BIOACTIVE PEPTIDE WITH ANTIOXIDANT ACTIVITY FROM HYDROLYSIS OF MUNG BEAN PROTEIN USING PROTEASE ENZYME FROM BACILLUS SUBTILIS B298

P Lestari[1*], Suyata[1], N I Muslihah[1], A M Istiqomah[1]
[1] Departement of Pharmacy, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia, 53123

Email: puji.lestari0611@unsoed.ac.id

Abstract. Mung bean protein can be used as a source to obtain bioactive peptides that have antioxidant activity. Research has been conducted to obtain bioactive peptides with antioxidant potential from mung bean protein by enzymatic hydrolysis using protease enzyme from *Bacillus subtilis* B298. The purpose of the study was to determine the antioxidant activity, IC_{50} value, AAI (Antioxidant Activity Index) value and percentage of hemolysis of the bioactive peptide. The research stages include: extraction of protease enzyme; hydrolysis of mung bean protein with protease enzyme at various hydrolysis times of 10, 20, 30, 40, 50, 60 minutes; antioxidant activity test; determination of IC_{50}; determination of AAI and percentage of hemolysis. The results of the study were: bioactive peptide hydrolysed for 10 minutes have the highest antioxidant activity of 57% with an IC_{50} value of 305 ppm and AAI of 0.065 so that it is still categorised as a weak antioxidant. The percentage of hemolysis obtained was 2% and categorised as little hemolysis towards red blood cell.

1. Introduction

Compounds known as antioxidants are vital to the body. Antioxidants can lessen free radicals and stop the oxidation process. Without supplemental antioxidant consumption, humans are unable to meet their own antioxidant needs. The body can obtain additional antioxidants through the consumption of fruits, vegetables, nuts, meat, and fish [1]. One of the substances that is currently being extensively researched for use as an alternate antioxidant intake is bioactive peptides [2].

Compounds with bioactive qualities that arise from the breakdown of proteins are known as bioactive peptides. Plant and animal proteins can be used to make bioactive peptides. Bioactive peptides from plant and animal dietary products have been demonstrated in several studies to have antioxidant activity [3]. Research in recent years has shown that some proteins sourced from plant-based food products have potential as antioxidant producers [4]. Mung beans are a source of vegetable protein that has a high protein content of around 21-24%, so it has the potential as a source of bioactive peptides with antioxidant activity.

Protein hydrolysis can be achieved chemically or enzymatically to produce bioactive peptides. Enzyme-based protein hydrolysis is more effective than chemical hydrolysis because it can yield protein hydrolysates without destroying specific amino acids, such as glutamine and tryptophan [5]. Protease enzymes are often used as bioactive peptide-producing biocatalysts. Protease enzymes can be produced by utilizing several types of bacteria. Bacteria are widely utilized as a source of enzymes because they are easier, faster, and more efficient in production. *Bacillus subtilis* is one of the bacteria that is often used in the production of protease enzymes.

Bacillus subtilis B298 is one of the bacteria of the genus Bacillus that produces protease enzyme [6]. This study was aimed to hydrolyse mung bean protein using protease enzymes from *B. subtilis* B298 bacteria to obtain bioactive peptides. The bioactive peptides obtained were tested for antioxidant activity, determined IC 50 value, AAI (Antioxidant Activity Index) and percentage of hemolysis.

2. Methods

This research was conducted in the Laboratory of Biochemistry Faculty of Mathematics and Natural Sciences, Universitas Jenderal Soedirman Purwokerto. The research stages carried out were: protease enzyme production, hydrolysis of mung bean protein to produce bioactive peptides, antioxidant activity test of bioactive peptides, determination of IC 50, AAI and percentage of hemolysis of bioactive peptides with the highest antioxidant activity.

2.1. Isolation of mung bean protein

Mung beans were washed with water and then dried in the oven. The dried mung beans were pulverized using a powder grinder and then sieved using a 60 mesh sieve. The mung bean flour obtained was then macerated using n-hexane in a ratio of 1:5 (b/v) for 10 hours, resulting in lipid-free mung bean flour. A total of 80 g of lipid-free mung bean flour was added to distilled water in a ratio of 1:10 (b/v), then stirred for 3 minutes and checked the initial pH. The flour solution was then added with 0.5 N NaOH until it reached pH 8.0. The dissolved protein was then centrifuged at 4000 rpm with a temperature of 4℃. The supernatant was then taken and lowered the pH using 2 N HCl until the pH showed the isoelectric pH of mung bean which is 4.81. The solution was then allowed to stand for 10 minutes at 30℃, then centrifuged again at 4000 rpm for 15 minutes at 4℃. The precipitate is mung bean protein isolate. The protein isolate was then dried in the oven at 40℃ for 8 hours [7].

2.2. Protease enzyme production from B. subtilis B298

A total of 1 ose of *B. subtilis* B298 culture from rejuvenation was put into 60 mL NB media. The NB media was then incubated using a shaker incubator at 37 ℃ for 9 hours. The incubated NB media was then transferred into 240 mL of NB media that had been supplemented with 1% mung bean protein isolate as an inducer, then incubated again on a shaker incubator at 37 ℃ for 12 hours. The media was then centrifuged at 4℃ for 15 minutes at 7000 rpm. The supernatant was the crude extract of protease enzyme.

2.3. Hydrolysis of mung bean protein isolate

Hydrolysis was carried out with a variety of hydrolysis times10; 20; 30; 40; 50 and 60 minutes. Mung bean protein isolate (4 grams) was added to distilled water (100 mL) while stirring. The protein solution was then adjusted to pH 7. The solution then added protease enzyme as much as 10 mL and then stirred until homogeneous using a shaker incubator. The solution was incubated in a shakerbath at 45 ℃ and 5 mL was taken for each incubation time, then centrifuged at 10,000 rpm for 20 minutes at 4 ℃. The supernatant obtained was protein hydrolysate as bioactive peptide.

2.4. Antioxidant activity test

Antioxidant activity test was conducted using DPPH method. Protein hydrolysate was dissolved in methanol with a concentration of 1 mg/mL. A total of 1 mL of mung bean protein hydrolysate solution was added with 2 mL of DPPH solution, then allowed to stand for 30 minutes. The blank was DPPH solution in 99% methanol. The sample solution and blank were measured for absorbance using spectrophotometer at λ 516 nm [6]. Antioxidant activity was calculated by the equation:

$$\% \text{ Inhibition} = \frac{\text{A blank-A sample}}{\text{A blank}} \times 100\% \quad (1)$$

A blank = Absorbance of DPPH solution in 99% methanol
A sample = Absorbance of reaction of DPPH solution with protein hydrolysate

2.5. Determination of IC_{50} and AAI (Antioxidant Activity Index)

The protein hydrolysate with the highest percentage inhibition was made into concentration series of 0.1; 0.2; 0.3; 0.4; and 0.5 mg/mL. Vitamin C was used as antioxidant standard and concentration series of 0.001; 0.002; 0.003; 0.004; and 0.005 mg/mL were made. Each concentration series was measured for antioxidant activity. A linear regression curve was constructed with the x-axis being concentration and the y-axis being antioxidant activity (% inhibition). The linear regression equation obtained was used to determine the IC_{50} (Inhibitory Concentration 50%) value. This was done by expressing the value of y as 50 while the resulting x value is expressed as IC_{50} [6]. The AAI value was calculated with the equation:

$$\text{AAI} = \frac{\text{concentration of DPPH (0.0197 mg/mL)}}{IC_{50} \text{ of sample (mg/mL)}} \quad (2)$$

2.6. Hemolysis test

Hemolysis test was conducted using chicken blood. Chicken blood was added oxalic acid to prevent coagulation. Chicken blood was washed with 0.01 M Tris-HCl pH 7.4 containing 0.15 M NaCl (Tris-salin), then centrifuged at 1000 rpm. The supernatant was discarded and the pellet was washed 2 times using Tris-saline bufer. A 0.1% red blood cell suspension was prepared by dissolving the red blood cell pellet using Tris-saline buffer. A total of 1 mL of protein hydrolysate was added with 1 mL of 0.1% red blood cell suspension. The solution was then incubated at 37℃ for 2 hours and then centrifuged at 4000 rpm for 5 minutes. The supernatant was measured for absorbance at λ 540 nm. Tween 80 was used as positive control with the percentage of hemolysis was 100%. Tris-saline was used as negative control with 0% hemolysis percentage [5]. The percentage of hemolysis of the sample was calculated using the following equation:

$$\% \text{ Hemolysis} = \frac{\text{Absorbance of sample-Absorbance control (-)}}{\text{Absorbance control (+)}} \times 100\% \quad (3)$$

3. Results and discussion

Antioxidant activity test was conducted on each protein hydrolysate from the hydrolysis of mung bean protein isolate by protease enzyme from *B. subtilis* B298. The result was shown in **figure 1**.

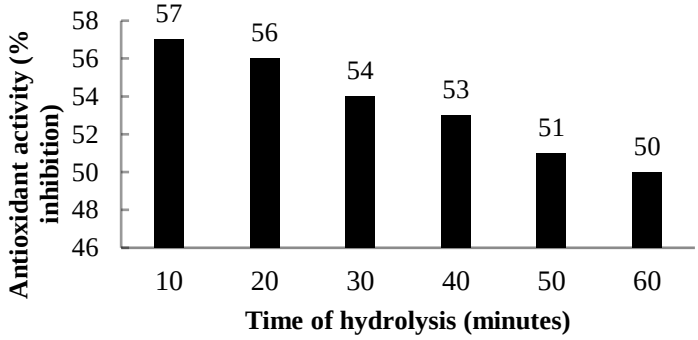

Figure 1. Antioxidant activity of each bioactive peptide fraction at various hydrolysis times.

The result showed that as the hydrolysis time increased, the antioxidant activity of bioactive peptides decreased. Bioactive peptide produced from mung bean protein hydrolysis for 10 minutes had the highest antioxidant activity with an inhibition percentage of 57%. The decrease of antioxidant activity along with the increase of hydrolysis time is due to the destruction of antioxidative peptide sequence [8]. The longer the hydrolysis time, the greater the amount of amino acids and small peptides that do not have antioxidant activity in their smallest molecular size [9]. Meanwhile, Ibrahim and Ghani stated that continuous protein hydrolysis process produced inactive free amino acids so that the antioxidant function is reduced [10].

The bioactive peptide with the highest antioxidant activity was further determined the IC_{50} and AAI. Vitamin C was used as antioxidant standard. The linear regression equation was determined to calculate the IC_{50} value, which is by entering the value of 50 in y, so that the x value is obtained as IC_{50}. The IC_{50} value represents the concentration bioactive peptide required to reduce DPPH by 50%. Furthermore, the IC_{50} value was used to calculate the AAI value. Results are shown in **table 1**.

Table 1. IC_{50} and AAI of bioactive peptide of mung bean protein and vitamin C.

Sample	Linear regression equation	IC_{50} (mg/mL)	AAI
Bioactive peptide	$y = 86x + 23.8$; $R^2 = 0.98$	0.305	0.07
Vitamin C	$y = 7600x + 27.6$; $R^2 = 0.99$	0.003	6.57

The strength of an antioxidant compound can be determined from its AAI value. The classification of antioxidants based on their AAI values are: weak if the AAI value is below 0.5; moderate if the AAI value is between 0.5-0.1; strong if the AAI value is between 0.1-2; very strong if the AAI value is above 2 [11]. Referring to the results shown in **table 2**, it is known that the AAI value of bioactive peptides from mung bean protein is smaller than the AAI value of vitamin C. This indicates that the bioactive peptide is still classified as weak, while vitamin C is classified as a very strong antioxidant. The weak antioxidant activity of bioactive peptides from mung bean protein is thought to be due to the lack of proper antioxidative amino acid sequence. This is probably because the protease enzyme used is still a crude extract so that its ability to hydrolyse mung bean protein to produce bioactive peptides is not optimal. Therefore, it is recommended that the protease enzyme be further purified, so that its ability to hydrolyse mung bean protein to produce bioactive peptides is better.

The bioactive peptide with the highest antioxidant activity was also determined for its hemolysis percentage. The hemolysis test is designed to evaluate the toxicity of bioactive peptide on erythrocyte cell (red blood cell) membrane damage. This test's basic idea involves seeing how bioactive peptide reacts with erythrocyte solution to determine how resistant it is to potential lysis. The principle of hemolysis test is the reaction between erythrocyte suspension and bioactive peptide to see the effect of erythrocyte resistance to the possibility of hemolysis. [5]. Results are shown in **table 2**.

Table 2. Hemolysis percentage of bioactive peptide of mung bean protein

Sample	% Hemolysis
Tween 80 (positive control)	100
Tris salin buffer (negative control)	0
Bioactive peptide	2

The resistance of erythrocytes to the possibility of hemolysis due to the presence of bioactive peptide compounds is categorised into 3 groups, namely: no hemolysis occurs if the percentage of hemolysis is below 2; slight hemolysis if the percentage of hemolysis is between 2-5; hemolysis occurs if the percentage of hemolysis is above 5 [12]. Based on the results of the hemolysis test shown in **table 2**, the bioactive peptide from mung bean protein is categorised as a little hemolysis against erythrocytes.

4. Conclusion

Bioactive peptides from mung bean protein that was hydrolysed for 10 minutes had the highest antioxidant activity with an inhibition percentage of 57%, an IC_{50} value of 0.305 mg/mL and an AAI of 0.07. Based on the AAI value, the bioactive peptide is still categorised as weak. The hemolysis test resulted in a hemolysis percentage value of 2%, so the bioactive peptide was classified as slightly lysing erythrocytes.

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