

Optimizing of Enzyme Hydrolysis Condition for Bitterness Suppression of Soybean Protein Using Response Surface Methodology (RSM)

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Abstract Enzyme hydrolysis of soybean protein can be adjusted according to purposes, but should be optimized for reaction time, concentration and substrate conditions to prevent bitterness caused by excessive hydrolysis. This study was carried out to find the optimal condition of the soybean protein hydrolysis process using a response surface methodology. The experiment was designed based on a central composite design, and the independent variables were the soybean protein extraction pH (X_1 , 10-12), enzyme concentration (X_2 , 0.1-0.5%) and hydrolysis time (X_3 , 150-210 minutes). The results of the degree of hydrolysis (Y_1), pH (Y_2) and soluble solid contents (Y_3) were fitted to a response surface methodology model ($R^2= 0.91, 0.98, \text{ and } 0.86$, respectively). The optimal hydrolysis condition for soybean protein hydrolysis was as follows; pH 12, enzyme concentration 0.27% and 187.88 min, respectively. While richness and sourness were increased with soybean protein hydrolysis, the saltiness was decreased. The bitterness of the hydrolysate prepared at the optimal condition did not show any difference compared with that of the soybean protein, whereas richness and sourness showed a significant increase. The enzyme hydrolysate at the optimal condition showed distribution of a molecular weight lower than the soybean protein did to form intense bands at 35, and 25 kDa. Therefore, this hydrolysate was expected to be used as a high-value plant protein food material.

Keywords: enzyme, hydrolysis, soybean protein, optimization, RSM

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1. Introduction

Globally, with the increasing vegetarian population, one-person households, and risk of allergies, plant proteins are attracting increased interest. Plant proteins such as soybean have a wide range of sources, low costs, and health promoting properties [1]. Soybean protein (SP) is an important plant protein resource that abounds in fatty acids and organic acids, and essential amino acids such as lysine and glutamic acid [2]. For this reasons SP is associated with the flavor components, and utilized as plant protein. Moreover, the internal moieties of soybean proteins are abundant in hydrophobic amino acid residues, which have potential binding ability to hydrophobic substances [3,4]. For example, soy protein isolates (SPI) have been used in the manufacture of coffee creamers, whipped toppings, and infant formulas to totally or partially replace, milk proteins [3,5]. Marengo et al. used E-tongue measurement and reported that soybean-enriched snacks are associated with increasing the umami

and salty taste with high soybean content [6]. These characteristics are because soybean protein denaturation broken down into amino acids or peptides, which give to useful food processing form and rich flavor [7,8].

In the East Asian Countries, including the Korea, have been traditionally used soybean sauce and soybean paste made by fermented soybean [7,8]. For utilizing plant protein sources in a variety of applications, it is important to examine data on their taste characteristics as well as functional characteristics. For this purpose, research on the protein extraction method for exploring and obtaining vegetable protein supply source must be preceded. Several studies have reported extraction methods to use plant proteins from sources such as soybean and rice. There are variety of processing methods using alkali, enzymes and physical treatments including homogenization with sonication and ultra-high pressure [9,10,11,12]. Another study was carried out to optimize the extraction condition to isolate of 11S and 7S globulin from soybean [13] and analysis of functional characteristics. A method using extremely alkaline treatment condition SP (pH-SP) [14] can also enhance hydrophobicity and molecular flexibility

[4]. Alkaline extraction results in the denaturation of soybean protein, release of structural bonds, and elution of the internal hydrophobic groups to the surface, potentially resulting in improved functional characteristics.

However, alkaline extraction method of the proteins is preparatory steps for setting protein hydrolysis conditions. Because hydrolysis of protein can act as factors to increase the functional properties and facilitate the production of low molecular weight peptides with structures showing both water-soluble and fat-soluble properties. Also, taste properties are decided according to hydrolysis of protein. Hydrolysis of the protein can be applied to a variety of methods according to purposes, adjusted the degree of hydrolysis. Among the methods of protein hydrolysis, enzymatic hydrolysis is an attractive approach for utilizing plant protein resource in soybean [15]. It depended on temperature, reaction time, concentration, substrate, and these factors contribute to the quality characteristics of hydrolysate [16,17]. Research has been conducted to hydrolysis to increase the availability production process of low molecular weight peptides and to increase the degree of hydrolysis [18]. At this time, in enzymatic hydrolysis, it is important to set an appropriate degree of hydrolysis because bitterness is observed when excessive degree of hydrolysis as increasing the free amino acids such as methionine (Met), leucine (Leu), phenylalanine (Phe) [19,20,21]. Therefore, the present study aimed to optimize the enzymatic hydrolysis conditions and to develop low molecular weight peptide material through limited hydrolysis of soybean protein by controlling soybean protein extraction pH, enzyme concentration, and reaction time which affect the structural modification of soybean protein.

2. Materials and Methods

2.1. Materials and Reagents

Soybean was 'Deadoo' variety that is widely used in Korea and was purchased from a commercial market (Jeollabuk-do, Republic Korea). L-leucine, 1 M NaOH, 1 M HCl, protease from *Bacillus* sp., and n-hexane, were of analytical reagent grade and were purchased from Sigma-Aldrich Co. Ltd., Korea. The 2, 4, 6-trinitrobenzene 1-sulfonic acid (TNBS solution, 1% w/v) was purchased from Thermo-Fisher Scientific. Reagents for SDS-PAGE gels (poly acryl amide concentration 10% and acrylamide: bisacrylamide = 29: 1), 10X Tris glycine, coomassie blue and other chemical reagents were purchased from Bio-Rad Lab., Inc., (California, USA).

2.2. Extraction of Soybean Protein

Soybean protein (7S fraction) was obtained according to the method of Puppo et al [10]. Soybean and n-hexane were mixed 1:5 w/v and then defatted over 3 h with magnetic stirring. The defatted soybean was air-dried overnight in a fume hood. Soybean protein was obtained using alkaline extraction and acid precipitation. The defatted soybean was dispersed in deionized water (1:10, v/v) and the pH (X_1) was adjusted to 12 from 10 with 1M

NaOH and was stirred for 3 h. The slurry was centrifuged at 8,000 rpm for 20 min. The supernatant was collected, and its pH was adjusted to 4.5 for pI precipitation with 1M HCl. The precipitated protein was obtained by centrifugation at 8,000 rpm for 20 min. The soybean protein (7S fraction) was then re-dispersed in deionized water for washing. It was then centrifuged thrice at 8,000 rpm for 10 min, and stored at -18°C with after freeze drying.

2.3. Modeling and Preparation of Soybean Protein Hydrolysates

Response surface methodology (RSM) was to optimize hydrolysis condition of soybean protein. Central composite design (CCD) was fitted regression model the relationship between the independent variables and response factors (Equation (1)). The predictive value under optimal condition was confirms with the actual experiment.

$$Y = \beta_0 + \sum_{i=1}^2 \beta_i x_i + \sum_{i=1}^2 \beta_{ii} x_i^2 + \sum_{i=1}^1 \sum_{j=i+1}^2 \beta_{ij} x_i x_j. \quad (1)$$

Extract pH (X_1) of soybean protein was to pH 12 from pH 10. Extracted soybean protein was dispersed in deionized water (1:10 w/v) and hydrolyzed using protease (X_2 , enzyme/substrate ratio, 0.1, 0.3, and 0.5%) at 55°C for X_3 (150, 180, and 210 min). To stop the reaction, the enzyme was inactivated by heating at 90°C for 30 min. After cooling down to the room temperature, the solutions were centrifuged at 8,000 rpm for 20 min, and the supernatants were filtered (Whatman No. 6, Whatman International Ltd, Kent, UK). The filtrate samples are stored at deep-freezer.

2.4. Degree of Hydrolysis (DH%)

The degree of hydrolysis was determined by measuring the chemical complex formed by the α -amino groups released during hydrolysis with 0.1% TNBS solution, following the Adler-Nissen protocol [16,22] with slight modifications. Sample dilutions (0.25 mL) were added to test tubes containing 1 mL of 0.2125 M sodium phosphate buffer, pH 8.2. Then, 1 mL of the 0.1% TNBS reagent was added to each tube, followed by mixing and incubation at 50°C for 60 min in a water bath. After incubation, the reactions were stopped by adding 2.25 mL of 0.1 N HCl. The samples were cooled down during 30 min and the absorbance was measured at 340 nm on a UV (ultra violet)-visible spectrophotometer (Varian, UK). L-leucine was used to determine the standard linear graph of the amino nitrogen content.

2.5. pH and Soluble Solid Contents

The pH of the solution in which the hydrolysate 5 g and deionized water 45 mL (1:10 w/v) were dispersed, was measured using pH meter (Easy pH titrator METTER TOLEDO, Japan). Soluble solid content (%) was analyzed using a digital refractometer (HI 96801, HANNA instruments, USA) and was expressed as percentage

(g of sugar/100 g of sample). All determinations were performed in triplicate.

2.6. Electronic Tongue

The electronic tongue was used to detect the taste of the overall sample rather than measuring the respective chemical components contained in the sample. Based on the sensitivity of each taste sensor, it was possible to compare the difference between the relative tastes. Therefore, the hydrolysate including the optimal condition and other samples were analyzed for the relative taste pattern compared with soybean protein using the electronic tongue (TS-5000Z, Intelligent Sensor Technology Inc., Tokyo, Japan). The samples were centrifuged at 12,000 rpm for 20 min after filtered through a vacuum tube top filter 0.45 μm (Corning Inc., Midland, MG). The electronic tongue was then used with modules for five different tastes: umami (AAE), saltiness (CT0), sourness (CA0), bitterness (C00) and astringency (AE1). Each sample was checked to ensure that the solution conductivity (above 0.4 mS/m) and pH (6-8) value for measurement were in the permissible range. The taste measurement was conducted using two-step washing according to the manufacturer's manual.

2.7. Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a vertical slab gel of 1.0 mm thickness using a modified method by Zhou et al. [1]. The tank buffer was 10X Tris glycine electrophoresis buffer (0.25M Tris, 1.92M glycine, 1% SDS, pH 8.3). Each sample and sample buffer mixed with 1: 1 was reacted for 10 min at 95°C. Then, 20 μL of each sample was loaded onto SDS-PAGE gels and the samples were electrophoresed at a constant voltage of 200 V. After electrophoresis, the gel was stained with Coomassie blue G250 solution and then destained using 2-3 changes of distilled water.

2.8. Statistical Analyses

Response surface methodology (RSM) was performed using Minitab (Minitab 16, Minitab Inc., State college, PA, USA). Means values were used for regression analysis. Polynomial regression modeling informs a mathematical relationship between the independent variables (X_n) and dependent variables (Y_n). To optimize the restrictive hydrolysis condition, we looked for the significant factors. The SPSS statistical program (ver. 22.0, IBM Corp., Armonk, NY, USA) was used to perform ANOVA and Duncan's multiple range test at a significance level $p < 0.05$. All the data of each sample were obtained for at least three measurements and were expressed as mean \pm standard deviation (SD). Principal components analysis (PCA) was performed using XLSTAT software (Addinsoft, Paris, France) and was applied for analyzing the E-tongue data to confirm the correlation of flavor among independent variables and hydrolysates based on their patterns.

3. Results and Discussion

3.1. Degree of Hydrolysis, pH and Soluble Solid Contents of Soybean Protein Hydrolysate

The degree of hydrolysis was analyzed according to enzyme hydrolysis conditions of soybean protein (Table 1). The degree of hydrolysis ranged from 8.0 to 10.65% and was found to be the highest (10.65%) with enzyme concentration 0.1%, soybean protein extraction pH 12, and 210 minutes (run 7). The degree of hydrolysis was the lowest conditions as 8.0% with soybean protein extraction pH 10, enzyme concentration 0.3%, and hydrolysis time of 180 minutes (run 16). The pH of the hydrolysate showed a range of 5.65-6.71 and showed neutral pH under run 14 (soybean protein extraction pH 12, enzyme concentration 0.1%, hydrolysis time 150 minutes). The soluble solids contents were measured higher as the soybean protein extraction pH and enzyme treatment concentration increased, resulting in a difference of 2.0 - 7.0% depending on the processing conditions. Enzymatic hydrolysis of proteins has a problem that it is difficult to react at high concentrations and to obtain complete hydrolysis. Singh et al. [16] have studied to improve the solubility as increased degree of hydrolysis in response to the enzyme by optimizing the enzyme hydrolysis temperature and time of rice bran protein. However, the hydrolysis reaction material, it may also be controlled to have a hydrolysis degree of 5-10% because that effected on the negative flavor characteristics [21]. Zhang et al. [17] used limited hydrolysis to improve structure and emulsification, and Kim, [21]'s study confirmed that after enzyme hydrolysis of the soybean protein isolate (SPI) was adjusted degree of hydrolysis to 10%, and that it was assessed to flavor property. However, previous studies had excluded the soybean protein extraction process or applied an analysis method to optimize the hydrolysis conditions. Therefore, in this study the production of hydrolysate was optimized using the response surface methodology (RSM) by including the soybean protein extraction. The result of RSM regression formula was expressed as a second order polynomial of soybean protein extraction pH (X_1), enzyme concentration (X_2), hydrolysis time (X_3) and consisted of a constant, and linear, quadratic, and interaction terms (Table 2).

$$Y_1 = -72.7 + 10.50 X_1 - 23.56 X_2 + 0.24 X_3 - 0.41 X_1^2 + 21.11 X_2^2 - 0.000606 X_3^2 + 0.681 X_1 X_2 - 0.00288 X_1 X_3 + 0.0219 X_2 X_3 \quad (2)$$

$$Y_2 = 9.64 - 0.66 X_1 + 0.15 X_2 - 0.025 X_3 + 0.0464 X_1^2 + 0.159 X_2^2 + 0.000052 X_3^2 - 0.0750 X_1 X_2 + 0.000500 X_1 X_3 + 0.00208 X_2 X_3 \quad (3)$$

$$Y_3 = -56.1 + 7.29 X_1 + 10.40 X_2 + 0.12 X_3 - 0.21 X_1^2 - 6.75 X_2^2 - 0.000039 X_3^2 + 0.42 X_1 X_2 - 0.00837 X_1 X_3 - 0.0556 X_2 X_3 \quad (4)$$

Table 1. Results of soybean protein hydrolysate under different conditions of the response surface analysis

Runs	Independent variables			Dependent variables		
	X ₁ Extraction pH	X ₂ Enzyme concentration (%)	X ₃ Hydrolysis time (min)	Y ₁ Degree of hydrolysis (%)	Y ₂ pH	Y ₃ Soluble solid contents (%)
1	11 (0)	0.3 (0)	180 (0)	9.11 ± 0.07 ¹¹⁾	6.07 ± 0.02 ^{1g}	4.33 ± 0.58 ^{cd}
2	12 (+1)	0.3 (0)	180 (0)	10.58 ± 0.05 ^b	6.53 ± 0.01 ^c	5.33 ± 0.57 ^b
3	11 (0)	0.3 (0)	210(+1)	9.20 ± 0.04 ^e	6.08 ± 0.01 ^{1g}	5.00 ± 0.00 ^{bc}
4	11 (0)	0.3 (0)	180 (0)	9.12 ± 0.01 ^f	6.07 ± 0.00 ^{1g}	5.00 ± 0.00 ^{bc}
5	10(-1)	0.5 (+1)	210 (+1)	8.01 ± 0.01 ^h	5.65 ± 0.01 ^m	3.00 ± 0.00 ^{1g}
6	12 (+1)	0.5 (+1)	210 (+1)	9.65 ± 0.02 ^d	6.54 ± 0.04 ^c	5.33 ± 0.58 ^b
7	12 (+1)	0.1(-1)	210 (+1)	10.65 ± 0.02 ^a	6.65 ± 0.01 ^b	5.33 ± 0.58 ^b
8	11 (0)	0.3 (0)	180 (0)	9.11 ± 0.01 ^f	6.07 ± 0.02 ^g	5.00 ± .00 ^{bc}
9	11 (0)	0.1(-1)	180 (0)	10.61 ± 0.02 ^{ab}	6.00 ± 0.01 ^h	5.00 ± 0.00 ^{bc}
10	11 (0)	0.3 (0)	150 (-1)	9.10 ± 0.01 ^f	6.13 ± 0.01 ^e	4.00 ± 0.00 ^{de}
11	12 (+1)	0.5 (+1)	150 (-1)	10.49 ± 0.02 ^c	6.50 ± 0.02 ^d	7.00 ± 0.00 ^a
12	11 (0)	0.3 (0)	180 (0)	9.14 ± 0.01 ^f	6.09 ± 0.02 ^f	4.67 ± 0.58 ^{bcd}
13	11 (0)	0.5 (+1)	180 (0)	10.48 ± 0.06 ^c	6.12 ± 0.01 ^e	3.53 ± 0.06 ^{ef}
14	12 (+1)	0.1(-1)	150 (-1)	10.47 ± 0.02 ^c	6.71 ± 0.01 ^a	5.00 ± 0.00 ^{bc}
15	11 (0)	0.3 (0)	180 (0)	9.14 ± 0.01 ^f	6.07 ± 0.02 ^g	4.67 ± 0.58 ^{bcd}
16	10(-1)	0.3 (0)	180 (0)	8.00 ± 0.02 ^h	5.67 ± 0.01 ^l	3.33 ± 0.58 ^{efg}
17	10(-1)	0.1(-1)	150 (-1)	8.03 ± 0.01 ^h	5.82 ± 0.01 ⁱ	2.00 ± 0.00 ^h
18	11 (0)	0.3 (0)	180 (0)	9.14 ± 0.01 ^f	6.07 ± 0.01 ^g	4.67 ± 0.58 ^{bcd}
19	10(-1)	0.1(-1)	210 (+1)	8.01 ± 0.02 ^h	5.75 ± 0.01 ^j	2.67 ± 0.58 ^g
20	10(-1)	0.5 (+1)	150 (-1)	8.09 ± 0.02 ^g	5.72 ± 0.01 ^k	3.00 ± 0.00 ^{1g}

^{1)a-m} Values with the same superscripts within a column are not significantly different.

Table 2. Analysis of variance for hydrolysis, pH and soluble solid contents of soybean protein hydrolysate

Response	Degree of hydrolysis (Y ₁)		pH (Y ₂)		Soluble solid contents (Y ₃)	
	F-value	P-value	F-value	P-value	F-value	P-value
Model	12.74	0.000	76.90	0.000	6.69	0.003
X ₁ ¹⁾	94.14	0.000	669.54	0.000	52.64	0.00
X ₂	0.82	0.386	5.74	0.038	0.93	0.358
X ₃	0.43	0.528	1.44	0.259	0.03	0.868
X ₁ X ₂	1.04	0.331	0.65	0.440	0.15	0.706
X ₁ X ₃	0.42	0.532	0.65	0.440	1.36	0.271
X ₂ X ₃	0.97	0.348	0.45	0.518	2.40	0.153
X ₁ ²	3.26	0.101	2.12	0.176	0.31	0.589
X ₂ ²	13.79	0.004	0.04	0.846	0.54	0.480
X ₃ ²	5.75	0.037	2.12	0.176	0.01	0.926
R ²	0.91 ²⁾		0.98		0.86	

¹⁾X₁; Extraction pH, X₂; Enzyme concentration (%), X₃; Hydrolysis time (min)

²⁾0 ≤ R² ≤ 1, close to 1 indicates that the regression line fits the model.

The degree of hydrolysis (Y₁) was a high value of R² = 0.91, soybean extraction pH (X₁) showed a significant influence on the constant (p < 0.001). However, the quadratic model of X₁ did not show a significant difference (0.101) on the degree of hydrolysis even though the p-values of enzyme concentration (X₂) and hydrolysis time (X₃) were significant at 0.004 and 0.037, respectively, in the quadratic model. The pH (Y₂) showed significant influence on X₁ (p < 0.001), and X₂ (p < 0.05) in the constant, quadratic

model was not significant although it had a very high R²=0.98. The R² of the soluble solid content (Y₃) was 0.86, and X₁ was significantly affected on the Y₃ at the constant.

3.2. Response Surface Plot of Soybean Protein Hydrolysate

The reaction surface plot to analyze the relationship of the independent variables for the hydrolysis condition optimization is shown in Figure 1. The degree of hydrolysis was found to be 10% at an extraction pH 12 and enzyme concentration 0.3%. When the hydrolysis time was 180 minutes, the degree of hydrolysis was about 10%. However, when the time increased (210 min), a lower degree of hydrolysis was obtained. Singh et al. [16] reported results similar for the degree of hydrolysis with rice bran protein. The initial phase of the hydrolysis reaction results low competition with the substrate and enzyme, generating high degree of hydrolysis. Prolonged hydrolysis time causes a low degree of hydrolysis, because peptides formed soluble solid derived from the substrate. Therefore, it is important to set an appropriate hydrolysis time to control the degree of hydrolysis. The pH of the hydrolysate is close to neutral (pH 7.0) when enzyme concentration is low and the hydrolysis time is less than 180 min. Whereas the soluble solid contents increased with higher enzyme concentration, decreased with longer hydrolysis time. We can predict that the soluble solid derived from the substrate contributed to the peptides formation.

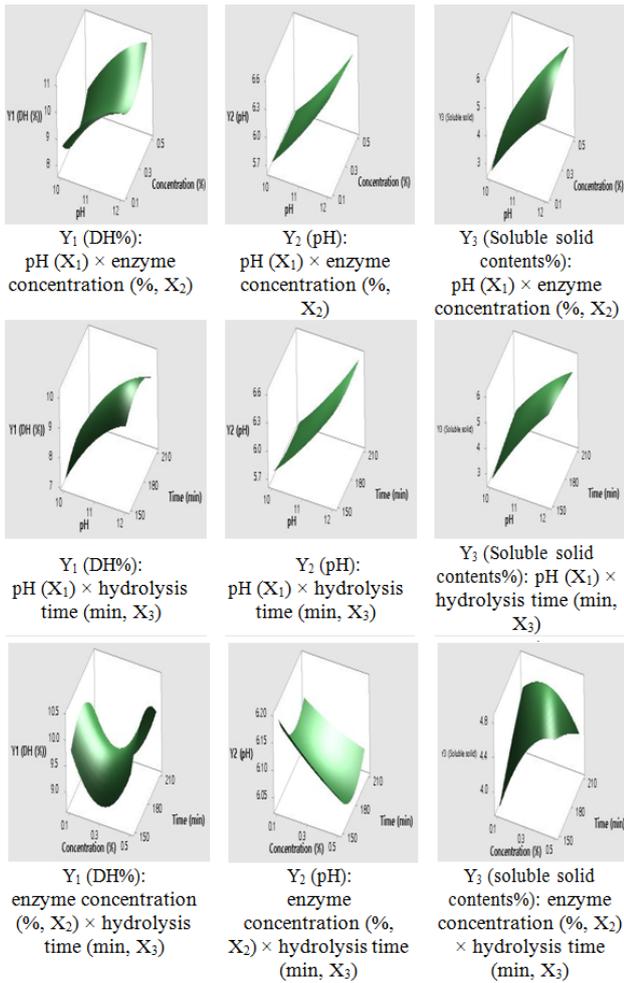


Figure 1. Response surface plots of the Y_1 ; degree of hydrolysis (DH%), Y_2 ; pH and Y_3 ; soluble solid contents (%)

3.3. Optimization of Enzyme Treatment for Soybean Protein Hydrolysate

Table 3. Optimum constraint values using three analytical methods in the object goal

Constraint name		Numerical optimization
Independent variables	pH (X ₁)	12
	Enzyme concentration (X ₂)	0.27 %
	Hydrolysis time (X ₃)	187.88 min
Predicted responses (dependent variables)	Degree of hydrolysis (Y ₁)	10%
	pH (Y ₂)	6.55
	Soluble solid contents (Y ₃)	5.75
Actual responses (dependent variables)	Degree of hydrolysis (Y ₁)	10.08±0.04
	pH (Y ₂)	6.42±0.01
	Soluble solid contents (Y ₃)	5.67±0.06

The optimal conditions for the production of a limited hydrolysis are shown in Table 3. The final goal of the degree of hydrolysis in limited hydrolysis was set as 10%; the conditions for the optimal hydrolysis production derived from soybean protein extraction were pH 12, enzyme concentration 0.27%, and hydrolysis time 187.88 minutes. Under these conditions, the predicted degree of

hydrolysis is 10%, pH is 6.55, and the soluble solid contents is 5.75%; the actual measured degree of hydrolysis was 10.08%, pH 6.42, and the soluble solid content was found to be 5.67%.

3.4. E-tongue Analysis of Soybean Protein Hydrolysate

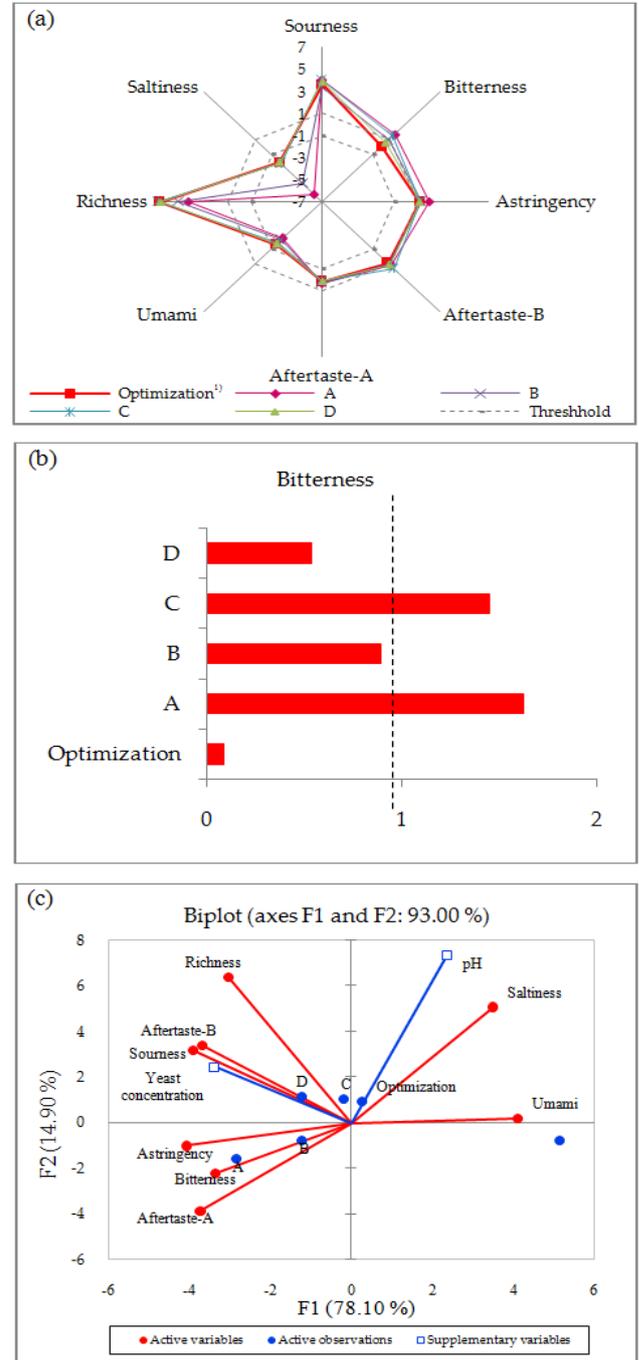


Figure 2. E-tongue analysis of soybean protein and hydrolysates (a) Flavor intensity of the hydrolysates (b) Bitterness intensity of the hydrolysates (c) PCA loading plot of the hydrolysates [1] Optimization; pH 12, enzyme concentration 0.27%, A; pH 10, enzyme concentration 0.3%, B; pH 12, enzyme concentration 0.5%, C; pH 12, enzyme concentration 0.3%, D; pH 12, 0.5%.]

The E-tongue was used to determine the change in the taste pattern according to the soybean protein extract pH or enzyme treatment concentration as shown in Figure 2a.

The taste characteristics were analyzed compare the hydrolysate the optimal enzyme hydrolysis condition with the others. Richness and sourness increased with the soybean protein hydrolysis, whereas saltiness showed a decrease. Upon hydrolysis of a protein, although the processing characteristics and digestibility improves by reducing the molecular weight, utilization of the material is difficult because bitterness is expressed by the hydrophobic amino acids released from the protein [19]. In this study, we optimized the limited enzyme hydrolysis conditions to inhibit bitter taste expression in order to use the product as a seasoning material. Based on the results, the bitterness of the optimal hydrolysate did not show a difference compared to the control group (soybean protein), even though richness and sourness were increased (Figure 2b).

The electronic tongue measurement results were analyzed through the PCA to establish the relationship between the sample and independent variables and taste (Figure 2c). Bitterness and astringency are related to samples A, and B, and were increased when applying the respective hydrolysis conditions. The optimal hydrolysate and samples C, and D were found to be associated with increasing richness, their negative flavor characteristics such as bitterness and astringency were analyzed to be lower than those of A and B. The soybean extraction pH 10 was associated with reduced saltiness whereas bitterness and astringency showed an increased result compared to pH 12. Thus, the expression of negative taste characteristics was low when the alkaline condition of the soy protein extraction was pH 12.

3.5. SDS-PAGE of Soybean Protein Hydrolysate

To determine the molecular weight distribution of the sample with minimized bitterness expression, the optimal enzyme hydrolysate was analyzed by SDS-PAGE (Figure 3). All samples formed a dark band at 75 kDa. The optimal enzyme hydrolysate formed an intense band distribution at 130, 75, and 35 kDa. The B and D samples with high enzymatic concentrations were formed a weak band at 130 kDa as compared to A and C. Lee et al. [23] have confirmed the reduced molecular weight distribution of fermented soybean protein with SDS-PAGE. When fermented using *Bacillus* sp., the molecular weight was decreased with the increase in fermentation time, forming a dark band at less than 30 kDa. This has been reported as a result of peptide formation as the protein is denatured by fermentation. This study thus showed a similar result, wherein alkaline extraction conditions and enzyme hydrolysis (by *Bacillus* sp.) were generated protein denaturation it effected on formation small peptide. Another study [15] investigated the SDS-PAGE analysis of black bean protein hydrolysate. Black bean protein presented a predominant band of 60-85 kDa, whereas its enzymatic hydrolysate showed an intense band of below 40 kDa. With similar molecular weight of the hydrolysate, the variance between the results for soybean and black bean was not large.

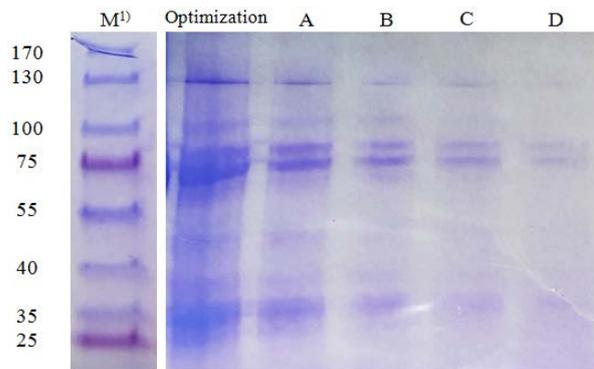


Figure 3. SDS-PAGE profile of soybean protein and hydrolysate [M⁽¹⁾: Marker; Optimization; pH 12, enzyme concentration 0.27%, A; pH 10, enzyme concentration 0.3%, B; pH 10, enzyme concentration 0.5%, C; pH 12, enzyme concentration 0.3%, D; pH 12, enzyme concentration 0.5%]

4. Conclusions

This study aimed to optimize the hydrolysis condition of soybean protein using a response surface methodology. Soybean protein extraction pH (X_1 , 10-12), enzyme concentration (X_2 , 0.1-0.5%), and hydrolysis time (X_3 , 150-210 minutes) were the independent variables in the analysis. The results of the degree of hydrolysis (Y_1), pH (Y_2) and soluble solid contents (Y_3) were fitted to a response surface methodology model ($R^2 = 0.91, 0.98, \text{ and } 0.86$, respectively). The optimal hydrolysis conditions were pH 12, enzyme concentration 0.27% and reaction time 187.88 min, respectively. Under these optimal conditions, the actual values were showed to be 10.08% (Y_1), 6.42 (Y_2), and 5.67% (Y_3) similar to the predicted values. While richness and sourness increased with the soybean protein hydrolysis, saltiness showed a decrease. The bitterness of the hydrolysate under optimal conditions did not differ compared to the control group (soybean protein); in contrast, richness was highly increased compared to other samples. The enzyme hydrolysate with optimized condition showed a lower distribution of molecular weight than the soybean protein to form an intense band at 35, and 25 kDa. Thus, the expression of bitterness was minimized through condition optimization for limited hydrolysis, and the richness was increased. Further, the formation of low molecular peptides compared with soybean protein was confirmed. The resulting product is thus expected to be utilized as a plant protein food material having high value.

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