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Antioxidant activity of bioactive peptides from peanuts protein hydrolysate

Maulidya Rachmawati, Wahyu Intan Prastiwi and Siti Narsito Wulan*

Department of Food Science and Biotechnology, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia

KEYWORDS

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Antioxidant activity
Degree of hydrolysis
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ABSTRACT

Peanut protein hydrolysate was produced from peanut protein isolate hydrolyzed using Alcalase® 2.4 L. Hydrolysis conditions were optimized using a response surface methodology. The two independent variables were the substrate/enzyme ratio and the hydrolysis time with lower and upper limits of (20:1-50:1 v/v) and (1-3 hours), respectively. Both were designed at three levels of central composite design from the response surface methodology and the degree of hydrolysis as the dependent variable. This study aimed to obtain the optimum conditions for enzymatic hydrolysis of peanut protein isolate using Alcalase to produce protein hydrolysate with the maximum degree of hydrolysis. The optimum hydrolysis conditions obtained were the substrate/enzyme ratio of 20:1 (v/v) and the hydrolysis time of 3 hours with a predicted degree of hydrolysis of 40.54%. The actual experimental results obtained a degree of hydrolysis of 40.35% ($p>0.05$), which showed no difference between the predicted and experimental results. The highest antioxidant activity was obtained from the combination of these treatments, indicated by IC_{50} of 5.61 ppm.

Introduction

Reactive oxygen species (ROS) is the result of oxygen metabolism during respiration in aerobic organisms which is unstable or highly reactive. ROS react quickly with DNA, membrane lipids, and proteins which can cause senescence, inflammation, and cell death (Abuine et al., 2019; Campos et al., 2010; Yoon et al., 2013). Even though the body has a defence system to fight ROS, oxidative damage cannot be completely prevented, therefore sufficient antioxidants are needed to ward off free radicals and reduce oxidative damage (Abuine et al., 2019; Campos et al., 2010; López-Barrios et al., 2014). Antioxidant deficiency is known to cause oxidative stress, which plays a role in the occurrence of hypertension, neurodegenerative disease, diabetes, and cell damage (Campos et al., 2010; Yoon et al., 2013).

Protein derivative products such as hydrolysates or bioactive peptides are widely studied because they have potential antioxidant activity. Peptides with antioxidant activity can be produced by enzymatic hydrolysis; where this method has several advantages because it does not use organic solvents and hazardous chemicals in the process and the final product. In addition, the peptides produced are more stable, enzymes are easily deactivated, and the hydrolysis process is simple (Cruz-Casas et al., 2021; Ulug et al., 2021). One of the protease enzymes that can be used is alcalase which has a wide specificity hence it can produce hydrolysate with a high degree of hydrolysis, low molecular weight, and is resistant to further degradation by gastric proteases (Hanafi et al., 2018; Wu et al., 2009; Wu et al., 2019). Alcalase is known to be suitable for protein hydrolysis from various food sources (Ulug et al., 2021).

Peanut is a good source of protein available worldwide, including in Indonesia, but is underutilized because of its low functional properties. Peanut flour is widely used as animal feed ingredients (Yu et al., 2021). The high protein content of peanuts compared to other legumes can be used as a protein source to be hydrolysed by using the Alcalase enzyme to produce protein hydrolysate with antioxidant activity.

*Corresponding author
E-mail address: wulan_thpub@ub.ac.id

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There are conditions to be considered when carrying out enzymatic hydrolysis such as the substrate/enzyme ratio, the hydrolysis time, the pH, and the ambient temperature. One of the parameters used to assess the hydrolysis process which indicates the percentage of broken peptide bonds during the hydrolysis is the degree of hydrolysis (Butré et al., 2014). The optimum degree of hydrolysis is associated with the production of low molecular-weight hydrolysates. Low molecular-weight hydrolysates is known to produce good antioxidant activity (Xie et al., 2019). To obtain optimum hydrolysis conditions to produce a high degree of hydrolysis of protein hydrolysate is by using the response surface methodology (RSM). This method can generate a mathematical and statistical model that relates the treatment and response to be observed (See et al., 2011). This study aimed to determine the optimum enzymatic hydrolysis conditions namely substrate/enzyme ratio and hydrolysis time of peanut protein isolate hydrolyzed with Alcalase enzymes to obtain hydrolysate with the high degree of hydrolysis and to analyze the antioxidant activity of the protein hydrolysates.

Materials and Methods

This study was conducted from May 2021 until January 2022 at the Laboratory of Food Chemistry and Biochemistry, Department of Food Science and Biotechnology, Faculty of Agricultural Technology, Universitas Brawijaya, Malang, Indonesia.

Materials

Peanuts were obtained from the Research Institute for Legumes and Tuber, Malang, Indonesia. Alcalase® 2.4L, an endopeptidase enzyme from *Bacillus licheniformis*, was purchased from Sigma-Aldrich. Formaldehyde 37% (Merck), petroleum ether (Merck), Kjeldahl tablets (Merck), aquadest (WaterOne, OneMed), NaOH (Merck), HCl (Merck), DPPH (Sigma-Aldrich), and ethanol (Merck) were used for the analysis.

Preparation of peanut protein isolate

The first step of the process was to make low-fat peanut flour using the method described by Yulifianti et al. (2015) with a few modifications. Peanut seeds were soaked in boiling water for 45 seconds and drained, then dried at 100°C in the oven for 120 minutes, cooled and peeled off the skin. Peanuts were ground into peanut flour and sifted using a 60-mesh sieve screen. Subsequently, defatting was carried out using a Soxhlet extractor with petroleum ether solvent (1:8 w/v) for 5 hours. Fat-free or defatted peanut flour was dried in an oven (Memmert UE500) at 100°C for 5 minutes to remove the solvent.

Preparation of peanut protein isolates was conducted using alkaline extraction and acid precipitation methods as previously applied in the soybean protein isolate extraction by Wulan et al. (2002) with slight modifications. Fat-free peanut flour was diluted in distilled water (1:5 w/v), and 1M NaOH was added to adjust the pH to 10.0, then stirred with a magnetic stirrer (Thermo Scientific Cimarec SP88857105) at 50°C for 1.5 hours. The mixture was centrifuged (Hettich EBA 200) at 6,000 rpm for 10 minutes. The supernatant containing dissolved protein was separated from its residue, and 1M HCl was added to pH 4.5, then centrifuged again for 10 minutes. The precipitated protein was separated from the supernatant and neutralized to pH 7.0 using 1M NaOH, then dried using a cabinet dryer (Aneka Mesin) at 50°C for 10 hours. The resulting protein isolate was then stored at -20°C.

Proximate analysis

Proximate analysis (protein, fat, water, ash, and crude fibre content) of whole peanuts, fat-free peanut flour, and peanut protein isolate were analyzed using the method of AOAC International (2016), whereas carbohydrates was determined by difference. Moisture content was determined using thermogravimetric analysis. Total crude protein was determined using Kjeldahl method with a nitrogen conversion factor of 6.25. Fat content in the samples was analyzed using Soxhlet extraction. Ash content was determined by burning the dried sample in a furnace (Thermo Scientific FB 1410M-33) at 640°C until white ash was formed. Crude fiber content was determined by hydrolysis of the sample using strong acid and subsequently using strong base.

Optimization of the conditions of enzymatic hydrolysis of peanut protein isolate

a. Experimental design for optimization

A central composite design with two variables was used to evaluate the effect of the independent variables, namely the ratio of substrates/enzymes (v/v, X1) and the hydrolysis time (h, X2) to the dependent variable, namely the degree of hydrolysis. The independent variables were arranged with three levels of values (-1, 0, and +1), the range of values obtained from several previous studies using Alcalase enzymes in the enzymatic hydrolysis process. This optimization design produces 13 runs, as shown in Table 1. It was assumed that the

mathematical function that shows the response of variable Y due to the influence of the independent variable was as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 \quad \ldots (1)$$

where β_0 represents a constant value of a model; βi, βii, and βij represent linear, quadratic, and interaction regression coefficients; X represents the independent variable. (i=variable 1 and 2; and j=variable 1 and 2).

b. Enzymatic hydrolysis (preparation of protein hydrolysates)

Peanut protein isolate (1 g) was suspended in 10 mL of phosphate buffer (pH 8.0), which was used as a substrate, and Alcalase was added to the substrate with the substrate/enzyme ratio according to the experimental design (Table 1). Hydrolysis was carried out at 50°C under constant stirring using a water-bath shaker (Memmert WNB45) with hydrolysis time according to the experimental design. The hydrolysis reaction was stopped by heating the mixture at 95°C for 30 minutes in a water bath, followed by centrifugation (Hermle Z326K) at 10,000 rpm at 4°C for 30 minutes. The supernatant was collected and stored at -20°C for further analysis. This hydrolysis method was in accordance with the method of Siow and Gan (2013).

c. Analysis of the degree of hydrolysis

The degree of hydrolysis of protein hydrolysate was determined using formol titration according to the method of Noman et al. (2018). One mL of protein hydrolyzate was added with 50 mL of distilled water, and the pH was adjusted to 7.0 using 0.1 N NaOH. Then the mixture was added with 10 mL of 37% formaldehyde and left for 5 minutes at room temperature, and the pH was measured again. Subsequently, titrated until the final pH was 8.5 using 0.1 N NaOH. The volume of NaOH was used to calculate the number of free amino groups. Total nitrogen was measured using the Kjeldahl method. The calculation was carried out by the equations below:

$$\%\ free\ amino\ groups = \frac{A\ x\ B\ x\ 14{,}007}{C\ x\ 1000} x100 \quad \ldots (2)$$

$$Degree\ of\ hydrolysis\ (\%) = \frac{\%\ free\ amino\ groups}{\%\ total\ nitrogen} x100 \quad \ldots (3)$$

where A=volume of NaOH used for titration (mL); B=NaOH concentration for titration (0.1 N); C= volume of sample (mL).

Antioxidant analysis

The scavenging ability of the sample towards 1,1,-diphenyl-2-picrylhydrazyl (DPPH) free radicals was measured according to the method of Xie et al. (2019). When DPPH meets a proton-donating compound such as an antioxidant, the free radical is bound, and its absorbance decreases. Aliquots (2 mL) of sample solutions at different concentrations were added with 2 mL of DPPH (0.2 mM in ethanol). The reaction mixture was incubated for 30 minutes at room temperature in the dark. The absorbance was measured at a wavelength of 517 nm using a UV-Vis spectrophotometer (Shimadzu). Ethanol was used as a control. The free radical scavenging ability of the sample was measured as the decrease in the absorbance of the DPPH radical and was calculated by the following equation:

$$DPPH\ inhibitory\ (\%) = \frac{(Absorbance_{control} - Absorbance_{sample})}{Absorbance_{control}} x100 \quad \ldots (4)$$

The IC_{50} value (x) was calculated by entering the value of 50 as Y in the linear regression equation (Y=ax+b) obtained from the log graph of the concentration against the percentage of DPPH inhibition.

Data analysis

Optimization analysis and analysis of variance (ANOVA) was done using Design Expert 11 software of RSM to obtain optimum hydrolysis conditions with a high degree of hydrolysis. The data obtained were processed through three stages of model selection. The first stage was a model selection based on the sum of the squares of the order of the model (Sequential Model Sum of Squares) with $p<0.05$. The second stage was testing the model's inaccuracy (Lack of Fit) with $p>0.05$. The third stage was an optimum treatment selection based on a summary of statistical models (Summary of Statistics) with an R value close to 1.00 to get the optimum point.

After obtaining the suggested optimum treatments for high degree of hydrolysis protein hydrolysate, verification was carried out to compare the actual degree of hydrolysis based on the suggested optimum conditions with the predicted degree of hydrolysis by conducting a T-Test analysis using the Minitab 17 application. To determine the relationship between the degree of hydrolysis and the antioxidant activity, samples with the optimum degree of hydrolysis, the first and the second highest, the middle and the lowest degree of hydrolysis were analyzed for antioxidant activity.

Table 1. Research design (coded and real values) to optimize the hydrolysis of peanut protein isolate using Alcalase

Run	Independent variables				Dependent variable
	Real		Coded		
	Substrate/Enzyme ratio (mL/mL)	Hydrolysis time (hours)	S/E ratio	Hydrolysis time	Degree of Hydrolysis (%)
1	20/1	3	-1	1	41.223
2	35/1	2	0	0	28.674
3	35/1	2	0	0	28.597
4	35/1	2	0	0	28.136
5	35/1	2	0	0	30.601
6	35/1	3.41	0	1.414	30.256
7	20/1	1	-1	-1	28.401
8	35/1	2	0	0	28.136
9	56.21/1	2	1.414	0	17.861
10	50/1	3	1	1	22.734
11	35/1	0.59	0	-1.414	14.327
12	50/1	1	1	-1	18.408
13	13.79/1	2	-1.414	0	37.791

Table 2. Chemical composition

Chemical characteristics	Whole peanuts	Defatted peanut flour	Peanut protein isolate
Protein (%)	23.71±0.73	48.66±0.55	87.38±0.18
Fat (%)	38.47±0.91	2.30±0.09	2.62±0.05
Water (%)	6.27±0.17	6.48±0.06	6.50±0.18
Ash (%)	2.49±0.02	4.75±0.12	2.38±0.16
Carbohydrates by difference (%)	29.07±0.33	37.81±0.64	1.12±0.20
Crude fiber (%)	5.59±0.34	11.34±0.14	1.71±0.12

Note: values represent means ± standard deviations from three measurement

Results and Discussion

Proximate composition of raw materials

The proximate composition showed that protein and fat are the main components in whole peanuts (Table 2). The protein content (23.71±0.73%) and fat content (38.47±0.91%) of whole peanuts were within the range of those reported by Bonku and Yu (2020) which were 20.7-25.3% and 31-46%, respectively. After defatting, the protein content increased up to 48.66±0.55%. These levels were the same as those reported by others, which ranging from 47–55% (Kain et al., 2009; Yu et al., 2007). When defatted flour was processed into protein isolate, the protein content increased up to 87.38±0.18%, which was higher than those reported by Kain et al. (2009), which amounted to 84.20%. The water (6.27±0.17%), ash (2.49±0.02%), and carbohydrate (29.07±0.33) content of whole peanuts had similar values as reported by Bonku and Yu (2020) being 4.9-6.8%, 1.2-2.3%, and 21-37%, respectively, while the crude fiber content (5.59±0.34%) was higher (1.4-3.9%) than the ones reported by the similar study. The defatted peanut flour had moisture (6.48±0.06%) and ash (4.75±0,12%) content close to those reported by Davara et al. (2022) being 5.64% and 4.76%, respectively, while the carbohydrate content (37.81±0.64%) was higher than the ones reported by the similar study (23.59%). Peanut protein isolate had higher fat (2.62±0.05%) and moisture content (6.50±0.18%) than reported by Kain et al., (2009) being 0.44% and 3.06% respectively, while the ash (2.38±0.16%) and carbohydrate content (1.12±0.20%) were lower than those reported by the similar study (4.02% and 4.11%, respectively). The difference in proximate composition is probably due to the different peanut varieties and the method of defatting and extracting protein to produce isolates (Liu et al., 2019).

The results of optimization of enzymatic hydrolysis of peanut protein isolate

a. The suggested model

The optimization results are shown in Table 1. The results of the analysis of the degree of hydrolysis response show that the suggested model to use was the quadratic model. The selection of the model was based on the sum of the squares of the

sequence of models (Sequential Model Sum of Squares), which was significant with a p-value of 0.0158 ($p < 0.05$) and the testing of model inaccuracy (Lack of Fit) which was not significant with a p-value of 0.061 ($p > 0.05$). In addition, the R^2 value of this model was 0.9685, which was close to 1, thus indicating that this quadratic model was appropriate for representing the real relationship of the selected hydrolysis parameters. The selection of this model was a suggestion from the results of the Fit Summary analysis in the Design Expert 11 application (data not included).

The model in this study met the requirements and was justified using ANOVA, as shown in Table 3. Thus, the statistical significance of the model equation was evaluated and the results showed that the model was significant with a 95% confidence level ($p<0.05$). Responses from hydrolysis conditions or factors, interactions between factors, and probability values (p-values) are also shown in the results of the ANOVA test. Table 3 shows X_1 (Substrate/Enzyme Ratio), X_2 (Hydrolysis time), X_1X_2 (Interaction of Substrate/Enzyme Ratio and Hydrolysis Time), and X_2^2 (Quadratic Hydrolysis Time) are factors that significantly influence the degree of hydrolysis with $p<0.05$. While X_1^2 (Substrate/Enzyme Quadratic) had no significant effect with $p>0.05$.

The lack of fit test was used to evaluate the fit or suitability of the model, with the expected result being $p>0.05$. The results were not significant ($p>0.05$) indicating that the model met the requirements or was adequate for predicting enzymatic hydrolysis conditions in peanut protein isolate. The regression equation of the response surface obtained by RSM was as follows:

$$Y = 15.94 - 0.24\ X_1 + 20.36\ X_2 + 0.00070\ X_1^2 - 2.61\ X_2^2 - 0.142\ X_1X_2 \quad \text{.....(4)}$$

where Y, X_1, and X_2 represent degree of hydrolysis (%), substrate/enzyme ratio (v/v), and hydrolysis time (h) respectively.

Figure 1 shows the 3D response surface generated by the proposed model (quadratic). This figure shows the effect of two independent variables (substrate/enzyme ratio and hydrolysis time) on the degree of hydrolysis. The results showed an increase in the degree of hydrolysis was parallel to an increase in hydrolysis time, but inversely with an increase in the substrate/enzyme ratio. This phenomenon was observed because when the substrate concentration was lower, the active sites available for the enzyme to hydrolyze the substrate would expand, thus, improving the disruption of the peptide bonds and increasing the degree of hydrolysis (Kurozawa et al., 2008).

b. Conditions for optimal response

The effect of substrate/enzyme ratio and hydrolysis time on the degree of hydrolysis was determined using RSM. The desirability value was used to determine the optimum value of each factor. Substrate/enzyme ratio and hydrolysis time were set in range values, while the degree of hydrolysis as the dependent variable was expected to have a maximum response (maximize).

The optimum conditions for enzymatic hydrolysis of peanut protein isolate using the Alcalase enzyme were 20:1 for the substrate/enzyme ratio and 3 hours for the hydrolysis time. The predicted value of the response was the degree of hydrolysis of 40.54% with a desirability of 0.975. The validation test was carried out to determine the predicted and actual experimental values. The actual experimental with optimum hydrolysis conditions produced peanut protein hydrolysate with the degree of hydrolysis of 40.35 ± 0.56%. Compared to the predicted degree of hydrolysis, these results showed no significant difference ($p> 0.05$), thus the model indeed predicted the correct value.

Table 3. ANOVA results on the model's ability to predict the degree of hydrolysis of peanut protein hydrolysate

Source	Sum of squares	df	MeanSquare	F-values	p-values	
Model	665.36	5	133.07	42.99	< 0.0001	Significant
X_1-Substrates/Enzymes	401.40	1	401.40	129.67	< 0.0001	
X_2-Hydrolysis Time	196.76	1	196.76	63.56	< 0.0001	
X_1X_2	18.05	1	18.05	5.83	0.0465	
X_1^2	0.1710	1	0.1710	0.0552	0.8209	
X_2^2	47.41	1	47.41	15.31	0.0058	
Residual	21.67	7	3.10			
Lack of Fit	17.49	3	5.83	5.58	0.0651	Not significant
Pure Error	4.18	4	1.04			

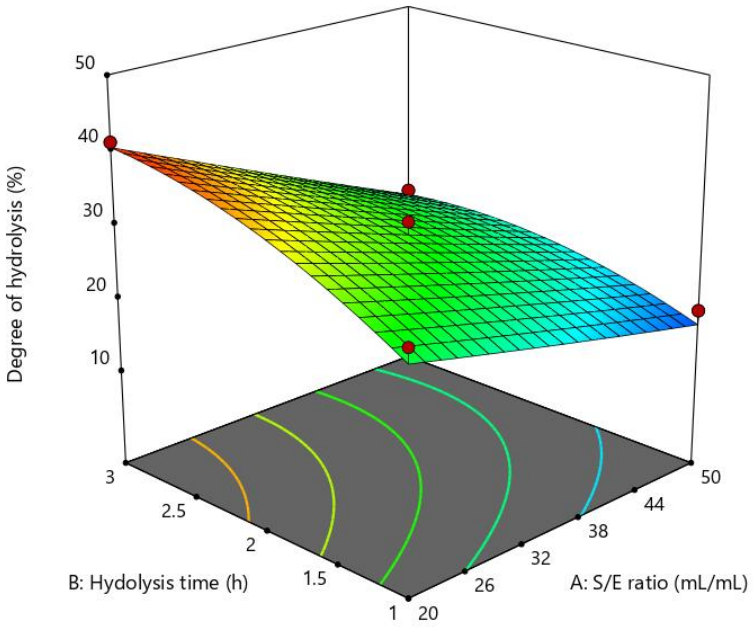

Figure 1. Response surface graph on the effect of substrate/enzyme ratio and hydrolysis time on the degree of hydrolysis of peanut protein hydrolysate

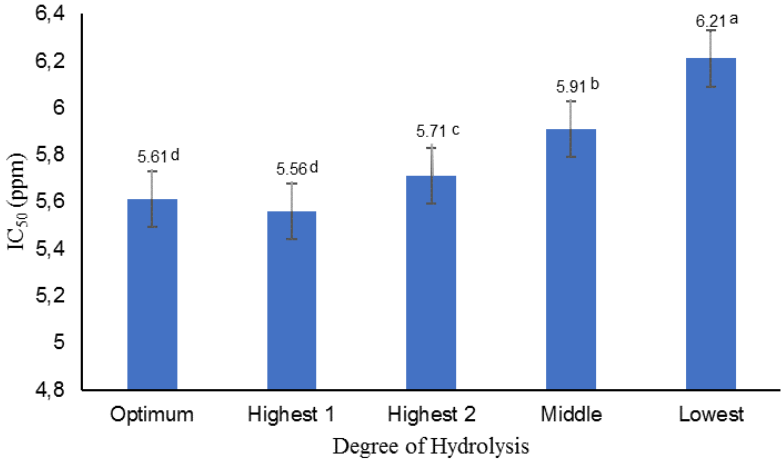

Figure 2. The antioxidant activity of peanut protein hydrolysate with various degrees of hydrolysis measured as IC50 (ppm). Data was shown with the mean values. Means with different letters indicate significant differences ($p<0.05$))

c. Results of analysis of antioxidant activity

The samples selected for analysis of their antioxidant activity were those with the optimum (Opt), the highest 1 (R1), the second highest or the highest 2 (R13), the middle (R7), and the lowest (R11) degree of hydrolysis. Figure 2 shows the effect of the degree of hydrolysis on antioxidant activity as determined by the ability to scavenge DPPH radicals measured as IC_{50} (ppm). The scavenging activity of peanut protein isolate prior to enzymatic hydrolysis could not be measured because the sample precipitated in ethanol.

The effect of the degree of hydrolysis on the IC_{50} value obtained from the DPPH antioxidant analysis showed that the hydrolysate with the optimum (Opt) and the highest degree of hydrolysis (R1) had a high antioxidant activity indicated by a low IC_{50} value (5.61 ppm and 5.56 ppm, respectively). In contrast, the hydrolysate with the lowest degree of hydrolysis had a low antioxidant activity with an IC_{50} value of 6.20 ppm. This was because the high degree of hydrolysis protein hydrolysate contained small-sized peptides with hydrophobic, aromatic, and positively

charged amino acids, which can activate antioxidant activity (Rezvankhah et al., 2021).

In this study, the degree of hydrolysis with a range of 14.33 to 41.22% had a fairly good DPPH scavenging activity with IC_{50} values ranging from 5.56-6.21 ppm, IC_{50} values below 50 ppm belonging to the category of very strong antioxidant activity (Herawati et al., 2022). IC_{50} values indicating the concentration of inhibitory peptides or hydrolysates needed to inhibit 50% of DPPH free radicals and low IC_{50} value is preferred because it shows higher antioxidant activity when compared to a high IC_{50} value (Abuine et al., 2019; López-Barrios et al., 2014; Sonklin et al., 2020). In this study, the increase in the degree of hydrolysis was proportional to the increase in DPPH scavenging activity. However, the high antioxidant activity with a degree of hydrolysis > 41.22% cannot be confirmed in this study. Previous studies reported that when the percentage of the degree of hydrolysis was too high, it could reduce the antioxidant activity. In the study by Rezvankhah et al. (2021), which hydrolyzed lentil protein using alcalase, the degree of hydrolysis produced was measured using the pHstat method and had the highest hydrolysis degree of >20%. Further increase in the degree of hydrolysis led to a reduction of antioxidant activity. Breaking too many peptide bonds can result in a very small molecular weight that has higher hydrophilicity, causing the interaction between DPPH and hydrolyzate to decrease therefore reducing scavenging activity (Martínez-Montaño et al., 2022; Rezvankhah et al., 2021; You et al., 2009). However, it is necessary to analyze the peptide sequences because differences in amino acid residues in each hydrolysate with various degrees of hydrolysis may potentially affect their antioxidant activity (Xia et al., 2020; Xie et al., 2019).

Conclusion

Peanut protein isolates with high protein content (87.38%) when hydrolyzed by Alcalase enzyme produced protein hydrolysate. Hydrolysis conditions had a significant effect on the degree of hydrolysis of protein hydrolysates. Based on the model, the optimum hydrolysis conditions were a combination of substrate/enzyme ratio of 20:1 (v/v) and a hydrolysis time of 3 hours producing peanut protein hydrolysate with the degree of hydrolysate of 40.35%. This protein hydrolysate had DPPH scavenging activity as IC_{50} of 5.61 ppm.

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Declarations

Conflict of interests. The authors declare no competing interests.

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