

Extraction, purification and characterization of papain enzyme from papaya

¹Khatun, M.N., ²Saeid, A., ³Mozumder, N.H.M.R. and ^{4,*}Ahmed, M.

¹Department of Food Technology, Chapainawabganj Polytechnic Institute, Chapainawabganj, Bangladesh

²Department of Nutrition and Food Engineering, Daffodil International University, Daffodil Smart City (DSC), Birulia, Savar, Dhaka-1216, Bangladesh

³Department of Food Science and Nutrition, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh

⁴Department of Food Processing and Preservation, Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh

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Abstract

Papain is one of the widely used enzymes in the industry. This study aimed to extract papain enzyme from various parts (i.e. latex, leaves, peels and seeds) of papaya using distilled water and citric phosphate buffer and subsequently purified with a two-step salt precipitation method. The optimum protein concentration of purified papain was obtained with citric phosphate buffer (0.159-0.319 mg/mL) and distilled water (0.119-0.347 mg/mL). The protein concentration was almost similar for crude extracted with both the solvent. The purification procedure enhanced up to 1.56 fold when extracted with distilled water and 0.83 fold when extracted with citric phosphate buffer. Latex, leaves, peels and seeds showed maximum enzyme activity at 60°C and pH 8.0 as well as better thermal stability at 60°C for 30 mins. Findings highlighted that extracted papain enzyme from waste parts of papaya could be utilized as a potential source of industrial application in terms of waste to value-added products.

1. Introduction

The papaya (*Carica papaya*) is one of the most popular and widely grown fruit crops in Bangladesh as well as in tropical and subtropical countries including India, Thailand and Central America and so on. It belongs to the family Caricaceae and Genus Carica (Anuar, 2008). According to Food and Agricultural Organization, about 13.58 million tons of fruit were produced worldwide and in Bangladesh 0.14 million tons (FAO, 2019). In Bangladesh, Rajshahi is well-known for the growth and fruiting of papaya (Zaman *et al.*, 2006). The pulp of the fruit is the edible portions whereas skin, seeds and peel are used as waste materials (Zaman *et al.*, 2006). Each part of the papaya has a rich source of nutritional value such as protein, fat, fibre, carbohydrates, minerals, vitamin C, thiamine, riboflavin, niacin, carotene and amino acids (Rehman *et al.*, 2003; Krishna *et al.*, 2008). Papaya is commonly consumed as a dessert or processed food in the form of jam, puree, or wine and green fruits are used as cooked vegetables (Ahmed *et al.*, 2002; Matsuura *et al.*, 2004).

The seeds, skin, leaves, latex and fruit of the papaya

contain several enzymes. Among them, the most important enzyme is papain and chymopapain (Aravind *et al.*, 2013). Papain is a natural enzyme and is industrially used as a source of proteolytic enzymes. Usually, crude papain is produced from papaya latex, leaves, seed and the skin of the fruit (Chaiwut *et al.*, 2007), while latex provides the maximum yield. Papain is a cysteine protease enzyme that consists of a single polypeptide chain with three disulfide bridges and a sulfhydryl group and it is necessary for the activity of the enzyme usually called vegetable pepsin (Bakar, 2010). Papain has a molecular weight of 23000Da (Ming *et al.*, 2002), optimal pH: of 3.0-9.0, and stability: 1 year at -20°C (Edwin and Jagannadham, 2000; Ghosh, 2005). Papain is widely used as a meat tenderizer (Krishna *et al.*, 2008), beer clarification (Margarita *et al.*, 2011), and preparation of protein hydrolysates (Monti *et al.*, 2000). It is also used for pharmaceutical purposes and assists in protein digestion in chronic dyspepsia, gastric, gastritis, removal of necrotic tissue and fermentable preparation of tyrosine derivatives for the treatment of parkinsonism, skin cleansing agents, acne treatment, preparation of tetanus vaccines and also used in dairy, textile,

*Corresponding author.

Email: anandhstu@yahoo.com

photographic, optical, tanning, cosmetic, detergents, food and leather industry (Parle, 2011).

Various methods for the extraction of enzymes can be used such as subcritical water extraction, microwave-assisted extraction method, soxhlet extraction, supercritical fluid extraction, extraction with buffer solution, pressurized solvent extraction and hot water extraction (Bakar, 2010). Previous works have used ethanol (Bianca *et al.*, 2014) and normal distilled water (Bakar, 2010) for extraction and also used ammonium sulphate (Saxena *et al.*, 2007) for the purification of protein. There are several methods of enzyme purification including the precipitation method, chromatographic methods including ion exchange, and covalent, or affinity chromatography. The best and most commercially important separation method is aqueous two-phase extraction for the purification of papain due to simple equipment requirements and low energy needs (Krishnan *et al.*, 2005). Several types of electrophoresis such as SDS-electrophoresis, PAGE-electrophoresis are also available for the purification of proteins (Monti *et al.*, 2000).

Generally, papaya peels and seeds are discarded from homes, restaurants and industries and leaves are also discarded from a plant. An accumulation of these wastes can become an environmental problem. Therefore, it is our interest to transform this waste into a valuable product like latex proteases which is commonly acceptable for many industrial uses. Therefore, in point of economic and technical aspects, it is necessary to find out the optimum extraction and purification process. To our knowledge, there is no information available about the extraction and purification of papain from leaves, latex, seeds and peel using the ammonium sulphate precipitation method. Therefore, the objective of the study was to extract papain enzyme from latex, leaves, peels and seeds from papaya and fix an optimal extraction process as well as to characterize the purified enzyme based on time, temperature and pH.

2. Materials and methods

2.1 Materials

Standard purified papain was purchased from Sigma (USA). Tetramethylethylenediamide (TEMED) was purchased from the local market. A molecular weight marker was purchased Bio-Rad (USA). Others reagents were analytical grade.

2.2 Samples collection and sample preparation

Full mature papaya (*Carica papaya*) fruits were collected from papaya orchards at Basherhat in Dinajpur, Bangladesh. Firstly papaya fruits and leaves were

washed with water to remove dirt and soil. Then papaya fruits were peeled and cut with a stainless still knife and seeds were collected manually. Subsequently, peels and leaves were cut into small pieces by a knife. On the other hand, latex was obtained by several longitudinal incisions with a blade on the unripe fruits and poured into plastic containers. Then seeds, leaves and peels were dried at 55°C in a cabinet dryer until the final moisture content reached below 10% and latex was concentrated in a cabinet dryer at 55°C. After that seeds, peels and leaves were ground in a blender and sieved through 60-mesh and stored at 4°C.

2.3 Papain extraction from seeds, peels, leaves and latex using distilled water and citric phosphate buffer

The papain extraction method was followed according to Chaiwut *et al.* (2007) with some modifications. Approximately 10 g of the dried samples were soaked in 190 mL of distilled water and 190 mL of 0.1 M citric phosphate buffer (pH 6.5) separately for 10 min containing 5 mM ethylenediaminetetraacetic acid (EDTA) and cysteine to prevent enzymatic activity such as polyphenol oxidase activity and to reduce oxidation air, respectively and then filtered. The filtrate was centrifuged at 4000 rpm at 4°C for 1 hr and supernatant was collected and finally stored at -8°C for further use.

2.4 Purification of papain

Papain was purified according to the method reported by Nitsawang *et al.* (2006). All samples were mixed with 40 mM cysteine at a ratio of 3:1 (w/v) and adjusted to pH 5.6 using 6M HCl and then stirred for 15 mins at 4°C. The mixture was filtered and pH was adjusted to 9.0 using 6M NaOH. Then, the filter was centrifuged at 4000 rpm for 30 mins at 4°C. After that, a few amounts of supernatant were used for protein determination and the remaining supernatant was precipitated with 45% saturated ammonium sulfate. Then, the precipitate was collected and mixed with 2 mL of 20 mM cysteine and 10% sodium chloride was added and stirred for 30 mins at 4°C. Then the solution was again centrifuged at 4000 rpm for 30 mins at 4°C. After that, the supernatant was removed and the precipitate was dissolved with water and citric buffer separately and then the solution was poured into a dialysis tube. Then the dialysis tube was soaked in distilled water and kept overnight at 4°C. Distilled water was changed subsequently over 12 hrs.

2.5 Determination of protein content

Protein content in the samples was determined by the Bradford method (Bradford MM, 1976) using bovine serum albumin (BSA) as standard.

2.6 Proteolytic activity assays

Proteolytic activity was determined according to Liggieri *et al.* (2009) with slight modification. A purified enzyme (0.1 mL) was mixed with 1.1 mL of 1% casein containing 12 mM cysteine in a 0.1 M Tris–HCl buffer (pH 8.5). Then incubated at 42°C for 2 mins and 1.8 mL of 5% trichloroacetic acid (TCA) was then added to stop the reaction. After that centrifuged at 3000 rpm for 20 mins and the absorbance of the supernatant was measured at 280 nm. The enzyme unit (Ucas) was defined as the amount of enzyme (g) that produces an increase of one absorbance unit per minute in the assay conditions (Hullikere *et al.*, 2014).

2.7 Characterization of papain enzyme

2.7.1 pH stability of papain enzyme

The reaction mixture was prepared by mixing 0.1 mL of the purified enzyme with 1.1 mL of 1% casein containing 12 mM cysteine in a 0.1 M Tris–HCl buffer (pH 6, pH 8 and pH 10) separately. The reaction was carried out at room temperature. Then incubated at 42°C and stopped the reaction after 2 mins with the addition of 1.8 mL of 5% trichloroacetic acid (TCA). Each test tube was centrifuged at 3000 rpm for 20 mins and the absorbance of the supernatant was measured at 280 nm. The enzyme unit (Ucas) was defined as the amount of enzyme (g) that produces an increase of one absorbance unit per minute in the assay conditions.

2.7.2 Thermal stability of papain enzyme

The purified papain (0.1mL) was first incubated at 50°C, 60°C and 70°C for 30, 60 and 90 mins respectively. Then the reaction mixture was prepared by mixing with 1.1 mL of 1% casein containing 12 mM cysteine in a 0.1 M Tris–HCl buffer (pH 8.5). The reaction was carried out at room temperature. Then incubate at 42°C and the reaction was stopped 2 mins later by the addition of 1.8 mL of 5% trichloroacetic acid (TCA). Each test tube was centrifuged at 3000 rpm for 20 mins and the absorbance of the supernatant was measured at 280 nm. The enzyme unit (Ucas) was defined as the amount of enzyme (g) that produces an increase of one absorbance unit per minute in the assay conditions.

2.8 Statistical analysis

All measurements were carried out in triplicate for each of the samples. Results are expressed as mean values with standard deviation. Data were statistically analyzed using MSTAT-C windows version 2.10. Duncan's multiple range test (DMRT) was performed to evaluate the significance of the difference between mean values at the level of $p \leq 0.05$.

3. Results and discussion

3.1 Purification and enzymatic activity of papain

The protein concentration of crude extracts latex ranged from 0.167-0.446 mg/mL (Table 1). The quantification results were consistent with the findings of Sebastian *et al.* (2004) who found a protein content of 0.276 mg/mL from the latex of *Asclepain fruticosa* fruit. The measured value was lower than the latex of *Araujia hortorum* fruits reported as 5.4 mg/mL (Priolo *et al.*, 2000). The protein concentration of purified papain ranged from 0.119-0.347 mg/mL. This finding was higher than the latex of *Asclepias curassavica L* fruits reported as 0.0075-0.025 mg/mL (Liggieri *et al.*, 2009). A similar protein concentration (0.07-0.2 mg/mL) was found in the latex of *Araujia hortorum* fruits (Obregon *et al.*, 2001). The protein concentration of papain extracted with citric phosphate buffer and distilled water ranged from 0.159-0.319 mg/mL and 0.119-0.347 mg/mL, respectively. This protein concentration was much higher than the latex of *Araujia hortorum* fruits (0.07-0.2 mg/mL) reported by Obregon *et al.* (2001). This variation may be due to the difference in source material used to extract enzymes. The highest protein concentration was found in latex (0.349±0.003 mg/mL) and lowest in seed (0.119±0.005 mg/mL) extracted with distilled water. This variation might be the differences in protease composition among leaves, seeds, latex and peels of papaya.

The specific activity of crude extract and purified papain ranged from 0.013-0.058 U/mg and 0.001-0.023 U/mg, respectively. A relatively higher specific activity (77.9 U/mg) was reported by Priolo *et al.* (2000) in the latex of *Araujia hortorum* fruit. The specific activity of papain extracted with distilled water was (0.001-0.012 U/mg) and with citric phosphate buffer (0.001-0.023 U/mg), respectively. The highest specific activity of papain enzyme was found in seed (0.023 U/mg) extracted with distilled water and lowest in peel (0.001 U/mg) extracted with both distilled water and citric phosphate buffer.

From Table 2 and Table 3, the value for the purification fold was found in the range of 0.032-1.565. The highest purification fold was obtained from seeds (1.565±0.832) extracted with distilled water. A relatively consistent purification fold was found by Rabelo *et al.* (2004) who purified bromelain using aqueous two-phase systems and obtained a purification of 1.25-fold. The lowest purification fold was found in peel (0.032±0.018) extracted with distilled water.

The per cent yield ranged from 1.95-46.66. The highest yield was obtained in leaves (46.66±17.04) and the lowest yield was found in peels (1.45±0.78 %) which were extracted with citric phosphate buffer. Our results

Table 1. Protein content of different samples extracted with distilled water and citric phosphate buffer

Samples	Distilled water						Citric phosphate buffer					
	Total volume (mL)		Protein (mg/mL)		Total protein (mg)		Total volume (mL)		Protein (mg/mL)		Total Protein (mg)	
	C.E	P.P	C.E	P.P	C.E	P.P	C.E	P.P	C.E	P.P	C.E	P.P
Leaves	90	5.5	0.179±0.029 ^{Ac}	0.188±0.003 ^{Ac}	16.08±2.568 ^{Ac}	1.034±0.015 ^{Ab}	90	6.0	0.178±0.007 ^{Ab}	0.159±0.004 ^{Bd}	16.077±0.653 ^{Ac}	0.958±0.022 ^{Bb}
Seeds	90	2.0	0.450±0.022 ^{Aa}	0.119±0.005 ^{Bd}	40.53±1.935 ^{Aa}	0.237±0.009 ^{Bd}	90	3.5	0.438±0.056 ^{Aa}	0.211±0.007 ^{Ab}	39.48±5.079 ^{Aa}	0.74±0.027 ^{Ac}
Peels	70	4.0	0.371±0.165 ^{Bb}	0.228±0.008 ^{Ab}	25.97±1.157 ^{Bb}	0.913±0.036 ^{Ac}	75	5.5	0.446±0.040 ^{Aa}	0.184±0.011 ^{Bc}	33.475±3.020 ^{Ab}	1.012±0.058 ^{Ab}
Latex	60	7.0	0.167±0.029 ^{Bd}	0.347±0.006 ^{Aa}	10.02±1.773 ^{Bd}	2.429±0.043 ^{Aa}	70	7.6	0.403±0.063 ^{Aa}	0.319±0.003 ^{Ba}	28.21±4.641 ^{Ab}	2.430±0.024 ^{Aa}

Values are presented as mean±SD of triplicate analysis. Values with different lowercase superscripts within the same column are significantly different among leaves, seeds, peels and latex. Values with different uppercase superscripts within the same row are significantly different among leaves seeds, peels and latex, between distilled water and citric phosphate buffer. C.E: Crude extract, P.P: Purified papain.

Table 2. Enzyme activity of different samples extracted with distilled water

Samples	Distilled water						Citric phosphate buffer					
	Enzyme activity (U/mL)		Total enzyme Activity (U)		Specific Activity (U/mg)		Purification fold		Yield (%)			
	C.E	P.P	C.E	P.P	C.E	P.P	C.E	P.P	C.E	P.P		
Leaves	0.007±0.002 ^{Ab}	0.001±0.002 ^{Ac}	0.63±0.208 ^{Ab}	0.004±0.001 ^{Bc}	0.037±0.001 ^{Ab}	0.004±0.001 ^{Bc}	1.0 ^{Aa}	0.117±0.014 ^{Ba}	100 ^{Aa}	12.5±2.16 ^{Bb}		
Seeds	0.007±0.001 ^{Ab}	0.003±0.001 ^{Ab}	0.6±0.051 ^{Ab}	0.005±0.002 ^{Bb}	0.015±0.001 ^{Ad}	0.023±0.011 ^{Aa}	1.0 ^{Aa}	1.565±0.832 ^{Aa}	100 ^{Aa}	41.27±21.99 ^{Aa}		
Peels	0.015±0.003 ^{Ba}	0.003±0.000 ^{Ac}	1.027±0.214 ^{Ba}	0.001±0.000 ^{Bd}	0.039±0.007 ^{Ba}	0.001±0.000 ^{Ad}	1.0 ^{Aa}	0.032±0.018 ^{Ba}	100 ^{Aa}	1.95±1.20 ^{Ab}		
Latex	0.004±0.003 ^{Bc}	0.003±0.002 ^{Aa}	0.26±0.173 ^{Bb}	0.030±0.010 ^{Aa}	0.028±0.020 ^{Bc}	0.013±0.004 ^{Ab}	1.0 ^{Aa}	1.298±1.79 ^{Aa}	100 ^{Aa}	22.60±17.91 ^{Ab}		

Values are presented as mean±SD of triplicate analysis. Values with different lowercase superscripts within the same column are significantly different among leaves, seeds, peels and latex. Values with different uppercase superscripts within the same row are significantly different among leaves seeds, peels and latex, between distilled water and citric phosphate buffer. C.E: Crude extract, P.P: Purified papain.

Table 3. Enzyme activity of different samples extracted with citric phosphate buffer

Samples	Citric Phosphate Buffer									
	Enzyme activity (U/mL)		Total enzyme Activity (U)		Specific Activity (U/mg)		Purification fold		Yield (%)	
	C.E	P.P	C.E	P.P	C.E	P.P	C.E	P.P	C.E	P.P
Leaves	0.005±0.00 ^{Ac}	0.001±0.000 ^{bAc}	0.48±0.226 ^{Ab}	0.007±0.000 ^{Ad}	0.030±0.015 ^{Bc}	0.007±0.000 ^{Ac}	1.0 ^{Aa}	0.281±0.118 ^{Aa}	100 ^{Aa}	46.66±17.04 ^{Aa}
Seeds	0.007±0.001 ^{Ac}	0.003±0.001 ^{Ab}	0.6±0.104 ^{Ab}	0.009±0.005 ^{Ab}	0.013±0.002 ^{Ad}	0.012±0.006 ^{Ba}	1.0 ^{Aa}	0.834±0.461 ^{Aa}	100 ^{Aa}	38.89±19.24 ^{Aa}
Peels	0.019±0.003 ^{Ab}	0.003±0.000 ^{Ac}	1.425±0.198 ^{Ab}	0.002±0.000 ^{Ac}	0.043±0.004 ^{Ab}	0.001±0.000 ^{Ad}	1.0 ^{Aa}	0.034±0.013 ^{Aa}	100 ^{Aa}	1.45±0.78 ^{Ab}
Latex	0.024±0.015 ^{Aa}	0.004±0.001 ^{Aa}	1.68±1.035 ^{Aa}	0.028±0.008 ^{Ba}	0.058±0.036 ^{Aa}	0.011±0.003 ^{Ab}	1.0 ^{Aa}	0.272±0.203 ^{Aa}	100 ^{Aa}	22.60±17.91 ^{Ab}

Values are presented as mean±SD of triplicate analysis. Values with different lowercase superscripts within the same column are significantly different among leaves, seeds, peels and latex. Values with different uppercase superscripts within the same row are significantly different among leaves seeds, peels and latex, between distilled water and citric phosphate buffer. C.E: Crude extract, P.P: Purified papain.

were contrasted with the earlier study of literature on L-Asparaginase which yields 85% in *Pseudomonas aeruginosa* (El-Bessoumy *et al.*, 2004).

3.2 Effect of pH on enzyme activity of purified papain extracted from leaves, seeds, peels and latex

Figure 1 shows the effect of pH on the purified papain enzyme from leaves, seeds, peels and latex. Enzyme activity was 0.001-0.007 U/mL for all samples. Higher enzyme activity was exhibited at pH 6.0 to 8.0 after that enzyme activity was decreased at pH 8.0 to 10. The highest enzyme activity was found at pH 8.0 for all samples. It was close to the observation of Walter *et al.* (2001) who obtained the highest activity at pH 8-9 but Hullikere *et al.* (2014) found the highest activity at pH 7. It was suggested that the enzymatic activity changes with changing pH levels due to changes in the ionization of the enzyme, the substrate, or the enzyme-substrate complex (Robinson, 2015)

It is also shown that the papain from the latex was more stable at pH 8.0 and 10.0 than that of leaves, seeds and peels. This result was different from Chaiwut *et al.* (2007) who reported the enzyme obtained from latex was less stable than the peels at pH 8.0-10.0. This may be due to compositional variations among peels, seeds, leaves and latex.

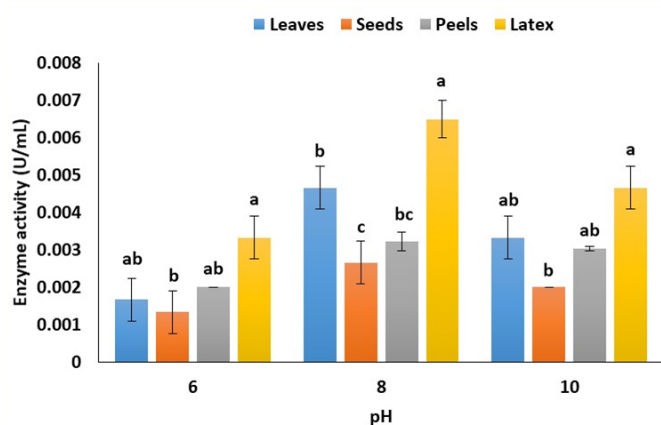


Figure 1. Effect of pH on enzyme activity of purified papain extracted from leaves, seeds, peels and latex. Bars with different notations are significantly different among leaves, seeds, peels and latex.

3.3 Effects of temperature on papain activity

The effect of the temperatures of purified papain on enzyme activity from leaves, seeds, peels and latex is shown in Figure 2. Maximum enzyme activity was obtained at 60°C. As the temperature increased, enzyme activity decreased for all samples. Priolo *et al.* (2000) suggested that a protective role played by the substrate would avoid self-digestion of the protease enzyme. A similar result was also found in Chaiwut *et al.* (2007) and Martins *et al.* (2014) who obtained good enzyme

activity up to 60°C for latex of *Araujia hortorum* fruits and bromelain from pineapple, respectively.

It was also revealed that papain from latex showed the maximum activity at all temperatures followed by leaves, seeds and peels and latex. Chaiwut *et al.* (2007) suggested that enzyme activity decreases more rapidly in latex than in peels. This difference occurred due to their inequivalent protease compositions (Chaiwut *et al.* 2007). Martin *et al.* (2014) reported that as the temperature increases, the kinetic energy of molecules increases and more molecules go through the reaction. When the temperature is raised above its optimum temperature, biochemical changes occur, and the energy of the system becomes so high that the peptide and disulfide bonds are disrupted and inactivate the enzymes.

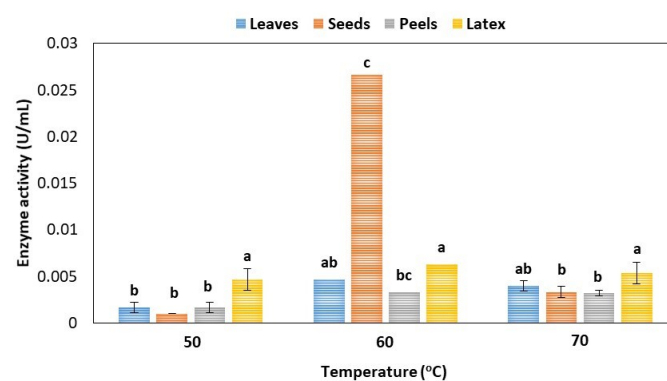


Figure 2. Effects of temperature on enzyme activity of purified papain extracted from leaves, seeds, peels and latex. Bars with different notations are significantly different among leaves, seeds, peels and latex.

3.4 Thermal stability of papain

The thermal stability of the papain enzyme with the combination of time and temperature is shown in Figure 3. With increasing incubation time, the enzyme activity of papain decreased. The highest activity was obtained at 60°C (about 43% of the remaining activity after 90 min). The loss of enzyme activity was getting higher with the incubation time (<30 mins) and a temperature (<60°C). A similar trend was reported by Liggieri *et al.* (2009) who obtained rapid loss of enzyme activity at 60-70°C in the latex of asclepaine II fruit. The deviation of enzyme activity with increasing temperature and incubation time might be due to the denaturation of the enzyme.

4. Conclusion

In the present study, extraction and purification of papain enzyme were completed from byproducts of papaya such as leaves, seeds, peels and latex using citric phosphate buffer and distilled water. The extraction process extended the knowledge that optimum protein concentration was obtained from citric phosphate buffer and distilled water. The specific enzyme activity was

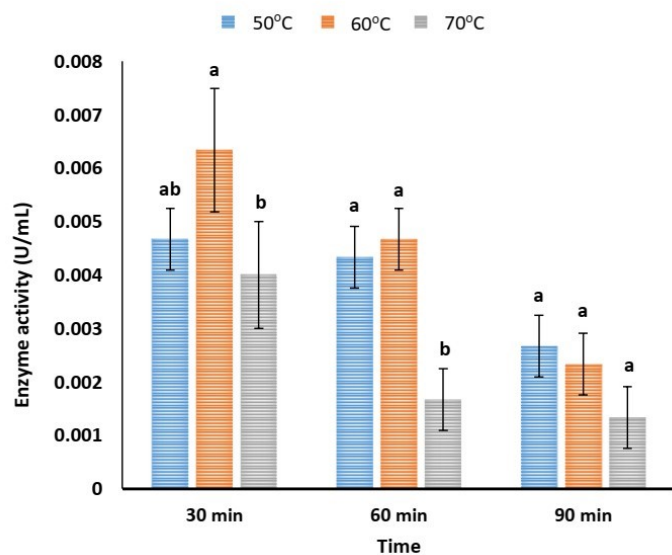


Figure 3. Effects of incubation time and temperature on enzyme activity. Bars with different notations are significantly different among temperature.

highest in seed followed by latex, leaves and peel. The purification method accomplished that the highest purification fold was achieved from seed extracted with distilled water whereas leaves were given the highest yield. Papain enzyme showed better thermal stability at 60°C with 30 mins of incubation time. Therefore, it can be concluded that various parts of papaya can be used as an excellent source of papain enzyme.

Conflict of interest

The authors declare no conflict of interest.

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