Profile of Bromelain Enzyme Extracts by Different Precipitation Methods and Effect of pH and Temperature on Protease Activity

Christian Suherman[1], *Muhammad Halim* Natsir[2*], *Eko* Widodo[2], and *Irfan H.* Djunaidi[2]

[1]Doctoral Student at the Faculty of Animal Husbandry, University of Brawijaya, East Java, Indonesia
[2]Department of Animal Nutrition and Feed Science, Faculty of Animal Husbandry, University of Brawijaya, East Java, Indonesia

Abstract, Bromelain is a proteolytic enzyme in pineapple that can be processed into a feed additive. This research used pineapple peel to obtain bromelain through extraction and precipitation, with twelve treatments: crude extract (T0), bromelain precipitation with ethanol 90%(T1), 80%(T2), 70%(T3), 60%(T4), 50%(T5), 40%(T6), 30%(T7), and ammonium sulfate 60%(T8), 50%(T9), 40%(T10), 30%(T11), 20%(T12). The results showed significant differences in total protein and enzyme activity. Treatments of T0 and T1 produced the highest total protein (p>0.05) but had considerable effect with other treatments (p<0.05). The results of the enzyme activity test showed treatments T0, T1, T2, and T8 did not have a significant effect (p>0.05) but had a considerable effect with other treatments (p<0.05). The crude enzyme extract has greater potential to be used as a feed additive because it is more efficient and affordable and has almost the same quality as 80%, 90% ethanol, and 60% ammonium sulfate treatment in terms of total protein and enzyme activity.

1 Introduction

Pineapple is a highly marketable product with significant global potential. As the second most popular plant globally, pineapple is used as a dietary staple and medicinal supplement [1]. Processing pineapple waste is a challenge for the government as a sustainable waste management strategy, given the increase in the amount of waste that continues to increase yearly. The byproducts of pineapple processing, including peels, cores, leaves, and crowns, can have a negative impact on the environment if not properly processed [2]. Approximately 60% of the total pineapple fruit is comprised of pineapple waste [3], of which the pineapple peel accounts for the most significant portion of about 30-40% [4]. Pineapple waste also contains crude fibre, protein, carbohydrates, vitamins, minerals, gallic acid, ascorbic acid, phenols and sugars [5], as livestock feed, and crop fertilizer [2]. In addition, pineapple waste contains bioactive substances such as organic acids, phenolic antioxidants and bromelain enzymes [6].

* Corresponding author: emhanatsir@ub.ac.id

Bromelain is a proteolytic enzyme or protease found in the fruit, peel, crown, core, stem, leaves and plants of the pineapple family Bromeliaceae [7]. Bromelain has attracted attention in various industrial applications as it has high commercial properties and value [8]. The increasing need for bromelain has led to steady annual growth [1]. Bromelain also has other functions, namely as an anti-inflammatory, anti-edematous, anti-thrombotic, anti-coagulation and fibrinolytic [9]. Giving bromelain as a feed additive to broilers reduces the population of *E. coli* bacteria in the intestine [10], can improve the morphology of the small intestine, can improve growth and performance [11].

The bromelain production process requires several steps, including extraction and precipitation. Enzyme precipitation is the highest-cost process, reaching about 70-90% of the production cost, so the precipitation process's economic and technical aspects are considered [9]. Factors such as extraction method, use of precipitants, production process, and type of raw material affect the quality of bromelain [1,12,13]. The choice of precipitant can affect the purity and quality of bromelain, the process of releasing enzymes from raw materials, the speed of extraction, and the number of chemicals used [14,15]. The discovery of simple, economical, and environmentally friendly extraction methods and precipitating agents in bromelain purification is important [8]. This can help produce more affordable products, reduce production costs, and adopt environmentally friendly practices [3,16]. Hence, the present study aims to evaluate different precipitation methods for extracting bromelain from pineapple peel, analyze the resulting bromelain profile, such as enzyme activity and total protein, and determine the optimal pH and temperature conditions for producing quality bromelain.

2 Materials and Methods

2.1 Material

The pineapple peels used came from almost ripe pineapple fruits, with a plant age of about 15 months from planting to harvesting in Babadan, Ngancar Subdistrict, Kediri Regency, East Java, Indonesia.

2.2 Research Methods for Bromelain Extract Profiles with Different Precipitation

The method used twelve treatments and three replications, as explained below:
T0: Crude extract
T1: Crude extract by precipitation using 90% Ethanol
T2: Crude extract by precipitation using 80% Ethanol
T3: Crude extract by precipitation using 70% Ethanol
T4: Crude extract by precipitation using 60% Ethanol
T5: Crude extract by precipitation using 50% Ethanol
T6: Crude extract by precipitation using 40% Ethanol
T7: Crude extract by precipitation using 30% Ethanol
T8: Crude extract by precipitation using 60% Ammonium sulfate
T9: Crude extract by precipitation using 50% Ammonium sulfate
T10: Crude extract by precipitation using 40% Ammonium sulfate
T11: Crude extract by precipitation using 30% Ammonium sulfate
T12: Crude extract by precipitation using 20% Ammonium sulfate

2.3 Implementation procedure

2.3.1 Extraction process to obtain bromelain enzyme crude extract

The bromelain enzyme crude extract procedure was carried out by 1) Pineapple peels are washed with clean water and draining, 2) peels are cut into small pieces and juiced with a hi-cook slow juicer, 3) centrifugation at 4,000 rpm for 20 minutes 4) take the supernatant part (crude bromelain enzyme extract) [4].

2.3.2 Precipitation Process

Crude extract of bromelain enzyme for treatment by precipitation using ethanol or ammonium sulfate was adapted from Sari et al. [7] precipitated bromelain enzymes using ethanol with different concentrations, namely 30, 40, 50, 60, 70, 80, and 90%. Deposition occurred at a temperature of 4°C for 24 hours. They were centrifuged at 4000 rpm for 20 minutes. The precipitate was resuspended in phosphate buffer (0.1 M, pH 7.0). Ammonium sulfate precipitation treatment with concentrations of 20, 30, 40, 50, and 60% was carried out according to the ethanol precipitation method.

2.4 Total Protein in bromelain extract

Protein determination of crude bromelain extract and its extracts with ethanol or ammonium sulfate precipitation treatment were performed using the micro-Kjeldahl (MK) method. This total protein analysis was tested at the nutrition laboratory of the Faculty of Public Health, Airlangga University, Surabaya, Indonesia. The nitrogen conversion factor to protein was 6.25 [17].

The amount of protein of crude bromelain extract and its extract with ethanol or ammonium sulfate precipitation treatment then ware tested again with the Bradford method (BM) using a standard bovine serum albumin (BSA) compound. Bradford reagent preparation using Coomassie Brilliant Blue (CBB) dye as much as 0.025g, added 12.5ml of 96% ethanol, added again with phosphoric acid as much as 25 ml, diluted again with distilled water as much as 250 ml and then filtered with filter paper. Samples were taken in as much as 1 ml, and then each was added 3 ml of Bradford reagent. It was determined at a wavelength (λ) of 595 nm [18].

2.5 Activity of Enzyme

The enzyme activity was determined using Banerjee et al. [19] with modifications. Crude bromelain extract with or followed by precipitation treatment, then 1 ml of sample was added to 0.65% casein substrate by weight/volume. The mixture was incubated for 10 minutes. The unhydrolyzed substrate was precipitated using 1.25 mL TCA (Trichloroacetic acid) for 10 minutes; the precipitate was then separated using Whatman No.1, then into the solution added 5 mL Sodium carbonate and 1ml folin-ciocalteu's phenol. It was determined at a wavelength of 275 nm. Bromelain activity was calculated on the L-tyrosine liberated in one minute per milliliter sample. The unit of bromelain activity was determined as 1 microgram of L-tyrosine liberated in one minute per milliliter of sample. Enzyme activity is calculated using the formula below equation 1:

$$\text{Enzyme Activity } \left(\frac{\text{CDU}}{\text{ml}}\right) = \frac{\text{µmol tyrosine liberated x total reaction volume (ml)}}{\text{Time (min) x volume analysed (ml)}} \quad (1)$$

CDU is for casein digestion units, and ml is for milliliters.

2.6 Effect of temperature and pH on bromelain enzyme activity

2.6.1 Research Methods

The method used was an experiment with three treatments, as described below:
T0: Bromelain crude extract
T1: Bromelain crude extract by precipitation treatment using 90% ethanol
T2: Bromelain crude extract by precipitation treatment using 60% ammonium sulfate

2.6.2 Optimal Temperature Testing

The chemicals used for the temperature test included 0.5 mL gelatin, 0.5 mL buffer phosphate pH 7 and 1 mL bromelain enzyme extract from treatments T0, T1 and T2. Each treatment required 5 (five) test tubes each and incubated for 10 minutes at different temperatures of 40, 50, 60, 70 and 80^{o}C using a Memmert water bath. It was measured at a wavelength (λ) of 595 nm [20].

2.6.3 Optimal pH Testing

The chemicals used for the temperature test included 0.5 mL of gelatin, 0.5 mL of buffer phosphate with different pH of 3, 4, 5, 6, 7, and 8 and 1 mL of bromelain enzyme extract from treatments T0, T1 and T2. Each treatment required 6 (six) test tubes, and then all treatment reactions were stopped by heating the water in boiling for 10 minutes. It was measured at a wavelength (λ) of 595 nm [20].

2.7 Statistical Analysis

The total protein data of the micro-Kjeldahl method (MK) was not tested statistically. However, the total protein data of the Bradford method (BM) and bromelain enzyme activity were tested by one-way ANOVA. Furthermore, if there were significant differences, Duncan's multiple range test was continued to compare the means between several experimental groups. Descriptive analysis was conducted for temperature and pH data.

3 Results

3.1 Total Protein and Enzyme Activity

The research results showed that the total protein content of the MK ranged from 4.24% to 9.21%. The total protein content of the BM ranged from 38.97 ± 0.037 to 94.89 ± 0.020 μg/ml (Table 1). The total protein of the bromelain enzyme showed ($p<0.01$) that there were very significant differences between all treatments, both using crude extracts of the bromelain enzyme and using ethanol and ammonium sulfate precipitation with various concentrations. Total protein in treatments T0 and T1 ($p>0.05$). However, when compared with other treatments, T0 and T1, it had a significant effect ($p<0.05$) (Table 1).

The enzyme activity results ranged from 4.51 ± 0.127 to 18.48 ± 0.054 U/ml (Table 1). Bromelain enzyme activity showed a significant difference ($p<0.01$). Enzyme activity on treatments T0, T1, T2, and T8 ($p>0.05$) However, compared to the other treatments, it had a significant effect ($p<0.05$) (Table 1).

Table 1. Quality Characteristics of Bromelain Enzyme

Treatment	Total Protein (MK) (%)	Total Protein (BM) (µg/ml)	Bromelain Activity (U/ml)
T0	9,21	94.89±0.020^{a}	15,55±0.046^{ab}
T1	7,22	89.34±0.015^{ab}	18,48±0.054^{a}
T2	5,03	82.35±0.029^{b}	15,80±0.016^{ab}
T3	4,89	70.87±0.025^{c}	10,79±0.084^{bcd}
T4	4,79	64.66±0.050^{cd}	8,58±0.096^{cd}
T5	4,68	54.40±0.063^{de}	5,88±0.053^{cd}
T6	4,59	50.43±0.036^{e}	4,72±0.100^{d}
T7	4,24	38.97±0.037^{f}	4,93±0.083^{d}
T8	6,93	82.69±0.069^{b}	15,79±0.067^{ab}
T9	6,17	70.38±0.020^{c}	11,76±0.132^{bc}
T10	5,27	55.56±0.037^{de}	7,80±0.155^{cd}
T11	5,20	47.91±0.053^{ef}	6,23±0.169^{d}
T12	5,08	39.74±0.082^{f}	4,51±0.127^{d}

Notes: Mean ± SD (n = 3). Values with distinct superscripts inside a column show a significant difference (p<0.05).

3.2 Effect of pH and temperature on bromelain enzyme activity

The results showed the effect of pH on bromelain enzyme activity using treatments T0, T1 and T2 (Figure 1).

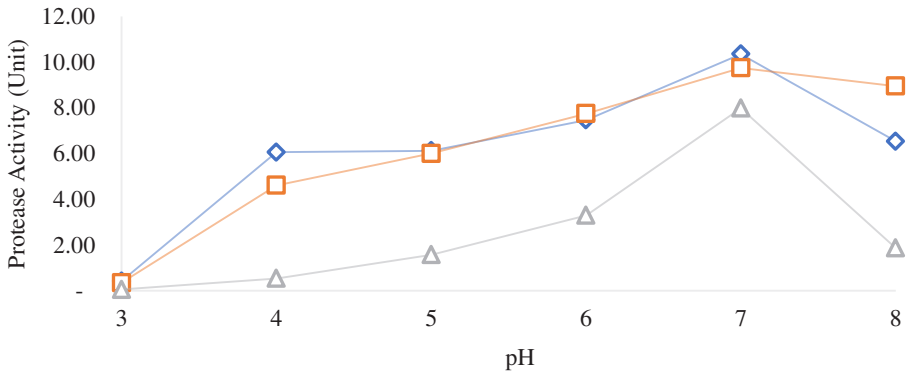

Figure 1: pH on bromelain enzyme activity ◊: T0; □: T1; △: T2

The optimum pH for all treatments occurred at pH 7. In the T0 treatment, enzyme activity reached 10.34 units, while for the T1 treatment, enzyme activity reached 9.74 units, and for the T2 treatment, enzyme activity reached 7.98 units (Figure 1).

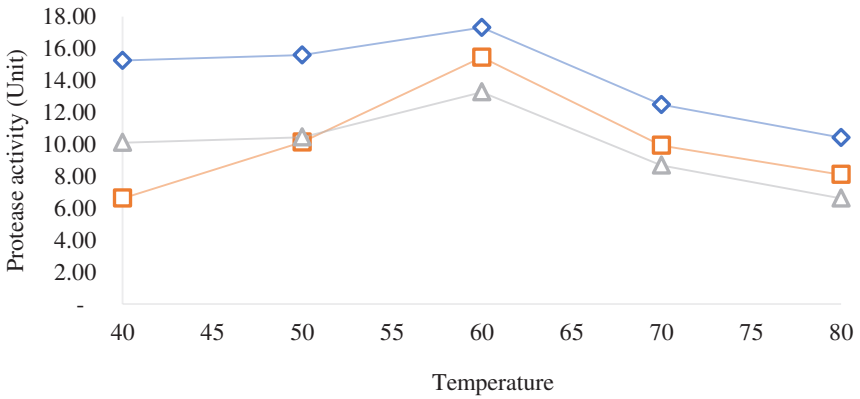

Figure 2: Temperature on bromelain enzyme activity. ◊: T0; □: T1; △: T2

The results of the study regarding the impact of temperature on the activity of enzymes showed that the optimal peak temperature of bromelain for all treatments occurred at 60°C. However, at temperatures above 60°C, enzyme activity began to decrease. The most significant bromelain activity temperature value was reported in the T0 treatment at 17.31 units, T2 at 15.10 units, and T3 at 13.28 units (Figure 2).

4 Discussion

The results of research on total protein align with the research of Silvestre et al. [17], found that the crude extract had the highest protein content (6.1 mg/mL), followed by the extract precipitated with 70% ammonium sulfate (2.9 mg/mL) and then 60% ammonium sulfate (2.7 mg/mL). Research conducted by Martins et al. [21] also showed results when ethanol concentration of 30% bromelain precipitation did not occur. However, ethanol concentrations higher than 65% showed recovery of enzymatic activity. Martins et al. [21] also said that the highest level of enzyme protein concentration was found in the crude extract. The 24-hour precipitation process can cause changes in the pH value and the autodigestive properties of the bromelain enzyme, which can affect its stability during the precipitation process and impact protein total and enzyme activity [17,22].

Protein precipitation often requires the addition of salts, polar and non-polar solutions, or manipulating temperature and pH [12]. Precipitation, also known as salting out, happens when the ability of proteins to dissolve in a solution diminishes as the quantity of precipitating chemicals increases. Adding ammonium salt would cause the salt ions to compete with the protein for binding to water molecules. Since the solubility of salt ions is greater than that of proteins, the proteins in the enzyme will form clumps and precipitate [14].

The results of the bromelain enzyme activity test in this study are almost the same as other studies, where the research of Sari et al. [7] showed the extraction of bromelain enzyme from pineapple core with 3 (three) treatments showed the highest protease activity in crude extract, namely 149.83 units followed by ethanol precipitation residue filtration treatment, namely 99.17 units and ammonium sulfate precipitation residue filtration

treatment, namely 44.71 units. Another study conducted by Soares et al. [15] on the extraction of bromelain enzyme from stem and peel showed high protease activity in the crude extract treatment of 2.86 ± 0.15 U/ml followed by the 30-70% ethanol precipitation treatment of 2.82 ± 0.04 U/ml. Silvestre et al. [17] said the extraction of bromelain enzyme from pineapple peel showed the highest enzyme activity in the crude extract treatment with a value of 21.4 ± 0.32 U/ml, followed by ammonium sulfate precipitation with a concentration of 50% with a value of 10.6 ± 0.44 U/ml. In another study conducted by Chaurasiya and Hebbar [23], bromelain enzyme with ammonium sulfate and acetone precipitation treatment showed the highest protease activity in 70% and 60% ammonium sulfate treatment with a value of 78.86 ± 1.23% and 78.35 ± 1.72%. Research conducted by Susanti et al. [24], bromelain from the crown of pineapple-type honi shows precipitation with ammonium sulfate concentration of 60% has the highest value of 1.61U/ml; this value is the highest when compared with other concentrations.

The effect of pH on bromelain enzyme activity in this study is in line with the research of Fissore et al. [8], which showed the optimum activity pH of the crude extract of pineapple flesh and pineapple core at pH 7. Similar findings were also found in the research of Saptarini et al. [25], which stated that the pH of the high activity of bromelain enzyme in the peel, core, and crown of pineapple was at pH 7, and the decrease occurred at pH 9.

A decreased enzyme activity indicates that the enzyme works less efficiently at acidic or alkaline pH. This decrease is due to pH changing the degree of ionization of the chain group (R) of amino acids in proteins, where the R group can act as an acid (accepting H+ protons) or base (releasing H+ protons). Changes in the R group of amino acid residues on the active side of the enzyme can change the enzyme's structure and cause it to lose its activity or denature. Significant changes in the R group of amino acid residues on the active side of the enzyme can interfere with the enzyme's ability to form enzyme-substrate complexes [26].

The effect of temperature on bromelain enzyme activity in this study is almost in line with the research of Banerjee et al. [19], showing activity of bromelain enzyme in their study occurred at 50^{o}C for crude bromelain and 60^{o}C for bromelain added with Glutaraldehyde to form cross-links. Martins et al. [21] also noted that the optimum temperature of bromelain enzyme occurs at 50°C for fractionation treatment with ethanol and 60°C for crude extract. Saptarini et al. [25] found that the optimum temperature for the peel and core was 55°C of the bromelain. Generally, the optimum bromelain enzyme extract temperature in the above discussion ranges from 50^{o}C to 60^{o}C.

5 Conclusion

Bromelain enzyme activity in the crude extract treatment, 80%, 90% ethanol precipitation treatment, and 60% ammonium sulfate precipitation treatment showed the best results compared to other treatments. The optimum temperature for enzyme activity occurs at 60°C, and the optimum pH is 7.

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