

Response surface optimization in determining hydrolysis reaction in soybean dregs using the Box Behnken design

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Article history:

Received: 8 January 2025

Received in revised form: 23 April 2025

Accepted: 22 May 2025

Available Online: 17 January 2026

Keywords:

Box Behnken design,
Glycine max (L.) Merr.
Hydrolysis,
Isolated soybean protein,
Soybean dregs

DOI:

[https://doi.org/10.26656/fr.2017.10\(1\).206](https://doi.org/10.26656/fr.2017.10(1).206)

Abstract

Protein hydrolysate from soybean dregs (*Glycine max* (L.) Merr.) was made through an enzymatic hydrolysis process using a protease enzyme from *Bacillus licheniformis*. The protein hydrolysis level was evaluated by measuring the hydrolysis degree (DH) value. This research aimed to obtain the best hydrolysis conditions for isolated soybean protein. The condition with the best hydrolysis degree (DH) was optimized using the response surface method (RSM) with Box Behnken Design (BBD) based on three independent variables, namely enzyme concentration, temperature, and hydrolysis time against DH, then checked for model optimization. The optimum condition of enzymatic hydrolysis of soybean dregs protein was obtained by adding 1.2% protease enzyme at 59°C for 2.6 h. The average value of this product's DH with three repetitions was 92.93%, and the yield was 10.39%. The equation describing the variables and correlation of DH is $DH = 92.84 + 6.32A - 0.4406B - 0.5108C$, where A, B, and C are E/S concentration, temperature, and time. In optimal conditions, alkaline protease hydrolysis yielded a 1.000 desirability value, with a 95.62%. The hydrolysate soybean protein's three highest amino acid contents are tyrosine 28.98 mg/g, lysine 1.17 mg/g, and methionine 0.36 mg/g. This study describes the possibility of utilizing soybean dregs to produce hydrolysate soybean protein with high yields by enzymatic hydrolysis with protease enzymes from the *Bacillus licheniformis* bacteria.

1. Introduction

Soybean pulp is a waste product from the tofu manufacturing industry that can be processed into protein hydrolysate with high economic value and used as a raw material for protein hydrolysate. Protein hydrolysate results from degrading proteins into peptides and amino acids using enzymes (Hariyanti *et al.*, 2024). The antioxidant activity of soybean pulp protein hydrolysate comes from peptides containing the amino acids tyrosine 0.20 mg/g and histidine 0.19 mg/g, which can neutralize free radicals. Meanwhile, its antibacterial activity comes from the amino acid phenylalanine 0.24 mg/g, by damaging bacterial cell membranes (Ratnayani *et al.*, 2022; Deng *et al.*, 2024).

Ratnayani *et al.* (2022) studied Okara protein hydrolysate production by isolating and hydrolysing it with papain enzyme at concentrations ranging from 3% to 7%. The study definitively found that the highest

antioxidant activity occurred at 4% papain concentration, achieving 98.86% using the DPPH method and 0.5 μ mol AAE/g using the FRAP method. It was based on previous studies that peanut protein hydrolysate, which has been previously hydrolyzed, must be in the form of a protein isolate extracted by the alkaline extraction method with a protein concentration of at least 90% based on the Codex standard.

Protein hydrolysate has a wide range of benefits in pharmaceutical and food applications. The utilization of soybean protein hydrolysate from tofu production has not been widely developed towards higher benefits that can increase the added value of soybean pulp as an ethnopharmaceutical product that can produce safe and natural traditional medicine products, dietary supplements, and cosmetics (Mehmet *et al.*, 2021). Studies have shown that hydrolysate protein increases the nutritional value and activity by converting low-

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consumption proteins into high-value protein products. Essential factors in enzymatic hydrolysis include pH, temperature, enzyme/substrate ratio, hydrolysis time, and enzyme type. As in previous studies, hydrolysis of proteins with enzymes can potentially increase bioactivity (Tekle *et al.*, 2022; Hariyanti *et al.*, 2024). This study aims to obtain the best hydrolysis conditions for isolated soybean protein using the response surface method (RSM) with Box Behnken Design (BBD).

2. Materials and methods

2.1 Materials

The material used was soybean dregs obtained from the tofu industry in the Pasar Minggu area, Jakarta Selatan, Indonesia. Other ingredients are Bradford reagent, trichloroacetate (TCA), P5380-100MG Alkaline protease 25 mg Type VIII, Aquadest, Bradford reagent, KH_2PO_4 , NaOH, distilled water, Phosphoric acid, Ethanol, and Bovine Serum Albumin. The chemical suppliers Sigma-Aldrich or Merck provided all the chemicals and solvents.

2.2 Flour manufacturing and soybean protein isolate

The first step in this study was to make flour by drying soybean dregs waste at a temperature of -103°C for 8 h using a vacuum pump with a pressure of 0.0004 mbar (Dong *et al.*, 2022). Soybean protein isolate was prepared by pouring 20 g of soybean pulp into a 500 mL flask, adding 200 mL of 0.2% NaOH, adjusting the measured pH to pH 12, and stirring the mixture on an orbital shaker for 60 min. The mixture was placed in a 15 mL centrifuge tube and centrifuged at 5000 rpm for 15 min at 4°C to dissolve the protein in the flour. The pellets from the extraction were dried in a freeze dryer at -103°C for 48-72 h (Edwards *et al.*, 2020).

2.3 Response surface methodology using Box-Behnken design

Each level of free variables in the BBD was coded, i.e., the angular point (-1) and (+1), the center point (0), and the axial point ($-\alpha$) and ($+\alpha$). This stage is influenced by three independent variables and one response, namely X_1 = enzyme concentration, X_2 = temperature, X_3 = hydrolysis time, and Y = degree of hydrolysis. The range of independent variables is shown in Table 1. Data processing uses Design Expert 13® to obtain the optimal condition of protein hydrolysate. The optimum conditions were tested for FTIR characteristics and amino acid analysis (Prandi *et al.*, 2024).

Table 1. The range of independent variable values.

Variable	-1	0	1
E/S (% v/b)	0.5	1	1.5
Temperature ($^\circ\text{C}$)	55	60	65
Time (h)	2	3	4

2.4 Preparation of formulation and design of response

This study's formulation and response design used the Design Expert 13.0® program to determine dependent and independent variables. The dependent variable in this analysis was the total isolate of hydrolyzed soybean protein. Meanwhile, the independent variables include enzyme concentration, temperature, and hydrolysis time. For randomization, trial and error obtain the minimum and maximum limits, as shown in Table 1, described in the Design Expert 13.0® program, RSM BBD. The 3 different factors (temperature, time, and E/S concentration) were applied at the same level (-1 and +1), and DH was the response variable (Y) (Mohammad *et al.*, 2015).

A 5% (w/v) soybean protein isolate solution was placed in a sealed 50-mL flask. Subsequently, the pH of the soybean protein isolate solution was adjusted to a range of 7-8 by the addition of 0.1 M NaOH. Protease enzymes (0.5, 1, 1.5%) were added at the predetermined temperature (55, 60, 65°C). Subsequently, a hydrolysis reaction was executed with a magnetic stirrer at a rate of 200 rpm for a predetermined duration (2, 3, 4 h). The initiation of the reaction occurs upon the mixing of the soybean protein isolate solution with enzymes. The hydrolysis reaction was terminated by increasing the temperature to 95°C for 15 min. Centrifugation was performed at a temperature of 25°C , at 8000 rpm, for 10 min. The protein hydrolysate obtained was subsequently collected. The protein hydrolysate obtained was then subjected to a freeze-drying process at a temperature of -104°C for 48 h (Phongphisutthinant *et al.* 2024).

After obtaining protein hydrolysate products in 15 treatments, the degree of hydrolysis (DH) analysis was carried out on each treatment (Table 2). After hydrolysis, 10 mg of soybean protein hydrolysate was added to 10 ml of 10% TCA and centrifuged at 3000 rpm for 15 min. The filtrate was mixed with 50 μL and 2.5 mL of Bradford reagent. The mixture was stirred for 1 min and incubated at 20°C for 5 to 55 min. Absorbance is measured at a wavelength of 595 nm and calculated by the following formula (Osukoya *et al.*, 2020; Hafifani *et al.*, 2021; Ningrum *et al.*, 2023).

$$\text{Degree Hydrolysis (\%DH)} = \frac{10\% \text{ TCA dissolved protein}}{\text{the total protein sample}} \times 100\%$$

2.5 Formulation stage and data analysis

Soybean protein isolate (from soybean pulp) was hydrolyzed at different enzyme concentrations (E/S), temperatures, and hydrolysis times to obtain the best DH. Linear, quadratic, 2FI, and cubic ANOVA tests each response variable. Models with significance in ANOVA and insignificance in lack of fit were used to study the

variables (Hariyanti *et al.*, 2024).

2.6 Optimization stage

The Design Expert 13.0® program optimizes DH's response. The program optimizes the variables and response measurement data. The optimization output recommends a new formula that is optimal and program-compliant. The best procedure is the variant with the maximum desired value, the value of the optimization objective function demonstrating the program's benefits to meet the desired requirements per the final product criteria. The value range ranges from 0 to 1, and a desired value close to 1 indicates a better ability to create the desired product (Joyjamras *et al.*, 2022).

2.7 Verification for optimum conditions

After optimization, the recommended solution for the optimum condition was obtained. From the optimum condition, enzymatic hydrolysis of the implied state was carried out three times. Next, the DH hydrolysate produced from hydrolysis was determined. RSM's prediction value was then compared with the experimental value obtained using a single-sample t-test (Hariyanti *et al.*, 2024).

2.8 Fourier transform infrared spectroscopy

The functional group detection of soybean protein hydrolysate powder and soybean protein isolate samples was analyzed by the Fourier-transform infrared (FTIR) technique. The functional group analysis of these samples was conducted within the wave number range of 4000 to 500 cm⁻¹. The functional group spectra of soybean protein hydrolysates and soybean protein isolates were analyzed by comparing them with reference data. Peaks were identified from the sample spectra based on the functional groups present in the samples.

2.9 Amino acid analysis

Amino acid profile test consists of 17 amino acid test parameters of MU-03/66 using high-performance liquid chromatography (HPLC) for both essential and non-essential amino acids. Essential amino acids include histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, and valine. Non-essential amino acids include aspartic acid, glutamic acid, serine, glycine, proline, alanine, tyrosine, arginine, and cysteine (Hariyanti *et al.*, 2024).

3. Results and discussion

Soybean dregs flour was a slightly yellowish powder. The yield of soybean pulp protein isolate from the flour was 34.06%. Soybean protein isolate has an

excellent protein content of 98.80%. The Box-Behnken design of RSM with 15 trials is the best approach for soybean protein hydrolysate research, especially when previous research supports this number of trials. This would save time and money (Hariyanti *et al.*, 2024).

Table 2 definitely shows the degree of hydrolysis for each treatment. BBD's mathematical model is proven. It guarantees optimal design. The linear model was selected using the Sequential Square Number Model based on the number of squares and order. The best model is the variant with a probability value of less than 5%. The linear model is the best candidate as it has the lowest probability value (p-value: 0.0500) (Table 3).

Table 2. The optimum conditions for the degree of hydrolysis in different enzyme concentrations, temperatures, and times.

No	E/S (% v/b)	Temperature (°C)	Time (Hour)	DH (%)
1	1.5	60	4	103.08
2	1.5	65	3	90.97
3	1	60	3	95.41
4	1	65	4	99.26
5	0.5	65	3	81.74
6	0.5	60	4	82.88
7	1	55	4	86.17
8	0.5	60	2	85.08
9	1	55	2	96.15
10	1	65	2	98.84
11	1	60	3	97.21
12	1	60	3	88.51
13	0.5	55	3	90.63
14	1.5	60	2	95.41
15	1.5	55	3	101.39

The non-conformance test with Design Expert 13.0® recommends a linear model, as shown in Table 4. The lack of conformity testing is based on model nonconformity, and the parameter applied to describe model nonconformity is the p-value. An insignificant mismatch value must be met for a good model that demonstrates the conformity of the DH response to the model (Heck *et al.*, 2022). The Linear test selects the best model according to a value close to 1. The adjusted R² test recommends the squared model as the best result, as shown in Table 5. The regression model is well-defined when R² > 0.80. A small R² indicates poor relevance of dependent variables in the model. In addition, the model can match the actual data when R² approaches one (Cao *et al.*, 2022).

The interaction between the answer and the hydrolysis process factor was examined using ANOVA. The significance of a factor is determined by the likelihood that the p-value should be <5%. This equation has 3 linear terms, 1-factor interaction term, and 1

Table 3. Sequential model sum of squares analysis.

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Mean vs Total	1.293E+05	1	1.293E+05			
Linear vs Mean	322.83	3	107.61	3.59	0.0500	Suggested
2FI vs Linear	52.02	3	17.34	0.4992	0.6931	
Quadratic vs 2FI	39.80	3	13.27	0.2786	0.8391	
Cubic vs Quadratic	195.96	3	65.32	3.10	0.2534	Aliased
Residual	42.14	2	21.07			
Total	1.300E+05	15	8664.48			

Table 4. Lack of fit test results using Design Expert 13.0® software.

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Linear	287.78	9	31.98	1.52	0.4593	Suggested
2FI	235.76	6	39.29	1.86	0.3894	
Quadratic	195.96	3	65.32	3.10	0.2534	
Cubic	0.0000	0				Aliased
Pure Error	42.14	2	21.07			

Table 5. Model summary statistics.

Source	Sequential p-value	Lack of Fit P-value	Adjusted R ²	Predicted R ²	
Linear	0.0500	0.4593	0.3567	-0.0149	Suggested
2FI	0.6931	0.3894	0.2550	-0.9832	
Quadratic	0.8391	0.2534	-0.0213	-3.9484	
Cubic	0.2534		0.5481		Aliased

Table 6. ANOVA model of the hydrolysis of soybean dregs.

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	322.83	3	107.61	3.59	0.0500	Significant
A-E/S	319.19	1	319.19	10.64	0.0076	
B-Temperature	1.55	1	1.55	0.0518	0.8242	
C-Time	2.09	1	2.09	0.0696	0.7968	
Residual	329.92	11	29.99			
Lack of Fit	287.78	9	31.98	1.52	0.4593	not significant
Pure Error	42.14	2	21.07			
Cor Total	652.75	14				

quadratic effect: A, B, and C (Table 6). The value of non-conformance is caused by interference because non-conformity is not as important as pure error potential. ANOVA's suggested model equation is: $DH = 92.84 + 6.32A - 0.4406B - 0.5108C$, where A, B, and C are E/S concentration, temperature, and time, respectively.

A 3-dimensional response surface constructed on a simple linear model can be used to visualize the impact of independent variables on the degree of hydrolysis (DH), which can be seen in Figure 1. Figure 1 shows a contour plot that represents the function of 2 factors: temperature and enzyme concentration. An increase in the enzyme concentration will increase the number of collisions between the enzyme and the substrate, so the hydrolysis reaction rate and DH also increase (Chiodza and Goosen, 2023). However, a continuous increase in enzyme concentration does not necessarily result in a linear increase in DH because temperature and time

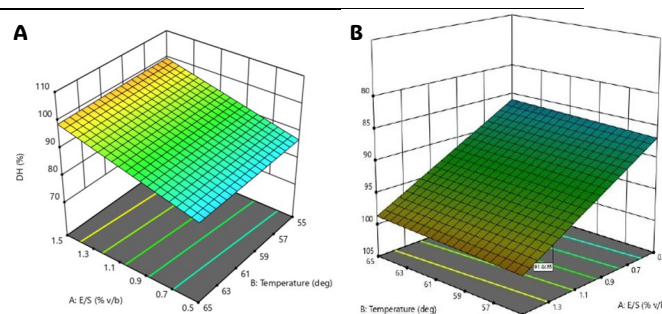


Figure 1. Response surface graph for the degree of hydrolysis as a function of A - temperature and time, B - temperature and E/S, during the hydrolysis of soybean dregs with protease enzyme.

factors contribute to the increase in degree value. An increase in temperature will generally increase the hydrolysis reaction rate as the molecule's kinetic energy increases. However, a temperature that is too high can cause enzyme denaturation, so its activity decreases, and

DH does not continue to increase. Too low a temperature will slow down the rate of the hydrolysis reaction (Magalhães *et al.*, 2022). The longer the hydrolysis time, the more peptide bonds are broken, so DH tends to increase. However, the enzyme can undergo denaturation and decrease its activity if the time is too long. A hydrolysis time that is too short will result in low DH because the hydrolysis reaction has not occurred optimally (Hariyanti *et al.*, 2024).

Based on the optimization results, DH at the point with temperature parameters of 59°C, time 2.58 h, and 1.2% enzyme was expected to have a DH of 95.62% (Figure 2). The desirability value obtained was 1.000, indicating the ideal health condition. A desirability value of 1 indicates excellent results, while 0 indicates an unsatisfactory reaction (Hariyanti *et al.*, 2024). The success rate for the desirability value of 1 response was 100.0%. Protein hydrolysate at an E/S concentration of 1.5%, a hydrolysis temperature of 60°C, and a hydrolysis time of 4 h had a hydrolysis degree of 103.08%. The hydrolysis degree (DH) value reaching 100% in protein hydrolysate indicates that all peptide bonds in the protein have been broken, producing free amino acids. A hydrolysis degree of 100% requires highly controlled conditions, including the correct type of enzyme, the optimum pH for hydrolysis of the protein, and the percentage of protein substrate to be hydrolyzed by the enzyme. DH of 100% results in amino acid damage, resistance to certain peptide bonds, and the need for optimal reaction conditions. Higher substrate concentrations can affect DH. Some enzymes show increased DH with increasing substrate concentration, while others experience decreased DH due to substrate inhibition (Deng *et al.*, 2018; Zapata-Montoya *et al.*, 2018). The DH value reaching 100% is also caused by extreme hydrolysis conditions, which are influenced by the use of very effective enzymes, optimal temperature, and pH conditions, which can increase the efficiency of protein breakdown to exceed 100% (Arteaga *et al.*, 2020).

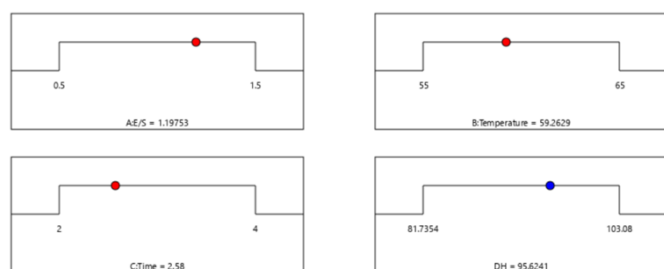


Figure 2. Solution optimization by Design Expert 13.0® software.

The degree of hydrolysis in soybean protein hydrolysate indicates the extent of the process of breaking down proteins into peptides. The degree of

hydrolysis is a key indicator of the final product's quality. An optimal degree of hydrolysis will result in hydrolysates with desirable functional properties such as solubility, viscosity, and biological activity. This method reliably and consistently replicates the hydrolysis process to ensure consistent results. The degree of hydrolysis is optimal under certain conditions, making it possible to repeat the production process to produce products with the same quality. Previous studies have shown that the degree of hydrolysis is often correlated with the biological activity of the hydrolysate, such as antioxidant, antimicrobial, or immunomodulatory activity. It can improve the desired biological activity by optimizing the degree of hydrolysis (Hou *et al.* 2022).

Table 7 shows that the predicted soybean protein hydrolysate is 95.62% with a desirability of 1.000, while the average value of the degree of hydrolysis obtained in this study was 92.93%. Table 8 shows that a two-sided test was carried out with an H_0 acceptance area (confidence interval = 95%), $82.9192 \leq t \leq 108.329$, with a two-sided % confidence interval of 95 %. The degree of hydrolysis obtained was 92.93%, where the value is still in the acceptance range of H_0 ; then H_0 is accepted, and H_a is rejected. This shows that there is no significant difference between the value of the degree of hydrolysis obtained and the value of the degree of hydrolysis predicted. This study also used the T-test to determine whether there is a significant difference between the average degree of hydrolysis obtained and the value of the degree of hydrolysis of the hydrolysate that is carried out. Table 9 shows the results of the T-test, with the result of the significance value obtained, which was 0.051. If the significance value of the t-test > 0.05, then H_0 is accepted and H_a is rejected; then the degree of hydrolysis obtained in this study and the value of the degree of hydrolysis of the desirability of 1.00 are not significantly different.

Table 7. Formulation obtained from the optimization process.

Type	E/S (% v/b)	Temperature (°C)	Time (H)	DH (%)
Predicted	1.1975	59.2629	2.58	95.62
Experimental	1.20	59.00	2.60	92.93

In this study, soybean protein hydrolysate and soybean protein isolate were tested by FTIR to identify compounds and detect their functional groups. This can be seen from the wave number of the two compounds, whether by the theoretical wave number of each functional group. The FTIR spectrum can be seen in Table 10. Table 10 shows that the spectrum of soybean protein isolate, after hydrolysis, decreases in the number of waves in the amide group A, namely in soybean protein isolate and hydrolysate. This is because, after hydrolysis, the N-H compound group binds more to

Table 8. Confirmation via two-sided test.

Analysis	Predicted Mean	Predicted Median	Std Dev	n	SE Pred	95% PI low	Data Mean	95% PI high
DH	95.6241	95.6241	5.47655	1	5.77236	82.9192	92.9319	108.329

Table 9. Confirmation via T-test.

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std Dev	SE Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Protein Hydrolysate -Protein RSM	-2.69203	1.09575	0.63263	-5.41403	0.02996	-4.255	2	0.051

Table 10. FTIR peak position of soybean protein hydrolysate and soybean protein isolate.

Functional group		Wavenumber (cm ⁻¹)	Wavenumber (cm ⁻¹)	
			Hydrolysate soybean protein	Isolate soybean protein
Amide A	N-H Stretching	3600-3000	3288.84	3278.86
Amide B	Asymmetrical Stretch of CH ₂	2935-2915	2923.73	2923.73
Amide I	C=O Stretching	1900-1600	1742.83	1741.40
Amide II	N-H Bending	1650-1500	1611.12	1615.90

hydrogen bonds than when it is still in soybean protein isolate (Yang *et al.*, 2023). The absorption area of Amide B has a wavenumber range of 2935-2915 cm⁻¹. In soybean protein isolate and hydrolysate, soybean protein has a wavenumber of 2923.73. Amide B showed the presence of symmetrical strain vibration from the CH₂ group (Mberato *et al.*, 2020).

The amide I absorption region in soybean protein isolate was obtained at 1742.83 cm⁻¹, and the soybean protein hydrolysate wave number was 1741.40 cm⁻¹. This was due to the telopeptide's large non-helix portion, which decreased the hydrogen intramolecular bond between C=O on the peptide and the hydrogen donor

relationship (Carolina *et al.*, 2024). Meanwhile, at the peak of amide II uptake in soybean protein isolate, which is 1611.12 cm⁻¹, and soybean protein hydrolysate, which is 1615.90 cm⁻¹, which is known to be directly related to C-N stretching and N-H bending bonds (Alarape *et al.*, 2024).

The amino acid analysis on soybean protein hydrolysate aims to determine the composition of amino acids contained in it. The quality of soy protein hydrolysate products was demonstrated by knowing the amino acid composition. Analysis of amino acids (AA) in soybean protein hydrolysate showed the proportion of AA contained in essential and non-essential AA. Table

Table 11. Amino acid content.

	Quantity (mg/g)		
	Hydrolysate soybean protein	Isolate soybean protein	Reference protein*
Essential amino acids	5.55 %	6.78 %	-
Histidine	Not detected	Not detected	16
Isoleucine	0.10	0.12	13
Leucine	0.03	0.06	19
Lysine	1.17	1.53	16
Methionine	0.36	0.35	17
Phenylalanine	0.04	0.04	-
Threonine	0.02	0.01	9
Valine	Not detected	Not detected	13
Non-essential amino acids	94.45 %	93.22 %	
Aspartic acid	0.10	0.11	-
Glutamic acid	0.04	0.03	-
Serine	0.03	0.02	-
Glycine	0.03	0.03	-
Proline	Not detected	Not detected	-
Cysteine	Not detected	Not detected	-
Alanine	0.10	0.10	-
Tyrosine	28.98	28.72	-
Arginine	Not detected	Not detected	-

*Suggested profile of essential amino acid requirements for adults (Joint FAO/WHO/UNU, 2007).

11 shows the existence of 17 types of 23 AAs. The AA with the highest concentration was tyrosine (28.98 mg/g), while the AA with the lowest concentration was threonine (0.02 mg/g). In Table 11, the amino acids in soybean protein hydrolysate from soybean pulp in this study have values that are not much different from those of soybean protein isolate. Tofu dregs are waste from tofu production, which still contains fewer amino acids than soybeans, and can be used as a source of alternative health raw materials. In contrast to previous studies that soybean protein hydrolysate from the primary raw material of soybeans (not soybean bagasse waste) hydrolyzed with alkaline enzyme, the highest AA obtained was lysine (40.20 mg/g), and the lowest was histidine (9.40 mg/g) (Islam *et al.*, 2022). The soybean protein hydrolysate in this study has a distinctive umami flavor. This distinctive umami flavor is obtained from aspartic acid (0.10 mg/g) and glutamic acid (0.04 mg/kg). The role of amino acids, namely glutamic acid and aspartic acid, contributes to providing a distinctive umami flavor (Ashaolu *et al.*, 2020; Shangguan *et al.*, 2024).

4. Conclusion

The hydrolysis condition of soybean protein with the best hydrolysis degree value of 92.93%, obtained at E/S 1.19%, 59.26°C, and 2.58 h, which was carried out in a two-sided test with an H_0 acceptance area (confidence interval = 95%), which was $82.9192 \leq t \leq 108.329$, showed that there was no significant influence.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We want to express our gratitude to the Ministry of Education, Culture, Research, and Technology, Republic of Indonesia, for providing the research grant for Master's Thesis Research No. 812/LL3/AL.04/2024,097/F.03.07/2024, as well as to the Research and Development Department of UHAMKA for their valued support.

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