two-phase systems. *Biochimica et Biophysica Acta (BBA)-General Subjects*, 1073(3), 451–455, 1991.

13. Fakhari, M. A., Rahimpour, F., & Taran, M. Response surface methodology optimization of partitioning of xylanase form Aspergillus Niger by metal affinity polymer-salt aqueous two-phase systems. *Journal of Chromatography B*, 1063, 1–10, 2017.
14. Grilo, A. L., Raquel Aires-Barros, M., & Azevedo, A. M. Partitioning in aqueous two-phase systems: fundamentals, applications and trends. *Separation & Purification Reviews*, 45(1), 68–80, 2016.
15. Gupta, R., Beg, Q., & Lorenz, P. Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology*, 59(1), 15–32, 2002.
16. Hatti-Kaul, R. Aqueous two-phase systems. *Molecular biotechnology*, 19(3), 269–277, 2001.
17. Huang, X. D., Liang, J. B., Tan, H. Y., Yahya, R., Khamseekhiew, B., & Ho, Y. W. Molecular weight and protein binding affinity of Leucaena condensed tannins and their effects on in vitro fermentation parameters. *Animal Feed Science and Technology*, 159(3-4), 81–87, 2010.
18. Hustedt, H., Kroner, K. H., & Papamichael, N. Continuous cross-current aqueous two-phase extraction of enzymes from biomass. Automated recovery in production scale. *Process Biochemistry*, 23(5), 129–137, 1988.
19. Hustedt, H., Kroner, K. H., Kula, M.R. (1985). Applications of phase partition in biotechnology. In Walter, H., Brooks D. E., Fisher, D. (Eds.), *Partitioning in Aqueous Two-Phase Systems; Theory, Methods, Uses and Application in Biotechnology*, pages 529-587. Academic press, Orlando, 1985.
20. Johansson, G. Partition of proteins and micro-organisms in aqueous biphasic systems. *Molecular and Cellular Biochemistry*, 4(3), 169–180, 1974.
21. Kula, M. R., Kroner, K. H., Hustedt, H., & Schutte, H. Scale-up of protein purification by liquid-liquid extraction. *Enzyme Engineering*, 69–74, 1982.
22. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227(5259), 680–685, 1970.
23. Ling, Y. Q., Nie, H. L., Su, S. N., Branford-White, C., & Zhu, L. M. Optimization of affinity partitioning conditions of papain in aqueous two-phase system using response surface methodology. *Separation and Purification Technology*, 73(3), 343–348, 2010.

24. Marcos, J. C., Fonseca, L. P., Ramalho, M. T., & Cabral, J. M. S. Variation of penicillin acylase partition coefficient with phase volume ratio in poly (ethylene glycol)–sodium citrate aqueous two-phase systems. *Journal of Chromatography B: Biomedical Sciences and Applications,* 711(1-2), 295–299, 1998.
25. Menge, U. (2000). Optimization of extractions in aqueous two-phase systems. In Hatti-Kaul,R. (Ed), *Aqueous Two-Phase Systems: Methods And Protocols*, pages 235-249. Humana Press, Totowa, NJ, 2000.
26. Naganagouda, K., & Mulimani, V. H. Aqueous two-phase extraction (ATPE): an attractive and economically viable technology for downstream processing of Aspergillus oryzae α-galactosidase. *Process Biochemistry*, 43(11), 1293–1299, 2008.
27. Nalinanon, S., Benjakul, S., Visessanguan, W., & Kishimura, H. Partitioning of protease from stomach of albacore tuna (Thunnus alalunga) by aqueous two-phase systems. *Process Biochemistry*, 44(4), 471–476, 2009.
28. Pathak, P. D., Mandavgane, S. A., & Kulkarni, B. D. Waste to wealth: a case study of papaya peel. *Waste and Biomass Valorization*, 10(6), 1755–1766, 2019.
29. Salabat, A., Abnosi, M. H., & Bahar, A. R. Amino acids partitioning in aqueous two-phase system of polypropylene glycol and magnesium sulfate. *Journal of Chromatography B,* 858(1-2), 234–238, 2007.
30. Singla, M., & Sit, N. Isolation of papain from ripe papaya peel using aqueous two-phase extraction. *Journal of Food Measurement and Characterization,* 17(2), 1685–1692, 2023.
31. Smith, E. L., Kimmel, J. R., & Brown, D. M. Crystalline papain: II. Physical studies; the mercury complex. *Journal of Biological Chemistry*, 207(2), 533–549, 1954.
32. Sumantha, A., Larroche, C., & Pandey, A. Microbiology and industrial biotechnology of food-grade proteases: a perspective. *Food Technology and Biotechnology*, 44, 211–220, 2006.
33. Sumathi, R., & Gowthami, M. Phytochemical analysis and in vitro antimicrobial activity of aqueous and solvent extracts of Carica papaya against clinical pathogens. *Int. J. Adv. Res. Biol. Sci,* 1, 73–77, 2014.
34. Tapadar, R., Nandi, M., Chatterjee, R., Mukherjee, K., & Majumder, K. Aqueous two phase system based downstream processing of endo-1, 4 B-D-xylanse from culture filtrate of aspergillus niger. *Global Journal of Bio-Science and Biotechnology,* 8(2), 168–173, 2019.

35. Tianwei, T., Qing, H., & Qiang, L. Purification of glycyrrhizin from Glycyrrhiza uralensis Fisch with ethanol/phosphate aqueous two phase system. *Biotechnology Letters*, 24(17), 1417–1420, 2002.
36. Tomar, R., Kumar, R., & Jagannadham, M. V. A stable serine protease, wrightin, from the latex of the plant Wrightia tinctoria (Roxb.) R. Br.: purification and biochemical properties. *Journal of Agricultural and Food Chemistry*, 56(4), 1479–1487, 2008.
37. Veide, A., Smeds, A. L., & Enfors, S. O. A process for large-scale isolation of β-galactosidase from E. coli in an aqueous two-phase system. *Biotechnology and Bioengineering*, 25(7), 1789–1800, 1983.
38. Vernau, J., & Kula, M. R. Extraction of proteins from biological raw material using aqueous polyethylene glycol-citrate phase systems. *Biotechnology and Applied Biochemistry*, 12(4), 397–404, 1990.
39. Vernet, T., Berti, P. J., de Montigny, C., Musil, R., Tessier, D. C., Ménard, R., Magny,M. C, Storer A.C. & Thomas, D. Y. Processing of the papain precursor: the ionization state of a conserved amino acid motif within the pro region participates in the regulation of intramolecular processing. *Journal of Biological Chemistry*, 270(18), 10838–10846, 1995.
40. Yogiraj, V., Goyal, P. K., Chauhan, C. S., Goyal, A., & Vyas, B. Carica papaya Linn: an overview. *International Journal of Herbal Medicine*, 2(5), 01–08, 2014.
41. Yue, H., Yuan, Q., & Wang, W. Purification of phenylalanine ammonia-lyase in PEG1000/Na2SO4 aqueous two-phase system by a two-step extraction. *Biochemical Engineering Journal*, 37(3), 231–237, 2007.
42. Zaslavsky, B. Y. *Aqueous two-phase partitioning: physical chemistry and bioanalytical applications*. CRC press, New York, USA, 1994.
43. Zhang, M., Hu, P., Liang, Q., Yang, H., Liu, Q., Wang, Y., & Luo, G. Direct process integration of extraction and expanded bed adsorption in the recovery of crocetin derivatives from Fructus gardenia. *Journal of Chromatography B*, 858(1-2), 220–226, 2007.