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ORIGINAL ARTICLE

Enzymatic Analysis and Characterization of Bromelain from Two Varieties of Pineapple (*Ananas comosus*) Fruit and Stem Extracts

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ABSTRACT – This study was designed to examine the presence of enzymatic activity of fruit and stem bromelain extracted from two varieties of pineapple (Ananas comosus): Morris and Sarawak. The bromelain crude extract obtained from fruits and stems were tested for their protein content via Bradford's assay, and both crude extract from Morris variety showed the highest value. Further analysis was done to investigate the proteolytic activity of the crude fruit and stem bromelain, resulting in Morris variety having the highest activity for fruit bromelain, while Sarawak variety having the highest activity for stem bromelain. The Gelatin Digestion Unit (GDU) analysis performed revealed both fruit and stem bromelain from Morris variety exhibited the highest activity. Furthermore, the temperature optimization showed that both fruit bromelain of Morris and Sarawak varieties were optimum at 35 °C, while stem bromelain from both pineapple varieties were optimum at 45 °C. Meanwhile, the optimum value of the pH for Sarawak variety extract of fruit and stem bromelain were found at pH 5.6, while Morris variety were optimum at pH 6.6. These results indicated both varieties of pineapple extracts of fruits and stems were having bromelain enzyme that can be further developed for application at industrial level.

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INTRODUCTION

Pineapple, or Ananas comosus has been widely known for its high nutritional value. These distinctive fruits may be found in many different nations, including China, Indonesia, Costa Rica, Malaysia, Philippines, Thailand, and Brazil [1]. The nutritional content of pineapple includes vitamins, sugar, protein, lipids, carbs, calcium, iron, magnesium, and other minerals. In addition, pineapple also rich with bromelain content. Bromelain is a combination of proteolytic enzymes derived from the *A. comosus*, and its high nutritional value drives market demand for pineapple. The main contributor to the high content of bromelain in *A. comosus* is the stem, which is followed by the fruit. There are various bromelain characteristics found in various *A. comosus* species. To promote the advantages of ingesting fresh pineapple, research has been conducted in Malaysia on the screening of bromelain in several varieties of *A. comosus*. Since 1894, people have been researching and studying the bromelain that was isolated from *A. comosus* [2].

There are several uses for bromelain across numerous industrial sectors. In therapeutic and pharmaceutical sector, bromelain is applied due to its generally non-specific action on proteins and in particular for the control of tumor development, improvement of antibiotic- and blood coagulation-related, and some malignant disorders [3], besides being effective in treating conditions including bronchitis, sinusitis, wounds, diarrhea, and different cardiovascular diseases, as well as some anti-cancerous properties [4]. In food sector, bromelain is frequently utilized for the baking process, meat tenderization, beer clarifying, food supplements, and apple juice browning prevention. Additionally, bromelain has been used to skin for pre-tanning, softening, and bating in the leather industry [5].

In Malaysia, pineapple has been widely cultivated for the commercialization of fruits. The Malaysian Pineapple Industrial Board (MPIB) reports that in 2006, an average of 8731 hectares of small-scale pineapple farming were registered, indicating a significant increase of the *A. comosus* industry in Malaysia [6]. The output of pineapples has gradually increased, resulting in more trash being produced. Given that it is frequently susceptible to microbial deterioration, the proper disposal of such waste poses an increasing challenge. Reusing pineapple trash would be a novel approach to solving this environmental issue [7].

Therefore, to counter this problem, the content of bromelain in pineapple wastes will be assessed. In this study, bromelain from fruit and stem of two pineapple variety; Morris and Sarawak, were investigated. The analysis involved the quantification of protein content within the crude fruit and stem bromelain as well as the determination of the enzyme activity of protease. Further analysis has been done to ascertain the enzyme activity of bromelain. The characterization of both crude bromelain on the effect of temperature and pH on their enzyme activity was also investigated.

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METHODOLOGY

Extraction of bromelain from the fruit and stem of *A. comosus*

The variety of pineapple used were Sarawak and Morris purchased from Tunas Mart at Gambang, Pahang. The extraction of bromelain from pineapple fruit and stem were done according to [8] with minor modifications. The pineapple fruit and stem were separated, cut into small pieces, and washed with tap water to remove any impurities. The fruit and stem were then homogenized separately using electric blender for the enzyme extraction. The juice obtained were filtrated with muslin cloth to remove the fibrous materials prior to centrifugation at 10,000 rpm at 4 ℃ for 10 minutes to remove any insoluble materials. The supernatant was collected as bromelain crude extract and stored at -20 ℃ until further use.

Determination of protein content via Bradford assay

The determination of protein content was done via standard curve of protein as reported by [9]. The stock solution of 0.1 mg/ml was prepared from 2.0 mg/ml bovine serum albumin (BSA) and applied in the range of 0-10 µg protein. A total of 0.5 ml fruit and stem bromelain crude extract was diluted with 0.5 ml distilled water protein content determination. Both BSA and bromelain crude extract were then mixed separately with 1.5 ml Bradford reagent and incubated at room temperature for 10 minutes. The absorbance was taken spectrophotometrically at 595 nm. The BSA standard curve was plotted, and the protein content of the bromelain crude extract was determined.

Determination of proteolytic enzyme activity of fruit and stem bromelain

The proteolytic activity of the fruit and stem bromelain crude extract was determined as described by [10] with slight adjustment. The protease assay was carried out by incubating a total of 5.0 ml of 0.65 % casein solution in 0.05 M phosphate buffer (pH 7.5) at 37 ℃ for 5 minutes. The reaction was initiated by addition of 1.0 ml fruit and stem bromelain crude extract to the solution, and the incubation was continued for another 10 minutes. The reaction was then stopped by adding 5.0 ml of 0.1 M trichloroacetic acid (TCA) and incubated for additional 30 minutes, prior to the centrifugation at 6,000 rpm for 5 minutes. A total of 2.0 ml of the supernatant was collected and mixed with 5.0 ml of 0.5 M sodium carbonate, followed by addition of 1.0 ml 0.4 M Folin-Ciocalteu reagent. The solutions were subjected to absorbance reading at 660 nm. The blank was prepared in the same trend, except the addition of fruit and stem bromelain crude extract was done upon addition of TCA. The tyrosine standard curve was done using different concentrations of tyrosine ranging from 10-100 µg/ml. One unit of protease activity was defined as the amount of the enzyme resulting in the release 1 µg/ml of tyrosine per minute under the assay conditions.

Determination of bromelain activity by GDU analytical assay

The determination of bromelain activity of fruit and stem crude extracts was carried out according to the previous method [11] with slight changes. A total of 25.0 ml of 5 % gelatin was incubated for 5 minutes at 45 ℃. Then, 1.0 ml of fruit and stem bromelain crude extract was added and incubated at 45 ℃ for 20 minutes, followed by the addition of 0.1 ml of 3 % H_2O_2 to the solutions and incubated for another 5 minutes. The pH of the solution was then adjusted to 6 using 0.1 N NaOH, followed by adding 10.0 ml of 37 % formaldehyde. The pH was adjusted to 9 using 0.1 N NaOH. The blank was prepared using the similar method, replacing bromelain crude extract with H_2O_2. The solution was titrated with 0.1 N NaOH and the titration volume was recorded. The fruit and stem bromelain crude extract activity were determined by using Equation (1):

$$\text{GDU/mg} = \frac{(\text{T}-\text{B}) \times 14 \times \text{N} \times 50}{\text{W (g)}} \quad (1)$$

where T is the test titer (volume in ml of 0.1 N NaOH used), B is the blank titer (volume in ml of 0.1 N NaOH used), 14 is the amount of nitrogen (mg) per mM nitrogen, and N is the normality of standardized NaOH.

Effect of temperature in fruit and stem bromelain activity

The effect of temperature on the enzyme activity was carried out at different temperatures ranging from 25-75 ℃. The bromelain crude extracts were pre-incubated without substrate at different temperatures (25, 35, 45, 55, 65, and 75 ℃). The residual bromelain activity was determined through proteolytic enzyme activity assay as described previously.

Effect of pH on fruit and stem bromelain activity

The effect of pH on the enzyme activity was done at different value ranging from pH 3.6-7.6. The bromelain crude extracts were pre-incubated without substrate at different pH values using acetate buffer (pH 3.6-5.6) and potassium phosphate buffer (pH 6.6-7.6). The residual bromelain activity was determined via proteolytic enzyme activity assay as described previously.

RESULTS AND DISCUSSION

Determination of protein content via Bradford assay

The protein content of crude extract of fruit and stem bromelain obtained from Morris and Sarawak variety of pineapple was investigated. Bradford assay was performed, and the result showed the crude bromelain from both fruit and stem of Morris variety has the highest protein contents (54.11 ± 0.95 and 42.90 ± 5.99 mg/ml) as compared to both crude fruit and stem bromelain of Sarawak variety (33.71 ± 2.60 and 21.43 ± 0.51 mg/ml). Figure 1 shows the different in protein content for both crude fruit and stem bromelain from both pineapple varieties.

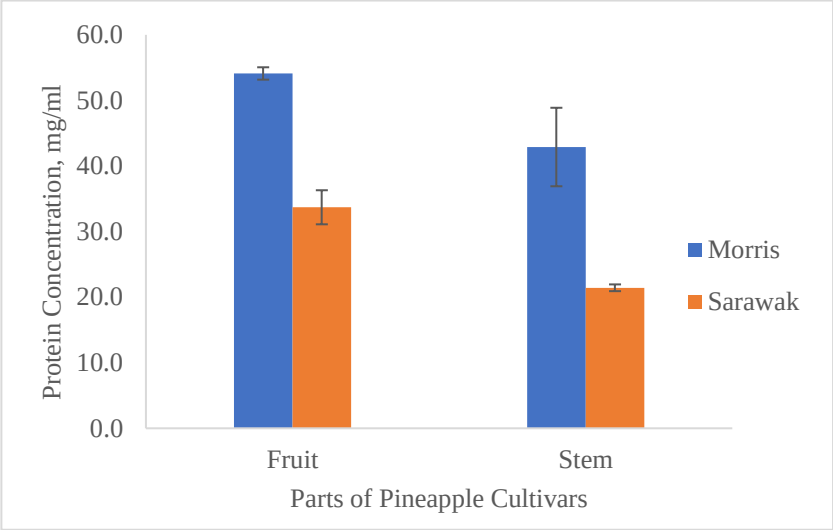

Figure 1. The protein concentration of different cultivars of pineapple.

The result obtained in this study was much higher compared to the previous study with only 1.11 µg/ml for fruit extract [12], and 1.24 mg/ml for stem extract [13]. This difference might be influenced by the variety of pineapple, the climate during culture, the stage of ripeness, the timing of harvest, and the extraction technique [14]. This was supported by [12] that stated freeze dry method for extraction works effectively for determining the natural content of an extract, due to the extremely low oxygen concentration in the vacuum, as it restricts the oxidative alterations of metabolites.

Determination of proteolytic enzyme activity of fruit and stem bromelain

The proteolytic activity of the enzyme was investigated via protease assay, in order to assess the ability of the bromelain to hydrolyse casein substrate. In this study, crude fruit bromelain from Morris variety and crude stem bromelain from Sarawak variety has the highest enzyme activity (0.82 ± 0.01 and 0.67 ± 0.003 U/ml, respectively). However, both crude fruit and stem bromelain from both pineapple variety were having moderate differences, as exhibited in Figure 2.

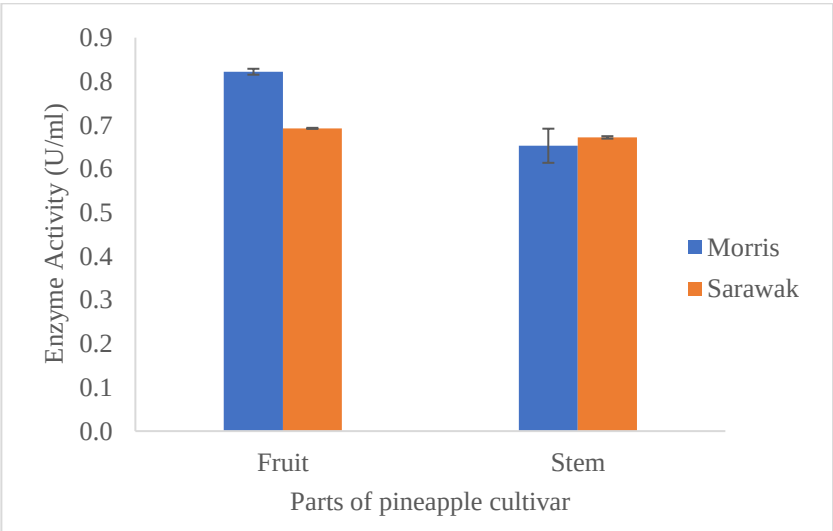

Figure 2. The enzyme activity of different cultivars of pineapple.

Previous study recorded partial purified extract of pineapple fruit has 0.0068 U/ml activity of enzyme [12], while [15] has its highest enzyme activity of pineapple stem at 0.0008 U/ml. However, it was reported crude fruit bromelain was having higher enzyme activity than the current one, which is 4.71 U/ml [8]. The diverse types of enzymes included in the pineapple, such as enzymes from the stem and from the fruit: ananain and comosain, are likely to be responsible for the variations in enzyme activity and protein quantity in each part of the pineapple [16, 17]. According to [18] the bromelain character is influenced by the differences in the pineapple parts (crown, fruit, peel, and stem) and the growth locations.

Determination of bromelain activity by GDU analytical assay

The GDU analytical assay were employed to measure the bromelain activity of the extract. Gelatin is employed as the substrate in a titrimetric test technique to measure the bromelain activity. Gelatin was chosen as the substrate because, in comparison to other substrates (skimmed milk and casein), it displays a larger quantity of the zone of clearance caused by protease hydrolysis [19]. In this study, the highest quantity of the bromelain activity was found on both Morris variety of fruit and stem with 158.95 ± 3.09 and 186.30 ± 20.9 GDU/g, respectively. Meanwhile, both crude fruit and stem bromelain from Sarawak variety were having slightly lower value with 153.07 ± 15.6 and 164.30 ± 7.59 GDU/g, respectively. Figure 3 shows the different in bromelain activity for both crude fruit and stem bromelain from both pineapple varieties.

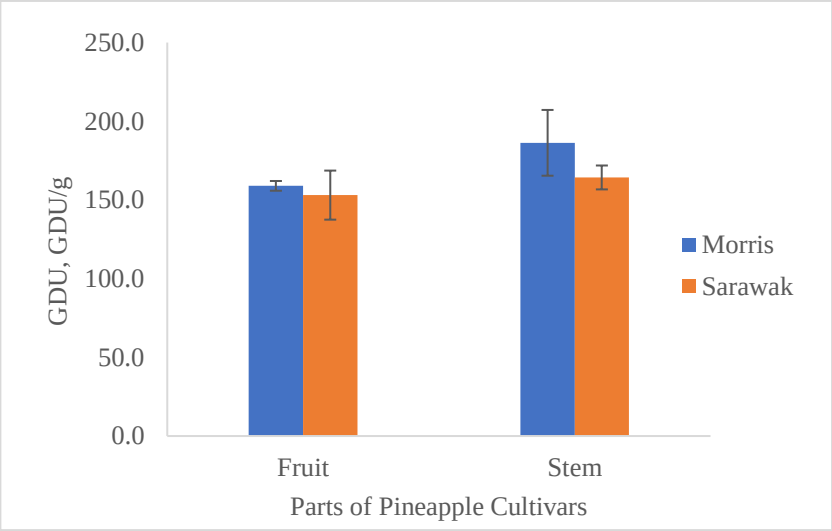

Figure 3. The GDU analysis of different cultivars of pineapple fruit and stem.

The current result was higher compared to previous study [20] where the fruit of three pineapple varieties; Mauritius, Kew, and Queen, were having 40.66 ± 1.04, 48.32 ± 1.00, and 39.36 ± 1.14 GDU/g, respectively. However, previous study recorded a higher bromelain activity for purified extract of fruit (437.5 GDU/mg) and stem (1750 GDU/mg) of local pineapple from Bangalore [11]. Both crude stem bromelain presently was having a much higher bromelain activity than the crude fruit bromelain from both pineapple varieties of Morris and Sarawak. The findings are consistent with those reported by [21], who found that the fruit bromelain had less enzymatic activity than the bromelain in the stem.

Effect of temperature on fruit and stem bromelain

The thermal profile of crude fruit and stem bromelain was determined at temperature ranging from 35 to 75 ℃. The enzyme activity against casein was calculated and presented in Figure 4. Both crude fruit bromelain from Morris and Sarawak variety showed highest enzyme activity at 35 ℃, denoting its optimum temperature, while both crude stem bromelain of the two varieties showed highest enzyme activity at 45 ℃. Upon reaching this point, the activity of the enzyme decreased as the incubation temperature is increased due to the disruption of peptide bonds and disulfide bonds of the inactivated enzymes [11, 22].

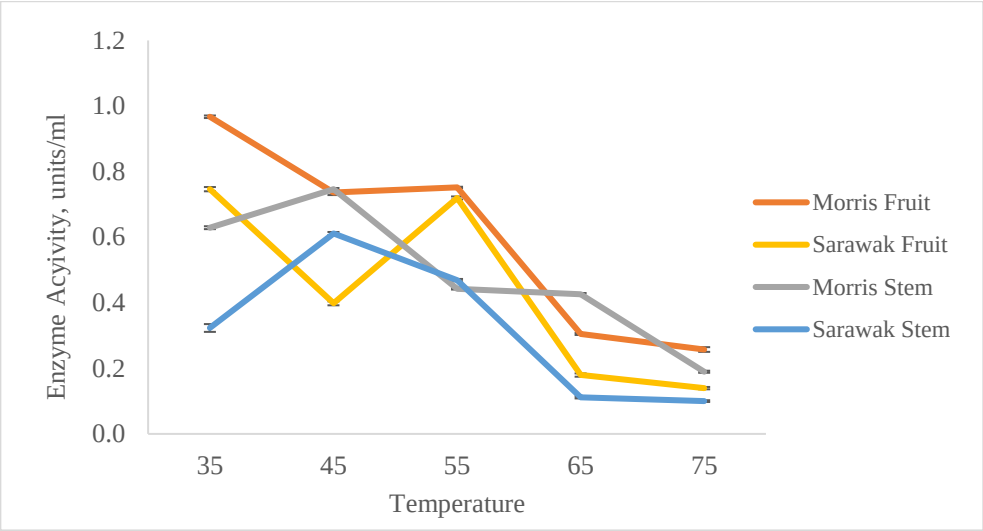

Figure 4. The enzyme activity of different cultivars of pineapple fruit and stem pre-treated at different temperatures

This outcome is similar and falls within the ideal bromelain fruit and stem temperature ranges of 37–70 °C [21, 24, 25] and 50–60 °C [8, 21, 23], respectively. The broad range of optimum temperature is due to the different molecular weights of bromelain, where the ideal temperature rises with increasing molecular weight because chemical links inside the bromelain structure make it more stable, which is caused by different methods of extraction [17]. The usage of the enzyme either directly or after modifications is investigated using the information of the ideal temperature. In contrast to most enzymes, which are destroyed or denatured between 40 and 60 °C, bromelain is exceptionally heat resistant, maintaining proteolytic activity [16].

Effect of pH on fruit and stem bromelain activity

The effect of pH in enzyme activity of crude fruit and stem bromelain from both Morris and Sarawak variety was measured. The pH ranged 3.6 to 7.6 were performed in each crude bromelain as presented in Figure 5. Crude fruit and stem bromelain from Morris variety has an optimum pH at 6.6, while Sarawak variety at pH 5.6.

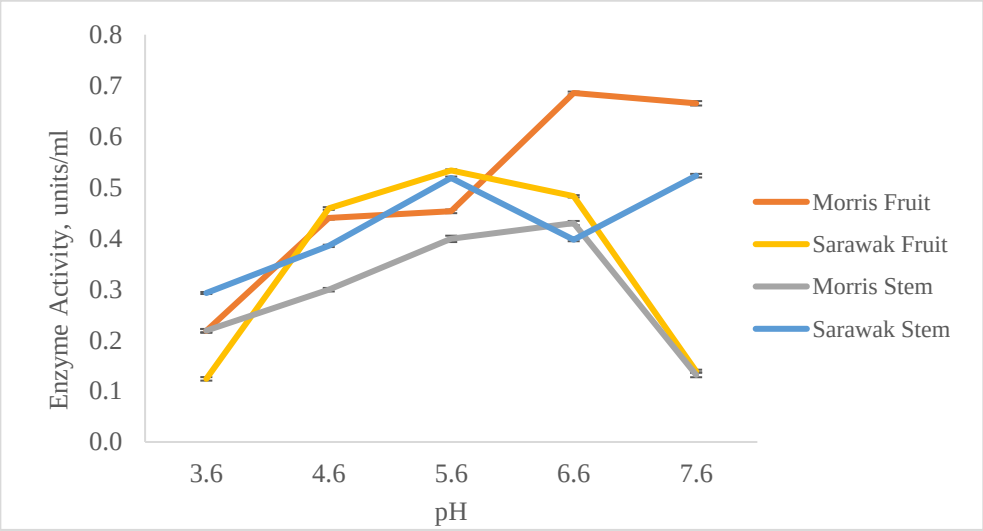

Figure 5. The enzyme activity of different cultivars of pineapple fruit and stem pre-treated at different pH

This result is in accordance with the fact that bromelain is sensitive to pH variation and works best in the range of 3-8 [13]. Bromelain activity reduces at low pH, whereas adsorption decreases at high pH [12]. The reduction in activity is due to the active side of the enzyme environment encountering proton deficit and a rise in pKa [26]. Previous studies reported partially purified fruit bromelain and crude stem bromelain were optimum at pH 7 [12, 13]. Other study showed that crude fruit bromelain was optimum at pH 4.5 [8]. The pH of the enzyme environment influences its activity in a variety of ways according to the study by [27]. First, the enzyme is stable within specific ranges both below and above its own optimal pH, which is where it operates at its highest efficiency. Second, ambient pH has an impact on the stability of the enzyme; at extremes of acidity or alkalinity, the enzyme may become denatured. Thirdly, the pH of the reaction mixture may have an impact on how the enzyme and substrate associate.

CONCLUSION

Identifying the presence of bromelain enzyme and their concentration from each part of pineapple of different varieties is crucial in order to determine which contain higher concentrations than the others. In this research, two parts of pineapple i.e., fruit and stem of different variety was investigated for their bromelain activity. Bradford's assay was used to determine the protein content of the crude bromelain extract collected from fruits and stems. Morris variety crude extract had the highest value. As a result of further investigation to determine the proteolytic activity of the crude stem and fruit bromelain, the Morris variety was found to have the greatest activity for stem bromelain and the Sarawak variety to have the highest activity for fruit bromelain. The Morris variety's fruit and stem bromelain showed the maximum activity, according to the Gelatin Digestion Unit (GDU) research. The results obtained indicated both pineapple varieties of Morris and Sarawak extracts of fruit and stem were having comparable bromelain enzyme activity, which can be further analysed and developed for their application at different fields, such as food and beverages, pharmaceuticals, and cosmetic purpose.

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