HYDROLYSIS OF SOYBEAN PROTEIN WITH ANTIOXIDANT ACTIVITY BY FRACTIONATED PROTEASE FROM *Bacillus subtilis B298*

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Abstract. Research has been carried out to obtain bioactive peptides with antioxidant potential from soybean protein. Bioactive peptides were produced by enzymatic hydrolysis using fractionated protease with the highest specific activity from *Bacillus subtilis* B298 bacteria. The aim of the research was to determine: the biochemical characteristics of fractionated protease with the highest specific activity, the effect of variations in hydrolysis time on the antioxidant activity of bioactive peptides by protease, the AAI (Antioxidant Activity Index) and the hemolysis percentage of bioactive peptide with the highest antioxidant activity. This research was carried out in stages: extraction and fractionation of the protease enzyme, characterization of the protease fraction with the highest specific activity, hydrolysis of soybean protein with varying incubation times of 10, 20, 30, 40, 50 and 60 minutes, antioxidant activity test, determination of AAI and percentage of hemolysis of bioactive peptide with the highest antioxidant activity. The results showed that the protease fraction with the highest specific activity was F15 with an optimum temperature of 45 °C and an optimum pH of 8. The highest antioxidant activity was obtained from bioactive peptide which was hydrolysed for 10 minutes with an inhibition percentage of 63.1%. The AAI was obtained at 0.119 and the hemolysis percentage was 3%.

Keywords: antioxidant, bioactive peptide, enzymatic hydrolysis, fractionated protease, soybean protein

A. Introduction

Antioxidants are compounds that play an important role in the body. Antioxidants can prevent the oxidation process and reduce free radicals. Lack of antioxidant compounds can cause high levels of free radicals that will have a negative effect on the body. Humans cannot meet their own antioxidant needs without additional antioxidant intake. Additional antioxidant intake in the body can be fulfilled by consuming fruits, vegetables, nuts, meat, and fish [1]. Bioactive peptides are one of the compounds that are currently being widely explored for application as an alternative antioxidant intake [2].

Bioactive peptides are compounds resulting from protein hydrolysis that have bioactive properties. Bioactive peptides can be obtained from plant and animal proteins [3]. Several studies have shown that bioactive peptides derived from plant and animal food products have potential as antioxidants. Soybeans can be one of the sources of bioactive peptides due to their high protein content. The protein content in soybeans is 35-40%. A number of bioactive peptides from soybean protein have been investigated to have physiological effects beneficial to human health including anti-obesity, anti-diabetes, anti-cancer, anti-inflammatory and antioxidant, with various experimental models [4]. Another study stated that redbeans protein

have a fairly high protein content of 16-33%, and also potential to be used as a source of bioactive peptides [5]. Bioactive peptides can be obtained by several methods, one of which is enzymatic hydrolysis using protease enzymes.

Bioactive peptides obtained from enzymatic hydrolysis using protease enzymes have the advantage of not damaging amino acids, safe use, and environmentally friendly [6]. Protease enzymes are obtained from animals, plants, and microbes. Alkaline protease enzymes can be produced by *Bacillus subtilis* at high levels [7]. This study used *Bacillus subtilis* B298 as a source of protease. *B. subtilis* B298 was isolated from the soil around potato roots [8]. Soybean protein hydrolysates obtained through enzymatic hydrolysis with fractionated protease from *B. subtilis* B298 has potential as an antioxidant agent in the body. In this study, hydrolysis of soybean protein using protease enzyme from *B. subtilis* B298 was carried out to obtain protein hydrolysate. The protein hydrolysate was then determined antioxidant activity using DPPH method. The protein hydrolysate that has the highest percentage of inhibition was determined the IC_{50} (Inhibitory Concentration 50%), Antioxidant Activity Index (AAI), and percentage of hemolysis.

B. Methods

The materials used were *Bacillus subtilis* B 298 isolate, Nutrient Agar (NA) (Merck), Nutrient Broth (NB) (Merck), soy bean, tyrosine (Merck), casein (Merck), DPPH (Merck), L-Ascorbic acid, distilled water, NaCl (Merck), H_2SO_4 (Merck), phosphate buffer pH 7, tris HCl buffer pH 8 and 9, borate buffer pH 10, TCA (Merck), Lowry's reagent, Follin ciocalteu reagent (Merck), methanol pa (Merck), skim milk powder, HCl (Merck), NaOH (Merck), n-hexane (Merck), Tween 80 (Merck). The tools used were laboratory glassware, Socorex 100-1000 L micropipette, autoclave Hirayama HVA-85 Sterilizer, Memmert water bath, magnetic stirrer, shaker incubator, pen type pH meter PH-009(I)A, UV-Vis Spectrophotometer Shimadzu UV-1800, Heraeus B6200 incubator, cold centrifuge, Buchner funnel, and Memmert oven.

1. Extraction and fractionation of crude extract protease

Protease extraction was carried out on a large scale (1000 mL) under optimum conditions. Rejuvenated *B. subtilis* B298 isolates from NA medium were inoculated into 40 mL of NB medium, incubated on a shaker incubator at 37 °C for 9 hours. The inoculum was transferred into 960 mL of NB medium supplemented with 1% (b/v) skim milk as an inducer, incubated in a shaker incubator at 37 °C for 12 hours. The mixture of medium and bacteria was centrifuged (5000 rpm, 15 minutes, 4 °C). The supernatant was the crude protease extract, then tested for protease activity using the Kunitz method [9]. The protease crude extract was multistage fractionated with ammonium sulfate salts at saturation levels of 0-15%, 15-30%, 30-45% and 45-60%. The fractionation results were F15 (0-15% protease fraction), F30, F45 and F60. All fractions were dialysed using cellophane bags with cold distilled water as dialysate. Each fraction was measured for activity, protein content, and determined for specific activity. The protease fraction with the highest specific activity was determined the optimum temperature and pH, and then used for the hydrolysis of soybean protein.

2. Hydrolysis of soybean protein

Isolation of soybean protein was carried out referring to the method [10]. A total of 4 grams of protein was dissolved with 100 mL of distilled water. The solution was then adjusted to the optimum pH by adding 0.5 N NaOH. A total of 16 ml of protease enzyme was added into the solution to hydrolyse the protein. The hydrolysis process was carried out with time variations of 0, 10, 20, 30, 40, 50 and 60 minutes. Samples with a hydrolysis time of 0 minutes were directly taken as much as 5 mL and then heated in water at 100 ℃ for 10 minutes. The remaining solution was incubated in a waterbath at the optimum temperature and 5 mL was taken for each

incubation time variation. The reaction was then stopped by heating at 100 ℃ for 10 minutes. The protein hydrolysate was then centrifuged at 7000 rpm for 25 minutes. The precipitate was discarded and the supernatant was stored in the refrigerator until use.

3. Antioxidant activity assay of protein hydrolysate

Antioxidant activity assay was carried out using DPPH method [11] with modification. DPPH solution was prepared by dissolving 1.98 mg DPPH into 100 mL 99% methanol. Each protein hydrolysate was added to methanol with a concentration of 1 mg/mL. The sample was taken 1 mL and then added 2 mL of DPPH solution. The solution was then allowed to stand for15 minutes.The blank used was DPPH solution in 99% methanol. Absorbance was measured at λ 515 nm. Antioxidant activity is expressed as inhibition percentage. The formula for determining antioxidant activity is:

$$\% \text{ Inhibition} = \frac{\text{A blank - A sample}}{\text{A blank}} \times 100\%$$

Description:
A blank = Absorbance of blank
A sample = Absorbance of DPPH solution with protein hydrolysate

4. Determination of AAI (Antioxidant Activity Index)

Determination of AAI was done using the method of [6]. Protein hydrolysate with the highest percentage of inhibition was made a concentration series of 100, 200, 300, 400 and 500 ppm. The linear regression obtained in the form of the equation y = bx + a was used to determine the IC_{50} (Inhibitory Concentration 50%) of sample by expressing the y value as 50, while the resulting x value is expressed as IC_{50}. The IC_{50} value states the concentration of the sample solution needed to reduce the DPPH radical by 50%. The AAI value is calculated with the equation:

$$\text{AAI} = \frac{\text{Concentration of DPPH (ppm)}}{IC_{50} \text{ of sample (ppm)}}$$

5. Hemolysis assay

Hemolysis assay was done using the method of [6]. Hemolysis test performed using chicken red blood cells. Chicken blood was added with oxalic acid to prevent coagulation. Chicken blood was washed with 0.01 M tris-HCl pH 7.4 containing 0.15 M NaCl, then centrifuged at 2500 rpm. The supernatant was discarded and the pellet was washed 2 times using tris-saline buffer. 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 0.01 mg/mL was added with 1 mL of 0.1% red blood cell suspension, then incubated for 2 hours at 37 ℃ and then centrifuged at 5000 rpm for 5 minutes. The supernatant was measured for absorbance at λ 540 nm. Tween 80 was used as positive control while tris-saline as negative control. The percentage of hemolysis can be obtained using the following equation:

$$\%\text{Hemolysis} = \frac{\text{A sample - A control (-)}}{\text{A control (+)}} \times 100\%$$

C. Results and Discussion

1. Fractionation of crude extract protease

Fractionation aims to separate protease enzyme proteins from other proteins using graded concentrations of salt saturation. Fractionation of crude extract protease was carried out by the addition of ammonium sulfate salts of increasing concentration under cold conditions. Fractionation results are shown in Table 1.

Table 1. Activity, specific activity and purification factor of protease fractions

Sample	Volume (mL)	Activity (U/mL)	Protein content (mg/mL)	Spesific activity (U/mg)	Purification factor (x CE)
CE	900	0.093	3.165	0.030	-
F15	50	0.104	0.121	0.859	28.60
F30	50	0.107	0.167	0.738	24.60
F45	50	0.070	0.275	0.254	8.46
F60	50	0.054	0.993	0.054	1.80

Notes: CE: crude extract; F15: protease fraction with 15% ammonium sulfate salt; F30: protease fraction with 30% ammonium sulfate salt; F45: protease fraction with 45% ammonium sulfate salt; F60: protease fraction with 60% ammonium sulfate salt

Fractionation is one of the stages of enzyme purification. Fractionation is declared successful in increasing the purity of an enzyme when the specific activity of the enzyme increases compared to the crude extract [12]. Based on the data in the table, it is known that all protease fractions have higher specific activity than the specific activity of protease crude extract. Therefore, it can be concluded that fractionation succeeded in increasing the purity of the protease enzyme. Protease fraction that has the highest specific activity is F15 with a value of 0.859 U/mg with a purification factor of 28.60 times the crude extract. Therefore, the optimum temperature and pH of F15 were determined. The optimum temperature was reached at 45 °C, while the optimum pH was reached at pH 8. Then F15 is used to hydrolyse soybean protein to produce protein hydrolysates.

2. Antioxidant activity of protein hydrolysates

Hydrolysis of soybean protein was carried out at a time variation of 10, 20, 30, 40, 50 and 60 minutes. The resulting protein hydrolysates were tested for antioxidant activity using the DPPH method. The results of the antioxidant activity test are shown in Table 2.

Table 2. Antioxidant activity values of each protein hydrolysate fraction

PH at the time of hydrolysis (minutes)	Antioxidant activity (% inhibition)
10	63.1
20	59.3
30	52.0
40	51.5
50	50.4
60	34.5

Note: PH; protein hydrolysate

Based on the results in Table 2, it was found that the amount of antioxidant activity of protein hydrolysate was inversely proportional to the hydrolysis time. The antioxidant activity decreased as the hydrolysis time increased. Increasing the hydrolysis time causes changes in the size of the peptides which become smaller and cause a decrease in the antioxidant activity of the protein hydrolysate [13]. Antioxidative peptide sequences formed in the early stages of the hydrolysis process may break down as the hydrolysis time increases and lead to decreased antioxidant activity [14]. The highest antioxidant activity was obtained in protein hydrolysate hydrolysed for 10 minutes with an inhibition percentage of 63.1%. The protein hydrolysate with the highest antioxidant activity was then determined for IC_{50}, AAI 9anyioxidant Activity index) and percentage of hemolysis.

3. AAI (Antioxidant Activity Index)

Determination of IC_{50} was done by measuring the antioxidant activity at concentration series of 100, 200, 300, 400 and 500 ppm. The results of the calculation of the percentage of inhibition of each concentration of protein hydrolysate made a linear regression curve to calculate the IC_{50} value. The IC_{50} value expresses the amount of antioxidant concentration required to reduce DPPH by 50%. Furthermore, the AAI value was calculated using the IC_{50} value obtained. and determined the antioxidant criteria. The results showed that the IC_{50} was 166 ppm and the AAI was 0.119. Referring to the antioxidant category based on the AAI value, soybean protein hydrolysate is categorized as a weak antioxidant [15].

4. Hemolysis

Hemolysis test is a test to determine the pathogenic properties of a sample by reacting to red blood cells. The principle of hemolysis test is the reaction between erythrocyte suspension and protein hydrolysate to see the effect of erythrocyte resistance to the possibility of hemolysis [6]. Hemolysis test results are shown in Table 3.

Table 3. Percentage hemolysis of soybean protein hydrolysate

Sample	Percentage of hemolysis (%)
Tris-saline (negative control)	0
Tween 80 (positive control)	100
PH of soybean protein	3

Note: PH: protein hydrolysate

Based on the results in the table, the percentage of hemolysis of soy bean protein hydrolysate is 3%. Referring to the category [16], it can be concluded that soy bean protein hydrolysate is categorized as causing little hemolysis in red blood cells.

D. Conclusion

The highest antioxidant activity was obtained from soy bean protein hydrolysate hydrolyzed for 10 minutes with an inhibition percentage value of 63.10%. The IC50 value obtained was 166 ppm (medium category). AAI value of 0.119. Judging from the percentage of hemolysis, the soy bean protein hydrolysate is categorized as a little hemolysis with a value of 3%.

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F. References

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