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Optimization of Enzymatic Hydrolysis for Producing Ferrous Binding Peptides from Horse Mackerel (*Trachurus japonicus*) Processing Byproducts

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ABSTRACT

Marine fish processing byproducts were considered as potential good protein resource for producing bioactive peptides by enzymatic hydrolysis. In this study, hydrolysates with high ferrous binding ability were prepared by enzymatic hydrolysis from mackerel (*Trachurus japonicus*) processing byproducts. In order to get hydrolysates of high ferrous binding ability and degree of hydrolysis, the enzymatic hydrolysis conditions were optimized, including proteases type, hydrolysis time, temperature, pH and enzyme to substrate ratio. The results showed that the hydrolysate by alcalase had the highest ferrous binding ability and degree of hydrolysis, reaching to 9.8 and 49.0%, respectively. The best hydrolysis conditions of alcalase were optimized using response surface methodology to be: pH 9.0, temperature of 50°C, enzyme substrate ratio of 160 mg/100 mL, hydrolysis time of 100 min. The ferrous binding ability and degree of hydrolysis were reached to 48.0 and 45.4%, respectively, at these optimized hydrolysis conditions.

Key words: Mackerel processing byproducts, ferrous binding peptides, enzymatic hydrolysis, response surface methodology

INTRODUCTION

Iron is one of the essential trace mineral elements for human, animal or plants. And it also is one of the components of heme groups in hemoglobin and myoglobin. Iron plays important role in life activities, function as regulators, activators, transmitters and controllers of various enzymatic reactions. About one-fifth of the world population has iron deficiency nutritional problems (Lee and Song, 2009). Iron acquired from food was absorbed by body only 15-35% from heme in meat (Monsen, 1988) or below 10% absorption rate from non-heme in plant-based diet (Martinez-Navarrete *et al*., 2002). Hence, iron fertilization in food is an effective defending method for iron deficiency. In order to uptake iron absorption rate, there have been many studies about non-heme iron binding bioactive peptides in recent years (Lee and Song, 2009; Huang *et al*., 2011; Torres-Fuentes *et al*., 2012; Kim *et al*., 2007).

Horse mackerel (*Trachurus japonicus*), a red flesh marine fish, was one of the most word catches fishes. As opposed to other marine fishes, mackerel was more likely perishable and lower commercial value. Except for iced fish, mackerel was mainly used for preparing fish oil. The fish oil processing produced many byproducts, such as frame, skin, head, viscera and others. These mackerel processing byproducts occupied about 50% of total weight and mainly are used for animal feeds (Cho *et al*., 2014). However, these processing byproducts were enriched in protein and might be good resource of proteins. These proteins could be prepared to bioactive peptides or complex amino acids by hydrolysis for functional food ingredients. A few functional components had been reported from various mackerel processing

byproducts, such as antibacterial peptides (Ennaas *et al*., 2015), rhamnose-binding glycoprotein (Terada *et al*., 2007), iron-binding peptides (Wang *et al*., 2013), fish oil (Sahena *et al*., 2010), gelatin (Khiari *et al*., 2011) and polyunsaturated fatty acids (Zuta *et al*., 2003).

In this study, the horse mackerel processing byproducts were chosen for producing ferrous binding peptides by enzymatic hydrolysis. Response Surface Methodology (RSM) was used for optimization of several enzymatic hydrolysis parameters, such as pH, temperature, enzyme to substrate ratio and hydrolysis time.

MATERIALS AND METHODS

Materials: The horse mackerel processing byproducts frame were obtained from Dongtou Fisheries Co., Ltd. (Wenzhou, China). The mackerel bypdroucts were transported to lab with ice and kept frozen at -20°C until used. The bypdroucts were washed by running water after thawed at 4°C for 24 h and then drained.

The trypsin (from bovine pancrease, type II, 2500 U mg^{-1}) and alcalase (200 U mg^{-1}) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Protemax (2000 U g^{-1}) and flavourzyme (1200 U g^{-1}) were purchased from Novozymes (China) Biotechnology Co., Ltd (Tianjin, China). All chemicals and reagents were of the highest grade available.

Enzymatic hydrolysis: The drained mackerel byproducts were homogenized into muscle minced with isopropanol in a 1:3 ratio (w/w). Then the mixture was incubated at 60°C for 6 h under stir at 200 rpm. The residue was obtained by air pump filtration and then dried under vacuum at 60°C. The dried sample was smashed, filtrated by 150 μm mesh and the defatted mackerel bypdroucts powder was obtained.

The defatted mackerel powder was added to 100 mM Tris-HCl buffer at various pH (8.5, 8.5, 7.1 and 7.1 for trypsin, alcalase, protemax and flavourzyme, respectively) and optimal temperature (37, 55, 50 and 502°C, respectively). At the end of hydrolysis process, the enzyme was inactivated by heating the hydrolysate at boiling water for 10 min. The hydrolysate was centrifuged at 10000 rpm for 30 min at 4°C. The supernatants were used for determining Ferrous Binding Ability (FBA) and Degree of Hydrolysis (DH).

Response surface methodology: The enzymatic hydrolysis parameters were optimized using RSM. The Central Composite Design (CCD) was employed in this regard. The pH (X_1), temperature (X_2), time of hydrolysis (X_3) and enzyme additive amount (ES, X_4) were chosen for independent variables and the ranges were given in Table 1, which were taken according to the results of preliminary experiments. The CCD composed by 21 experiments was carried out. Design of experiments and dependent variable values were presented in Table 2. The ferrous binding ability and degree of hydrolysis were selected as the response for the combination of the independent variables given in Table 2.

Table 1: Coded and uncoded settings of the process parameters for mackerel byproducts hydrolysis, according to a central composite rotatable design

	Level				
Process parameter	-1.68 (-α)	-1	0	1	+1.68 (+α)
X_1: pH	7.7	8.0	8.5	9.0	9.3
X_2: Temperature (°C)	46.6	50.0	55.0	60.0	63.4
X_3: Time (min)	86.0	100.0	120.0	140.0	154.0
X_4: ES (mg/100 mL)	53.0	80.0	120.0	160.0	187.0

Table 2: Experimental design used in RSM studies by using three independent variables with six center points showing observed DH (Y_{DH}) and FBA (Y_{FBA})

Coded levels of variables					
X_1	X_2	X_3	X_4	Y_{DH} (%)	Y_{FBA} (%)
0	0	0	0	59.9±4.3	24.6±2.6
0	0	0	0	60.5±2.7	23.1±3.3
-1	-1	1	-1	45.3±2.5	10.1±1.3
1	1	1	-1	55.9±1.3	19.7±2.4
1	1	-1	-1	52.7±4.0	39.5±3.0
0	1.68	0	0	55.7±3.9	22.6±3.3
0	0	0	0	59.9±2.8	25.2±1.6
0	0	0	1.68	55.7±2.7	23.4±2.6
0	0	0	-1.68	57.1±0.4	28.6±3.7
0	-1.68	0	0	51.9±1.3	19.8±1.8
0	0	0	0	60.3±2.3	24.5±2.9
0	0	0	0	60.5±4.9	24.5±2.8
-1	1	1	1	47.7±0.5	10.8±2.0
-1	-1	-1	-1	50.7±1.9	14.4±3.3
1	-1	1	1	52.0±0.9	28.3±4.3
0	0	1.68	0	56.7±4.6	17.3±3.4
-1	1	-1	1	57.4±1.8	7.0±1.6
1.68	0	0	0	49.2±2.1	39.3±1.5
1	-1	-1	1	45.2±2.1	49.4±3.1
-1.68	0	0	0	53.5±2.4	10.7±4.5
0	0	-1.68	0	57.2±1.3	20.4±4.1

RSM: Response surface methodology, DH: Degree of hydrolysis and FBA: Ferrous ability binding

Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses. The behavior of the system was explained by the following quadratic Eq. 1:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii} x_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3}\beta_{ij} x_i x_j \quad (1)$$

where, Y was the predicted value of FAB or DH, β_0 is a constant term, β_i, β_{ii}, β_{ij} are the linear, quadratic and cross-product regression coefficients and x_i, x_j are levels of the independent variables. The model evaluated the effect of each independent variable on response. Analysis of the experimental design and calculation of predicted data were carried out using Design Expert Software (V8.05) to estimate the response of the independent variables. Subsequently, three additional verified experiments were conducted to verify the validity of the statistical experimental strategies.

Determination of ferrous binding ability: The Ferrous Binding Ability (FBA) was determined according to the method of Decker and Welch (1990) with some modifications. One milliliter of sample was added to 3.0 mL of distilled water. Then 0.1 mL of 1 mmol L^{-1} $FeSO_4$ was added to the mixture and they were stirring at 100 rpm under room temperature for 30 min. Then mixture was reacted with 0.2 mL of 5 mmol L^{-1} 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) solution for 20 min at room temperature. Then the absorbance was read at 562 nm. The control was prepared in the same manner except that distilled water was used to instead of the sample. The FBA was calculated by the following equation.

$$FBA(\%) = (1 - \frac{A_s - A_0}{A_{Fe}}) \times 100$$

where, A_{Fe} and A_0 were the absorbance of the control the sample blank, respectively. The A_s was the absorbance of the sample in the presence of samples.

Determination of degree of hydrolysis: Degree of Hydrolysis (DH) was defined as the percentage of small peptides cleaved from proteins, which was calculated from the ratio of trichloroacetic acid (TCA) soluble peptides to total nitrogen. Mixed 500μL hydrolysate with 20% (w/w) TCA solution and kept at 4°C for 30 min. Then, the mixture was centrifuged at 10000 rpm for 30 min at 4°C. The supernatants were used for determining TCA soluble peptides by Folin-phenol method (Lowry *et al*., 1951). The total nitrogen was determined by Kjeldahl method (Ren *et al*., 2008).

Statistical analysis: All data presented were the Mean±standard error of three determinations. Statistical comparisons were performed by one-way analysis of variance (ANOVA) and the differences were calculated by Duncan's multiple range test. Statistical significance was measured at $p<0.05$.

RESULTS AND DISCUSSION

Choice of the enzyme: Suitable enzymes can be selected for marine protein hydrolysis according to various criteria, such as high DH (Dey and Dora, 2014), bioactive peptides (Chabeaud *et al*., 2009; Naqash and Nazeer, 2012; Dadzie *et al*., 2013), or reduction of bitterness (Nilsang *et al*., 2005; Sumaya-Martinez *et al*. 2005). In this study, ferrous binding ability was selected to evaluate the hydrolysis effective of various proteases for horse mackerel processing byproducts. Four proteases were used to hydrolysis the mackerel byproducts protein for 2 h at optimized pH and temperature. The results were shown in Fig. 1. The flavorzyme, trypsin and alcalase were good choices for producing ferrous binding peptides from mackerel byproducts

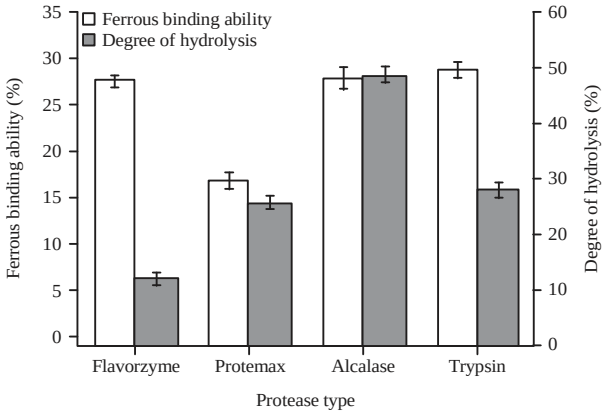

Fig. 1: Effects of protease type on degree of hydrolysis and ferrous binding ability of mackerel byproducts hydrolysates

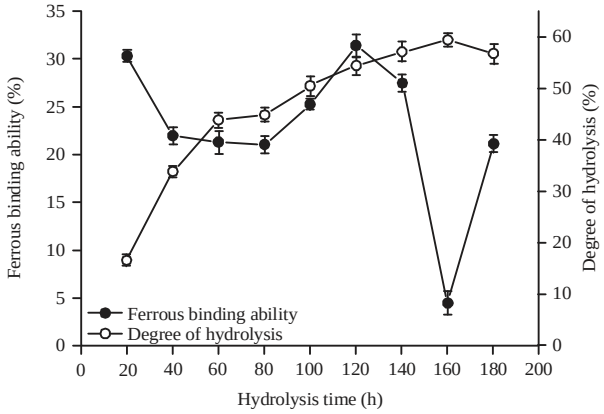

Fig. 2: Effects of hydrolysis time on degree of hydrolysis and ferrous binding ability of mackerel byproducts hydrolysates

protein. However, the alcalase had the best hydrolysis efficiency and the DH reached to 49.0%. Therefore, alcalase was chosen for next experiments.

Effects of hydrolysis time and enzyme additive amount: Hydrolysis time and enzyme substrate ratio were two of important key factors during protein enzymatic hydrolysis. During alcalase hydrolysis of horse mackerel byproducts, DH increased with hydrolysis time and reached to maximum value of 58% at 140 min (Fig. 2). However, the FAB did not show regular change during hydrolysis. With enzymatic hydrolysis, more and more small peptides produced. But many reports showed that the iron or ferrous binding ability of peptides did not show positive or negative relationship with molecular weight of peptides (Wu *et al*., 2012; Huang *et al*., 2011; Kim *et al*., 2014). Therefore, high FAB hydrolysis time might be one of any points during whole hydrolysis process. In this study, the highest FAB was appeared at hydrolysis time of 120 min. The effects of alcalase substrate ratio (alcalase additive amount) on FAB and DH were shown in Fig. 3. With

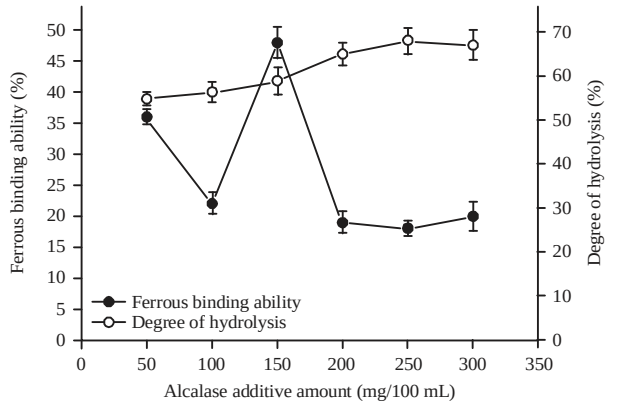

Fig. 3: Effects of alcalase additive amount on degree of hydrolysis and ferrous binding ability of mackerel byproducts hydrolysates

alcalase additive amount from 50-300 mg/100 mL, the DH increased step by step and reached to about 70%. However, the FAB did not show similar changes and the FAB reached the highest value of 46% at alcalase additive amount of 150 mg/100 mL.

Response surface methodology: In terms of preliminary experiments and alcalase properties, the center points of alcalase hydrolysis by RSM were chose at pH 8.5, temperature of 52°C, hydrolysis time of 120 min and enzyme addition of 120 mg/100 mL. Total 21 experiments were conducted and the results were given in Table 2. The resulting DH and FBA fluctuated from 45.2-60.5 and 7.0-49.4%, respectively (Table 2).

A second-order polynomial regression model was selected to predict DH and FBA responses. All terms with a statistical significance with multiple regression analysis below 5% probability level were listed in Table 3 and 4. The following two quadratic models for DH and FBA, shown in Eq. 2 and 3, explained the experimental data.

$$Y_{DH}=-1212.08144+206.24448X_1+14.33347X_2-1.23158X_3+1.36999X_4-0.040354X_1X_2+0.31225X_1X_3-0.070722X_1X_4-0.010025X_2X_3-0.00938185X_2X_4-0.00009375X_3X_4-13.85712X_1X_1-0.10392X_2X_2-0.0036711X_3X_3-0.00104817X_4X_4 \quad (2)$$

$$Y_{FBA}=0.23+0.085X_1+0.008324X_2-0.034X_3-0.015X_4-0.030X_1X_2-0.051X_1X_3+0.040X_1X_4+0.012X_2X_3-0.033X_2X_4+0.008500X_3X_4 \quad (3)$$

The R^2 of predicted model for FAB and DH were 0.9865 and 0.9345, respectively. The model F value of 31.2 for DH and 14.27 for FAB. These two points implied that the two predicted models were significant and might be used for predicting the hydrolysis process. There was only a 0.01% chance that a "Model F value" this large could occur due to noise. The $p<0.05$ indicate the two models terms were significant. The independent variables of pH and temperature had significant influence in FBA at $p<0.05$, as well as the interactions between these two factors to other two independent variables.

Table 3: ANOVA for DH (Y_{DH}) response surface quadratic model

Sources	Sum of squares	Df	Mean square	F-value	p-value Prob> F
Model	467.17	14	33.370	31.200	0.0002*
X_1	9.29	1	9.290	8.680	0.0257*
X_2	7.49	1	7.490	7.000	0.0382*
X_3	2.45	1	2.450	2.290	0.1812
X_4	0.9	1	0.900	0.840	0.3949
X_1X_2	0.034	1	0.034	0.032	0.8649
X_1X_3	78	1	78.000	72.930	0.0001*
X_1X_4	6.63	1	6.630	6.200	0.0472*
X_2X_3	8.04	1	8.040	7.520	0.0337*
X_2X_4	11.67	1	11.670	10.910	0.0163*
X_3X_4	0.045	1	0.045	0.042	0.8443
X_1^2	179.35	1	179.350	167.700	<0.0001*
X_2^2	100.87	1	100.870	94.320	<0.0001*
X_3^2	32.23	1	32.230	30.130	0.0015*
X_4^2	42.03	1	42.030	39.300	0.0008*
Lack of fit	6.05	2	3.020	32.940	0.0033

*Significant, DH: Degree of hydrolysis, DF: Degree of freedom

Table 4: ANOVA for FBA (Y_{FBA}) response surface quadratic model

Sources	Sum of squares	Df	Mean square	F-value	p-value Prob> F
Model	0.2	10	0.02	14.27	0.0001*
X_1	0.041	1	0.041	28.87	0.0003*
X_2	0.000392	1	0.000392	0.28	0.6103
X_3	0.016	1	0.016	11.23	0.0073*
X_4	0.00135	1	0.00135	0.95	0.3516
X_1X_2	0.00302	1	0.00302	2.13	0.1747
X_1X_3	0.02	1	0.02	14.4	0.0035*
X_1X_4	0.00526	1	0.00526	3.71	0.0830
X_2X_3	0.0011	1	0.0011	0.78	0.3980
X_2X_4	0.00336	1	0.00366	2.58	0.1392
X_3X_4	0.000578	1	0.000578	0.41	0.5373
X_1^2	0.014	6	0.00232	38.88	0.0017*
X_2^2	97.02	1	97.02	91.78	<0.0001*
X_3^2	28.32	1	28.32	29.31	0.0018*
X_4^2	38.77	1	38.77	37.2	0.0018*
Lack of fit	5.98	2	3.03	31.65	0.0041

*Significant, FBA: Ferrous binding ability, DF: Degree of freedom

The 3D response surfaces of the response using Eq. 2 and 3 when two of the variables were fixed at the central point and the other two were allowed to vary were shown in Fig. 4 and 5. Contour plots were generated from the polynomial model to illustrate the effect of each pair of independent variables on the DH and FBA. An increase in FBA was achieved by a decrease in pH and a medium value of temperature, enzyme additive amount. When temperature, pH and enzyme additive amount values were fixed, time seemed to have a negligible effect on the generation of antioxidant peptides. The protuberant shapes of the 3D response surfaces show that there is a maximum value for this model. The optimal conditions were optimized by Design Expert Software with its optimization menus: pH 9.0, temperature of 50°C, enzyme additive amount of 160 mg/100 mL, hydrolysis time

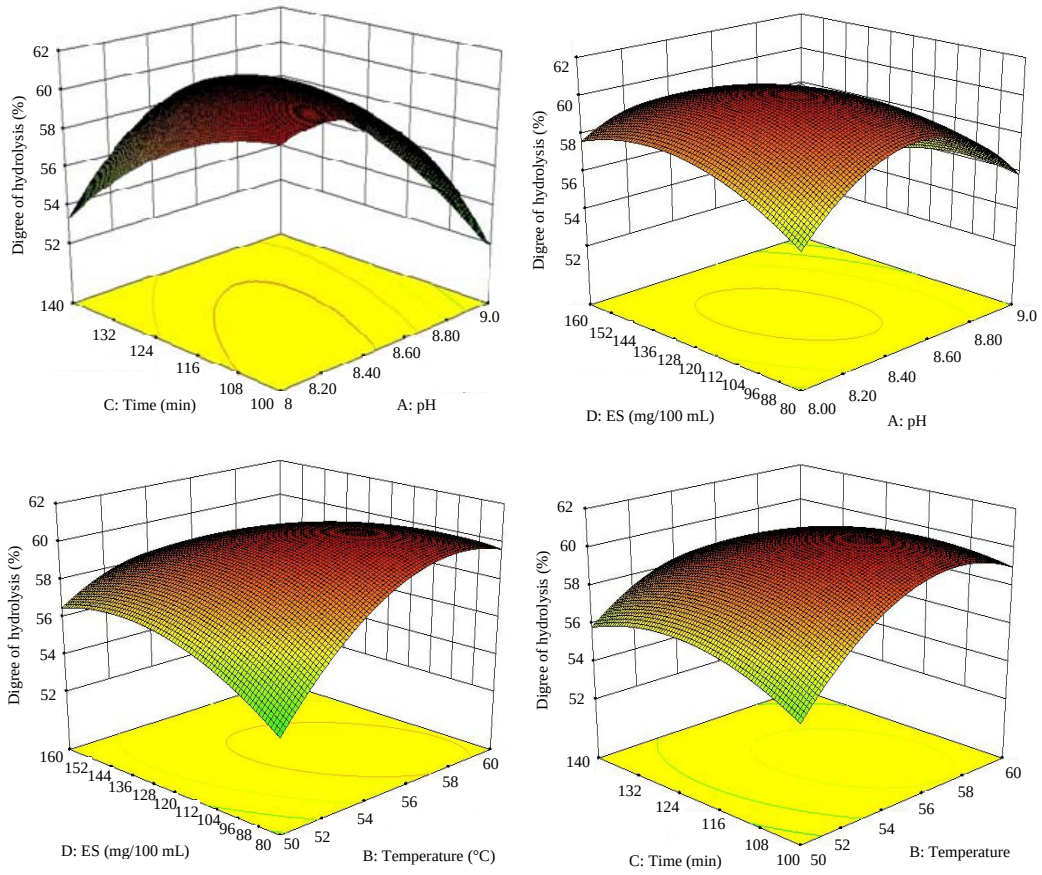

Fig. 4: Response surface graphs of degree of hydrolysis, other two of the variables were fixed at the central point

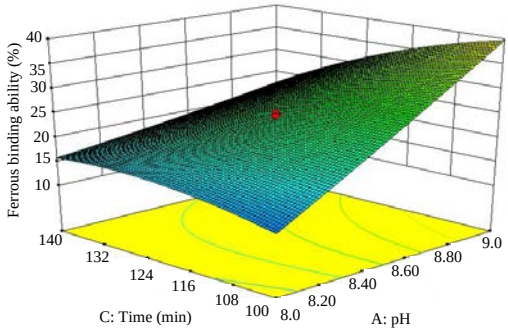

Fig. 5: Response surface graphs of ferrous binding ability at temperature 55°C and enzyme additive amount of 160 mg/100 mL

of 100 min. To confirm the validity of the statistical experimental strategies and to gain a better understanding of DH and FBA, three additional verification experiments were conducted. The measured FBA of 46.5% were very close to the predicted values of 48.0% using RSM model. The average DH of three confirmation experiments was 45.4%. These results were obviously in close agreement with the model prediction and approved the predictability of the model for the enzymatic hydrolysis of the mackerel byproducts protein in the experimental condition used.

CONCLUSION

The horse mackerel byproducts hydrolysates with flavourzyme, alcalase and trypsin had stronger ferrous binding ability. However, only alcalase hydrolysate had high degree of hydrolysis. Alcalase was the best choice for ferrous binding peptides from mackerel byproducts. The alcalase hydrolysis conditions were optimized by RSM using a central composite design. The optimal enzymatic hydrolysis conditions for producing ferrous binding peptides from mackerel byproducts were as following: temperature of 50.0°C, hydrolysis time duration 120 min, pH 9.0, enzyme addition of 160 mg/100 mL. The FBA and DH of hydrolysate at optimal conditions were 48.0 and 45.4%, respectively. The quadratic model predicted well about the actual measured value.

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