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Optimization of Hydrolysis Conditions for the Production of Iron-Binding Peptides from Scad (*Decapterus maruadsi*) Processing Byproducts

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Abstract: The scad (*Decapterus maruadsi*) processing byproduct (SPB) was hydrolyzed by four commercial enzymes, namely, trypsin, flavourzyme, protamex and alcalase, for preparing high Iron-Binding Capacity (IBC) hydrolysate. Alcalase was the best choice for obtaining high IBC hydrolysate from SPB. Response surface methodology using a central-composite design was employed to optimize the enzymatic hydrolysis conditions with alcalase to obtain a maximum hydrolysate yield from the SPB with high iron-binding capacity. The best alcalase hydrolysis conditions were as following: Hydrolysis temperature of 46°C, enzyme substrate ratio of 6040 U/g-protein and hydrolysis time of 66 min, respectively. Under these optimal hydrolysis conditions, the predicted iron-binding capacity was 317.2 μg g^{-1}, which was consistent with the average of three replicates of 296.2 μg g^{-1} obtained in the validation experiments. The IBC of hydrolysate did not displayed linear relationship with antioxidative ability or the Degree of Hydrolysis (DH). Results indicated that the alcalase hydrolysate from scad processing byproduct can be developed into iron supplant ingredients in functional foods.

Keywords: Decapterus Maruadsi, Scad, Byproducts, Enzymatic Hydrolysis, Response Surface Methodology, Iron-Binding Capacity, Antioxidative

Introduction

The scad (*Decapterus maruadsi*), belongs to the family of mackerel, plays an important role in Chinese marine fishing industry economics and its production in China reached 540,000 tons in 2009 (Zhong *et al*., 2012). In Southern China, the peak of scad harvest is during the summer. Because of dark skin, summer high temperature, easy to oxidation and off-putting flavor (Thiansilakul *et al*., 2007), this fish is limited to process high-valued products. Therefore, the scad is usually processed into fish feedstuffs or fertilizer or used for producing fish oil. When it was used for fish oil, a lot of byproducts, mainly composited of head, skin, bone and others, would be included. These Scad Processing Byproducts (SPB) contained more than 50% protein (dry basis). Utilization of these marine low-value fish or fish processing byproducts to produce biological active peptides has become a research hotspot, such as antioxidative activity (Jiang *et al*., 2014), antihypertensive activity (Forghani *et al*., 2016), antimicrobial activity (Battison *et al*., 2008), antifreeze activity (Wang *et al*., 2014b), anticoagulant activity (Jung and Kim, 2009), metal elements chelating activity (Jung and Kim, 2007), anti-cancer activity (Picot *et al*., 2006), HIV-1 protease inhibitory activity (Lee and Maruyama, 1998) and appetite suppressant activity (Cudennec *et al*., 2008). The SPB contained high content of protein and suitable for producing high-value biological activity products. Two antioxidative peptides (His-Asp-His-Pro-Val-Cys and His-Glu-Lys-Val-Cys) had been isolated and purified from round scad muscle protein commercial proteases hydrolysates (Jiang *et al*., 2014). Thiansilakul *et al*. (2007) also studied the composition of defatted round scad protein hydrolysate with antioxidative activity. Two endogenous proteases, cathepsin L and MBSP, were isolated from blue scad skeletal muscle (Zhong *et al*.,

2012). However, no information regarding the iron-binding peptides from scad processing byproduct hydrolysate has been reported.

Iron is one of the most essential trace elements for human. Iron deficiency will cause many diseases. Iron is absorbed in the human intestine and many factors influence iron absorption. Generally, iron in plant foods is less absorbed than that in animal foods. Many animal proteins enhance iron absorption because they are digested into small peptides with the special amino acids or free amino acids, such as aspartic acid, histidine, glutamic acid and cysteine (Swain *et al*., 2002). These amino acids in free or small peptides can bind iron to form soluble complexes, which were stable under human acid stomach conditions or alkaline intestinal conditions and enhance iron absorption. The iron-peptide complex entered into enterocyte brush border membrane as soluble iron form and therefore, the iron was transported with blood through ferroportin 1, which enhanced iron absorption (Fuqua *et al*., 2012; Wang *et al*., 2014a). In recent years, many researchers had good interested in small peptides with high iron-binding ability prepared from food sources.

Various edible protein hydrolysates had high Iron-Binding Capacity (IBC) or iron bioavailability, such as spirulina protein (Kim *et al*., 2014), mackerel processing byproducts (Zhang *et al*., 2015), shrimp processing byproducts (Huang *et al*., 2012), anchovy muscle (Wu *et al*., 2012), lactoglobumin (Wang *et al*., 2014a), soybean (Zhang *et al*., 2014), fish collagen (Huang *et al*., 2015) and chickpea protein (Torres-Fuentes *et al*., 2012). However, there was no literature about enzymatic hydrolysate with iron-binding capacity from scad processing byproduct.

In this study, the preparation of hydrolysate with high iron-binding capacity from scad processing byproduct was investigated. Response Surface Methodology (RSM) using a central composite design was employed to optimize the enzymatic hydrolysis conditions with alcalase to obtain a maximum iron-binding capacity from the SPB, including temperature, pH, time and enzyme addition quantity.

Materials and Methods

Material

The SPB were obtained from Zhoushan city, P.R. China. Samples were washed by tap water, drained and then chopped into slurry. The slurry was mixed with isopropyl alcohol and heated reflux at 80°C for 6h in order to wipe off fat. After reflux, it was dried at 65°C to violate the isopropyl alcohol. The defatted SPB was smashed and stored at -20°C. The commercial proteases including alcalase (200 U/mg), flavourzyme (1200 U/mg), protamex (2100 U/mg) and trypsin (50 U/mg) were obtained from local chemical company. All other chemicals were of analytical grade.

Preparation of Enzymatic Hydrolysates

Defatted SPB powder was mixed with 0.05 M Tris buffer and solid content was fixed at 5% (w/v) based on protein content. The buffer pH was adjusted according to the selected commercial proteases and the solution was heated and waved at 150 r/min and proteases' recommendatory temperatures (Table 1) for 10 min. Then various commercial proteases were added into the solution at 7500 U/g based on protein content. The hydrolysis reaction was sustained for 4 h and then the mixture was boiled for 15 min to inactivate the hydrolase and then it was centrifuged at 12,000×g for 20 min to collect supernatants. The Degree of Hydrolysis (DH), protein content, IBC and Diphenyl-picryl hydrazyl radical Scavenging Ability (DSA) were determined.

Experimental Design

In the primary stage experiments confirm, the reaction time, temperature and enzyme addition quantity (E/S) were influenced significantly on the iron-binding capacity of hydrolysate. Therefore, these three independent hydrolysis conditions were investigated to obtain the maxium iron-binding ability by a Central Composite Design (CCD) in RSM. The 2nd-order designs were used in response surface methodology as acceptable approximation of true responses. The CCD design was very popular 2nd-order designs because of its extremely simple to use and allowing estimation of all the parameters in a full second-order model. Also, the CCD design was widely used for optimization of enzymatic hydrolysis of protein (Guo *et al*., 2009; Castro and Sato, 2015). Total 20 groups of parallel test were designed (with six reduplicative at the centre of the design). The axial distance α was chosen to be 1.68 in order to make this design orthogonal. The independent variables were shown in Table 2 and they were set according to preliminary experiment's conclusion. The codified values were labeled in Table 3. From this design, these 20 experiments were executed. For the 3 levels CCD system, the 2nd-order polynomial equation was applied to fit IBC (Y_1) and DH (Y_2):

$$\mathrm{Y}k = a_0 + \sum_{i=1}^{4} a_i X_i + \sum_{i=1}^{4} a_{ii} X_{ii} + \sum_{i \neq j=1}^{4} a_{ij} X_{ij} \quad (1)$$

where, Y_k is predicted value, a_0 is the intercept, a_i, a_{ii} and a_{ij} are coefficients of the model. X_i or X_j are the coded factors. The data were analyzed by the Design Export (Statsoft, USA). The 3-D charts were constructed to reveal the interactive effects of the three factors.

Table 1. Enzymatic hydrolysis conditions for tested proteases

	Parameters for enzymatic hydrolysis			
Enzyme	E/S (U/g)	Time (h)	pH	Temperature (°C)
Flavourzyme	7500	4	7	50
Trypsin	7500	4	8	37
Protamex	7500	4	8	37
Alcalse	7500	4	8	50
Flavourzyme	7500	4	7	50

Table 2. Coded levels of the hydrolysis parameters according to a central composite design for scad processing byproduct

	Coded level					
Hydrolysis parameters	Symbol	-1.68(-α)	-1	0	1	1.68(+α)
Temperature (°C)	X_1	36.6	40	45	50	53.4
Time (min)	X_2	9.6	30	60	90	110.4
E/S (U/g)	X_3	4150	5000	6250	7500	8350

Table 3. The iron-binding capacity and DH results of the CCD experiments

	Factors				
Experiment no.	Temperature (°C, Temp.)	Time (min)	E/S (U/g, ES)	Iron-binding capacity (ug/g)	DH (%)
1	0	0	-1.68	265.7±1.6	61.8±4.9
2	0	0	0	328.9±9.2	52.9±2.8
3	-1.68	0	0	223.3±13.1	58.5±3.4
4	0	0	0	318.3±4.1	60.0±4.2
5	1	-1	1	256.9±17.8	61.8±2.4
6	0	-1.68	0	279.6±5.6	58.8±3.4
7	0	1.68	0	261.8±3.7	64.4±2.8
8	1.68	0	0	261.9±6.8	63.2±5.2
9	0	0	0	339.1±19.5	51.5±4.1
10	1	1	-1	287.1±3.8	50.1±3.2
11	-1	-1	-1	233.7±2.5	48.7±4.4
12	-1	-1	1	259.9±9.0	51.9±4.6
13	1	1	1	284.8±2.5	58.1±1.1
14	0	0	0	306.2±3.7	57.3±3.3
15	0	0	0	294.7±3.0	56.4±4.5
16	0	0	1.68	275.9±1.4	53.5±5.6
17	-1	1	1	276.1±7.3	59.8±4.0
18	1	-1	-1	273.7±15.4	56.1±4.1
19	-1	1	-1	251.5±16.3	57.1±8.1
20	0	0	0	308.3±5.0	56.1±4.2

Determination of the DH

The DH was tested by Peričin *et al*. (2009) method with a little modification. Simply, 0.5 mL hydrolysate and 0.5 mL 20% trichloroacetic acid was mixed to make the protein to precipitate at 4°C for 3 h. Thereafter, the mixture solution was centrifuged at 10000×g for 20 min and the trichloroacetic acid soluble protein was determined by Folin-phenol method (Lowry *et al*., 1951). The DH value was expressed as the Equation 2:

$$DH(\%) = \frac{trichloroacetioacid\ soluble\ protein}{total\ protein} \times 100\% \qquad (2)$$

Determination of IBC

The IBC was determined using Chaud *et al*. (2002) method. When Fe^{3+} ions and thiocyanate ions in the solution were mixed, an intense red color developed. However, peptides in the solution compete with thiocyanate ions to bind free Fe^{3+} and the red color will fade. The sample was diluted by distilled water to 1.0 mg mL^{-1} based on protein content. The 0.1 mL of 0.5% (w/w) $FeCl_3$ was added to the mixture of 4.2 mL sample solution and 0.1 mL of 1.0 M KSCN. The reaction solution was incubated at 20°C for 20 min. The OD value was then read at 484 nm and the iron concentration was obtained. The sample was replaced by distilled water to use as the control. The IBC was

expressed as the bond iron qualities (μg) per gram peptides in the solution.

Determination of DSA

The DSA was defined as Diphenyl Picryl Hydrazinyl (DPPH) radical scavenging activity and the determination was according to Sánchez-Moreno (2002) method with a little modification. The sample was diluted into 1.0 mg mL^{-1} based on protein content. A 1.0 mL sample was added to a test tube containing 3.0 mL ethanol and then 1.0 mL DPPH ethanol solution (250 μM) was added. The mixture was incubated at 20°C for 30 min in dark in order to react completely and then the OD value was obtained at 517 nm. The DSA was expressed as Equation 3:

$$DSA(\%) = (1 - \frac{A - A_1}{A_0}) \times 100\% \quad (3)$$

where, A_0 was the OD value of the blank, A_1 was back ground OD value and A was the OD value of the sample.

Statistical Analysis

Three parallel experiments were designed and the data were analyzed using ANOVA. The results were expressed as the means of triplicates ± Standard Deviations (SD). The RSM results were statistically analyzed by Design-Expert (V8.0.5) software. The means differences were run by Least Significant Difference (LSD) at 5% confidence level.

Results and Discussion

Choice of Hydrolase

Four kinds of hydrolysis enzymes, flavourzyme, protamex, trypsin and alcalase, were selected to prepare small peptides from SPB with iron-binding ability. The hydrolysis conditions were listed in Table 1. After hydrolysis for 4 h, the hydrolysate was used to determine DH and IBC and the result was shown in Fig. 1. The DH had no significant difference (P>0.05) between selected four commercial hydrolases and the DH was ranged at 39 to 42%. However, the kinds of enzyme had significant effects on IBC. The IBC of hydrolysate with alcalase hydrolysis was significantly higher than the other three enzymes at P<0.05. As an endopeptidase, alcalase had a broad specificity of peptide bonds for protein hydrolysis, preferentially aromatic amino acids residues formatting peptide bonds, especially large uncharged residues (Peksa and Miedzianka, 2014). Therefore, alcalase was widely used for producing bioactive peptides from various proteins, such as ACE inhibitory peptides (Wu *et al*., 2016), antioxidative peptides (Xia *et al*., 2012; Najafian and Babji, 2014), mineral-binding peptides (Kim *et al*., 2007), antimicrobial peptides (Liu *et al*., 2008) and anticoagulant peptides (Ren *et al*., 2014). So, alcalase was selected for next step to optimize hydrolysis conditions for producing small peptides from SPB with high iron binding ability.

Effects of Hydrolysis Conditions

During enzymatic hydrolysis process, more and more small peptides were liberated from protein and also the molecular weight of peptides was decreased. The bioactivity of hydrolysate, including iron-binding activity, changed during enzymatic hydrolysis. The changes in iron-binding ability and DH during alcalase hydrolysis of SPB were shown in Fig. 2a. During hydrolysis, the DH increased slowly, but its iron-binding ability did not always increased and it reached a peak at 60 min. Then, the iron-binding ability significantly decreased, possibly due to peptides with high iron-binding ability hydrolyzed by alcalase into smaller peptides with lower iron-binding ability. This phenomenon also appeared in producing other biological peptides during enzymatic hydrolysis. The hydroxyl radical scavenging activity of Flavourzyme hydrolysate from barley glutelin protein first increased then decreased during 4 h hydrolysis process (Xia *et al*., 2012). Castro and Sato (2015) also reported that there had similar changes in antioxidative ability of egg white protein hydrolysates. Therefore, 60 min was selected as the hydrolysis time for further experiments.

The effects of hydrolysis temperature from 40 to 60°C on DH of SPB and iron-binding capacity of hydrolysate were determined at pH 8.0, shown in Fig. 2b. The optimum temperature for hydrolysis of SPB by alcalase was at 45°C. Also, the hydrolysate showed maximum IBC at 45°C. Hence 45°C was chosen as center point in the next RSM experiments.

In order to study the effects of enzyme addition quantity on the iron-binding capacity, the hydrolysis process was carried out using different enzyme concentrations: 5000, 6250, 7500, 8750 and 10000 U/g, results shown in Fig. 2c. The increase in E/S ratio caused an increasement of DH but not resulted in improved iron-binding ability and the iron binding ability swung between 100 and 200 μg g^{-1}. Therefore, 6250 U/g was sufficient to obtain maximum iron-binding ability.

To study the effects of initial pH on the iron-binding ability of hydrolysate, the initial pH of hydrolysate was stabled at pH 7.0, 7.5, 8.0, 8.5 and 9.0. The result was shown in Fig. 2d. During alkaline pH, the hydrolysate had higher DH because the alcalase had optimal pH at alkaline range. However, the IBC did not show the same tendency. The hydrolysate showed maximum iron-binding ability at pH 7.0 though it did not show the highest DH at this pH. In order to obtain the high iron-binding ability, the initial pH of substrate was fixed at pH 7.0.

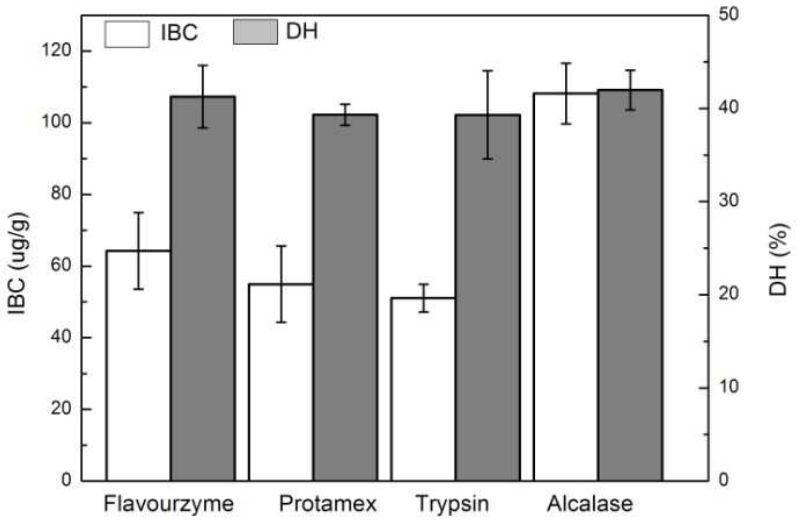

Fig. 1. The Iron-Binding Capacity (IBC) and Degree of Hydrolysis (DH) of scad processing byproduct with commercial proteases hydrolysis. The data was obtained by triplicate experiments

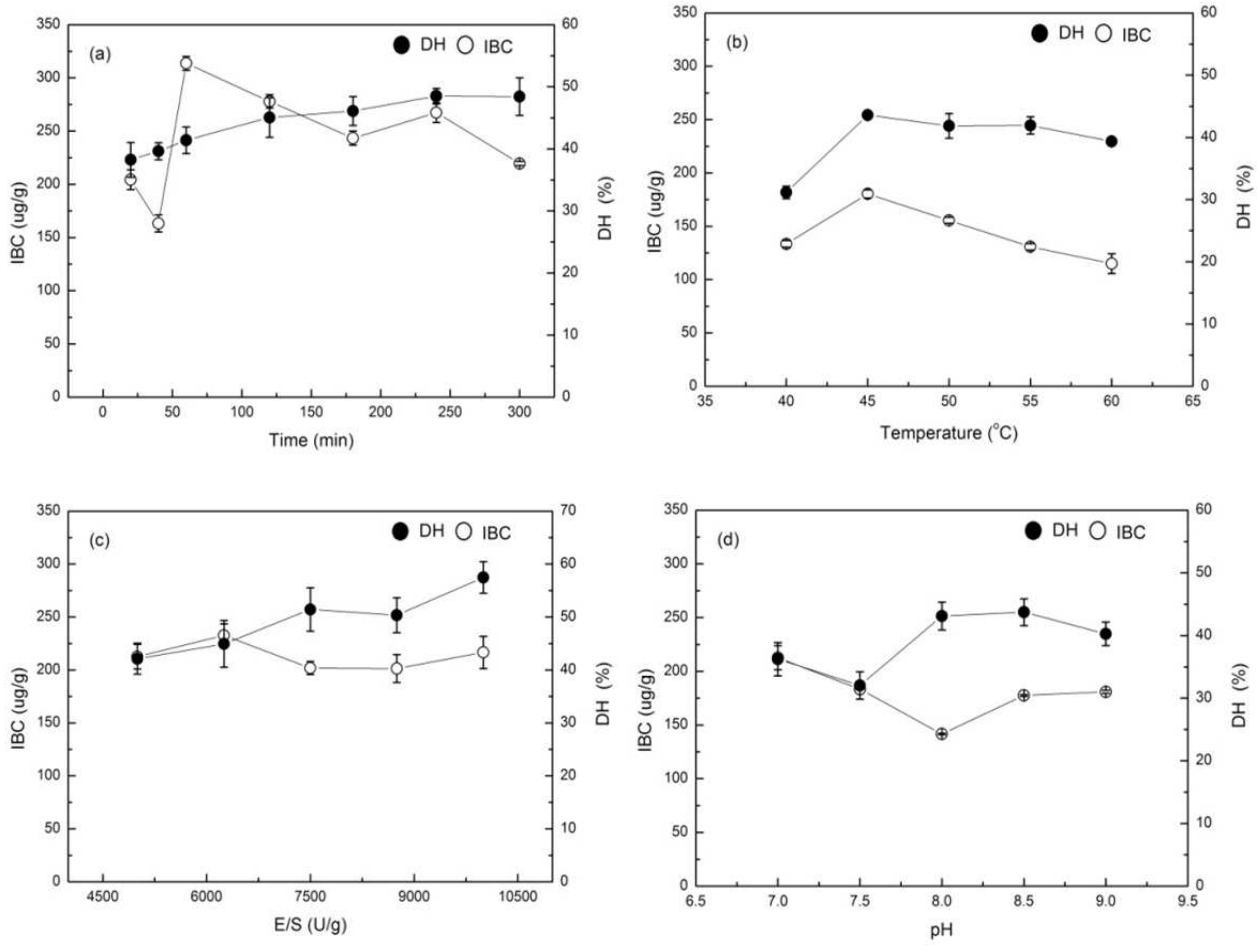

Fig. 2. Effects of hydrolysis time (a), hydrolysis temperature (b), enzyme substrate ration (E/S) (c), and hydrolysis pH (d) on IBC and DH. The data was obtained by triplicate experiments

Optimization by RSM

The RSM experiments were carried out to optimize the alcalase hydrolysis conditions to get the maximum IBC hydrolysate. By the results of preliminary experiments, the temperature of 45°C, time of 60 min and E/S ratio of 6250 U/g were selected to be the center points of alcalase hydrolysis conditions. The CCD was applied to investigate the interactional effects of hydrolysis temperature, time and enzyme addition quantity on the IBC of SPB hydrolysate during alcalase hydrolysis. Independent coded variables were shown in Table 2. Total 20 runs were performed and the DH and IBC of hydrolysates were analyzed. The data were given in Table 3.

The multiple regression analysis was used to analyze the experimental data to obtain the parameters of the predicted model. A quadratic model as following explained the experimental data:

$$IBC(\%) = 315.73 + 18.02X_1 + 3.32X_2$$
$$+3.59X_3 + 1.52X_1X_2 - 14.66\,X_1X_3$$
$$+1.60\,X_2X_3 - 68.90\,X_1^2 - 14.43\,X_2^2 - 14.40\,X_3^2$$

The regression equation for IBC is highly significant shown in Table 4 ($F = 7.36$, $p<0.05$). In trials, the first degree term X^2 (time) and quadratic term X_1^2 (temperature), X_2^2, X_3^2 (E/S) influenced significantly on IBC. However, interactive items did not affect significantly on the IBC. This result showed that during the process of the preparation of iron binding peptide, the influence of various factors on the IBC was not a simple linear relationship. The hydrolysis time, temperature and alcalase addition quantity were significantly affect on IBC. The interaction between various factors on the IBC did not have a significant impact.

The coefficient of R^2 equals to 0.8688 indicated that the model has good precision. The R^2 (adj) of IBC was 0.7508, indicating that 75.08% of the response on IBC could be explained by the model. Only 24.92% of total variation could not be explained by this mode. With the model of alcalase hydrolysis process of preparing iron binding peptide optimization was appropriate. The ANOVA shown in Table 4 also improved the model's lack of fit was not significant ($P>0.05$), which conversely validated that the model was stable and could be used to predict the influence of various factors on the IBC. The 3-D charts were constructed to reveal the interactive effects of the three factors (Fig. 3). Though, the cross-interaction effects of time, temperature and alclase addition quantity (E/S) were not significant (Table 4), yet the suitable ranges of time, temperature and E/S would be known from Fig. 3 in order to obtain high IBC.

The optimal alcalase hydrolysis conditions were obtained by the predicted model as following: Hydrolysis temperature of 45.6°C, E/S of 6338 U/g, hydrolysis time of 66.3 min. The highest IBC by the predicted model was 317.2 μg g^{-1}. According to these optimal hydrolysis conditions, we choose hydrolysis temperature of 46°C, E/S of 6340 U/g, hydrolysis time of 66 min as a condition of hydrolysis, which was consistent with the average of three replicates of 296.2 μg g^{-1} obtained in the validation experiments. The IBC of validated experiments was uniformity to that of predicted value. Therefore, the predict model for IBC during SPB enzymatic hydrolysis might be used for preparing hydrolysate with high IBC.

Relationship of DH, DSA and IBC

During enzymatic hydrolysis process, more and more small peptides are liberated from protein, that is to say, the DH of protein increased. Also the molecular weight of peptides was decreased. The bioactivity of hydrolysate, including iron-binding activity, varied during enzymatic hydrolysis. The relationships of DH and IBC, DH and DSA, IBC and DSA during hydrolysis process were shown in Fig. 4. The IBC of hydrolysate displays with the DH of protein during alcalase hydrolysis change was not consistent. The linearly dependent coefficient r was only -0.3724 (Fig. 4a) and, the IBC did not display consistent with the DSA of hydrolysate and the linearly dependent coefficient r was only -0.4499 (Fig. 4b). Also, the DSA did not show good relationship with DH and the linearly dependent coefficient r was only 0.03371 (Fig. 4c). The DSA of hydrolysate had a little relationship to peptides molecular, but more depends on the amino acids compositions and sequence. Many studies had improved that high DSA of hydrolysate did not appeared at high DH point (Xia *et al*., 2012; Castro and Sato, 2015). Generally, the transition metal ions, including Fe^{2+} and Cu^{2+}, promoted the oxidation of free radical. Therefore, the transition metal ions in solution chelated by other substance, such as EDTA and peptides, would decrease the oxidation of free radical. So, we investigated the hydrolysate with high IBC whether or not had high DSA. Because many enzymatic hyrolysate with high antioxidative ability had good transition metal ions chelating activity (Thiansilakul *et al*., 2007; Klompong *et al*., 2007; Agrawal *et al*., 2016). However, the antioxidative ability (DPPH radical scavenging activity) did not display linear relationship with IBC (Fig.4b) in this study, which might be because of the too narrow range of iron-binding ability (only from 251 to 349 μg g^{-1}) and there were no data of IBC or DSA at lower value. In the next study, this should be considered.

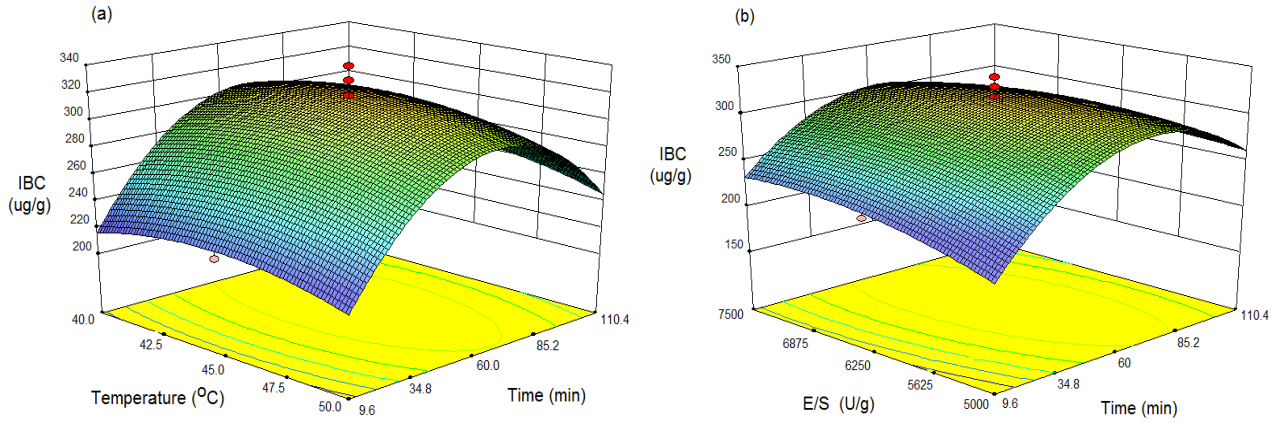

Fig. 3. The 3-D plots for the effects of variables on iron-binding capacity (IBC): (a) temperature and time; (b) time and E/S ratio

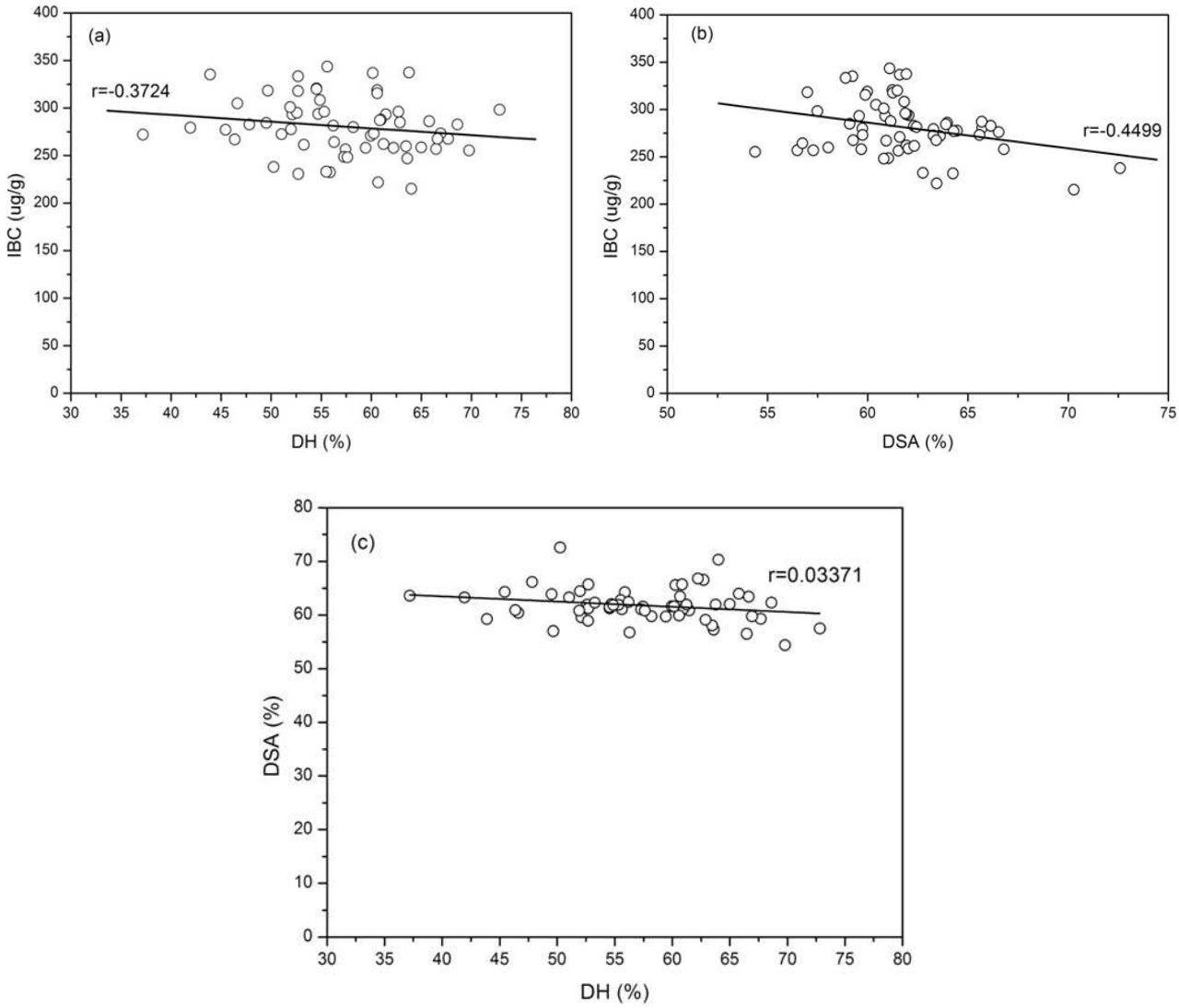

Fig. 4. The relationships of Iron-Binding Capacity (IBC), Degree of Hydrolysis (DH) and DPPH radical scavenging activity (DSA)

Table 4. ANOVA for IBC

Item	Sum of squares	df	*F* Value	*p*-value
Model	14921.15	9	7.36	0.0022*
X_1: Temperature	150.28	1	0.67	0.4331
X_2: Time	1568.45	1	6.96	0.0248*
X_3: E/S	175.91	1	0.78	0.3976
X_2X_1	6.58	1	0.029	0.8677
X_2X_3	608.24	1	2.7	0.1314
X_1X_3	20.6	1	0.091	0.7686
X_2^2	8550.98	1	37.96	0.0001*
X_1^2	3000.05	1	13.32	0.0045*
X_3^2	2986.4	1	13.26	0.0045*
Residual	2252.91	10		
Lack of fit	935.51	5	0.71	0.6418
Pure error	1.32E+03	5		
Cor total	17174.06	19		

Conclusion

The peptide-iron complexes, as a new type of iron supplement agents, increased the iron absorption in intestinal. The enzymatic hydrolysate with high iron-binding capacity from scad processing byproducts was investigated. The alcalase was the best protease within the four commercial enzymes for preparing high iron-binding capacity hydrolysate from SPB. The best hydrolysis conditions were optimized by RSM to be as following: Hydrolysis temperature of 46°C, enzyme substrate ration of 6040 U/g and hydrolysis time of 66 min, respectively. The predicted iron-binding capacity was 317.2 μg g^{-1} at these optimal hydrolysis conditions, which was consistent with the average of three replicates of 296.2 μg g^{-1} obtained in the validation experiments. Future researches about iron-binding peptides purification, stability of iron-peptide complex and actual absorption in the intestinal cells are needed in order to improve the application as a new iron supplement agent.

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Author's Contributions

Wenting Zhang: Performed the experiments and wrote the manuscript.

Yanan Li and Jie Zhang: Participated in partial experiments.

Guangrong Huang: Developed the idea and analyzed the data.

Ethics

The authors declare that they had no conflict of interest.

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