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Purification of bromelain enzyme from MD2 hybrid pineapple core by ultrafiltration and its antioxidative potential

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ABSTRACT

Bromelain, a protease enzyme found in pineapple, is commonly recognized for its therapeutic applications. This study aimed to investigate the antioxidant activity of bromelain from MD2 pineapple core using the ultrafiltration method and assess bromelain antioxidant activities in correlation with its purity. A partially purified bromelain (PPB) was obtained using ammonium sulfate (50%), followed by centrifugal ultrafiltration as a purifying step for ultrafiltrate bromelain (UFB). The antioxidant activities of bromelain were evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and ferric ions reducing antioxidant power methods. Using the protein content of crude extract as a reference, the purification fold of PPB was 0.20-fold, while UFB was 3.25-fold. PPB and UFB showed DPPH scavenging activities with IC_{50} values of 67.60±9.55 µg/mL and 42.11±2.55 µg/mL, respectively. At a concentration of 1000 µg/mL, the reducing power of PPB and UFB was 30.89%±2.03% and 35.09%±1.59%, respectively, which were lower than that of ascorbic acid. The result shows an increase in bromelain antioxidant activities after the ultracentrifugation, concluding that ultrafiltration effectively preserves antioxidant potentials in bromelain. Bromelain is a medium antioxidant with medium potential as a free radical scavenger but has poor reducing power.

Keywords: antioxidant activity; bromelain; MD2 pineapple; ultrafiltration; DPPH; FRAP

1. INTRODUCTION

Free radicals are unstable molecules with unpaired electrons formed by homolytic fission of covalent bonds and redox reactions (Saptarini et al., 2019a). Living cells produce free radicals, called reactive oxygen species (ROS), as part of regular cellular metabolism (Prabakar et al., 2021). ROS include superoxide, hydroxyl, nitrogen dioxide, lipid peroxyl, and peroxyl. However, uncontrolled or excessive ROS production will lead to oxidative stress, affecting cell membrane permeability and fluidity and causing harm to biomolecules, including DNA, proteins, and lipids (Lee et al., 2018). Eventually, the damage to cellular biomolecules, especially DNA, causes mutagenic changes, cellular changes, and various degenerative diseases, which have gained significant attention over the past year among scientists. The presence of ROS causes oxidative stress, which occurs when there is a lack of antioxidants (Lee et al., 2018).

Antioxidants are substances that donate their electrons to free radicals, neutralizing the free radicals and inhibiting oxidative stress (Saptarini et al., 2019a). Exploring the potency of natural antioxidants from plant species is gaining attention since 80% of the world population depends on plant-based formulations (Prabakar et al., 2021). Natural antioxidants, such as ascorbic acid, exhibit antioxidant properties by suppressing free radicals produced by the xanthine oxidase enzyme, scavenging ROS by stabilizing free radicals and stimulating the antioxidant system (Gęgotek and Skrzydlewska, 2022). Natural enzymatic antioxidants, such as superoxide dismutase, glutathione peroxidase, and catalase, are readily available in various vegetables and fruits. Tomatoes are especially preferred as a rich source of antioxidant in cooking as it has the highest capability to retain enzymatic antioxidants after thermal treatment (Ergüder et al., 2007). Conversely, prolonged intake of synthetic antioxidants causes many side effects (Alam et al., 2017). Common synthetic antioxidants are butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Venkatesh and Sood, 2011). BHA is classified as Group 2B, "possibly carcinogenic to humans," by the International Agency for Research on Cancer (Felter et al., 2021), and BHT could disrupt the endocrine system (Pop et al., 2013). Thus, using natural antioxidants derived from a plant's secondary metabolites is favorable. Due to its therapeutic benefits and medical significance, pineapple has been a traditional medicine for centuries (Baidhe et al., 2021; Prabakar et al., 2021). According to the Malaysian Pineapple Industry Board, most pineapple cultivars in Malaysia are Moris, N36, Sarawak, Moris Gajah, Gandul, Yankee, Josapine, Masapine and MD2 (Thalip et al., 2015). The MD2 variety was created to meet consumer demand for fresh pineapples that were superior to other cultivars in terms of sweetness, vitamin C content, color, and shelf life (Thalip et al., 2015). Pineapples are a major source of bromelain, extracted from its waste products, such as cores and peels (Indrajeet et al., 2017). The MD2 variety has higher bromelain content in its cores than other cultivars (Banerjee et al., 2022). Bromelain is a protease enzyme with various clinical properties, including antioxidant properties (Colletti et al., 2021). Bromelain exhibits antioxidative potential by scavenging reactive radicals and inhibiting lipid peroxidation, thus ending the oxidative chain reaction (Lee et al., 2018). Bromelain extracted from pineapple core waste is a potential antioxidant; therefore, finding effective extraction and purification methods is essential to preserve its therapeutic properties (Hale, 2004; Hale et al., 2006).

Researchers are developing methods to economically extract highly pure bromelain without difficulties. Among promising modern techniques in bromelain extraction is membrane filtration developed by Lopes et al. (2009). Various studies have applied membrane filtration in extracting bromelain (Abreu and Figueiredo, 2019). However, limited studies measured the antioxidant activities of bromelain using this technique, especially bromelain from the cores of MD2 pineapples. Thus, this study primarily aimed to determine the effectiveness of ultracentrifugation membrane filtration in preserving protein content and antioxidant activities of the bromelain from MD2 pineapple cores. This research may improve the use of pineapple cores as an alternative source of natural antioxidants in the food or pharmaceutical industries. The antioxidant activity of the bromelain samples was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and ferric ions reducing antioxidant power (FRAP).

2. MATERIALS AND METHODS

2.1 Plant material

MD2 hybrid pineapple, which ripens with an average of 18–20 days after harvest, was identified and provided by SMART KJ Agro (Asia) Plt in Sungai Petani, Kedah, Malaysia.

2.2 Chemicals

Ammonium sulfate and casein were purchased from Nacalai Tesque (Japan). Absolute ethanol, bovine serum albumin (BSA), Bradford reagent, Folin-Ciocalteau's reagent, ferric chloride, potassium ferricyanide, phosphate buffer tablets pH 7, sodium carbonate, trichloroacetic acid, L-tyrosine, L-ascorbic acid, 2, and DPPH were purchased from Sigma Aldrich (Saint Louis, MO, USA). All solvents and standards used were analytical grade and did not require purification.

2.3 Bromelain purification

To compare the effectiveness of the ultracentrifugation extraction technique, three samples were obtained: Crude sample, partially-purified bromelain (PPB), and ultra-filtrate bromelain (UFB). The MD2 pineapples were dissected to separate their core and cut into small pieces. The cores were then blended with a mechanical blender until a smooth consistency was achieved and filtered using a muslin cloth sieve to remove fibrous materials from the juice. Then, it was centrifuged at 12,000 rpm at 4 °C for 20 min. The liquid supernatant was collected, the "Crude sample." Enzymatic pre-treatment was conducted by adding 0.01% (w/v) pectinase into the crude to lessen its viscosity by breaking down pectin (Nor et al., 2018). The bromelain at 50% saturation was precipitated using ammonium sulfate salt. Pinch by pinch, 60.21 g of ammonium sulfate salt was added in 200 mL crude and was left to precipitate overnight at 4 °C. The precipitated solution was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was discarded, and the tubes containing the pellet were kept in an inverted position and blotted onto the tissue to eliminate extra liquid, which comprises salt and contaminants (Chaurasiya and Umesh Hebbar, 2013). The pellet was redissolved in 5 mL of 5% ethanol to obtain PPB (Triastuti et al., 2021). The PPB was then filtered using a 0.22 µm syringe filter before centrifuging using Amicon (Merck Millipore, Germany) centrifugal ultrafiltration tube with a 10 kDa pore membrane. The ultrafiltration tube was centrifuged at 7000 rpm for 20 min. The pellet in the membrane of the filter tube was resuspended using 0.1M phosphate buffer, pH 7.0, to obtain the final UFB.

2.4 Enzymatic activity measurement

Proteolytic activity was measured using a casein digestion unit according to the Sigma-Aldrich universal protease activity assay protocol with some modifications. The measurement used 1.1 mM L-tyrosine and 0.65% casein as standard and substrate accordingly. The absorbance of L-tyrosine standards of varying concentrations was read

using a spectrophotometer (T80+, PG Instrument Ltd, UK) at 660 nm wavelength. Data was plotted to generate a standard curve. The proteolytic activity of the crude sample, PPB, and UFB was analyzed by preparing 0.65% casein stock solution in 0.05 M phosphate buffer pH 7 dissolved at 37 °C. First, 2.5 mL of 0.65% casein was added into all tubes and equilibrated for 5 min at 37 °C. Then, 500 µL of test samples (crude, PPB, and UFB) were mixed and incubated at 37 °C for 10 min. Next, 2.5 mL of 0.11 M trichloroacetic acid was added to all samples before incubating for 30 min at 37 °C. All solutions were then filtered using a 0.45 µm syringe filter, and 1 mL of each filtered solution was pipetted into 2.5 mL of sodium carbonate. 500 µL of Folin's reagent was added immediately and then mixed thoroughly before incubating for 30 min at 37 °C. After incubation, absorbance was taken using a spectrophotometer at 660 nm wavelength. Proteolytic activity was obtained by using the following formula:

$$\text{Activity (U/mL)} = \frac{\text{Tyrosine equivalent released} * \text{total volume of reaction}}{\text{volume of enzyme sample} * \text{reaction time} * \text{cuvette volume}} \quad (1)$$

2.5 Protein quantification

Protein content was measured using bradford assay to compare the bromelain recoverability between the samples. Bovine serum albumin (0.05–2 mg/mL) was used as the standard to generate the standard curve. 100 µL of the crude sample, PPB, and UFB, were mixed with 1 mL Bradford's reagent and incubated for 10 min. The absorbances were read in triplicates using a spectrophotometer at 595 nm. The yield, purification fold, and specific activities for PPB and UFB were calculated to compare the bromelain content in PPB and UFB with the bromelain content in crude sample:

$$\text{Yield (\%)} = \frac{\text{Total enzymatic activity of bromelain sample(U/mL)}}{\text{Total enzymatic activity of crude sample(U/mL)}} \quad (2)$$

$$\text{Specific Activity (CDU/mg)} = \frac{\text{Total enzymatic activity (CDU/mL)}}{\text{Protein content (mg/mL)}} \quad (3)$$

$$\text{Purification Fold} = \frac{\text{Specific activity of bromelain sample (CDU/mg)}}{\text{Specific activity of crude sample(CDU/mg)}} \quad (4)$$

2.6 DPPH assay

DPPH scavenging activity of PPB and UFB was conducted following a previous study (Saptarini et al., 2019b). Using ethanol, bromelain extracts and ascorbic acid were prepared at various concentrations (15.63, 31.25, 62.50, 125, 250, 500, and 1000 µg/mL). An equal volume of 0.002% DPPH (in 99.5% ethanol) and samples were mixed thoroughly and incubated for 30 min in a dark condition to prevent oxidation. The ascorbic acid (AA) sample was used as the standard. A mixture of ethanol and DPPH (1:1) was used as a negative control, while 96% ethanol was used as blank. The absorbances were then measured at 517 nm using a spectrophotometer. In a DPPH assay, the anti-oxidant donates an electron to the DPPH radical, changing the color to pale yellow (Figure 1a). The percentage of scavenging activity was calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance control} - \text{Absorbance sample}\ )}{\text{Absorbance control}} \text{ x } 100 \quad (5)$$

2.7 FRAP assay

The reducing power of purified Bromelain extract was determined using a previous study with modifications (Vijayalakshmi and Ruckmani, 2016). Ascorbic acid (1 mg/mL) was prepared as standard and positive control. 250 µL of PPB, UFB, and ascorbic acid at various concentrations (7.81, 15.63, 31.25, 62.50, 125, 250, 500, 1000 µg/mL) were added to 625 µL of 0.2 M phosphate buffer pH 6.6 and 625 µL of 1% w/v potassium ferri-cyanide. All solutions were mixed thoroughly and incubated at 50 °C for 20 min before adding 625 µL of 10% trichloroacetic acid and centrifuging at 3000 rpm for 10 min. An equal amount of 625 µL supernatant and distilled water was mixed before 125 µL of 0.1% w/v ferric chloride was added. Absorbance was read at 700 nm. Distilled water was used as blank. In a FRAP assay, the reducing power is indicated by the change of color from yellow to blue or green (Figure 1b). The reducing power of PPB, UFB, and the standard was calculated using the following calculation (Güder and Korkmaz, 2012):

$$\text{Reducing power (\%)} = \frac{\text{Absorbance sample}}{\text{Absorbance standard ascorbic acid}} \text{x} 100 \quad (6)$$

2.8 Data analysis

Data were expressed as mean ± standard error mean (SEM). All tests were performed in triplicate, and the results are shown as the mean values of the three independent experiments. Analysis of variance was used to compare means between the three experimental groups from data of triplicate measurement by using IBM SPSS software (Version 28.0). The significance level was set at p-value<0.05.

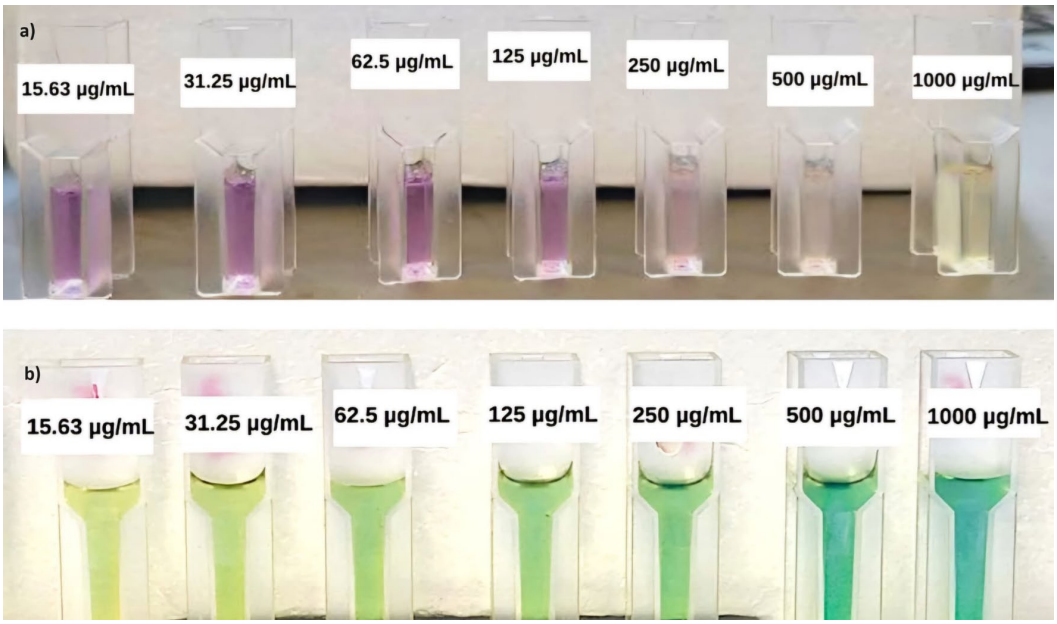


Figure 1. (a) The reduction color of DPPH from purple to pale yellow and (b) the reducing power in FRAP assay from yellow solution to various shades of greenish blue color

3. RESULTS AND DISCUSSION

3.1 Bromelain purification

Bromelain crude extract was partially purified by ammonium sulfate salt at 50% saturation. The ammonium sulfate precipitation method aims to acquire a concentrated protein pellet by fractionation (Soares et al., 2011) after the centrifugation, as shown in Figure 2a. Typically, in a solution, protein forms hydrogen bonds with water molecules. Ammonium sulfates are small, highly charged ions, and when in high concentrations, these ions compete with protein to bind to the water molecules. Removing water molecules from the protein decreases its solubility, resulting in precipitation (Wingfield, 2001). Centrifugal ultrafiltration method was applied to separate excess ammonium sulfate salt from the solution to obtain bromelain extract with higher purity (Figure 2b).

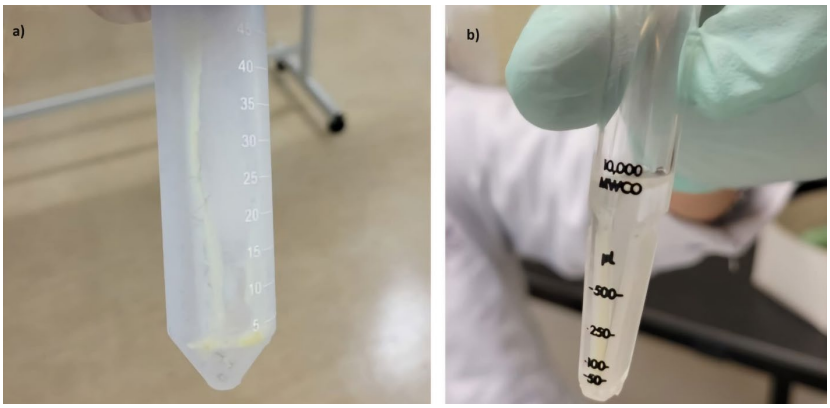


Figure 2. (a) Protein pellet obtained from ammonium sulfate precipitation after centrifugation and (b) bromelain retentate retained in centrifugal ultrafiltration filter membrane (10 kDa membrane pores)

In this study, bromelain was extracted from pineapple core by ammonium sulfate precipitation, followed by centrifugal ultrafiltration to obtain purified bromelain enzyme. Principally, ammonium sulfate salt will increase the surface tension of the water and enhance the hydrophobic interaction between the target protein and water, causing salting out (Setiasih et al., 2019). 50% ammonium sulfate concentration was chosen based on the research done by Gul et al. (2021), which found that 50% concentration had the highest recovery of bromelain (215.22%). However, without an appropriate technique for the desalting process, excess ammonium salt in the extract solution would interfere with the protein binding mechanism, thus underestimating the true value of the assays performed, especially colorimetric assays. Using 10 kDa pore membrane ultrafiltration was proven viable for bromelain extraction by Lopes et al. (2009), which showed a recovery of 100% of proteolytic activity and a 10-fold in bromelain yield. Centrifugation using ultrafiltration tube forced the interference materials to pass through the 10 kDa membrane, while bromelain was retained in the membrane chamber as the molecular weight of bromelain

was approximately 24–37 kDa (Bala et al., 2012). Removing excess ammonium sulfate salt and other impurities increases the purity and bromelain yield.

3.2 Purification yield and enzymatic activity

Table 1 shows the protein content, proteolytic activity, specific activity, purification fold, and yield for crude, PPB, and UFB. The protein content of crude, PPB, and UFB was 1.40±0.007 mg/mL, 1.69±0.019 mg/mL, and 2.41±0.069 mg/mL, respectively. The proteolytic activity for crude, PPB, and UFB was 0.79±0.001 CDU/mL, 0.67±0.000 CDU/mL, and 4.40±0.004 CDU/mL, respectively. The specific activity for crude, PPB, and UFB was 0.56±0.001 CDU/mL, 0.40±0.004 CDU/mL, and 1.83±0.051 CDU/mL, respectively. The protein content of the sample was increased after each purification step. Conversely, the proteolytic activity and specific activity of PPB are lower than that of crude even though the crude had higher protein content than PPB. The low proteolytic and specific activity of PPB was due to the presence of excess ammonium sulfate salt that hinders the enzyme's active site, causing less substrate binding to the active site (Junaidi et al., 2017; Koteshwara et al., 2021). The purification fold of PPB was decreased with a folding of 0.20, whereas UFB has a purification fold of 3.25. Principally, the purification fold should increase more than one after the purification method has been applied (Devi and HemaLatha, 2014). A low purification fold suggests salt interference in the solution. Hence, ultrafiltration technique was conducted to completely remove ammonium sulfate salt and purify the bromelain to increase the purity of the enzyme. The effectiveness of the centrifugal ultrafiltration method was indicated by increasing purification fold and bromelain yield, equivalent to that in the study conducted by Gul et al. (2021). The yield is the amount of enzyme activity preserved in the purified sample compared with the original sample, the crude. The percentage of recovered enzymatic activity in enzyme purification is frequently used to demonstrate the effectiveness of the purification technique in capturing the target enzyme. The bromelain yield was low at approximately 65% after the ammonium sulfate precipitation step and significantly increased to 222% after the ultrafiltration method was applied (Table 1).

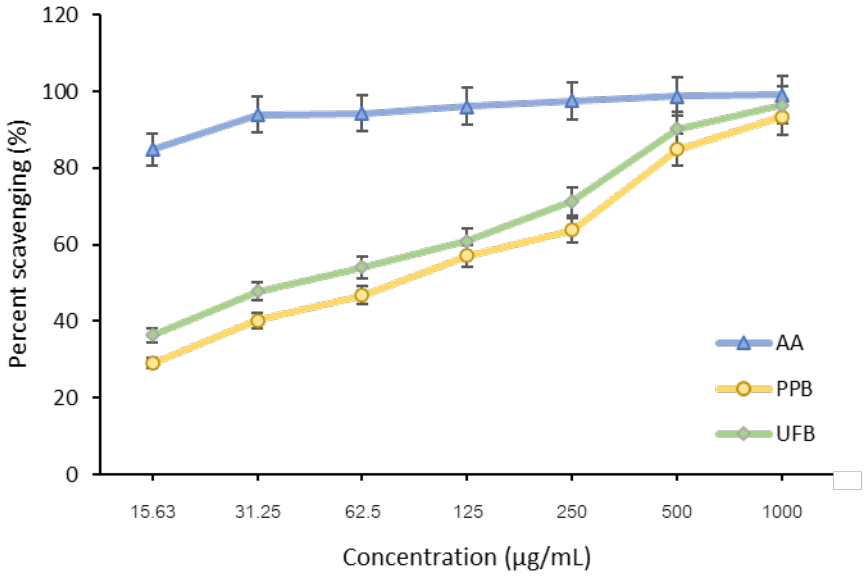
Table 1. Evaluation of bromelain activity parameters at purification step by ammonium sulfate fractionation and centrifugal ultrafiltration method

Sample	Protein content (mg/mL)	Proteolytic activity (CDU/mL)	Specific activity (CDU/mg)	PF	Yields (%)
Crude	1.40±0.007[a]	0.79±0.001[a]	0.56±0.001[a]	1	100
PPB	1.69±0.019[b]	0.67±0.000[b]	0.40 ±0.004[a]	0.20	65.40
UFB	2.41±0.069[c]	4.40±0.004[c]	1.83±0.051[b]	3.25	222.48

Note: All values represent the mean ± SEM of three independent experiments conducted in triplicate.
Means that do not share a common letter in a column were significantly different at $p \leq 0.05$.
PPB = partially purified bromelain, UFB = ultrafiltrate bromelain, PF = purification fold

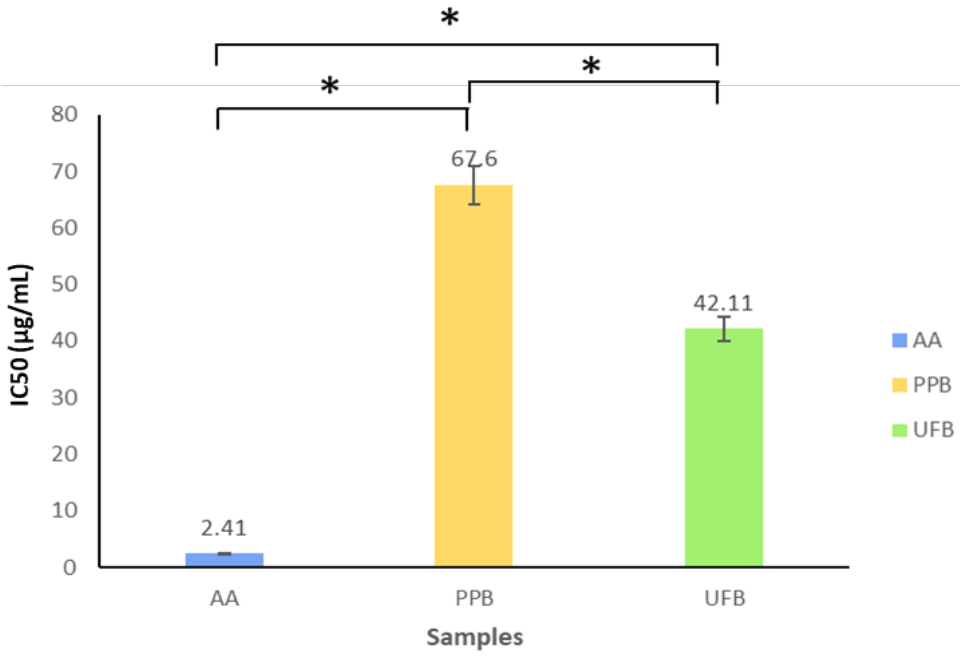
3.3 Antioxidant activity

The antioxidative potential of each bromelain sample was identified using DPPH radical scavenging and FRAP assay. Bromelain comprises many thiol endopeptidases and is a plant protease with various therapeutic and clinical effects (Varilla et al., 2021). Manivasagan et al. (2013) reported that protease is an enzyme that exhibits antioxidant potentials, and Prabakar et al. (2021) specifically tested bromelain as an antioxidant. However, the exact mechanism of action is still vague. In this current study, UFB demonstrates a higher antioxidative value than PPB. Figure 3 shows the result of the DPPH scavenging activity of AA, PPB, and UFB. At 1000 µg/mL concentration, the scavenging activity of PPB and UFB was 93.40% and 96.46%, respectively, which compared favorably with AA (99.05% at a concentration of 1000 µg/mL). Figure 4 presents the IC_{50} value of the DPPH assay. A lower IC_{50} value indicates a higher antioxidant activity. The IC_{50} value of the DPPH assay AA, PPB, and UFB is 2.41±0.27 µg/mL, 67.60±9.55 µg/mL and 42.11±2.55 µg/mL, respectively. The results of the current findings vary compared to that of previous studies. Rathnakumar et al. (2017) reported that the IC_{50} values of pineapple core and peel were 38.65 µg/mL and 738.3 µg/mL, respectively, whereas Abbas et al. (2021) reported an IC_{50} of 13.158 µg/mL, 24.13 µg/mL, 23.33 µg/mL, and 113.79 µg/mL from peel, fruit, stem, and crown, respectively. In contrast, Azizan et al. (2020) reported 353.10 µg/mL, 296.31 µg/mL, and an undetermined value for peel, crown, and cores, respectively. The previous result differs greatly, which might be attributed to the type of cultivars and the maturation of the pineapples (Ding and Syazwani, 2015; Ferreira et al., 2016). In addition, the purity of the bromelain might also contribute to the antioxidant level (Devi and HemaLatha, 2014; Saptarini et al., 2019a). The IC_{50} value of UFB was lower than that of PPB. The presence of impurities may cause low scavenging activity and thus explains the high IC_{50} value of PPB, as it contains excess ammonium sulfate salt (Manosroi et al., 2014). Thus, the findings from this study proved that further purification of bromelain by centrifugal ultrafiltration method increased bromelain purity.



Note: All values represent the mean ± SEM of three independent experiments conducted in triplicate.
PPB = partially purified bromelain, UFB = ultrafiltrate bromelain, AA = ascorbic acid

Figure 3. Percent of DPPH scavenging activity of bromelain samples

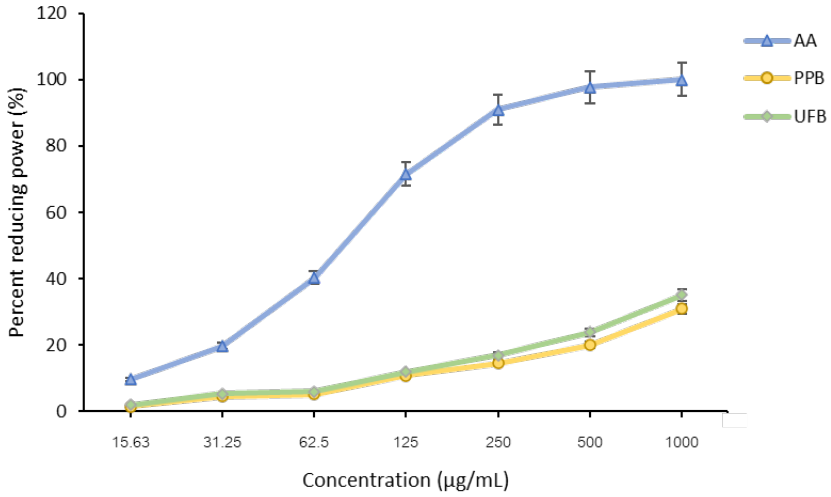


Note: All values represent the mean ± SEM of three independent experiments conducted in triplicate.
Asterisk (*) denotes a significant difference at $p \leq 0.05$
PPB = partially purified bromelain, UFB = ultrafiltrate bromelain, AA = ascorbic acid

Figure 4. IC_{50} value of bromelain samples

The reduction potential of bromelain was measured by FRAP assay. FRAP assay revealed that the reducing power of 1000 µg/mL PPB and UFB was 30.89% and 35.09%, respectively, which is less potent when compared with AA (97.69%) (Figure 5). As of date, no research illustrates the reducing power of bromelain. Reducing power assay is measured by reducing ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). The reducing power of UFB at 1000 µg/mL was 35.09%, indicating poor antioxidant activity as a higher concentration than that is needed to possess at least 50% of reducing power. The low antioxidant activity of bromelain in the FRAP assay could be attributed to reduced proteolytic activity in the presence of metal ions (Fe^{3+}). Potassium ferricyanide can inhibit approximately 59% of bromelain proteolytic activity (Priya et al., 2012). Meanwhile, Meng et al. (2017) declared that Fe^{3+} and Fe^{2+}

could inhibit enzymatic activity; as such, Fe^{2+} would inhibit bromelain activity when the concentration was higher than 0.75 mM, while the inhibitory effect of Fe^{3+} was proportional to the concentration of Fe^{3+}. The inhibitory effect of heavy metal ions decreases α-helices content, β-turns, and β-sheets and increases the random coil content, altering the active site of bromelain (Meng et al., 2017). Thereby, decreasing proteolytic activity will influence the antioxidant activity since the bromelain's protective action is based on its proteolytic activity (Hale, 2004; Hale et al., 2006).



Note: All values represent the mean ± SEM of three independent experiments conducted in triplicate. PPB = partially purified bromelain, UFB = ultrafiltrate bromelain, AA = ascorbic acid

Figure 5. Percentage of FRAP's reducing power in bromelain samples

The results demonstrated bromelain's capability to scavenge free radicals and reduce ferric ions in a dose-dependent manner. The control and samples were statistically significant (p<0.05) between each other shown in Figure 4. The radical scavenging activity of the bromelain could be from the amino acid contents by the mechanism of hydrogen atom donation to stabilize the DPPH molecule (Manosroi et al., 2014). It was reported that the presence of amino acids in the active site of bromelain, including histidine, phenylalanine, tryptophan, tyrosine, and methionine, could contribute to its antioxidant activity (Husain and Lowe, 1968; Murachi, 1964). The potency of UFB was approximately 0.06 times that of ascorbic acid, concluding it as a medium to poor antioxidant. However, the antioxidant activities of UFB are higher than that of PPB, proving that ultracentrifugation is effective in recovering and preserving the antioxidant capabilities of bromelain. With the increased protein content, specific activity, and yield, ultracentrifugation can be concluded as a good purifying step to preserve antioxidant activities by purifying bromelain enzyme.

4. CONCLUSION

Bromelain has been proven to be a potential source of a natural antioxidant compound for therapeutic purposes. Bromelain exhibited medium radical scavenging activity; however, in the presence of metal ions (Fe^{3+}), bromelain demonstrates poor reducing power as metal ions inhibit the proteolytic activity of bromelain. Using centrifugal ultrafiltration positively impacts the recovery of protein content, proteolytic activity, and antioxidant activities of bromelain. Hence, the ultrafiltration method by membrane separation may be advantageous for bromelain production from pineapple wastes. Comparison between the antioxidant activities from ultrafiltration and other methods should be done to further understand the efficiency of ultracentrifugation in antioxidant potential recovery.

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