Influencing Factors of Conditions for Autolysis of Perna Viridis Protein

Xin Chen¹*, Feng Xu¹, Deke Chen², Hua Chen² and Huili Sun²

¹ School of Environment and Chemical Engineering, Foshan University, Foshan, Guangdong, China
² Nanhai Institute of Chinese Academy of Sciences, Guangzhou, Guangdong, China

*Corresponding author e-mail: 670511263@qq.com

Abstract. The optimal enzymatic conditions obtained by L9 (34) orthogonal experiments were a hydrolysis time of 5 h, hydrolysis temperature of 50 ℃, initial pH of 7, and solid/liquid ratio of 1:3. The autolysis conditions of P. viridis protein were studied as a function of the interactions of multiple factors. The reaction pH was the main factor that affected the degree of hydrolysis, and the degree of hydrolysis reached a maximum at pH 7. Changing the pH made a significant difference in the degree of hydrolysis, followed by time and the solid/liquid ratio, with the effect of temperature on the degree of hydrolysis being minimal. The comprehensive analyses revealed that the optimal reaction conditions were a pH of 7, time of 5 h, solid/liquid ratio of 1:3.5, and temperature of 50 °C. Under these conditions, the degree of hydrolysis of the autolytic hydrolysate of P. viridis protein was 23.37%, and the protein recovery was 87.90%.

1. Introduction
The Asian green mussel (Perna viridis) is an important Chinese herbal medicine. It is rich in protein, glycogen, and a variety of amino acids and vitamins. It can nourish yin and kidney, and nourish the blood for regulating menstruation. It has beneficial effects in improving blood lipids, resisting fatigue, and improving immunity and other functions [1-2].

As such, relying on fresh sales is insufficient, and it is necessary to further process P. viridis. In addition to fresh sales, P. viridis is sometimes dried or frozen. Because these products are made using traditional methods, they are not processed in a cost-effective manner [3-4].

The optimal conditions for the autolysis of P. viridis were determined using orthogonal experiments to achieve the optimal hydrolysis effect, which provided basic data for the high-value utilization of this material.

2. Experimental materials and methods

2.1 Determination of the free amino nitrogen content
The content of free amino acids was determined by neutral formaldehyde potentiometric titration according to the method of Zhao [5]. First, 10.0 g of P. viridis enzymatic solution was weighed and placed in a 200-mL beaker. Then, 60 mL of distilled water was added, a magnetic stirrer was turned on, and the resulting solution was titrated with a standard solution of sodium hydroxide (0.05 M) until the pH reached 8.2. Next, 10.0 mL of 20% neutral formaldehyde solution was added to the solution
with stirring. The solution was further titrated with a standard sodium hydroxide solution (0.05 M) until the pH was 9.2, and the volume (V1) of the sodium hydroxide standard titration solution (0.05 M) used was recorded.

Meanwhile, 80 mL of distilled water was added to another 200-mL beaker. First, a standard sodium hydroxide solution (0.05 M) was added dropwise into the solution until the pH reached 8.2, and then 10.0 mL of 20% neutral formaldehyde solution was added with stirring. The standard sodium hydroxide solution (0.05 M) was then added until the pH reached 9.2, and the volume (V2) of the standard sodium hydroxide standard solution was recorded. This was used as the reagent blank experiment. After completing the procedure, the amino nitrogen content was calculated according to the following Formula 1.

\[
\text{Amino acid nitrogen (mg/L) = } \frac{(V_1 - V_2) \times c \times 0.014 \times 100}{m \times 20/100}
\]

where \( V_1 \) (mL) is the volume of the standard sodium hydroxide solution consumed by the sample dilution after titrating the sample dilution with formaldehyde to the end point (pH 9.2); \( V_2 \) (mL) is the volume of the standard sodium hydroxide solution consumed by the blank test after titrating with formaldehyde to the end point (pH 9.2); \( C \) is the concentration (M) of standard sodium hydroxide; \( m \) (g) is the solution equivalent to the mass of the sample; \( M \) (g) is the sample solution corresponding to the mass of the sample; and 0.014 (mg/mL) is the millimol mass of nitrogen atom.

2.2 Determination of the total protein content

The total protein content of \( P. \) \textit{viridis} was determined using the Kjeldahl method [6]. The principle of the Kjeldahl method involves heating and digesting the sample with concentrated sulfuric acid and catalyst to decompose protein. Carbon and hydrogen are oxidized into carbon dioxide and water, and organic nitrogen is converted into ammonia and sulfuric acid to form ammonium sulfate. Next, alkali is added to the sample and distilled to remove ammonia, and the sample is titrated with a standard hydrochloric acid solution or sulfuric acid solution after absorbing with boric acid. Finally, the protein content is calculated based on the standard acid consumption.

First, 1 g of sample was weighed (accurate to 0.001 g) and placed in a dry Kjeldahl flask to prevent sticking to the inner wall of the bottleneck, and 10 g of mixed catalyst and 25 mL of sulfuric acid were added to the flask. The flask was shaken slightly to completely moisten the sample, and the flask was slowly heated in a digestion oven, and then quickly heated to boiling after the foam in the bottle disappeared. When no charcoal was observed on the wall of the bottle and the liquid in the bottle was clear and light green, the solution was then continuously heated for 30 min until it was completely decomposed.

The sample solution, which was digested and cooled to room temperature, was fixed in a Kjeldahl flask at the alkali outlet of the nitrogen analyzer. First, 20 mL of 2% boric acid solution and 2–3 drops of the mixed indicator solution were added to the 100-mL receiving bottle, and the receiving bottle, whose lower mouth was immersed into the boric acid receiving solution, was placed at the end of the condenser tube. After opening the nitrogen analyzer, 20–30 mL of 40% sodium hydroxide solution (excess) was added to the Kjeldahl flask, and then steamed for 5 min. The receiving bottle was positioned lower to remove the end of condenser tube from the liquid surface, and then it was continuously distilled for 1 min. The end of condenser was then rinsed with a small amount of distilled water, the washing solution was finally collected in the receiving bottle, and the receiving bottle was removed.

A standard 0.01 M hydrochloric acid or sulfuric acid solution was used to titrate the liquid in the receiving bottle just until a grayish purple color appeared, and the consumed volume (mL) of the standard 0.01 M hydrochloric acid or sulfuric acid solution was recorded. At the end of the experiment, the total protein content was calculated according to the following formula:
\[ X = \frac{(V_1 - V_2) \times c \times 0.014}{W} \times 100 \]  

(2)

where \( X \) (%) is the protein content of the sample; \( W \) (g) is the quality of the sample; \( V_1 \) (mL) is the volume of the salt standard solution consumed by the titration sample; and \( V_0 \) is the volume (mL) of the standard solution of hydrochloric acid consumed by the blank titration:

- \( C \)-concentration of standard solution of hydrochloric acid (mol/L);
- 0.014: 1mL, 1mol/L - the quality of hydrochloric acid is equal to that of Equation 2.

2.3 Calculation of the degree of hydrolysis
The content of free amino nitrogen and total protein was substituted into Equation 3 to obtain the degree of hydrolysis.

\[ \text{Degree of hydrolysis (\%)} = \frac{\text{Free amino nitrogen content}}{\text{Total protein content}} \times 100 \]  

(3)

2.4 Determination of the protein recovery rate
According to a previous study [7], 5 mL of \( P. \) viridis protein hydrolysate was centrifuged, and the total protein content in the supernatant was determined using the Kjeldahl method. In addition, 5 mL of \( P. \) viridis protein hydrolysate was sampled without centrifugation, and the total protein content was determined. The two results were substituted into Equation 4 to calculate the protein recovery.

\[ \text{Protein recovery rate (\%)} = \frac{\text{Total protein content in the supernatant}}{\text{Total protein content in the hydrolysate before centrifuging}} \times 100 \]  

(4)

3. Experimental results and analysis

3.1. Orthogonal experimental design to determine the optimal conditions for the autolysis of \( P. \) viridis protein
According to the experimental parameters obtained for the studied factors in the single-factor experiments, the autolysis conditions of \( P. \) viridis protein were studied as a function of the interactions of multiple factors. The temperature, pH, time, solid/liquid ratio, and optimal autolysis conditions at three levels of four factors were determined in orthogonal experiments in Table 1.

| Experiment No | Time (h) | Temperature (°C) | pH | Solid/liquid ratio |
|---------------|----------|------------------|----|-------------------|
| 1             | 4        | 40               | 6  | 1:2.5             |
| 2             | 5        | 50               | 7  | 1:3.0             |
| 3             | 6        | 60               | 8  | 1:3.5             |

3.2. Results of the orthogonal study
Taking the degree of hydrolysis (%) as the index and using the time (A), pH (B), temperature (C), and solid/liquid ratio (D) as the controlling factors, an orthogonal experiment was conducted to optimize the degree of hydrolysis. The results are shown in Table 2 and Figure 1.

| Table 2. Results of the \( L_9(3^4) \) orthogonal experiment on the hydrolysis conditions of \( Perna \) viridis autolysis. |
| Experiment No. | A | B | C | D | Degree of hydrolysis (%) |
|---------------|---|---|---|---|---------------------------|
| 1             | 4 | 6 | 40| 1:2.5 | 20.98                     |
| 2             | 4 | 7 | 50| 1:3.0 | 22.62                     |
| 3             | 4 | 8 | 60| 1:3.5 | 21.30                     |
| 4             | 5 | 6 | 50| 1:3.5 | 22.96                     |
| 5             | 5 | 7 | 60| 1:2.5 | 23.28                     |
| 6             | 5 | 8 | 40| 1:3.0 | 21.55                     |
| 7             | 6 | 6 | 60| 1:3.0 | 21.60                     |
| 8             | 6 | 7 | 40| 1:3.5 | 22.92                     |
| 9             | 6 | 8 | 50| 1:2.5 | 20.75                     |
| K₁            | 64.9 | 65.54 | 65.45 | 65.01 |
| K₂            | 67.79 | 68.82 | 66.33 | 65.77 |
| K₃            | 65.27 | 63.6 | 66.18 | 67.18 |
| k₁            | 21.63 | 21.85 | 21.82 | 21.67 |
| k₂            | 22.60 | 22.94 | 22.11 | 21.92 |
| k₃            | 21.76 | 21.20 | 22.06 | 22.39 |
| Range         | 0.96 | 1.74 | 0.29 | 0.72 |

Rank of affecting factors: \( B > A > D > C \)

Optimization level: \( B2A2D3C2 \)
Figure 1. Results of the $L_9(3^4)$ orthogonal experiment on the hydrolysis conditions of *Perna viridis* autolysis.

Seen from Table 2 and Figure 1, the reaction pH was the main factor that affected the degree of hydrolysis (Table 2), and the degree of hydrolysis reached a maximum at pH 7. Changing the pH made a significant difference in the degree of hydrolysis, followed by time and the solid/liquid ratio, with the effect of temperature on the degree of hydrolysis being minimal. The comprehensive analyses revealed that the optimal reaction conditions were a pH of 7, time of 5 h, solid/liquid ratio of 1:3.5, and temperature of 50°C. The confirmatory experiments showed that when using the optimal reaction conditions, the degree of *P. viridis* protein hydrolysis was 23.37%, which was higher than the maximum values obtained in the single-factor experiments. At the same time, by testing the protein recovery rate of the hydrolysate in the experiments measuring the autolytic enzyme hydrolysis of *P. viridis* protein based on the optimal reaction conditions, the protein recovery was as high as 87.90%, indicating the feasibility of identifying the optimal reaction conditions.

4. Conclusions

The orthogonal experiments were carried out to optimize the autolysis conditions, and comprehensive results of the effects on the rate of hydrolysis were determined. The optimum conditions for autolysis of *P. viridis* were a temperature of 50°C, time of 5 h, liquid/solid ratio of 1:3.5, and pH of 7. Under the optimum reaction conditions, the extent of hydrolysis of *P. viridis* protein was 23.37% and the recovery rate of protein was 87.90%.

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