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Effects of Precipitation Methods on the Properties of Protease Extracted from Starfruit (*Averrhoa carambola* L.) of Different Maturity Index

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ABSTRACT

Proteases were extracted from starfruit at maturity Index 2 (unripe, light green) and Index 7 (very ripe, orange) and partially purified using acetone and 40% ammonium sulfate precipitations. Higher yield and proteolytic activity were observed for proteases purified using acetone than 40% ammonium sulfate. As for maturity index, yield and protein concentration of proteases from Index 2 were higher than those from Index 7. SDS-PAGE result showed intense bands for acetone proteases while a distinct band at 50 kDa was observed in all the proteases. Enzyme activity decreased during the seven days storage at 4°C with minimum relative activity of 70% achieved for acetone proteases at day seven. This study suggested that acetone precipitation is more effective method for purifying starfruit protease based on the yield and proteolytic activity compared to using 40% ammonium sulphate precipitation. In order to obtain higher protein concentration and proteolytic activity, starfruit at the unripe stage, Index 2 is a better raw material than Index 7 to be used for protease production.

Keywords: *starfruit (Averrhoa carambola L.), protease, purification, acetone, ammonium sulfate*

INTRODUCTION

Starfruit (*Averrhoa carambola L.*), categorized under Oxalidaceae family, is one of the widely grown tropical fruits in Malaysia especially in Selangor,

Negeri Sembilan and Johor. The fruit which is sweet and slightly acidic, succulent and juicy with attractive flesh and distinctive flavor is usually eaten fresh, also served as fresh juices or used as flavor ingredients in juice blends [1].

Proteases are enzymes that breakdown protein. They are classified according to their sources (i.e., animal, plant, microbial), catalytic action (i.e., endopeptidase or exopeptidase) and nature of the catalytic site [2]. Some fruits have already been known to contain high amount of protease, for example in young fruit of papaya (*Carica papaya*); the protease is abundantly found in the latex in the form of papain, chymopapain and papaya peptidase A [3]. Protease is also found in fig (ficin) as well as fruit and stem of pineapple (bromelain) [4]. Proteases are routinely used in cheese making, baking and meat tenderization. Most plant proteases are active over a wide range of pH.

Protease has been purified by several methods including salt precipitation and chromatography [5], three-phase partitioning (TPP) [6], extraction by homogenizing in Tris-HCl buffer followed by purification in ammonium sulfate [7, 8] and extraction using phosphate buffer and subsequent purification with acetone precipitation [9, 10]. In this study, proteases from the unripe and ripe starfruit were extracted, purified using acetone and ammonium sulfate precipitation and the effects of the purification methods on the proteolytic activity, molecular weight distribution and storage stability of the extracted and purified proteases were determined.

MATERIALS AND METHOD

Plant Materials and Chemicals

Starfruit was purchased from Malaysian Agricultural Research and Development Institute (MARDI), Jelebu, Negeri Sembilan. Starfruits with maturity indices 2 (unripe, light green) and 7 (very ripe, orange) were used in this study. All chemicals and reagents used were of analytical grade.

Extraction of Protease from Starfruit

The fruit was cut and the seeds were removed before being ground in a juice extractor. The juice was then filtered through a layer of muslin cloth and stored at 4°C.

Purification of Protease

Two different purification methods were used to purify the crude extract comprising of acetone and ammonium sulfate precipitation. Acetone precipitation was performed according to the method of He *et al.* [9]. Cold acetone (-20°C) was slowly added into the crude extract and the mixture was gently agitated to allow precipitation. This was followed by centrifugation using a centrifuge (Model 5420 Kubota, Japan) at 10,000 rpm for 15 min. The precipitate was then dissolved in phosphate buffer (50 mM), pH 7.2 and dialyzed. Ammonium sulfate precipitation was performed according to Wang *et al.* [7]. One hundred milliliter crude extract was mixed with ammonium sulfate to a concentration of 40% (w/v) followed by 4 hrs incubation at 4°C to precipitate the protease. This was followed by centrifugation at 10,000 RCF for 10 min using a centrifuge (Model 5420 Kubota, Japan). The precipitate was collected and then dissolved in 0.02 M Tris-HCI buffer, pH 7.5 and dialyzed at 4°C for 12 hrs.

Protein Content and Protein Concentration

Protein content in starfruit was determined using Kjeldahl method [11]. Protein concentration was determined using Bradford method [12].

Total Activity of Proteases

Proteolytic activity assay was performed according to the method of Kaneda & Uchikoba [13] with slight modification. Protease at 0.1 mL was added into 0.9 mL of 1% (w/v) casein dissolved in 0.2M sodium phosphate buffer solution at pH 7. The mixture was incubated at 38° C for 20 min. 3 mL trichloroacetic acid (5% w/v) is added. After 30 min, the precipitate was removed by centrifugation at 10,000 RCF for 20 min using a centrifuge (Model 5420, Kubota, Japan). The absorbance of the supernatant was measured at 280 nm using UV-vis spectrophotometer.

Protease Specific Activity

Protease unit per ml divided by protein in mg/ml concentration indicated the specific protease activity [14] where:

$$\text{Specific activity (CDU/mg)} = \frac{\text{enzyme units (CDU)/ml}}{\text{protein in mg/ml}}$$

Molecular Weight Distribution

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with gel electrophoresis unit (Invitrogen Novex, United States) using 12% resolving and 4% stacking gel. 20 µl samples were loaded into each well of the gel and the electrophoresis was then run at 200V for 35 min. The gel was then washed in deionized water, stained in Coomassie brilliant blue and destained until the zones of blue background cleared. Bench mark protein ladder ranging from 10 to 220 kDa was used as the marker.

Effect of pH, Temperature and Storage Stability of Starfruit Protease

Protease was incubated for 24 hrs in different buffers in the pH range of 2.0 to 12.0 before the determination of proteolytic activity. In order to determine the effect of temperature, the protease was incubated at different temperatures in the range of 20 to 80°C at 10°C interval. An aliquot of the protease was analyzed for proteolytic activity. Storage stability of the proteases were determined daily during one week storage at 4°C.

RESULTS AND DISCUSSION

Protein Content, Yield, Protein Concentration and Specific Activity of Starfruit Proteases

Protein content in starfruit was found to be 0.73%, which closely agreed with reports by Ashok *et al.* [15] and USDA [16] where the protein contents are 0.81% and 0.60%, respectively. Proteases purified with acetone resulted in higher yield compared to those purified using 40% ammonium

sulfate (Table 1). In addition, yield of proteases from Index 2 (unripe stage) was also higher than those from Index 7 (ripe stage) for both purification methods which is in-line with protein concentration. In Chaurasiya & Hebbar [17] studies, higher protein was obtained in bromelain extracted from partially ripe fruits (12.15 mg/ml) than the fully ripe fruits (11.75 mg/ml). Their results also showed that as ripening progresses bromelain activity decreases. According to Barraclough *et al.* [18], acetone precipitation is more efficient to concentrate the protein. In starfruit protease study, the efficiency of acetone to precipitate the protein can be seen by the higher yield obtained.

Table 1: Yield (%), Protein Concentration (mg/ml) and Specific Activity (CDU/mg) of Purified Starfruit Proteases Prepared Using Different Purification Methods

Purification Method	Maturity Index	Yield (%)	Protein concentration (mg/ml)	Specific activity (CDU/mg)
Acetone	2	1.50	0.054	5407.90
40% ammonium sulfate	2	0.88	0.065	4883.80
Acetone	7	1.20	0.010	26, 667
40% ammonium sulfate	7	0.65	0.018	14,715.74

Molecular Weight Distribution

Figure 1 shows that protein bands with molecular weight range from 10 to 220 kDa are present in Index 2 proteases purified using both acetone and 40% ammonium sulfate. As for Index 7, there is lesser protein bands, which appear extremely, faint than those of Index 2. Between the two purification methods, acetone proteases showed more obvious bands than 40% ammonium sulfate precipitation regardless of maturity stages while protein band at 50 kDa existed in all the proteases. According to Fleischmann *et al.* [19], enzymatically active protein fractions of *Averrhoa carambola* fruit skin consists of four protein bands ranging from 12 to 90 kDa. Wang *et al.* [7] and Siti Balqis & Rosma [20] obtained a single protein band at 60 and 69 kDa, respectively for bitter gourd and *Artocarpus integer* proteases whereas Chaiwut *et al.* [6], obtained protein bands with molecular weights lower than 18.3 kDa for papaya peel extracts and crude latex. In this study, based on the distinct protein band at 50 kDa, most of

the proteolytic activity of starfruit proteases might have been contributed by protein characterized by 50 kDa. Presence of very faint bands in the ammonium sulfate proteases indicated that protein denaturation might have occurred during the purification process as proposed by Maldonado *et al.* [21], who claimed that the used of different purification methods might affect the physical or chemical environment of specific proteins differently, thus changing the protein stability or solubility. According to Koay & Gam [22], acetone precipitation is a good method to purify and concentrate protein.

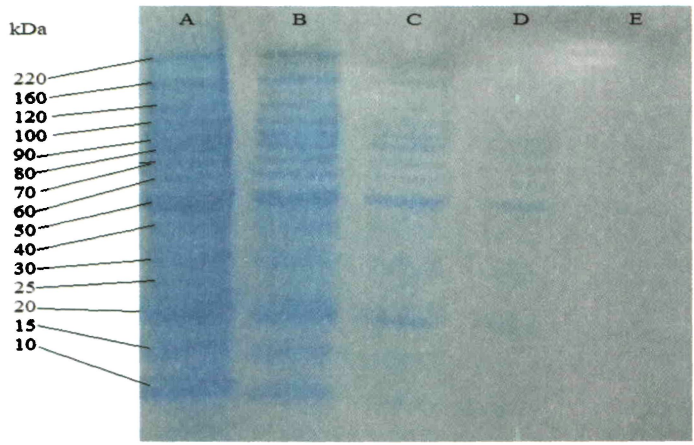

Figure 1: Electrophoretic Profile of Starfruit Proteases at Index 2 and 7 Purified Using Different Purification Methods. A: Protein Marker; B: Index 2 (Acetone Purified Protease); C: Index 2 (40% Ammonium Sulfate Purified Protease); D: Index 7 (Acetone Purified Protease); E: Index 7 (40% Ammonium Sulfate Purified Protease)

Effect of pH on Proteolytic Activity

The proteolytic activity of the Index 2 purified protease was higher compared to the crude extract with the activity range from 100 to 710 CDU for purified protease and 15 to 110 CDU for crude extract while proteolytic activity of acetone purified protease was higher than those purified with 40% ammonium sulfate only up to pH 6 (Figure 2a). Proteolytic activity of crude extract is constant at all pH whereas the purified protease showed maximum activity at pH 8. Higher proteolytic activity of the purified proteases than the crude extract is similarly observed for Index 7 (Figure

2b). In contrary, protease purified with 40% ammonium sulfate was higher than those of acetone up to pH 6. Maximum activity was achieved at pH 8 and 6 for acetone and 40% ammonium sulfate proteases, respectively. pH had no effect on the proteolytic activity of crude extract.

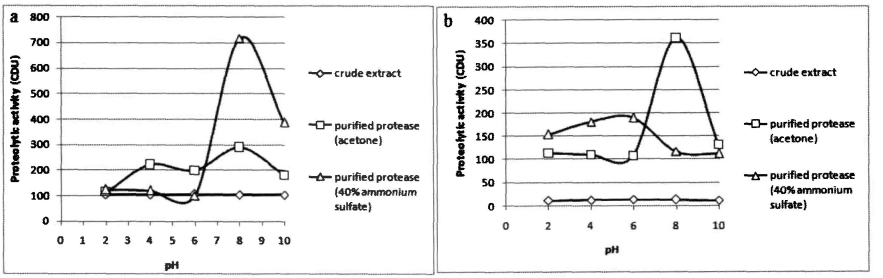

Figure 2: Effect of pH on Proteolytic Activity of Index 2 (a) and Index 7 (b), Starfruit (*Averrhoa carambola L.*) Crude Extract, Protease Purified with Acetone and Protease Purified with Ammonium Sulfate

Maximum bromelain activity of green and ripe pineapple purified using acetone are reported to be at pH 7.5 and 6.5, respectively [23]. Prajapati *et al.* [24] study found that the optimal pH for high enzyme activity for lapsi leaf protease was at pH 7 as compared to pH 8 for the fruit protease. In Bruno's *et al.* [25] study on unripe *Bromelia hieronymi* Mez (Bromeliaceae) purified with cold acetone, the maximum proteolytic activity achieved was at pH 8.5 to 9.5. Corzo *et al.* [26] found that the optimum enzyme activity showed a sharp peak at pH 7.7 for crude bromelain. The use of casein was limited to a neutral to basic pH range because of the low solubility of casein under acidic conditions. Therefore, the optimal pH obtained from an analysis may reflect more on the susceptibility of a substrate to the enzyme at a given pH rather than on the actual activity of the enzyme [20].

Effect of Temperature on Proteolytic Activity

Effect of temperature on protease activity was determined by incubating the starfruit proteases from 20 to 80°C at the interval of 10°C for 15 minutes. Figure 3a shows that the proteolytic activity of the purified proteases are higher than the crude extract while the proteolytic activity of acetone protease is higher compared to those from 40% ammonium sulfate purification method. Maximum activity is achieved at 50°C for acetone protease and 60°C for ammonium sulfate protease. Similar trend is

observed for Index 7 except that the proteolytic activity of protease purified with 40% ammonium sulfate is higher than those from acetone purified protease (Figure 3b). Maximum proteolytic activity is achieved at 60°C for 40% ammonium sulfate purified protease and 50°C for acetone protease.

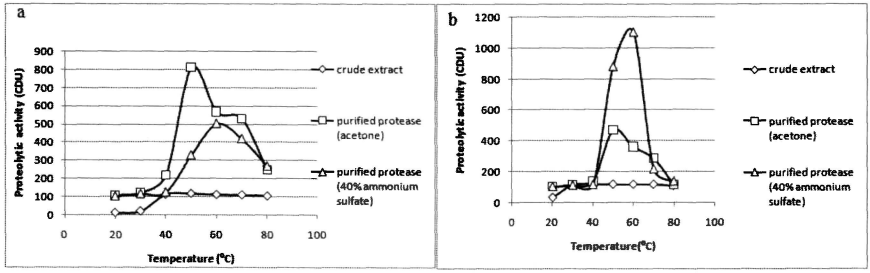

Figure 3: Effect of Temperature on Proteolytic Activity of Index 2 (a) and Index 7 (b), Starfruit (*Averrhoa carambola L.*) Crude Extract, Protease Purified with Acetone and Protease Purified with Ammonium Sulfate

Siti Balqis & Rosma's [20] study on *Artocarpus integer* showed that proteolytic activity steadily increased with the temperature up to 40°C while high activity occurred at 70°C. Priya *et al.* [23] obtained an optimum bromelain activity at 55°C for both unripe and ripe fruit. Corzo *et al.* [26] reported the optimum bromelain activity at 59°C for ripe pineapple. Ibrahim *et al.* [27] study on *Jatropha curcas leaves* protease showed that the optimum temperature for high enzyme activity was at 45°C. Valles *et al.* [28] reported that the optimum bromeliaceae activity was at 60°C for ripe fruits of *Bromelia antiacantha Bertol* while in Asif-Ullah *et al.* [29], the optimum activity for kachri (*Cucumis trigonus* Roxburghi) fruit was at 70°C.

Stability of Proteolytic Activity During Storage

Starfruit proteases of Index 2 maturity stage were incubated for one week at 4°C and the relative proteolytic activity was determined (Figure 4). The proteolytic activity of protease purified with 40% ammonium sulfate rapidly decreased during the first day of storage and slowly decreased thereafter until the relative activity is approximately 57% at day seven. However, for acetone protease, the relative activity remained higher than ammonium sulfate purified protease throughout the seven day storage. Similarly, the activity gradually decreased during storage with relative activity of 70% at day seven. Valles *et al.* [28] reported that the enzyme

activity of the protease from ripe fruits of *Bromelia antiacantha* Bertol (Bromeliaceae) was 100% when stored for 180 days at -20°C. Most probably, if starfruit proteases were stored at temperature lower than 4°C a higher relative activity could be restored and the rate of decrease would be lesser.

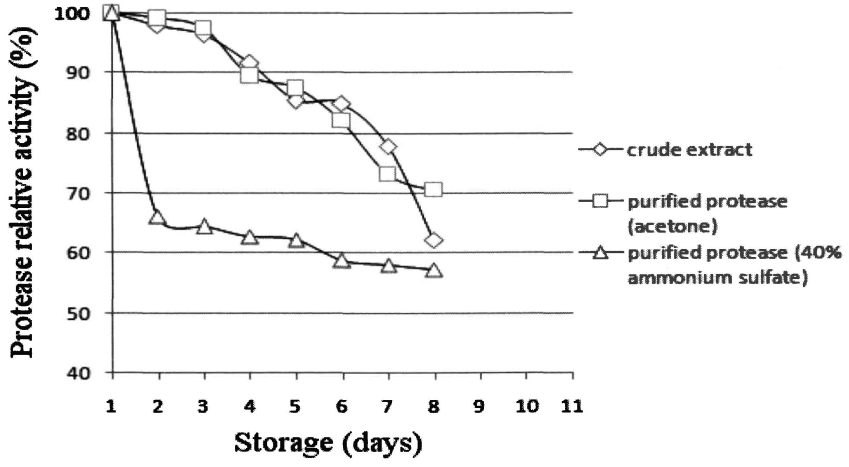

Figure 4: Protease Relative Activity (%) of Index 2 Starfruit (*Averrhoa carambola L.*) Crude Extract, Protease Purified with Acetone and Protease Purified with Ammonium Sulfate During Seven Days Storage at 4°C

CONCLUSION

Protease from starfruit has been successfully extracted and purified by two purification methods comprising of acetone and 40% ammonium sulfate precipitation. Data indicated that the purified protease was stable in the alkaline region with an optimal pH recorded at pH 6 and 8 and at 50 and 60°C. Maturity Index 2 contains higher protein concentration compared to maturity Index 7. The yield for both purification methods ranges from 0.65% to 1.50%. The presence of protein band at 50 kDa indicated that this protein band could have contributed to the proteolytic activity of starfruit protease. The protein bands for protease purified with acetone are more intense compared to those purified with 40% ammonium sulfate. This study suggests acetone precipitation is a better method to purify starfruit protease than ammonium sulfate precipitation. Higher protein concentration and

proteolytic activity in Index 2 than 7 suggested that Index 2 starfruit was a better source of protease than Index 7. Therefore, starfruit may serve as another alternative source of plant protease.

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