

Research Paper

Extraction of Papain from Papaya (*Carica papaya* L.) Fruit Latex and Its Application in Transforming Tannery Raw Trimming

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Abstract

The leather industry is one of the major polluting industries in Ethiopia. Environment protection has prompted industrialists and scientists to re-look at transformation of tannery solid waste materials to valuable products using enzymatic hydrolysis. Acid and liming process of gelatin production generate large amount of wastes and used limited raw materials. This study, therefore, aimed to extract papain from papaya fruit latex and use it for transforming raw trimming to gelatin. Extraction and purification was carried out using Three Phase Partitioning (TPP). Protein content and protease activity were determined using lowry and casein hydrolysis method, respectively. Effect of agitation time, crude extract to t-butanol ratio, ammonium sulphate concentration and temperature on purification fold and activity recovery of papain was evaluated and process was optimized. At its optimized parameters, the intermediate phase of TPP showed 94.14 % of protease activity recovery and 8.31 purification fold. At its optimized parameters, bottom phase showed 75.15% percentage of protease activity recovery and 6.61 purification fold. Enzymatic hydrolysis of raw trimming produced 68% and 64% (weight by weight) gelatin recovery from the use of crude and intermediate phase papain, respectively. Relatively low gel strength of gelatin with bloom value of 95 g and 87 g were obtained from hydrolysis of raw trimming by intermediate and crude papain, respectively. This study showed that the use of crude extract for transforming raw trimming to gelatin was found good as it showed comparable protease activity. The TPP can be implemented for the extraction and purification of proteases from papaya fruit for industrial application that requires it in partial pure form.

Keywords: - *Gelatin; Optimization; Papain; Papaya latex; Tannery waste.*

1. Introduction

The leather industry is one of the major polluting industries in Ethiopia since it generates large amount of liquid and solid wastes (Solomon, 2011). Tanning is the process of converting raw hides and skins into imputrescible substance. The reduction, leveling and purification operations generate untanned and tanned proteinous wastes. These solid wastes pose major environmental problem if not managed effectively (Sundar et al., 2011). It releases obnoxious smell because of degradation of proteinous molecules of skin and emits greenhouse gases such as NH₃, H₂S, CH₄ and

CO₂ (Solomon, 2011). Therefore, stringent enforcement of the environment protection has prompted industrialists and scientists to re-look at the decontamination of wastes and transform tannery solid waste materials to valuable products (Sundar et al., 2011). Conventional chemical-thermal process of solid waste treatment from the tannery industry needs high amounts of energy and consumes much time. An alternative enzyme treatment process is more favorable in terms of environment protection and can be carried out at low cost of energy and temperature. Furthermore,

protein products obtained can be used as fertilizers or for other agricultural purposes (Sundar et al., 2011).

Gelatin is used in many traditional applications such as in the food, pharmaceutical, photographic, and cosmetic industries. The enzymatic hydrolysis of several natural materials has been extensively studied and several substantial yield improvements of gelatin has achieved (Guerard et al., 2001; Park et al., 2001). In gelatin manufacturing industry, the major source of raw material comes from the tannery industry. Rawhide is the treated split of large animals (after fleshing) such as cows and buffalos used as raw materials. It is composed of a large amount of structural fibrous proteins called collagen. Acid and liming gelatin production processes have several disadvantages, especially large amount of wastes generated and the type limitation of raw materials (Damrongsakkul et al., 2007).

Proteolytic enzymes from plant latexes receive added attention due to broad substrate specificity and activity in wide range of pH and temperature, and in presence of organic compounds and other additives (Tomar, 2008). Unripe green papaya fruit as well as other part of papaya tree is concentrated with latex, which is composed of several valuable enzymes: papain, chymopapain, caricain and glycyl endopeptidase. To make industrially useable state, these enzymes need to be extracted and purified from green papaya fruits. It is crucial to isolate and purify papain in the native crystalline state from fresh latex (Paul et al., 2013). For isolation and purification of papain, aqueous two phase system (Nitsawanget al., 2006; Li et al., 2010) and TPP system has been reported as common method. But, TPP is a simple, inexpensive and easily scalable technique which can be directly used with crude suspensions (Vetal and Rathod, 2015). So far, the application of TPP for the purification of papain enzyme from papaya fruit latex and the enzymatic hydrolysis of solid protein waste of tannery by the extracts are rarely found in the literature. Therefore, this study aimed to extract papain from fruit latex using TPP and utilize it for transforming raw trimming waste from tannery to gelatin.

2. Methods and Materials

2.1. Latex Extraction and Collection

The latex of papaya was collected and extracted as described by Rathi and Gadevar (2007). Unripe

matured fruit was tapped early in the morning and finished by mid-late morning. Vertical cut of 1-2 mm deep was made using a stainless steel knife. A plastic dish was used to collect the latex. The latex was then scraped into a polythene lined box with a close fitting lid. Latex adhering to the fruit was carefully scraped off and transferred to the collecting box using plastic spoon. Collected latex was mixed with potassium metabisulphite (Kms) ($K_2S_2O_5$) by ratio of 0.5% W/W.

2.2. Latex Drying and Storage

Latex was dried as described by Puig et al. (2008). Collected latex was arranged in aluminum trays and dried in a tray drier at 40°C, 746.6 mbar pressures for 2hr. After drying, the latex was transferred to a plastic bottle and stored at -20°C until used for analysis (Nitsawang et al., 2006).

2.3. Preparation of Crude Papain and Three Phase Partitioning

Preparation of crude papain and three phase partitioning was carried out following modified methods of Vetal and Rathod, (2015). Crude papain was prepared by dissolving 10 g of thawed latex in 100 mL of 0.1 M phosphate buffer of pH 7. The homogenate was filtered through Whatman No.1 and was centrifuged at 5,000 rpm for 30 min. After centrifugation supernatant was collected and analyzed for protein content and activity, while pellet was discarded.

For three phase partitioning, 10 mL of crude papain was added into 50 mL flask, mixed with 10 mL t-butanol followed by addition of 8 g of ammonium sulfate (= 40%, w/v) at 45°C. Then, the pH of mixture was adjusted to 7 and stirred at 200 rpm for 100 min and was centrifuged at 5000 rpm for 20 min to facilitate the separation of phases. Finally, mixture was kept at room temperature in a separating funnel for 1hr for phase separation. Three distinct phases was carefully observed and separated. Upper phase was discarded while intermediate and bottom phase was used for further study. The intermediate phase was dissolved in 0.1 M phosphate buffer of pH 7 by ratio of 1:0.5 (v/v), respectively. Protein content, enzyme activity, purification fold and percentage activity recovery were determined for the phases.

2.4. Measurement of Protein Content

Concentration of the protein was determined by lowry assay using Folin reagent (Lowry et al., 1951). Papain extract samples solution were mixed well using vortex and 1.5 mL of the sample solution was transferred to 10 mL capacity centrifuge tubes. Then, 2.2 mL of fresh lowry solution was added to each sample tubes and the solution was mixed gradually decreasing the speed of vortex. After 20 min incubation at room temperatures in dark, 0.3 mL of 0.5 mM of Folin reagent was added to each tube and mixed immediately using vertex. The solutions were incubated for 30 min at room temperatures in dark. Then, the solutions were mixed briefly using vortex and 4mL of each was transferred to cuvette. The absorbance was measured at 660 nm. Bovine serum albumin (BSA) was used as standard. The stock solution of 10 mg/ml was prepared using buffer solutions. The stock was diluted to 0.5, 1, 1.5, 2, 2.5 mg/mL concentrations.

2.5. Measurement of Protease Activity

Assay for protease activity was conducted using protocol described by Cupp-Enyard (2008). Five mL of 0.65% casein solution was added into 15 ml capacity sample and blank test tube, and equilibrated in a water bath at 37°C for 5 min. Then, 500 µL papain extract sample solution was added to the sample test tube, but not to the blank test tube. The solution was mixed by swirling and incubated for 37°C for 10 min. After incubation, 500 µL of the papain extract sample was added into the blank test tube and simultaneously, 5 mL of the Trichloroacetic acid (TCA) solution was added into both test tubes to stop the reaction. The reaction solutions were incubated for 30 minutes. After incubation, 2 mL of the solution was filtered using filter paper into centrifuge tubes of 10 mL capacity. Thereafter, 5 mL sodium carbonate was added into centrifuge tubes and mixed by swirling followed by immediate addition and mixing of 1ml Folin's reagent. Then, the tubes were incubated at 37°C for 30 min. The absorbance of the sample solutions was measured by a spectrophotometer using a wavelength of 660 nm. Tyrosine was used as standard and its stock solution (10 mg/ml) was prepared using buffer solution. The stock was diluted to 200 µg/mL, 400 µg/mL, 600 µg/mL and 800 µg/mL concentrations. One protease unit was

defined as the amount of casein hydrolyzed to produce color equivalent to 1.0 µmole (181 µg) of tyrosine per min at pH 7 and 37°C (color by Folin and Ciocalteu's reagent) and was calculated as:

$$\frac{\text{Units}}{\text{mL of enzyme}} = \frac{(\mu\text{mol of Tyrosine}) (V_T)}{(V_g)(t) (V_c)}$$

Where V_T is the total assay volume in mL, V_E is the volume of the enzyme used mL, t is the reaction time taken in min, and V_C is the volume used in the colorimetric reaction in mL.

After determination of protein content and protease activity, activity recovery percentage and purification fold were calculated using the following equations.

$$\text{Activity recovery percentatge} = \left(\frac{\text{Protease activity of the phase papain}}{\text{Protease activity of the crude papain}} \right) \times 100$$

$$\text{Purification fold} = \left(\frac{\text{Specific activity of the phase papain}}{\text{Specific activity of the crude papain}} \right)$$

Where specific activity was calculated using equation:

$$\text{Specific activity} = \left(\frac{\text{Protease activity of the sample papain extract}}{\text{Protein content of the sample papain extract}} \right)$$

2.6. Process Optimization

Process optimization of three phase partitioning for crude papain was considered agitation time taken, crude extract to t-butanol ratio, ammonium sulphate concentration and temperature parameters. First, the effect of agitation time taken was determined according to Vetal and Rathod, (2015). Optimization of time taken was done by varying it from 60 to 100 min at interval of 10 min. The experimental parameters were as follows: 1:1 ratio of crude extract to t-butanol, (40% w/v) ammonium sulphate solution, pH 7 buffers, 45°C, 200 rpm agitation speed. The agitation time taken that gave the highest protease activity recovery was chosen for further study.

Second, the effect of ratio of crude papain extract to t-butanol was determined by methods described by Chawuit et al. (2010). Optimization of t-butanol ratio was done by varying it from 0.5 to 1.5 mL at interval of 0.25 mL. The experimental parameters used were the ratio of crude papain extract to t-butanol of 1:0.5, 1:0.75,

1:1, 1:1.25, and 1:1.5 (v/v), (40% w/v) ammonium sulphate , pH 7 buffer , 45°C , 200 rpm agitation speed and optimized agitation time. The ratio of crude papain extract to t-butanol that gave the highest protease activity recovery was chosen for further study.

Third, the effect of ammonium sulfate concentration was determined by methods described by Chawuit et al., (2010). Optimization ammonium sulfate concentration was done by varying it from 30 to 50 % at interval of 5. The experimental parameters were as follows: 45°C temperature, pH 7 buffer, 200 rpm agitation speed, optimized agitation time taken and ratio of crude papain extract to t-butanol. The ammonium sulfate concentration providing the highest protease activity recovery was chosen for further study.

Fourth, the effect of temperature was investigated. The optimization of temperature was done by varying it from 45 to 85°C (Pitpreech and Damrongsakkul, 2006) at interval of 5. The experimental parameters were as follows: pH 7 buffer, 200 rpm agitation speed, optimized agitation time, ratio of crude papain extract to t-butanol and ammonium sulfate concentration. The temperature providing the highest protease activity

recovery was chosen for purification fold and enzyme activity recovery of optimized process.

2.7. Enzymatic Hydrolysis of Proteineous Tannery Waste

Gelatin production was carried out as described by Pitpreecha and Damrongsakkul (2006). Raw trimming waste was obtained from Modjo Tannery Sharing Company. The trimming was washed, dried and ground. Slurry was prepared by dissolving 75 g of ground raw trimming in 300 mL of phosphate buffer (pH = 7.0) by 1:4 (w/v) ratio. The solution was well mixed and the formed slurry was then heated to 75 °C temperature. Then, 0.035 mL of intermediate and crude papains was mixed with 100 mL of slurry. This enzymatic hydrolysis reaction mixture was heated at 75°C for 90 min. Then, reaction mixture was heated to 90°C for 15 min to deactivate enzyme, centrifuged at 9000 rpm for 5 min and filtered to separate gelatin solution from residue. Protein concentration of filtered gelatin solution was measured and percentage of gelatin recovery was calculated based on the following equation:

Table 1: The process of three phase partitioning optimization

Independent factor Optimization order	Independent factor values	Controlled factors value
1. Agitation time (min)	60, 70, 80, 90, 100	Crude extract: t-butanol = 1:1 (V/V) (NH ₄) ₂ SO ₄ = 40% (w/v) T ⁰ = 45 °C pH = 7 Speed = 200 rpm
2. Crude extract: t-butanol (v/v)	1:0.5, 1:0.75, 1:1, 1:1.25, 1:1.5	Optimized agitation time (NH ₄) ₂ SO ₄ = 40% (w/v) T ⁰ = 45 °C pH = 7 Speed = 200 rpm
3. (NH ₄) ₂ SO ₄ (w/v %)	30, 35, 40, 45, 50	Optimized agitation time Optimized Crude extract: t-butanol (v/v) T ⁰ = 45 °C pH = 7 Speed = 200 rpm
4. Temperature (°C)	45, 55, 65, 75, 85	Optimized agitation time Optimized Crude extract: t-butanol (v/v) Optimized (NH ₄) ₂ SO ₄ (w/v %) pH = 7 Speed = 200 rpm

$$\text{Percentage of gelatin recovery} = \frac{P_g}{\text{pf} \left(\frac{w}{v} \right)} \times 100$$

where, P_g is protein concentration of gelatin solution in g/mL, P_f is protein fraction of raw trimming 0.6362 (Pitpreech, 2006), W is the weight (g) of raw trimming powder used to make slurry and V is the volume (mL) of buffer used to dissolve the raw trimming powder for slurry formation.

The left gelatin solution was dried for yield and gel strength measurement. Gel strength of gelatin was determined according to Pitpreecha and Damrongsakkul (2006) and Damrongsakkul et al. (2007). The dried gelatin was dissolved in distilled water making concentration of 12.5% gel (w/v) at 60°C. The solution was cooled at 4°C (maturation temperature) for 16 hrs. The gel strength of gelatin was determined by using a texture analyzer, equipped with 1.27 cm diameter hemisphere head cylindrical at a cross-head speed of 0.8 mm/s. The force required was measured at the center of gel surface vertically to a depth of 4 ± 0.01 mm. The measured gel strength was in the double bloom standard. Yield was determined according to Mad-Ali et al. (2016).

$$\text{Yield (\%)} = \frac{\text{weight of dried gelatin solution (g)}}{\text{weight of raw trimming powder (g)}} \times 100$$

2.8. Data Analysis

All the experiments were performed in triplicate. The data was expressed as mean \pm standard deviation. The significance differences of the different factors was evaluated by analysis variances (ANOVA) using SPSS version 20. A single factor ANOVA was used for the statistical analysis. The p-value less than 0.05 ($p < 0.05$) considered to be statistically significant.

3. Results and Discussion

3.1. Enzymatic Activity of Crude Papain and Two Phase of TPP

The preliminary three phase partitioning was conducted at parameters of 1:1 ratio of crude extract to t-butanol, at 40 % ammonium sulfate saturation, 45°C, pH = 7 and at 200 rpm agitation for 100 min and Crude papain was extracted from the papaya fruit latex by using phosphate buffer at pH=7. Three phase

partitioning uses t-butanol and ammonium sulfate to precipitate enzymes and proteins from aqueous solution. Tertiary butanol is having complete miscibility with water, but after addition of ammonium sulfate, its solubility changes, forming top t-butanol layer, middle enzyme/protein precipitate layer and bottom aqueous layer (Pike and Dannison, 1989; kalyani et al., 2006). The target enzymes get partitioned into either of phases depending upon its molecular properties and operational conditions (Pike and Dannison, 1989). T-butanol is also played role in removing some small molecular weight contaminant compounds such as lipids, phenolics and some detergents (Roy and Gupta, 2001).

The result of the study showed that protein content of crude extract was significantly higher than that of both phase of TPP at initial and optimum parameters. Additionally, protease activity of crude extract was higher than that of both phases of TPP at initial and optimum parameters, but not significantly different ($p > 0.05$) (Table 2). Higher protease activity of crude extract is might be due to maintaining of pH of the solution and impact of t-butanol. It was reported that the phosphate buffer efficiently maintained the system pH (Chaiwut et al., 2010). Generally, many studies showed that t-butanol does not cause denaturation of the partitioned enzyme as it is unable to permeate inside the folded three dimensional structure of protein due to its larger molecular size. However, it was reported that some enzymes may lose their activity in the presence of high amount of t-butanol (Gagaoua and Hafid, 2016) and that t-butanol can also inhibit activities of some enzymes (Dannison et al., 2000).

The study result showed that optimized parameters of three phases partitioning for intermediate phase were 90 min agitation time, 1:1.5 crude extract to t-butanol ratio, 50% ammonium sulphate concentration at 65°C and for bottom phase were 80 min agitation time, 1:0.75 crude extract to t-butanol ratio, 30% ammonium sulphate concentration at 75°C.

At optimized parameters, protease activity recovery and purification fold (94.14%, 8.31), respectively, of intermediate phase were higher that of bottom phase protease activity recovery and purification fold (75.15%, 6.61), respectively (Table 2). A different trend was reported by Chaiwut et al. (2010). They reported that at optimized first TPP with 20% (w/v) $(\text{NH}_4)_2\text{SO}_4$

and 1:0.5 ratio of extract to t-butanol, papaya fruit peel bottom phase showed higher purification fold and activity recovery (15.8, 253.5%), respectively, than that of intermediate phase purification fold and activity recovery (4, 4%), respectively. The difference might be due to different papaya part used for protease extraction, and chemical structure and composition of protease papaya peel and fruit latex. Chaiwut et al. (2010) obtained most proteases from papaya peel extract partitioned in the aqueous phase of TPP while in present study in intermediate phase of fruit latex extract. This implied the difference in chemical structure and composition of cystein protease from peel and latex.

3.2. Three Phase Partitioning Process Optimization

Three Phase Partitioning (TPP) is affected by various process conditions, such as ammonium salt concentration and ratio of crude enzyme extract to t-butanol (Chaiwut et al., 2010; Duman and Kayan, 2013 and 2014; Kulkarni and Rathod, 2013; Vet al and Rathod, 2014), temperature (Chaiwut et al., 2010; Kulkarm and Rathod, 2013), pH (Duman and Kayan, 2013; Kulkarni and Rathod, 2013; Vet al and Rathod, 2014), agitation time (Vetal and Rathod, 2014). In this study, different values or intervals of ammonium salt concentration, ratio of crude enzyme extract to t-butanol, agitation time was investigated. The effects of these parameters on purification fold and activity recovery of papain purified by TPP from fruit latex were given in Figure 1- 4. As purification fold and activity recovery are very important to determine the optimum parameter at which TPP system works best for the

purification of enzymes, they were put on Y-axis of the same graph for each independent factors affecting TPP. In this study, In order to determine the best TPP system for the purification of papain, the parameters that gave the highest % activity recovery and purification fold was considered for stepwise optimization process.

3.2.1. Effect of Agitation Time on Protease Purified by TPP

Agitation time is one of the key factors as far as economy of the process is concerned. Time taken required for completion of any industrial reaction or process should be the minimum to make it economically feasible (Vetal and Rathod, 2014). Hence, agitation time was optimized to get maximum possible activity recovery and purification fold in small possible duration. As shown in Figure 1, optimization of time was done by varying it from 60 to 100 min and keeping other experimental parameters constant. The result of study showed that highest protease activity recovery and purification fold of papain was occurred at 80 min and 90 min for bottom and intermediate phase, respectively. Activity recovery (94.4%) and purification fold (6) were obtained for intermediate phase while Activity recovery (84.2%) and purification fold (4.7) were obtained for bottom phase, respectively. A higher purification fold (12.14), but lower activity recovery (71.43%) was reported for peroxidase extracted from orange peel (Vetal and Rathod, 2014). As saturation was achieved in 80 min for bottom phase and 90 min for intermediate phase, they were selected as optimum time for the subsequent experiments.

Table 2: Enzymatic parameters of different extracts of papain from papaya fruit latex

Extraction steps	Product	Protease activity (U/ml)	Protein content (mg/mL)	Specific activity (U/mg)	Activity recovery(%)	Purification fold
Crude	Crude	$11.88 \times 10^{-2} \pm 0.06^a$	16.44 ± 3.02^d	$7.68 \times 10^{-3} \pm 0.01^a$	-	-
I-TPP	I-phase	$8.86 \times 10^{-2} \pm 0.06^a$	10.44 ± 1.34^c	$8.12 \times 10^{-3} \pm 0.00^a$	71.42 ± 6.03^a	1.12 ± 0.12^a
	B-phase	$7.56 \times 10^{-2} \pm 0.03^a$	7.26 ± 0.59^b	$10.50 \times 10^{-3} \pm 0.00^a$	67.70 ± 7.77^a	1.59 ± 0.41^a
O-TPP	I-phase	$11.14 \times 10^{-2} \pm 0.05^a$	2.10 ± 0.89^a	$68.03 \times 10^{-3} \pm 0.06^a$	94.14 ± 1.16^b	8.31 ± 1.15^b
	B-phase	$8.47 \times 10^{-2} \pm 0.03^a$	2.02 ± 0.82^a	$47.80 \times 10^{-3} \pm 0.03^a$	75.15 ± 7.46^a	6.61 ± 1.38^b

I-TPP is Three Phase Partitioning at initial parameters and O-TPP is at optimized parameters. I is intermediate and B is bottom. The numbers are in mean and standard deviation. Letter compares means in the column. Means with different letters are significantly different at $p < 0.05$.

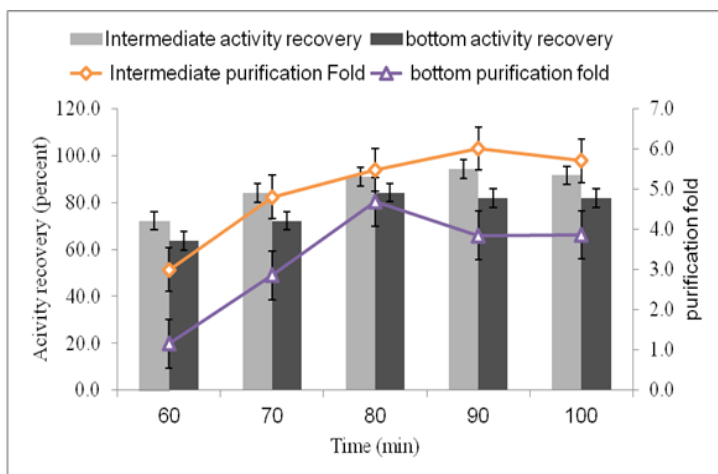


Figure 1: The effect of time on activity recovery and purification fold of papain from papaya fruit latex

3.2.2. Effect of Crude Extract to t-Butanol on Protease Purified by TPP

The effect of t-butanol volume that was required for protease partitioning by TPP was shown in Figure 2. The ratio of the crude extract to t-butanol was varied from 1:0.5, 1:0.75, 1:1, 1:1.25 and 1:1.5 (v/v) with a constant of 40% (w/v) $(\text{NH}_4)_2\text{SO}_4$ saturation. The highest protease activity recovery (92.6%) and purification fold (5.7) were obtained from the intermediate phase at 1:1.5 ratios. For bottom phase, the highest protease activity recovery (66.7%) and purification fold (4.1) were obtained at 1:0.75 ratios. A similar highest protease recovery (67.3%) and a different purification fold (2.4) were reported for bottom phase from papaya peel at ratio of 1: 0.5 (Chaiwut et al., 2010). The result of this study showed that an increase in t-butanol volume decreases the activity recovery and purification fold in the bottom phase. Chaiwut et al. (2010) were reported similar trend and described that if the t-butanol content is high (>1:1), the denaturation of the protein is more likely. For intermediate phases, an increase of t-butanol volume resulted in increase of activity recovery and purification fold. In supporting this result, Vetal and Rathod, (2014) found that increased amount of t-butanol increased salt concentration in aqueous phase causing precipitation of enzyme at intermediate phase. The ratio of crude extract to t-butanol of 1:0.75 and 1:1.5 were selected for bottom and intermediate phase, respectively, for investigating the effect of $(\text{NH}_4)_2\text{SO}_4$ concentration.

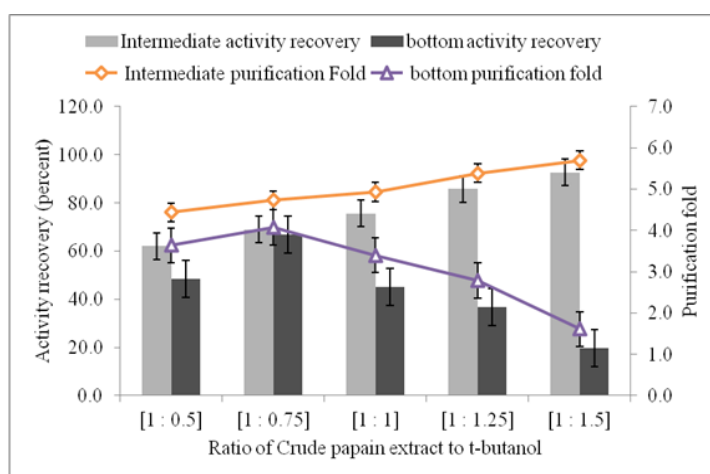


Figure 2: The effect of ratio of crude extract to t-butanol on activity recovery and purification fold of papain from papaya fruit latex

3.2.3. Effect of Ammonium Sulfate Concentration on Protease Purified by TPP

Ammonium sulfate concentration plays a major role in TPP as it is responsible for protein-protein interaction and precipitation. It causes protein precipitation by salting out mechanism. Protein solubility is influenced by the ionic strength of the solution which subsequently depends on salt concentration. On addition of sufficient salt, proteins precipitation occurs. This is known as salting out effect (Narayan et al., 2008; Chaiwut et al., 2010; Vetal and Rathod, 2014). In this study, the effect of $(\text{NH}_4)_2\text{SO}_4$ was studied by varying the concentration of $(\text{NH}_4)_2\text{SO}_4$ from 30 to 50% (w/v). It was indicated that minimum salt concentration to starts with for optimization of TPP is 30% (w/v) so as to obtain the maximum amount of the desired protein in the interfacial precipitate (Chaiwut et al., 2010). This study result showed that at the concentration of 50% $(\text{NH}_4)_2\text{SO}_4$, the highest protease activity recovery (104.5%) and the highest purification fold (5.8) were obtained for intermediate phase, and the highest protease activity recovery (97.7%) and the highest purification fold (4.4) were obtained for bottom phase at (30%) (Figure 3). Opposing present result, most proteases from papaya peel extract partitioned in the aqueous phase of TPP and the highest protease recovery (121.4%) and the highest purification fold (5.4) at concentration of 20% $(\text{NH}_4)_2\text{SO}_4$ were reported in the bottom phase of the TPP system (Chaiwut et al., 2010).

In this study, increasing % $(\text{NH}_4)_2\text{SO}_4$ concentration resulted in an increase of protease activity recovery and purification fold for intermediate phase and a decrease of protease activity recovery and purification fold for bottom phase. Similar trend has been reported by Chaiwut et al. (2010) in the extraction of protease from papaya peel for both phases. In line with this, it was reported that at higher salt concentration, water molecules are attracted by salt ions result in stronger protein–protein interactions and the protein molecules coagulate through hydrophobic interactions at intermediate phase (Narayan et al., 2008; Chaiwut et al., 2010; Vetal and Rathod, 2014). As maximum activity recovery and purification fold was achieved at 50% $(\text{NH}_4)_2\text{SO}_4$ concentration for intermediate phase and at 30% $(\text{NH}_4)_2\text{SO}_4$ for bottom phase, 50% and 30% $(\text{NH}_4)_2\text{SO}_4$ selected as optimum value for temperature optimization.

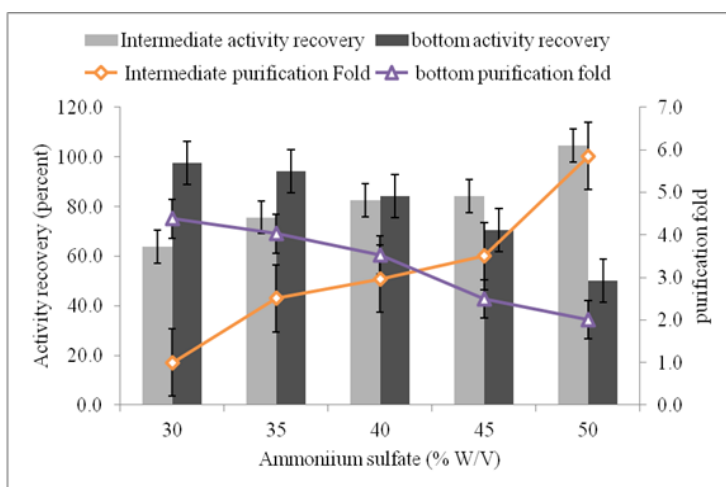


Figure 3: The effect of ammonium sulfate concentration on activity recovery and purification fold of papain from papaya fruit latex

3.2.4. Effect of Temperature on Protease Purified by TPP

Temperature is an important parameter that affects the enzyme configuration and overall stability (Vetal and Rathod, 2014). Effect of temperature was studied by varying water bath temperature from 45 to 75 °C. This temperature was selected considering appropriate temperature for gelatin production. Other parameters were kept at optimized value. Figure 4 illustrated the effect of temperature on partitioning behavior of papain in TPP. Activity recovery and purification fold of the

intermediate phase enzyme was increased with an increase in the temperature from 45 °C to 65 °C. At 65 °C temperature, maximum activity recovery (97.7%) and purification fold (4.9) were found. For bottom phase, maximum activity recovery and purification fold was increased with an increase in the temperature from 45 °C to 75 °C. At 75 °C temperature, maximum activity recovery (96.0%) and purification fold (4.5) were found. Decrease in activity recovery and purification fold at higher temperature (above 65 and 75 °C for intermediate and bottom, respectively) was may be thermal deactivation of enzymes. In line with this, Chaiwut et al. (2006) reported that papaya peel and latex protease showed good stability to temperatures up to 70 °C and latex protease activity decrease rapidly at temperature > 80 °C.

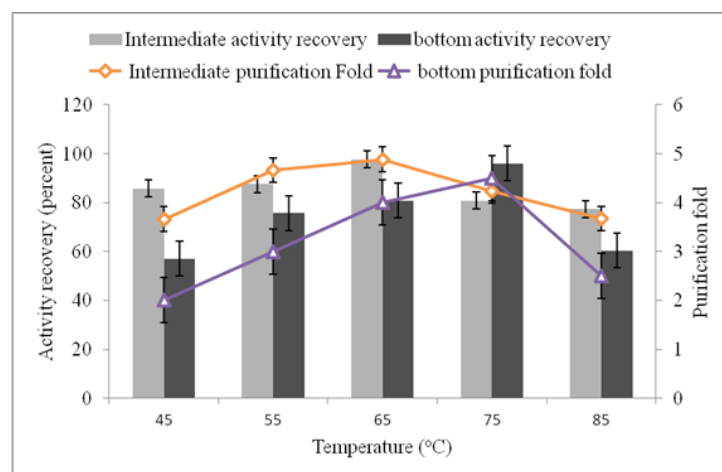


Figure 4: The effect of temperature on activity recovery and purification fold of papain from papaya fruit latex

3.3. Enzymatic Hydrolysis of Raw Trimming

The hydrolysis of raw trimming was carried out using the optimized temperature and time parameters by Pitpreecha and Damrongsakkul (2006). The crude extract and optimized intermediate phase papain was used for enzymatic hydrolysis of raw trimming. The gel yield, recovery and strength of gelatin obtained from hydrolysis of raw trimming by crude papain and intermediate phase is depicted in Figure 5. From the hydrolysis reactions, percentage of gelatin recovery from crude extract (crude papain) hydrolysis was 68 wt%. In the case of optimized intermediate phase papain, the percentage of gelatin recovery was 64 wt%.

The higher gelatin recovery percentage for crude papain was may be due to the higher rate of enzyme hydrolysis reaction. Compared to present study, Pitpreecha and Damrongsakkul (2006) reported a higher 72 wt% and 71 wt% of gelatin recovery from hydrolysis of raw hide using crude papaya latex extract and commercial papain respectively, at 75 °C. Similarly, Damrongsakkul et al. (2007) reported significantly higher gel recovery of (85 wt %) gelatin produced by hydrolysis of raw hide using papain, EC 3.4.22.2, (0.51 Unit/mg). Gelatin extraction yield (on dry basis) from both hydrolysis of raw trimming by crude and intermediate phase papain were expressed as the weight of dried gelatin solution (g) with respect to the weight of dried powder of raw trimming (g). The amount of gelatin extracted by using crude papain was similar with amount of gelatin extracted by using intermediate phase.

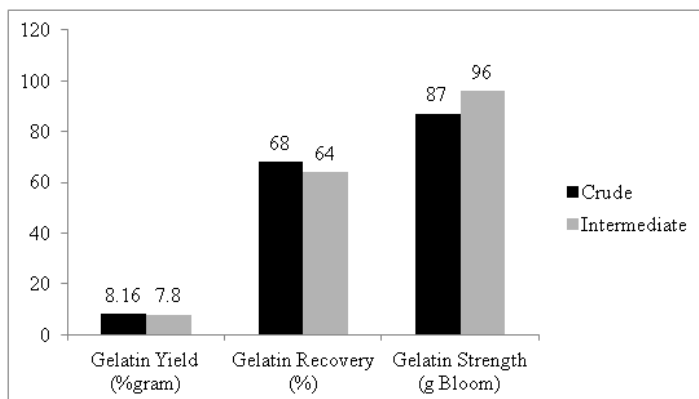


Figure 5: Gelatin yield, recovery and strength from hydrolysis of raw trimming

Hydrolysis of raw trimming by crude papain resulted in a lower gel strength (bloom value of 87 g) as compared to the gel strength of gelatin produced by intermediate (bloom value of 95 g). This was due to a higher protease activity of crude papain. Similar trend was reported by Pitpreecha and Damrongsakkul (2006) in which the lowest gel strength was observed at optimum temperature of 75 °C at which enzyme activity

is the highest. Gelatin with higher molecular weight showed greater bloom value was reported by Du et al. (2014). Pitpreecha and Damrongsakkul (2006) described that at the condition for highest enzyme activity, short chain gelatin or low molecular weight gelatin was obtained from the hydrolysis whereas, high molecular weight gelatin could be obtained from the hydrolysis at the condition corresponded to low enzyme activity.

4. Conclusion

Proteolytic enzymes contained in papaya fruit latex can be simply extracted using phosphate buffer pH 7 as the activity of crude papain was greater than that of papain purified by Three Phase Partitioning (TPP). The extraction and purification of papain at optimized parameters of TPP increased activity recovery and purification fold of papain from 71.42% to 94.14% and 1.12 to 8.31, respectively, for intermediate phase. As well as, increment in activity recovery and purification fold of papain for bottom phase was from 67.70% to 75.15% and 1.59 to 6.61, respectively, at optimized parameters of TPP. The gelatin recovery from both hydrolysis reactions is related to the gel strength of the obtained gelatin. The gel strength of the obtained gelatin from both hydrolysis reactions was relatively low. The results of hydrolysis of raw trimming tannery waste indicated that crude papain proteolysis could be used to produce gelatin from raw trimming. As well as the intermediate phase could be used alternatively in production of gelatin incase purity is needed.

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