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Alcalase hydrolysis of bioactive peptides from soybean

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ABSTRACT

Soybean is very important for vegetarians and vegans because of its high protein content and abundance of vitamins, minerals, and fiber. Soybean-based foods contain an array of biologically active compounds that can confer important health benefits such as antioxidant effects. 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 peptides from soybean by 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 peptides with biochemical and microbial characteristics to ensure the best nutrion and safety for human consumption.

Keywords: *Soybean, bioactive peptides, hydrolization, alcalase*

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is one of the oldest cultivated crops of the Far East. For centuries, the Oriental people, including 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, improvement in arterial compliance and endothelial function, insulin resistance, and weight loss in obesity[6,7,9,14,22]. Food proteins from both plant and animal sources have been used to obtain a wide range of bioactive peptides[30]. Peptides and protein hydrolysates derived from food sources such as milk, egg, fish, meat, and soybeans[24]. 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]. Studies on bioactive peptides derived from major human milk proteins, such as caseins, α-lactalbumin and lactoferrin, during gastrointestinal digestion have been reviewed[29]. Soybean meal was first solid state fermented with different strains of Lactic Acid Bacteria (LAB). Among the strains used, Lactobacillus plantarum Lp6 was selected for further studies because of its highest Degree of Hydrolysis (DH) of protein (2.49±0.08%) in soybean meal after 72 h fermentation[18]. Marco Malaguti *et al.*[13], 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, pH, temperature, hydrolizing time to hydrolize bioactive peptides from soybean by alcalase 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 peptides with biochemical and microbial characteristics to ensure the best nutrition and safety for human consumption.

MATERIAL AND METHOD

Material

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

Research method

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

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.	

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* (QCVN 5518 -1: 2007), *S. aureus* (QCVN 4830 -1: 2005), *L. monocytogenes* (QCVN 7700 – 2: 2007), *Salmonella* (QCVN 4829: 2005).

Statistical analysis

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

RESULT AND 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. This value is similar to one by Ajay K. Dixit[1] (36% protein and 19% on dry basic). Moisture in soybean is 11.8% which is adequated for investigation.

Activity of alcalase

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

Fig.1: Calibration curve of Tyrosine

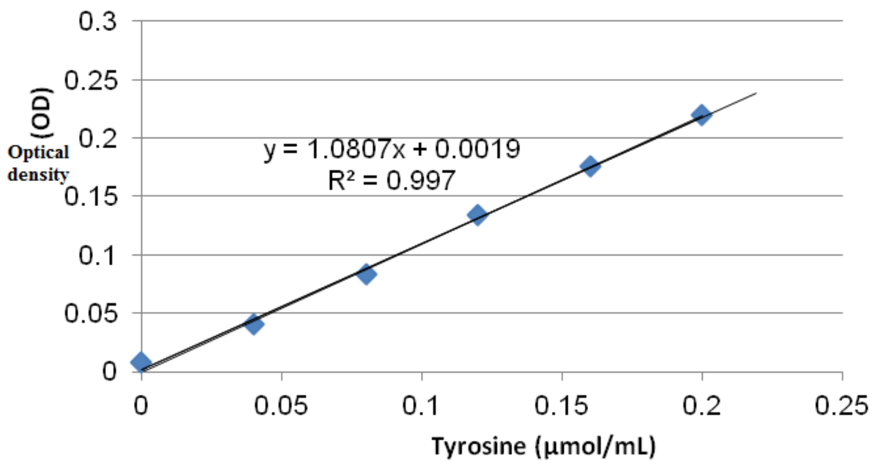


Table 4. Alcalase activity before and after investigation

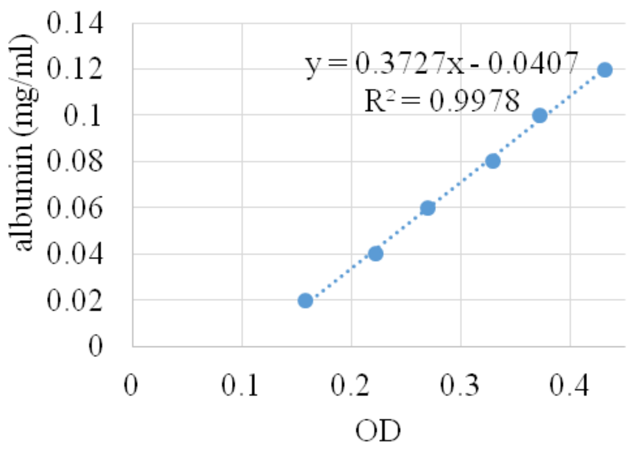
	Equivalent mol Tyrosin (μmol/Ml)	Activity (UI/g)
Before	0.1019	1630.6
After	0.1013	1620.4

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

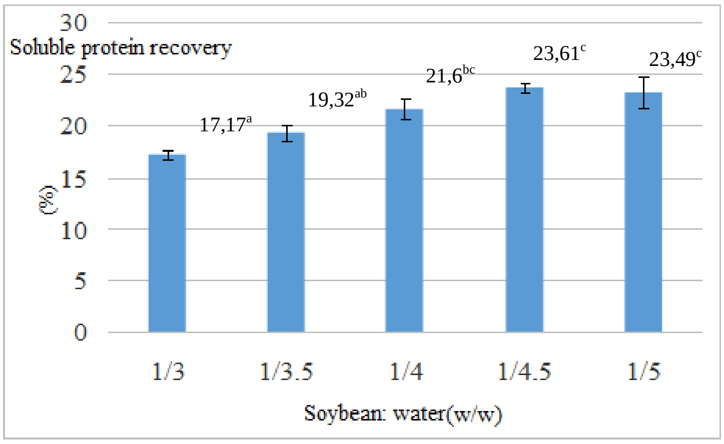
Fig.2: Albumin calibration curve



Hydrolysis by alcalase

Effect of soybean: water

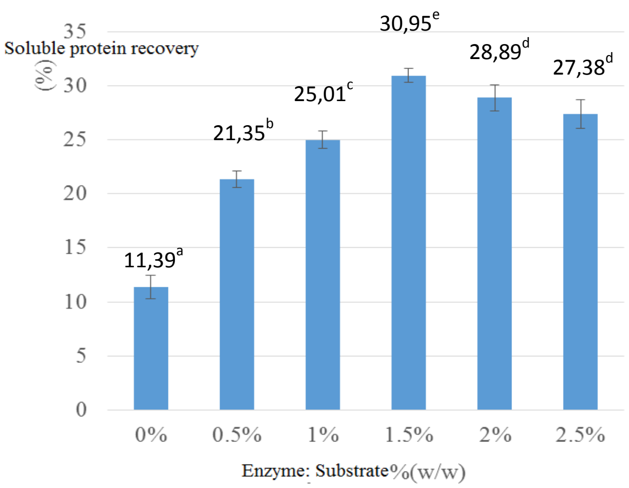
Fig.3: Effect of soybean: water to hydrolysis by alcalase



From figure 3, with soybean: water ratio 4.5%, we get the highest soluble protein recovery at 95% significant difference.

Effect of enzyme/ substrate

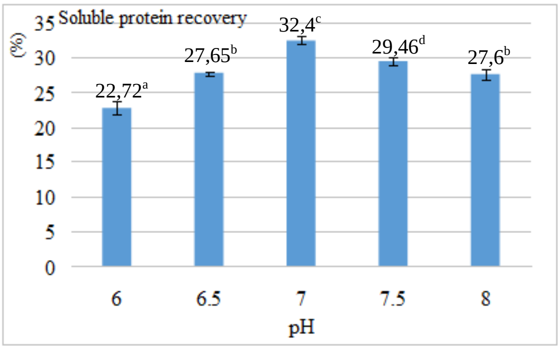
Fig.4: Effect of enzyme: substrate to hydrolysis by alcalase



At ratio of enzyme: substrate 1.5% (w/w) we get the highest soluble protein recovery at 95% significant difference.

Effect of pH

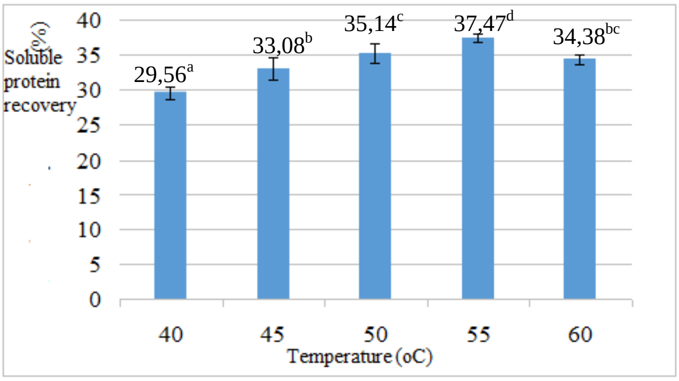
Fig.5: Effect of pH to protein hydrolysis by alcalase



We can see that pH 7 is optimal for protein hydrolysis

Effect of hydrolysis temperature

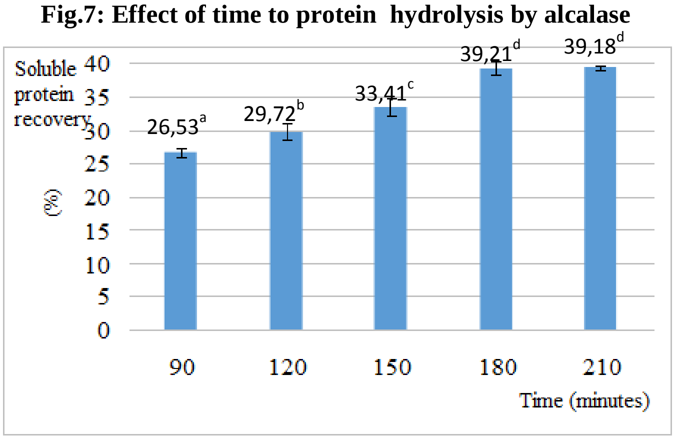
Fig.6: Effect of temperature to protein hydrolysis by alcalase



Hydrolysis temperature 55^{0}C is adequated to get the highest soluble protein recovery.

Effect of hydrolysis time

Fig.7: Effect of time to protein hydrolysis by alcalase



At 180 minutes we get the highest soluble protein recovery so this value is chosen for further research.

Screening the impact factor and optimizing the hydrolysis by alcalase

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, 1.5%; pH: 7; temperature: 55^{o}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 $\in$ [4;5], core 4.5% ; enzyme : substrate $\in$ [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 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
+−−−+	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 7. Impact factor of the examined functions in Plackett – Burman model by alcalase

Impact factor	Impact value	Reliability
Temperature	6.18	0.0008*
Enzyme: substrate	3.92	0.0078*
pH	0.04	0.0909
Time	-0.88	0.4114
Soyebean: water	-2.01	0.9730

From matrix Plackett – Burman we get the protein recovery 25.020% to 36.903%. Among impact factors, temperature has the strongest impact to the soluble protein recovery (6.18) following enzyme / substrate (3.92). Time, soybean: water and pH have not much 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 hydrolisation by alcalase

No	Root	X_1	X_2	Y
1	M1	1.0	50	30.7959
2	M2	2.0	50	38.3184
3	M3	1.0	60	36.1931
4	M4	2.0	60	37.4720
5	M5	0.8	55	32.4246
6	M6	2.2	55	35.9800
7	M7	1.5	48	25.0197
8	M8	1.5	62	31.5037
9	M9	1.5	55	41.0957
10	M10	1.5	55	40.2431
11	M11	1.5	55	40.8825

Table 9. Values of the regression equation by alcalase

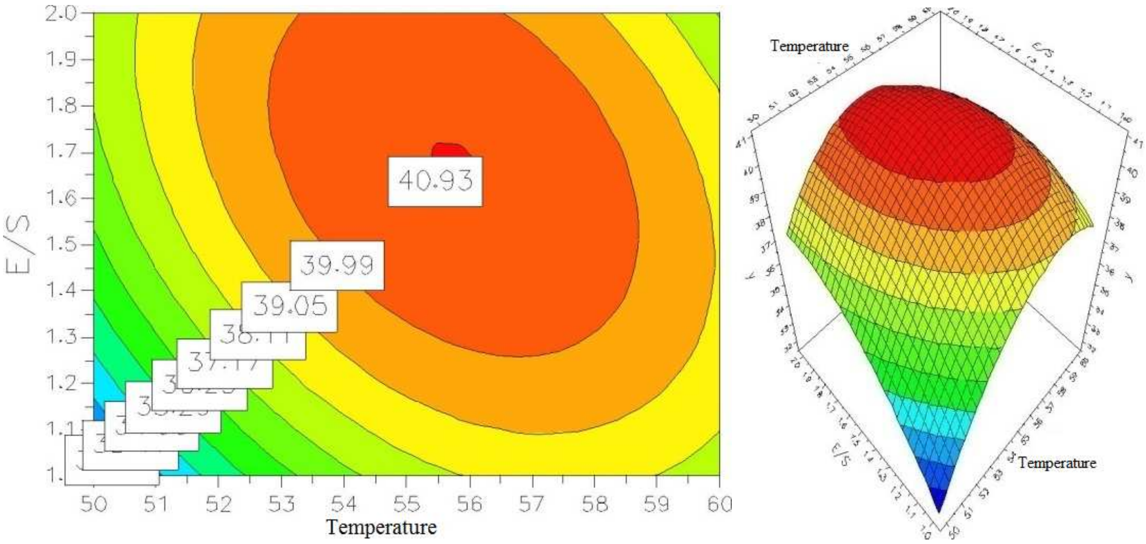
	Value the regression equation	Standard deviation	P	Conf. int(±)	Remark
Constant	40.6177	0.553357	8.89E-09	1.42245	
X_1	1.32601	0.311391	0.008027	0.800458	Accepted
X_2	1.4599	0.311391	0.005393	0.800458	Accepted
X_1*X_1	-1.62687	0.245528	0.001179	0.63115	Accepted
X_2*X_2	-3.11202	0.245528	5.43E-05	0.63115	Accepted
X_1*X_2	-1.56089	0.539346	0.034026	1.38643	Accepted
N = 11	Q^2 =		0.773	Cond. no. =	38.788
DF = 5	R^2 =		0.977	Y-miss =	0
	R^2Adj. =		0.954	RSD =	10.787
				Conf. lev. =	0.95

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

$$Y = 40.62 + 1.33X_1 + 1.46X_2 – 1.63X_1^2 – 3.11X_2^2 – 1.56X_1X_2$$

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

Fig.8: Effect of alcalase concentration and temperature during hydrolysis to the soluble protein recovery in 3-dimension view



From calculation, the soluble protein recovery is estimated at 40.93%. However, in three replications we get the soluble protein recovery 41.32 ± 0.13%.

Degree of hydrolysis

Table 10. Degree of hydrolysis by alcalase

Enzyme	Degree of hydrolysis	Average
Alcalase	35.417	35.73 ± 0.55 %
	36.363	
	35.417	

Quality of protein powder

Molecular size of hydrolized soybean protein powder

By electrophoresis, we see that the molecular size of peptide hydrolized by alcalase is below 8.5kDa. Short peptides entering human body is easily metabolized as functional food[26]. There are several research demonstrated the functional health effect of bioactive peptides. Sui Xiaonan, Jiang Lianzhou[25] proved that alcalase can produce many bioactive peptides having anti-oxidation property. After 5 hours of activation in prevention OH^- 36.43%, ROO^- 46.24%, to eliminate O_2^-[25]. Kim et al[23]., demonstrated bioactive peptide originated from soybean protein to treat cancer[23]. Nakashima[19] showed the short peptides to prevent blood pressure. Bioactive peptides had tiny molecular size effective in absorption[20]. 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[5]. Bioactive peptide below 1kDa was suitable to treat allergy[16].

Identification and quantification of acid amin in the hysrolized protein powder

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

Acid amin	Content
	Enzyme alcalase (g/100g)
Glycine	0.55
Valine	0.46
Leucine	0.96
Isoleucine	0.44
Threonine	0.44
Serine	1.44
Proline	0.85
Aspartic acid	1.44
Methionine	0.09
Trans-4-Hydroxyproline	0.06
Acid glutamic	1.89
Phenylalanine	0.88
Lysine	1.06
Histidine	0.60
Tyrosine	0.24
Cystine (C-C)	0.05
Glycine	0.55
Valine	0.46
Total acid amin	12.03

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 32.2% regarding to alcalase. So the hydrolized protein powder by alcalase was appropriated as supplementation for patient[4,12] . 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. Iwasawa et al[10]. 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. 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[11,21,28] and food nutrition for patient[10]. Bioactive peptide can be considered as a good food source for enteral tube feeding[17,27].

Physio-chemical characteristics of the hydrolized protein powder

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

Testing parameter	Enzyme alcalase
Lipid	2.25%
Carbohydrat	68.8%
Total	61.5%
Moisture	3.9%
Protein	22.5%
Peroxide	Not detected

The hydrolized protein powder has low moisture content 3.9% so that is ideal for storage. According to QCVN 5-2/2010, moisture in protein powder should be below 5%. Lipid content 2.25% are quite low. Comparing to QCVN 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 result from the hydrolized protein powder, the protein content wass 22.5%. This ratio was quite high. Moreover, molecular size of protein powder hydrolized by alcalase was below 8.5kDa so that is suitable for metabolism in patient meal[15].

Microorganism in the hydrolized protein powder

Table 13. Microorganism in the hydrolized protein powder by alcalase

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 QCVN 5-2/2010/BYT. Moreover, the pleasant taste is evaluated on the hydrolized protein powder which quite differs with product investigated by Heidi Geisenhoff[8].

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 soluble protein recovery by alcalase is 41.32 ± 0.13%. The electrophoresis executed by alcalase shows the short bioactive peptide 8.5kDa. Composition of acid amin in the hydrolized protein powder by alcalase is leucine: isoleucine: valine by ratio 3: 1: 1.

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