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Extraction and Partial Purification of Protease from Bilimbi (*Averrhoa bilimbi* L.)

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ABSTRACT

Unripe and ripe bilimbi (*Averrhoa bilimbi* L.) were ground and the extracted juices were partially purified by ammonium sulfate precipitation at the concentrations of 40 and 60% (w/v). The collected proteases were analysed for pH, temperature stability, storage stability, molecular weight distribution, protein concentration and protein content. Protein content of bilimbi fruit was 0.89 g. Protease activity of both the unripe and ripe fruit were optimum at pH 4 and 40°C when the juice were purified at 40 and 60% ammonium sulfate precipitation. A decreased in protease activity was observed during the seven days of storage at 4°C. Molecular weight distribution indicated that the proteases protein bands fall between 10 to 220 kDa. Protein bands were observed at 25, 50 and 160 kDa in both the unripe and ripe bilimbi proteases purified with 40% ammonium sulfate, however, the bands were more intense in those from unripe bilimbi. No protein bands were seen in proteases purified with 60% ammonium sulfate. Protein concentration was higher for proteases extracted with 40% ammonium sulfate at both ripening stages. Thus, purification using 40% ammonium sulfate precipitation could be a successful method to partially purify proteases from bilimbi especially from the unripe stage.

Keywords: *bilimbi, Averrhoa bilimbi* L., *protease, purification, ammonium sulfate*

INTRODUCTION

Bilimbi (*Averrhoa bilimbi* L.) belongs to Oxalidaceae family, is a small tree growing up to 10 m with a trunk diameter can be up to 30 cm. Normally, it is planted as a fruit tree although sometimes growing wild in tropical countries [1]. The fruit has a torpedo-like shape measuring 4 to 10 cm long and 2.5 cm width [2]. Bilimbi is also known as bilimbi, cucumber tree, tree sorrel, pickle tree in English; kamias, camias, pias in Philippines; ta ling pling in Thailand; huangguashu in Chinese; belimbing buluh, belimbing asam in Malaysia; bilimbim, biri-biri, limao decaiena, azedinha in Brazil; vilimbipuli, irumpanpuli in Malayalam; khetay in Vietnamese; and bilimbi in India [1-2]. Bilimbi contains 20.8 to 60.9 mg vitamin C, 0.61 g protein, 15 mg calcium and 11.1 mg phosphorus [1]. The fruit which is generally regarded as too acidic if eaten raw is sometimes cooked in curry or used to prepare refreshing drink, chutney, salad, jam, sherbet and pickles [1]. In medicinal field, bilimbi has been shown to have antibacterial and antidiabetic effects [3-4].

Proteolytic enzymes or proteases are a class of proteins ubiquitously found in all organisms; they act as catalysts and perform diverse vital functions [5]. Proteases have several applications in food industries for example in accelerating the dough making process in which by adding protease, wheat gluten is partially hydrolysed [6]. Bacterial neutral proteases may be used in the production of cookies and crackers [7]. In the mashing stage of brewing and general cereal processing the added protease helps to increase the volume of filterable extract and to obtain the desired level of nitrogen nutrient [8]. Proteases like papain, bromelain and ficin are also useful for removing chill haze in beer and as meat tenderising agent. Rennin is commonly used as milk clotting enzyme in cheese making industry and helps develop flavor and texture in ripened cheese [9].

Enzyme can be extracted from animal, microorganism and plant. Enzyme extracted from animal can be from non-halal source which is a big issue by the Muslim consumer. By extracting protease from plant resources, the utilization of such resources can be diversified in wider range of food application. Enzyme from bilimbi has many advantages than animal and microorganism because the source used is a local product, easy to obtain and the cost of source material is cheap. The usage of bilimbi is not fully

discovered and there is not much research have been done to develop and promote the fruit. Due to its food value and medicinal uses, it has high potential for exploitation in the future especially as source of protease. Thus, the objectives of this research are to partially purify protease from the unripe and ripe bilimbi using ammonium sulfate precipitation methods and to determine the proteolytic activity and storage stability of the protease.

MATERIALS AND METHOD

Materials

Plant Materials

Bilimbi (*Averrhoa bilimbi* L.) was bought at Sijangkang, Selangor. The fruit was selected for uniformity and free from defects, washed thoroughly and cut into dice. Two stages of fruit maturity was selected which were unripe and ripe.

Chemicals

All chemicals used were of analytical grade.

Methods

Extraction of Proteases

Bilimbi juice was extracted using a juice extractor and subsequently filtered through four layers of muslin cloth into a beaker cooled with ice to prevent thermal degradation.

Purification

The collected juice was purified according to the method by Wang *et al.*, [10]. Two different concentrations of ammonium sulfate solution, 40% and 60% (w/v) were added until precipitates were formed. The purified extract was then centrifuged at 12,000 g for 10 minutes to separate the precipitate from the supernatant which was then dialyzed against Tris-HCL buffer (0.02 M, pH 7.5).

Proteolytic Activity

Proteolytic activity was measured according to Siti Balqis and Rosma [11]. Protease (0.1 ml) was mixed with 0.9 ml casein dissolved in 0.2 M

sodium phosphate buffer (1.0% w/v, pH 7). The mixture was incubated at 38°C for 20 minutes. After 20 minutes, the reaction was terminated by the addition of 3 ml trichloroacetic acid (5%, w/v) and then centrifuged at 6000 g for 20 minutes. The supernatant was collected and absorbance was measured at 280 nm.

Total Activity of Proteases

The total activity of the protease was determined using the following formula:

$$CDU = \frac{E_t - E_b}{E_s} \times 50 \times \frac{11 \times DF}{10}$$

where

E_t = Absorbance of enzyme sample
E_b = Absorbance of blank
E_s = Absorbance of tyrosine standard
DF = Dilution factor

Proteases Specific Activity

Proteases unit per ml divided by protein in mg/ml concentration indicated the specific proteases activity [12].

Protein Concentration

Protein concentration was measured according to Field and Field [13] using bovine serum albumin as a standard. Samples were placed into microcentrifuge tubes and brought to a volume of 800 µl with deionized water. 200 µl of 5x Bradford reagent was added to each sample to bring it to 1 ml in volume. The samples were then analysed in a spectrophotometer to determine their absorbance at 595 nm.

Protein Content

Protein content of the bilimbi flesh was measured using Kjeldahl method [14].

Proteases Temperature Stability

Stability of the purified protease was evaluated by incubating 1.0 ml protease solution (1% v/v) at different temperature ranging from 20 to 80 °C at the interval of 10 °C for 15 minutes. Protease solution was

then removed and rapidly cooled in an ice bath prior to the determination of proteolytic activity.

Proteases pH Stability

Protease was incubated in different buffers for 24 hours at 4 °C, with pH ranging from 2 to 12 for determination of pH stability. The buffer solutions used were glycine-HCL (pH 2.0 to 3.5), sodium acetate (pH 4.0 to 5.5), sodium phosphate (pH 6.0 to 7.5), Tris-HCL (pH 8.0 to 10.0) and sodium carbonate (pH 10.5 to 11.5). The molarity for all buffers used was 0.05 M.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Molecular weight distribution of the proteases was determined using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Deionized water (1 ml) was added into 1 mg of proteases and vortexed to dissolve the proteases. Simply blue staining (10 μL) was added into the proteases. The solution was then boiled for 10 minutes at 70 °C. 10 μL sample or marker was loaded into the gel. The electrophoresis was carried out using Invitrogen Novex bis-tris gel instrument. 4% stacking gel and 12% resolving gel were used. Bench mark protein ladder ranging from 10 to 220 kDa was used as the marker. The electrophoresis was run at 200 V constant current for 35 minutes at room temperature.

RESULTS AND DISCUSSION

Purification of the Protease

Purification of proteases from the unripe and ripe bilimbi using 40% (w/v) ammonium sulfate precipitation resulted in higher yield compared to those purified with 60% (w/v) ammonium sulfate (Table 1). This finding was in close agreement with Chaiwut *et al.* [15] who obtained lowest yield when papaya peel and latex proteases were purified with 60% ammonium sulfate precipitation as compared to ethanol, methanol and 2-propanol purification methods. Wang *et al.* [10] stated that the bitter gourd protease was enriched in the fractionation using 40% ammonium sulfate. According to Narayan *et al.* [16], the use of an increase concentration of ammonium sulfate reduced the degree of precipitation thereby decreasing the yield. The yield of bilimbi proteases ranged from 0.84 to 3.2%.

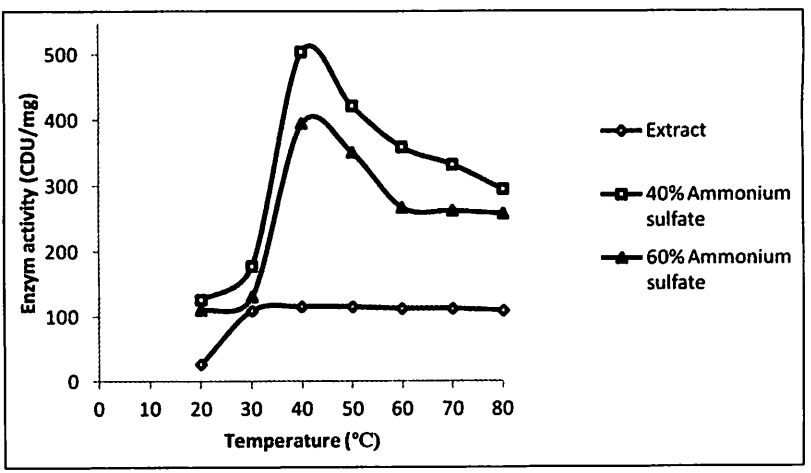
Table 1: Yield of Proteases from Bilimbi (*Averrhoa bilimbi* L.) at 40 and 60% of Ammonium Sulfate Precipitation

Ripening stage of bilimbi	Concentrations of ammonium sulfate (%)	Yield of proteases (%)
Unripe	40	3.20
	60	1.80
Ripe	40	1.12
	60	0.84

Temperature Stability

The effect of temperature on protease activity was evaluated by varying the incubation temperature from 20 to 80 °C for 10 minutes. Protease activity against casein was calculated and presented in Figures 1a and b. Protease purified in ammonium sulfate showed higher activity than the crude extract. The activity of the proteases in the extract was constant at approximately 110 CDU/mg beginning from 30 °C. Both unripe and ripe purified bilimbi proteases showed the same trend where the protease activity was maximum at 40 °C reaching up to 800 CDU/mg. The activities started to decline thereafter.

a)



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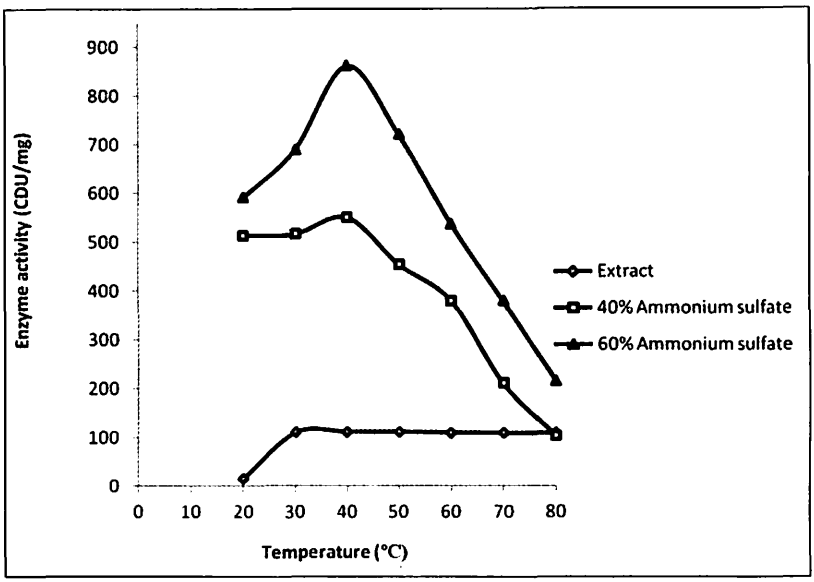
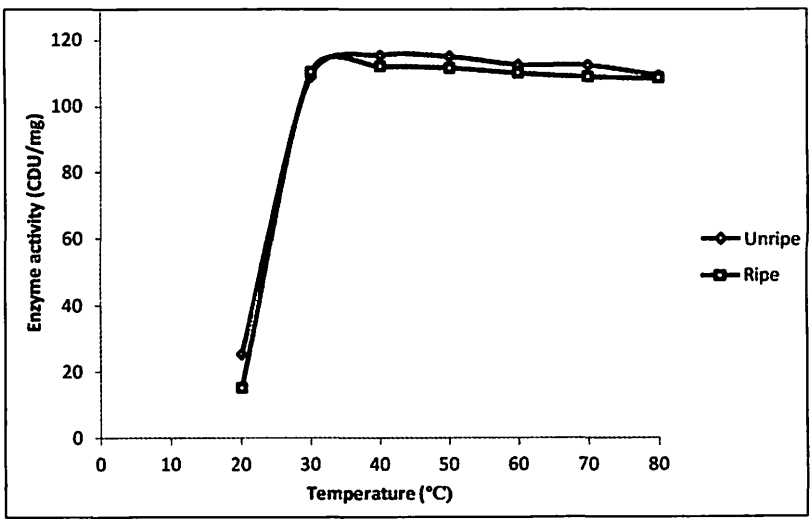


Figure 1: Effect of Temperature on the Extract, 40% and 60% Ammonium Sulfate Precipitation of Proteases from Unripe (a) and Ripe (b) Bilimbi (*Averrhoa bilimbi* L.)

According to Klomklao *et al.* [17] enzyme is inactivated at higher temperature due to partial unfolding of its molecule. Ketnawa *et al.* [18] obtained high activity at 50 to 60 °C for bromelain in the crude extract of pineapple waste from *Nang Lea* and *Phu Lae* varieties. As the incubation temperature increased to 70 °C, the proteolytic activity constantly decreased until the lowest point at 90 °C. Hashim *et al.* [19] stated that the activity of purified enzyme from ginger increased with increase in temperature up to 60 °C which is the optimum temperature. Siti Balqis and Rosma [11] studies showed that protease from *Artocarpus integer* leaves had higher enzyme activity at 40 °C. However a sharp decline was observed with further increases in temperature beginning from 60 °C. Study conducted by Jiang *et al.* [20] showed that the optimum temperature for parsley proteases was at 37 °C and the activity significantly decreased as the temperature rose above 40 °C. They concluded that parsley protease is sensitive to high temperature. From the result, bilimbi proteases had an optimum activity close to *Artocarpus integer* and parsley which is at 40°C.

Effect of temperature on enzyme activity was compared between the crude extract, 40 and 60% ammonium sulfate precipitation of unripe and ripe bilimbi (Figures 2a, b and c). Proteases from unripe and ripe bilimbi extract showed the same trend (Figure 2a). Enzyme activity increased rapidly from 20 to 30 °C. When it reaches 40 °C, the activity became constant and levelled off until 80 °C. The activity was approximately 110 CDU/mg. As for 40 and 60% ammonium sulfate precipitation, ripe bilimbi had higher enzyme activity compared to the unripe (Figures 2b and c). Ripe bilimbi showed slow increase in activity until 40 °C while the unripe bilimbi showed marked increase from 30 to 40 °C for 40% ammonium sulfate precipitation. The activity declined rapidly beginning from 50 °C. Ripe bilimbi proteases showed higher activity compared to the unripe at both concentrations of ammonium sulfate used. This suggested that protease from the ripe bilimbi was more thermostable. Meanwhile for the crude extract, both maturity stages had the same level of enzyme activity.

a)



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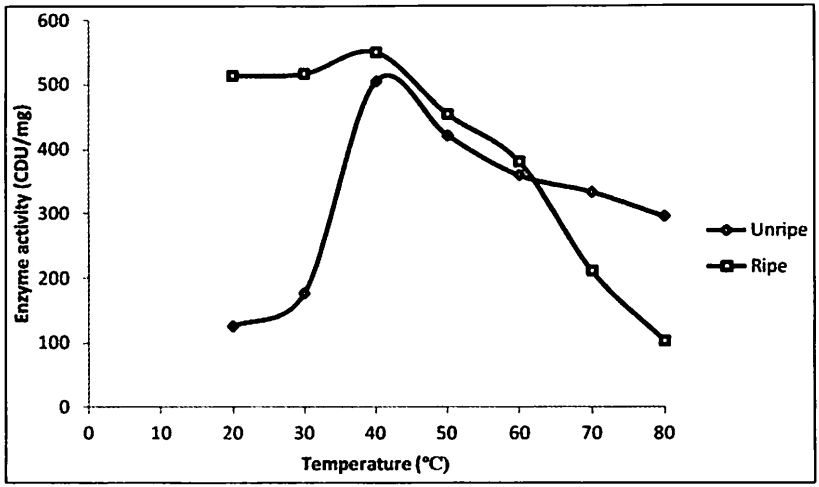

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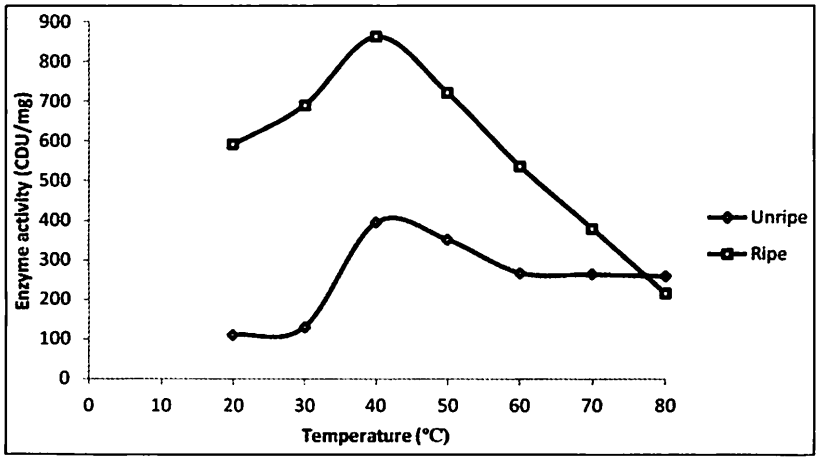


Figure 2: Effect of Temperature on the Extract (a), 40% (b) and 60% (c) Ammonium Sulfate Precipitation of Proteases from Unripe and Ripe Bilimbi (*Averrhoa bilimbi. L*)

pH Stability

The effect of pH on the proteolytic activity of bilimbi proteases from both ripening stages was measured at pH ranges from 2 to 10. The relative proteolytic activity against casein was calculated and showed in Figures 3a and b. Purified proteases showed higher activity than the extract. The enzyme activity was accelerated as the pH increased from 2 to 4. Sharp decline was observed starting from pH 6. Maximum activity occurred at pH 4.

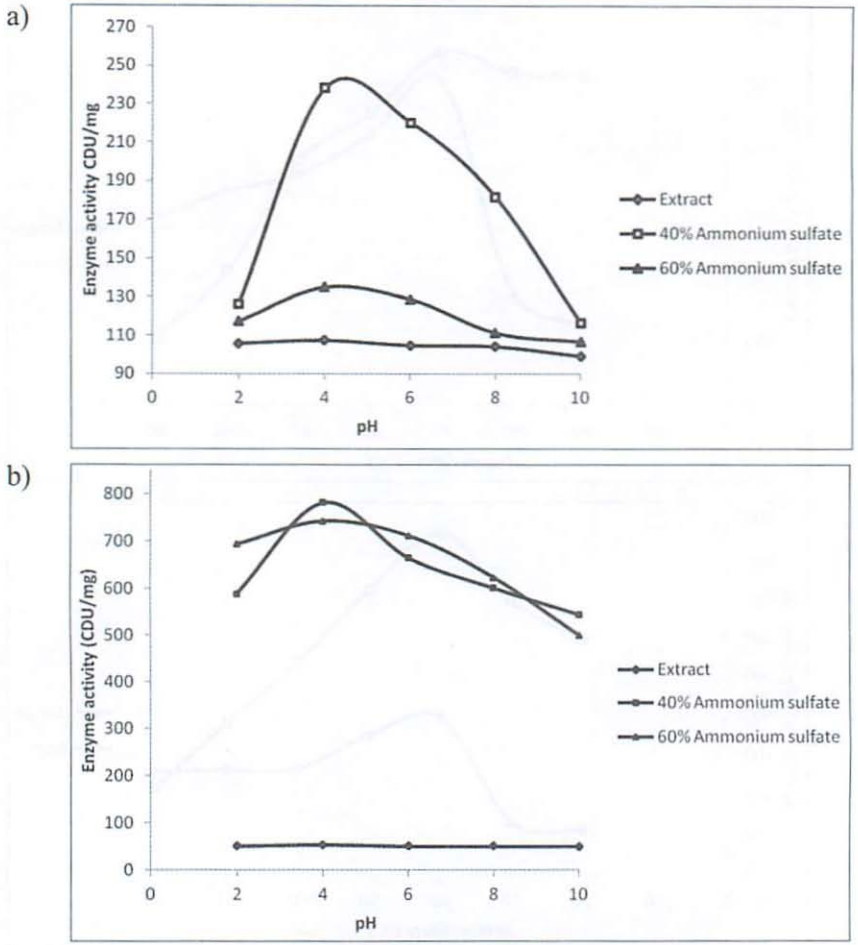


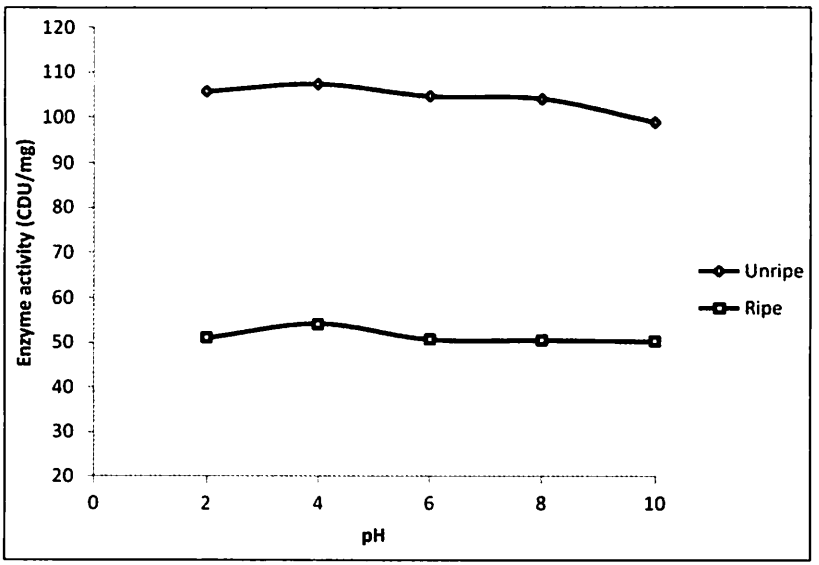
Figure 3: Effect of pH on the Extract, 40 and 60% Ammonium Sulfate Precipitation of Proteases from (a) Unripe and (b) Ripe Bilimbi (*Averrhoa bilimbi* L.)

Research done by Ketnawa *et al.* [21] showed that bromelain extracted from *Nang Lea* and *Phu Lae* had higher activity at pH 3.6 and 3.7, respectively. According to Rowan *et al.* [22] the optimum pH for stem and fruit bromelain were around 4.0 to 4.5 and 6.8, respectively. While Hashim *et al.* [19] stated that proteases from ginger acted optimally at pH 5.5 where further increased in pH from pH 7 resulted in a decline in cysteine proteases activity.

The study by Siti Balqis and Rosma [11] showed that protease activity of *Artocarpus integer* was accelerated as the pH was increased up to pH 10. A sharp decline in activity was observed beyond the maximal point which is pH 11. Parsley protease had maximum activity at pH 7 to 8, with a gradual decrease when the pH was higher or lower than this level [20]. They also observed some activity at pH 4. From the result, bilimbi proteases are considered to be more acidic compared to *Artocarpus integer*, ginger and parsley.

Extract and purified proteases from different ripening stages of bilimbi showed different activity (Figures 4a, b and c). Protease activity of the crude extract from unripe bilimbi was higher compared to those from the ripe bilimbi. But both ripening stages showed a similar pattern where the activity was approximately constant at all pH studied. Meanwhile for 40% and 60% ammonium sulfate precipitation, the protease activity was much higher for ripe bilimbi (Figures 4b and c). This suggested that ripe bilimbi protease was more pH stable than the unripe. The purified bilimbi proteases had activity reaching up to approximately 800 CDU/mg.

a)



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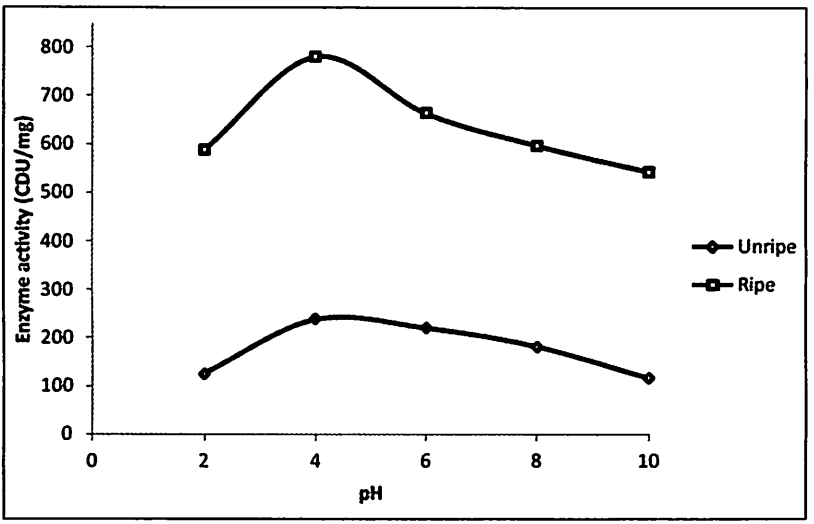

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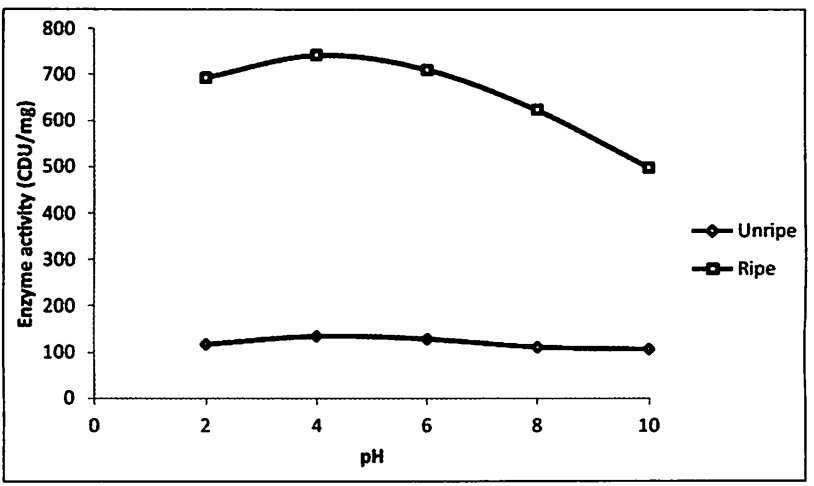


Figure 4: Effect of pH on the Extract (a), 40% (b) and 60% (c) Ammonium Sulfate Precipitation of Proteases from Unripe and Ripe Bilimbi (*Averrhoa bilimbi* L.)

Storage Stability

The storage stability study of bilimbi proteases was carried out at 4 °C to slow down the rate of enzyme damage as well as to prevent from thermal degradation. Both ripening stages and different concentrations of ammonium sulfate showed a decline trend in activity (Figures 5a and b). The activity

declined with storage time when stored at 4°C for both proteases extracted from the unripe and ripe bilimbi. Protease activity also decline regardless of ammonium sulfate concentration. However, enzyme activity of the purified proteases maintained higher than those of the extract, throughout the seven days storage period.

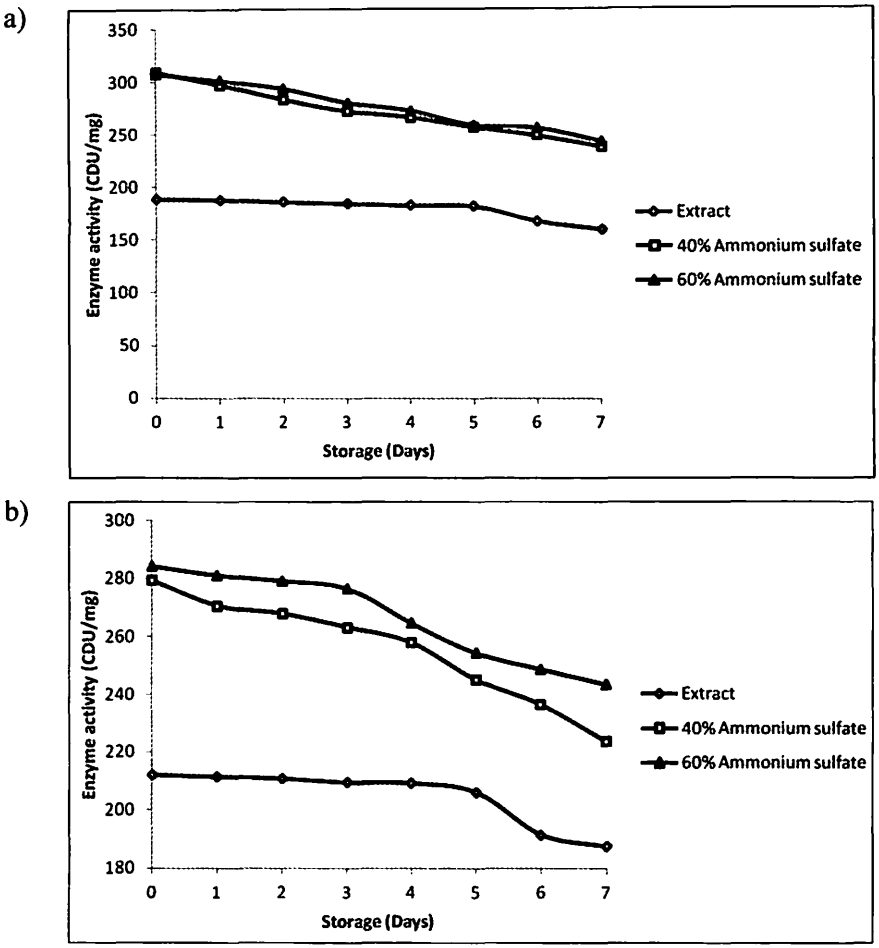
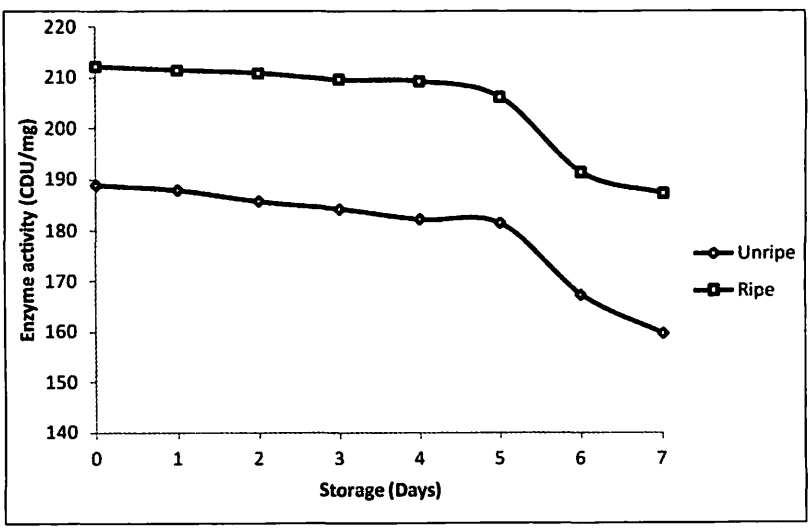


Figure 5: Effect of Storage on the Extract, 40% and 60% ammonium Sulfate Precipitation of Proteases from Unripe (a) and ripe (b) bilimbi (*Averrhoa bilimbi* L.)

Proteases purified using 40% ammonium sulfate showed loss of activity of less than 20% starting from day 3. Ripe bilimbi protease extracted

using both 40 and 60% ammonium sulfate showed a slight decreased until third day of storage followed by a gradual decreased thereafter. This resulted in the loss of approximately 5 to 10% of activity. Adultayam and Apenten [23], obtained 80% loss of activity after four days storage at 5 °C for crude ginger proteases. Amid *et al.* [5] observed a direct correlation between the storage stability of the enzyme and enzyme activity. Unripe and ripe bilimbi proteases from the extract, 40 and 60% ammonium sulfate precipitation showed different activities (Figures 6a, b and c). Both ripening stages showed a decline in protease activity beginning from day 5 for the extract (Figure 6a). For 40 and 60% ammonium sulfate purified proteases, those from the unripe bilimbi showed higher activity (Figures 6b and c). The activity gradually decreased with storage which could be due to protease deactivation during storage. According to Adulyatham and Apenten [23], there are two mechanisms contribute to protease deactivation; the browning reaction and autolysis. Protease may undergo browning reaction where the product; quinone reacts with amine, sulfhydryl and other nucleophilic residues within the protease thus causing the deactivation. Browning is due to polyphenol oxidase-mediated oxidation of phenolic compounds present in the tissue [24]. Bilimbi fruits have been reported to contain a significant amount of phenolic compounds ranging from 50.23-68.67 mg [25]. Therefore, protease extracted from bilimbi is prone to browning which contribute to its gradual deactivation during storage.

a)



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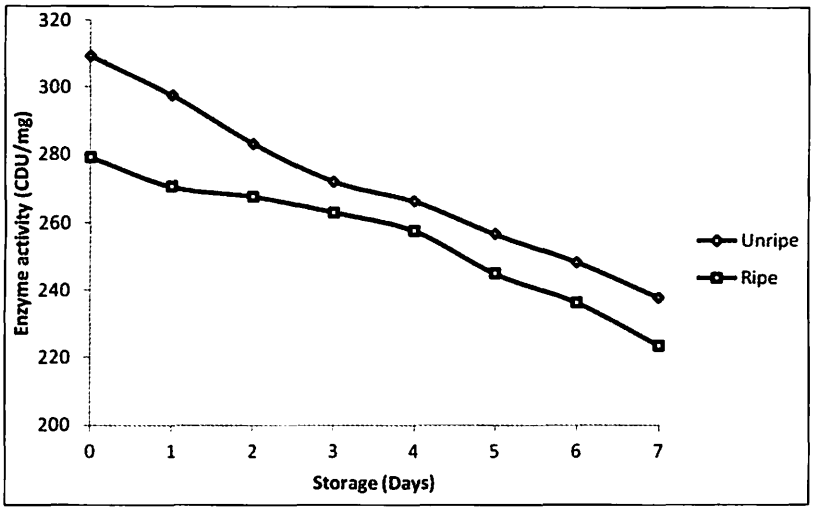

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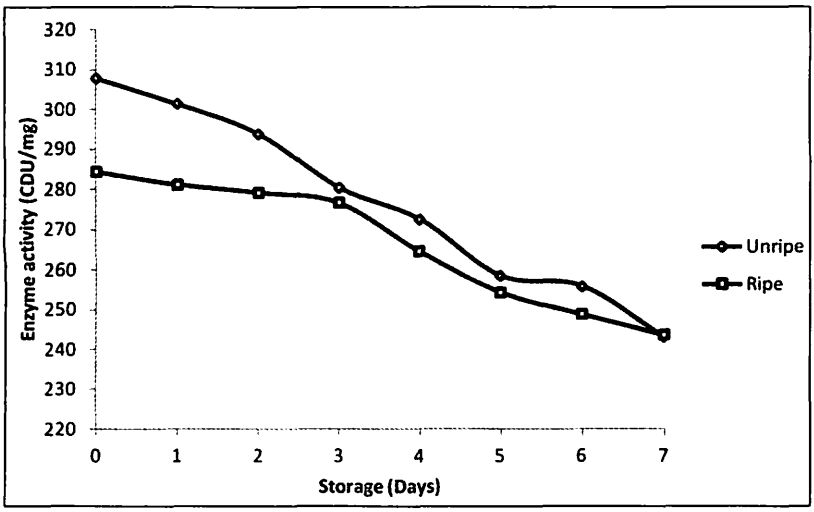

Figure 6: Effect of Storage on the Extract (a), 40% (b) and 60% (c) Ammonium Sulfate Precipitation Proteases from of Unripe and Ripe Bilimbi (*Averrhoa bilimbi* L.)

Protein Concentration

Different concentrations of ammonium sulfate used during purification resulted in different protein concentration for both ripening stages of bilimbi (Table 2). Proteases purified by 40% ammonium sulfate had higher protein

concentration probably due to higher degree of purification. Previous report by Narayan *et al.* [16] showed that higher concentration of ammonium sulfate resulted in the decrease of the degree of purification. Thus purification with 40% ammonium sulfate produced high amount of proteases compared to 60% ammonium sulfate.

Table 2: Protein Concentration (mg/ml) of Proteases from Bilimbi (*Averrhoa bilimbi* L.) at 40 and 60% Ammonium Sulfate Precipitation

Ripening stage of bilimbi	Concentration of ammonium sulfate (%)	Protein concentration, (mg/ml)	Specific activity (CDU/ mg)
Unripe	40	1.55	199.57
	60	1.23	250.20
Ripe	40	0.49	606.63
	60	0.34	836.09

Protein Content

Bilimbi protein content was found to be 0.89 g which is slightly higher than the value reported by Love and Paul [2] which is 0.61 g for bilimbi. Waste from different parts of pineapple had different protein content. As reported by Ketnawa *et al.* [18], whose study showed that pineapple crown had higher protein which is 220.5 mg for *Nang Lea* and 141.0 mg for *Phu Lae* varieties followed by peel 132.4 and 70.7 mg, respectively. The core and stem of pineapple had the lowest protein content for both varieties. Research done by Chaiwut *et al.* [26] on fresh papaya peels showed that it contains more protein (48.59 mg) compared to that of dried peels (20.93 mg).

SDS-Page (Molecular Weight Distribution)

Molecular weight distribution of bilimbi proteases was approximately in the range of 10 to 220 kDa for both proteases extracted from the ripe and unripe bilimbi (Figure 7). However, very faint protein bands were observed at 25, 50 and 160 kDa for proteases purified with 40% ammonium sulfate from ripe bilimbi. No protein bands can be seen in proteases purified by 60% ammonium sulfate precipitation at both unripe and ripe stages. Less intense bands in the ripe bilimbi protease could be due to the lower protein

concentration in the ripe bilimbi (0.49 mg/ml) compared to the unripe (1.55 mg/ml).

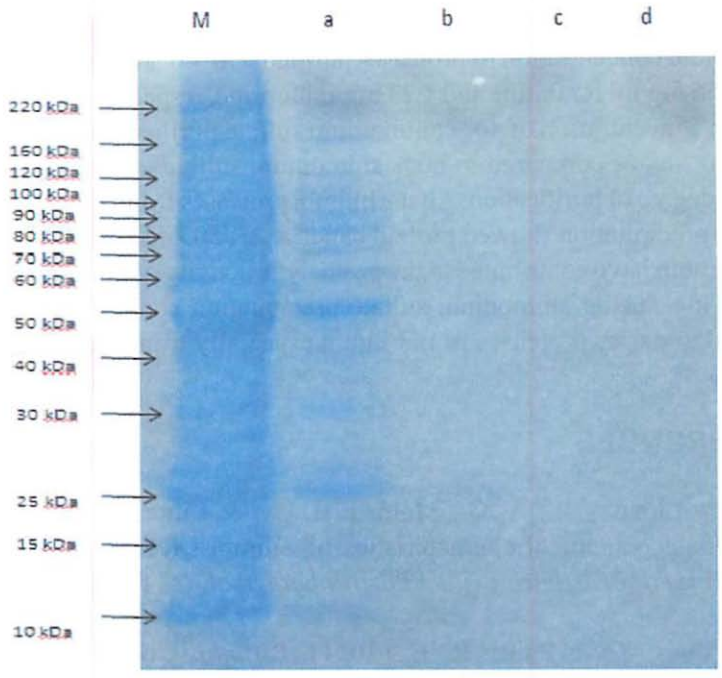

Figure 7: Electrophoretic Profile for Proteases Extracted and Purified from Unripe (a) and Ripe Bilimbi (b) at 40% Ammonium Sulfate Precipitation, Unripe (c) and Ripe Bilimbi (d) at 60% Ammonium Sulfate Precipitation. M is the Protein Marker

Ketnawa *et al.* [21] observed a major protein band with a molecular weight of around 28 kDa for bromelain from pineapple. Electrophoresis study by Thomas *et al.* [27] showed an intense band at 23 to 25 kDa for papain which corresponded to papain content in the sample. Molecular weight for ginger proteases was 35.1 to 36.4 kDa compared to 29.2 kDa for papain [23].

CONCLUSION

Protease extracted from bilimbi had an optimum activity at pH 4 and optimum temperature at 40 °C. Protein content of bilimbi was 0.89 g while the protein concentration of proteases purified with 40% ammonium sulfate was 1.55 mg/ml for unripe and 1.23 mg/ml for ripe, respectively. The higher protein concentration of 40% ammonium sulfate purified proteases at both ripening stages compared to 60% ammonium sulfate could be due to the higher degree of purification. All the bilimbi proteases from 40% ammonium sulfate precipitation showed protein bands at 25, 50 and 160 kDa and these bands could have contributed to the proteolytic activity of bilimbi proteases. Purification using ammonium sulfate precipitation could be a successful method to purify proteases from bilimbi especially from the unripe stage.

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