

## Research Article

# Optimization of Hydrolysis Conditions for the Production of Iron-Binding Peptides from Mackerel Processing Byproducts

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**Abstract:** The aim of this study was focused on optimization of enzymatic hydrolysis conditions for the production of iron-binding peptides from marine mackerel processing byproducts. The marine mackerel processing byproducts protein were hydrolyzed using trypsin, Protamex, Flavourzyme, Alcalase and Neutrase. Alcalase and Protamex proteolytic hydrolysates exhibited the highest iron-binding capacity; however, Alcalase proteolytic hydrolysate had higher degree of hydrolysis than that of Protamex. A four-factor-three-level composition central design experiment in response surface methodology was used to optimize the enzymatic hydrolysis conditions of Alcalase. The optimal enzymatic hydrolysis conditions were temperature of 46.0°C, time of 2.01 h, pH 8.35 and enzyme to substrate 6460 U/mL. The quadratic model predicted well about the actual measured value. The average iron-binding capacity of three verification experiment was 6.62 mg-EDTA/g-protein, which was much closed to model predicted value of 6.69 mg-EDTA/g-protein.

**Keywords:** Alcalase, hydrolysis, iron binding peptides, mackerel processing byproducts, response surface methodology

## INTRODUCTION

As one of the essential trace mineral elements, iron is not only involved in the various kinds of biochemistry reactions, also has regulation, activation, transfer function in the human metabolic process. It was essential components of cytochromes, hemoglobin, myoglobin and some enzymes. According to the world health organization, iron deficiency was one of the world's most common deficiency problems (Guilbert, 2003). What the reason was that iron in food mainly trivalent form, could not be directly absorbed by organism. Currently, biosynthesis iron supplements and synthetic iron supplements are widely used, however, the absorption and utilization rate is low and leads to certain side effects. The potential adverse effects of these iron supplements have stimulated their replacement by natural iron supplements derived from dietary sources. Some food derived polysaccharides and peptides were recently reported to have metal binding capacity (Debon and Richard, 2001; Freire-Nordi *et al.*, 2005; Lee and Song, 2009; Wang *et al.*, 2012; Torres-Fuentes *et al.*, 2012) and their iron-bound complexes might be one of the best choices of replacement of these biosynthesis iron supplements.

It is well know that natural protein can be hydrolyzed to generate small peptides, the latter with trace elements combine to form a complex can be directly absorbed by the small intestine. And a number of peptides with significant biological activities have

been identified through enzymatic hydrolysis process of various marine tissue proteins and mineral carrier peptides are one kind of these, such as calcium-binding peptides (Inoue *et al.*, 2004; Jung and Kim, 2006; Jung *et al.*, 2006), iron-binding peptides (Lee and Song, 2009), copper-binding peptides (Megias *et al.*, 2007), zinc-binding peptides (Wang *et al.*, 2012) and so on. In recent years some small peptides from other sources have been reported to possess high iron binding capacity or increased bioavailability of iron, include porcine blood plasma protein (Lee *et al.*, 2009), chickpea protein (Torres-Fuentes *et al.*, 2012) and shrimp processing byproducts (Huang *et al.*, 2011).

As a kind of low-value fish, mackerel has a rich annual production. However, the aquatic product leftovers utilization focuses on the production of feed, fish sauce, hydrolyzed protein. It is a waste of high quality protein resources and the process is complicated with high cost. Enzymatic hydrolyzed proteins or peptides from mackerel protein had been reported to have biological activity, such as antioxidative ability (Wu *et al.*, 2003; Xiong *et al.*, 2011), inhibition of lipoxygenase and thinning effects (Chuang *et al.*, 2000). However, there is little information regarding to iron-binding peptides from Mackerel Processing Byproducts (MPB). Therefore, this study was aimed to choose the optimum enzyme and hydrolysis conditions for producing the iron-binding peptides from MPB.

Table 1: Optimum conditions of enzymatic hydrolysis for tested enzymes

Enzyme	Buffer	Time (h)	pH	Temperature (°C)
Blank	50mMTris-HCl	4	7.0	50
Trypsin	50mMTris-HCl	4	8.0	37
Protamex	50mMTris-HCl	4	8.0	45
Flavourzyme	50mMTris-HCl	4	7.0	50
Neutrase	50mMTris-HCl	4	7.0	50
Alcalase	50mMTris-HCl	4	8.0	50

Table 2: Independent variables and their level in the central composite design for optimizing the enzymatic hydrolysis condition of mackerel processing byproduct

Independent variables	Symbol	Coded level				
		-1.68(- $\alpha$ )	-1.0	0.00	+1	+1.68(+ $\alpha$ )
Temperature (°C)	Temp	42.0	46.0	50.0	54.0	58
Time (h)	Time	1.00	2.00	3.00	4.00	5
pH	pH	6.80	7.20	7.60	8.00	8.4
Enzyme to substrate (U/mL)	ES	5000	6250	7500	8750	10000

## MATERIALS AND METHODS

**Materials:** Mackerel processing byproducts were bought from aquatic product market in Lianyungang City, Jiangsu Province, China. They were dried and grounded into fine powder and then stored at -50°C until used. Trypsin (2,500 U/mg), Flavourzyme (500 U/mg), Neutrase (500 U/mg), Alcalase (250 U/mg) and Protamex (50 U/mg) were purchased from Shanghai Kayyon Biological Technology Co., Ltd. (Shanghai, China). All other chemicals used in the experiments were from commercial resource and of analytical grade.

**Preparation of MPB hydrolysate:** The Mackerel Processing Byproducts (MPB) was defatted with petroleum ether at 50°C for 8h by reflux extraction. Then the residue was dried at 60°C under reduced pressure. To prepare MPB hydrolysates, enzymatic hydrolysis was performed in 50 mmol/L Tris-HCl buffer at the optimal conditions of pH and temperature for each enzyme, listed in Table 1. The proteases were used as 20:1 of enzyme to substrate and the substrate was dissolved in buffer at 5% level. And the mixtures were stirred to enzymatic hydrolysis at optimum temperature for 4h. The reaction was terminated by boiling in water bath for 10 min and then the hydrolysate was centrifuged at 12000×g for 10 min at 4°C. The supernatant was collected and stored at -50°C before further analysis.

**Determination of DH:** The Degree of Hydrolysis (DH) of MPB hydrolysate was determined according to the method of Pericin *et al.* (2009) with some modifications. A 0.5 mL 20% (w/w) Trichloroacetic Acid (TCA) was added to an equal volume of hydrolysates. Then the mixture was kept for 30 min at 4°C. Thereafter, the mixture was centrifuged at 12,000×g for 10 min. The peptide content in the supernatant and the original protein content before hydrolysis were determined by method of Lowry *et al.* (1951) and using Bovine Serum Albumin (BSA) as the standard protein. The DH was calculated as the ratio of TCA-soluble peptide to total protein before hydrolysis in the mixture, expressed as a percentage.

**Determination of iron-binding capacity:** The Iron Binding-Capacity (IBC) 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 1mmol/L FeSO<sub>4</sub> was added to the mixture and they were stirring at 100 rpm under room temperature for 10 min. Then mixture was reacted with 0.2 mL of 5 mmol/L 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. And Ethylene Diamine Tetraacetic Acid (EDTA) was used as a positive control. The IBC was calculated by the following formula:

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

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

**Experimental design for optimization:** Response Surface Methodology (RSM) as a generic method for optimization was applied to optimize enzymatic hydrolysis. The optimization was designed based on a four-factor central composite rotatable design with a total of 30 experimental runs which involved 6 replicates at the center points. Based on the single factor experiments and our previous studies, four formulation parameters which included temperature (*Temp*), time (*Time*), pH (*pH*) and Enzyme to Substrate (*ES*) were identified as key factors responsible for iron-binding capacity. In view of feasibility of the single factor experiments, the ranges of four factors were designed in Table 2. In order to minimize the effects of unexpected variability in the observed response, randomized experimental runs were carried out. After enzymatic hydrolysis process, the mixture was centrifuged at 12000×g for 10 min. The supernatant

was used to determination the DH and iron-binding capacity.

**Statistical analysis:** All samples were prepared and their activities were measured in triplicate and the data obtained were subjected to statistical analysis using ANOVA. By the way, the Least Significant Difference (LSD) with a confidence interval of 95% was used to compare the means. The RSM was statistically analyzed by Design-Expert (V8.0.5) software. The behavior of the system was explained by the following quadratic equation:

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

where,  $Y$  is the iron-binding capacity of hydrolysate;  $\beta_0$  is a constant term;  $\beta_i, \beta_{ii}, \beta_{ij}$  are the linear, quadratic and cross-product regression coefficients, respectively. And  $x_i$  and  $x_j$  are levels of the independent variables. The model evaluated the effect of each independent variable to a response. Analysis of the experimental design and calculation of predicted data were carried out using Design-Expert (V8.0.5) software to estimate the response of the independent variables. Subsequently, three additional confirmed experiments were conducted to verify the validity of the statistical experimental strategies.

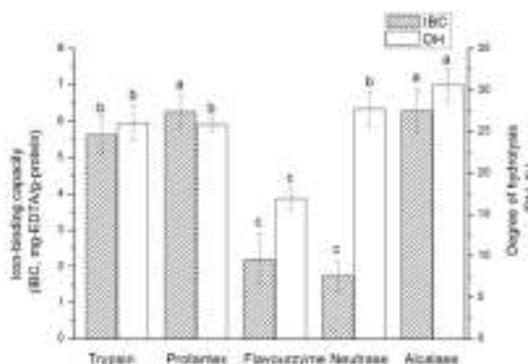


Fig. 1: The Iron-Binding Capacity (IBC) and Degree of Hydrolysis (DH) of mackerel processing byproducts hydrolysates with different proteases hydrolysis. Each observation is a mean of three replicate experiments ( $n = 3$ ) and the values with different lowercase differ significantly at  $p < 0.05$

## RESULTS AND DISCUSSION

**Effect of enzyme type on iron-binding capacity:** In order to select the best protease to hydrolyze the MPB for producing iron-binding peptides, the material was independently hydrolyzed by trypsin, Protamex, Flavourzyme, Neutrase and Alcalase, respectively. The iron-binding capacity, as well as DH, was determined after enzymatic hydrolysis. The results were shown in

Table 3: Experimental results of the central composite design for optimization of enzymatic hydrolysis of mackerel processing byproduct

Factors						
Runs	Temperature (°C, Temp)	Time (h, Time)	Enzyme to substrate (U/mL, ES)	Ph (pH)	Iron-binding capacity (mg-EDTA/g-protein)	
1	-1	1	-1	1	6.68±0.95	
2	-1	1	1	1	6.78±1.11	
3	1	-1	-1	1	2.54±0.94	
4	1	-1	1	-1	3.33±1.06	
5	1	1	-1	-1	2.32±1.01	
6	-1	-1	1	-1	7.31±1.2	
7	-1	-1	1	1	8.0±1.28	
8	0	0	0	-1.68	5.8±1.37	
9	0	0	0	0	3.16±0.97	
10	1.68	0	0	0	3.37±0.91	
11	0	0	0	2	4.87±1.5	
12	1	-1	1	1	2.59±1.04	
13	1	1	-1	1	1.8±0.93	
14	-1	1	-1	-1	7.1±0.89	
15	0	0	0	0	6.73±2.25	
16	0	0	-1.68	0	4.58±1.51	
17	0	0	0	0	2.99±0.96	
18	1	-1	-1	-1	2.09±1.07	
19	1	1	1	-1	2.14±1.1	
20	-1.68	0	0	0	2.69±0.83	
21	-1	1	1	-1	2.84±1.04	
22	0	0	1.68	0	6.01±1.06	
23	-1	-1	-1	-1	7.98±0.97	
24	-1	-1	-1	1	8.5±0.96	
25	0	0	0	0	4.77±1.11	
26	0	0	0	0	4.22±0.98	
27	0	1.68	0	0	4.9±1.11	
28	0	-1.68	0	0	4.8±0.98	
29	1	1	1	1	1.48±0.97	
30	0	0	0	0	6.49±1.64	

Fig. 1. The mackerel processing byproducts hydrolysates with Alcalase and Protamex had the higher iron-binding capacity than that of other three hydrolysates. And the iron-binding capacity of these two hydrolysates had not significantly difference at  $p < 0.05$ . The DH of hydrolysate by Alcalase had significantly higher than that of other four hydrolysates. This result in our experiments agreed with the findings that the bioactive activities of protein hydrolysate depended on the types of enzymes used (Venugopal and Shahidi, 1995). Also, as a typical endo-protease, Alcalase was effective hydrolysis enzyme to muscle proteins and had been widely used to produce bioactive peptides from various marine proteins, such as oyster protein (Liu *et al.*, 2008), rotifer protein (Lee *et al.*, 2009), *Chlorella ellipsoidea* protein (Ko *et al.*, 2012), tuna and halibut skin gelatin (Alemán *et al.*, 2011), cuttlefish and sardine protein (Kechaou *et al.*, 2009). Therefore, the Alcalase was selected to be used for producing iron-binding peptides from mackerel processing byproducts in the future studies.

**Optimization by response surface methodology:** The optimization experiments were designed to find the enzymatic hydrolysis conditions of the maximum iron-binding capacity. In terms of preliminary experiments (data not shown), the center points of Alcalase hydrolysis were chose at pH7.6, 50°C, 3h and 7500U/mL. Independent variables and their level in the central composite design were listed in Table 2. The results were given in Table 3. A full factorial Central Composite Design (CCD) was performed to study the combined effects of these four factors on the Iron-Binding Capacity (IBC) of MPB hydrolysate during Alcalase hydrolysis. The parameters of the predicted model were obtained by multiple regression analysis of the experimental data. The following quadratic model explains the experimental data:

$$\begin{aligned} \text{IBC}(\%) = & -10.85396 + 3.41948 \text{Temp} - 4.95021 \text{Time} \\ & - 0.007672 \text{ES} - 6.03542 \text{pH} - 0.026875 \text{Temp}^2 + 0.025 \\ & \text{Time}^2 + (8.72\text{E}-008) \text{ES}^2 + 0.91406 \text{pH}^2 + 0.087188 \\ & \text{Temp} \times \text{Time} + (7.65\text{E}-005) \text{Temp} \times \text{ES} - 0.24219 \text{Temp} \\ & \times \text{pH} - (2.39\text{E}-004) \text{Time} \times \text{ES} + 0.22187 \text{Time} \times \text{pH} + \\ & (4.0\text{E}-004) \text{ES} \times \text{pH} \end{aligned}$$

The optimal hydrolysis conditions by Alcalase were extracted by Design-Expert software with their optimization menus:  $\text{pH} = 8.35$ ,  $\text{Temp} = 46.0^\circ\text{C}$ ,  $\text{ES} = 6460 \text{ U/mL}$ ,  $\text{Time} = 2.01 \text{ h}$ . The highest iron binding activity prediction by this model at the optimal hydrolysis conditions was 6.69 mg-EDTA/g-protein. In order to confirm the accuracy of the model predictions, three parallel experiments were carried out for verification experiments under the predicted optimal hydrolysis conditions. For convenience, the hydrolysis

pH and time were set as pH8.3 and 2.0 h, respectively. The average iron-binding capacity of three verification experiment was 6.62 mg-EDTA/g-protein. The result was obviously in close agreement with the model prediction and confirm the predictability of the model for the enzymolysis of the MPB in the experimental condition used. Therefore, the regression model was applicable to predict MPB enzymatic hydrolysis process.

## CONCLUSION

The Protamex hydrolysate of mackerel processing byproduct had higher iron-binding capacity; however, the Alcalase hydrolysate of mackerel processing byproduct had both of DH and IBC. Therefore, Alcalase was the best choice for producing iron-binding peptides from mackerel processing byproducts. The optimal hydrolysis conditions with Alcalase were optimized by RSM using a central composite design and the optimal enzymatic hydrolysis conditions were temperature of 46.0°C, time of 2.01 h, pH 8.35 and enzyme to substrate 6460 U/mL. The quadratic model predicted well about the actual measured value.

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