Study on optimization of extraction process and resistance to oxidation of Polypeptide from sea cucumber waste liquid

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Abstract. The protease hydrolysis technology is used to extract the polypeptides from the sea cucumber waste liquid. The enzymatic hydrolysis ability of protein in sea cucumber waste liquid by seven proteases shows that the best protease is compound protease by taking the hydrolysis degree as the index. Furthermore, the effects of enzyme dosage, pH, temperature and time of enzymatic hydrolysis on the hydrolysis degree are studied, and Box-Behnken central composite test and response surface analysis method are used to optimize the enzymatic hydrolysis conditions. The results show that the optimal enzymatic hydrolysis conditions are: enzyme dosage: 647 U/g; pH: 6.48; temperature: 52.5°C; enzymatic hydrolysis time: 3.44h; protein hydrolysis degree under the best condition: 25.9%. The polypeptides extracted from the sea cucumber waste liquid have high antioxidant activity, and the DPPH· scavenging activity increases with the polypeptide concentration.

1. Introduction
There are various active ingredients with nutritional and medicinal value in the sea cucumber, which can reinforce kidney, benefit marrow, dissolve phlegm, generate vein blood, and treat ulcer [1, 2]. There is autolytic enzyme in fresh sea cucumber body, so the enzyme shall be deactivated with boiling water during processing to preserve [3]. However, many nutritional and functional ingredients are lost during sea cucumber cooking, and the waste liquid from processing is discharged as waste liquid, which not only causes resource waste, but also brings serious environmental pollution. The research results show that protein in sea cucumber waste liquid has a higher quality than sea cucumber protein [4], and has some special functions that sea cucumber protein does not have. This paper optimizes the enzymolysis technology of protease during the sea cucumber waste liquid protein extraction, determines enzyme type, enzyme dosage, pH, temperature and time of enzymatic hydrolysis, and conducts a response surface analysis. Furthermore, the optimal conditions of the enzymolysis technology are analyzed by taking the hydrolysis degree of sea cucumber protein as the response optimization value, and the resistance to oxidation of the hydrolyzed polypeptide is studied, providing the basis for the extraction of sea cucumber polypeptides from waste liquid and related research.

2. Materials and methods

2.1. Materials
The sea cucumber (Apostichopus japonicus Selenka) waste liquid from processing is provided by Homey Group (Weihai China), which is concentrated and lyophilized to obtain solid for further test;
Compound protease, aquatic protease and trypsin are provided by Nanning DOING-HIGHER Biotechnology Co., Ltd.; neutrase, flavourzyme and alcalase are provided by Novozymes Biotechnology Co., Ltd.; nematolyt is provided by Nanning Pangbo Biological Engineering Co., Ltd.

2.2. Test method

2.2.1. Screening test of different proteases. It is necessary to prepare 5% sea cucumber waste liquid freeze-dried powder solution, and take 50ml of each enzymatic hydrolysis sample. Compound protease, nematolyt, aquatic protease, neutrase, flavourzyme, trypsin and alcalase are selected to carry out the enzymatic hydrolysis under appropriate conditions, and 500 U/g amount of protease is added as the substrate. After 4h of enzymatic hydrolysis, the enzyme is deactivated at 100°C for 10min, and centrifuged at 4000r/min for 10min, and the supernatant is collected for the determination of hydrolysis degree. The enzymatic hydrolysis for each kind of protease should be conducted three times, and the protease with the highest hydrolysis degree is selected as the index for the subsequent optimization study.

2.2.2. Single factor test. The effects of enzyme dosage, pH value, temperature and enzymatic hydrolysis time on the hydrolysis degree in the sea cucumber waste liquid are investigated. The five items of each factor are tested. After the hydrolysis, the enzyme is deactivated at 100°C for 10min, and centrifuged at 4000r/min for 10min, and the supernatant is collected for the determination of hydrolysis degree.

2.2.3. Response surface test. On the basis of single factor test and Box-Behnken central composite test design principle, Design Expert 8.0 software is used to design the four-factor and three-level test by taking enzyme dosage, pH, temperature and enzymatic hydrolysis time as independent variables, and taking the hydrolysis degree as response value. The optimal enzymatic hydrolysis conditions are optimized through the regression analysis and diagraph analysis by taking the hydrolysis degree as the response value.

2.2.4. Determination of hydrolysis degree. Refer to the TNBS method for the determination of hydrolysis degree [5, 6].

\[
DHI = \frac{H_2 - H_1}{H_0 - H_2} \times 100\%
\]

where \(H_2\): amino nitrogen concentration after reaction /mmol·g\(^{-1}\); \(H_1\): amino nitrogen concentration before reaction /mmol·g\(^{-1}\); \(H_0\): protein concentration per gram of waste liquid powder /mmol·g\(^{-1}\) (7.7mmol·g\(^{-1}\) measured in the test)

2.2.5. Determination of resistance to oxidation. After the enzymatic hydrolysis for the sea cucumber waste liquid protein is conducted through the compound protease, the polypeptide is obtained from the enzymatic hydrolysate by ultrafiltration and purification, and the solid sample is obtained after freeze-drying. DPPH free radical scavenging rate is used to determine the resistance to oxidation of polypeptide from sea cucumber waste liquid [7, 8].

\[
AOA = \left( \frac{A_x - A_0}{A_0} \right) \times 100\%
\]

where AOA: free radical scavenging rate /%; \(A_x\): sample absorbance; \(A_0\): absorbance of blank group; \(A_0\): absorbance of control group.
3. Results and discussion

3.1. Effects of protease type on hydrolysis degree

The enzymatic hydrolysis for the sea cucumber is conducted through all proteases under their own suitable conditions. The protein hydrolysis degree is different due to the different specificity of different proteases for sea cucumber waste liquid protein and different enzyme cutting sites. The results are shown in Fig. 1. It can be seen from Fig. 1 that under the same conditions, the hydrolysis degree of compound protease is the highest, followed by alcalase. In order to achieve the best enzymatic hydrolysis effect, compound protease with the highest hydrolysis degree is selected for the subsequent test of the enzymatic hydrolysis conditions.

![Figure 1. Hydrolysis effect of different proteases. A: Complex protease; B: Papain; C: Aquatic protease; D: Neutral protease; E: Flavourzyme; F: Trypsin; G: Alkaline protease](image)

3.2. Single factor test analysis

In order to study optimal enzymatic hydrolysis conditions of compound protease, the single factor test is conducted to investigate the effects of enzyme dosage, pH, temperature and enzymatic hydrolysis time on the hydrolysis degree of sea cucumber protein (Fig. 2 a-d).
Figure 2. Effects of different conditions on hydrolysis degree. a. Enzyme dosage; b. pH; c. Temperature; d. Time.

Figure 2a shows the effects of enzyme dosage on the substrate hydrolysis. The results indicate that when the enzyme dosage is 600-800 U/g, the substrate hydrolysis effect is the best, because the contact opportunities between the substrate and the enzyme will increase with the increase of the enzyme dosage, and the enzymatic reaction will also increase gradually when the substrate is fixed. When the concentration of enzyme substrate is saturated, the enzymatic reaction rate reaches the maximum value, and then there will be a stable period when the concentration of enzyme increases, namely, the zero-order reaction of enzymatic reaction. If the enzyme dosage is increased continuously, the system fluidity will be poor, which will affect the diffusion movement of molecules, and the reaction rate will show a downward trend [9].

Figure 2b shows the effects of pH on the enzymatic reaction. The optimal pH value is 6.6, under which hydrolysis degree decreases greatly because the enzyme is a special protein, and the enzymatic reaction rate is closely related to pH. When pH reaches a certain value, the enzyme activity is the highest, and the enzymatic reaction rate is the maximum. When pH is higher or lower than this value, the natural conformation of the enzyme and the dissociative state of the enzyme and the substrate will be affected, thus affecting the stability, inhibiting the enzyme activity, and even inactivation [10]. The temperature can also affect the enzyme activity.

It can be seen from Fig. 2c that at 30-50 °C, the substrate hydrolysis degree increases rapidly with the increase of temperature, and decreases when the temperature is higher than 50°C because in a certain temperature range, enzyme activity will increase, and enzymatic reaction will accelerate with the increase of temperature. The enzymatic hydrolysis rate reaches the maximum value, and then continues to rise the temperature when the temperature reaches the optimal enzymatic reaction temperature. The enzyme activity decreases and the natural conformation is destroyed, leading to the denaturation, even permanent inactivation of enzyme, and reducing the enzymatic hydrolysis rate [11].

Fig. 2d shows that the enzymatic hydrolysis time also affect substrate hydrolysis degree. In the first 3h, the hydrolysis degree increases rapidly with the increase of the enzymatic hydrolysis time, and then increases slowly after 3h because the enzyme and substrate gradually combine with the continuous reaction, and catalytic reaction is in the positive direction. When the active site of the enzyme is saturated with the substrate, the reaction tends towards equilibrium, but when the reaction time continues to increase, the concentration of the enzymatic hydrolysis product will increase, and competitive inhibition will be stronger, so the enzymatic reaction will begin to decline [11]. Therefore, the optimal hydrolysis conditions can be obtained by single factor test: enzyme dosage: 600 U/g; pH: 6.8; temperature: 50°C; hydrolysis time: 3h.
3.3. Analysis and optimal process results of response surface analysis

3.3.1. Response surface design and analysis. Based on the single factor test results, Box-Behnken central composite test design principle is used to design the four-factor and three-level response surface test (27 test points in total) by taking enzyme dosage (A), pH (B), temperature (C) and enzymatic hydrolysis time (D) as test factors, and taking hydrolysis degree as response value.

Table 1. Response Surface Analysis Scheme and Test Results

| S/N | Enzyme dosage (A) | pH (B) | Temperature (C) | Time (D) | Hydrolysis degree (y) | S/N | Enzyme dosage (A) | pH (B) | Temperature (C) | Time (D) | Hydrolysis degree (Y) |
|-----|------------------|--------|-----------------|---------|-----------------------|-----|------------------|--------|-----------------|---------|-----------------------|
| 1   | 400              | 5.8    | 50              | 3       | 20.58                 | 15  | 600              | 7.4    | 40              | 3       | 22.86                 |
| 2   | 400              | 7.4    | 50              | 3       | 22.5                  | 16  | 600              | 7.4    | 60              | 3       | 24.6                  |
| 3   | 800              | 5.8    | 50              | 3       | 23.49                 | 17  | 400              | 6.6    | 40              | 3       | 18.78                 |
| 4   | 800              | 7.4    | 50              | 3       | 23.1                  | 18  | 400              | 6.6    | 60              | 3       | 22.83                 |
| 5   | 600              | 6.6    | 40              | 2       | 20.22                 | 19  | 800              | 6.6    | 40              | 3       | 23.82                 |
| 6   | 600              | 6.6    | 40              | 4       | 23.1                  | 20  | 800              | 6.6    | 60              | 3       | 22.92                 |
| 7   | 600              | 6.6    | 60              | 2       | 22.8                  | 21  | 600              | 5.8    | 50              | 2       | 22.17                 |
| 8   | 600              | 6.6    | 60              | 4       | 24.45                 | 22  | 600              | 5.8    | 50              | 4       | 24.63                 |
| 9   | 400              | 6.6    | 50              | 2       | 20.1                  | 23  | 600              | 7.4    | 50              | 2       | 25.14                 |
| 10  | 400              | 6.6    | 50              | 4       | 22.5                  | 24  | 600              | 7.4    | 50              | 4       | 23.07                 |
| 11  | 800              | 6.6    | 50              | 2       | 22.95                 | 25  | 600              | 6.6    | 50              | 3       | 25.17                 |
| 12  | 800              | 6.6    | 50              | 4       | 24.95                 | 26  | 600              | 6.6    | 50              | 3       | 25.8                  |
| 13  | 600              | 5.8    | 40              | 3       | 19.44                 | 27  | 600              | 6.6    | 50              | 3       | 25.5                  |
| 14  | 600              | 5.8    | 60              | 3       | 24.45                 |     |                  |        |                 |         |                      |

Design-Expert is used to carry out the multiple regression fitting of the test data, and the quadratic multiple regression model of four factors selected for the proteolysis of sea cucumber waste liquid is obtained:

\[
Y = 25.59 + 1.16A + 0.54B + 1.15C + 0.78D - 0.58AB - 1.24AC - 0.1AD - 0.82BC - 1.13BD - 0.31CD - 1.91A^2 - 0.97B^2 - 1.69C^2 - 0.96D^2, R^2=0.9282.
\]

The model fitting is good. Table 2 shows the variance analysis results of the regression equation of the response surface test. It can be seen from Table 2 that the model \(P<0.0001\), which is extremely significant, and the mismatch \(P>0.05\), which is not significant, indicating that the model is relatively stable. A, B, C, D, AC, BC, BD, A2, B2, C2 and D2 have a significant effect on the enzymatic hydrolysis, but the interaction items (AB, AD and CD) have no significant effect on the enzymatic hydrolysis. The effect order of each factor on protein hydrolysis degree is as follows: enzyme dosage (A) > temperature (C) > enzymatic hydrolysis time (D) > pH (B).
Table 2. Variance Analysis of Regression Equation

| Source | Sum of Squares | df | Mean Square | F Value | P Value | P Value   |
|--------|---------------|----|-------------|---------|---------|-----------|
| Model  | 86.18         | 14 | 6.16        | 11.09   | <0.0001 | significant |
| A      | 16.19         | 1  | 16.19       | 29.17   | 0.0002  |           |
| B      | 3.53          | 1  | 3.53        | 6.36    | 0.0268  |           |
| C      | 15.94         | 1  | 15.94       | 28.71   | 0.0002  |           |
| D      | 7.24          | 1  | 7.24        | 13.04   | 0.0036  |           |
| AB     | 1.33          | 1  | 1.33        | 2.40    | 0.1470  |           |
| AC     | 6.13          | 1  | 6.13        | 11.04   | 0.0061  |           |
| AD     | 0.040         | 1  | 0.040       | 0.072   | 0.7929  |           |
| BC     | 2.67          | 1  | 2.67        | 4.82    | 0.0486  |           |
| BD     | 5.13          | 1  | 5.13        | 9.24    | 0.0103  |           |
| CD     | 0.38          | 1  | 0.38        | 0.68    | 0.4252  |           |
| A^2    | 20.43         | 1  | 20.43       | 36.86   | <0.0001 |           |
| B^2    | 5.53          | 1  | 5.53        | 9.96    | 0.0083  |           |
| C^2    | 16.12         | 1  | 16.12       | 29.03   | 0.0002  |           |
| D^2    | 5.46          | 1  | 5.46        | 9.84    | 0.0086  |           |
| Residual | 2.06      | 12 | 0.56        |         |         |           |
| Lack of Fit | 2.06   | 10 | 0.64        | 4.83    | 0.1834  | not significant |
| Pure Error | 0.053   | 2  | 0.13        |         |         |           |
| Cor Total | 92.84     | 26 |             |         |         |           |

3.3.2. Response surface graph analysis. Figure 3a-f shows the effects of the interaction of enzyme dosage (A), pH (B), temperature (C) and enzymatic hydrolysis time (D) on the enzymatic hydrolysis of the protein in the sea cucumber waste liquid. The result analysis indicates that the optimal enzyme dosage, pH, temperature and time are all within the set test range, and the optimal test conditions can be obtained by using Design-Experpt software optimization and selecting maximum response value within the range of test factors: enzyme dosage: 647 U/g; pH: 6.48; temperature: 52.5°C; enzymatic hydrolysis time: 3.44h; predicted optimal hydrolysis degree: 25.9%.
3.3.3. Proof test. In order to verify the reliability of the predicted regression equation results, it is necessary to carry out the proof test under the optimal enzymatic hydrolysis conditions (enzyme dosage: 647 U/g; pH: 6.48; temperature: 52.5°C; enzymatic hydrolysis time: 3.44h). In view of the practical operation feasibility, the optimal enzymatic hydrolysis conditions are as follows: enzyme dosage: 650 U/g; pH: 6.5; temperature: 53°C; enzymatic hydrolysis time: 200 min. The test is conducted 3 times. Actual hydrolysis degree under this condition is 25.8%, which is basically consistent with the theoretically predicted value of 25.9%, indicating that the predicted model value is highly consistent with the actual situation, and the response surface analysis method is accurate and feasible for the prediction of the optimal process.
3.4. Study on resistance to oxidation of polypeptide

Figure 4 shows the DPPH· scavenging rate of different concentrations of polypeptides in sea cucumber waste liquid. DPPH scavenging activity increases with the increase of polypeptide concentration within the range of mass concentration of 0-120 mg/mL. When the polypeptide concentration increases to 80 mg/mL, DPPH· scavenging rate reaches 58.1%, and it will increase slowly as the polypeptide concentration continues to increase.

![Figure 4. DPPH· Scavenging Rate of Different Concentration of Polypeptides](image)

4. Conclusions

In this paper, enzymatic hydrolysis of protein in sea cucumber waste liquid is studied to determine that compound protease is the best enzyme. The single factor test, Box-Behnken central composite test and response surface analysis are used to optimize the enzymatic hydrolysis conditions, and the results show that the optimal enzymatic hydrolysis conditions are: enzyme dosage: 647 U/g; pH: 6.48; temperature: 52.5°C; enzymatic hydrolysis time: 3.44h; protein hydrolysis degree under the best condition: 25.9%. The optimal enzymatic hydrolysis conditions are obtained through the test, which will be helpful for the further utilization of sea cucumber waste liquid. The polypeptides from the enzymatic hydrolysis have high antioxidant activity, and DPPH· scavenging activity increases with the increase of polypeptide concentration. When the polypeptide concentration is 80 mg/mL, DPPH· scavenging rate will reach 58.1%.

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