Journal of Science and Technology ***55*** *(2) (2017) 137-149*

DOI: 10.15625/0866-708X/55/2/7778

RELEASE BIOACTIVE PEPTIDES FROM ENZYMATIC HYDROLYSATED SOYBEAN BY ALCALASE AND PROTAMEX USING RESPONSE SURFACE METHODOLOGY

Nguyen Thi Quynh Hoa*, Dong Thi Anh Dao*

HCMC-VNU University of Technology, 268 Ly Thuong Kiet Street, Ward 14, District 10, Ho Chi Minh city, Viet Nam

*Email: *nguyenthiquynhhoa2016@gmail.com; dtanhdao@hcmut.edu.vn*

Received: 19 February 2016; Accepted for publication: 21 January 2017

ABSTRACT

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions and conditions and may ultimately influence health. The objective of this paper is to study the enzymatic hydrolysis process of soy protein to produce bioactive peptides. To study the action of Alcalase and Protamex on the proteins of soybean, the influence of the temperature, pH, substrate concentration, enzyme concentration and hydrolysis time on the soluble protein recovery of the soy-proteins was evaluated. The soy protein was hydrolyzed by two different enzymes. Response surface methodology (RSM) was applied to optimize the hydrolysis conditions using Alcalase and Protamex. The result showed Alcalase 2.4 L has the stronger hydrolysis capacity. The protein recovery was also higher with Alcalase, the soluble protein recovery by Alcalase was 41.32 ± 0.13 %, by Protamex was 33.91 ± 0.17 %. The highest soluble protein recovery for soy protein was obtained with a [E/S] of 1.5 % (w/w) and 2.0 % (w/w) for Alcalase and Protamex, respectively. For soy protein the conditions to get the highest soluble protein recovery were: 55 ^{o}C, pH 7, the ratio of soybean: water, 1.0 : 4.5 and reaction time of 180 mins, for both enzymes. The dried hydrolysate was low to medium molecular weight bioactive peptides (predominantly < 8.5 kDa for Alcalase and < 20 kDa for Protamex). The results of amino acid analysis showed that the composition of amino acid of soy protein and its hydrolysates obtained under the optimized conditions was considerably enriched in essential amino acids and ensure the nutrition and safety for human consumption.

Keywords: Alcalase, hydrolization, Protamex, process optimization, soybean protein.

1. INTRODUCTION

Soybean [Glycine max (L.) Merrill] is one of the oldest cultivated crops of the Far East. For centuries, the Chinese, Japanese, Koreans, and Southeast Asians, have used soybean as a staple source of dietary protein and oil. Soybean-derived bioactive peptides have many beneficial properties, including hypolipidemic and hypocholesterolemic effects, hypotensive effects, improvements in arterial compliance and endothelial function [1]. Soy hydrolysate and the soy-

fermented foods, natto and tempeh, were dephosphorylated, deglycosylated and digested with a variety of endoproteases (pronase, trypsin, Glu C protease, plasma proteases and kidney membrane proteases) to generate oligopeptides. The peptides were purified and characterized. They demonstrated a range of biological activities – angiotensin converting enzyme (ACE) inhibitory, anti-thrombotic, surface tension and antioxidant properties [2]. Soy milk, an aqueous extract of soybean, and its fermented product have great biological properties and are a good source of bioactive peptides [3]. Marco M. et al. [4] focused on bioactive peptides identified in cereals and legumes, from an agronomical and biochemical point of view, including considerations about requirements for the design of appropriate clinical trials necessary for the assessment of their nutraceutical effect *in vivo*.

The main purpose of this research is to investigate the favourable conditions such as water, enzyme/substrate ratio, pH, temperature, hydrolysis time to hydrolyze bioactive peptides (< 20 kDa) from soybean by Alcalase and Protamex so that the highest protein recovery can be achieved. From that the optimal extraction procedure was chosen. Finally, the hydrolyzed soybean powder was made by spray drying.

2. MATERIALS AND METHODS

2.1. Materials and enzyme

The soybean used in this study was cultivated in Dong Nai province, Vietnam and purchased at Long Tan Phu co., Ltd, Ho Chi Minh City, Vietnam. Alcalase® 2.4 L (a bacterial endoprotease of *Bacillus licheniformis*) and Protamex® (EC 3.4.24.28, from *Bacillus subtilis*), were obtained from Novozymes (Denmark). Alcalase® 2.4 L optimum conditions operate in a wide range, temperatures between 55 – 70 °C and pH from 6.5 to 8.5 depending on the specific substrate conditions. The unit activity of Alcalase is 2.4 AU-A/g. In the acidic environment of pH 4.0, Alcalase® 2.4 L can be inactivated at 50 °C for 30 minutes and when pH 8.0, it will be inactivated when the temperature 85 ^{o}C for 10 minutes [5]. Protamex® was active at all pH values from 5.0 - 11.0 with 100 % activity at 8.0. Optimal pH for protein stability is determined to be at 7.0 and Protamex® works best at pH from 5.5 to 7.5 while the optimal temperature for stability is at 35 – 60 °C with 95 % activity maintained. Protamex® is inactivated at temperatures of 85 °C for 10 minutes. Proteolytic activity of enzyme preparations was determined according to Anson [6]. Alcalase and Protamex were stored at 4 ^{o}C until used for the hydrolysis experiment. All chemical reagents were of analytical grade.

2.2. Research methods

In this research, soybean protein was hydrolyzed by Alcalase and Protamex. Target functions included the optimal hydrolysis conditions for soybean substrate, biological characteristics of the hydrolyzed products, the degree of hydrolysis, the composition of amino acids and the ratio of branched amino acids were showed in Table 1.

2.3. Analytical methods

The total crude protein (N×6.25) in raw materials was determined using the Kjeldahl method (AOAC 2005). The total lipid in the sample was determined by Soxhlet extraction (AOAC 2005). The ash content was estimated by charring a pre-dried sample in a crucible at 600 °C until a white ash was formed (AOAC 2005). The protein recovery was calculated as the

amount of protein present in the hydrolysate relative to the initial amount of protein in the reaction mixture [7], the peroxide value by titration; the total soluble protein by Lowry method ; the degree of hydrolysis by comparing the linkage of cut peptides with the total linkage of peptides; molecular size by electrophoresis (SDS-PAGE); protease activity by Anson method; amino acids by gas chromatography GC-FID (EZ-Faast); microorganism: *E. coli* (QCVN 5518 - 1: 2007), *S. aureus* (QCVN 4830 -1: 2005), *L. monocytogenes* (QCVN 7700 – 2: 2007), *Salmonella* (QCVN 4829: 2005).

Table 1. Target functions investigated during soybean protein hydrolysis by Alcalase and Protamex.

Examined functions		Fixed functions	Target functions
Soybean : water	1.0:3.0; 1.0:3.5; 1.0:4.0; 1.0:4.5; 1.0:5.0 (w/w)	Ratio of E/S: 1 %; pH 7; Temperature 50 ^{o}C; Time 180 mins	Soluble protein recovery (%)
Ratio of E/S (both Alcalase and Protamex)	0; 0.5; 1.0; 1.5; 2.0; 2.5 (% w/w)	Ratio of soybean : water in the previous experiment; pH 7; Temperature 50 ^{o}C, Time 180 mins	
pH	5.0; 5.5; 6.0; 6.5; 7.0	Ratio of soybean : water in the previous experiment Ratio of enzyme: substrate in the previous experiment Temperature 50 ^{o}C; Time 180 mins	
Temperature	40; 45; 50, 55; 60 (^{o}C)	Ratio of substrate concentration, E/S, pH in the previous experiments, Time 180 mins	
Time	60; 90; 120; 150, 180; 210 (mins)	Ratio of soybean: water, E/S, pH, temperature in the previous experiments.	

2.4. Optimization experiments and statistical analysis

All experiments were repeated three times and the data of experiments conducted computer error and analysis of variance ANOVA a factor (one-way ANOVA) to determine the difference of the data with the meaning and the standard error of $P < 0.05$ software Statgraphics Centurion XV.I aimed to test the reliability of the results obtained from these experiments. The result was expressed in the form the mean standard deviation. Then, JMP software 9.0 and Modde 5.0 were used to analyze the data. The experiment was designed according to Plackett-Burman matrix with 5 factors and 12 experiments. The hydrolysis conditions were optimized using response surface methodology (RSM) with a completely randomized factorial design.

3. RESULTS AND DISCUSSION

3.1. Compositions of soybean

From the Table 2, soybean had a protein content of 37.76 % on dry basic. This value was similar to the results of Ajay K. Dixit et al. [8] (36 % protein and 19 % on dry basic). Moisture content in soybean was about 11.80 % which was adequate for following experiments.

Table 1. Nutrient compositions (per 100 g) of raw soybean.

Parameter	Calculated on wet basic (%)	Calculated on dry basic (%)
Moisture	11.80	-
Total protein	33.30	37.76
Total lipid	10.27	11.64

3.2. The hydrolysis of Alcalase

3.2.1. Effect of ratio of soybean : water to soluble protein recovery by Alcalase

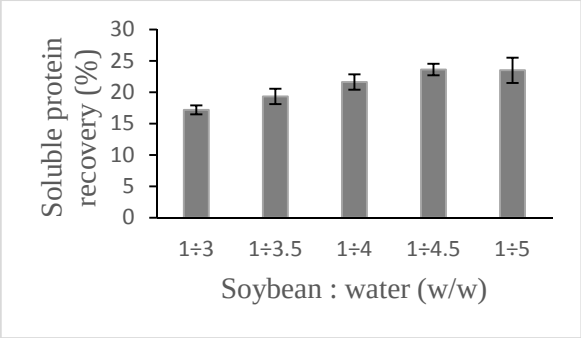

Figure 1. Effect of soybean : water to soluble protein recovery by Alcalase.

From Fig.1, when the concentration of soybean substances was increased from 1: 3 to 1: 4.5 of soybean : water ratio, the recovery soluble protein also increased from 17.17 % to 23.61 %. However, when the concentration of the substances increased continuously from a ratio of 1: 4.5 to 1: 5, the recovery protein had lightly decrease from 23.61 % to 23.49 %. According to the analysis of Anova and LSD, at the ratio of 1: 3, 1: 3.5, 1: 4, 1: 4.5, 1: 5, the recovery performance of dissolved proteins had significantly statistical differences ($p < 0.05$) at the 95% confidence level. From the above results, the soybean: water (1:4.5, w/w) was chosen to get the highest protein recovery.

3.2.2. Effect of enzyme activity/substrate [E/S] to the process of hydrolysis of soy protein

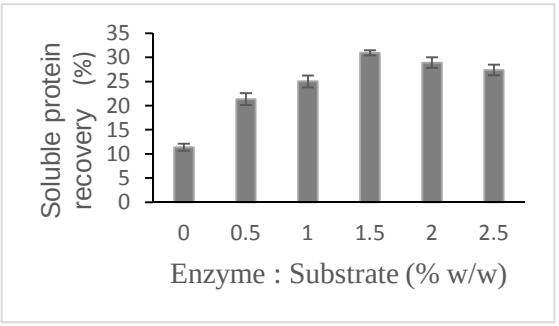

Figure 2. Effect of [E/S] to soluble protein recovery by Alcalase.

When the E/S ratio increased from 0.5 to 1.5 %, the soluble protein recovery increased from 21.35 % to 30.95 %, but when this E/S ratio increased from 1.5 % to 2.5 %, the soluble protein recovery reached equilibrium (Fig. 2). According to the analysis of Anova and LSD, the soluble protein recovery was difference significantly at the 95 % confidence level at 0.5 %; 1 %; 1.5 %; 2 %; 2.5 % E/S ratio. From above result, the E/S at 1.5 % (w/w) was chosen to get the highest protein recovery for subsequent experiments. With the same concentration of organic substance, when increasing the amount of the enzyme used, the mixture would have more exposure and enzyme hydrolysis of organic substance, so the products created even more. It could be explained that when enzyme activation increased from 0.5 % to 1.5 %, protein recovery performance was also proved.

3.2.3. Effect of pH to the process of hydrolysis of soy protein

When pH increased from 6 to 7, the soluble protein recovery also increased from 22.72 % to 32.40 %. However, when the pH increased from 7 to 8, the soluble protein recovery decreased from 32.40 % to 27.60 %. According to the analysis of Anova and LSD, the soluble protein recovery was of significantly statistical difference at the 95 % confidence level at different pH values 6; 6.5; 7; 7.5; 8. From Fig. 3, the pH at 7 was the optimal value for protein hydrolysis and was used for following experiments. pH affects the hydrolysis reaction by the process of ionization or muscle enzymes. This process can form the link causes the substance becomes tight and difficult than hydrolysis. In addition, the process of ionization can also make product change and affect the durability of the enzyme. This process also affects amino acid carboxyl groups by influencing and amine, to change the spatial structure of proteins and affect the ability of enzyme activity [9].

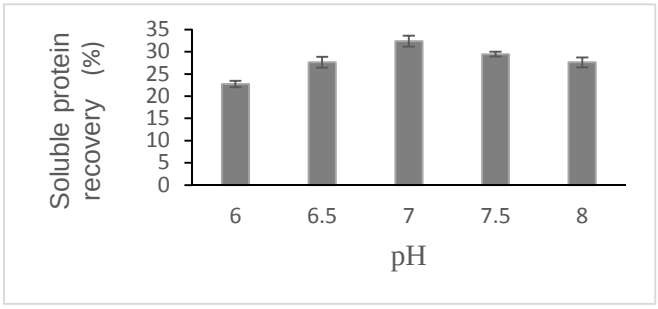

Figure 3. Effect of pH to soluble protein recovery by Alcalase.

3.2.4. Effect of hydrolysis temperature to the process of hydrolysis of soy protein

When the temperature increased from 40 to 55 ^{o}C, the soluble protein recovery also increased from 29.56 % to 37.47 % (Fig. 4). However, the soluble protein recovery reduced when the temperature reached 60 ^{o}C. It can be explained that each enzyme only performs high activity within a certain temperature range. High temperature no biochemical reaction velocity increases but also denature any reversible enzyme should affect the efficiency of hydrolysis. According to the analysis of ANOVA and LSD, the value of soluble protein recovery at these temperatures represented the significantly statistical differences ($P < 0.05$) at the 95 % confidence level. At 55 ^{o}C temperature, the protein retrieval performance achieved the highest (37.47 %). Therefore, the hydrolysis temperature at 55 ^{o}C was adequated to get the highest soluble protein recovery and was chosen as the appropriate temperature for the following experiments.

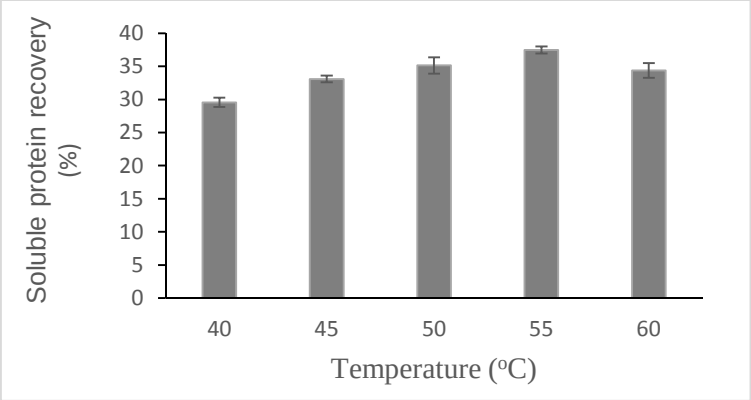

Figure 4. Effect of temperature to soluble protein recovery by Alcalase.

3.2.5. Effect of hydrolysis time to the process of hydrolysis of soy protein

From Fig. 5, at 180 mins, the highest soluble protein recovery was obtained. Therefore, this value was chosen for further research.

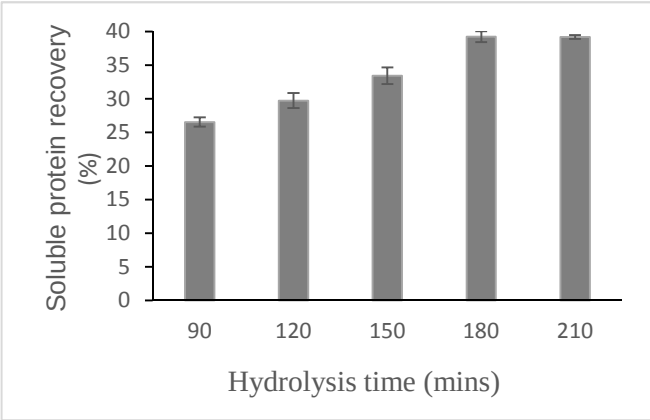

Figure 5. Effect of time to soluble protein recovery by Alcalase.

3.3. Screening the impact factor and optimizing the hydrolysis by Alcalase

3.3.1. Screening the impact factor by model Plackett –Burman

From above experiments, some optimal hydrolysis parameters were drawn out, such as: Soybean: water ratio, 1.0:4.5; the enzyme Alcalase: substrate, 1.5 %; pH: 7; temperature: 55 ^{o}C; time: 180 mins. The Plackett –Burman model with above five factors in 12 experiments was conducted to screen the impact factors for the soluble protein recovery. In Plackett – Burman model, the adjacent value of impact peak at the high (+1) and low (-1) was examined with the hydrolyzing conditions of 5 impact factors: soybean : water $\in$ [4, 5], core 4.5 %; [E/S] $\in$ [1, 2], core 1.5 %; pH $\in$ [6.5; 7.5], core 7; temperature $\in$ [50; 60], core 55 ^{o}C; time $\in$ [150; 210], core 180 mins. The soluble protein recovery (%) was the target function for all the experiments (Table 3).

Table 2. Plackett – Burman model according to 5 impact factors.

Code	Soybean : water	E/S	pH	Temperature	Time	Soluble protein recovery (%)
+−−−+	5	1	6.5	50	210	25.236
++−−−	5	2	6.5	50	150	26.316
+++−−	5	2	7.5	50	150	28.909
−−+−−	4	1	7.5	50	150	26.964
−−+−+	4	1	7.5	50	210	25.020
−+−−+	4	2	6.5	50	210	28.044
−+−++	4	2	6.5	60	210	36.687
+−−+−	5	1	6.5	60	150	31.069
+++++	5	2	7.5	60	210	34.527
−−−+−	4	1	6.5	60	150	31.934
+−+++	5	1	7.5	60	210	27.180
−+++−	4	2	7.5	60	150	36.903

Table 3. Impact factors of the examined functions in Plackett – Burman model by Alcalase.

Impact factors	Impact value	Reliability
Temperature	6.18	0.0008*
E/S	3.92	0.0078*
pH	0.04	0.0909
Time	-0.88	0.4114
Soybean: water	-2.01	0.9730

From matrix Plackett – Burman, the protein recovery ranged from 25.02 % to 36.90 % respectively. Among mentioned impact factors, temperature had the strongest impact to the soluble protein recovery (6.18) and followed by the E/S (3.92) (Table 4). Other factors (time, soybean: water and pH) had not much influence to the soluble protein recovery. From above results, two most influence factors (E/S and temperature) were optimized with the soluble protein recovery as the target function according to RSM - CCC model on Modde 5.0.

3.3.2. Optimizing the hydrolysis by the experimental planning matrix

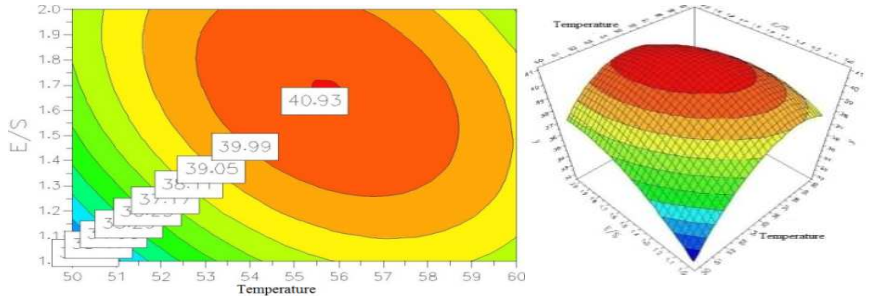

Figure 1. Effect of Alcalase concentration and temperature during hydrolysis to the soluble protein recovery in 3-dimension view.

Experiment was conducted in the same two factors: enzyme (X_1) and hydrolysis temperature (X_2). After that, the rule of these impacts to the soluble protein recovery (Y%) was drawn out. From this basic, the optimal value for each factor was chosen. Numbers of experiments were $3^2 = 9$, in which only one experiment in core. The core experiment was performed in triplicate to verify the significance of these ratios in the regression equation. From these experiments, the regression equation for expressing the correlation between enzyme concentration and temperature for the hydrolysis was determined as:

$$Y = 40.62+1.33X_1 + 1.46X_2 – 1.63X_1^2 – 3.11X_2^2 – 1.56X_1X_2, (Q^2 = 0.773, R^2 = 0.977).$$

The regression equation was expressed on 3 dimensional axis and response surface. From calculation, the soluble protein recovery was estimated at 40.93 %. However, in three replications the soluble protein recovery was 41.32 ± 0.13 % and the degree of hydrolysis by Alcalase was 35.73 ± 0.55 %.

3.4. Determination of the protein hydrolysis by Protamex

Similar to the process of determining all factors affecting the hydrolysis by Alcalase such as the effect of soybean: water, the effect of E/S, the effect of pH, the effect of hydrolysis temperature and the effect of hydrolysis time to the process of hydrolysis of soy protein by enzyme Protamex to get the highest protein recovery were the soybean: water (1:4.5, w/w), E/S at 2 % (w/w), pH 7, temperature 55 ^oC, 180 mins, respectively.

3.5. Screening the impact factor and optimizing the hydrolysis by Protamex

3.5.1. Screening the impact factor by model Plackett – Burman

From the above experiments, screening the impact fators by model Plackett Burman was conducted in the same way as one by enzyme Alcalase above. By examining the hydrolysis conditions of 5 impact factors such as soybean: water ∈ [4; 5], core 4.5 %; E/S ∈ [1; 2], core 1.5 %; pH ∈ [6.5; 7.5], core 7; temperature ∈ [50; 60], core 55 ^oC; time ∈ [150; 210], core 180 mins. Soluble protein recovery (%) was the target function for all the experiments. The results was shown in Table 5.

Table 4. Impact factors of the examined functions in Plackett – Burman model by Protamex.

Impact factors	Impact value	Reliability
Temperature	6.17	0.0008*
E/S	4.87	0.0028*
Soybean: water	-1.86	0.1124
pH	1.20	0.2751
Time	-0.25	0.8085

From matrix Plackett – Burman, the protein recovery ranged from 19.66 % to 32.36 %, respectively. Among these impact factors, temperature has the strongest impact to the soluble protein recovery (6.17) and E/S (4.87). Time, soybean:water and pH had not much influence to the soluble protein recovery. From above results, two factors (E/S and temperature) were optimized for the soluble protein recovery as the target function, according to RSM - CCC model on Modde 5.0.

3.5.2 Optimizing the hydrolysis by the experimental planning matrix

Experiment was conducted in the same way as the optimizing the hydrolysis by Alcalase. After that, the experimental planning matrix of two factors: enzyme/substrate and temperature was conducted. And the regression equation to express the correlation between enzyme concentration and temperature to hydrolysis was:

$$Y = 33.81 + 0.6X_1 + 0.93X_2 - 1.38X_1^2 - 2.41X_2^2 - 1.27X_1X_2 \ (Q^2 = 0.865, R^2 = 0.979).$$

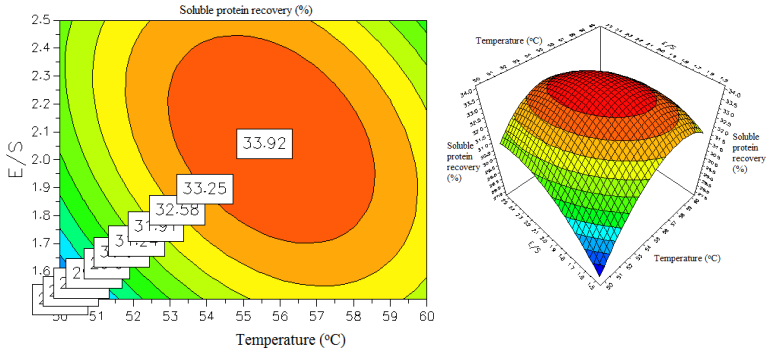

Figure 7. Effect of Protamex concentration and temperature during hydrolysis to the soluble protein recovery in 3-dimension view.

From the regression equation, the hydrolysis degree was affected by the E/S (X_1) and hydrolysis temperature (X_2). Optimal results of the regression equation, as shown in Fig. 7, were as follow: E/S: 2.13 % (w/w); hydrolysis temperature: 55.46 ^oC; hydrolysis time: 180 mins; soybean: water: 1.0/ 4.5 (w/w); pH: 7. From theoretical calculation, the soluble protein recovery was estimated at 33.92 %. However, the soluble protein recovery value was 33.91 ± 0.17 % after three replications. The degree of hydrolysis by Protamex was 15.33 ± 0.68 %.

3.6. Quality of protein powder

3.6.1. Molecular size of hydrolyzed soybean protein powder

Molecular weight patterns of hydrolysates obtained by the sequential hydrolysis of Alcalase and Protamex determined by SDS gel electrophoresis were shown in Figure 8.

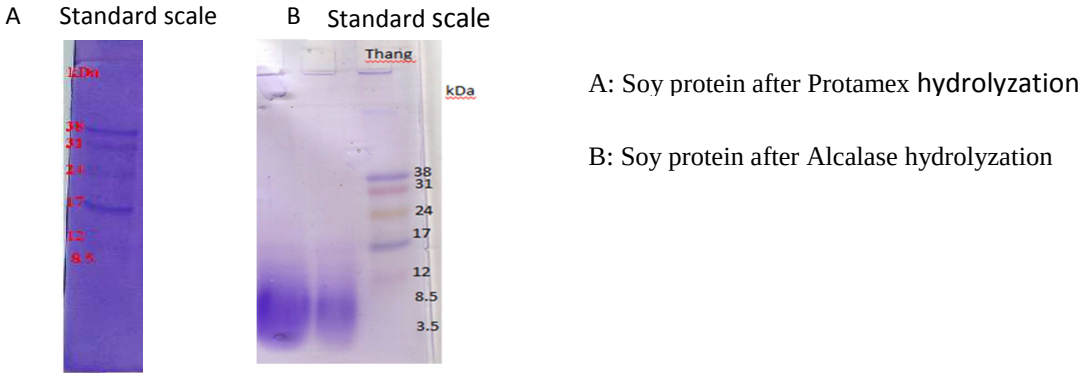

Figure 8. The result analysis of electrophoresis of hydrolysates by the hydrolysis of Alcalase and Protamex.

The electrophoretic patterns showed that the hydrolysates by Alcalase were composed of many peptides with molecular weights below 8.5 kDa. The electrophoretic pattern also indicated that the hydrolysates by Protamex comprised peptides of higher molecular weight (< 20 kDa). Hydrolysates obtained with Alcalase and Protamex, on the other hand, showed a broad range of medium-size and low molecular weight polypeptides. These short peptides after entering human body will be easily metabolized as functional food [10]. Several researchs also demonstrated the functional health effect of bioactive peptides. Sui X. et al. [11] proved that Alcalase can produce many bioactive peptides with anti-oxidation property. Song EK. et al. [12] demonstrated that bioactive peptides originated from soybean protein have strong ability for cancer treatment. Y Nakashima et al. [13] proved that some short peptides can lower the blood pressure. Low molecular weight peptides were also studied for their antioxidative effects in different in vitro oxidative systems. Medium bioactive peptides (molecular size 2-5 kDa) were suitable for functional food and bioactive peptides in size 1-2 kDa were appropriated for sportman or patient [14].

3.6.2. Identification and quantification of amino acid in protein powder

The amino acid compositions of soybean protein powder were analyzed by gas chromatography (GC/FID) and presented in Table 6.

From the Table 6, the essential amino acids (Val, Leu, Ile, Thr, Met, Phe, Lys) from soybean protein powder have the high percentage, 33.2 % regarding to Alcalase and 38.8 % regarding to Protamex. So the hydrolyzed protein powder by Protamex and Alcalase is appropriated as supplementation for patients [15]. Branched chain amino acids (BCAA) originated from Alcalase includes: leucine 0.96 g/100 g, isoleucine 0.44 g/100 g, valine 0.46 g/100 g equivalent to leucine: isoleucine: valine at 2:1:1. Iwasawa et al. 1991 examined the branched chain amino acids of leucine: isoleucine: valine at ratio 0.5:1:1, 1:1:1, 2:1:1 and 4:1:1. They found that the optimal ratio for the branched chain amino acid of leucine: isoleucine: valine was 1:1:1 and 2:1:1 [16]. Leucine, isoleucine and valine were also investigated for prevention of liver cancer and food nutrition for patient [17]. Bioactive peptide can be considered as a good food source for enteral tube feeding [18]. The ratio of branched chain amino acids (leucine : isoleucine : valine) in the hydrolyzed protein powder by Alcalase is 2: 1:

1, by Protamex is 4 :1 :1. Moreover branched chain amino acids are the substrates that can be utilized in some peripheral or wounds tissue as a energy source. Specific nutrients are found in the so-called “immune-enhancing diets” includes BCAA, either individually or in combination with other nutrients [19]. BCAA may improve mental state in patients with hepatic encephalopathy and a higher proportion of BCAAs are suitable for hepatic failure and hepatic encephalopathy patients [20].

3.6.3. Physical-chemical characteristics of the hydrolyzed protein powder

Table 6. The amino acid composition of soybean protein powder hydrolyzed by Alcalase and Protamex (g/100g).

Amino acid	Content by enzyme Alcalase(g/100g)	Content by enzyme Protamex (g/100g)
Glycine	0.55	0.68
Valine	0.46	0.34
Leucine	0.96	1.15
Isoleucine	0.44	0.31
Threonine	0.44	0.49
Serine	1.44	1.05
Proline	0.85	1.00
Aspartic acid	1.44	1.62
Methionine	0.09	0.16
Trans-4-Hydroxyproline	0.06	0.07
Acid glutamic	1.89	2.00
Phenylalanine	0.88	0.82
Lysine	1.06	1.29
Histidine	0.60	0.62
Tyrosine	0.24	0.20
Cystine (C-C)	0.05	0.05
Glycine	0.55	0.68
Valine	0.46	0.34

Table 7. Physical-chemical characteristics of the hydrolyzed protein powder by Alcalase and Protamex.

Testing parameter	By Alcalase	By Protamex
Lipid	2.25 %	3.67 %
Carbohydrate	68.80 %	69.20 %
Moisture	3.90 %	3.22 %
Protein	22.50 %	22.90 %
Peroxide	Not detected	Not detected

From the Table 7, the hydrolyzed protein powder had low moisture content (3.90 % by Alcalase and 3.22 % for Protamex) so that was ideal for storage. According to TCVN 5-2/2010, moisture in protein powder should be below 5 %. Lipid content 2.25 % and 3.67 % was quite low. Comparing to TCVN 5-2:2010/BYT lipid content should be 1.5 to 2.6 %. Peroxide was in limit 10 meq/kg so it can prevent oxidation. As the analyzed result from the hydrolyzed protein powder, the protein contents were 22.50 % and 22.90 % and these ratios were quite high. Moreover, molecular size of protein powder hydrolyzed by Alcalase was below 8.5 kDa so that is suitable for metabolism in patient meal [21].

3.6.4. Microorganism in the hydrolyzed protein powder

The hydrolyzed protein powder was suitable to standard of Vietnam TCVN 5-2/2010/BYT. Moreover, the pleasant taste was evaluated on the hydrolyzed protein powder which was quite different from product investigated by Heidi Geisenhoff et al. [22].

Table 8. Microorganism in the hydrolyzed protein powder by Alcalase and Protamex.

Microorganism	Detection limit	Result by Alcalase	Result by Protamex	Unit
E. coli	10 cfu/g	2	2	cfu/g
S. aureus	100 cfu/g	Not detected	Not detected	cfu/g
L. monocytogenes	100 cfu/g	Not detected	Not detected	cfu/g
Salmonella	Not detected	Not detected	Not detected	cfu/g

4. CONCLUDING REMARKS

The results of amino acid analysis showed that the composition of amino acid of soy protein and its hydrolysates obtained under the optimized conditions was considerably enriched in essential amino acids. The electrophoresis executed by Alcalase showed the short bioactive peptide 8.5 kDa and peptide < 20 kDa for Protamex. The ratio of branched chain amino acids in the hydrolyzed protein powder by Alcalase was leucine:isoleucine:valine by ratio 2:1:1, and by Protamex was 4:1:1. The highest soluble protein recovery for soy protein was obtained with a [E/S] of 1.5 % (w/w) and 2.0 % (w/w) for Alcalase and Protamex and the soluble protein recovery hydrolyzed was 41.32 ± 0.13 % by Alcalase, 33.91 ± 0.17 % by Protamex, respectively. For both enzymes, the optimization of enzymatic hydrolysis of soybean to get the highest soluble protein recovery and the bioactive protein fragments were at 55^{o}C, pH 7, the ratio of soybean: water, 1.0:4.5 and reaction time of 180 mins.

REFERENCES

1. Hermansen K., Hansen B., Jacobsen R., Clausen P., Dalgaard M. Dinesen B., Holst J. J., Pedersen E., and Astrup A. - Effects of soy supplementation on blood lipids and arterial function in hypercholesterolaemic subjects. European Journal of Clinical Nutrition **59** (7) (2005) 843–850.
2. Bernard F. G., Alexandre Z., Robert M., Catherine M. - Production and characterization of bioactive peptides from soy hydrolysate and so.y-fermented food. Food Research International **37** (2) (2004) 123-131.
3. Brij P. S., Shilpa V., Subrota H. - Functional significance of bioactive peptides derived from soybean. Peptides **54** (2014) 171-179.

4. Marco M., Giovanni D., Emanuela L., Valeria B., Sara B., Arrigo F. G. C., and Silvana H. - Bioactive Peptides in Cereals and Legumes: Agronomical, Biochemical and Clinical Aspects. Int. J. Mol. Sci., **15** (2014) 21120-21135.
5. Wemer A., Hahn M,, Klade M., Seebacher U., Spök A., Wallner K., Witzani H. - Collection of information of Enzyme. European Communities. Chem. Eng. Technol. (2002) 523-553.
6. Rukhlyadeva A. P., Polygalina G. V. - Methods for determination of activity of hydrolytic enzymes. Moscow, Legk. Pishch. Prom (1981) 118-124
7. Ovissipour M., Abedian A. M., Motamedzadegan A., Rasco B., Safari R., Shahiri H. - The effect of enzymatic hydrolysis time and temperature on the properties of protein hydrolysates from the Persian sturgeon (*Accipenser persicus*) viscera, Food Chem **115** (2009) 238-242.
8. Ajay K. D. - Soybean constituents and their function benfits. Opportunity, Challenge and Scope of Natural Products in Medicinal Chemistry (2011) 367 – 383.
9. Vom Fachbereich Chemie, 2002. Enzymatic hydrolysis of renewable vegetable proteins to amino acids.
10. Tomiya T. - Leucine stimulates the secretion of hepatocyte growth factor by hepatic stellate cells. Biochem. Biophys. Res. Commun. **297** (2002) 1108-1111.
11. Xiaonan S., Yang L., Lianzhou J., Shengnan W. - Optimization of the aqueous enzymatic extraction of pine kernel oil by response surface methodology, Procedia Engineering **15** (2011) 4641–4652.
12. Kim S. K. - Purification and characterisation of antioxidative peptides from enzymeatic hydrolysates of venison protein. Food Chem. **114** (2009) 1365-1370.
13. Nakashima Y. - Antihypertensive activities of peptides derived from porcine skeletal muscle myosin in spontaneously hypertensive rats. J. Food Sci. **67** (2002) 434-437.
14. Niranjan R. - Purification and in vitro antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. The Journal of Nutritional Biochemistry **16** (9) (2005) 562-9.
15. Brosnan J. T., Brosnan M. E. - Branched-chain amino acids: Enzymee and subtrate regulation, American society for Nutrition **6** (2006) 207-211.
16. Yasuo I., Tetsuya K., Motoyo M., Keiko I., Hideaki S., Tadashi S. - Optimal Ratio of Individual Branched-Chain Amino Acids in Total Parenteral Nutrition of Injured Rats, Journal of Parenteral and Enteral Nutrition **15** (1991) 612-618.
17. Song E. K. - Anticancer activity of hydrophobic peptides from soy proteins. Journal of Food Science **12** (14) (2000) 151–155.
18. Valenzuela A. - Natural antioxidants in functional foods: From food safety to health benefits. Grasas Aceites **54** (2003) 295-303.
19. Jonkers C. F. - Diets for enteral nutrition, in Basics in Clinical Nutrition. Prague, Czech Republic: Galen (2004) 201.
20. Clemmesen J.O., Kondrup J., Ott P. - Splanchnic and leg exchange of amino acids and ammonia in acute liver failure. Gastroenterology **118** (2000) 1131-1139.
21. Miona M. Belović. Potential of bioactive Proteins and peptides for prevention and treatment of mass non-communicable diseases. Food & Feed Research, Journal **38** (2) (2011) 51-62.
22. Heidi G. - Bitterness of soya protein hydrolysates according to molecular weight of peptides. Graduate Theses and Dissertations (2009) 109-13.