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Optimization of Enzymatic Hydrolysis of Freeze-Dried Sea Cucumber Powder with Response Surface Methodology and its Antioxidant Evaluation

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Abstract: In this study, freeze-dried sea cucumber powder was used as raw material and the preparation processing conditions were studied for hydrolysis of sea cucumber powder with neutral protease using Response Surface Methodology (RSM) to obtain a high concentration of sea cucumber polypeptide. The effects of hydrolysis temperature, pH, enzyme dosage and hydrolysis time on the degree of hydrolysis (DH) were studied. Based on the single factor experiment, Box-Benhnken center composite experiment was carried out with three factors and three levels, including hydrolysis temperature, pH and enzyme dosage. RSM was used to determine the effect of prime factors on the DH and establish a regression model. The results showed that the optimal hydrolysis conditions were as follows: enzyme dosage of 5753 U g^{-1} dry matter, hydrolysis temperature of 52°C, pH of 8.3, substrate concentration of 5% and hydrolysis time of 5 h. Under the optimal conditions, the DH was up to 16.01%, which matched well with the predicted value. With VC as the positive control, the scavenging ability of the hydrolyzate on DPPH radicals, hydroxyl radicals and superoxide radicals were very powerful, which mean that the hydrolyzate of sea cucumber powder has good antioxidant activity.

Keywords: Enzymatic Hydrolysis, Neutral Protease, Response Surface Methodology, Antioxidant Activity, Sea Cucumber Powder

Introduction

As the traditional healthy seafood in East Asian countries, sea cucumber is an important marine resource of food and drugs (Farouk *et al.*, 2007; Bai *et al.*, 2013). The major edible and medicinal parts of sea cucumber are its body walls mainly consisting of polypeptide, mucopolysaccharide, sterols, saponins, fatty acids and other biologically active substances (Cui *et al.*, 2007; Yu *et al.*, 2015). Those active substances have physiological activity such as anti-tumor, reducing blood pressure, anti-oxidation, improving immunity, reducing joint pain and anti-aging (Zhao *et al.*, 2007). Sea cucumber polypeptide is the degradation product of protein, which can be used as the supplements of protein and amino acid, on account of its small molecular weight, water-soluble and easy to digest (Zhu *et al.*, 2012). As the typical high-protein, low-fat, low-cholesterol and rich in minerals and vitamins high-quality seafood, sea cucumber is an ideal raw material for the preparation of bioactive peptides.

Under the condition of vacuum and low temperature, vacuum freeze-drying technology made the moisture in frozen material sublimate directly, which can greatly limited the maintenance of the original color, shape, aroma, flavor, active material and nutrients (Alibas, 2007) and effectively inhibited the proliferation of bacteria. At present, enzymatic hydrolysis was usually used for the preparation of sea cucumber polypeptide, protease substrate specificity, the enzyme sites are different for various proteases. Neutral protease opens the peptide bond of protein mainly internally, which can position hydrolytic cleavage to produce a specific peptide in mild conditions, with the characteristics of moderate hydrolysis potency, high safety, does not destroy the

amino acid structure and can keep nutrients effectively (Tavano, 2013).

In this study, neutral protease was used to hydrolyze the vacuum freeze-dried sea cucumber powder. On the basis of preparative experiment, the effect parameters of the degree of hydrolysis such as hydrolysis temperature, pH and enzyme dosage were optimized with the Response Surface Methodology (RSM) employing a three-variable, three-level Box-Behnken Design (BBD). With VC as the positive control, the scavenging effect of the DPPH radicals, hydroxyl radicals and superoxide radicals by enzymatic hydrolyzate were measured, which would be used to provide reference for the application and further study of sea cucumber polypeptide.

Materials and Methods

Sea cucumbers (*Stichopus japonicus*) were purchased from the local market in Qinhuangdao, Hebei Province, China. Neutral protease (60000 U g^{-1}) and TRIS were purchased from Beijing solarbio science and technology Co., Ltd. DPPH was purchased from Shanghai Yuanye Biological Technology Co., Ltd. All the other chemical and solvents used were of analytical grade and purchased from Tianjin Tianli Chemical Reagents Co., Ltd.

Preparation of Sea Cucumber Powder

Fresh sea cucumber was washed extensively with distilled water after evisceration and then heated to boiling 10 min at 100°C to ensure that the autolysis enzyme of the body wall was inactivated. After steeping 48 h in stilled water at 4°C，the body wall of sea cucumber was cut into thin slices about 5 mm and then put the frozen slices in a vacuum freeze dryer (LG-1.5, Shenyang Areo Space Xinyang Quick freezing Equip. Manuf. Co., Ltd, China). Under the condition of 30 Pa degree of vacuum and heating plate temperature at 40°C, the sea cucumber was dried around 12 h, then smashed into a high-speed multi-function mill (DC-100, WuyiDingcang Metal Factory, Zhejiang, China) to obtain sea cucumber powder and then kept in storage at 4°C.

Preparation of Sea Cucumber Hydrolyzate

About 1.0 g sea cucumber powder was added to distilled water according to a certain substrate concentration and mixed with neutral protease, then hydrolyzed it in a water bath (HH-4, Shanghai Bilang Instrument Co., Ltd, China) at an appropriate pH (adjusting the suspension pH by 0.1 mol L^{-1} NaOH or HCl with the pH meter, STARTER2100, Shanghai Ohaus Instrument Co., Ltd, China) for a period of time to keep solution temperature stable. To make sure the neutral protease was inactivated, kept the hydrolyzate in a boiling water bath for 10 min and then the cooling mixture was centrifuged at 6000 r/min for 10 min with a high speed refrigerated centrifuge (Neofuge 15 R, Shanghai Lishen Scientific Instrument Co., Ltd, China) to collect the supernatant as sea cucumber polypeptide and determined the content of amino nitrogen to calculate the degree of hydrolysis.

Determination of the Degree of Hydrolysis

The degree of hydrolysis (DH) is the percentage of hydrolyzed peptide bonds and the total number of peptide bonds in the process of hydrolysis, indicating the extent of peptide bonds cleavage during the hydrolysis. Total nitrogen content of the sample measured with the kjeldahl determination and the determination of amino nitrogen of hydrolyzate in accordance with formaldehyde titration. The calculation of the degree of hydrolysis was as follows (Liu, 2014):

$$DH = \left[\left(B - C \right) / \left(A - C \right) \right] \times 100\%$$

where, DH is the degree of hydrolysis (%), A is the total amino nitrogen content of the sample (g/100g), B is the amino nitrogen content of the hydrolysis (g/100g) and C is the free amino nitrogen content of raw materials (g/100g).

Optimization of Hydrolysis Design

The degree of the hydrolysis was influenced by various factors, such as hydrolysis temperature, pH, enzyme dosage, hydrolysis time and the concentration of substrate. With the increasing of substrate concentration, the sample generated steric hindrance with enzyme, which was not conducive to hydrolysis and it was difficult and inaccurate to adjust the pH if the concentration of the sticky mixture is too high, so that the substrate concentration was 5% in this experiment. In order to improve the degree of hydrolysis to obtain a higher concentration of polypeptide, the single factor experiment was carried out respectively in different hydrolysis temperature, hydrolysis time, pH and enzyme dosage to measure the degree of hydrolysis, each level was repeated 3 times to take the mean value.

Response Surface Methodology (RSM) is more advantageous than the traditional single parameter optimization method (Iqbal and Khan, 2010). On the basis of single factor experiment, a three-factor, three-level Box-Behnken Design (BBD) with Design-Expert 8.0.5 Trial was carried out, where Y is the predicted DH and A, B and C are the coded values for temperature, pH and enzyme dosage, respectively. The design of experiment factors and levels are shown in Table 1.

Table 1. Variables and levels in the Box-Behnken experimental design

	Coded levels		
Variables	-1	0	1
A-temperature (°C)	40	50	60
B-pH	7	8	9
C-enzyme dosage (U/g)	4000	5000	6000

Determination of Antioxidant Activity of Hydrolyzate

Added ethanol to the collected hydrolyzate up to 60% ethanol degree, after 12 h, the solution was centrifuged at 6000r/min for 10 min to remove polysaccharides and impurities. In order to reduce the interference for the determination of antioxidant activity came from ethanol, used a vacuum rotary distillation (RE52CS, Shanghai Yarong Biochemical Instrument Factory, China) to remove ethanol of the supernatant, then diluted it into different concentrations and measured the oxidation resistance (Liu and Wang, 2011) as follows.

Determination of Scavenging Rate of the Hydrolyzate on DPPH Radicals

DPPH radicals is a kind of steady-state nitrogen-centered free radical, which ethanol solution is purple, antioxidants can make a combination or replace with it to reduce the number of free radicals and change color shallow, at the same time, the absorbance decreases until it reaches stable (Zhang *et al*., 2015). Thus, the absorbance can be measured with a UV-visible Spectrophotometer (752, Shanghai Jinghua Technology Instrument Co., Ltd, China) at 517 nm and calculate the scavenging rate of hydrolyzate on DPPH radical. Added 2 mL 0.2 mmol L^{-1} DPPH-ethanol solution to different concentration of 5, 10, 15, 25, 50 and 100% of the 1mL hydrolyzate, mixed to react 30 min in the dark room at room temperature. Instead of DPPH-ethanol solution, equal volume of anhydrous ethanol was used as a blank and with equal volume of anhydrous ethanol in place of sample solution as the control. With the same as other operations, absorbance values was measured at 517 nm and to calculate the clearance rate. With VC as the positive control, the concentration of VC solution were 0.02, 0.04, 0.06, 0.08, 0.10, 0.12 mg mL^{-1} and then the scavenging rate was measured according to the following formula, respectively:

$$\text{Scavenging rate on DPPH radicals} = [1-(A_{sample}-A_{blank})/A_{control}] \times 100\%$$

Determination of Scavenging Rate of the Hydrolyzate on Hydroxyl Radicals

Hydroxyl radical (•OH) is the most toxic and damaging radicals to organisms produced by the body in the metabolic process. 2 mL 6 mmol L^{-1} $FeSO_4$ and 2 mL 6 mmol L^{-1} H_2O_2 were added into different concentration of 5, 10, 15, 25, 50 and 100% of the 1mL hydrolyzate, mixed and placed 10 min. And then added 6 mmol L^{-1} sodium salicylate 2 mL and reacted 30 min. Instead of sodium salicylate, equal volume of distilled water was as a blank and with equal volume of distilled water in place of sample solution as the control. With the same as other operations, absorbance values was measured at 510 nm and to calculate the clearance rate. With VC as the positive control, under the concentration of VC solution of 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 mg mL^{-1}, the scavenging rate was measured according to the following formula, respectively:

$$\text{Scavenging rate on hydroxyl radicals} = [1-(A_{sample}-A_{blank})/A_{control}] \times 100\%$$

Determination of Scavenging Rate of the Hydrolyzate on Superoxide Radicals

Pyrogallic acidauto-oxidize toproduce superoxide ($\bullet O_2^-$) under alkaline conditions, antioxidants are able to make a reduction for the highly oxidizing radicals to scavenging free radicals. 5.7 mL 50 mmol L^{-1} Tris-HCl and 6 mmol L^{-1} pyrogallic acid 0.1 mL were added into different concentration of 5, 10, 15, 25, 50 and 100% of the 0.2 mL hydrolyzate, mixed and reacted 4 min, then added 2 drops of HCl to stop the reaction. Instead of pyrogallic acid, equal volume of distilled water was as a blank and with equal volume of distilled water in place of sample solution as the control With the same as other operations, absorbance values was measured at 320 nm and to calculate the clearance rate. With VC as the positive control, the concentration of VC solution of 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 mg mL^{-1}, the scavenging rate was measured according to the following formula, respectively:

$$\text{Scavenging rate on superoxide radicals} = [1-(A_{sample}-A_{blank})/A_{control}] \times 100\%$$

Statistical Analysis

The results obtained were presented as mean ± SD, while analysis of variance was carried out by ANOVA Method (SPSS 13). The RSM and multiple regression analysis was performed by the Design-Expert 8.0.5.

Results and Discussion

Effects of pH on the DH

The pH is one of the factors that can affect the degree of hydrolysis. Under the condition that the hydrolysis temperature was 50°C, neutral protease dosage was 6000 U g^{-1} and hydrolysis time was 5 h, the effect on the DH was studied when the pH were 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. As shown in Fig. 1, the degree of hydrolysis increased rapidly when the pH ranged from 5.0 to 8.0 and then the DH decreased with increasing of pH. The result of ANOVA test showed that there was an obvious significant difference among different levels ($p<0.05$). When the pH was 8.0, the DH reached a maximum value, which indicated that the optimal pH of neutral protease was around 8.0. Based on these results, 8.0 was considered to be the optimum pH in the present experiment.

Effects of Temperature on the DH

Temperature is another factor that can affect the degree of hydrolysis. The effect of hydrolysis temperature on DH was studied with the pH 8.0, hydrolysis time 5 h and the neutral protease dosage 6000 U g^{-1}. The temperature was set at 30, 40, 50, 60 and 70°C. From Fig. 2, it could be found that the DH increased as temperature ascended from 30 to 50°C and then decreased with increasing temperature. The result of ANOVA test showed that there was an obvious significant difference among different levels ($p<0.05$). It was well known that the effect on enzymatic reaction efficiency comes from the speed of protease catalytic reaction and the stability of the protease. However, high temperature led to the disintegration of the secondary bond which maintains the molecular structure of enzyme, causing the degeneration of neutral protease and making enzyme activity weakened. Thus, the DH decreased when the hydrolysis temperature was over 50°C. Therefore, 50°C was considered to be the optimum temperature.

Effect of Enzyme Dosage on the DH

To study the effect of enzyme dosage on the degree of hydrolysis, extraction process was carried out using the different enzyme dosage of 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000 U g^{-1}. The hydrolysis temperature was fixed at 50°C, pH was fixed at 8.0 and extraction time was fixed at 5 h. As shown in Fig. 3, the DH increased significantly when the enzyme dosage ranged from 1000 to 5000 U g^{-1}, but there was no obvious change on the DH as the enzyme dosage continued to increase. The result of ANOVA test showed that enzyme dosage had an significant effect on the DH ($p<0.05$). A larger enzyme dosage implied greater concentration between neutral protease and mixture and the hydrolysis occurred more quickly. However, when the enzyme dosage reached a certain extent, the enzyme and substrate binding reached saturation. Therefore, the DH increased slowly when the enzyme dosage increased from 5000 to 8000 U g^{-1}. Considering the cost of production and avoiding the wasting consumption of neutral protease, 5000 U g^{-1} was chosen as the optimum enzyme dosage.

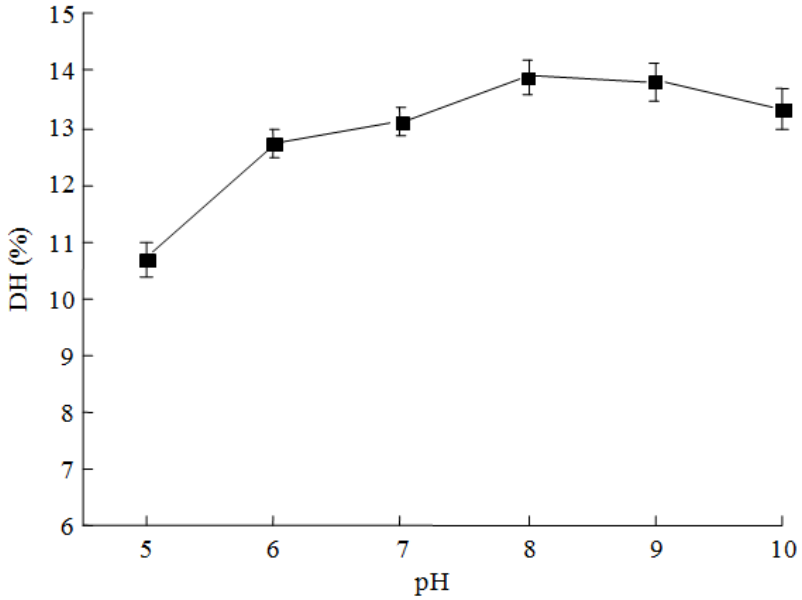

Fig. 1. The effect of pH on DH

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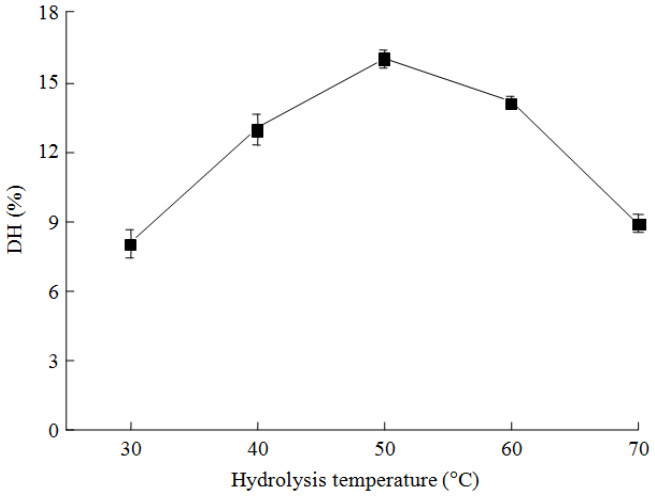

Fig. 2. The effect of temperature on DH

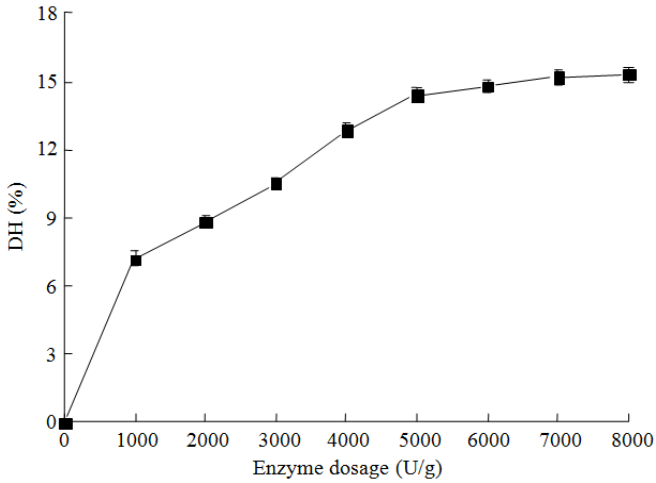

Fig. 3. The effect of enzyme dosage on DH

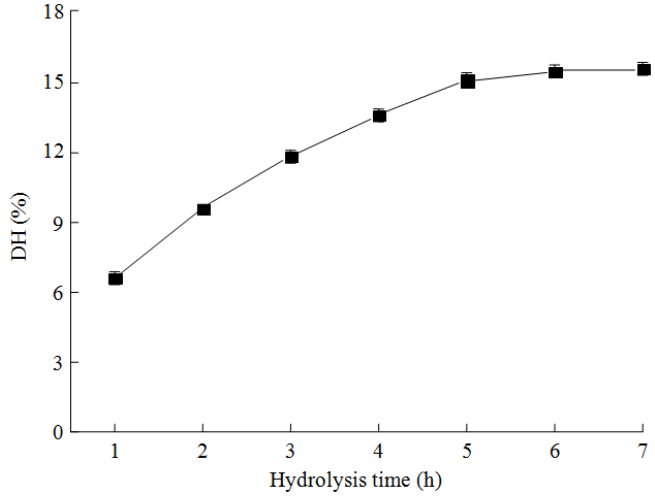

Fig. 4. The effect of hydrolysis time on DH

Effect of hydrolysis time on the DH

Hydrolysis time is another factor that would influence the degree of hydrolysis. Fig. 4 showed the effect of hydrolysis time on the DH and other conditions were fixed as follows: Hydrolysis temperature 50°C, pH 8.0 and enzyme dosage 5000 U g^{-1}. Fig. 4 showed that the DH increased with extending hydrolysis time and then increased slowly when the time exceeded 5 h. According to the ANOVA test, hydrolysis time had an significant effect on the DH ($p<0.05$). With the proceeding of hydrolysis, protein substrates gradually were reduced and enzymolysis products were accumulated, which led to inhibitory effect on the enzyme. Otherwise, long hydrolysis time wasted the energy and reduced the production efficiency. Thus, the DH had no obvious change as the hydrolysis time over 5 h and the optimum time was 5 h.

Response Surface Optimization Results

Based on the results obtained in the single factor experiment, RSM was applied to monitor the degree of hydrolysis and to determine the optimum conditions. A total of 17 runs were conducted for optimizing the temperature, pH and hydrolysis time three parameters in the current Box-behnken design. The design of the variables coded with the experimental and predicted values was shown in Table 2. The zero point tests were repeated 5 times to improve the sensitivity and accuracy of the test error estimation and each test was repeated 3 times at the same time.

The data were analyzed by multiple regression analysis. Y is the DH that was taken as the dependent variable, A, B and C represented temperature, pH and enzyme dosage respectively, which were regarded as the independent variables. The second-order polynomial stepwise equation is as follows:

$$Y = 15.75 + 0.60A + 0.20B + 0.87C + 0.54AB$$
$$+0.17AC + 0.027BC - 2.31A^2 - 0.49B^2 - 0.60C^2$$

The credibility analysis of the regression equation is shown in Table 3. The coefficient of determination (R^2) indicated the proportion of the variability in the data explained or accounted for by the model (Xu *et al.*, 2013). As can be seen, the coefficient of determination (R^2) was 0.9923, indicating that the model was credible and only 0.77% of the total variations was not explained by the regression model. The adjusted determination coefficient (R^2_{Adj}) was 0.9823, suggested that the model was highly correlation and it can commendably describe and predict the hydrolysis process of sea cucumber powder. Meanwhile, the degree of precision and reliability of the experiment value were rather high, which we can see from the low score 1.44 of the Coefficient of the Variation (CV) (Zhu *et al.*, 2015).

Table 2. Design and results of response surface experiment

	Levels				
Test number	A	B	C	Experimental DH (%)	Predicted DH (%)
1	-1	0	-1	11.34	11.54
2	0	0	0	15.92	15.75
3	0	1	-1	14.10	13.96
4	0	0	0	15.68	15.75
5	1	0	1	14.67	14.47
6	1	1	0	14.10	14.29
7	0	0	0	15.83	15.75
8	-1	0	1	12.87	12.92
9	1	0	-1	12.45	12.40
10	0	-1	-1	13.62	13.61
11	0	-1	1	15.15	15.29
12	-1	1	0	12.06	12.00
13	-1	-1	0	12.87	12.68
14	0	0	0	15.53	15.75
15	1	-1	0	12.75	12.81
16	0	0	0	15.77	15.75
17	0	1	1	15.74	15.75

Table 3. Credibility analysis of regression model

Item	Value	Item	Value
Standard deviation	0.20	R^2	0.9923
Mean	14.14	Adj R^2	0.9823
CV	1.44	Pre R^2	0.9106
PRESS	3.33	AdeqPrecisior	27.0340

Table 4. Results of regression analysis

Source	SS	DF	MS	F-Value	P-Value	significant
Model	37.01	9	4.11	99.75	<0.0001	**
A	2.92	1	2.92	70.73	<0.0001	**
B	0.32	1	0.32	7.86	0.0264	*
C	5.99	1	5.99	145.19	<0.0001	**
AB	1.17	1	1.17	28.29	0.0011	**
AC	0.12	1	0.12	2.89	0.1331	-
BC	3.025×10^{-3}	1	3.025×10^{-3}	0.073	0.7943	-
A^2	22.48	1	22.48	545.2	<0.0001	**
B^2	1.01	1	1.01	24.57	0.0016	**
C^2	1.53	1	1.53	37.13	0.0005	**
Residual	0.29	7	0.041			
Lack of fit	0.2	3	0.067	2.99	0.1585	-
Pure error	0.089	4	0.022			
Cor total	37.3	16				

**: Highly significant, $p<0.01$; *: Significant, $p<0.05$; -: Not significant

The analysis of variance (ANOVA) of the regression model is shown in Table 4. The P-value was used to check the significant of the regression equation and model coefficients and the smaller the P-value was, the more significant the corresponding coefficient was (Guo *et al.*, 2010). It can be seen from Table 4 that the model F-value of 99.75 with a low probability P-value (<0.0001) implied high significance of the model. Lack of fit of the model was used as a tool to assess the not fitting probability between model predictions and actual values. The P-value of the lack of fit was 0.1585 indicated not significant, the fit of the equation to test was preferably and unknown factors had small interfere with the results, so the test methods are reliable. In addition, the results in this table show that the linear coefficients (A and C), the cross product coefficient (AB) and the quadratic term coefficients (A^2, B^2, C^2) were extremely significant, the linear coefficient (B) was significant, whereas the factors with no significant effect on the results were the cross product coefficients (AC and BC). F-value of each factor reflected the importance of various factors on the test indicators and the larger the F-value was, the greater importance the test indicator was. Thus, the factors influencing the response value of the DH was as follows: Enzyme dosage > hydrolysis temperature >pH.

Response surface is a three-dimensional space constitute from the specific response value with the corresponding factors, mapping on the two-dimensional flat to form contour plots, which can intuitively reflect the interaction of various factors and the impact on the response value and also can analyze the sensitivity of response value for different independent variables. According to the analysis of regression equation, the three-Dimensional (3D) response surface and two-Dimensional (2D) contour plots are shown in Fig. 5 to 7. The shape of contour reflects the strength of the interaction between two factors, the oval indicates strong interaction and the circle indicates weak interaction between two factors (Xu *et al.*, 2008).

Fig. 5 shows the effect of interaction between hydrolysis temperature and pH on the DH. As can be seen from Fig. 5, the oval contour plot reflected the strong interaction between temperature and pH, which also verified the significant effect of temperature and pH on the DH by the variance analysis. Keeping enzyme dosage at 0 level, the response surface and the contour plot in Fig. 5 show that the DH of sea cucumber powder increased obviously when the temperature increased from 40 to 52°C, but the DH changed slowly when it exceeded 52°C. The DH increased evidently with the increasing of pH from 7.0 to 8.0, but beyond 8.0, the DH increased slowly as the pH ascended. The response surface and the contour plot in Fig. 6 show the effect of interaction between hydrolysis temperature and enzyme dosage on the DH. It can be seen from Fig. 6 that the DH increased as enzyme dosage ascended from 4000 to 5500 U g^{-1} when the pH remains unchanged, but it became slowly when the enzyme dosage beyond 5500 U g^{-1}. Fig. 7 shows the effect of interaction between pH and enzyme dosage on the DH. From Fig. 7, it can been seen that when the hydrolysis temperature was at a certain value, the DH increased rapidly with pH added from 7.0 to 8.0, but then decreased slowly when the pH beyond 8.0.

Optimization and Verification

The Design Expert 8.0.5 software was used to optimize and solve the regression equation. The optimum condition for the hydrolysis of sea cucumber powder was as follows: Hydrolysis temperature, 51.98°C; pH, 8.34; and enzyme dosage, 5752.64 U g^{-1} and the model predicted values for the maximum degree of hydrolysis was 16.1652%. For the convenience of practical operation, the actual conditions are hydrolysis temperature, 52°C; pH, 8.3; and enzyme dosage, 5753 U g^{-1}. Under these conditions, a mean value of 16.01% was gained, which in agreement with the predicted value from the regression model within a relative error of 0.96%, which indicated the correctness of the mathematical regression model.

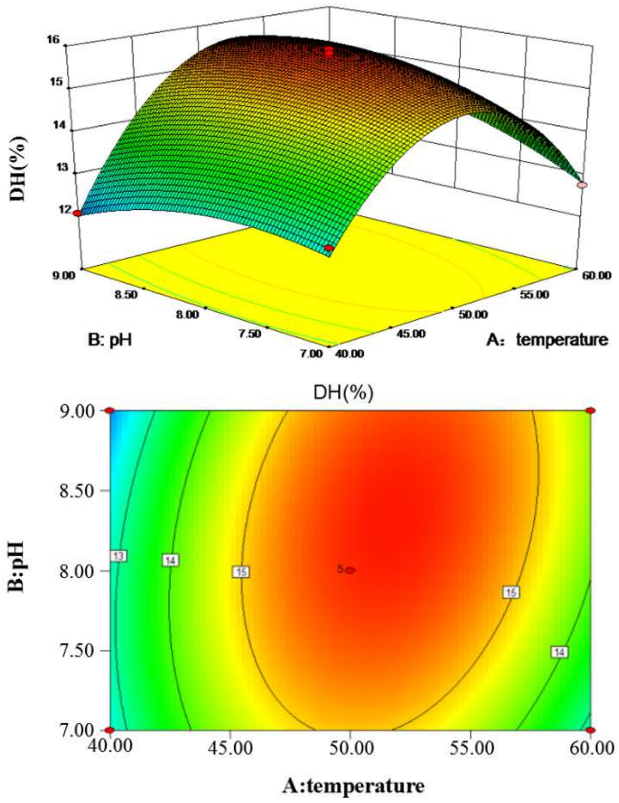

Fig. 5. Responsive surfaces and contour plot of the combined effects of temperature and pH on DH

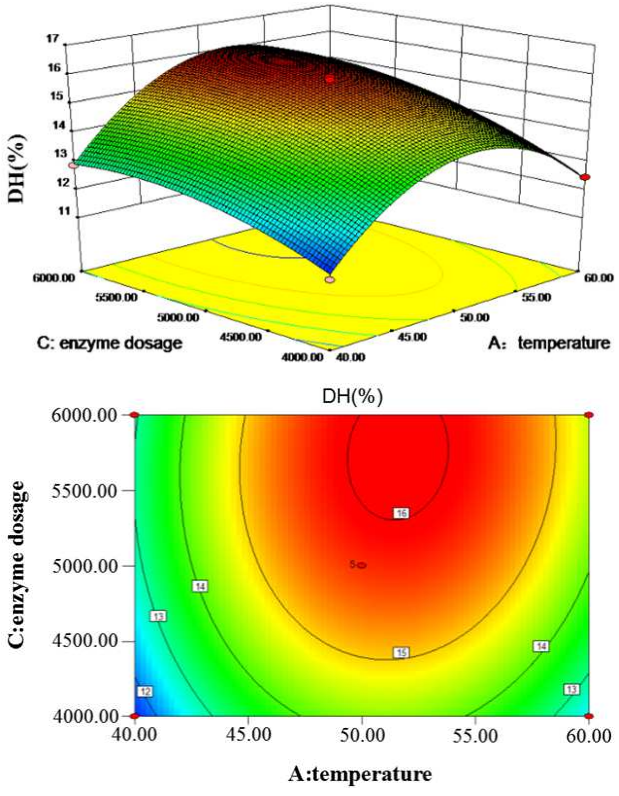

Fig. 6. Responsive surfaces and contour plot of the combined effects of temperature and enzyme dosage on DH

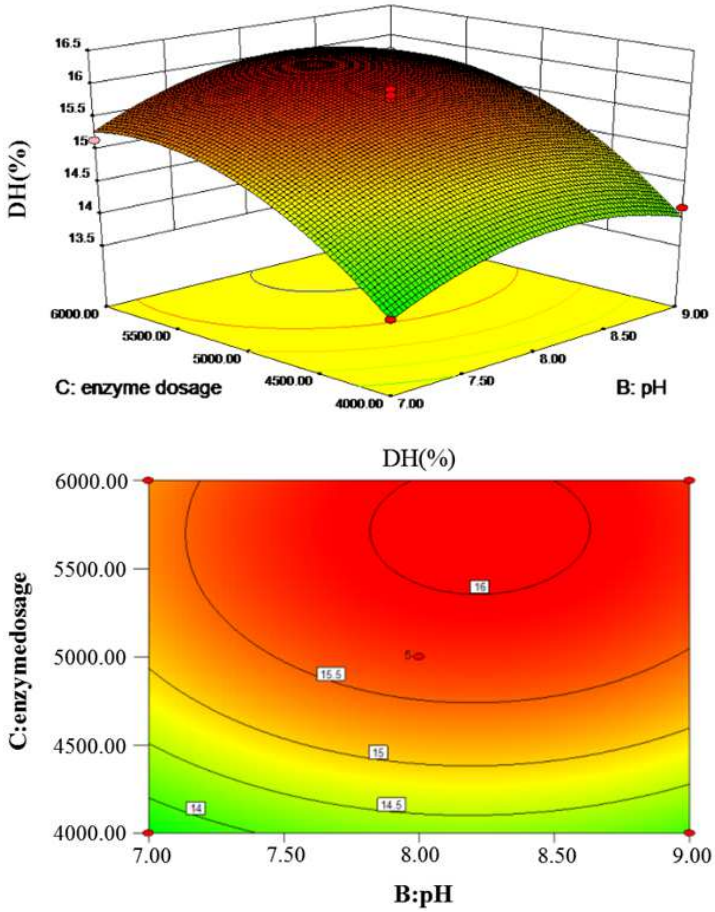

Fig. 7. Responsive surfaces and contour plot of the combined effects of pH and enzyme dosage on DH

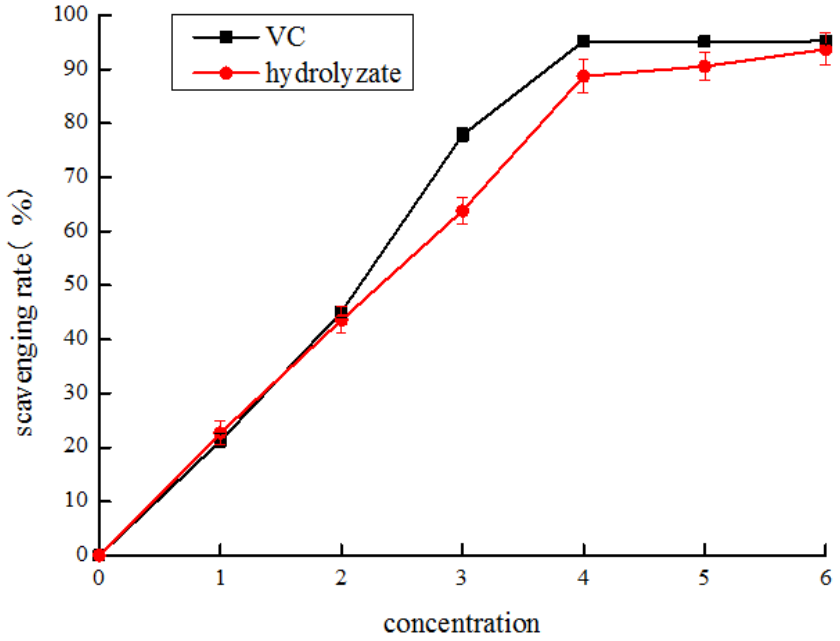

Fig. 8. The scavenging effect of different concentrations of hydrolyzate and ascorbic acid on DPPH

Antioxidant Properties of the Hydrolyzate

Scavenging Ability of the Hydrolyzate on DPPH Radicals

It is well known that DPPH is generally used to determine the free-radical scavenging ability of many materials (Wang *et al.*, 2009). From Fig. 8, the abscissa values of 0, 1, 2, 3, 4, 5 and 6 represent the concentration of 0, 5, 10, 15, 25, 50 and 100% of the hydrolyzate and 0.00, 0.02, 0.04, 0.06, 0.08, 0.10, 0.12 mg mL^{-1} of VC solution, respectively. With VC as the positive control, the scavenging rates of different concentration of VC solutions on DPPH radicals were shown in Fig. 8. The ANOVA test showed that the hydrolyzate concentration had significant effect on its scavenging ability ($p<0.05$). When the concentration ranged from 0 to 25%, the scavenging rate of hydrolyzate on DPPH radicals rapidly increased to 88.72%, which was higher than 78.56% obtained by other study (Raheleh *et al.*, 2015), because of the difference of the protease used in the two studies. In addition, antioxidant ability of sea cucumber hydrolyzate was also affected by its species (Althunibat *et al.*, 2009). In this study, the result of the scavenging rate of undiluted hydrolyzate was 93.71%, which roughly equivalent to the VC solution at the concentration of 0.08 mg mL^{-1}. Thus, the hydrolyzate had strong scavenging ability on DPPH radicals.

Scavenging Ability of the Hydrolyzate on Hydroxyl Radicals

From Fig. 9, the abscissa values of 0, 1, 2, 3, 4, 5 and 6 represent the concentration of 0, 5, 10, 15, 25, 50 and 100% of the hydrolyzate and 0.0, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 mg mL^{-1} of VC solution respectively. With VC as the positive control, the scavenging rates of different concentration of VC solutions on hydroxyl radicals were shown in Fig. 9. It can be seen from Fig. 9 that the scavenging rate of hydrolyzate on hydroxyl radicals was around 40% and there was no obvious change ($p>0.05$) as the concentration increased. The scavenging rate of undiluted hydrolyzate on hydroxyl radicals was 55.45%, which was equal to the VC solution at 1.0 mg mL^{-1}. Combined with other study (Liu *et al.*, 2012), it can be concluded that the source of scavenging ability of the hydrolyzate on hydroxyl radicals was from the polysaccharide content of the cucumber. Therefore, the hydrolyzate should be concentrated if we want to obtain the stronger scavenging capacity for hydroxyl radicals.

Scavenging Ability of the Hydrolyzate on Superoxide Radicals

The abscissa values in Fig. 10 have the same meaning as Fig. 9. With VC as the positive control, the scavenging rates of different concentration of VC solutions on superoxide radicals were shown in Fig. 10. The ANOVA test showed that the hydrolyzate concentration had significant effect on Scavenging ability of superoxide radicals ($p<0.05$). Within the scope of the test concentration, the scavenging rate of hydrolyzate on superoxide radicals increased significantly with increasing concentration and the undiluted hydrolyzate had the same ability with the 1.5 mg mL^{-1} VC solution for the scavenging on superoxide radicals, both around 68%, which verified that the scavenging ability on superoxide radicals of hydrolyzate was rather strong.

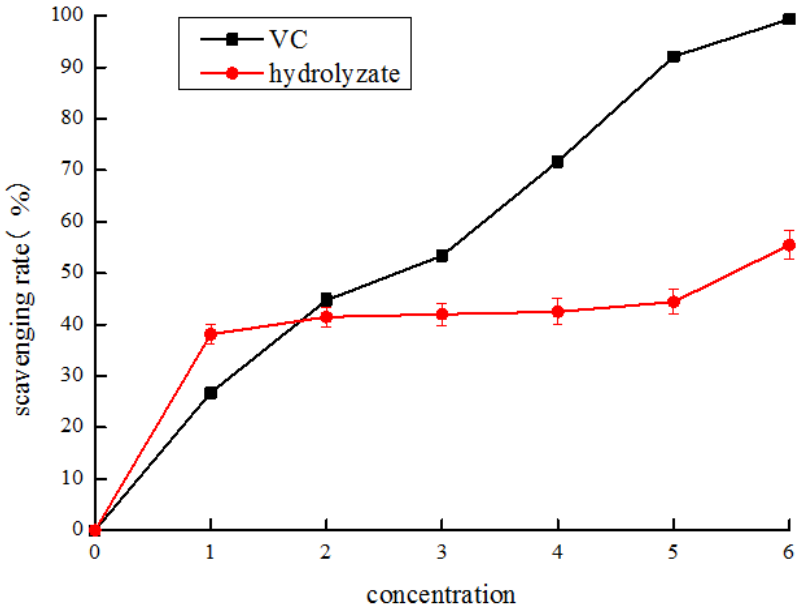

Fig. 9. The scavenging effect of different concentrations of hydrolyzate and ascorbic acid on •OH

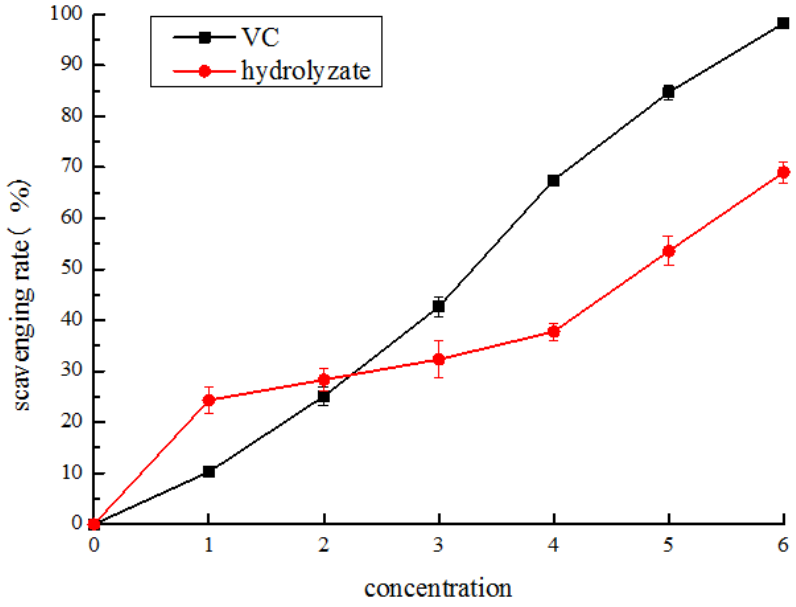

Fig. 10. The scavenging effect of different concentrations of hydrolyzate and ascorbic acid on $\bullet O_2^-$

Many contents of the sea cucumber hydrolyzate can affect its antioxidant ability (Zeng *et al.*, 2007; Zou *et al.*, 2016), which needs further study.

Conclusion

In this study, the DH of vacuum freeze-dried sea cucumber powder was performed with a three-variable, three-level Box-Behnken design based on the RSM. The statistical analysis showed that the optimal hydrolysis conditions were as follows: Enzyme dosage of 5753 U g^{-1} dry matter, hydrolysis temperature of 52°C, pH of 8.3, substrate concentration of 5% and hydrolysis time of 5 h. Under the optimal conditions, the DH was up to 16.01%, which matched well with the predicted value.

Removing polysaccharide and impurity after ethanol precipitation, the hydrolyzate of sea cucumber powder was diluted into different concentrations of polypeptide mixture and then with VC as the positive control, determined the scavenging ability on DPPH radicals, hydroxyl radicals and superoxide radicals. It was shown that the hydrolyzate has strong scavenging ability on these three radicals, the hydrolyzate that without diluting had the same scavenging ability with 0.08 mg mL^{-1} of VC solution on DPPH radicals and the scavenging capacity on hydroxyl radicals and superoxide radicals were equivalent to the 1.0 and 1.5 mg mL^{-1} of VC solution, respectively. The hydrolyzate which has a good potential for further exploitation and utilization has strong antioxidant activity and it would be used in health care products, cosmetics, medicines and other areas as the antioxidant.

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Author's Contributions

Yahui Pang: Involved in study design, sample preparation , performing in the laboratory work and writing the draft.

Jianfeng Sun: Involved in study design, manuscript writing and revising and scientific discussion.

Qian Liu: Involved in antioxidant evaluation study.

Conflict of Interest

This article is original containing unpublished materials. All authors have read and approved the manuscript and no ethical issues involved.

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