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Extraction, Partial Purification and Characterization of Bromelain from Pineapple (Ananas Comosus) Crown, Core and Peel Waste

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HIGHLIGHTS

- Bromelain was extracted from crown, core, and peel waste of pineapple.
- Ammonium sulphate $(NH_4)_2SO_4$ precipitation method was used as a simple and cost-effective approach for partial purification of bromelain.
- A purification of 4.34, 2.75 and 2.59-fold was achieved for crown, core and peel bromelain samples respectively.
- Enzyme activity recovery of 170.41 %, 192.37 % and 337.59 % was achieved for crown, core, and peel bromelain, respectively.

Abstract: Ananas Comosus (also known as pineapple) is a part of Bromeliaceae family and it is consumed as food as well as folk medicine for the treatment of various diseases. It is reported that pineapple is a rich source of bromelain, a cysteine protease and it is considered as an important enzyme in different industries due to its significant therapeutic and industrial applications such as anticancer, anti-inflammatory and meat tenderizing. Bromelain is mostly present in fruit and stem of pineapple, but it is reported that crown, core, and peels, which constitute the waste of the pineapple plant, also contain bromelain but limited data is available. Therefore, the proposed study aimed at utilizing pineapple waste for the extraction and characterization of bromelain. Firstly, crude bromelain was extracted with phosphate buffer (pH 7), then it was subjected to partial purification using different fractions of ammonium sulphate $(NH_4)_2SO_4$ such as 30, 40, 50 and 60% followed by desalting and concentration. Enzyme activity was calculated by using casein digesting unit (CDU) method. The results demonstrated that the crown bromelain showed highest purification of 4.34-fold at 30%

$(NH_4)_2SO_4$ saturation, whereas core and peel bromelain showed highest purification of 2.75 and 2.59-fold at 40% $(NH_4)_2SO_4$ saturation. The molecular weight of crude and partially purified bromelain was determined by SDS-PAGE analysis and found to be 26 KDa. The pH and thermal stability of all the parts of pineapple showed maximum stability at pH 7 and at 35^oC temperature.

Keywords: proteases; SDS-PAGE; enzyme activity; ammonium sulphate; zymography.

INTRODUCTION

Bromelain is defined as a natural complex mixture of various proteolytic enzymes which has a cysteine amino acid side chain. It is also stated that bromelain is a combination of various thiol endopeptidases and other constituents like peroxidase, phosphatase, cellulase, glucosidase and several protease inhibitors [1]. Bromelain is mainly derived from pineapple (Ananas comosus), which is a well-known plant of Bromeliaceae family that is widely cultivated in tropical and subtropical areas [2]. Thailand is one of the major cultivators and exporter of pineapple around the world [3]. It is used for edible purposes and the fruit has been popularly used in different cuisine. Moreover, pineapple is considered as a nutrient rich plant and has been traditionally known for having a wide array of potential biological compounds among which bromelain is of great importance [2]. Bromelain has been used in folk medicine for so many years to address various health problems which causes persistent interest in this protease [4]. It possesses a wide range of therapeutic applications like fibrinolytic, anti-thrombotic, anti-cancer, anti-inflammatory and anti-bacterial activity. In addition to this, bromelain has various industrial applications also that include pharmaceutical, food, textile, and cosmetic industry [5].

Bromelain has captured attention in vast range of industrial applications due to its higher commercial values and potential properties. The proteases including bromelain holds approximately 60% share of the global enzyme market. This growth is associated with the increasing general awareness about the protection of environment from the harmful impacts of chemical industrialization [6]. The robust protein degrading activity of bromelain has formed a widespread interest in a number of applications, mainly in the tenderization of meat in food industry. The pharmaceutical and the food and beverages industries have the largest applications of bromelain for a variety of purposes, both of these sections throughout the forecast period of 2017-2025, have mutually expected to hold more than 85% of the global bromelain market share [7]. The boundless interest in a wide variety of bromelain applications has encouraged many researchers to isolate and characterize the protease enzyme from pineapple waste or juice. It is reported that the stem of ripped pineapple has the highest concentration of bromelain. However, pineapple waste parts such as peel, core, and crown which serve as a byproduct in the pineapple processing industry are also rich in bromelain and are still not utilized properly. The commercial bromelain, most of the time is extracted from pineapple stem by means of centrifugation, ultrafiltration, lyophilization and two-step Fast Protein Liquid Chromatography (FPLC) [8].

Moreover, with the increase in pineapple production worldwide, pineapple waste (which includes peel, leaves, stem, core, and crown) are also proportionally increasing [3]. These waste parts have low commercial importance, and, in most cases, they are discarded, and this waste is occasionally utilized as compost for soil fertilization [9]. Therefore, proper waste disposal is the biggest concern around the world as throwing them as garbage causes serious environmental problems as well as it is usually prone to microbial spoilage which later can cause biohazards [3]. Considering the wide range of bromelain application in various industries, and the potential of pineapple waste for the extraction of bromelain, the aim of the present study was to extract and partially purify bromelain using ammonium sulphate precipitation method using pineapple waste (crown, core and peel) as an alternate and cheap source for this enzyme and to perform biochemical characterization studies of the extracted bromelain enzyme which will serve as the basis for further studies.

MATERIAL AND METHODS

Raw materials

The pineapple (phu-Lae) variety was purchased from the local fruit market in Thailand. The research study was carried out in DRIBBS (Dow Research Institute of Biotechnology and Biomedical Sciences) at Dow University of Health Sciences, Karachi, Pakistan. The pineapple was washed thoroughly using distilled water until it was cleaned and dried properly. The crown, core and peels of the fruit were separated, cut into small

pieces and weighed separately was washed, airdried and its waste parts (crown, core and peel) were collected, and stored at 4°C for further use.

Chemicals

All the reagents used for the experiment were research grade. Ammonium sulfate $(NH4)_2SO_4$, casein, bovine serum albumin (BSA), L-tyrosine, trichloroacetic acid (CCl_3COOH), hydrochloric acid (HCL), ethylene diaminetetraacetic acid (EDTA), betamercaptoethanol (ME), sodium dodecyl sulfate (SDS), Coomassie brilliant blue G250, stem bromelain (lyophilized powder), folin dye, sodium carbonate, sodium potassium tartrate and copper sulphate were purchased from Sigma-Aldrich Co., LLC, USA. Sodium hydroxide (NaOH) was procured from Reidel-de-haen, Seelze, Germany and molecular weight marker was obtained from Molequle on, Auckland, New Zealand.

Crude Enzyme Preparation

The crown, core and peel waste were washed thoroughly using distilled water until it was cleaned and dried properly. Each waste part was cut into small pieces weighed, and 100 grams of each part was blended with 100mL of ice-cold phosphate buffer (0.05M, pH 7). After blending, the mixture was filtered using cheese cloth and filter paper. The filtrate was then centrifuged at 10,000 × g for 20 min at 4°C. The supernatant containing crude bromelain enzyme was collected and stored at -20°C for further use.

Ammonium Sulfate $(NH_4)_2SO_4$ Precipitation

The partial purification of bromelain was done using ammonium sulfate precipitation (NH4)2SO4 method. The crude bromelain extracts were saturated with 30%, 40%, 50% and 60% $(NH_4)_2SO_4$ (w/v). The amount of ammonium sulfate required to attain the desired concentrations was calculated via online application named as $(NH_4)_2SO_4$ calculator by EnCor Biotechnology Inc. Briefly, the crude extracts of crown, core and peel were transferred to a beaker containing a magnetic bead and then the beaker was placed on a magnetic stirrer at 4oC. While stirring, calculated amount of $(NH_4)_2SO_4$ was added slowly to the extracts and then the crude extracts were left overnight at 4°C and then the precipitated extracts were subjected to centrifugation at 10,000 × g for 20 min at 4°C. After centrifugation, supernatant was carefully discarded and removed and the pellet was resuspended in 0.05M phosphate buffer at pH 7. The partially purified bromelain was concentrated and de-salted using Amicon centrifugal filter with minimum pore size of 10 KDa. The concentrated samples were then stored at -20°C for later use.

Proteolytic activity measurements

The proteolytic activity of bromelain was assessed using casein digestion unit (CDU) method by Cupp-Enyard (2008) with some modifications [10]. L-tyrosine and casein were used as a standard and substrate, respectively. Briefly, 0.5% Casein stock solution was prepared in 0.05M phosphate buffer (pH 7). Two sets of tubes were used in the assay, one as control and the other as test in duplicates. 0.8ml of 0.05M phosphate buffer was added to each tube along with 0.2mL of activator solution (0.006M EDTA and 0.03M L-cysteine in 0.05 phosphate buffer). Then 1ml casein stock solution (0.5%) was added to both test and control tubes and incubated in water bath for 10 min at 37°C. The crude and partially purified bromelain enzyme (100µL) were added to the tubes except the control tubes and were further incubated for 15 min at 37°C and then 3mL of 5% Trichloroacetic acid (TCA) was added to all the tubes and incubated for 20 min. Finally, 100µl of enzyme solution was added to control tubes and all the tubes were centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was collected, and absorbance was measured at 280nm. Absorbance values of the bromelain enzyme were plotted in tyrosine standard curve and concentration of bromelain was determined by calculating the amount of tyrosine released during the reaction. The bromelain activity i.e. units/ml was determined by using the following formula:

$$\text{Enzyme activity} = \frac{(\text{Total µg/mL tyrosine released} \times \text{total volume of reaction})}{(\text{Volume of sample} \times \text{reaction time} \times \text{volume of cuvette})} \quad (1)$$

Determination of total Protein content

The protein estimation method by Lowry and coauthors. [11] was used for quantifying the protein concentration of the crude extract and precipitated samples. Briefly, 50µL of enzyme was added to 950µL of

distilled water to make up 1000µL final volume, then 5mL of Lowry reagent was added to each test tube and the tubes were then incubated for 15 min at room temperature. After incubation, 0.5mL of folin dye (1X) was added to each tube and the tubes were shaken so that the dye and the solution get mixed thoroughly and then the tubes were incubated for 15 min at room temperature in the dark. After incubation, tubes were taken out and absorbance was measured at 650nm. The calibration curve was constructed using bovine serum albumin (BSA) in the range of 25-250 µg/mL.

Effect of pH

The pH stability of bromelain from crown, core, and peel was determined by incubation of enzyme for 2 hours at room temperature with different pH buffers ranging from 3, 5, 7 and 9 by adding sodium-citrate buffer (pH 3 and 5), phosphate buffer (pH 7) and glycine buffer (pH 9) with the final ratio of 1:1. After the completion of incubation period, the aliquots of bromelain were taken and assayed at optimal assay conditions to determine the residual bromelain activity using casein digestion unit (CDU) method.

Effect of Temperature

The temperature stability of the partially purified bromelain from crown, core and peel sample was determined by incubating the enzyme in water baths at 25°C, 35°C, 45°C, 55°C and 65°C for 2 hours. The tubes were sealed and capped to avoid any contamination and evaporation of the enzyme. After the completion of incubation period, the aliquots of bromelain were taken and assayed as mentioned above.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Protein pattern

The molecular weight of crude and precipitated bromelain samples from crown, core and peel waste was measured by SDS-PAGE according to Laemmli method [12] using 12% separating and 4% stacking gel (30% acrylamide/bisacrylamide). The crude and precipitated crown, core and peel bromelain samples were mixed at a ratio of 1:1 with the sample buffer containing β-mercaptoethanol (βME) and heated at 95°C for 5 min. Then 10µg of crude and partially purified crown, core and peel bromelain samples were loaded into the wells and subjected to separation at 110mA. After separation, the gel was stained with Brilliant Blue G250 dye and then the gel was destained and the bands of proteins were observed and compared with the reference ladder. The commercial stem bromelain was used as a control to compare the size of bromelain from different waste parts.

Zymography

The catalytic activity of crown, core and peel bromelain in the protein bands that were separated on SDS PAGE was determined via activity staining or zymography (using casein as a substrate). The method described by Garcia-Carreno and coauthors. [13] was followed with slight modification. The wells were loaded with 10µg of bromelain samples and after completion of electrophoresis, the gel was soaked in 50mL of 2% casein (w/v) solution of pH 7. The soaked gel was placed at 4^{o}C for 45 min with continuous agitation. The gel was then washed with deionized water and placed in staining solution for 60 min. After staining, the gel was immersed in destaining solution and left overnight with constant agitation at room temperature. The activity of bromelain was evaluated by observing the development of clear zones against a dark background.

Statistical analysis

All the experiments were performed in triplicates and results are represented as average of three independent experiments. The data is presented as mean± standard deviation.

RESULTS AND DISCUSSION

Ammonium sulphate $(NH_4)_2SO_4$ precipitation of pineapple waste parts and its effect on purification fold and percent recovery

The crude bromelain from the waste parts mentioned above were partially purified with a range of 30-60% $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ saturation that showed highest purification fold in case of crown bromelain sample was 30% that gave 4.34 purification fold with 170.41% activity recovery (Table 1 and Figure 1). After

30% saturation, the purification fold was decreased, hence, 30% saturation of crown sample was used for further characterization. The study reported by Singh and coauthors [14] demonstrated highest purification fold of 2 with 98% activity recovery at 37% $(NH_4)_2SO_4$ saturation. In the core sample, the purification fold initially increased from 30 to 40% then started to decrease after 40% salt saturation. Therefore, for further characterization studies, we selected 40% saturated core samples with a purification fold of 2.75 and percent recovery of 192.37% respectively (Table 1 and Figure 1). Similar findings were reported by Chaurasiya and Hebbar [15] in which 2.6-fold purification was observed at 40% precipitation of core bromelain. The peel sample demonstrated increase in purification fold after 30 percent salt saturation and at 40 percent highest purification fold was observed which was 2.59 with percent recovery of 337.59% and after that we observed decrease in purification fold (Table 1 and Figure 1). Therefore, further characterization was done using 40% as final saturation percentage. These result lies close to the findings of Benefo and Ofosu [16] that also demonstrated 2.47-fold purification at 40% $(NH_4)_2SO_4$ saturation. Bresolin and coauthors. [17], in which 2.2-fold purification with 75% activity was achieved in the range of 40-80% saturation. Study reported by Mohapatra and coauthors [18] also demonstrated highest purification fold at 44.4% $(NH_4)_2SO_4$ saturation i.e. 4.191 purification fold and 242.6% activity, respectively. Difference in the results of the study could be due to the difference in pineapple specie, use of different extraction buffer for instance in the study of Mohapatra and coauthors. [18] sodium acetate buffer was used instead of phosphate buffer which is used in our study. Overall, these results suggest that the crown waste part of pineapple has the highest purification fold in comparison to the core and peel samples.

Table 1. Ammonium sulphate precipitation of bromelain from pineapple crown, core, and peel and its purification and recovery profile.

Sample	$(NH_4)_2SO_4$ (% w/v)	Activity (U/mL)	Total Protein (mg/mL)	Specific Activity (U/mg)	Purification fold	Recovery (%)
Crown	Crude	161.92 ± 11.58[1]	3.14 ± 0.49	51.51 ± 15.54	1.00 ± 0.00	100.00 ± 0.00
	30	275.93 ± 4.49	1.23 ± 0.13	223.48 ± 15.71	4.34 ± 1.00	170.41 ± 30.23
	40	278.25 ± 5.79	2.22 ± 0.51	125.11 ± 66.11	2.43 ± 0.07	171.84 ± 17.61
	50	365.90 ± 28.38	2.64 ± 0.47	138.56 ± 47.08	2.69 ± 0.10	225.97 ± 2.75
	60	214.35 ± 23.17	3.34 ± 0.21	64.20± 53.35	1.25 ± 0.57	132.38 ± 48.05
Core	Crude	181.62 ± 30.47	1.63 ± 0.26	111.51 ± 0.90	1.00 ± 0.0	100.00 ± 0.00
	30	127.75 ± 10.88	0.62 ± 0.05	204.65 ± 0.03	1.84 ± 0.02	70.34 ± 18.05
	40	349.38 ± 45.71	1.14 ± 0.16	306.76 ± 85.27	2.75 ± 0.79	192.37 ± 58.27
	50	390.94 ± 74.01	1.54 ± 0.13	254.56 ± 26.08	2.28 ± 0.22	215.25 ± 4.70
	60	223.17 ± 2.18	2.12 ± 0.52	105.17± 27.53	0.97 ± 0.24	122.88 ± 19.70
Peel	Crude	104.66 ± 26.12	1.09 ± 0.32	96.45 ± 4.32	1.00 ± 0.00	100.00 ± 0.00
	30	135.44 ± 30.47	1.17 ± 0.03	115.64 ± 23.02	1.20 ± 0.29	129.41 ± 3.28
	40	353.32 ± 10.76	1.41 ± 0.03	250.12 ± 12.63	2.59 ± 0.25	337.59 ± 76.34
	50	212.40 ± 17.41	2.43 ± 0.25	87.23 ± 16.27	0.90 ± 0.21	202.94 ± 35.10
	60	120.05 ± 47.89	2.20 ± 0.06	54.69 ± 20.31	0.57 ± 0.23	114.71 ± 17.68

[1]Means± Standard deviation from triplicate measurements

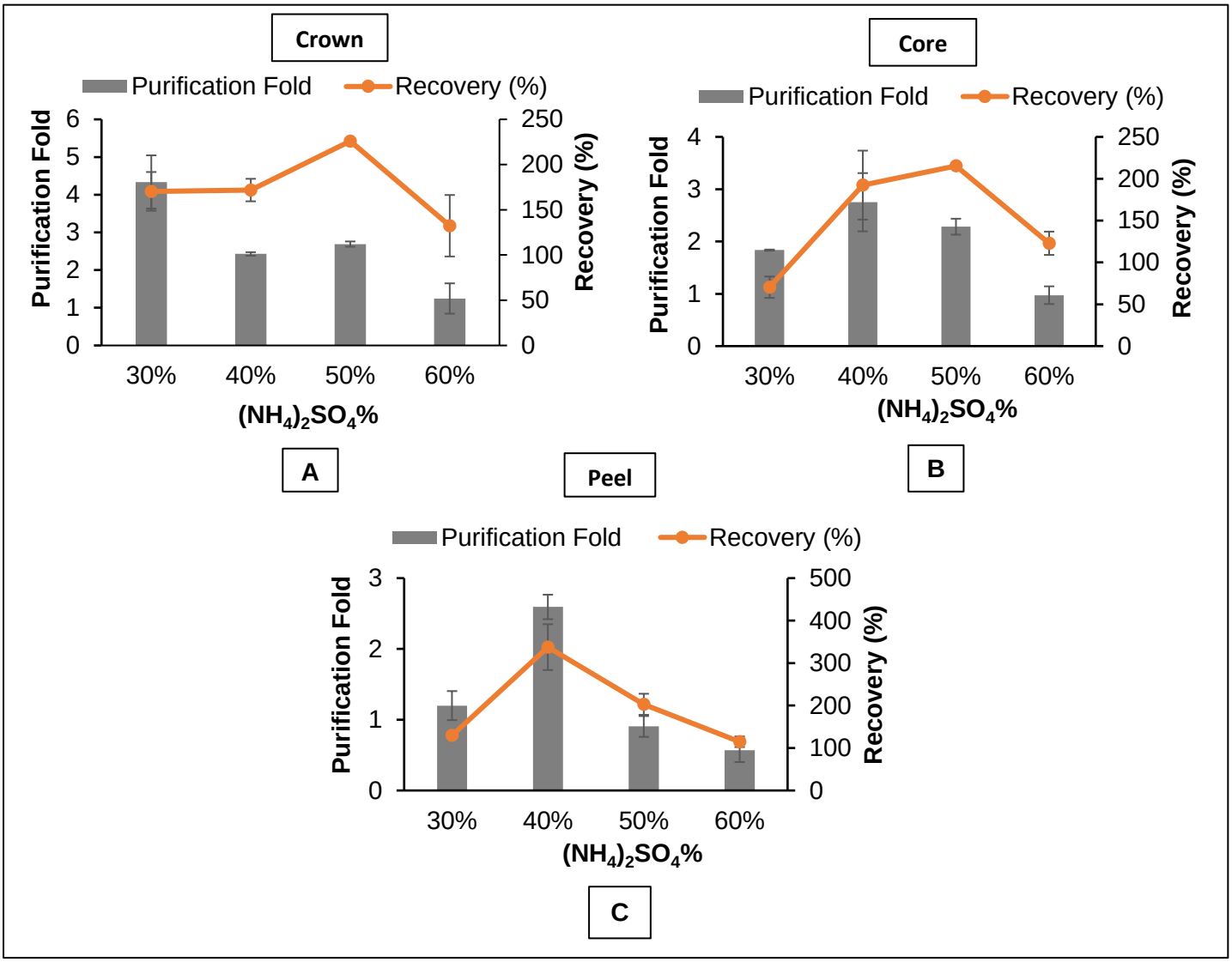

Figure 1. Effect of different $(NH_4)_2SO_4$ saturations on the purification and percent recovery of crown, core and peel bromelain. The crude extract of crown, core and peel waste was subjected to 30-60% (W/V) $(NH_4)_2SO_4$ saturation and then analysed for purification fold and percent recovery. (A). The crown bromelain showed highest purification fold. At 30% salt saturation *i.e.* 4.34-fold with 170.41% activity recovery. (B) At 40% $(NH_4)_2SO_4$ saturation, core bromelain demonstrated 2.75-fold purification with 192.37% activity recovery. (C). In peel bromelain, maximum purification of 2.59-fold with 337.59% activity recovery was observed.

SDS-PAGE and Activity Staining Analysis

The molecular weights observed after SDS-PAGE were ~26 KDa for all the fractions. This was found to be similar to the molecular weight of bromelain *i.e.* 26 KDa reported by Hebbar and coauthors. [19]. Moreover, the MW was also approximated to the range of 24.5-37 KDa and 24–28 KDa reported by Bala and coauthors. [20] and Ketnawa and coauthors [3] respectively. Zymography or activity staining analysis showed clear zones in gel. These results correspond to the findings reported by Larocca and coauthors. [21], in which 2-D zymography of pineapple extract was done using casein and gelatin as a substrate and clear spots of bromelain were observed. In the study by Ketnawa and coauthors. [3], clear spot against dark blue background was observed at the bromelain band of 28 KDa (Figure 2).

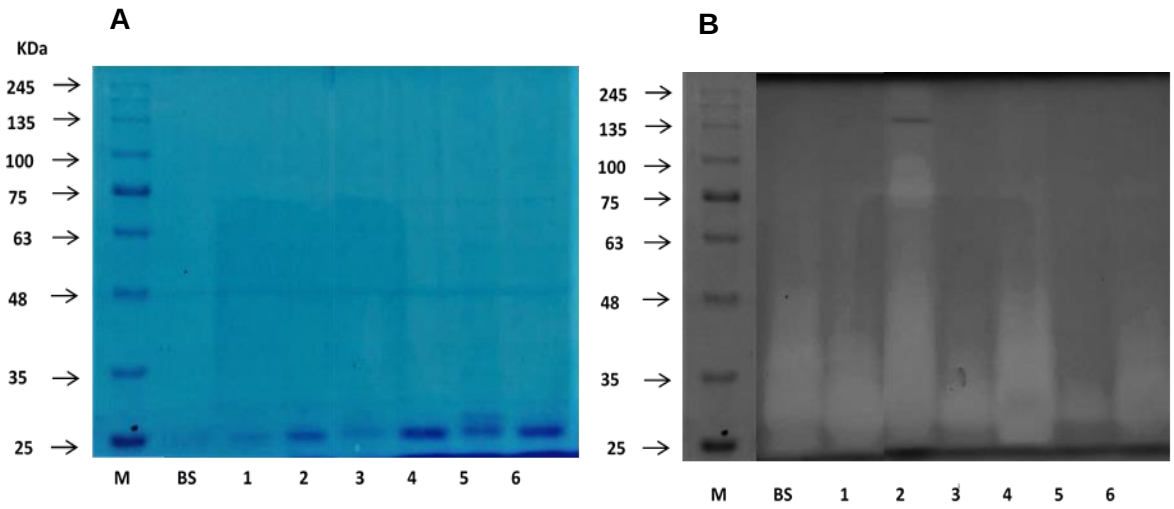

Figure 2. Protein pattern (A) and Activity staining (B) of crown, peel and core bromelain. M: molecular marker, BS: bromelain standard, 1: crude crown bromelain, 2: 30% crown bromelain, 3: crude core bromelain, 4: 40% core bromelain, 5: crude peel bromelain and 6: 40% peel bromelain. 10 µg of sample was loaded in each well.

pH and thermal stability analysis of bromelain from crown, core and peel samples

Effect of pH

The stability analysis of bromelain at different pH conditions demonstrated measurable difference in relative activity of crown, core, and peel bromelain across the range. The pH stability assay was carried out in buffers of various pH ranges from 3, 5,7 and 9. In crown sample, the enzyme was stable with more than 80 percent relative activity in all pH range, but the highest activity was observed at pH 7 and slight decrease in activity was observed at pH 9 as mentioned in Figure 3. In core samples, the highest activity was observed at pH 7 and then it dropped to 74.6% at pH 9 (Figure 3). The peel bromelain also showed highest relative activity at pH 7 and at pH 9 more than 50% reduction in the relative enzyme activity was observed (Figure 3). It was observed that bromelain from all waste parts worked best at pH 7. These results are in correspondence to various studies that have also reported similar findings that bromelain is stable within the range of pH 6.5-8.0 with highest activity at pH 7 [3,22].

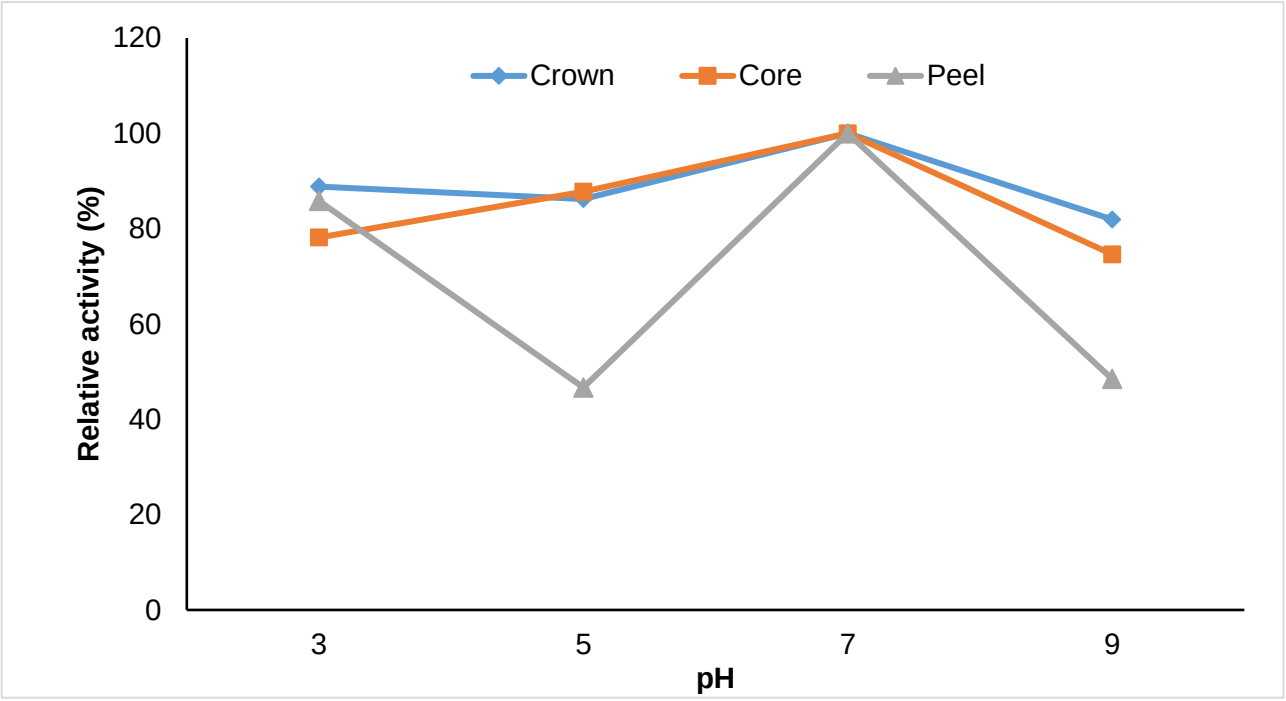

Figure 3. pH stability profile of crown, core, and peel bromelain. The relative activity was calculated after incubation of crown, core, and peel bromelain at different pH buffers for 2 hours at room temperature.

Effect of temperature

Thermal stability assay was carried out at various range of temperature i.e. 25°C, 35°C, 45°C, 55°C and 65°C. The bromelain from all waste parts was incubated for 2 hours and then activity was determined. In the crown sample, the highest activity was observed at 35°C as mentioned in Figure 4. The relative activity decreased to approximately 50% at 45°C and then further decreased to 14% at 55°C and finally no activity was observed at 65°C. The highest activity of core bromelain was observed at 35°C and decrease in activity was observed at 45°C onwards and at 65°C no activity was observed as mentioned in Figure 4. The peel bromelain showed highest relative enzyme activity at 35°C and at 45°C the activity was decreased to 17% and at 55°C and 65°C no activity was observed (Figure 4). It was observed that the optimum temperature for the activity of bromelain was 35°C and it started to decrease at the temperature above 45°C except core bromelain which showed 50% activity at 55°C but no activity at 65°C. These results showed similarity to the studies reported by Xue and coauthors. [23], which demonstrated that most enzymes are unstable at increasing temperature. Generally, above 65°C, enzymatic activities are severely inhibited. Khan and coauthors [24] validated that bromelain showed maximum activity and stability at 30°C and the activity decreased by 17% when the temperature was increased to 40-60°C. Omotoyinbo and coauthors [25] also presented that the optimum temperature for bromelain activity in all parts of pineapple was observed at 40°C. Moreover, bromelain was stable with approximately 50% relative enzyme activity at 30-60ºC temperature range unless it finally began to lose its activity at 70°C. Furthermore, Febriani and coauthors [26] reported 37°C as the optimum temperature for bromelain activity. Results of this research depicted that at temperature above 60°C, activity of bromelain was completely lost while in studies that are mentioned above, it is concluded that activity is decreased by varying temperature at the range of 40-70°C but is not lost. The reason could be the prolonged incubation time of 2 hours for thermal stability analysis due to which enzyme started to degrade and lost its activity.

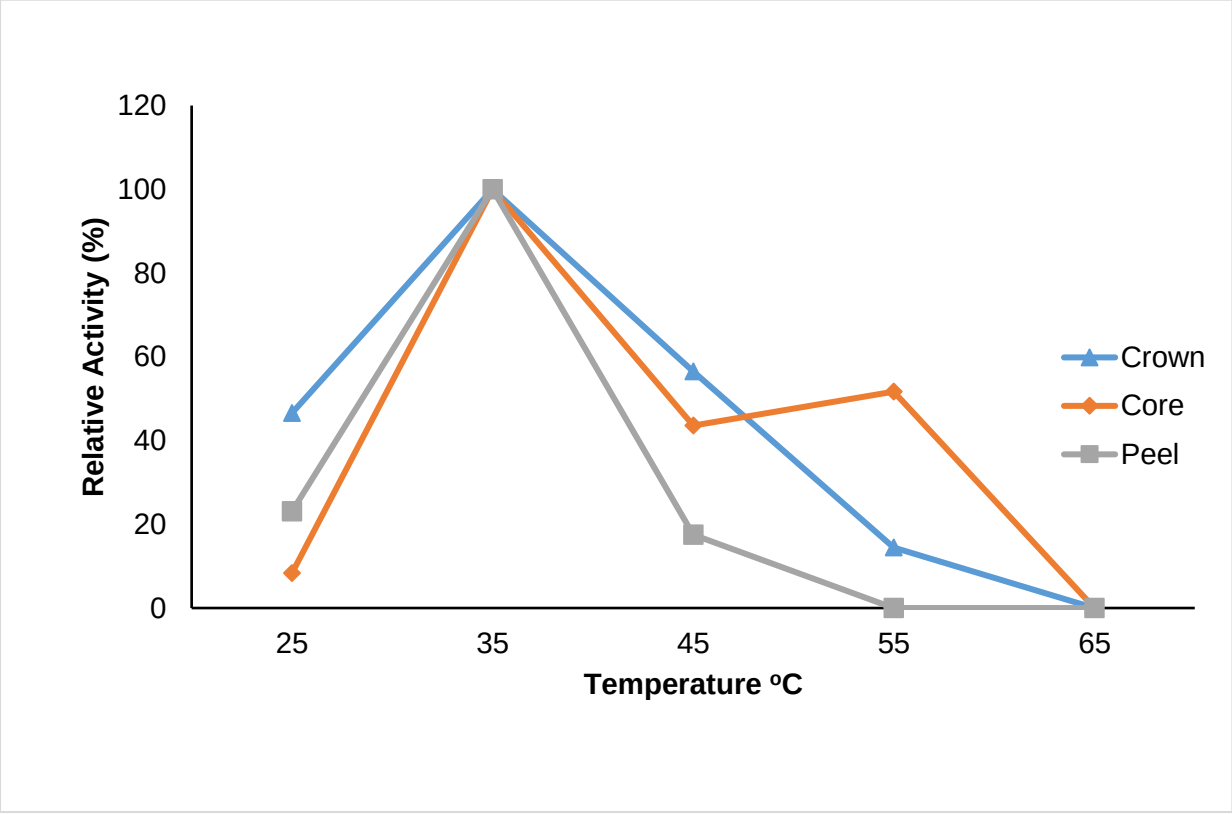

Figure 4. Thermal stability profile of crown, core, and peel bromelain. The relative activity was calculated after incubation of partially purified bromelain from waste parts at different temperature for 2 hours.

CONCLUSION

The present study has resulted in partially purified bromelain from crown, core and peel waste parts of the pineapple using $(NH4)_2SO_4$ precipitation method as a simple and cost-effective approach for partial purification of enzyme. The 30% salt saturation of crown bromelain and 40% core and peel bromelain showed highest purification fold whereas bromelain from all the waste parts mentioned above showed highest stability

at pH 7. All the extracts showed highest thermal stability at 35°C. Moreover, the molecular weight of all the fractions was found to be 26 KDa approximately. The present study can be considered as a preliminary study which can serve as the basis for the development of more efficient purification methods that will lead to the utilization of purified bromelain from pineapple waste in food, beverage, pharmaceutical, textile and other relevant industries.

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Conflicts of Interest: "The authors declare no conflict of interest."

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