Antibacterial activity of hydrolysate protein from Etawa goat milk hydrolysed by crude extract bromelain

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Abstract. A study has been conducted on the potential of hydrolysate protein from Etawa goat milk protein which is hydrolysed by bromelain crude extract enzyme. The bromelain enzyme is extracted from the leaf of honey pineapple fruit. The research steps are: bromelain extraction, bromelain characterization, preparation of goat milk protein, hydrolysis of goat milk protein with bromelain and antibacterial activity test of protein hydrolysate fractions against Escherichia coli and Staphylococcus aureus bacteria by diffusion method. The bromelain crude extract enzyme has the following characteristics: optimum pH is 6, optimum temperature is 55 °C, activity is inhibited by EDTA metal chelating compound and Cu²⁺ and Zn²⁺ metal ions, whereas Ca²⁺ and Mg²⁺ metal ions increase activity. Inhibition of activity by EDTA shows that the bromelain enzyme is a metalloenzyme. Ca²⁺ and Mg²⁺ metal ions act as activators, while Cu²⁺ and Zn²⁺ metal ions are inhibitors. Protein preparation of goat milk produces two types of protein namely casein and whey. The result of hydrolysis of both protein types with characterized bromelain enzymes are hydrolysate proteins, which are then tested for their antibacterial activity. The results showed that the hydrolysate protein was able to inhibit the growth of Escherichia coli and Staphylococcus aureus bacteria.

1. Introduction

Hydrolysate proteins or bioactive peptides are defined as special fragments of proteins with amino acid sequences that have biological activity in the body such as antioxidants, antihypertensive, antithrombotic, antimicrobial, anti-inflammatory, opioid and immunomodulatory effects [1]. Bioactive peptides have a positive impact on body function or condition and can affect health. The peptide has 2-20 amino acids with a molecular weight of less than 6000 Da. The bioactivity of the peptide is determined by the composition and sequence of amino acids [2]. Bioactive peptides can be obtained from plant and animal proteins. Sources of bioactive peptides from plants generally come from groups of grains such as wheat, rice, oats, rye, corn, and groups of legumes such as soybeans, peas and chickpeas [3]. Bioactive peptide sources from animals are also very potential, such as meat, eggs, fish and milk [4].

Goat milk is one of the sources of hydrolyzate protein that is feasible to be developed because it has high nutritional value and is easy to digest. The high protein content in goat milk and the result of hydrolysis in the form of peptides has a broad biological function [5]. The protein content of goat milk reached 3.4 %, higher than cow milk (3.2 %) [6]. Etawa goat milk is milk produced by Etawa breed goats and has a nutritional composition that is richer than other types of goat milk. The protein content in Etawa goat milk is 8.7 grams per 100 grams of milk.

Research on bioactive peptides derived from cow milk, especially those that function as antibacterial, has been widely carried out, but exploration of milk bioactive peptides from other sources such as goat
milk as antibacterial is still limited. Some bioactive peptides from the enzymatic hydrolysis of goat milk that have been identified are antibacterial peptides [7], antioxidants [8] and inhibitors of Angiotensin Converting Enzyme (ACE) [9]. Peptides from whey protein of goat milk hydrolysed with human gastrointestinal enzymes are known to inhibit the growth of *Escherichia coli* K12, *Bacillus cereus* RT INF01, *Listeria monocytogenes* and *Staphylococcus aureus* ATCC 25923 [8]. Milk hydrolysate protein is obtained by enzymatic hydrolysis using protease enzymes.

In this study, antibacterial activity of protein hydrolysate will be tested from Etawa goat milk protein. The protease enzyme that is used for the hydrolysis of goat milk protein is bromelain from the leaf of honey pineapple. The bromelain enzyme from the leaf of honey pineapple has never been studied before. Bromelain from the leaf of honey pineapple is extracted then characterized. The characterized bromelain is used to hydrolyse Etawa goat milk protein. Two types of Etawa goat milk protein, i.e. casein and whey are hydrolysed and resulted hydrolysate protein fractions. Antibacterial activity was tested for each fraction towards *Staphylococcus aureus* and *Escherichia coli* bacteria. The hydrolysate protein fraction which has the largest diameter of clear zone is then determined the MIC value (Minimum Inhibitory Concentration).

2. Materials and methods

2.1. Materials

Leaf of honey pineapple from Beluk Village, Belik District, Pemalang Regency, Etawa goat milk from local farmer, *E. coli* and *S. aureus* bacteria (collection of Biochemistry Laboratory, Department of Chemistry, Faculty of Mathematics and Natural Sciences, University of Jenderal Soedirman), HCl, NaOH, chloramphenicol 250 mg (Generic), Nutrient Broth (NB) (Merck), Nutrient Agar (NA) (Merck), TCA, tyrosine, Na2CO3 (Merck), Sodium acetate buffer pH 5.0, Sodium phosphate buffer pH 6.0 and 7.0, Tris-HCl buffer pH 8.0 and 9.0, casein (Merck) EDTA (Merck), CaCl2 (Merck), MgCl2 (Merck), ZnCl2 (Merck), CuCl2 (Merck), CoCl2 (Merck), NiCl2 (Merck).

2.2. Extraction of crude bromelain

A total of 100 grams of pineapple leaf were mashed with a homogenizer, then the filtrate was added with 0.1 M Sodium phosphate buffer pH 7. The filtrate then was centrifuged at 3,500 rpm for 15 minutes. The obtained filtrate was bromelain crude extract enzyme.

2.3. Determination of bromelain activity

The bromelain activity was determined spectrophotometrically according to [10]. A total of 0.5 mL casein substrate (10 mg/mL) in 0.1 M Sodium phosphate buffer pH 7 was preincubated at 35°C for 5 minutes. The enzyme reaction began with adding of 0.1 mL of the enzyme solution to the substrate and incubating at 35°C for 30 minutes. The reaction was stopped by adding 4.3 mL of a TCA solution in cold condition then centrifuging at 3,500 rpm, for 10 minutes. The absorbance of peptides in the supernatant resulting from hydrolysis was measured using a spectrophotometer at a wavelength of 275 nm. The control solution was made in the same way, but the TCA solution added to the enzyme was carried out after 5 minutes of preincubation. Tyrosine solution (0-1 mg/mL) was used as a standard for measurement of proteolytic activity. One unit of bromelain activity was defined as the amount of 1 μmol product (tyrosine) per minute.

2.4. Characterization of crude extract bromelain

2.4.1. Determination of the optimum temperature. Determination of the optimum temperature was conducted according to [11], over a temperature range of 30-65°C in Sodium phosphate buffer pH 7. The substrate used was casein solution of 10 mg/mL. The activity was measured using the method described before.
2.4.2. Determination of the optimum pH. Determination of optimum pH was carried out according to Kamelia et al. [12] over a pH range from 5 to 10 at the temperature of 37 °C. The substrate used was casein solution of 10 mg/mL. Various pH solutions were used, i.e.: 0.1 M Sodium acetate buffer for pH 5, 0.1 M Sodium phosphate buffer for pH 6 and 7, 0.1 M Tris-HCl buffer for pH 8, 9 and 10. The activity was measured using the method described before.

2.4.3. Determination the effect of EDTA and metal ions on the activity of bromelain [13]. The effect of EDTA and metal ions on protease activity was determined by adding EDTA solution and metal ions with a concentration of 10 mM into the sample solution at optimum temperature and pH. Metal ions used were: Ca²⁺, Zn²⁺, Cu²⁺, Ni²⁺, Co²⁺ and Mg²⁺. The final EDTA and metal ions concentration in the enzymatic reaction mixture were 10⁻³ M. The control solution was made without the addition of EDTA and metal ions.

2.5. Goat milk preparation
Preparation of goat milk was conducted according to [7]. Fresh Etawa goat milk preparations were centrifuged at 2000 rpm, 4°C for 30 minutes to separate the fat component. Casein isolation was done by adding 2N HCl at 40°C to the pasteurized milk until it reached the isoelectric point (pH 4.6), then centrifuged at 3500 rpm for 30 minutes. It aims to separate casein and whey. The pellet is casein and the filtrate is whey. The casein then was rinsed with 1x distilled water. The whey was neutralized with 1 N NaOH then concentrated by freeze drying until the volume shrinks to 1/5 of the initial volume.

2.6. Hydrolysis of goat milk
Preparation of 10 mg/mL casein: solid casein was dissolved in 0.05 M Sodium phosphate buffer pH 7. Casein and whey were hydrolysed using bromelain enzyme with the ratio of enzymes and substrate (1:4 v/v) under optimum temperature and pH, with intervals of incubation time 30, 45, 60, 75, and 90 minutes. Non-hydrolysed casein was used as a control. Hydrolysis was stopped by heating at 80°C for 15 minutes. Each fraction obtained was filtered using filter paper and tested for antibacterial activity.

2.7. Test for antibacterial activity
The test of antibacterial activity was done by diffusion method against E. coli and S. aureus bacteria, according to [14]. One ose of bacteria from the culture stock was taken and incubated in 10 mL of liquid medium (Nutrient Broth) for 18-24 hours at 37°C, and shaking using a shaker with a speed of 100 rpm. As much as 5 mL of bacterial culture was taken and the absorbance was measured at λ 620 nm. A 50 µL culture was then spread in a sterile petri dish containing 15 mL of Nutrient Agar medium which had been allowed to solidify. The medium was perforated using a cork drill with a diameter of ± 7 mm, then into each hole was inserted 50 µL of hydrolysate protein fraction with a concentration of 1000 ppm, then incubated at 37°C for 24 hours, with positive control being chloramphenicol, and negative control was distilled water. The clear zone seen around the hole indicates the presence of antibacterial activity, then the diameter of the clear zone formed was measured using a calliper. The fractions with the highest diameter of the clear zone were assayed for Minimum Inhibition Concentration (MIC).

2.8. Minimum Inhibition Concentration (MIC) assay
Hydrolysate protein fractions which has the highest antibacterial activity then determined the Minimum Inhibition Concentration (MIC) on the tested bacteria. The variation of hydrolysate protein fraction concentration used was 1000, 500, 250, 125, 65, 30, 15, 10, 5 and 1 ppm. Each concentration of 50 µL was tested by inserting into the hole of Nutrient Agar medium which was inoculated with the tested bacteria, then incubated at 37°C for 24 hours. The antibacterial activity was obtained by measuring the diameter of the clear zone by using a calliper.
3. Results and discussion

3.1. Determination of optimum temperature

Determination of the optimum temperature is done to get the temperature with optimum activity. The temperature variations used are 30-60°C with interval of 5°C. The result is shown at Fig. 1.

![Figure 1. Effect of Various Temperatures on Bromelain Activity.](image)

Based on the results shown in Fig. 1, the bromelain activity of pineapple leaf increased with increasing temperature and reached optimum activity at 55°C with an activity of 8.333 U/mL and at temperatures above 55°C, enzyme activity decreased. The results of research conducted by [15] on bromelain isolated from pineapple stems showed that the optimum temperature was achieved at a temperature of 55°C with an activity value of 4.05 U/mL. The low enzyme activity at temperatures below the optimum temperature is due to the low activation energy available. Activation energy is needed to produce active complexes in both enzyme and substrate molecules. Increased energy in substrate molecules increases the rate of enzyme reactions. At optimum temperature, collisions between enzymes and substrates are very effective so that the formation of enzyme substrate complexes is easier and the products formed increase. Temperatures above the optimum temperature cause large kinetics of enzyme molecules to break secondary bonds which maintain the three-dimensional structure of the enzyme, causing damage to secondary and tertiary structures so that enzyme activity decreases.

3.2. Determination of optimum pH

Determination of bromelain optimum pH was carried out to determine the optimum activity of bromelain by measuring its activity on variations of pH 5-10 using casein substrate at 37°C. The optimum pH determination curve is shown at Fig. 2. Based on the results of the research shown in Fig. 2, it shows that the bromelain activity of pineapple leaf increased with increasing pH and reached optimum at pH 6 with an activity of 8.905 U/mL. The bromelain activity decreased above the optimum pH. At pH 6 bromelain reaches the highest activity because the enzyme is at the level of ionization that is most suitable for binding to the substrate. Enzyme conformation in a stable form is when enzymes and substrates form stable enzyme and substrate complexes.
The effect of pH can also be related to an isoelectric point. The isoelectric point is the pH that causes a protein to have the same amount of positive charge and negative charge. At pH 5, 7, 8, 9 and 10, bromelain activity is lower than in pH 6. This is because at pH lower or higher than the optimum pH, the side chains of some amino acids act as weak acids or bases that perform functions critical on the active side of the enzyme so that the enzyme becomes inactive.

3.3. Effect of EDTA and metal ions towards bromelain activity

Determination of the effect of the addition of EDTA and metal ions is to determine whether the enzyme bromelain extracted from honey pineapple leaf is a metalloenzyme, and type of metal ions that act as an activator or inhibitor. The result is shown at Fig. 3.

Based on the results of the study shown in Fig. 3, the addition of EDTA decreases bromelain activity from honey pineapple leaf with a relative activity of 75.21 % compared to control. The decrease in bromelain activity with the addition of EDTA shows that the bromelain enzyme from honey pineapple leaf is classified as metalloenzyme. Metalloenzyme activity decreases with the addition of EDTA because the metal ions found on the catalytic side of the enzyme bind to EDTA thereby altering the three-dimensional structure of the enzyme and causing the enzyme to lose its catalytic activity. According to [16], metalloenzyme is an enzyme that require metal ions in the active site for catalytic ability. If addition of EDTA decreased enzyme activity compared to control, the enzyme including metalloenzyme.
Figure 3. Effect of Addition of EDTA and Metal Ions on Bromelain Activity.

The addition of Mg$^{2+}$, Ca$^{2+}$, Ni$^{2+}$ and Co$^{2+}$ metal ions increases their relative activity by 171.11 $\%$, 180.85 $\%$, 110.56 $\%$ and 134.39 $\%$ compared to control. This means that all four metal ions are cofactors for bromelain from honey pineapple leaf. Among these four metal ions, calcium ion has the highest relative activity. Whereas the addition of Cu$^{2+}$ and Zn$^{2+}$ metal ions decreases the relative activity of bromelain respectively by 86.15 $\%$ and 69.54 $\%$.

3.4. Antibacterial activity test
Peptide antibacterial activity test resulted from casein and whey hydrolysis were to determine the hydrolysate protein fraction which has the ability to inhibit the growth of gram negative bacterium *Escherichia coli* and gram positive bacterium *Staphylococcus aureus* using well diffusion method. Before hydrolysing, the milk protein must be separated to casein and whey in order to obtain different hydrolysate protein fractions. The results are displayed at table 1 and table 2.

| Time of hydrolysis (minutes) | Clear zone (mm) |
|-----------------------------|------------------|
|                             | *S. aureus* | *E. coli* |
| 0                           | 0.00         | 0.00      |
| 30                          | 2.05         | 2.04      |
| 45                          | 2.56         | 2.04      |
| 60                          | 2.05         | 2.18      |
| 75                          | 2.07         | 2.29      |
| 90                          | 2.06         | 3.05      |
Table 2. Antibacterial activity of hydrolyzate protein fractions from whey

| Time of hydrolysis (minutes) | Clear zone (mm) |
|-----------------------------|-----------------|
|                            | S. aureus | E. coli |
| 0                           | 0.00      | 0.00    |
| 30                          | 2.68      | 2.05    |
| 45                          | 2.56      | 2.06    |
| 60                          | 2.18      | 2.53    |
| 75                          | 2.05      | 2.67    |
| 90                          | 1.67      | 2.92    |

Based on the tests that have been carried out, peptides resulting from casein and whey hydrolysis in various incubation times have antibacterial activity shown by the formation of different clear zones. In the antibacterial activity test of the hydrolysate protein fractions of casein against S. aureus, it was found that the fraction which was hydrolysed at an incubation time of 45 minutes yielded the largest clear zone of 2.56 mm. While the test against E. coli showed that the fraction which was hydrolysed at 90 minutes’ incubation time resulted in the largest clear zone of 3.05 mm. As for the antibacterial activity of hydrolysate protein fractions of whey against S. aureus, it was found that the fraction which was hydrolysed at 30 minutes’ incubation time produced the largest clear zone of 2.68 mm. While the test towards E. coli showed that the fraction hydrolysed at 90 minutes’ incubation time produced the largest clear zone of 2.92 mm. Bioactive peptide milk can have antibacterial activity because of the interaction between the peptide and the bacterial membrane which is then followed by membrane damage, membrane physiological disturbances such as cell wall biosynthesis, cell division or translocation across the membrane to interact with the cytoplasm. The target cell is generally assumed that the positive pole of peptides interact with the negative poles of the lipids on the outer surface or cytoplasmic membrane, then the peptides insert with a parallel position orientation on the bilayer, into the cytoplasmic membrane which then results in the release of lipids [17]. Furthermore, the fractions with the largest clear zone diameter values are then determined the MIC value.

3.5. Determination of Minimum Inhibitory Concentration (MIC)

The results of determining the MIC value from casein and whey hydrolysate fractions with the largest clear zone are shown at Fig. 1 and 2. Based on the results shown at Fig. 1, it is known that the MIC of hydrolysate protein from casein is 15 ppm for S. aureus with the clear zone value is 1.31 mm. Meanwhile, the MIC for E. coli is also 15 ppm with the clear zone value is 1.05 mm. The MIC of hydrolysate protein from whey is 10 ppm for S. aureus with the clear zone value is 1.06 mm, and 30 ppm for E. coli with the clear zone value is 1.05 mm. Antibacterial peptides from casein and whey hydrolysis can be categorized as antimicrobial agents with very strong activity because they have MIC values of less than 100 ppm [18]. MIC is the lowest antibacterial concentration that can inhibit bacterial growth. Determination of MIC from an antibacterial compound is very important because it is intended to increase the effectiveness of antibacterial compounds, and to prevent the emergence of problems of bacterial resistance due to excessive use of doses so that bacterial cells become immune over time.
Based on the results shown at figure 4, it is known that the MIC of hydrolysate protein from casein is 15 ppm for \textit{S. aureus} with the clear zone diameter value is 1.31 mm. Meanwhile, the MIC for \textit{E. coli} is also 15 ppm with the clear zone diameter value is 1.05 mm. The MIC of hydrolysate protein from whey is 10 ppm for \textit{S. aureus} with the clear zone diameter value is 1.06 mm, and 30 ppm for \textit{E. coli} with the clear zone diameter value is 1.05 mm. Antibacterial peptides from casein and whey hydrolysis can be categorized as antimicrobial agents with very strong activity because they have MIC values of less than 100 ppm. MIC is the lowest antibacterial concentration that can inhibit bacterial growth. Determination of MIC from an antibacterial compound is very important because it is intended to increase the effectiveness of antibacterial compounds, and to prevent the emergence of problems of bacterial resistance due to excessive use of doses so that bacterial cells become immune over time.

4. Conclusion
Bromelain that is extracted from pineapple leaf could be used to hydrolyse casein and whey protein from Etawa goat milk. Hydrolysate proteins resulted from hydrolysis of casein and whey have the antibacterial activity. The MIC of casein hydrolysate protein for \textit{S. aureus} and \textit{E. coli} is 15 ppm, meanwhile the MIC of whey hydrolysate protein is 10 ppm for \textit{S. aureus} and 30 ppm for \textit{E. coli}. All the
MIC values are below 100 ppm; it means that they are categorized as antimicrobial agents with very strong activity.

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