Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env.Pharmacol. Life Sci., Vol 4 [7] June 2015: 10-19
©2014 Academy for Environment and Life Sciences, India
Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE

Bioactive Peptides from Soybean Hydrolysis Using Protamex

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ABSTRACT
Soybean meal, a co-product after oil extraction from seeds, is rich in protein. Dietary proteins can be important sources of bioactive peptides with specific biological activities. As such, they can potentially be used in the prevention and treatment of agerelated chronic diseases like cardiovascular disease, cancer and obesity. Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions and conditions and may ultimately influence health. Main purpose of this research is to optimize favourable conditions such as water, enzyme/substrate, pH, temperature, hydrolizing time to hydrolize bioactive peptide from soybean by protamex enzymes so that the highest protein recovery can be achieved. The soluble protein recovery by protamex is 33.91 ± 0.17%.
Keywords: *Soybean, bioactive peptide, hydrolization, protamex*

Received 23.04.2015 Revised 03.05.2015 Accepted 02.06.2015

INTRODUCTION

Many peptides that are released *in vitro* or *in vivo* from animal or plant proteins are bioactive and have regulatory functions in humans beyond normal and adequate nutrition. Different health effects have been attributed to food-derived peptides, including antimicrobial properties, blood pressure-lowering (ACE inhibitory) effects, cholesterol-lowering ability, antithrombotic and antioxidant activities, enhancement of mineral absorption and/or bioavailability, cyto- or immune modulatory effects, and opioid activities. Numerous products are already on the market or under development by food companies that exploit the potential of food-derived bioactive peptides and which ascribe scientifically evidenced health claims to consumption of these functional foods.

Proteins and peptides from food have been found to be physiologically active or bioactive, either in a direct manner through their presence in the undisturbed food itself or after their release from the respective host proteins by hydrolysis *in vivo* or *in vitro* [17].

Many peptides of plant and animal origin with relevant bioactive potential have been discovered, with by far the most being isolated from milk-based products. Candidate proteins containing these latent biological activities are found in rice, broccoli, milk, eggs, meat and fish as well as in different plant protein sources such as soy, wheat, and so on [5, 6, 12, 16, 19, 20].

The main purpose of this research is to investigate the favourable conditions such as water, enzyme/substrate, pH, temperature, hydrolizing time to hydrolize bioactive peptides from soybean by protamex enzyme so that the highest protein recovery can be achieved. From that we can choose the optimal extraction procedure. Finally, we manufacture the hydrolized soybean powder, degree of hydrolization, molecular size of hydrolized bioactive peptide with biochemical and microbial characteristics to ensure the best nutrion and safety for human consumption.

MATERIAL AND METHOD

Material

Soybean is collected in HCM City, Vietnam. Protamex enzyme is originated from Novozymes – Denmark.

Research method

In this research, we examine soybean hydrolysis by protamex. Target functions include optimal hydrolyzing conditions on soybean substrate, biological characteristics of the hydrolized products, degree of hydrolization, composition and content of acid amin.

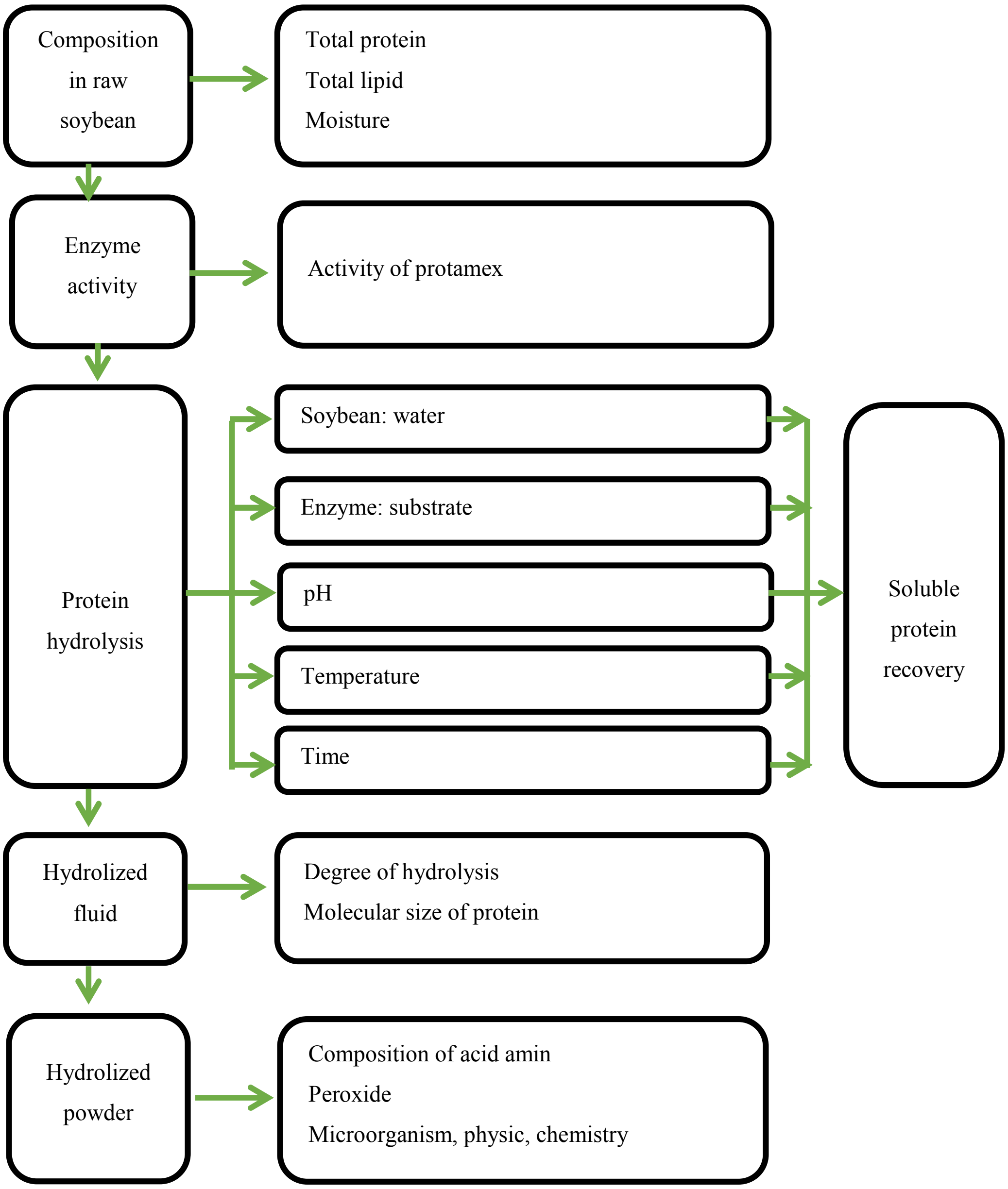

Figure 1. Flow chart of the experiments

Table 1. Target functions to investigate during soybean protein hydrolysis

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 enzyme: substrate 1% pH 7 Temperature 50^0C Time 180 minutes	Soluble protein recovery
Ratio of enzyme/ substrate	0; 0.5; 1.0; 1.5; 2.0; 2.5 (% w/w)	Ratio of soybean : water in the previous experiment pH 7 Temperature 50^0C Time 180 minutes	
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 50^0C Time 180 minutes	
Temperature	40, 45, 50, 55, 60 (^{0}C)	Ratio of substrate concentration, enzyme: substrate, pH in the previous experiments. Time 180 minutes	
Time	60, 90, 120, 150, 180, 210 (minutes)	Ratio of soybean: water, enzyme: substrate, pH, temperature in the previous experiments.	

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Testing method

We determine the total protein by Kjeldahl method; the moisture content by drying to constant weight; the total lipid by Sholext method; peroxit 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; acid amin by gas chromatography GC-FID (EZ-Faast); microorganism: *E. Coli (*TCVN 5518 -1: 2007*), S. aureus (*TCVN 4830 - 1: 2005*), L. monocytogenes (*TCVN 7700 – 2: 2007*), Salmonella (*TCVN 4829: 2005*).*

Statistical analysis

All data are processeed by ANOVA, Statgraphics, RSM (Response Surface Method) on Modde 5.0.

RESULT & DISCUSSION

Composition on soybean

Table 2. Composition in raw soybean

Parameter	Calculated on wet basic (%)	Calculated on dry basic (%)
Moisture	11.8	-
Total protein	33.3	37.76
Total lipid	10.27	11.64

From the above table, soybean has protein content 37.76% on dry basic. Moisture in soybean is 11.8% which is adequated for investigation.

Activity of protamex

Table 3. Calibration curve of Tyrosine

Tyrosine concentration (μmol/mL)	0	0.04	0.08	0.12	0.16	0.20
Optical density (OD)	0.0080	0.0403	0.0829	0.1338	0.1755	0.2193

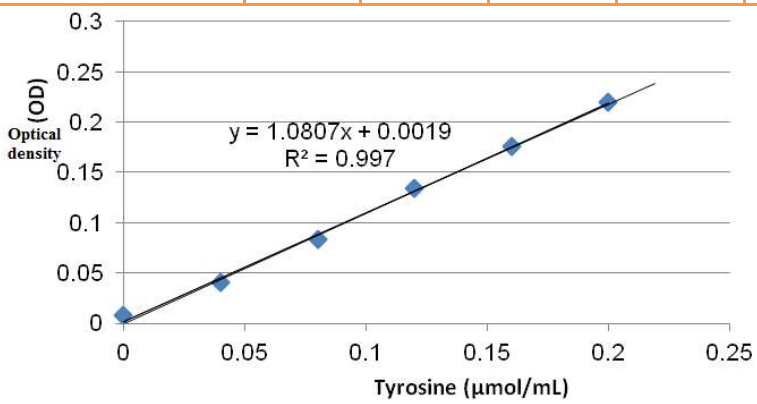

Figure 2. Calibration curve of Tyrosine

Table 4. Protamex activity before and after investigation

	Equivalent mol Tyrosin (μmol/Ml)	Activity (UI/g)
Before	0.1222	1072.3
After	0.1217	1067.2

During experiments, enzyme activity should be examined carefully as well as protected from light, high temperature, air etc.

Table 5. Albumin concentration (mg/ml)

Optical density OD	**0.158**	**0.222**	**0.27**	**0.329**	**0.372**	**0.431**
Albumin (mg/ml)	0.02	0.04	0.06	0.08	0.10	0.12

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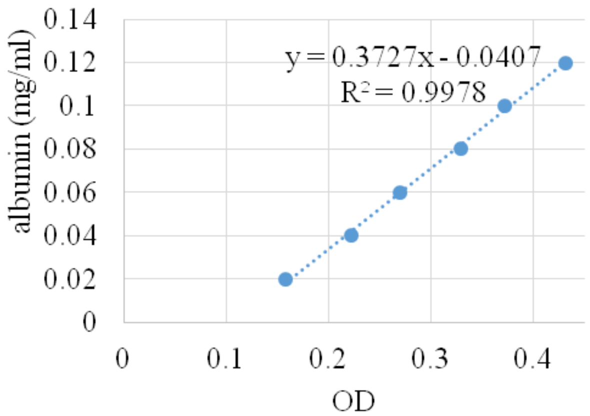

Figure 3. Albumin calibration curve

Protein hydrolysis by protamex

Effect of soybean: water

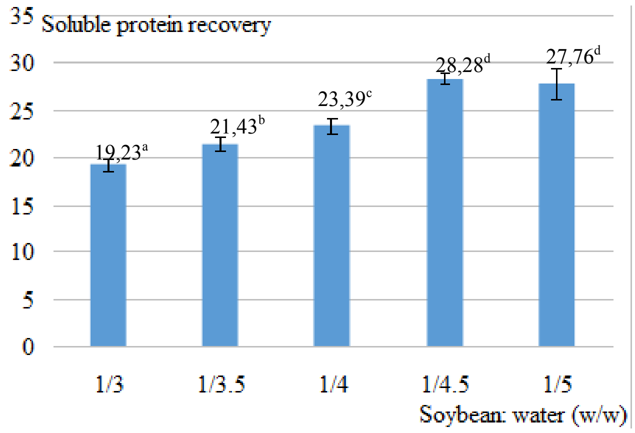

Figure 4. Effect of soybean:water to protein hydrolysis by protamex.

From above result, we choose soybean: water (1:4.5, w/w) to get the highest protein recovery.

Effect of enzyme/ substrate

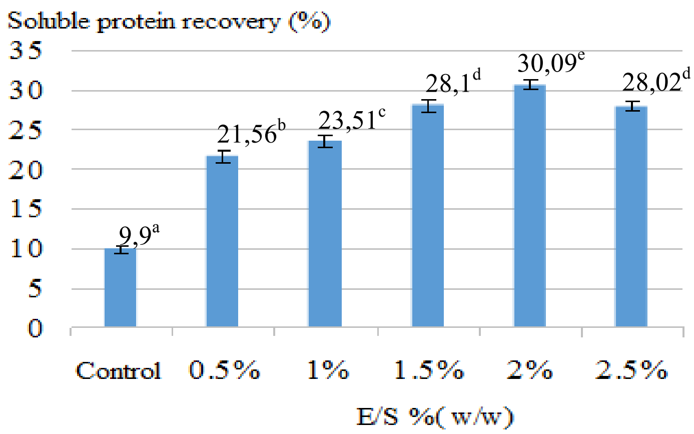

Figure 5. Effect of enzyzme/ substrate to protein hydrolysis by protamex

From above result, we choose E/S at 2% (w/w) to get the highest protein recovery.

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Effect of pH to protein hydrolysis

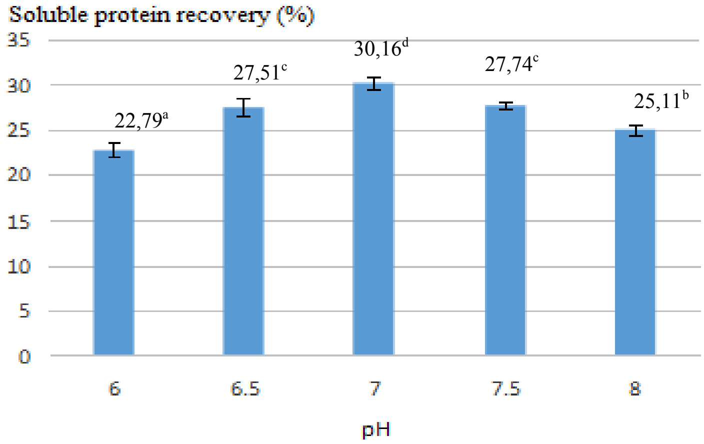

Figure 6. Effect of pH to protein hydrolysis by protamex

pH 7 is optimal for enzyme activity so we choose this value for further research.

Effect of hydrolysis temperature

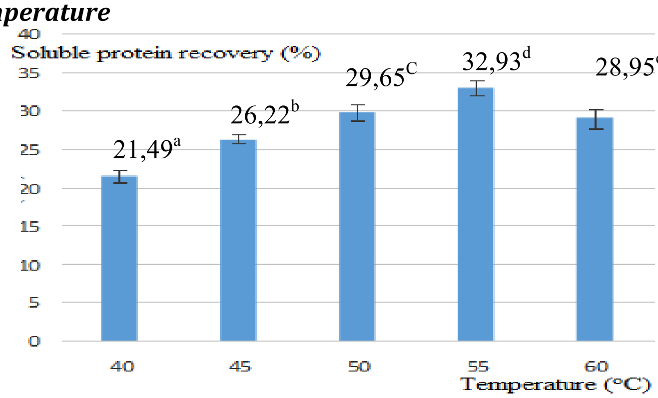

Figure 7. Effect of temperature to protein hydrolysis by protamex.

The optimal temperature is 55°C for enzyme activity so we choose this value for further research.

Effect of hydrolysis time

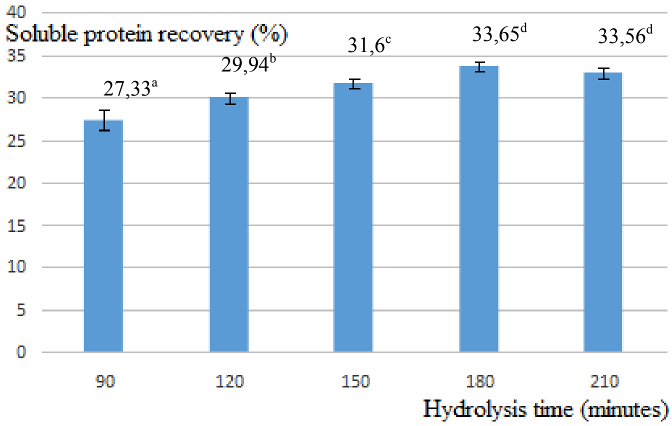

Figure 8. Effect of hydrolysis time to protein hydrolysis by protamex

The optimal time for hydrolysis is 180 minutes.

Screening the impact factor and optimizing the hydrolysis by protamex

Screening the impact factor by model Plackett – Burman

From above experiments, we draw out some optimal hydrolysis parameters such as soybean: water, 1.0:4.5; enzyme: substrate, 2.0%; pH: 7; temperature: 55°C; time: 180 minutes. We conduct the Plackett – Burman model with above five factors in 12 experiments to screen the factors impact to the soluble protein recovery. In Plackett – Burman model, we examine the adjacent value of impact peak at the high (+1) and low (-1). By examining the hydrolyzing conditions of 5 impact factors soybean : water ∈ [4;5], core 4.5% ; enzyme : substrate ∈ [1;2], core 1.5% ; pH ∈ [6.5;7.5], core 7 ; temperature ∈ [50;60], core 55°C ; time ∈ [150;210], core 180 minutes ; target function is the soluble protein recovery (%).

Table 6. Plackett – Burman model according to 5 impact factors

Code	Soybean: water	Enzyme : substrate	pH	Temperature	Time	Soluble protein recovery	Code	Soybean: water
1	+−−−+	5	1.5	6.5	50	210	0.208	21.346
2	−−+−−	4	1.5	7.5	50	150	0.201	19.659
3	−+−−+	4	2.5	6.5	50	210	0.225	25.097
4	−−+−+	4	1.5	7.5	50	210	0.215	22.900
5	+++−−	5	2.5	7.5	50	150	0.212	21.911
6	++−−−	5	2.5	6.5	50	150	0.226	24.896
7	+−+++	5	1.5	7.5	60	210	0.228	25.322
8	−−−+−	4	1.5	6.5	60	150	0.229	25.535
9	+++++	5	2.5	7.5	60	210	0.246	29.159
10	−+++−	4	2.5	7.5	60	150	0.261	32.356
11	−+−++	4	2.5	6.5	60	210	0.256	31.290
12	+−−+−	5	1.5	6.5	60	150	0.224	24.469

Table 7. Impact factor of the examined functions in Plackett – Burman model by protamex

Impact factor	Impact value	Reliability
Temperature	6.17	0.0008*
Enzyme/ substrate	4.87	0.0028*
Soyeban: water	-1.86	0.1124
pH	1.20	0.2751
Time	-0.25	0.8085

From matrix Plackett – Burman we get the protein recovery 19.656% to 32.356%. Among impact factors, temperature has the strongest impact to the soluble protein recovery (6.17) following enzyme / substrate (4.87). Time, soybean: water and pH have not many influences to the soluble protein recovery. From above results, we optimize two factors (enzyme/ substrate and temperature) with the soluble protein recovery as the target function according to RSM - CCC model on Modde 5.0.

Optimize the hydrolysis by the experimental planning matrix

Experiment is conducted in the same two factors enzyme (X_1) and hydrolysis temperature (X_2). From that we draw out the rule of these impacts to the soluble protein recovery (Y%). From this basic, we choose the optimal value for each factor.

Numbers of experiments are $3^2 = 9$, in which there is one experiment in core. The core experiment is performed in triplicate to verify the significance of these ratios in the regression equation.

Table 8. The experimental planing matrix of two factors and hydrolysis by enzyme protamex

No	Root	X_1	X_2	Y
1	M1	2.0	48	25.165
2	M2	1.5	50	24.529
3	M3	2.5	50	28.554
4	M4	1.3	55	24.529
5	M5	2.7	55	29.825
6	M6	2.0	55	33.214
7	M7	2.0	55	33.85
8	M8	2.0	55	34.273
9	M9	1.5	60	28.766
10	M10	2.5	60	27.495
11	M11	2.0	62	28.130

Table 9. Values of the regression equation by protamex

Y	Value the regression equation	Standard deviation	P	Conf. int(±)	
Constant	33.8113	0.392035	3.97E-09	1.00776	
X_1	0.602418	0.220611	0.041247	0.567099	Accepted
X_2	0.926083	0.220611	0.008507	0.567099	Accepted
X_1*X_1	-1.38421	0.173948	0.000505	0.44715	Accepted
X_2*X_2	-2.41133	0.173948	3.51E-05	0.44715	Accepted
X_1*X_2	-1.27075	0.382109	0.020879	0.982244	Accepted
N = 11	Q^2 =		0.865	Cond. no. =	3.8788
DF = 5	R^2 =		0.979	Y-miss =	0
	R^2Adj. =		0.958	RSD =	0.7642
				Conf. lev. =	0.95

From above data, we draw out the regression equation to express the correlation between enzyme concentration and temperature to hydrolysis.

$$Y = 33.81 + 0.6X_1 + 0.93X_2 - 1.38X_1^2 - 2.41X_2^2 - 1.27X_1X_2$$

The regression equation is expressed on 3 dimensional axis and response surface.

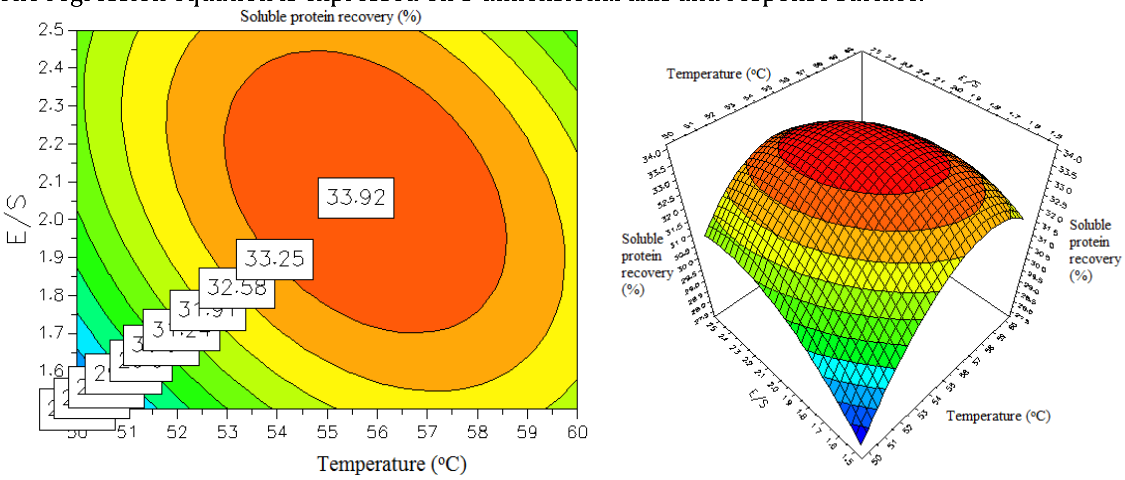

Figure 9. Effect of protamex concentration and temperature during hydrolysis to the soluble protein recovery in 3-dimension view

From the regression equation we see that the enzyme/ substrate (X_1) and hydrolysis temperature (X_2) affect to the hydrolysis degree. Optimal results of the regression equation are as follow: enzyme/ substrate: 2.1327 %(w/w); hydrolysis temperature: 55.4687 ^{0}C ; hydrolysis time: 180 minutes; soybean: water: 1.0/ 4.5 (w/w); pH: 7. From calculation, the soluble protein recovery is estimated at 33.92%. However, in three replications we get the soluble protein recovery 33.91 ± 0.17 %.

Degree of hydrolysis

Table 10. Degree of hydrolysis by protamex

Enzyme	Degree of hydrolysis	Average
Protamex	15.942	15.33 ± 0.68 %
	14.599	
	15.441	

Quality of protein powder

Molecular size of hydrolized soybean protein powder

By electrophoresis, we see that the molecular size of peptide hydrolized by protamex is below 20kDa. Short peptides entering human body is easily metabolized as functional food [21]. There are several research demonstrated the functional health effect of bioactive peptides. They proved that alcalase can produce many bioactive peptides having anti-oxidation property [22]. After 5 hours of activation in prevention OH^- 36.43%, ROO^- 46.24%, to eliminate O_2^- . They demonstrated bioactive peptide originated from soybean protein to treat cancer [18]. They showed the short peptides to prevent blood pressure [14]. Bioactive peptides had tiny molecular size effective in absorption [15]. Medium bioactive peptide having molecular size 2-5 kDa was suitable for functional food. Bioactive peptides in size 1-2 kDa was appropriated for sportman or patient [1]. Bioactive peptide below 1kDa was suitable to treat allergy [10].

Identification and quantification of acid amin in protein powder

Table 11. Acid amin content in soyeban protein powder hydrolized by protamex

Acid amin	**Content**
	Enzyme protamex (g/100g)
Glycine	0.68
Valine	0.34
Leucine	1.15
Isoleucine	0.31
Threonine	0.49
Serine	1.05
Proline	1.00
Aspartic acid	1.62
Methionine	0.16
Trans-4-Hydroxyproline	0.07
Acid glutamic	2.00
Phenylalanine	0.82
Lysine	1.29
Histidine	0.62
Tyrosine	0.20
Cystine (C-C)	0.05
Glycine	0.68
Valine	0.34
Total acid amin	12.47

Acid amin in protein powder is analyzed by gas chromatography (GC/FID). Protein powder from soybean contains 20 kinds of acid amin necessary for direct consumption. Acid amin irreplacable (Val, Leu, Ile, Thr, Met, Phe, Lys) having the high percentage 33.8% regarding to protamex. So the hydrolized protein powder by proteamex was appropriated as supplementation for patient [8]. Branch acid amin originated from alcalase had leucine 0.96g/100g, isoleucine 0.44g/100g, valine 0.46g/100g equivalent to leucine: isoleucine: valine at 2:1:1. They examined the branch acid amin of leucine: isoleucine: valine at ratio 0.5:1:1, 1:1:1, 2:1:1 and 4:1:1 [3]. They found that the optimal ratio for the branch acid amin of leucine: isoleucine: valine as 1:1:1 and 2:1:1. Leucine, isoleucine and valine were investigated to prevent liver cancer [4, 13, 23] and food nutrition for patient [3]. Bioactive peptide can be considered as a good food source for enteral tube feeding [11].

Physio-chemical characteristics of the hydrolized protein powder

Table 12. Physio-chemical characteristics of the hydrolized protein powder by protamex

Testing parameter	**Enzyme protamex**
Lipid	3.67%
Carbohydrat	69.2%
Total	60.9%
Moisture	3.22%
Protein	22.9%
Peroxide	Not detected

The hydrolized protein powder has low moisture content 3.9% and 3.22% so that is ideal for storage. According to TCVN 5-2/2010, moisture in protein powder should be below 5%. Lipid content 2.25% and 3.67% are quite low. Comparing to TCVN 5-2:2010/BYT lipid content should be 1.5 to 2.6%. Peroxide is in limit 10 meq/kg so it can prevent oxidation. Analyzed results from the hydrolized protein powder, the protein content were 22.9%. This ratio was quite high. Moreover, molecular size of protein powder hydrolized by protamex was below 8.5kDa so that is suitable for metabolism in patient meal [9].

Microorganism in the hydrolized protein powder

Table 13. Microorganism in the hydrolized protein powder by protamex

Microorganism	Detection limit	Result		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

The hydrolized protein powder is suitable to standard of Vietnam TCVN 5-2/2010/BYT. Moreover, the pleasant taste is evaluated on the hydrolized protein powder which quite differs with product investigated by another research [2].

CONCLUSION

Peptides with biological activities, released during gastrointestinal digestion or food processing, play an important role in metabolic regulation and modulation, suggesting their potential use as nutraceuticals and functional food ingredients for health promotion and disease risk reduction. The electrophoresis executed by protamex shows the short bioactive peptide 8.5kDa. Composition of acid amin in the hydrolized protein powder by protamex is leucine: isoleucine: valine by ratio 3: 1: 1.

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CITATION OF THIS ARTICLE

Nguyen Phuoc Minh. Bioactive Peptides from Soybean Hydrolysis Using Protamex. Bull. Env.Pharmacol. Life Sci., Vol 4 [7] June 2015: 10-19