Characterization and Purification of Protease extracted from Guava (*Psidium guajava)* peel

*Normah Ismail and Mariyah Faizal

Abstract— **This study was carried out to extract, partially purify and characterise protease from guava peel. The extracted protease was purified using 60% ammonium sulfate precipitation method followed by gel filtration chromatography. The obtained proteases were analysed for protein concentration, proteolytic activity, total proteolytic activity, specific activity, percent recovery, purification fold, molecular weight distribution, optimum temperature and pH. Guava peel contains 7.10% protein. The optimum temperature and pH of the protease was achieved within the range of 40 to 60°C and pH 4 to 6, respectively, where maximum activity identified was 50°C and pH 5. Total activity decreased with the purification steps involving 60% ammonium sulfate precipitation and dialysis but subsequently increased after being subjected to gel filtration chromatography. This study suggested that further purification using gel filtration chromatography increases the proteolytic activity of guava peel protease.**

Index Terms— **Ammonium Sulfate Precipitation, Guava (*Psidium guajava*), Protease, Purification,**

I. INTRODUCTION

Guava (*Psidium guajava)*, grows as a large spreading shrub or a small tree up to 15 metres high. Commonly known as guava, guyava and kuawa, guava is widely cultivated and thought to be a useful medicinal plant [1, 2]. It has been used to make products such as guava juice, candy or even sold in cut form. Guava is usually consumed fresh or processed into canned slices, concentrate, dehydrated products and juice [3,4]. On the average, the fruit contains 74 to 78% moisture, 13 to 26% dry matter, 0.5 to 1% ash, 0.4 to 0.7% fat and 0.8 to 1.5% protein [5]. In addition, guava contains alkaloids, glycosides, steroids, flavanoids, tannins and saponins [6, 7, 8]. India is the world major producer of guava. In Malaysia, Perak is the largest area for guava plantation [9].

Protease is mainly produced from animals, plants and microorganisms with microbial protease production accounts for about 60% of total output throughout the world [10]. Proteases derived from plants are extensively employed in food industries due to its excellent solubility, substrate specificity, activity over a wide pH and temperature range and high stability in extreme conditions [11].

Peel forms a major portion of whole fruit. At present, peels are a waste product and their disposal has become a great problem [12] and this ''waste material'' produces ecological problems related to the proliferation of insects and rodents [13]. According to Joseph and Priya [14], the parts of the guava used are the fruit, leaves, bark, root, seeds and twigs. The fruit is used for direct consumption while the leaves have been found to be a source of antioxidants in addition to having the anti-inflammatory properties. The bark contains polyphenols, resin as well as calcium oxalate crystals [15]. The roots are a source of tannin, leukocyanidins and sterols, gallic acid, carbohydrates, salts and tannic acid [16, 17]. The seeds are a source of proteins, starch, oils, and flavonoid compounds whereas the twigs are a source of calcium, magnesium, phosphorus, potassium, sodium, fluoride, copper, iron, zinc, manganese and lead [15, 18]. Based on this information, guava peel was not emphasized which indicates the possibility that guava peel is not being fully explored. Therefore, this study was carried out to extract, partially purify and characterise protease from guava peel so that the utilization of guava peel waste could be diversified and problem associated with discards from guava peels could be overcome.

II. MATERIALS AND METHOD

Raw materials and chemicals
Guava (*Psidium guajava*) peels were obtained from a cut fruit seller in Shah Alam, Selangor. The chemicals and reagents used were of analytical grade purchased from Sigma Aldrich Sdn Bhd.

Methods

(a) Protease extraction
Protease was extracted according to Amid *et al.,* [11]. Guava peels were initially cut into small pieces and then blended for 2 minutes with a minimum amount of cold (4°C) sodium phosphate buffer (pH 7.5). The obtained crude enzyme extract was then filtered through two layers of muslin cloth followed by centrifugation (KUBOTA, Model 5420, Japan) at 8000 g for 15 minutes at 4°C. The collected supernatant was then purified.

(b) Purification
Purification was performed according to the method by Normah and Nur' Ain [19]. Ammonium sulfate was added up to 60% into the crude extract to form precipitates. The mixture was then centrifuged (KUBOTA, Model 5420, Japan) at 12000 g for 10 minutes. The collected precipitate was then dialysed against Tris-HCL buffer (0.02 M, pH 7.5) overnight. The dialysed enzyme was further purified by gel filtration chromatography by using Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, California). The elution was carried out at a flow rate of 20 mL/h and fractions of 3 mL were collected for an elution profile. Absorbance at 280 nm was measured using a spectrophotometer.

Department of Food Technology, Faculty of Applied Sciences, Universiti Teknologi MARA, Shah Alam, 40450, Selangor, Malaysia

✉ Normah Ismail
   *norismel@salam.uitm.edu.my

(c) Protein concentration

The protein concentration was measured using Lowry method [20]. The absorbance of the mixture was measured at 750 nm using UV-visible spectrometer. A standard curve was constructed from bovine serum albumin (BSA) prepared with concentrations ranging from 0 to 100 (mg/L).

(d) Protein content

Protein content of the guava peel was measured by Kjeldahl method according to AOAC [21].

(e) Proteolytic activity

The protease activity was measured using casein as the substrate [22, 23]. An enzyme solution of 0.1 mL was added to 0.9 mL of 1% (w/v) casein in 0.2 M sodium phosphate buffer solution (pH 7.0) and incubated at 38°C in a heating block for 20 minutes. The reaction was stopped by the addition of 3 mL of 5% (w/v) trichloroacetic acid (TCA) and then centrifuged at 6000 g for 20 minutes. The absorbance of the supernatant was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme catalysing the production of 1 µmol of tyrosine per minute at 38 °C. A tyrosine standard was prepared based on the absorbance obtained.

(f) Total Proteolytic Activity

The total protease activity was determined according to Ahmad *et al.,* [24] as follows:

$$\text{Total activity (U)} = IU \times 100 / V_t \times 1 / 0.1 \times DF$$

IU = µmole tyrosine/minute
V_t = volume of sample in mL
0.1 = volume of enzyme (in mL) used
DF = dilution factor

(g) Specific Activity

The protease specific activity was determined according to Ngo *et al.*, [25].

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

(h) Protease recovery

Protease recovery was determined according to El-Beltagy *et al.,* [26].

$$Percent\ recovery\ (\%) = \frac{Total\ activity}{Total\ activity\ of\ crude\ extract} \times 100$$

(i) Protease Purification Fold

The protease purification fold was determined according to Amid *et al.*, [27] by using the following formula:

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

(j) Determination of optimum temperature

The temperature stability of the purified proteases was evaluated by incubating 1.0 mL enzyme solution at different temperatures ranging from 20 to 90°C with interval of 10°C for 15 minutes. The enzymes were then removed and cooled in an ice bath. The aliquots were utilised to examine the proteolytic activity.

(k) Determination of optimum pH

The pH stability of protease was conducted according to Nadaroglu and Demir [28]. The protease was incubated at different pH ranging from pH 2.0 to 10.0 for 24 h. Glycine-HCl (pH 2.0 to 3.0), acetate buffer (pH 4.0 to 5.0), sodium phosphate (pH 6.0 to 8.0) and glycine-NaOH buffer (pH 9.0 to 10.0) were used.

(l) Determination of molecular weight by SDS-polyacrylamide gel electrophoresis

Molecular weight distribution of the proteases was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [29]. 10µL of simply blue staining was added into the proteases. The solution was then boiled for 10 minutes at 70°C. The electrophoresis was carried out in an Invitrogen NovexBis-Tris gel instrument. A 10µL marker with the range of 10 to 220 kDa was initially loaded into the gel (10x10cm) comprising of 10% resolving and 4% stacking gel. This was followed by loading the sample. The electrophoresis proceeded at 200V constant current for 30 minutes.

(m) Statistical analysis

All tests were conducted in triplicate and the data was averaged. Statistical analysis was carried out using statistical analysis system [30]. The data was subjected to Analysis of Variance (ANOVA). Duncan's Multiple Range Test was carried out in order to compare differences between means at a significant difference of 95% confidence level ($p < 0.05$).

III. RESULTS AND DISCUSSION

Protein content

Protein content was determined by Kjeldahl method according to AOAC [21]. Guava peel contains 7.10% protein. The flesh, seed and juice protein content as reported in the previous studies were 6.80 to 7.00%, 7.90 to 9.60% (dry weight basis) and 7.50%, respectively [31, 32, 33]. Protein concentrations of the proteases are as shown in Table 1. Protein concentration decreased at each purification step with protease obtained after being subjected to gel filtration chromatography having the lowest amount.

Table 1 Protein concentration of guava (*Psidium guajava*) peel at each step of purification

Samples	Protein concentration (mg/mL)
Crude extract	0.96
60% ammonium sulfate precipitation	0.80
Gel filtration chromatography	0.26

Proteolytic activity

Guava peel protease was purified using 60% ammonium sulfate precipitation and further purified using gel filtration chromatography before the analysis of proteolytic activity. Total activity and specific activity increased as the protease was further purified beginning from ammonium sulfate precipitation to gel filtration chromatography. Specific activity increased up to 76.75% after gel filtration chromatography which is from 24.22 U/mg to 104.18 U/mg as shown in Table 2.

Table 2 Proteolytic activity of guava (*Psidium guajava*) peel at each purification stage

Samples	Volume (mL) per 50 g	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	26.5	25.44	614.57	24.16	1.00	100
60% ammonium sulfate precipitation	21.0	16.80	406.85	24.22	1.00	66.20
Gel filtration chromatography	17.0	4.42	460.48	104.18	4.31	74.93

Fig. 1 Effect of temperature on proteolytic activity of guava (*Psidium guajava*) peel protease

In the previous study, polygalacturonase-inhibitory proteins were purified and characterised from guava fruit (*Psidium guajava Linn.)* where the fruit was subjected to gel filtration chromatography [34]. Fraction collected with the highest peak showed specific activity of 3490 U/mg with a purification fold of 28.3 and a recovery of 10.2%. According to a study by Chaiwut *et al.,* [35] on the extraction and three-phase partitioning behavior of proteases from papaya peels, the total protein, total activity and specific activity was higher in dried papaya peels than fresh papaya peels when the proteases were extracted with 50 mM phosphate buffer (pH 7) and water. The total protein, total activity and specific activity for protease extracted with buffer were 48.90 mg, 3450 U, and 70.6 U/mg, respectively whereas extraction with water resulted in 20.93 mg, 2796 U and 70.6 U/mg, respectively. They suggested that upon cutting of peels, the proteases in the fresh material were possibly still in the laticifer as pro-enzymes, however, during drying, these pro-enzymes were transformed to a mature type and subsequently higher proteolytic activity were observed.

Optimum temperature for guava peel protease
Temperature stability for guava peels was determined by incubating 1.0 mL enzyme solution at different temperatures ranging from 20 to 90°C with the interval of 10°C for 15 minutes. The effect of temperature on the proteolytic activity is shown in Figure 1. Proteolytic activity increased gradually from 20 to 40°C before rapidly increasing at 50°C which was identified as the optimum temperature for the protease. However, a slight decline in proteolytic activity was observed to occur at 60°C followed by a sharp decrease at 70°C. This reduction suggested that the enzyme started to denature with the recorded activity of 20U at 90°C. A study on protease from *Artocarpus interger* leaf showed that proteolytic activity increased steadily to a temperature of 40°C [22]. Since the temperature stability of serine protease in mango (*Mangifera indica cv. chokanan*) has a direct correlation with its total activity, an increase in the total activity leads to an increase in temperature stability of the protease [11].
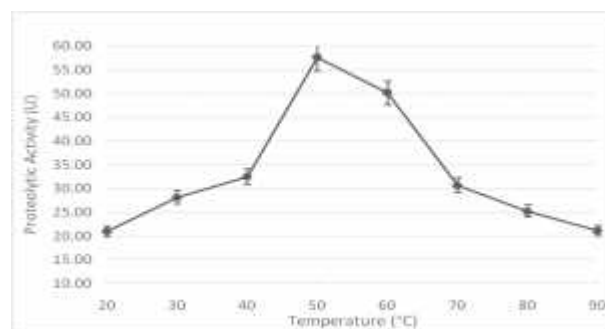
pH stability
The pH range at which an enzyme shows highest activity is called pH stability. Increase or decrease in pH above or below the stability range leads to decrease in enzyme activity [36]. The pH stability of purified guava peel was determined by incubating the enzyme at different pH ranging from pH 2.0 to 10.0 for 24 h. The relationship between pH and proteolytic activity of the enzyme is shown in Figure 2. The proteolytic activities of the protease were extremely low at pH 2 and 3. The proteolytic activity gradually increases up to pH 6 which was the optimum pH for guava peel protease and the enzyme starts to denature gradually before declining sharply at pH 8. Proteases activity depends on the pH [37]. The decline in proteolytic activity could be associated with the loss of activity in the alkaline condition [38]. Guava peel protease was stable between pH 5 to 7. Amid *et al.,* [27] studied the effect of pH on the purification factor of serine protease from mango (*Mangifera indica cv. chokanan*) peels. They obtained a significant increase in purification fold when pH was increased from 6 to 8 where a purification fold of 11.6 was achieved at pH 8.
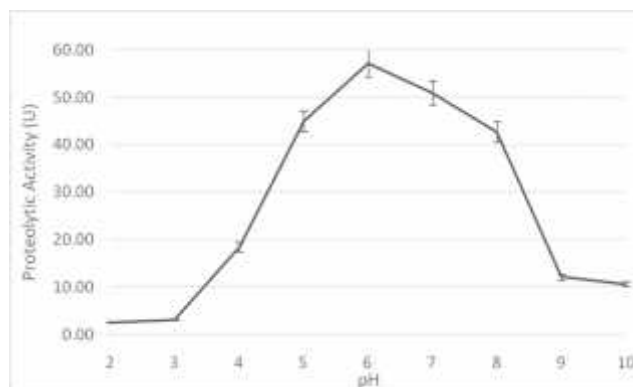

Fig. 2 Effect of pH on proteolytic activity of guava (*Psidium guajava*) peel protease

Molecular weight distribution
The molecular weight distribution is as shown in Figure 3. Guava peel protease purified using 60% ammonium sulfate precipitation showed clear bands between 3 to 188 kDa. The crude extract and dialysed protease also had bands within similar range, however, the bands were unclear. Protein bands for guava peel protease subjected to gel filtration chromatography were very much lesser in number and can hardly be visualised.
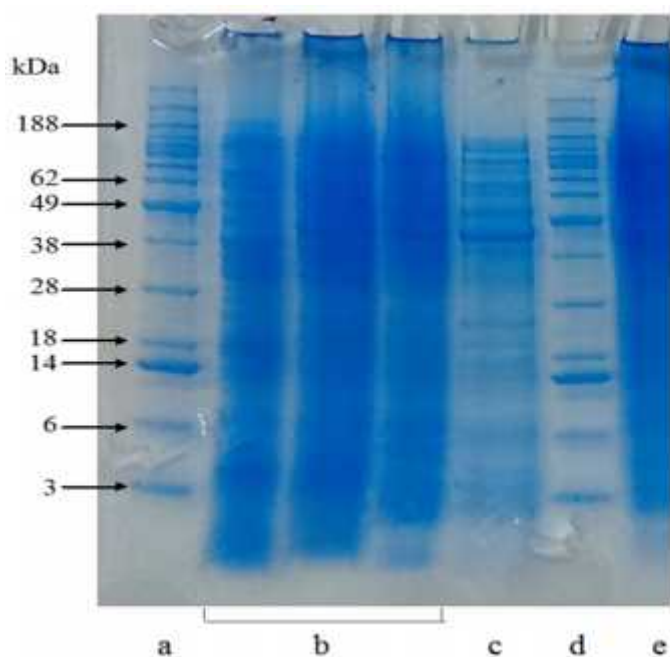
Fig. 3 Electrophoresis pattern of protease extracted from guava (*Psidium guajava*) peel; a) and d) protein marker b) dialysed protease c) protease purified by 60% ammonium sulfate precipitation (e) protease purified by gel filtration chromatography (f) crude extract.

IV. CONCLUSIONS

Proteases extracted from guava (*Psidium guajava*) peels were purified by 60% ammonium sulfate precipitation and gel filtration chromatography. Based on the data obtained, maximum enzyme activity was at 50°C, pH 5. The protease was stable at 40 to 60°C and pH 5 to 7. The protein concentration in the crude extract was higher than those of the other purification levels which was 0.96 mg/mL. For the proteolytic activity of guava peel protease, it was found that the total activity decreased in the purification steps involving 60% ammonium sulfate precipitation and dialysed enzyme but later increased after being subjected to gel filtration chromatography. This study suggested that ammonium sulfate precipitation method could be a useful method to partially purify protease from guava peel.

REFERENCES

[1] M.N. Norazmir, M.Y. Ayub. Malaysian J. Nut. 2010; 16 (1), 171-185.
[2] Y. P. Zheng, K.P. Yuan. Fd. Sci.(China). 2003; 24, 256–259.
[3] M. Amid, C. P. Tan, H. Mirhosseini, N. A. Aziz, T. C. Ling. Fd. Chem. 2011; 124 (2), 666-671.
[4] N. Verma, M. C. Bansal, V. Kumar. Bioresources. 2011; 6 (2), 1505-1519.
[5] A. Bernardino-Nicanor, A. A. Scilingo, A. A. Añón, G. Dávila-Ortíz. LWT-Fd. Sci. Tech. 2006; 39 (8), 902-910.
[6] B. Joseph, M. Priya. Int. J. Pharma and Bio. Sci. 2011; 2 (1), 53-69.
[7] H. M. Burkill. The useful plants of west tropical Africa. Royal Bot. Garden Kew, 1997; 138-140.
[8] M.M. Iwu. Handbook of African Medicinal Plants. CRC Press. 1993.
[9] E. Quisumbing. Medicinal Plants of the Philippines. Manila: Katha Pub. Co. 1978.
[10] D. E. Okwu, O. Ekeke. Global J. Pure and App. Sci. 2003; 9 (2), 235-238.
[11] I. Normah, M. K. Nur' Ain. 2013; Scientific Res. J., 10 (2):29-50.
[12] Pierre, S., Toua, V., T. Fernand-N, N.N. Alexandre-Michel, M. Jean. J. Ecol. Nat. Env. 2011; 3(3), 104-117.
[13] O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall. J. Bio. Chem. 1951; 193(1), 265-275.
[14] AOAC (Association of Official Analytical Chemist) (1990). Official methods of analysis (15th ed.). Washington, VA: AOAC.
[15] Z. Siti Balqis, A. Rosma. Fd. Chem. 2011; 129 (4), 1523-1529.
[16] M. Kaneda, K. Arima, H. Yonezawa, T. Uchikoba. Phytochem. 1994; 35 (6), 1395-1398.
[17] M. N. Ahmad, S. L. Liew, M. A. Yarmo, M. Said. Biosci., Biotech. Biochem. 2012; 76 (8), 1438-1444.
[18] L.T.A. Ngo, T.L. Pham, V.V.M. Lee. Int. Fd. Res. J. 2008; 15, 135-140.
[19] A. El-Beltagy, T. El-Adawy, E. Rahma, A. El-Bedawey. Fd. Chem. 2004; 86, 33-39.
[20] M. Amid, M. Shuhaimi, M. Z. I. Sarker, M. Y. A. Manap. Fd. Chem. 2012; 132 (3), 1382-1386.
[21] H. Nadaroglu, N. Demir. Euro. Fd. Res. Tech. 2012; 234 (6), 945-953.
[22] U. K. Laemmli. Nature. 1970; 227 (5259), 680-685.
[23] J. Jagtiani, H. T. Chang, W. S. Sakai. Guava. In Tropical Fruit Processing, 1988; Academic Press, New *York*.
[24] SAS Institute Inc. SAS user's guide to the statistical analysis system. 2004; SAS Institute Inc. Raleigh, NC.
[25] D. O. Ali, A. R. Ahmed, E. B. Babikir, B. Gangaiah, H. Singh, B. G. Shivakumar, A. J. Dass. Agri-Fd. App. Sci. 2014; 2(2), 27-30.
[26] Y. P. Chang, M. P. Tan, W. L. Lok, S. Pakianathan, Y. Supramaniam. Plant Foods for Human Nut. 2014; 69 (1), 43-49.
[27] V. Patil, A. K. Chauhan, R. P. Singh. Powder Tech. 2014; 253, 230-236.
[28] A. Deo, N. V. Shastri. Plant Sci. 2003; 164 (2), 147-156.
[29] P. Chaiwut, P. Pintathong, S. Rawdkuen. Process Biochem. 2010; 45 (7), 1172-1175.
[30] A. B. Bello, M. S. Sule, A. J. Alhassan, J. Bayero. Pure App. Sci. 2011; 4 (1), 26-31.
[31] R. A. van der Hoorn. Annual Rev. Plant Bio. 2008; 59, 191-223.
[32] J. Smith-Marshal, K.D. Golden. J. Scientific Res. 2012; 4 (2), 445-456.
[33] D. M. Kadam, P. Kaushik, R. Kumar. Int. J. Fd Sci. Nut. Eng. 2012; 2 (1), 7-11.
[34] H.F. Chin, H.S. Yong. Malaysian food in colour. Tropical Press, 1980; Kuala Lumpur, Malaysia.
[35] E. J. Cho, T. Yokozawa, D. Y. Rhyu, S. C. Kim, N. Shibahara, J. C. Park. Phytomed. 2003; 10 (6), 544-551.
[36] K. R. Narayana, M. S. Reddy, M. R. Chaluvadi, D. R. Krishna. Indian J. Pharmacology. 2001; 33 (1), 2-16.
[37] Y. A. Geidam, A. G. Ambali, P. A. Onyeyili. Int. J. Pharmacology. 2007; 3 (1), 68-73.
[38] L. T. Kwee, K. K. Chong. Guava in Malaysia. In Production, Pests and Diseases. Tropical Press. 1990; Kuala Lumpur, Malaysia.