

Characterization and Purification of Protease Extracted from Two Maturity Stages of 'Noni' (*Morinda citrifolia L.*) fruit

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ABSTRACT

Protease was extracted from two maturity stages of noni fruits (Morinda citrifolia L.), unripe (stage 1) and ripe (stage 5). The crude extract was partially purified by acetone precipitation method followed by dialysis, gel filtration chromatography and freeze drying. Protein concentrations, proteolytic activity, molecular weight distribution, pH stability, temperature stability and storage efficiency of the resulting protease were evaluated. The unripe and ripe noni fruit contains 0.65 and 0.35% protein, respectively. Molecular weight of the proteases from both stages ranged approximately between 3 to 28 kDa based on the SDS-PAGE results. The optimum activity were at pH 7s and 6, temperatures of 40 and 50°C, respectively for proteases obtained from the unripe and ripe fruit. Analysis from the freeze dried protease indicated that protease from ripe noni fruits had higher protein concentration and specific activity compared to those from unripe fruit. However, it is more sensitive to pH and temperature and less stable during storage as it shows lower proteolytic activity compared to protease from unripe fruit. Based on its high proteolytic activity reaching up to 70.31 U/mg and storage stability (30% lost of activity), noni fruit could be an alternative source of plant protease.

Keywords : protease, noni, *Morinda citrifolia L.*, proteolytic activity, acetone precipitation

INTRODUCTION

Protease is a proteolytic enzyme that hydrolyses the peptide bonds. It is commonly used in leather, brewing and detergent. In the food processing industry, protease is regarded as one of the most important groups of industrial enzymes. An example of such an enzyme is chymosin, a milk clotting enzyme is particularly important in cheese production while treatment by protease such as papain is one of the popular methods for meat tenderization [1, 2]. Furthermore, other studies have been carried out to develop a method for improving tenderness and overall quality of tough meat by using plant proteolytic enzymes from different sources [3].

Morinda citrifolia L. or commonly known as noni is a species of Rubiaceae family [4]. The fruit was categorized into five maturity stages. It appears as dark green and is very hard in its early stage while it is translucent grayish and soft when matured [5]. Based on skin colour, at stage one the appearance was dark green followed by green yellow during the second stage, pale yellow for the third and fourth stages and translucent grayish in its last stage [5]. The flesh is juicy, bitter in flavor and comprises of numerous seeds [6]. Despite its rancid and unpleasant cheese odor when fully ripe, the fruit is also known for its medicinal benefits [7, 6, 8]. The fruit juice is rich in antioxidant, anthraquinones, flavonoids, saponins and scopoletin [9].

Protease from noni plant can be purified by several methods. Smith-Marshall and Golden [10] partially purified a bromelain-like enzyme from noni fruit by applying 40% ammonium sulfate precipitation, gel filtration and ion exchange chromatography. Ishartani *et al.*, [11] purified proteases from noni fruit and leaves by using saturated ammonium sulfate precipitation and electrophoresis under denaturing conditions. Some studies revealed that protein bands molecular weight were similar to those of papain and bromelain. The present investigation was undertaken to evaluate the proteolytic activity of proteases extracted and partially purified during two different maturity stages of noni fruit. The physicochemical characteristics of the proteases were also determined.

MATERIALS AND METHODS

Materials

Noni fruits at two different maturity stages; unripe (stage 1; dark green) and ripe (stage 5; translucent grayish) were obtained from a local market in Klang, Selangor. All chemicals and reagents used were of analytical grade.

Methods

Proteases Extraction

Protease was extracted according to Siti Balqis and Rosma [12]. Noni flesh weighs two hundred gram was homogenized in a minimum amount of cold sodium phosphate buffer (pH 7) containing 5% NaCl (w/v) and 5% ascorbic acid (w/v). Polyvinylpyrrolidone (PVPP) at 0.45% (w/w) was added. The homogenate was filtered through two layers of muslin cloth before centrifugation at 5000 rcf for 10 minutes (Kobuta 7800, Japan). The supernatant also referred as the crude extract was collected and kept for further analysis.

Proteases Purification

Noni protease was partially purified by acetone precipitation method according to He *et al.*, [13] and Abirami *et al.*, [14]. Cold acetone (-20°C) was slowly added to the crude extract and agitated gently for 15 min before centrifugation at 5000 rcf for 10 min at 4°C. The collected pellet was resuspended with a minimum volume of sodium phosphate buffer (pH 7.0) and dialysed with the same buffer for 24 hrs to remove the remaining salt. The dialysed protease was passed through Sephadex G-25 column equilibrated with the same buffer and eluted at 3 mL/hr. Protease fraction of 3 mL each was collected and subjected to freeze drying in a freeze drier (Alpha, 1-4 LD Plus, Martin Christ, Germany) before analysis.

Protein Content

Protein content of noni fruit was determined by Kjeldahl method [15]. Protein content in noni proteases were determined by Bradford method [16].

Proteolytic Activity

Proteolytic activity was determined according to Chanda *et al.*, [17] with slight modifications. 3 mL protease was added into 1.5 mL of 1% (w/v) casein in 0.1 M sodium phosphate buffer (pH 7). The mixture was incubated at 50 °C for 20 minutes and then terminated by the addition of 4.5 mL of 5% (w/v) trichloroacetic acid. The solution was left to stand at room temperature for 30 minutes and then filtered through Whatman No. 40 filter paper. Proteolytic activity of the enzyme was measured at wavelength 280 nm using a spectrophotometer (Hellos alpha Thermscientific, England). One activity unit refers to the amount of enzyme that cause an increment at wavelength 280 nm absorbance equivalent to 1 µL tyrosine per minutes [18].

Proteases Total Activity

Protease total activity was determined by spectrophotometric method according to the method of Ketnawa *et al.*, [19] and calculated as casein digestion unit :

$$\text{Casein Digestion Unit (CDU)} = \frac{E_a - E_b}{E_s} \times 50 \times \frac{11}{10} \times \text{DF}$$

E_a = Absorbance of sample

E_b = Absorbance of blank

E_s = Absorbance of standard (tyrosine)

DF = Dilution Factor

Tyrosine standard was prepared by dissolving tyrosine in 5% trichloroacetic acid solution [14].

Proteases Specific Activity

Specific activity of the protease was determined according to Bisswanger [20] where:

$$\text{Specific activity (U/mg)} = \frac{\text{Total activity (U/mL)}}{\text{Total protein (mg/mL)}}$$

Proteases Purification Fold

Proteases purification fold was determined according to Amid *et al.*, [21] as follows:

$$\text{Purification fold} = \frac{\text{Specific activity}}{\text{Specific activity of crude extract}}$$

Molecular Weight Determination

Molecular weight was determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) with Invitrogen Novex Bis-Tris gel containing 10% polyacrylamide gel. 50 μL of each sample was mixed with 5 μL NuPAGE LDS sample buffer (4x), 2 μL NuPAGE reducing agent (10x) and 5 μL deionized water. The sample was heated in a waterbath (70°C) for 20 minutes. 10 μL sample was then loaded into each well of Xcel Surelock Mini-Cell (10 cm x 10 cm) electrophoresis set (Bio-Rad laboratories, Hercules, CA, USA). Blue staining solution and bench mark protein ladder (3 to 188 kDa) was used as staining agent and marker, respectively. Electrophoresis was run for 1 hr at 125 mA/gel.

Effect of pH

Protease was incubated in different pH ranging from pH 2.0 to 10.0 for 24 hrs [22]. Glycine-HCL (pH 2.0-3.0), acetate buffer (pH 4.0-5.0), sodium phosphate (pH 6.0-8.0) and glycine-NaOH buffer (pH 9.0-10.0) were used as buffers.

Effect of Temperature

The effect of temperature on the proteolytic activity was determined according to Chaiwut *et al.*, [23]. The protease was incubated at different temperatures ranging from 20 to 80°C and the activity was determined at every 10°C interval.

Storage Stability

The protease was stored for three weeks at 26°C and the activity was determined every three days interval. Storage stability which is also storage efficiency was calculated according to Li *et al.*, [24] (2009):

$$\text{Storage efficiency (\%)} = \frac{\text{Proteolytic activity after storage}}{\text{Initial proteolytic activity}} \times 100$$

Statistical Analysis

All measurements were carried out in triplicate and results were subjected to analysis of variance (ANOVA) using SPSS software [25]. All values were expressed as mean \pm standard deviation. The minimum level of significant difference was at $p < 0.05$.

RESULTS AND DISCUSSION

Protein Content in Noni Fruits

The crude protein content for the unripe (stage 1) and ripe fruit (stage 5) were 0.65% and 0.35%, respectively. According to Satwadhari *et al.*, [26] matured noni fruit contains 0.39% protein while West *et al.*, [27] reported 0.55%. Ishartani *et al.*, [11] stated that the protein content of noni fruits were 0.76% for stage 2 and 0.72% for stage 4. Most fruits have low amount of protein and the protein is mostly concentrated in the seeds [28].

Protein Concentration

Crude extract, acetone precipitates and dialysed proteases of unripe noni fruits (stage 1) exhibited higher protein concentration (0.25, 0.38 and 0.63 mg/mL) compared to ripe fruit (stage 5); 0.16, 0.29 and 0.42 mg/mL; respectively. The results obtained indicated a similar trend with Ishartani *et al.*, [11] findings where stage 2 noni fruits crude extract have higher protein concentration (0.67 mg/mL) compared to stage 4 crude extract (0.51 mg/mL). According to Kulkarni and Aradhya [29], protein concentration of pomegranate fruits increased during development and will decreased with increased in maturity stages. This was also observed in guava fruits and muskmelon [30, 31]. Proteases such as bromelain extracted from the core of partially ripe pineapple also showed slightly higher protein concentration (12.115 mg/ml) compared to those extracted from the fully ripe pineapple (11.75 mg/ml) [32]. Protein concentration significantly increased ($p < 0.05$) after purification with acetone and dialysis for both stages, however, the amount decreased after gel filtration column chromatography and freeze drying (Table 1). No significant difference ($p > 0.05$) was exhibited by freeze dried proteases at both stages.

Table 1: Protein concentration (mg/ml) of noni fruit proteases at unripe (stage 1) and ripe (stage 5) maturity stages

Purification Steps	Unripe (stage 1) (mg/ml)	Ripe (stage 5) (mg/ml)
Crude extract	0.25 ± 0.02^{Ac}	0.16 ± 0.03^{Be}
Acetone precipitates	0.38 ± 0.04^{Ab}	0.29 ± 0.04^{Bc}
Dialysed	0.63 ± 0.05^{Aa}	0.42 ± 0.03^{Ba}
Gel filtration	0.27 ± 0.01^{Ac}	0.32 ± 0.02^{Ac}
Freeze dried	0.10 ± 0.01^{Ad}	0.22 ± 0.02^{Ad}

Values represent mean \pm standard deviation (n=3). Different small letter within column indicates significant difference at $p < 0.05$ between purification steps. Different capital letters within row indicates significant difference at $p < 0.05$ between maturity stages.

Proteolytic Activity

Specific activity of the proteases from ripe fruit increased significantly ($p < 0.05$) from 28.92 U/mg when subjected to acetone precipitation to 70.31 U/mg after freeze dried (Table 2). However, the protease from unripe fruit showed fluctuation which is 35.33, 13.76 and 21.55 U/mg for acetone precipitation, dialysis and gel filtration, respectively. Specific activity increased upon freeze drying (25.52 U/mg). According to Devakate *et al.*, [33] by freeze drying 96% of proteases activity could be recovered. Ishartani *et al.*, [11] studies showed that specific activity for noni fruits at stage 2 decreased from 3.79, 2.88 and 1.94 U/mg for crude extract, ammonium sulfate precipitates and dialysed protease, respectively. However, for stage 4, the specific activity increased as follows; crude extract (1.83 U/mg), ammonium sulphate precipitates (2.11 U/mg) and dialysed (3.18 U/mg). This is in agreement with Koak *et al.*, [18] suggestion that over matured fruits would undergo increases in specific activity for about 16.9%.

Table 2: Proteolytic activity of noni fruits proteases at each purification step.

Purification step	Unripe (Stage 1)					
	Volume (ml)	Total protein (mg)	Activity (U)	Total activity (U)	Specific activity (U/mg)	Purification fold
Crude extract	29.25	7.43	20.06±4.88 ^{Aa}	586.90±8.27 ^{Aa}	78.99±1.11 ^{Aa}	1.00
Acetone ppt	20.40	7.84	13.58±0.14 ^{Ab}	276.99±2.90 ^{Ab}	35.33±0.37 ^{Ab}	0.45
Dialysed	18.80	11.81	8.64±0.12 ^{Bc}	162.51±2.28 ^{Bc}	13.76±0.19 ^{Be}	0.17
Gel filtration	20.00	5.49	5.92±0.11 ^{Bcd}	118.31±2.10 ^{Bd}	21.55±0.38 ^{Bd}	0.27
Freeze dried	2.18	0.23	2.69±0.00 ^{Bd}	5.87±0.00 ^{Be}	25.52±0.017 ^{Bc}	1.85
Purification step	Ripe (Stage 5)					
	Volume (ml)	Total protein (mg)	Activity (U)	Total activity (U)	Specific activity (U/mg)	Purification fold
Crude extract	37.50	5.87	8.65±0.02 ^{Bc}	324.38±0.53 ^{Bc}	55.26±0.09 ^{Bb}	1.00
Acetone ppt	25.00	7.34	8.49±0.02 ^{Bcd}	212.27±0.39 ^{Bd}	28.92±0.05 ^{Be}	0.52
Dialysed	22.60	9.42	16.31±0.13 ^{Ab}	368.60±2.92 ^{Ab}	39.13±0.31 ^{Ac}	0.71

Gel filtration	20.00	6.38	21.82±0.25 ^{Aa}	436.4±4.90 ^{Aa}	68.36±0.77 ^{Aa}	1.24
Freeze dried	3.12	0.69	15.55 ±0.03 ^{Ad}	48.51±0.09 ^{Ae}	70.31±0.13 ^{Ad}	1.27

ppt=precipitates

Values are mean ± standard deviation (n=3). Different small letter within column indicates significant difference at $p < 0.05$ between purification steps.

Different capital letters within column indicates significant difference at $p < 0.05$ between maturity stages.

Protease activity may be involved in protein turn over and particularly during the final stages of senescence by protein catabolism, which is responsible for increase in free amino acids and amides [34]. As for other fruits, the specific activity of tomato pulp crude extract was 128.2 U/mg and increased to 458.33 U/mg after dialysis while latex of *Plumeria rubra* L. showed increased in specific activity from the crude extract (4.56 U/mg) to Sephadex G-200 gel filtration (153.16 U/mg) [35, 17]. In general, purification fold increased with purification steps for both stages (Table 2).

Molecular Weight Determination

Molecular weight distribution of proteases for unripe (stage 1) noni fruits showed that more bands appeared in the purified proteases compared to the crude extract (Figure 1a). The bands were within the range of 3 to 28 kDa. No protein band observed in crude extract could be due to the nature of the crude extract which is very dilute. According to Chaiwut *et al.*, [23], dark band of protein appear rather than clear zone due to lower caseinolytic activities of plant proteases. Similar pattern was observed for ripe (stage 5) noni fruits where the bands also appeared within 3 to 28 kDa (Figure 1b). Ishartani *et al.*, [11] observed the protein bands of noni fruit proteases at approximately 25, 27, 37 and 38 kDa and they claimed that bands at 25 and 29 kDa were similar to papain and bromelain's molecular weight. According to Ketnawa *et al.*, [36], pineapple proteases also showed very clear protein band approximately at 28 kDa while Chaiwut *et al.*, [37] observed major protein band of *C. procera* latex at 11 to 38 kDa.

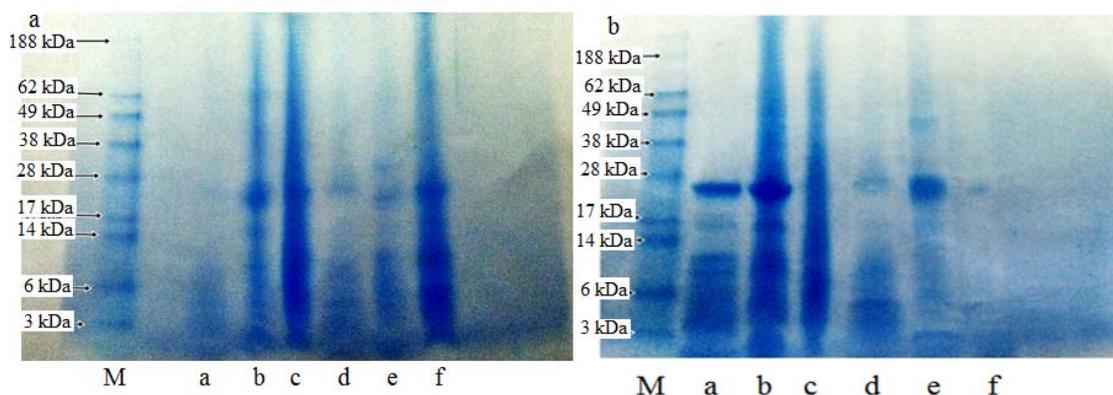


Figure 1: SDS-PAGE of proteases from a) unripe noni fruit and b) ripe noni fruit; Lanes represent M: protein marker; a: crude extract; b: acetone precipitate; c: dialysed; d: freeze dried; e: gel filtration; and f: noni fruit.

Effect of pH

The effect of pH on noni protease was determined based on the freeze dried proteases. The proteolytic activity against casein was calculated and the result is shown in Figure 2a. Protease from unripe fruit had optimum pH range from 6 to 8 and ripe fruit from 5 to 7 with maximum proteolytic activity at pH 7 (62.19 U) and 6 (52.75 U), respectively. From the results, ripe fruit noni protease activity was optimum at slightly acidic pH compared to unripe fruit. This was in agreement with Assanga *et al.*, [38] (2013) findings who also suggested that the variation in proteolytic activity may be due to different cultivars and extraction methods. Low protease activity was observed at extreme pH which is at pH 2 and 10 with approximately 2.49 to 7.85 U, respectively. The decrease in activity was due to the lost of activity caused by the alkalinity of the reaction mixture [10]. Proteases activity depends on the pH [39]. Protease which is bromelain like enzymes in noni fruits according to Smith-Marshall and Golden [10] findings have an optimal pH at 7.1 with activity of 274.77 $\mu\text{mol}/\text{min}/\text{ml}$. Other plants such as ginger displayed an optimal proteases activity at pH at 5.5, *Euphorbia nivulia* (Sabar) at pH 6.6, *Calotropis procera* (Ait) at pH 7.0, *Carica papaya* L. at pH 6.5 and *Ficus carica* L. (Anjir) at pH 6.6 [40, 41].

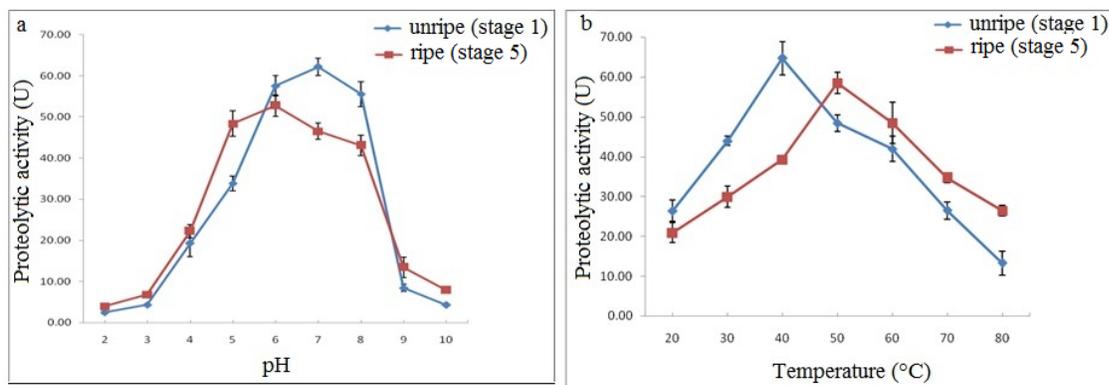


Figure 2: Effect of a) pH and b) temperature on proteolytic activity of freeze dried protease from unripe (stage 1) and ripe (stage 5) noni fruits.

Temperature Stability

Effect of temperature on freeze dried noni proteases is shown in Figure 2b. As temperature increases proteolytic activity of proteases from both maturity stages also increased, however, the activity gradually reduced as they reached an optimal temperature. According to He [42], change in protease activity as the temperature increases was not correlated with decline in protein but it was due to the heat injury [42]. Optimum temperature range were between 30 to 50°C for protease from unripe fruit and 40 to 60°C from ripe fruit with the maximum activity at 40°C (64.74 U) and 50°C (58.47 U), respectively. Smith-Marshall and Golden [10] found that bromelain-like noni proteases displayed highest activity (600.55 $\mu\text{mol}/\text{min}/\text{ml}$) at its optimum temperature (35°C). Other plant proteases such as *Euphorbia nivulia* (Sabar) have an optimal temperature at 45°C, *Carica papaya* L. (papaya) at 60°C, *Calotropis gigantea* L. (Ruie) at 55°C and *Ficus carica* L. (Anjir) at 50°C [41].

Storage Efficiency

Storage efficiency of freeze dried noni protease during the three weeks storage at 26°C showed that protease from ripe fruit displayed a lost of only 38% of proteolytic activity which is slightly greater lost than unripe fruits (stage 1) which is 33% (Figure 3). Freeze drying is one of the important process intended to conserve enzyme, extend shelf life and retains its purity [43].

However it may destabilize and deactivate the enzyme due to different types of stress generated such as low temperature stress, dehydration stress and ice crystal formation [44]. It is suitable for various types of goods including enzymes, however, its applications in food industry are still limited due to its high cost [45, 46].

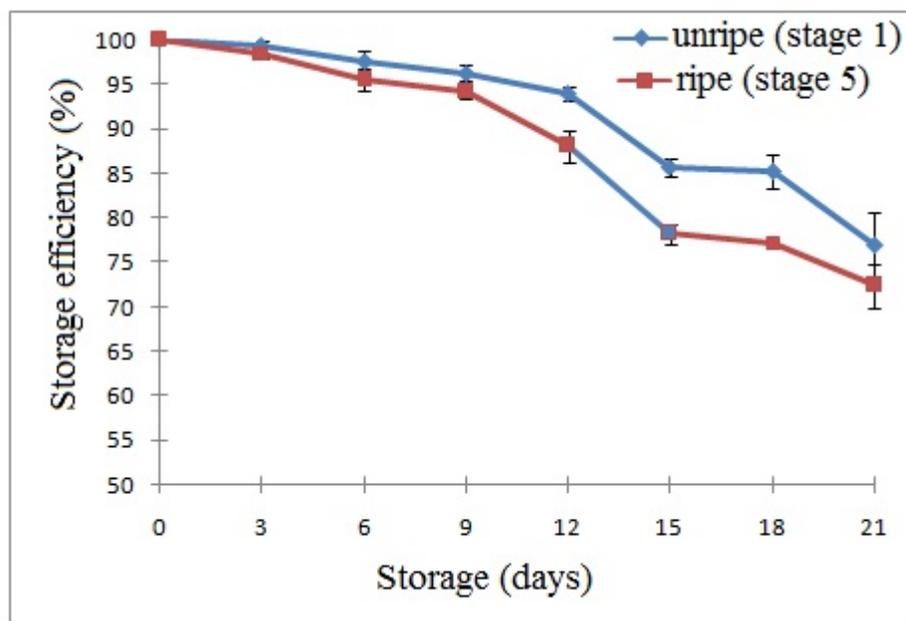


Figure 3: Storage efficiency of freeze dried protease from unripe (stage1) and ripe (stage 5) noni fruits during the three weeks storage at 26°C.

CONCLUSIONS

Protease extracted from unripe and ripe noni fruits had an optimal activity at pHs 7 and 6, 40 and temperatures of 50°C, respectively. Major protein bands were observed within 3 to 28 kDa for all the proteases. Freeze dried protease from ripe noni fruits displayed higher protein concentration and higher specific activity compared to those from unripe fruit. However, it is more sensitive to pH and temperature and less stable during storage as it shows lower proteolytic activity compared to protease from unripe fruit. Nevertheless, proteases from unripe noni fruits had better purification fold than those from ripe noni fruit. Freeze drying step during the partial purification of noni fruit improved the specific activity and purification fold of both proteases while the freeze dried proteases lost their activity at approximately only 30% during the three weeks storage at

26°C. Therefore, noni fruit can be a potential source of new plant protease in view of its proteolytic activity and stability during storage.

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