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Isolation of Proteolytic Enzyme from Pineapple Crown

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ABSTRACT

The pineapple waste from the pineapple industry has contributed to an increase in waste in Malaysia and worldwide every year. A major type of endopeptidase enzymes found in pineapple is fruit bromelain, stem bromelain, ananain, and comasain. This study aims to extract and purify protease from the crown of MD2 pineapple. Protease was extracted and purified using anion exchange chromatography, gel filtration, and desalting before being identified using liquid chromatography-mass spectrometry (LC-MS). Proteolytic activity was determined using the well diffusion method and Casein Digestion Unit. In the present study, the proteolytic assay showed that 1 kg crown of MD2 cultivar produced an activity of 126.0 ± 3.86 U/ml, a specific activity of 3937.50 U/mg. In the present study, the proteolytic assay showed that 1 kg crown of MD2 cultivar produced an activity of 126.0 ± 3.86 U/mL, a specific activity of 3937.50 U/mg and the total activity of 3.94×10^9 U. The molecular weight of the purified enzyme was in the range of 25 to 35 kDa under the optimum condition of pH 7 and 37°C. Purification of the extract yielded a band at the molecular weight of 20–25 kDa at the optimum pH of 3 and 9 at 60°C. From LC-MS analysis, the purified enzyme from the crown extract was similar to ananain under accession number A0A199VSS3 (according to Uniprot). It had five unique peptides and covered 97/356 amino acids (44.9% coverage). The ananain (EC 3.4.22.31) is classified in the subfamilies of cysteine protease C1A (clan CA, family C1), a peptidase family related to papain. In conclusion, protease was extracted and identified as an ananain-like protease from the crown.

Keywords: Ananain, bromelain, crown, MD2, proteolytic activity

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INTRODUCTION

Pineapple (*Ananas comosus*), locally known as 'nanas,' is a member of the Bromeliaceae family. It is the most common fruit cultivated worldwide, particularly in China, Indonesia, Malaysia, Thailand, Kenya, the Philippines, and India (Ramli et al., 2017). Today, world pineapple production continues to increase yearly, including in Malaysia. Three common cultivars that are commercially cultivated in Malaysia are Spanish (also known as Josapine, Maspine, and hybrid pineapple), Queen, and Smooth Cayenne (Sarawak pineapple) (Hidayat et al., 2012). The introduction of MD2 (hybrid variety) into the pineapple market in Malaysia has increased demand and a gradual shift from the export of Smooth Cayenne to MD2. Among pineapples, MD2 and N36 are cultivated for export due to their longer shelf life, whereas Moris, Sarawak, and Josapine are grown for the local market. Therefore, pineapple cultivars MD2 are a benchmark in developing future cultivars for fresh consumption. The MD2 pineapple weight is uniform, approximately 1.5 kg each, and has a shelf life of 30 days, like papaya (Falah et al., 2015; Thalip et al., 2015).

According to the Malaysia Pineapple Industry Board (MPIB), in 2017, Malaysia had a total cultivation area of 13,733 ha with a production of 432,812 metric tonnes and import of USD 24 million, while exports of USD 47 million for all varieties of pineapple (Lembaga Perindustrian Nanas Malaysia [LPNM], 2018). After durian and banana, the pineapple industry is one of Malaysia's most important fruit sectors. The production is expected to reach 1.7 million metric tonnes by 2025. For MD2, the production has increased and reached 3,600 metric tonnes by the end of the year 2017. The MD2 pineapple production at 3,800 metric tonnes by 2019 saw an increase of 200 metric tonnes. Malaysia currently targets USD 42 million to export pineapple products, with a total production of 253,227 metric tonnes by 2023. Three hundred sixty hectares of MD2 pineapple plants are being grown commercially in Ulu Tiram, Kluang, and most recently, in Tanah Abang, Mersing, Johor. The plan was to expand the crop to 1,000 ha by 2021. The aim is to produce 100 containers together with 1,800 tonnes of pineapple per month, including 50 containers or 900 tonnes of pineapple would be exported. Growing pineapple crops in Malaysia generates millions of tons of plant waste. During harvesting activities, a large amount of leftover from pineapple growing, known as agro-waste, is created in the agricultural process. According to Nor et al. (2015), the waste proportions are twice the proportion of fruit for the flesh, core, peel, and crown of the Smooth Cayenne pineapple. In addition, approximately one billion tons of agricultural waste are produced globally, with 1.2 million tonnes of agro-waste disposed of into landfills in Malaysia each year (Neh & Ali, 2020). In Malaysia, agricultural waste is projected to be 0.210 ($kg \cdot cap^{-1} \cdot day^{-1}$) in 2025 (Ngoc & Schnitzer, 2009). Generally, harvest leftovers from crops such as pineapple waste are usually just burnt or left to rot, releasing carbon dioxide and methane gas

into the atmosphere. Recovery of nutrients from fruit processing wastes could minimize environmental issues (Mirabella et al., 2014). It was revealed that some parts of the pineapple waste, such as the core, crown, and peel, were sources of protease enzyme (Ketnawa et al., 2012).

Pineapple waste has been discovered as a possible source of proteases like bromelain (Abreu & Figueiredo, 2019). Bromelain comprises proteases such as fruit bromelain (EC 3.4.22.33), stem bromelain (EC 3.4.22.32), ananain (EC 3.4.22.31), and comosain, whereas non-proteases components consist of carbohydrates, peroxidases, cellulases glucosidases, glycoproteins, and phosphatases (Sahoo & Das, 2017). Therefore, it suggested that protease enzymes extracted from pineapple can be utilized to fulfill the demand in industrial processes, including the foods, textile, and medical cosmeceutical industries. Protease enzyme has been shown to aid in the tenderization of meat (M. S. Arshad et al., 2017), the relaxation and suppression of shrinkage in baking industries (Sahoo & Das, 2017), the improvement of silk and wool quality (Z. I. M. Arshad et al., 2014), and the removal of tooth stains in tooth whitening products (Munchow et al., 2016).

In addition, the topical application of protease for the debridement of skin wounds and burns has been introduced (Muhammad & Ahmad, 2017). It acts as a fibrinolytic agent, which increases fibrinolysis by activating the conversion of plasminogen to plasmin (Kwatra, 2019), an anti-cancer agent by suppressing carcinogenesis during the cancer development (Rathnavelu et al., 2016), and an anti-inflammatory agent (Barrera-Núñez et al., 2014). As a result, there is an increasing demand to exploit the sustainable utilization of pineapple wastes for industrial applications that can be commercialized as an environment-friendly alternative for carbon source utilization. However, enzyme purity is necessary for industrial and commercial applications (Nor et al., 2015). Since a high degree of purity is required, entire downstream processing is necessary, including extraction, purification using ion-exchange chromatography, and identification of purified enzyme. Therefore, pineapple stems, fruits, and waste products such as crowns, leaves, peel, and core are extracted and purified with different homogenization processes and the addition of different extraction buffers to produce beneficial products (Wan et al., 2016; Youryon et al., 2018).

MATERIALS AND METHODS

Plant Materials

Six pineapple wastes (crown and stem) from different cultivars were brought from a local market at Taman Universiti, Skudai, Johor, Malaysia (Figure 1). The six pineapple cultivars included Madu Kaca, Josaphine, Morris, Yankee, N36, and MD2. All the pineapples were verified by Hj Musliman bin Tasim from Lembaga Pekan Nanas Malaysia (LPNM). The wastes (crown and stem) were directly taken to the laboratory (Plant Biotechnology Lab, T02 UTM) for enzyme assay. However, the wastes (MD2 crown) were taken from LPNM,

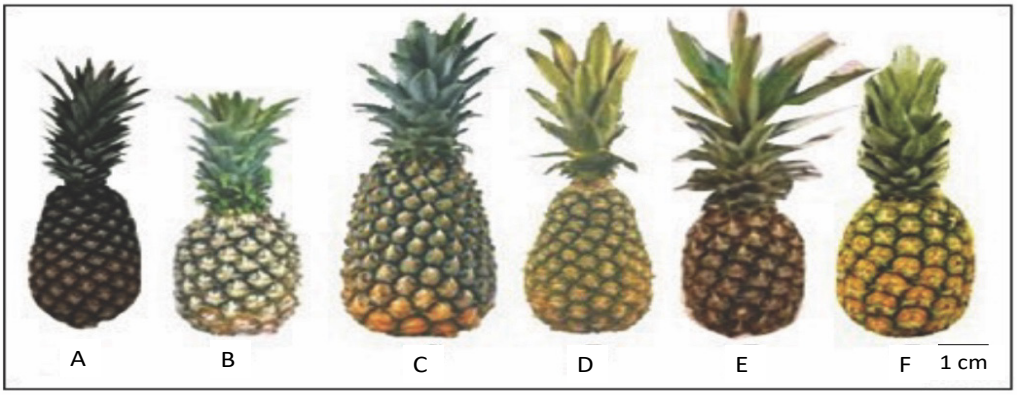

Figure 1. Six pineapple cultivars: (A) Madu Kaca; (B) Josaphine; (C) Morris; (D) Yankee; (E) N36; and (F) MD2

Pekan Nanas, to the laboratory (Plant Biotechnology Lab, T02 UTM) a day before the purification step. All the samples were kept at room temperature.

Preparation of Crude Protease Extract from the Pineapple Waste

A known quantity (1 kg) of each crown was ground into a fine piece using a blender (Panasonic MX-GM1011 G, Malaysia). Then 100 mM sodium acetate buffer solution (pH 7.0) (Bio-Rad, USA) was added and left at room temperature for 3 min. Next, the mixture was filtered through a filter (Minisart PES 28, Sartorius, Germany) and centrifuged at 10,108 x *g* for 20 min at 4°C. After that, the supernatant (crude enzyme extract) was collected, measured, and stored at -20°C before the experiment.

Determination of Proteolytic Activity Using Casein Digestion Unit (CDU)

The CDU method determined the enzyme activity (Murachi, 1976). This study chose casein as substrate, and L-tyrosine was used as standard. In this method, the proteolytic activity of hydrolyzed casein was measured. One unit of proteolytic activity was defined as 1 g of tyrosine released in 1 min/ml of a sample when casein was hydrolyzed under the standard conditions at 37°C and pH 7.0 for 10 min. The activity of the protease was estimated by the following Equation 1:

$$Proteolytic\ activity\ \left(\frac{Units}{ml}\right) = \frac{\mu moles\ of\ tyrosine\ \times\ Reaction\ vol}{Sample\ vol\ \times\ Reaction\ time\ \times\ Vol\ assayed} \quad (1)$$

Determination of Proteolytic Activity Using Well Diffusion Method

The enzyme activity is used well diffusion as described by Vijayaraghavan and Vincent (2013) with a minor modification. About 1% (w/v) casein skimmed milk (Sunlac, New Zealand) was added into the agar and autoclaved at 121°C for 15 min. Then, the agar was poured into disposable Petri dishes and solidified for 30 min. Approximately 8 mm diameter was punched into the solidified agar using tip collars of 1 ml pipette tips. A 70 µl crude supernatant volume was pipetted

into the holes and incubated overnight at 37°C. A 70 µl of acetate buffer (Bio-Rad, USA) was used as a negative control.

Protein Concentration

The concentration of the crude enzyme extract was determined as described by the Bradford method (Bradford, 1976) and bovine serum albumin (BSA) as a standard. Coomassie Brillant Blue G-250 (Bio-Rad, USA) was a dye bound to an unknown protein and formed a complex detected spectrophotometrically at 595 nm.

Specific Activity of Enzyme

The specific activity of the enzyme was determined by the following formula and expressed as U/mg protein.

$$Specific\ activity\left(\frac{Units}{mg}\right) = \frac{Proteolytic\ activity}{Protein\ concentration} \quad (2)$$

Purification of Protease Enzyme

The purification steps were constructed according to Costa et al. (2014). However, the buffers used in each stage of purification, on the other hand, are slightly different. Firstly, the resuspended extracts were transferred into centrifugal filter devices (Amicon Ultra-15 10K, Merck, Germany) for fast ultrafiltration and centrifuged at 1,792 x *g* for 20 min at 4°C. The concentrate was collected from the filter device sample reservoir using a pipette. The extract was concentrated using the Amicon Ultra-15 (Merck, Germany) before being loaded onto a 5 ml HiTrap Q HP (5 × 5 ml) (Cytiva, USA) column pre-equilibrated with buffer A (50 mM Tris hydrochloride [Tris-HCL] buffer, pH 8.0) (Bio-Rad, USA). The protein was eluted using buffer B (50 mM Tris-HCL, pH 8, and 1 M sodium chloride (NaCl) (Bio-Rad, USA). The eluate was collected in a 96-deep well plate container after elution at a flow rate of 2 ml/min. Aliquots of anion exchange chromatography were collected and subjected to gel filtration chromatography purification. The Sephadex 200 column (Merck, Germany) was prepared with pH 8 buffer (25 mM Tris + 150 mM NaCl + 0.5 mM ethylenediamine tetraacetic acid (EDTA) (Bio-Rad, USA). The sample was eluted at a flow rate of 0.5 ml/min. The purified enzyme was loaded onto a 5 ml HiTrap Q HP (5 × 5 ml) (Cytiva, USA) equilibrated with sodium acetate buffer (pH 7) (Bio-Rad, USA) and then eluted at a flow rate of 3 ml/min. All procedures were conducted at 4°C. The desalted enzyme was stored at 4°C.

Determination of Protein Molecular Weight by SDS-PAGE

The sodium dodecyl-sulfate polyacrylamide gel (SDS-PAGE) electrophoresis estimated the molecular weight of the purified protein with different gel concentrations of 12 and 15% (w/v). First, each 20 µl protein sample was mixed with 10 µl loading buffer (Bio-Rad, USA) comprising 0.2 M Tris-HCl of pH 6.8, 20% (v/v) glycerol, 10% (w/v) SDS, 10 mM beta-mercapto-ethanol, and 0.05% (w/v) bromophenol blue. Then, the protein sample was heated at 95°C for 15 min. Then,

the protein sample was electrophoresed at 150 V for 120 min and stained with 0.02% Comassive Brilliant Blue G-250 (Bio-Rad, USA) for 30 min. Finally, the gel was detained with a mixture of acetic acid and methanol solution for 1 hr till the bands were visible. The molecular weight markers used were Precision Plus Protein™ Dual Color Standards (Bio-Rad, USA).

pH and Temperature Characterizations

The pH profile of protease was determined by assaying its proteolytic activity at various pHs ranging from 3 to 10 according to the previous method (Ketnawa et al., 2012). The residual proteolytic activity was measured and expressed as the relative proteolytic activity. In addition, the effect of temperatures (30, 37, 40, 50, 60, 70, 80, and 90°C) on proteolytic activity was performed for 10 min. All assays were carried out using casein as the substrate. The caseinolytic activity was expressed as the relative proteolytic activity compared to the control.

LC-MS

Amino acid sequence analysis was recorded using LC-MS. The purified enzymes were digested with ammonium bicarbonate which consisted of 12.5 ng/µl mass spectrometry grade Trypsin Gold. The Liquid Chromatography analysis was carried out in Dionex Ultimate 3000 RSLCnano, and full scan spectra were collected using orbital MS (OTMS) following the parameters of scan range of 310–1800 m/z, resolving power of 120000, AGC target of 4.0 e5 (400 000), and maximum injection time of 50 ms. LC-MS data were investigated using Thermo Scientific™ Proteome Discoverer™ 4 Software Version 2.1.

Phylogenetic Analysis

Five peptide sequences obtained from LC-MS analysis were subjected to sequence analyses using National Center for Biotechnology Information (NCBI-Bethesda, Maryland, USA) BLASTP. Then, the full sequences of ananain of *Ananas comosus* under the accession number AOA199VSS3 were retrieved from the NCBI Genbank database. Ten sequences with 100% sequences identity (AOA199UL32, AOA199UUY2, AOA199W8N4, AOA199VSB5, AOA199W3D2, AOA199W9F2, AOA199W8R4, O24641, and P0074.8) to AOA199VSS3 were aligned using Clustal Omega. Ten aligned sequences were used to construct the phylogenetic tree using the MEGA-X version software. The domain was identified using InterProScan against multiple databases, including Pfam, PRINTS, SMART, PROSITE, ProDom, Panther, GO lookup, TMHMM, and SignalP_EUK with pathway.

Statistical Analysis

All data obtained from three or more independent variables were analyzed using One-way ANOVA of Statistical Package for Social Science (SPSS) version 22. The Bonferroni method evaluated differences between means of treatments by the post-hoc test. The test was used to compare the means at a significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Proteolytic Activity in Crown and Stem of Pineapple from Different Cultivars

Major endopeptidase enzymes found in bromelain extract are stem bromelain (EC 3.4.22.32), fruit bromelain (EC 3.4.22.32), ananain (EC 3.4.22.31), and comasain (Kahiro et al., 2017; Ketnawa et al., 2012). These enzymes were found in pineapple fruit and industrial waste, such as the crown, core, leaves, and peel (Z. I. M. Arshad et al., 2014). However, each extract's enzyme activity and protein content are influenced by different proteolytic enzymes (Ketnawa et al., 2012). This study revealed that the MD2 crown obtained higher proteolytic activity than the MD2 stem, and different protease enzymes were found in both the crown and stem extracts.

The extracts from the MD2 crown (A) recorded higher proteolytic activity than stem (B), with values of 126.27 ± 3.86 and 118.5 ± 1.19 U/ml in 100 g of pineapple wastes, respectively (Figure 2). This study recorded that the MD2 crown exhibited a higher yield than the MD2 stem (p<0.05). The MD2 crown also showed a larger diameter of the halo zone in casein and skim milk plates, where it was 9.3 mm and 11.0 mm, respectively (Figures 3A and 3C). The halo zone diameters of the stem in the casein and skimmed milk plates were 6.0 mm and 9.7 mm, respectively (Figures 3B and 3D). The resulting halo zone may be related to the amount of protease presented in the crude extract. Similar findings also showed that the crown of *Ananas comosus* L. contained higher proteolytic activity compared to other parts (Hebbar et al., 2008, 2011, 2012; Kahiro et al., 2017; Ketnawa et al., 2012; Neta et al., 2012; Z. I. M. Arshad et al., 2014). According to Neta et al. (2012), proteolytic activity depends on the part of the plant from which the sample was extracted.

Furthermore, some researchers also found that crown extracts from both cultivars (*Nang Lae* and *Phu Lae*) contained bromelain as the major protease enzyme (Ketnawa et al., 2012; Neta et al., 2012)—pineapple cv. *Vitória* has high proteolytic activity than other cultivars (Costa et al., 2014). *Ananas comosus* also showed the highest proteolytic activity than other varieties of pineapples (Chaurasiya & Hebbar, 2013; Martins et al., 2014). In addition, the expression of the proteolytic enzyme was influenced by environmental conditions such as salt concentration, temperature, soil moisture, and agroecological zone (Kahiro et al., 2017). Thus, it shows that the proteolytic activity varies depending on the cultivar and the part of the examined and extracted plant. The MD2 cultivar showed the highest proteolytic activity in this study, with the crown being the best part. The MD2 crown was purified for further characterization. In conclusion, cultivars and different parts of plant extracts gave differences in enzyme activity and protein content.

Purification of Protease Enzyme

Protease purification using anion exchange chromatography yielded 147.09 U/ml of proteolytic activity, 171.03 U/mg of specific activity, and a purification

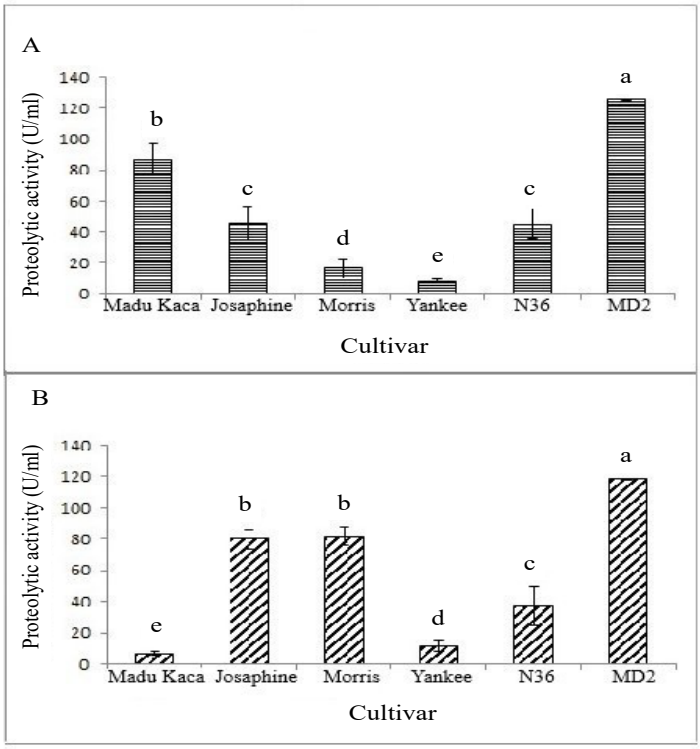

Figure 2. Comparison of proteolytic activity (U/ml) in (A) crown and (B) stem of different pineapple cultivars

Note. Different alphabetical letters indicate the significant differences (p>0.05)

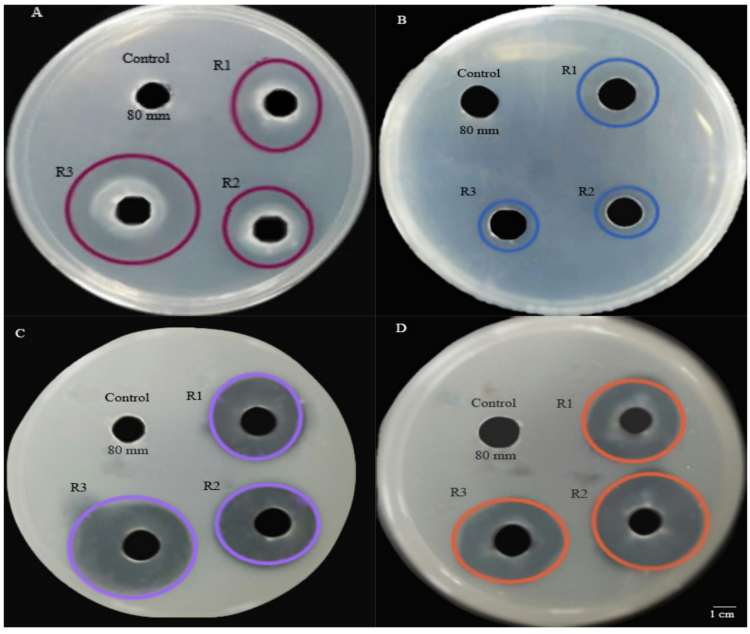

Figure 3. Halo zone formation on casein plate by extracts from the crown (A) and stem (B) and halo zone formation on skim milk plates by extracts from the crown (C) and stem (D)

fold of 4.33 (Table 1). The proteolytic and specific activities were higher than previously reported bromelain purification by Costa and co-workers (2012). They reported the purification of bromelain from carboxymethyl cellulose (CMC) that exhibited specific enzyme activity with a purification fold of 69.50 U/mg and 3.01, respectively. It indicated that the purified protease in this study showed higher proteolytic activity than other reported pineapple proteases using anion exchange chromatography. The high proteolytic activity obtained in this study could be attributed to using 50 mM Tris-HCl buffer (pH 8) instead of 1 M acetate buffer (pH 4.5) at the ion exchange chromatography step. Novaes et al. (2016) reported that bromelain exhibited an enhanced capacity to bind to acid 8-aniline-1-sulfonic acid without tertiary contact at pH 8. Optimization of the extraction conditions is necessary to extract the protease enzyme from the main contaminants, thus greatly enhancing the purity of the enzyme extracted (Vicente et al., 2016).

Table 1

Purification steps of crown extract

Steps	Vol (ml)	Total protein (mg)	Total activity (Units)	Protein concentration (mg/ml)	Proteolytic activity (U/ml)	Specific activity (U/mg)	Fold purification	% yield
Step 1 (After crude extract)	200	640	25200	3.20	126.27	39.46	1.00	100
Step 2 (After anion exchange chromatography)	150	129	22060.50	0.86	147.09	171.03	4.33	0.87
Step 3 (After gel filtration chromatography)	0.50	0.24	75.47	0.48	150.90	314.38	1.84	0.03
Step 4 (After desalt)	0.50	0.42	72.01	0.84	144.02	171.45	0.55	0.95

After ion exchange chromatography, fractions pooled I (fractions D12-E6), II (fractions 2 A10-2B9), and III (fractions 2 C12-2 D9) (Figure 4) were used for the gel filtration chromatography step. The gel filtration chromatogram showed that the C10/C11 peak was eluted at 17 ml (Figure 5). After the gel filtration chromatography step, the concentration, proteolytic activity, and specific activity of the purified enzyme were 0.48 mg/ml, 150.90 U/ml, and 314.38 U/mg, respectively (Table 1). Based on these results, high solution concentrations make the enzyme more stable, resulting in decreased proteolytic activity, whereas low concentrations make the enzyme less stable, resulting in increased proteolytic activity. According to Hale (2005), low bromelain concentrations are more susceptible to spontaneous inactivation of proteolytic activity than concentrated bromelain solution. In addition, stem bromelain

was more stable than fruit bromelain and ananain. Plus, the higher number of steps involved in purification could remove more natural protease enzyme inhibitors, causing activity to rise (Bala et al., 2012).

Initially, during the purification process, it was observed that purified protease did not show a clear and distinct band on SDS-PAGE gel even though proteolytic activity was detected when assayed. It indicates that the purified proteases were actively being degraded and most likely were self-degrading. During gel filtration chromatography, ethylene diamine tetra acetic acid (EDTA) was added to the buffer to chelate metal ions by forming a stable complex with divalent ions, such as calcium ions (Ca^{2+}). EDTA is a cation-chelating agent that eliminates essential cations required for proteases. That way, EDTA inactivates protease activity so that the purification process will proceed with low or negligible proteolytic degradation, and the purified enzyme can be detected on an SDS-PAGE gel. However, EDTA must be removed before catalytic activity characterization so the protease can be activated again. Removal of EDTA from the purified sample was achieved by using desalting chromatography.

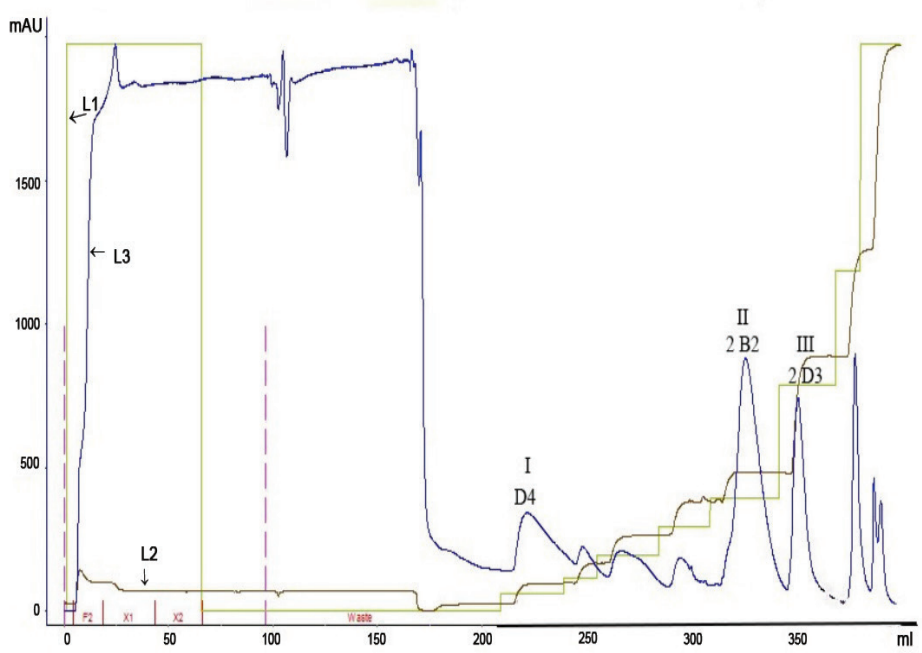

Figure 4. Anion exchange chromatography using step gradient for the crown extract. Green (L1), brown (L2), and blue (L3) lines represent the percent concentration of buffer B (%), the conductivity of protein (mS/cm), and protein sample flow. Y-axis represents the intensity of absorbance (mAU), and the x-axis represents the volume of the sample (ml)

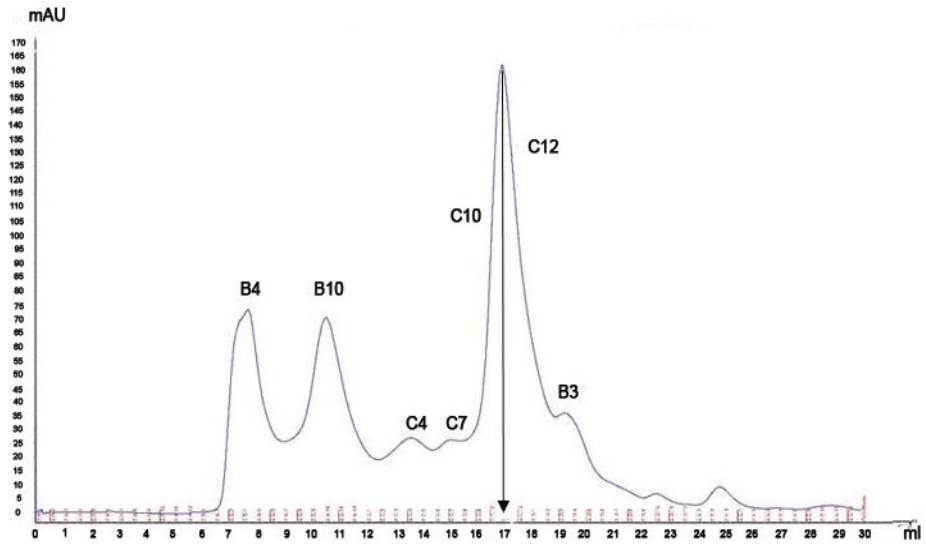

Figure 5. Gel filtration chromatography of crown extract. Y-axis represents the intensity of absorbance (mAU), and the x-axis represents the sample volume (ml). The arrow indicated the elution of purified enzyme at 17 ml

After desalting chromatography purification, the purified enzyme's proteolytic activity and specific activity decreased to 144.02 U/ml and 171.45 U/mg, respectively. The reduced activity could be due to the loss of enzyme during the desalting chromatography step and the inhibitory effect conferred by residual EDTA that may still be in the sample. Figure 6A depicts SDS-PAGE with 15% acrylamide for improved separation. Lane C was the crude sample, D4, D3, and B2 were from anion exchange chromatography peaks, and C10/11 was from gel filtration chromatography. The C10/C11 fraction has high protein band intensities with 25–32 kDa MW. The purified enzyme was then desalted, and a single band on SDS-PAGE in Lane I showed an MW of 25–32 kDa (Figure 6B). It demonstrates that it is a single polypeptide, and the protease was purified to apparent homogeneity. According to certain studies, protease enzyme isolated from the crown has a molecular weight of 25–27 kDa and a more intense band of 28–30 kDa (Bresolin et al., 2013; Ketnawa et al., 2012; Krishnan & Gokulakrishnan, 2015).

pH and Thermal Profiles

Based on a comparison among cultivars, crowns from the MD2 cultivar showed the highest proteolytic activity. Thus, the purified protease enzyme from the MD2 crown was used for further characterization. The effect of pH on proteolytic activity from the crown was measured and expressed as a relative protease activity against control. Purified protein from the MD2 crown exhibited the highest proteolytic activity at pH 7. Lower proteolytic activity was detected in acidic conditions (pH 4–5) and alkaline conditions (pH 8) (Figure 7). The

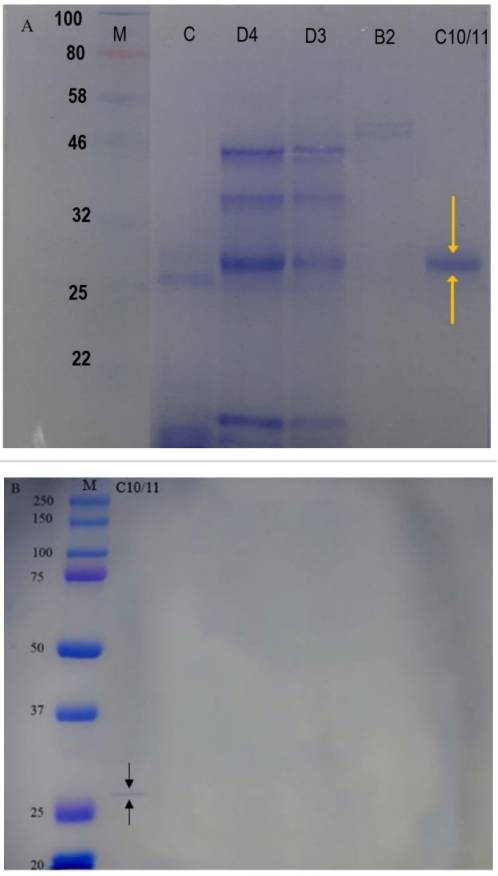

Figure 6. (A) 15% SDS-PAGE; C is the crude extract, D4, D3, and B2 are from anion exchange chromatography and C10/11 from gel filtration. (B) Desalting step of purified protein in the lane I. The arrow shows the band of the purified enzyme

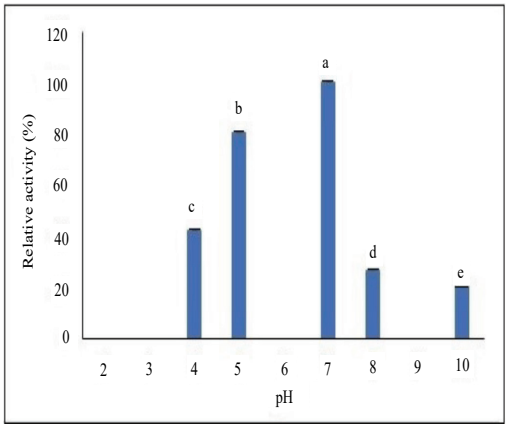

Figure 7. Effect of pH on the proteolytic activity of purified protein extracted from crown MD2 cultivar

Note. Different alphabetical letters indicate the significant differences (p>0.05)

major components of protease enzyme in pineapple were stem bromelain (80%), fruit bromelain (10%), and ananain (5%) (Kahiro et al., 2017). The types of protease enzymes depend on the extracted part of the fruit. Some researchers found that the activation of protease enzyme was at the pH range of 3–7 for all parts of pineapple and showed high proteolytic activity and specific activity at pH 7 and gradually declining after attaining the optimum pH (Ketnawa et al., 2012; Krishnan & Gokulakrishnan, 2015; Novaes et al., 2016; Omotoyinbo & Sanni, 2017). However, the proteolytic activity of fruit bromelain was found to be optimum at pH 4.5 with 4.429 U/ml (Mohan & Sivakumar, 2016), whereas stem bromelain recorded the optimum pH in the range of 7.5–10 (Han et al., 2018). In addition, peel and pulp extracts exhibited optimum pH at 7.5 and slightly decreased when pH changed to alkali or acidic (Indrajeet et al., 2017). Pineapple leaf and pulp also recorded the maximum activity at pH 6 and 8, respectively (Ramalingam et al., 2012). It revealed that different extracted parts of pineapple possessed different optimum pH due to the surface charge on the adsorbent materials (Omotoyinbo & Sanni, 2017).

However, no activity was recorded at pH 3, 6, and 9. Different pHs affect the enzyme's structure and enzyme activity by changing the ionization state of basic or acidic amino acids. Basic amino acids

have amine-containing functional groups, whereas acidic amino acids have carboxyl functional groups on their side chains. The ionic bonds hold three dimensional of a protein; therefore, if the ionized state of the amino acids in the protein changes, the protein structure will also alter. It may result in the inactivation of enzymes or alterations in protein function. Thus, extremes of acidity (pH 2 to 3) or alkalinity (pH 9) can cause the total loss of enzyme activity. Aside from that, no activity was shown at pH 6. However, adjusting the pH of the reaction mixture to the optimal pH restored the highest activity observed at pH 7. The pH of the reaction mixture may promote substrate dissociation and hence impact activity by its action on the substrate (Piper & Fenton, 1965).

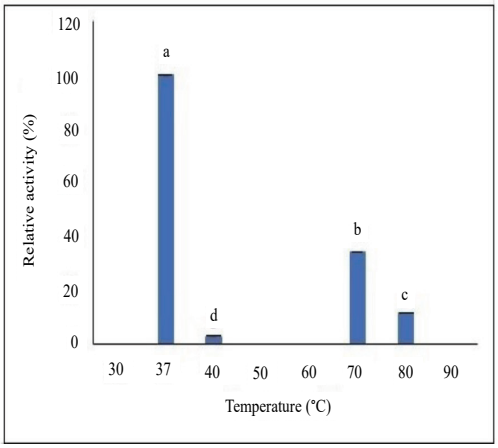

Figure 8. Effect of temperature on the proteolytic activity of purified protein extracted from crown MD2 cultivar

Note. Different alphabetical letters indicate the significant differences (p>0.05)

The temperature condition for activity was between 30–40°C (Figure 8). As the temperature rises, more molecules have adequate kinetic energy to react up to the optimal point, when the peptide bonds begin to degrade, resulting in protein misfolding and structural alteration (Han et al., 2018; Ketnawa et al., 2012). As a result of the irreversible inactivation of the enzyme, the proteolytic enzyme, and its function decreased (Ataide et al., 2018; Soares et al., 2012). Hence, bromelain has optimum activity ranging from 30 and 40°C. As a result, the purified enzyme showed an optimum pH of 7 and temperature between 30–40°C with a molecular weight of 25 to 32 kDa. However, the fruit bromelain showed an optimum pH of 7 and a temperature between 30 and 40°C with a molecular weight of 24.5 kDa (Ferreira et al., 2011). Even though the purified protein seemed to resemble fruit bromelain, the activity was drastically reduced at temperatures ranging from 40 to 60°C. It showed that the purified protein was not a glycosylated enzyme (Mamo & Assefa, 2019). Generally, stem and fruit bromelains consist of 1–2 sites of glycosylation (Ramli et al., 2018). Thus, the purified protein was most likely to be ananain as it was a non-glycosylated protein, while comosain differed from ananain but had similar carbohydrate composition closely resembling stem bromelain (Napper et al., 1994). Unlike stem bromelain and fruit bromelain, the purified protein from the crown more resembled ananain. The purified protein was then identified via LC-MS.

LC-MS

The use of LC-MS is to provide protein identification information and quantitative measurements in proteomic research. From this analysis, five unique sequences (Query ID: 55577, 46136, 15596, 35225, and 36722) were subjected to sequence analyses using NCBI BLASTP, which revealed that 100% of the amino acids sequences were identical with the sequences of ananain of *Ananas comosus* from the family Bromeliaceae under accession number OAY80104.1 (according to NCBI) or AOA199VSS3 (according to Uniprot) (Table 2). The five unique peptide sequences (in colors) show that 100% of sequences were identified with the full sequences of ananain of *Ananas comosus* from the family Bromeliaceae under accession number AOA199VSS3 (Figure 9). It covered 97/356 amino acids with 44.9% coverage. In addition, this study revealed that the ananain from *Ananas comosus* (AOA199VSS3) had the closest relationship with ananain (accession number AOA199UL32, AOA199UUY2, and AOA199W8N4), fruit bromelain (AOA199VSB5, AOA199W3D2, AOA199W9F2, and AOA199W8R4), bromelain (O24641), and papain (P0074.8).

Thus, the phylogenetic relationships of 10 proteases were constructed (Figure 10). Based on the MEROPS database (the peptidase database), the evolutionary tree indicated that family C1 was categorized into two subfamilies: C1A and C1B, which are closely related to the papain and bleomycin hydrolase subfamily, respectively. Ananain (EC 3.4.22.31) was classified in the subfamilies of C1A, a peptidase family related to papain (Figure 10). The molecular weight of ananain is in the range of 20 to 35 kDa and, thus, grouped in the clan CA (superfamily cysteine protease). In conclusion, a comparative analysis of this sample revealed that the purified enzyme had highly matched with the sequences of ananain from *A. comosus* under accession number A0A199VSS3 (according to Uniprot). It has five unique peptides and covers 97/356 amino acids (44.9% coverage). Thus, the purified enzyme extracted from pineapple crown was ananain-like protease, one type of cysteine protease like other papain family members.

Table 2

Identification of five unique peptides sequences from the purified enzyme

No.	Query ID	Sequences	Read length	Total score	% identity
1.	55577	KGILEPLSEQQVLDCAK	17	56.6	100 % OAY80104.1 (Ananain)
2.	46136	GVASGAIYPYK	11	37.1	100 % OAY80104.1 (Ananain)
3.	15596	QPITVAVDANANFQYYK	17	58.7	100 % OAY80104.1 (Ananain)

Table 2 *(Continue)*

No.	Query ID	Sequences	Read length	Total score	% identity
4.	35225	SGVFNGPCGTSLNHAVTAIGYGQDSNGKK	29	91.8	100 % OAY80104.1 (Ananain)
5.	36722	DVSSSSGICGIAIDSLYPTLESR	23	73.6	100 % OAY80104.1 (Ananain)

MAWKVQLVFLFLFLCVMWASPSAASADEPSDPMMKRFEEWMVEYGRVYKDNDEKMRRFQIFKNNV
NHIETFNSRNENSYTLGINQFTDMTNNEFIAQYTGGISRPLNIEREPVVSFDDVDISAVPQSIDWRDYGA
VTSVKNQNPCGACWAFAAIATVESIYKIKKGILEPLSEQQVLDCAKGYGCKGGWEFRAFEFIISNKGVAS
GAIYPYKAAKGTCKTNGVPNSAYITGYARVPRNNESSMMYAVSKQPITVAVDANANFQYYKSGVFNGP
CGTSLNHAVTAIGYGQDSNGKKYWIVKNSWGARWGEAGYIRMARDVSSSSGICGIAIDSLYPTLESRAN
VEAIKMVSESRSSV

Figure 9. The full sequences of ananain of *Ananas comosus* under the accession number AOA199VSS3. The colors indicated the five unique peptides sequences (Query ID: 55577, 46136, 15596, 35225, and 36722)

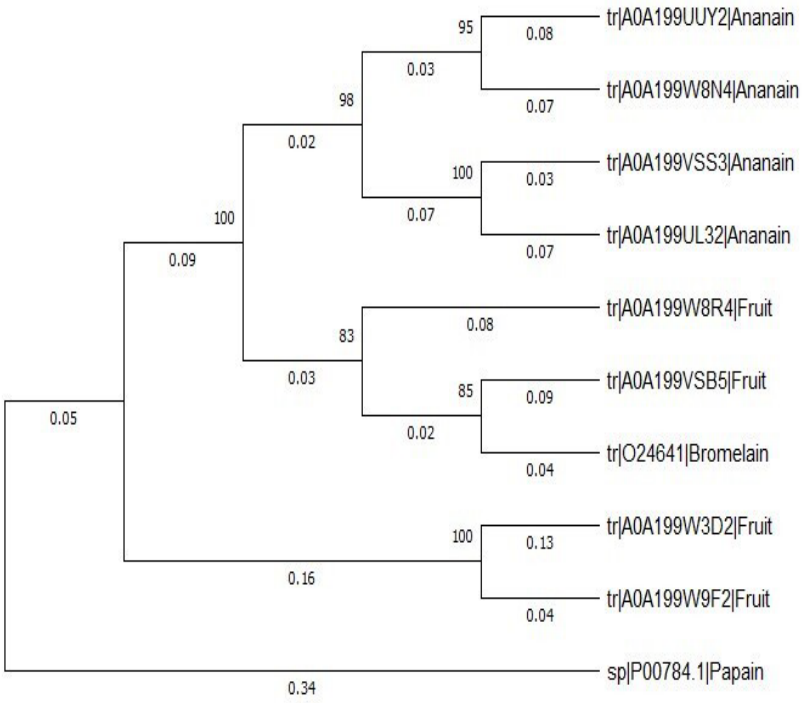

Figure 10. The phylogenetic relationship of ananain from *Ananas comosus*

CONCLUSION

The proteolytic activity of the MD2 crown was 126.0 ± 3.86 U/ml, the specific activity was 3937.50 U/mg, and the specific activity in 1 kg of waste was 3.94×10^9 U. Therefore, in 50 tonnes of a crown, it is possible to obtain 1.97×10^{14} U of bromelain. MD2 crown recorded the highest proteolytic activity, as shown by larger halo zone formation on both casein and skim milk plates. As a result, the MD2 cultivar was the best cultivar with the most proteolytic activity. The purified enzyme (ananain) extracted from the pineapple's crown contained high proteolytic activity and specific activity of 144.02 U/ml and 171.43 U/mg, respectively. Purification of the extract yielded a band at a molecular weight of 25–32 kDa MW. The optimum conditions for the proteolytic activity of this purified enzyme were pH 7 at 37°C. The purified protein was characterized, and LC-MS confirmed that it resembled more ananain than comosain, stem bromelain, or fruit bromelain. The sample highly matched with ananain from *A. comosus*, under accession number A0A199VSS3 (according to Uniprot), and it has five unique peptides and covered 97/356 amino acids (44.9 percent coverage). Ananain (EC 3.4.22.31) was classified in the subfamilies of C1A, a peptidase family related to papain. The molecular weight of ananain is in the range of 20 to 35 kDa and, thus, grouped in the clan CA (superfamily cysteine protease). Thus, the purified enzyme extracted from pineapple crown was ananain-like protease, one type of cysteine protease like other papain family members.

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