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ORIGINAL RESEARCH PAPER

Optimization of Three Phase Partitioning Purification of Papain from *Carica papaya* Latex by Response Surface Methodology

Shreya Chhetry Mahatara[1] and Bunty Maskey*[2]

[1]*Department of Food Technology, Central Campus of Technology, Tribhuvan University, Dharan, Nepal*

[2]*Central Department of Food Technology, Tribhuvan University, Dharan, Nepal*

Abstract

Three phase partitioning (TPP), a novel and non-chromatographic method was effectively applied for the extraction and recovery of papain from Carica papaya latex. The effect of change in ammonium sulfate concentration, crude extract to solvent ratio and pH on extraction efficiency was studied, and these parameters were optimized by response surface methodology (RSM) using Box-Behnken Design. The protease was found to be concentrated more at the aqueous phase (AP) with 51.08% ammonium sulfate precipitation, crude extract to tert-butanol ratio of 1:0.78 (v/v), and pH 6.05 resulting 9.242 purification fold and 159.806% activity recovery. Compared to the intermediate phase (IP), the AP demonstrated higher recovery and purification fold. Purified papain showed significantly (p<0.05) greater activity at a temperature of 60°C and pH of 6.0. It also demonstrated stability within the temperature range of 40 to 70°C and pH range of 6.0 to 8.0. Enzyme retained maximum milk clotting activity when stored under freezing (-20°C) for 3 weeks. This study showed that the TPP can be used to extract and purify papain from papaya latex economically and it can also be employed as a vegetable coagulant for cheesemaking.

Keywords:
Latex protease
TPP purification
RSM optimization
Milk clotting activity
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*Corresponding author at:
Central Department of Food Technology
Tribhuvan University
Hattisar, Dharan, Nepal.
E-mail address: bunty.maskey@cdft.tu.edu.np

Introduction

Proteases are crucial enzymes with various industrial applications such as pharmaceuticals, food technology, detergents, and the leather industry (Feijoo-Siota & Villa, 2011). These enzymes are present in a diverse range of species, from prokaryotes to complex organisms like plants and mammals. In plants, cysteine proteases are the most prevalent, followed by serine and aspartic proteases (Troncoso David et al., 2022).

Papain is a naturally occurring cysteine protease of 212 amino acids that is obtained from unripe papaya fruits, stem, and the latex, and it is stable as well as functional under diverse conditions (Mitchel et al., 1970; Cohen et al., 1986; Mamboya & Amri, 2012). It is employed in various applications, including, soft unripened cheese preparation (Maskey & Shrestha, 2020), enhancing the meltability and stretchability of Nabulsi cheese (Hejazin & El-Qudah, 2009), addressing dyspepsia and digestive disorders (Huet et al., 2006), serving as a protein modifier in the meat and beer industries (Khanna & Panda, 2007), and fulfilling diverse food and non-food purposes (Mamboya & Amri, 2012). Papaya latex has been reported to have good milk clotting property and used to substitute rennet in manufacturing of cottage cheese (Rana et al., 2017) and Dangke, an Indonesian cheese made in Enrekang, South Sulawesi province (Prasetyo et al., 2015).

Papain has been purified from papaya latex using conventional precipitation methods (Baines & Brocklehurst, 1979). Nevertheless, the enzyme still remained contaminated with other substances (Braia et al., 2013). Purification by other methods, such as chromatography requires a lot of steps, are expensive, time-consuming, and difficult to scale up (Ramos, 2011). Considering that proteins can contribute to over 70% of downstream production expenses (Gupta et al., 2002; Najafpour, 2007), adopting a purification approach like three phase partitioning (TPP) results in more economical extraction, concentration and enzyme recovery (Gagaoua & Hafid, 2016).

The TPP method involves mixing a crude extract with an organic solvent (typically tert-butanol) and an aqueous anti-chaotropic

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salt, usually ammonium sulfate [$(NH_4)_2SO_4$] to separate into three phases during centrifugation: upper (butanol), interfacial (protein-rich), and the lower phase (aqueous) (Dennison & Lovrien, 1997). It offers high enzyme recovery, and can be enhanced with methods like ultrasound, microwave assistance, micro affinity ligand-facilitated reactions, and ionic liquid-based reactions (Chew et al., 2019). It has been successfully applied in purifying various plant proteases, including bromelain (Gul et al., 2022), ficin (Gagaoua et al., 2014), actinidin (Maskey & Karki, 2023) and calotropin (Maskey et al., 2023).

In general, the classical approach to process optimization, changing one variable at a time, is complicated and often yields imprecise results due to a lack of parameter interaction consideration (Gokhale et al., 1991; Cochran, 1992). However, response surface methodology (RSM), which first requires an experimental design and then fitting experimental data into an empirical model equation to determine the optimal conditions, offers an efficient alternative. It is able to provide the data required for design and process optimization, as well as information regarding the interactions between factors. RSM can minimize development time and costs, guarantee greater conformity to output criteria, enhance product yields and purity, and lower process variability in purifying processes (Box, 1978; Haaland, 1989; Cochran, 1992). Hence the objective of this study was to optimize the TPP parameters for purification of papain from *C. papaya* latex using RSM.

Materials and Methods

Materials

Fresh papaya latex was collected from Itahari, Nepal. Ammonium sulfate, Skimmed milk powder, casein (purified), BSA (bovine serum albumin) and L-cysteine were purchased from the HiMedia Laboratories Pvt. Ltd., India, TCA (trichloroacetic acid) and tert-butanol from Thermo Fisher Scientific Pvt. Ltd., India and EDTA from the Merck, India. All chemicals were of analytical grade and solutions were prepared using distilled water.

Latex collection

Latex from unripe *Carica papaya* fruits was extracted through vertical incisions (2-3 mm deep) using a stainless steel knife, following Nitsawang et al. (2006) methodology. The collected latex was allowed to flow down the fruit, collected in a plastic beaker, placed on ice, and stored at -18°C until further use.

Preparation of crude dialyzed papaya extract

Extraction of papaya protease was carried out according to Gagaoua et al. (2015) with slight modifications. Latex was thawed, blended with fresh distilled water (1:1, w/v), stirred (4°C, 45 min), and then centrifuged (DLAB D3024R, China) at 2500 rpm (4°C, 10 min) to remove insoluble particles. The supernatant was saturated with 30% ammonium sulfate, followed by centrifugation. Further saturation with 80% ammonium sulfate, centrifugation, and pellet dissolution in sodium phosphate buffer (pH 7.0) were performed. The crude extract was dialyzed (12 kDa cut-off) with three changes of pH 7 phosphate buffer, and purification was carried out using TPP method.

Three phase partitioning of crude dialyzed extract

TPP purification was conducted in accordance with Gagaoua (2018). The crude extract (CE) was first concentrated at 25°C with ammonium sulfate, followed by the addition of tert-butanol and pH adjustment. After gentle vortexing, the mixture was let stand at room temperature for about 45 min and was then centrifuged at 4000 rpm (10 min, 4°C) for phase separation. The upper butanol phase was removed, and the remaining interfacial phase (IP) and lower aqueous phase (AP) were carefully separated. The IP and AP fractions were dissolved in phosphate buffer (pH 7.0), followed by overnight dialysis. Protein content and caseinolytic activity (CA) were assessed in both dialyzed fractions. The concentrations of ammonium sulfate were determined using a calculation tool (www.encorbio.com/protocols/AM-SO4.htm).

Optimization of TPP parameters

RSM was used to optimize the parameters of TPP for purification of papain. The Box-Behnken Design was used by the application of Design Expert (Version 13.0, Stat-Ease Inc., USA). The three coded levels were -1, 0 and +1 as shown in Table 1. In order to find out maximum response variables (purification fold and activity recovery), ammonium sulfate concentrations (40, 50 and 60%), ratio of CE to tert-butanol (1:0.5, 1:0.75 and 1:1), and pH (5, 6 and 7) were selected based on previous research performed by Hafid et al. (2020).

Table 1
Range of factors for RSM

Factors	*-1*	*0*	*+1*
$(NH_4)_2SO_4$ concentration (%)	40	50	60
CE to tert-butanol ratio	1.0:0.5	1.0:0.75	1.0:1.0
pH	5	6	7

The response variables for various experimental combinations were related to the coded variables (Xi, i = 1 and 2) by a second-degree polynomial equation 1.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1{}^2 + \beta_{22} X_2{}^2 + \beta_{12} X_1 . X_2 + \varepsilon \quad (1)$$

The polynomial coefficient was depicted by β_0 (constant), β_1, β_2, (linear effects); β_{12}, (interaction effects); β_{11}, β_{22}, (quadratic effects); and ε (random error). Multiple regression analysis was used to model the data, and analysis of variance (ANOVA) was used to determine each term's statistical significance

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Caseinolytic activity determination

Caseinolytic activity (CA) was assessed following the method given by Ladd & Butler (1972). 1% casein (w/v) in pH 7.0 phosphate buffer (50 mM) was used as substrate. Equal volume (1 ml) of substrate and diluted enzyme were incubated at 37°C for 30 min. The reaction was halted by adding 3 ml of 10% trichloroacetic acid (TCA) and incubating on ice for 1 h. After centrifugation at 5000 rpm for 10 min, the supernatant absorbance was measured at 280 nm using tyrosine as a reference by a double beam spectrophotometer (Agilent Cary 60 UV-Vis, US). In blank, TCA was used first to inac he enzyme and later the substrate was added. One CA uni ed 1 mg of tyrosine under standard test conditions. CA was calculated using equation 2.

$$CA\left(\frac{U}{ml}\right)=\text{Tyrosine}(\mu g)\times\frac{\text{Dilution factor}\times V_T}{V_E\times t} \quad (2)$$

Where V_T and V_E are the total assay volume and volume of the enzyme used (ml) respectively, t is the incubation time (min).

Determination of milk-clotting activity

Milk-clotting activity (MCA) of CE and fractions was assessed using procedure mentioned by International Dairy Federation (IDF, 2007). Reconstituted milk (pH 6.5) in a 2 ml vial was incubated at 37°C for 5 min, followed by the addition of 200 µl of enzyme. The vial was frequently rotated (every 10 s) to check for any indications of clotting. Coagulation time was recorded, and MCA was calculated using equation 3. One MCA unit was defined as enzyme needed to coagulate 10 ml reconstituted skim milk at 37°C in 40 min (Berridge, 1952).

$$MCA\left(\frac{U}{ml}\right)=\frac{2400\times V_S}{T\times V_E} \quad (3)$$

Where V_S and V_E is the volume of milk and enzyme (ml) respectively. T is the time needed for clotting (s).

Determination of protein

Bradford protein-dye binding technique was used to determine the protein content of CE and fractions (Bradford, 1976). Bovine serum albumin (BSA) solutions (0.2-1.5 mg/ml) were used to create the calibration curve. Protein was estimated using the BSA standard curve and absorbance was measured at 595 nm using a double beam spectrophotometer.

Characterization of TPP purified papain

Temperature effect on enzyme activity

The standard assay protocol was carried out by incubating purified enzyme with 1% (w/v) casein at various temperatures (30-80°C) to determine the papain temperature profile (Gagaoua et al., 2017). Thermal stability of the TPP-purified protease was evaluated after 2 h of incubation at the same temperature range by measuring residual caseinolytic activity (Rajagopalan & Sukumaran, 2018).

pH effect on enzyme activity

The impact of pH was assessed from pH 4.0 to 9.0 using 50 mM citrate buffer for pH 4.0-5.0, 50 mM phosphate buffer for pH 6.0-7.0 and 50mM Tris-HCl buffer for pH 8.0-9.0. Residual activity under the same conditions after a 2 h incubation was determined by measuring CA (Gagaoua et al., 2015).

Storage stability

Storage stability of TPP-purified papain was tested for 3 weeks at both refrigerated (4°C) and frozen (-20°C) condition. The results were expressed as MCA (U/ml).

Statistical analysis

Data was analyzed using GenStat 12th edition, created by VSN International Limited ANOVA at a 5% significance level, with mean differences tested using Tukey's HSD. Graphs were created with Microsoft Excel (2016).

Results and Discussion

Three phase partitioning of papaya latex protease

In order to achieve high purification fold and activity recovery of papain from papaya latex, the crude dialyzed extract was purified by TPP method utilizing ammonium sulfate and tert-butanol. This technique concentrates the partitioned biomolecules in the IP fraction while separating impurities into the upper tert-butanol and lower AP fractions (Gagaoua et al., 2014; Gagaoua et al., 2015; Gagaoua et al., 2017). However, it was discovered that papain was concentrated into the AP fraction, with greater enzymatic activity as compared to IP fraction. This outcome parallels the findings reported by Hafid et al. (2020) during papain purification from *C. papaya* latex, where papain was also concentrated in AP. Thus, the AP fraction was used for further analysis to optimize the TPP parameters using RSM. The CA and protein content of each AP fraction were assessed to determine the specific activity, purification fold, and activity recovery (%).

RSM analysis

The experimental outcomes are shown in Table 2 where purification fold and percentage recovery ranged from 5.03-9.24 and 76.22-161.39% respectively. The experimental results of measured values of purification fold and activity recovery from Box Behnken design were fitted with the second order polynomial equation. The significance of the response models was assessed through detailed analysis using ANOVA, as illustrated in Table 3.

The purification fold and activity recovery were represented by the coded equations 4 and 5:

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$$\text{Purification fold} = 9.22 + 0.6506A + 0.1442B + 0.1555C + 0.0558AB - 0.0843AC - 0.0200BC - 2.35A^2 - 0.6199B^2 - 0.9445C^2 \quad (4)$$

$$\text{Activity recovery} = 160.19 + 10.20A + 2.85B + 2.23C + 2.6AB - 0.3436AC + 0.1406BC - 48.41A^2 - 13.52B^2 - 22.53C^2 \quad (5)$$

Where A, B and C are coded values of ammonium sulfate concentration, CE to butanol ratio and pH respectively. A^2, B^2, C^2 and AB, AC and AB are model terms.

Table 2
Box Behnken design with experimental values

Run	*A: $(NH_4)_2SO_4$ concentration*	*B: CE to tert-butanol ratio*	*C: pH*	*Purification fold*	*Activity recovery (%)*
1	50	0.75	6	9.21261	159.663
2	50	0.75	6	9.242	160.568
3	50	0.75	6	9.224	159.951
4	60	0.5	6	6.678	103.215
5	40	0.75	5	5.032	76.2189
6	50	1	7	7.9254	129.596
7	40	1	6	5.7	88.0989
8	50	0.75	6	9.223	161.385
9	60	0.75	7	6.6398	101.598
10	50	1	5	7.642	125.095
11	50	0.5	7	7.7035	122.902
12	40	0.5	6	5.4966	88.3145
13	60	1	6	7.1045	113.41
14	50	0.75	6	9.1836	159.405
15	50	0.5	5	7.34	118.964
16	60	0.75	5	6.51	97.5997
17	40	0.75	7	5.49895	81.5918

From purification fold regression model analysis, the concentration of ammonium sulfate (A) had a positive significant ($p<0.05$) impact on purification fold, followed by the CE to butanol ratio (B) and pH (C) showing a positive significant ($p<0.05$) impact. The interaction term of A and C (AC) had a negative significant impact ($p<0.05$), whereas that of B and C (BC) demonstrated a negative non-significant impact ($p>0.05$). The interaction term of A and B (AB) had a positive significant impact ($p<0.05$), and the quadratic terms (A^2, B^2 and C^2) had a statistically significant negative impact ($p<0.05$) on purification fold. The regression model was fitted well, as indicated by the value less than 0.0001, and the lack of fit was not significant ($p>0.05$). With an adjusted R^2 of 0.9998, the predicted R^2 value of 0.9991 was in reasonable agreement. Thus, the purification fold was analyzed and predicted using this regression model.

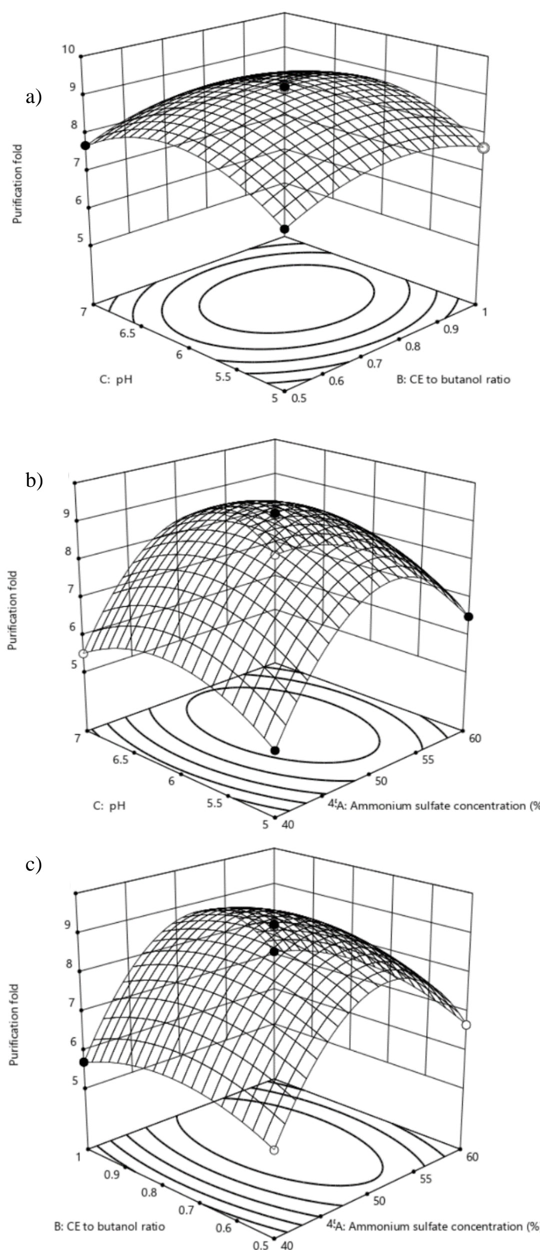

Figure 1
Response surface plot showing the effect of ammonium sulfate concentration, CE to tert-butanol ratio and pH on purification fold

From activity recovery regression model analysis, A, B and C had a positive significant ($p<0.05$) impact on activity recovery. AB had a positive significant effect ($p<0.05$), whereas AC had a negative non-significant impact ($p>0.05$). BC had a positive non-significant impact ($p>0.05$), and A^2, B^2 and C^2 had a negative

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significant ($p<0.05$) effect on activity recovery. A value less than 0.0001 indicated that the regression model was fitted well, and the lack of fit was not statistically significant ($p>0.05$). The estimated R^2 value of 0.9983 was in reasonable agreement with an adjusted R^2 of 0.9994. Thus, the activity recovery was analyzed and predicted using this regression model.

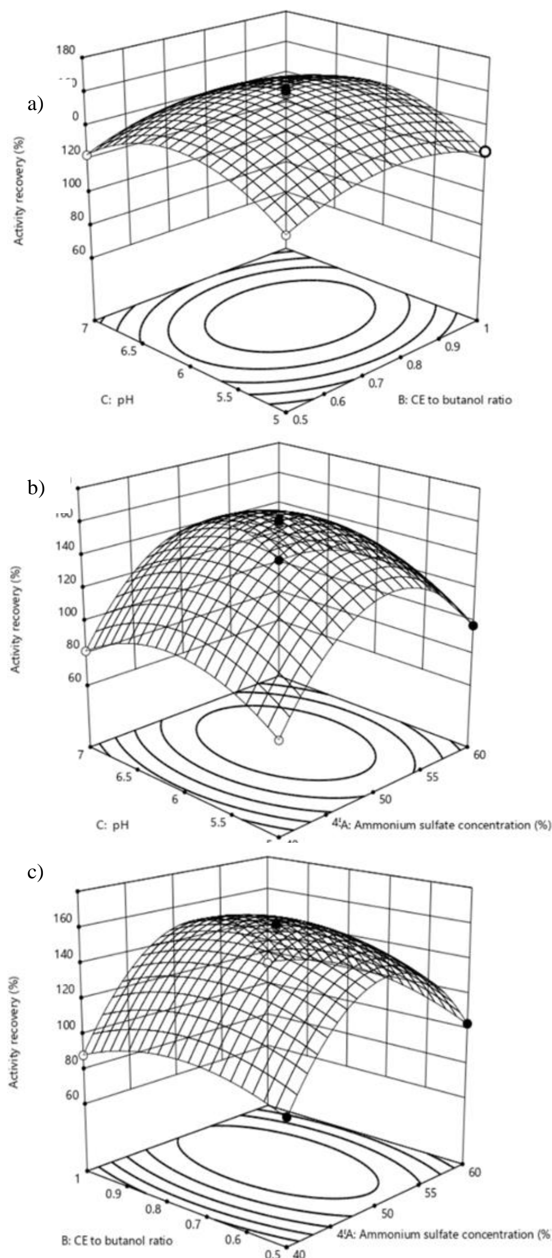

Figure 2
Response surface plot showing the effect of ammonium sulfate concentration, CE to tert-butanol ratio and pH on activity recovery

Figures 1 and 2 represent 3D response graphs for purification fold and activity recovery respectively. Each figure showed the impact of two variables while keeping the other variable at its intermediate level. The steepness of the response surface plot indicates the degree to which this component influences the purification fold and activity recovery, and the optimal condition for each factor is displayed at the apex of surface (Chen et al., 2020). The effects of the variables are related to each other and to one another separately when almost every interaction produced is a nearly spherical variance function (Kumar et al., 2011). This study also demonstrated similar effects, thus making it possible to predict the optimum concentration levels for maximizing purification fold and activity recovery.

Ammonium sulfate concentration is crucial for protein precipitation. Higher salt concentrations reduce purification fold and activity recovery due to irreversible protein denaturation (Dhananjay & Mulimani, 2008). Lower salt concentrations fail to alter the hydrophobic surface of papain. With the increase in salt concentration, the surface and interfacial tensions of the TPP system rise (Kiss et al., 1998). Salting-out process in TPP by SO_4^{2-} is dependent on ion concentration effects, exclusion crowding, kosmotropy, osmotic stresses and SO_4^{2-} binding to the cationic receptors of targeted protease (Tschesche, 2011; Gagaoua, 2018).

The volume of tert-butanol significantly influences the efficiency of TPP. Tert-butanol is mainly used for partitioning because it helps to stabilize protein structures. Because of its size and branch structure, it cannot penetrate within the protein molecules at the optimal volume, preventing protein denaturation (Dennison & Lovrien, 1997; Tschesche, 2011; Gagaoua et al., 2016; Gagaoua et al., 2017). Lower solvent ratios might hinder the efficient combination with ammonium sulfate (Sharma & Gupta, 2004; Özer et al., 2010; Tschesche, 2011), while larger ratios could induce protein denaturation and impede precipitation (Chaiwut et al., 2010; Tschesche, 2011). Increasing tert-butanol volume raises solution viscosity, reducing molecular mobility interaction (Yan et al., 2018). The partitioning of protease in TPP is influenced by solution pH, driven by electrostatic interactions between charged residues in proteins and involved phases (Gagaoua, 2018). The system predominantly relies on the protein's isoelectric point (pI) (Pike & Dennison, 1989). Below pI, the positive charges separate in the IP; while above pI, the proteins with negative charges accumulate in the AP (Gagaoua, 2018). In the study of Hafid et al. (2020), recovered papain primarily had a pI around 4.3, resulting in partitioning in the AP. This aligns with the recovery of similar cysteine proteases such as zingibain (Gagaoua et al., 2015), calotropin (Rawdkuen et al., 2010) and papain from dried papaya leaves (Chaiwut et al., 2010) in the AP. Gagaoua et al. (2015) purified zingibain at pH 7.0 in the bottom phase because the reported pI of zingibain was approximately 4.38.

Numerical optimization

Numerical optimization approach was used to identify the ideal combinations of ammonium sulfate concentration, CE to solvent ratio and pH for maximum purification fold and activity recovery. The various parameters for optimization are shown in Table 4.

The optimum combinations were found to be 51.08% ammonium sulfate concentration, 1:0.78 CE to tert-butanol ratio and 6.05 pH which reported the purification fold of 9.274 and activity recovery of 160.967% with the desirability of 0.998.

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Table 3
ANOVA for response surface quadratic models of purification fold and activity recovery

Source	*Purification fold*					*Activity recovery*				
	Sum of Squares	*df*	*Mean Square*	*F-value*	*p-value*	*Sum of Squares*	*df*	*Mean Square*	*F-value*	*p-value*
Model	34.64	9	3.85	7282.42	<0.0001	14780.1	9	1642.23	3013.51	<0.0001
A	3.39	1	3.39	6407.30	<0.0001	832.30	1	832.30	1527.27	<0.0001
B	0.1664	1	0.1664	314.87	<0.0001	65.01	1	65.01	119.29	<0.0001
C	0.1933	1	0.1933	365.82	<0.0001	39.66	1	39.66	72.77	<0.0001
AB	0.0124	1	0.0124	23.55	0.0019	27.10	1	27.10	49.72	0.0002
AC	0.0284	1	0.0284	53.77	0.0002	0.4723	1	0.4723	0.8667	0.3829
BC	0.0016	1	0.0016	3.04	0.1250	0.0791	1	0.0791	0.1451	0.7146
A^2	23.30	1	23.30	44088.43	<0.0001	9867.92	1	9867.92	18107.69	<0.0001
B^2	1.62	1	1.62	3061.23	<0.0001	770.09	1	770.09	1413.13	<0.0001
C^2	3.76	1	3.76	7106.59	<0.0001	2137.54	1	2137.54	3922.39	<0.0001
Residual	0.0037	7	0.0005			3.81	7	0.5450		
Lack of fit	0.0019	3	0.0006	1.34	0.3795	1.29	3	0.04310	0.6838	0.6069
Pure error	0.0018	4	0.0005			2.52	4	0.6304		
Cor Total	34.64	16				14783.91	16			

Table 4
Parameters for TPP optimization

Parameters	*Goal*	*Lower limit*	*Upper limit*
$(NH_4)_2SO_4$ ***concentration***	In range	40	60
CE to tert-butanol ratio	In range	0.5	1.0
pH	In range	5	7
Purification fold	Maximize	5.032	9.242
Activity recovery	Maximize	76.2189	161.385

Model verification

Additional three experimental runs were carried out to verify the adequacy of model regression equations. The summary of the findings that were obtained from the confirmatory testing is shown in Table 5.

The observed mean values of purification fold and recovery activity were close to the predicted values, which supported the verification of regression model for the prediction of the optimal values. Hence, the latex protease was purified by TPP method under these optimum conditions.

Overall recovery profile of papain

Table 6 presents the overall purification profile of TPP purified papain. The highest recovery achieved was 159.806% with a purification fold of 9.242. In contrast, Hafid et al. (2020) reported a lower recovery of 134% but a higher purification fold of 11.45 using TPP for papain purification from *C. papaya* latex in single factor experiments. This difference could be attributed to variations in plant species and fruit maturity stage (Gul et al. (2021).

Table 5
Predicted and observed value of responses

Response	*Optimal conditions*			*Mean value*	
	$(NH_4)_2SO_4$ concentration	**CE to butanol ratio**	**pH**	**Predicted**	**Observed**
Purification fold	51.08%	1:0.78	6.05	9.274	9.242
Activity recovery	51.08%	1:0.78	6.05	160.967	159.806

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Table 6
Overall purification profile of papain

Portions	*Total activity (U)*	*Total protein (mg)*	*Specific activity (U/mg)*	*Purification fold*	*Activity recovery (%)*
Crude	325.13	6.47	50.25	1	100
IP	267.90	3.83	69.95	1.39	82.39
AP	519.58	1.119	464.325	9.242	159.806

Table 7
Comparison of various papain extraction and purification methods from different papaya plant parts

Plant source	*Methods*	*Purification fold*	*Activity recovery (%)*	*References*
Unripe green papaya fruits	Reversed phase expanded bed adsorption chromatography	7.04	74.98	He et al. (2014)
Dried Papaya peel	Two-step TPP	15.8	253.5	Chaiwut et al. (2010)
		10.1	89.4	
Papaya leaves	Three step purification using 70% Ammonium sulfate, DEAE-Cellulose column chromatography and Sephadex G-25 column chromatography	1.64	9.78	Babalola et al. (2023)
Latex	Aqueous two-phase system		88	Nitsawang et al. (2006)
	Two step salt precipitation		49	
	Ion exchange chromatography		10.7	
	Fractionation using 60% ammonium sulfate and ion exchange chromatography		6.3	Purwanto (2016)
	Two-phase affinity extraction using Alginate as affinity macro-ligand	2.41	72	Rocha et al. (2016)
	TPP (by single factor experiments)	11.45	134	Hafid et al. (2020)
	TPP (by RSM)	9.242	159.806	Present study

Different methods of papain extraction from different parts of papaya plant are compared with the TPP method in Table 7. The TPP method demonstrated maximum recovery and purification fold, attributed to a synergistic interaction between salt concentration and solvent volume under ideal purification conditions. This interaction may reduce interfering enzymes during pre-dialysis and activate enzymes during ammonium sulfate precipitation (Rajagopalan & Sukumaran, 2018). Comparable results were found in previous TPP research on plant proteases by Chaiwut et al. (2010), Özer et al. (2010), Rawdkuen et al. (2010), Duman & Kaya (2013), Gagaoua et al. (2015), Gagaoua et al. (2017), Hafid et al. (2020), Gul et al. (2022), and Maskey & Karki (2023).

Characterization of TPP purified papain

Impact of temperature on the activity and stability

Figure 3 shows the impact of temperature (30-80°C) on the activity and stability of partitioned papain. The papain showed significant ($p<0.05$) activity across a broad temperature range, where activity peaking at 60°C, consistent with previous research by Sugiura & Sasaki (1974), Priolo et al. (2000) and Gagaoua et al. (2014). Over a 2-h period, papain exhibited stability, retaining about 85% activity at 60°C but declining above 70°C. This aligns with findings by Hashim et al. (2011), that enzyme activity initially rises with temperature but declines after reaching peak activity. Cysteine proteases typically operate optimally between 40 and 60°C (Dubey & Jagannadham, 2003; Gagaoua et al., 2014).

Impact of pH on the activity and stability

Figure 4 presents the effect of pH (4.0-9.0) on papain activity and stability. Papain activity increased from pH 4.0 to 6.0, peaking significantly ($p<0.05$) at pH 6.0. This pH profile aligns with previous research by Purwanto (2016) who used ion exchange chromatography to purify papain. The enzyme retained over 60% activity across a broad pH range, from 5.0 to 8.0. This optimal pH range of 5 to 8 aligns with findings from Blumberg et al. (1970), Pendzhiev (2002) and Hafid et al. (2020). After a 2-h incubation, papain demonstrated significant stability ($p<0.05$) across a wide pH range, with its peak stability observed at pH 7.

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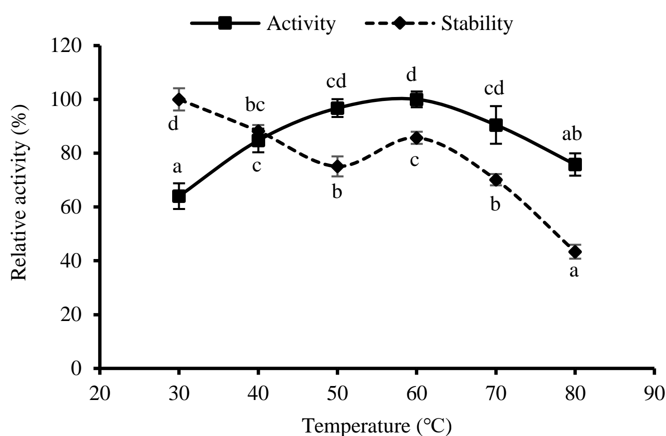

Figure 3
Effect of temperature on the activity and stability of purified papain

Note: Values bearing similar superscript are not significantly different at 5% level of significance. Data represents mean ± standard deviation.

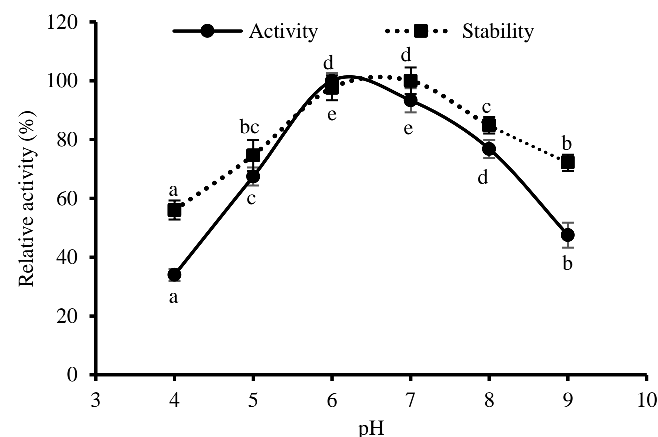

Figure 4
Effect of pH on the activity and stability of purified papain

Note: Values bearing similar superscript are not significantly different at 5% level of significance. Data represents mean ± standard deviation.

Storage stability

Figure 5 shows the storage stability of purified papain at refrigerated and frozen conditions. The activity of recovered papain was preserved better at lower temperatures, retaining 100% for the first 9 days and losing only 10.71% after 21 days at frozen condition (-20°C). At refrigerated condition (4°C), 64.11% activity was retained for the first 9 days, decreasing to 91% after 21 days. This decrease in enzyme activity over time may result from molecular rearrangements, protein-protein interactions, or degradation (Gagaoua et al., 2015). Autolysis, influenced by enzyme concentration, pH, incubation duration, temperature, and activator presence, is common in proteases. This MCA profile resembles that of cucumisin (Gagaoua et al., 2017).

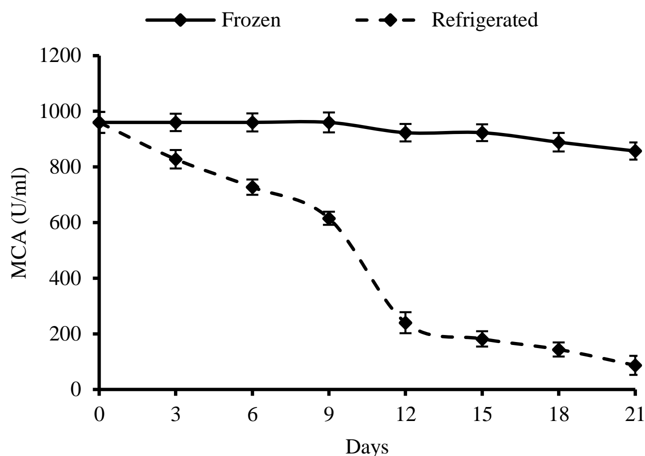

Figure 5
Effect of storage temperature on the activity of purified papain

Conclusions

TPP is an efficient process for purifying papain from papaya latex when compared to chromatographic and other aqueous system approaches. The papain was recovered in the aqueous phase using an optimized one-step TPP system to 9.242 purification fold and 159.806% activity recovery. The optimum conditions obtained using RSM were 51.08% $(NH_4)_2SO_4$, 1.0:0.78 CE to tert-butanol ratio and pH 6.05. The characterization of the purified papain revealed enhanced activity and stability over a wide temperature and pH range. Hence, TPP could be a promising method to purify papain from latex, especially for the food industry. However, more research, especially in cheese preparation, is needed for practical viability in dairy industries.

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Compliance with Ethical Standards

Conflict of Interest

The authors declare no conflict of interest.

Ethical approval

The work did not involve any animal study.

References

Babalola, B. A., Akinwande, A. I., Gboyega, A. E., & Otunba, A. A. (2023). Extraction, purification and characterization of papain cysteine-proteases from the leaves of *Carica papaya*. *Scientific African*, *19*, e01538. https://doi.org/10.1016/j.sciaf.2022.e01538

Baines, B. S., & Brocklehurst, K. (1979). A necessary modification to the preparation of papain from any high-quality latex of *Carica papaya* and evidence for the structural integrity of the enzyme produced by traditional

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methods. *Biochemical Journal*, *177*(2), 541-548. https://doi.org/10.1042/bj1770541

Berridge, N. (1952). 488. An improved method of observing the clotting of milk containing rennin. *Journal of Dairy Research*, *19*(3), 328-329.

Blumberg, S., Schechter, I., & Berger, A. (1970). The purification of papain by affinity chromatography. *European Journal of Biochemistry*, *15*(1), 97-102. https://doi.org/10.1111/j.1432-1033.1970.tb00981.x

Box, G. H., W.; Hunter, J (1978). *Statistics for experimenters: An introduction to design, data Analysis, and model building*. Wiley and Sons.

Bradford, M. M. (1976). 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. https://doi.org/10.1016/0003-2697(76)90527-3

Braia, M., Ferrero, M., Rocha, M. V., Loureiro, D., Tubio, G., & Romanini, D. (2013). Bioseparation of papain from *Carica papaya* latex by precipitation of papain–poly (vinyl sulfonate) complexes. *Protein Expression and Purification*, *91*(1), 91-95. https://doi.org/10.1016/j.pep.2013.07.008

Chaiwut, P., Pintathong, P., & Rawdkuen, S. (2010). Extraction and three-phase partitioning behavior of proteases from papaya peels. *Process Biochemistry*, *45*(7), 1172-1175. https://doi.org/10.1016/j.procbio.2010.03.019

Chen, W., Jia, Z., Huang, G., & Hong, Y. (2020). Global optimization for simultaneous extraction of oil and polysaccharides from *Schizochytrium limacinum* by enzyme-assisted three-phase partitioning. *Journal of Food Processing and Preservation*, *44*(10), e14824. https://doi.org/10.1111/jfpp.14824

Chew, K. W., Ling, T. C., & Show, P. L. (2019). Recent developments and applications of three-phase partitioning for the recovery of proteins. *Separation & Purification Reviews*, *48*(1), 52-64. https://doi.org/10.1080/15422119.2018.1427596

Cochran, W. G. C., G.M. (1992). *Experimental Designs* (2nd ed.). Wiley Classics Library.

Cohen, L. W., Coghlan, V. M., & Dihel, L. C. (1986). Cloning and sequencing of papain-encoding cDNA. *Gene*, *48*(2-3), 219-227. https://doi.org/10.1016/0378-1119(86)90080-6

Dennison, C., & Lovrien, R. (1997). Three phase partitioning: concentration and purification of proteins. *Protein Expression and Purification*, *11*(2), 149-161. https://doi.org/10.1006/prep.1997.0779

Dhananjay, S., & Mulimani, V. (2008). Purification of α-galactosidase and invertase by three-phase partitioning from crude extract of *Aspergillus oryzae*. *Biotechnology Letters*, *30*, 1565-1569. https://doi.org/10.1007/s10529-008-9722-5

Dubey, V. K., & Jagannadham, M. (2003). Procerain, a stable cysteine protease from the latex of *Calotropis procera*. *Phytochemistry*, *62*(7), 1057-1071. https://doi.org/10.1016/S0031-9422(02)00676-3

Duman, Y. A., & Kaya, E. (2013). Three-phase partitioning as a rapid and easy method for the purification and recovery of catalase from sweet potato tubers (Solanum tuberosum). *Applied Biochemistry and Biotechnology*, *170*, 1119-1126. https://doi.org/10.1007/s12010-013-0260-9

Feijoo-Siota, L., & Villa, T. G. (2011). Native and biotechnologically engineered plant proteases with industrial applications. *Food and Bioprocess Technology*, *4*, 1066-1088. https://doi.org/10.1007/s11947-010-0431-4

Gagaoua, M. (2018). Aqueous methods for extraction/recovery of macromolecules from microorganisms of a typical environments: A focus on three phase partitioning. In *Methods in Microbiology* (Vol. 45, pp. 203-242). Elsevier.

Gagaoua, M., Boucherba, N., Bouanane-Darenfed, A., Ziane, F., Nait-Rabah, S., Hafid, K., & Boudechicha, H.-R. (2014). Three-phase partitioning as an efficient method for the purification and recovery of ficin from Mediterranean fig (*Ficus carica* L.) latex. *Separation and Purification Technology*, *132*, 461-467. https://doi.org/10.1016/j.seppur.2014.05.050

Gagaoua, M., & Hafid, K. (2016). Three phase partitioning system, an emerging non-chromatographic tool for proteolytic enzymes recovery and purification. *Biosensors Journal*, *5*(1), 100134. https://doi.org/10.4172/2090-4967.1000134

Gagaoua, M., Hafid, K., & Hoggas, N. (2016). Data in support of three phase partitioning of zingibain, a milk-clotting enzyme from *Zingiber officinale* Roscoe rhizomes. *Data in Brief*, *6*, 634-639. https://doi.org/10.1016/j.dib.2016.01.014

Gagaoua, M., Hoggas, N., & Hafid, K. (2015). Three phase partitioning of zingibain, a milk-clotting enzyme from *Zingiber officinale* Roscoe rhizomes. *International Journal of Biological Macromolecules*, *73*, 245-252. https://doi.org/10.1016/j.ijbiomac.2014.10.069

Gagaoua, M., Ziane, F., Rabah, S. N., Boucherba, N., El, A. A. K. E.-H., Bouanane-Darenfed, A., & Hafid, K. (2.). Three phase partitioning, a scalable method for the purification and recovery of cucumisin, a milk-clotting enzyme, from the juice of *Cucumis melo* var. reticulatus. *International Journal of Biological Macromolecules*, *102*, 515-525. https://doi.org/10.1016/j.ijbiomac.2017.04.060

Gokhale, D., Patil, S., & Bastawde, K. (1991). Optimization of cellulase production by *Aspergillus niger* NCIM 1207. *Applied Biochemistry and Biotechnology*, *30*, 99-109. https://doi.org/10.1007/BF02922026

Gul, A., Khan, S., Arain, H., Khan, H., Ishrat, U., & Siddiqui, M. (2022). Three-phase partitioning as an efficient one-step method for the extraction and purification of bromelain from pineapple crown waste. *Journal of Food Processing and Preservation*, *46*(11815 & 157), e16973. https://doi.org/10.1111/jfpp.16973

Gul, A., Siddiqui, M., Arain, H., Khan, S., Khan, H., & Ishrat, U. (2021). Extraction, partial purification and characterization of bromelain from pineapple (*Ananas comosus*) crown, core and peel waste. *Brazilian Archives of Biology and Technology*, *64*. https://doi.org/10.1590/1678-4324-2021200639

Gupta, R., Beg, Q., Khan, S., & Chauhan, B. (2002). An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Applied Microbiology and Biotechnology*, *60*, 381-395. https://doi.org/10.1007/s00253-002-1142-1

Haaland, P. D. (1989). *Experimental Design in Biotechnology*. Marcel Dekker.

Hafid, K., John, J., Sayah, T. M., Domínguez, R., Becila, S., Lamri, M., Dib, A. L., Lorenzo, J. M. & Gagaoua, M. (2020). One-step recovery of latex papain from *Carica papaya* using three phase partitioning and its use as milk-clotting and meat-tenderizing agent. *International Journal of Biological Macromolecules*, *146*, 798-810. https://doi.org/10.1016/j.ijbiomac.2019.10.048

Hashim, M. M., Mingsheng, D., Iqbal, M. F., & Xiaohong, C. (2011). Ginger rhizome as a potential source of milk coagulating cysteine protease. *Phytochemistry*, *72*(6), 458-464. https://doi.org/10.1016/j.phytochem.2010.12.002

He, Y., bin Tuan Chik, S. M. S., & Chong, F. C. (2014). Purification of papain from unclarified papaya juice using reversed phase expanded bed adsorption chromatography (RP-EBAC). *Journal of Industrial and Engineering Chemistry*, *20*(6), 4293-4297. https://doi.org/10.1016/j.jiec.2014.01.035

Hejazin, R. K., & El-Qudah, M. (2009). Effect of proteases on meltability and stretchability of Nabulsi cheese. *American Journal of Agricultural and Biological Sciences*, *4*(3), 173-178. https://doi.org/10.3844/ajabssp.2009.173.178

Huet, J., Looze, Y., Bartik, K., Raussens, V., Wintjens, R., & Boussard, P. (2006). Structural characterization of the papaya cysteine proteinases at low

© 2023 CDFT. All rights reserved.

pH. *Biochemical and Biophysical Research Communications*, *341*(2), 620-626. https://doi.org/10.1016/j.bbrc.2005.12.210

IDF. (2007). *Milk-Determination of total milk-clotting activity of bovine rennets*. International Dairy Federation, Brussels.

Khanna, N., & Panda, P. (2007). Effect of papain on tenderization and functional properties of spent hen meat cuts. *Indian Journal of Animal Research*, *41*(1), 55-58.

Kiss, E., Szamos, J., Tamas, B., & Borbas, R. (1998). Interfacial behavior of proteins in three-phase partitioning using salt-containing water/tert-butanol systems. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *142*(2-3), 295-302. https://doi.org/10.1016/S0927-7757(98)00361-6

Kumar, V. V., Sathyaselvabala, V., Kirupha, S. D., Murugesan, A., Vidyadevi, T., & Sivanesan, S. (2011). Application of response surface methodology to optimize three phase partitioning for purification of laccase from *Pleurotus ostreatus*. *Separation Science and Technology*, *46*(12), 1922-1930. https://doi.org/10.1080/01496395.2011.583306

Ladd, J., & Butler, J. (1972). Short-term assays of soil proteolytic enzyme activities using proteins and dipeptide derivatives as substrates. *Soil Biology and Biochemistry*, *4*(1), 19-30. https://doi.org/10.1016/0038-0717(72)90038-7

Mamboya, E. A. F., & Amri, E. (2012). Papain, a plant enzyme of biological importance: A review. *American Journal of Biochemistry & Biotechnology*, *8*(2), 99-104. https://doi.org/10.3844/ajbbsp.2012.99.104

Maskey, B., & Karki, D. B. (2024). Efficient three phase partitioning of actinidin from kiwifruit (*Actinidia deliciosa*) and its characterization. *Preparative Biochemistry & Biotechnology*, 54(1), 95-102. https://doi.org/10.1080/10826068.2023.2209877

Maskey, B., Karki, D. B., & Shrestha, N. K. (2023). Optimization of three-phase partitioning system for the purification of protease from *Calotropis gigantea* latex using response surface methodology. *Chemical Papers*, 1-11. https://doi.org/10.1007/s11696-023-03259-4

Maskey, B., & Shrestha, N. K. (2020). Optimization of crude papaya (*Carica papaya*) protease in soft-unripened cheese preparation. *Journal of Food Science and Technology Nepal*, *12*(12), 1-8.

Mitchel, R. E., Chaiken, I. M., & Smith, E. L. (1970). The complete amino acid sequence of papain: additions and corrections. *Journal of Biological Chemistry*, *245*(14), 3485-3492. https://doi.org/10.1016/S0021-9258(18)62954-0

Najafpour, G. (2007). Downstream processing. In *Biochemical Engineering and Biotechnology* (1st ed., pp. 170-198). Elsevier Science.

Nitsawang, S., Hatti-Kaul, R., & Kanasawud, P. (2006). Purification of papain from *Carica papaya* latex: Aqueous two-phase extraction versus two-step salt precipitation. *Enzyme and Microbial Technology*, *39*(5), 1103-1107. https://doi.org/10.1016/j.enzmictec.2006.02.013

Özer, B., Akardere, E., Çelem, E. B., & Önal, S. (2010). Three-phase partitioning as a rapid and efficient method for purification of invertase from tomato. *Biochemical Engineering Journal*, *50*(3), 110-115. https://doi.org/10.1016/j.bej.2010.04.002

Pendzhiev, A. (2002). Proteolytic enzymes of papaya: medicinal applications. *Pharmaceutical Chemistry Journal*, *36*(6), 315-317. https://doi.org/10.1023/A:1020832807958

Pike, R., & Dennison, C. (1989). Protein fractionation by three phase partitioning (TPP) in aqueous/t-butanol mixtures. *Biotechnology and Bioengineering*, *33*(2), 221-228. https://doi.org/10.1002/bit.260330213

Prasetyo, H., Kartawiria, I. S., & Marpaung, A. M. (2015, November 25-26). *Optimization of papain enzyme utilization in non-rennet cream cheese production*. Proceedings of the ICONIET Conference, BSD City, Indonesia.

Priolo, N., Del Valle, S. M., Arribére, M. C., López, L., & Caffini, N. (2000). Isolation and characterization of a cysteine protease from the latex of *Araujia hortorum* fruits. *Journal of Protein Chemistry*, *19*, 39-49. https://doi.org/10.1023/A:1007042825783

Purwanto, M. G. M. (2016). The role and efficiency of ammonium sulphate precipitation in purification process of papain crude extract. *Procedia Chemistry*, *18*, 127-131. https://doi.org/10.1016/j.proche.2016.01.020

Rajagopalan, A., & Sukumaran, B. O. (2018). Three phase partitioning to concentrate milk clotting proteases from *Wrightia tinctoria* R. Br and its characterization. *International journal of biological macromolecules*, *118*, 279-288. https://doi.org/10.1016/j.ijbiomac.2018.06.042

Ramos, O. S., & Malcata, F. X. (2011). Food-Grade Enzymes. In M. Moo-Young (Ed.), *Comprehensive Biotechnology* (2nd ed., Vol.3, pp. 555-569). Academic Press.

Rana, M. S., Hoque, M. R., Rahman, M. O., Habib, R., & Siddiki, M. S. R. (2). Papaya (*Carica papaya*) latex-an alternative to rennet for cottage cheese preparation. *Journal of Advanced Veterinary and Animal Research*, *4*(3), 249-254. https://doi.org/10.5455/javar.2017.d218

Rawdkuen, S., Chaiwut, P., Pintathong, P., & Benjakul, S. (2010). Three-phase partitioning of protease from *Calotropis procera* latex. *Biochemical Engineering Journal*, *50*(3), 145-149. https://doi.org/10.1016/j.bej.2010.04.007

Rocha, M. V., Di Giacomo, M., Beltramino, S., Loh, W., Romanini, D., & Nerli, B. B. (2016). A sustainable affinity partitioning process to recover papain from *Carica papaya* latex using alginate as macro-ligand. *Separation and Purification Technology*, *168*, 168-176. https://doi.org/10.1016/j.seppur.2016.05.025

Sharma, A., & Gupta, M. N. (2004). Oil extraction from almond, apricot and rice bran by three-phase partitioning after ultrasonication. *European Journal of Lipid Science and Technology*, *106*(3), 183-186. https://doi.org/10.1002/ejlt.200300897

Sugiura, M., & Sasaki, M. (1974). Studies on proteinases from *Ficus carica* var. Hōraishi. V. Purification and properties of a sugar-containing proteinase (Ficin S). *Biochimica et Biophysica Acta (BBA)-Enzymology*, *350*(1), 38-47. https://doi.org/10.1016/0005-2744(74)90200-9

Troncoso David, F., Alberto Sánchez, D., & Luján Ferreira, M. (2022). Production of plant proteases and new biotechnological applications: an updated review. *ChemistryOpen*, *11*(3), e202200017. https://doi.org/10.1002/open.202200017

Tschesche, H. (2011). *Methods in Protein Biochemistry* (1st ed.). Walter de Gruyter.

Yan, J.-K., Wang, Y.-Y., Qiu, W.-Y., Ma, H., Wang, Z.-B., & Wu, J.-Y. (2018). Three-phase partitioning as an elegant and versatile platform applied to nonchromatographic bioseparation processes. *Critical Reviews in Food Science and Nutrition*, *58*(14), 2416-2431. https://doi.org/10.1080/10408398.2017.1327418

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