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Purification and Characterization of Bromelain from Pineapple Variety Josapine

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Abstract: Pineapple (*Ananas comosus (L) Merr*) is a plant that contains the bromelain enzyme which can be found in the crown, flesh, stem, and leaves of the pineapple plant. The bromelain enzyme has the ability to break down the structure of protein into amino acids. Pineapple peels waste from processing are usually discarded and not utilized properly. This study aimed to purify and characterize the bromelain enzyme from pineapple variety Josapine's peels. The production of bromelain powder in this study was done in four steps, the extraction of crude bromelain from pineapple peel extracts, the purification of bromelain using ammonium sulphate precipitation, desalting using the diafiltration process and, finally, freeze drying using a freeze dryer. The protease activity was determined using casein digestion unit (CDU), meanwhile the protein content was determined using Lowry's Method. Results showed that the highest bromelain activity was observed in maturity index 5 in powder form with 2132.78±18.01 CDU/ml, the highest protein content was observed in maturity index 5 in crude extract form with 4.19±0.17 mg/ml and the highest specific enzyme activity was observed in maturity index 5 in powder form with 15234.14 CDU/mg protein. The agroindustry waste product, Josapine pineapple peel, has potential as a future alternative bromelain source.

Keywords: *Purification, bromelain activity, protein content.*

1. Introduction

The pineapple is believed to originate from Brazil. Once discovered, the pineapple was imported to Europe. Christopher Columbus and his men are thought to have tasted the pineapple initially. The word pineapple started to be used in English in 1938 which refers to the organs of conifer trees (Jungle Dragon, 2024). The European pioneers named the fruit as a 'pineapple' according to what is known as a pinecone. The term pinecone was first recorded in 1694, that is to supplant the importance of the pineapple (Hoque *et al*., 2019). According to Sun, (2015), the pineapple or *Ananas comosus L. Merr*. is widely accepted to have come from South America, Argentina, and Paraguay. The pineapple is known by the people of South America just before Christopher Columbus arrived in 1493. The European pilgrims use the word pineapple to present the natural product as to have come from pinecones. Meanwhile, the word Ananas is the initial name of the natural product that comes from the word for pine 'nanas' and 'comosus' referring to the tuft of the stem of the natural product (Jungle Dragon, 2024).

Pineapple is a significant nourishment crop which is planted widely in the tropical and sub-tropical regions of the world. It is a significantly produced natural product for business in Malaysia and is, for the most part, utilized as organic ingredients for pastry or to produce canned pineapple as cuts or rings, squeezes, and sticks. There are five assortments of pineapples in Malaysia; these include the Morris, Sarawak, Gandol, Josapine and N36 (Lasekan & Hussein, 2018). Pineapple processing such as canning and juice produces wastes including pineapple peels. Discharge of pineapple peels during this process will produce waste and lead to serious environmental pollution. In artificial practices, pineapple waste is either used as animal feed or disposed to the soil as waste. Natural enzyme contained in pineapple is called bromelain. Bromelain is an enzyme which is believed to have numerous benefits and is veritably promising to the development of food and medicinal diligence. Bromelain can be found in several parts of the pineapple. The specific part from which it is extracted lends its name to the enzyme; thus, we have fruit bromelain and stem bromelain (Bala *et al*., 2012). Bromelain activity has been reported to be within a pH range of 3 to 8 and a temperature range of 30–70 ºC (Kumar *et al*., 2011;Liang *et al*., 2012;Ramli *et al*., 2018). The natural bromelain enzyme has been used as a meat tenderizer, anti-browning agent and in the processing of formulas for babies (Tochi et al., 2008). As a protease, it hydrolyses proteins in these formulas, therefore making amino acids more readily available to babies. Bromelain is utilized in some applications including cosmetic and pharmaceutical and in textile industry as reported by (Aehle, 2007;B.K. Bhattacharrya, 2008;Ataide *et al*., 2018;Sancesario *et al*., 2018).

Some successful methods have been used for bromelain extraction and purification. Purification of enzymes is important to determine the three-dimensional structure of an enzyme and its impact on the functionality of the enzyme (A. Illanes, 2008). The successful methods in bromelain purification include aqueous two phase systems (Ketnawa *et al*., 2010;Ferreira *et al*., 2011), ammonium sulphate precipitation (G & Viswanathan,

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2013;Gautam *et al*., 2010), ethanol precipitation (Martins *et al*., 2014), ion exchange chromatography (G & Viswanathan, 2013;Gautam *et al*., 2010; Bresolin *et al*., 2013), membrane separation (Seguí & Fito Maupoey, 2018) and adsorption using functionalized Santa Barbara Acid-15 (Arumugam & Ponnusami, 2013).

The pineapple variety Josapine was chosen because it is abundantly planted in Malaysia which covers about 41.5% or 6725 hectares. This shows that the waste produced by the pineapple industry is large at about 150 000 kg. Other than that, the pineapple variety Josapine was easily obtained aIB, 2022). The main reason to select the maturity index 5 and 7 is to determine whether there is a significant difference of bromelain activity among them. As a justification, the maturity index 3 and 4 is not chosen because their difference is too narrow while maturity index 5 is in the middle and maturity index 7 is overripe. Maturity index 1 is too young while maturity index 2 is usually selected for harvesting. Thus, the aim of this study was to determine the bromelain activity, protein content and specific enzyme activity of bromelain extracted from pineapple variety Josapine peel with maturity indices 2, 5 and 7.

2. Materials and Methods

Preparation of Samples

Pineapple of the Josapine variety with different maturity indices of 2, 5 and 7 used in this study were purchased from a local market in Seksyen 6, Shah Alam, Selangor, Malaysia. Table 1 shows the seven-maturity index of pineapple according to the standard specification by Federal Agriculture Marketing Authority (FAMA) (FAMA, 2020). The pineapple peel with maturity index 2, 5 and 7 were cut into small pieces and crushed in a food processor (Model PB-3203L, Brand Pensonic, Country Malaysia) with the addition of water at a ratio of 1:1 to produce pineapple peel extract. Extraction was done in a chilled temperature (4^{o}C). Next, the extract was filtered through a muslin cloth to remove the solid parts. The filtrate was collected and kept in a freezer (-20^{o}C) to be used in further experiments (Gautam *et al*., 2010).

Table 1. Maturity Indices of Pineapple

Index	Characteristics
1	Young or immature. The entire surface of the skin or the eye is slightly oval-shaped upward, dark green with reddish bractea. Flesh is very solid, firm, and sour. Not yet ready for harvest.
2	Early maturity stage. All eyes are quite rounder with glossy dark green and traces of yellow between eyes at the base. The bractea is whitish in colour and dry. Flesh is solid, firm, and sourish. Inedible flesh. Suitable to be harvested for export.
3	Mature fruit. All eyes are green with 1-2 eyes yellowish green at the base. Flesh is solid, crunchy, and sourish. Edible flesh but not palatable. Suitable to be harvested for export.
4	Underripe. An average of 25% of the eyes from the base of the fruit turns into yellowish-orange. Flesh is solid, firm, crunchy and sweet sour. Edible flesh. Suitable to be harvested for domestic market
5	Early ripe. Almost 50% of the eyes are yellowish-orange from the base of the fruit. Flesh is less spongy, yet juicy, sweet, and fragrant. The best level to be eaten fresh.
6	Ripe. Over 75% of their eyes are yellowish-orange. Flesh is a little flaccid, yet spongy, very juicy, sweet, and fragrant. Less suitable to be harvested for export but more suitable for domestic market.
7	Overripe. The whole eyes are yellowish-orange. Flesh is flaccid, soft, very juicy, and sweet sour. Still edible flesh. Not suitable for any domestic market or export.

Production of Bromelain Powder

Purification of Bromelain Extract

Firstly, the purification of the crude bromelain extract was carried out using the ammonium sulphate precipitation method. About 10 mL of crude bromelain extract was transferred into a centrifuge tube and placed in an ice bath. Then, about 6 g of ammonium sulphate was added pinch by pinch. Then, the sample was left for incubation for 1 hour until the appearance of precipitation is observed. Then, the cold incubated sample was centrifuged (Model 5420, Brand Kubota, Country Japan) at 3500 rpm for 30 mins. Then, the pellet was collected by dissolving in 10 mL Tris buffer, while the supernatant was removed. The purified bromelain was stored in a chiller for further analysis (Devakate *et al*., 2009).

Desalting

The dialysis process was performed using a diafiltrator machine. Firstly, about 1 L of deionised water was allowed to run into the diafiltrator machine for washing. Then, before running the sample, the initial salinity of purified extract was checked using a salinity meter (Model RSA0100, Brand Trans Instruments, Country Singapore). Then, the purified extract sample was allowed to run into the diafiltrator machine, until the salinity percentage reached 0%. The purified extract sample was dialysed using the deionised water. After the salinity reached 0%, the dialysed sample was stored in a freezer for further analysis (Devakate *et al*., 2009).

Drying of Desalted Bromelain

Desalted bromelain was dried using a vacuum freeze dryer (Model Alpha 1-4 LD Plus, Brand Christ, Country Germany) which took 4 days for complete drying at -60^{o}C. The desalted bromelain was frozen at -20^{o}C before undergoing the freeze-drying process to provide a necessary condition for low temperature drying (Devakate *et al*., 2009).

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Protein Content

Preparation of Reagents

Firstly, two solutions were prepared namely solution A and solution B. For solution A, 50 ml of 2% (w/v) of sodium carbonate (Na_2CO_3) solution was mixed with 50 ml of 0.1 N sodium hydroxide (NaOH) solution. For solution B, 10 ml of 1.56% (w/v) of copper sulphate ($CuSO_4.5H_2O$) solution was mixed with 10 ml of 2.37% (w/v) of sodium potassium tartarate ($C_4H_4O_6KNa.4H_2O$) solution. Then, 100 ml of solution A and 2 ml of solution B were mixed to prepare Lowry's solution. To prepare the Folin-Ciocalteau reagent, about 2 ml of Folin-Ciocalteau was mixed with 2 ml of distilled water.

Protein Assay (Lowry's Method)

Firstly, about 1 ml of the sample was transferred into a test tube. Then, about 5 ml of Lowry solution was added into the test tube. The test tube was vortexed and incubated at room temperature for 10 minutes. Then, about 0.5 mL of Folin was added into the test tube. Then, the test tube was incubated in the dark for 30 minutes at room temperature. Next, the sample was transferred into a beaker. Afterwards, the absorbance of sample was measured at 595 nm using a UV-Visible spectrophotometer (Model Lamda 35, Brand Perkin Elmer, Country USA). The amount of protein content was determined using the BSA standard calibration curve (Ketnawa *et al.*, 2012).

Bromelain Activity

Bromelain activity in the sample was determined using the casein digestion unit (CDU) method (Enzyme Development Corporation, 2015). Firstly, approximately 5 ml of the casein substrate was transferred into a test tube. The test tube was then immersed in a water bath at 37°C for 10 minutes. Next, about 5 mL of trichloroacetic acid (TCA) stopping reagent and 1 mL of the enzyme solution were added to the blank test tube, while only about 1 mL of the enzyme solution was added to the sample test tube. The test tubes were vortexed and placed back into the water bath at 37°C for 10 minutes. Subsequently, about 5 mL of the TCA stopping reagent was added to both the blank test tube and sample test tube, and they were vortexed again before being placed back into the water bath at 37°C for 30 minutes. Afterwards, the blank test tube and sample test tube were removed from the water bath and left to cool to room temperature. The blank test tube and sample test tube were then filtered using filter paper, and the filtrate was collected in a beaker. The absorbance was measured at 280 nm using a UV-Visible spectrophotometer (Soares *et al*., 2012). The bromelain activity was calculated using the formula.

$$CDU/mg = [(Et - Eb)/Es] \times 50 \times (11/10) \times DF$$

Where;
DF: Dilution Factor
Et = Absorbance of enzyme sample tube
Eb = Absorbance of blank sample tube
Es = Absorbance of standard Tyrosine

Standard Tyrosine

A standard solution containing exactly 50µg/ ml of L-Tyrosine was prepared in 0.1N HCl. The absorbance of the standard was measured and recorded at 275 nm using distilled water for the blank test tube (Enzyme Development Corporation, 2015).

Specific Enzyme Activity

The specific enzyme activity in pineapple peels will be calculated using the following formula.

$$\text{Specific enzyme activity} = \frac{\textit{Bromelain Activity}}{\textit{Protein Content}}$$

Data Analysis

All experiments were performed in triplicate. The data obtained were analyzed using the software, Statistical Package for the Social Sciences (SPSS). The analysis of variance (ANOVA) tests was performed.

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Table 2. Activity of pineapple Josapine

Sample	Bromelain activity (CDU/ml)	Protein content (mg/ml)	Specific bromelain enzyme activity (CDU/mg protein)	Purification level
Index 2				
Pineapple peel extract	659.16±10.14^{d}	3.72±0.11^{a}	177.19^{d}	1.00^{d}
Purified bromelain	791.66±33.18^{c}	0.80±0.05^{b}	989.57^{c}	5.58^{c}
Desalted bromelain	1004.95±5.02^{b}	0.21±0.01^{c}	4785.47^{b}	27.00^{b}
Bromelain powder	1440.63±63.87^{a}	0.14±0.00^{d}	10290.21^{a}	58.07^{a}
Index 5				
Pineapple peel extract	877.89±35.71^{d}	4.19±0.17^{a}	209.52^{d}	1.00^{d}
Purified bromelain	1574.43±24.64^{c}	0.74±0.05^{b}	2127.60^{c}	10.15^{c}
Desalted bromelain	1728.31±63.49^{b}	0.19±0.01^{c}	9096.36^{b}	43.41^{b}
Bromelain powder	2132.78±18.01^{a}	0.14±0.01^{d}	15234.14^{a}	72.70^{a}
Index 7				
Pineapple peel extract	505.96±14.49^{d}	3.84±0.14^{a}	131.76^{d}	1.00^{d}
Purified bromelain	1012.36±9.64^{c}	1.15±0.06^{b}	880.31^{c}	6.68^{c}
Desalted bromelain	1281.75±16.32^{b}	0.22±0.01^{c}	5826.13^{b}	44.21^{b}
Bromelain powder	1611.83±8.57^{a}	0.13±0.01^{d}	12398.69^{a}	94.10^{a}

Means within each column with different superscript are significantly different at $p < 0.05$

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3. Results and Discussion

In this study, bromelain powder was produced in four steps. The production steps included the extraction of crude bromelain from pineapple peel extract, followed by bromelain purification using ammonium sulphate precipitation. Then, a desalting process was performed using a diafiltration machine, and, finally, freeze drying was done using a freeze dryer. Subsequently, the samples obtained from each processing step were analysed for bromelain activity, protein content, and specific bromelain enzyme activity.

The bromelain activity measures the quantity of active enzymes present in the sample, while the protein content measures the total level of protein in a solution. The specific bromelain enzyme activity measures the enzyme purity in a mixture. The purification level, on the other hand, shows whether the sample became purer following the purification technique.

The bromelain activity, protein content, and specific bromelain enzyme activity were tested for pineapple peel extract, purified bromelain, desalted bromelain, and bromelain powder at maturity indices 2, 5, and 7. Table 2 shows the bromelain activity, protein content, and specific enzyme activity for each sample.

For maturity index 2, the bromelain activity, protein content, and specific enzyme activity of pineapple peel extract were 659.16 CDU/ml, 3.72 mg/ml, and 177.19 CDU/mg of protein, respectively. This step was to isolate most of the protein present in the pineapple peel extract since it could affect the bromelain activity as well as protein content in the following steps. The value of bromelain activity, protein content and specific bromelain enzyme activity in the purified bromelain were 791.66 CDU/ml, 0.80 mg/ml, and 989.57 CDU/mg of protein, respectively. For the desalted bromelain, its value of bromelain activity, protein content and specific bromelain enzyme activity were 1004.95 CDU/ml, 0.21 mg/ml, and 4785.47 CDU/mg of protein, respectively. While the value of bromelain activity, protein content and specific bromelain enzyme activity in the bromelain powder were 1440.63 CDU/ml, 0.14 mg/ml, and 10290.21 CDU/mg of protein, respectively.

Similarly, for maturity index 5, the bromelain activity, protein content, and specific enzyme activity of pineapple peel extract were 877.89 CDU/ml, 4.19 mg/ml, and 209.52 CDU/mg of protein, respectively. The value of bromelain activity, protein content and specific bromelain enzyme activity in the purified bromelain were 1574.43 CDU/ml, 0.74 mg/ml, and 2127.60 CDU/mg of protein, respectively. For the desalted bromelain, its value of bromelain activity, protein content and specific bromelain enzyme activity were 1728.31 CDU/ml, 0.19 mg/ml, and 9096.36 CDU/mg of protein, respectively. While the value of bromelain activity, protein content and specific bromelain enzyme activity in the bromelain powder were 2132.78 CDU/ml, 0.14 mg/ml, and 15234.14 CDU/mg of protein, respectively.

Lastly, for maturity index 7, the corresponding values of bromelain activity, protein content, and specific enzyme activity of pineapple peel extract were 505.96 CDU/ml, 3.84 mg/ml, and 131.76 CDU/mg of protein, respectively. The value of bromelain activity, protein content and specific bromelain enzyme activity in the purified bromelain were 1012.36 CDU/ml, 1.15 mg/ml, and 880.31 CDU/mg of protein, respectively. For the desalted bromelain, its value of bromelain activity, protein content and specific bromelain enzyme activity were 1281.75 CDU/ml, 0.22 mg/ml, and 5826.13 CDU/mg of protein, respectively. While the value of bromelain activity, protein content and specific bromelain enzyme activity in the bromelain powder were 1611.83 CDU/ml, 0.13 mg/ml, and 12398.69 CDU/mg of protein, respectively.

The determination of bromelain activity, protein content and specific enzyme activity were important to know the trend loss of bromelain enzyme during the purification processing studied. Every step of the process gives different results for bromelain activity, protein content and specific enzyme activity. The purification level was estimated by dividing the specific enzyme activity at each purification step with that of crude pineapple peel extract.

Table 2 shows that the bromelain activity was significantly higher in the bromelain powder at the 5% level followed by those in the purified bromelain, desalted bromelain, and pineapple peel extract of those maturity indices. Bromelain activity increased after the purification process. This is because only bromelain was purified from the pineapple peel extract. A previous study done by Nadzirah *et al*., (2012) on the extraction of bromelain form pineapple crown variety N36 by using distilled water as the extraction medium found that the bromelain activity was significantly higher in bromelain powder (529.77 CDU/ml), followed by purified (501.08 CDU/ml), desalted (485.78 CDU/ml), and pineapple extract (426.49 CDU/ml). Furthermore, Misran *et al*., (2019) conducted a study on pineapple variety Morris and found that the highest bromelain activity was observed in the peel (229.64 CDU/ml). Another study by Nor *et al*., (2015) found that the bromelain activity for pineapple peel variety Smooth Cayenne is 429.70 CDU/ml. In this study, the values of bromelain activity ranges between 505.96 to 2132.78 CDU/ml which was higher than the findings by Nadzirah *et al*. (2012) and Nor *et al*. (2015) which ranged between 229.64 to 529.77 CDU/ml. The different results obtained from this study compared to the previous findings may have been due to the difference in species of pineapple used. Different varieties of pineapples will influence the quality of bromelain extracted. Environmental factors such as climatic growth conditions, growth, ripening stage, temperature, duration of storage and thermal treatment may have influenced the bromelain activity (Ramli & Munir, 2022). Therefore, pineapple peel variety Josapine can provide a better source of bromelain enzyme compared to N36 and Smooth Cayenne variety.

In this study, the protein content was significantly higher in the pineapple peel extract while specific enzyme activity was significantly higher in the powdered bromelain. A previous study done by Gul et al., (2021) found that protein content in pineapple variety Phu Lae (0.215 mg/ml) and Nang Lae (0.337 mg/ml). Another study by Misran et al., (2019) found that the protein content for pineapple peel variety Morris was 83.33 mg/ml.

Furthermore, Nor et al., (2015) found that the protein content of pineapple peel variety Smooth Cayenne was 1.37 mg/ml. To compare, the protein content obtained in this research study was lower than the above previous studies. The range of protein content obtained in this study was between 0.13 to 4.19 mg/ml meanwhile the protein content for the above previous studies was between 0.2 to 83.33 mg/ml. According to Ramli & Munir, (2022) the difference in the protein content is influenced by the variety of pineapple, climate during culture, the stage of ripeness, the timing of harvest, and the extraction technique.

Lastly, regarding specific enzyme activity, (Gul *et al*., 2021) studied the specific enzyme activity of pineapple variety Phu Lae extract purified with 60% ammonium sulphate. The highest specific enzyme activity was observed in the core (105.17 CDU/mg), followed by the crown (64.20 CDU/mg) and peel (54.69 CDU/mg). Additionally, Umesh Hebbar *et al*., (2008) examined the specific enzyme activity of pineapple variety Kew, and the results showed that the highest specific enzyme activity was observed in the crown (49.41 CDU/mg), followed by the core (40.32 CDU/mg), stem (14.72 CDU/mg), and peel (10.22 CDU/mg). For comparison, the specific bromelain enzyme activity obtained from this research study was higher than reported in the previous studies. The range for specific bromelain enzyme activity for this research was between 131.76 to 15234.14 CDU/mg protein meanwhile for the reported above previous studies was between 10.22 to 105.17 CDU/mg protein. According to Susanti *et al*., (2022) the differences in specific bromelain enzyme activity may due to the number of impurities decreasing after the purification steps. This shows that the level of purity value of bromelain enzyme is higher when the value of specific bromelain enzyme activity is higher.

For pineapple peel extract, it has lower bromelain activity, specific bromelain enzyme activity and purification level compared to bromelain powder, but it has the highest protein content. High protein content indicates the presence of other proteins in the sample while low protein content indicates the presence of target bromelain in sample (Nadzirah *et al*., 2012) From the data in Table 2, the purification of enzyme through the drying process (powdered bromelain) yielded the highest purification level at 58.07, 72.70 and 94.10 for maturity index 2, 5 and 7 respectively. The increasing trend in purification level showed that the samples are successfully purified at each stage.

4. Conclusion

The extraction process to produce bromelain was carried out consecutively. Therefore, one step might affect the following steps. Bromelain activity and purification level in powdered bromelain was significantly higher compared to those from pineapple peel extract, purified bromelain, and desalted bromelain.

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