INVESTIGATION INTO ANTIOXIDANT ACTIVITY OF PROTEIN HYDROLYSATE DERIVED FROM WHITE LEG SHRIMP HEAD (*LITOPENAEUS VANNAMEI*)

KHẢO SÁT HOẠT TÍNH KHÁNG OXY HÓA CỦA DỊCH ĐẠM THỦY PHÂN TỪ ĐẦU TÔM THẺ CHÂN TRẮNG (*LITOPENAEUS VANNAMEI*)

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Abstract - The antioxidant activity of protein hydrolysate from white leg shrimp head (WLSH) is investigated in this research. Firstly, chemical composition of the shrimp head is analyzed. Then, the effect of WLSH: water ratio on protein recovery yield and effect of enzyme type, pH, temperature, enzyme to substrate (E:S) ratio and hydrolysis time on antioxidant potential of the proteolysate are examined. Next, response surface methodology (RSM) is employed to optimize hydrolysis through the E:S ratio and hydrolysis time. The result show that the WLSH contains 81.4 ± 0.3% moisture, 55.9 ± 0.6% protein, 4.3 ± 0.2% lipid and 23.1 ± 0.2% ash (on dry weight basis). The protein recovery yield achieves 4.25 ± 0.14% with the WLSH: water ratio of 1:4 (w/v). The optimal hydrolysis condition includes Flavourzyme, pH 7, 45^{o}C, E:S ratio of 54.87 U/g protein, hydrolysis time of 4.23 hours and the DPPH scavenging activity of the protein hydrolysate reaches 80.74%. This study suggests a new way of utilizing the WLSH, antioxidant proteolysate which could be applied as a functional food or natural antioxidant additive replacing synthetic compounds.

Key words - white leg shrimp head; antioxidant activity; proteolysate; protein hydrolysate; enzymatic hydrolysis.

Tóm tắt - Nghiên cứu này khảo sát hoạt tính kháng oxy hóa của dịch thủy phân protein từ đầu tôm thẻ chân trắng (*Litopenaeus vannamei*) (WLSH). Trước tiên, thành phần hóa học của WLSH được xác định. Tiếp theo, ảnh hưởng của tỷ lệ WLSH:nước đến hiệu suất thu hồi protein và ảnh hưởng của loại enzyme, pH, nhiệt độ, tỷ lệ enzyme:cơ chất (E:S) và thời gian thủy phân đến hoạt tính kháng oxy hóa được khảo sát. Phương pháp bề mặt đáp ứng được sử dụng để tối ưu hóa tỷ lệ E:S và thời gian nhằm thu dịch có hoạt tính kháng oxy hóa cao nhất. Kết quả cho thấy, WLSH chứa 81,4 ± 0,3% ẩm, 55,9 ± 0,6% protein, 4,3 ± 0,2% lipid và 23,1 ± 0,2% tro (theo hàm lượng chất khô). Hiệu suất thu hồi protein đạt 4,25 ± 0,14% với tỷ lệ WLSH : nước 1:4 (w/v). Với điều kiện thủy phân tối ưu, hoạt tính nhốt DPPH đạt 80,74%. Nghiên cứu này đề xuất hướng sử dụng mới cho WLSH như dịch thủy phân có hoạt tính kháng oxy hóa, có thể dùng như thực phẩm chức năng hoặc phụ gia kháng oxy hóa tự nhiên thay cho hợp chất tổng hợp.

Từ khóa - Đầu tôm thẻ chân trắng; kháng oxy hóa; dịch thủy phân; hoạt tính sinh học; thủy phân enzyme.

1. Introduction

In certain circumstances, antioxidant defense systems fail to protect the body against reactive radicals, leading to oxidative stress which causes numerous diseases such as cancer, atherosclerosis, diabetes, arthritis, coronary heart disease, and Alzheimer's disease. In food industry, free radicals shorten the shelf life and reduce the quality as well as the safety of food product through causing the oxidation of lipid, releasing undesirable secondary lipid peroxidation products [1]. These negative effects could be prevented by using antioxidant compounds which could delay or inhibit oxidation of a substance. Although synthetic antioxidant substances including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), t-butylhydroquinone (TBHQ) and propyl gallate are cost-effective and show high antioxidant potential, they display several toxic and hazardous effects and their use is restricted in some countries. Hence, the search for safe, natural antioxidant compounds has attracted many scientists. Peptides from aquatic product and by-product have been clearly demonstrated to possess the capability of scavenging free radicals and reactive oxygen species or preventing oxidative damage by interrupting the radical chain reaction of lipid peroxidation [1].

Huge amounts of shrimp head which accounts for approximately 35–45% of the whole shrimp weight [2] are discarded each year from shrimp processing industries. Only a little quantity of the byproduct is actually used in some ways, mostly for animal feed or paste while the remainder represents an environmental problem. Recently, shrimp head has been considered to be a rich source of protein, chitin, carotenoid and enzymes, which show potential to be recovered as marketable products [2].

The objectives of this study are to (i) analyze the chemical composition of the WLSH; (ii) investigate the effect of WLSH: water on protein recovery yield and effect of hydrolysis condition on the antioxidant activity of protein hydrolysate; (iii) optimize the hydrolysis through E:S ratio and hydrolysis time for maximizing the antiradical activity of the proteolysate using RSM.

2. Materials and methods

2.1. Materials

2.1.1. WLSH

WLSH used in this study is provided by a local shrimp processing plant in Long An province, Vietnam. The by-products are transported on ice to the Biochemical laboratory of Ho Chi Minh City University of Technology within 4 hours, packed in polyethylene bags, labelled and stored -20^{o}C until used.

2.1.2. Enzyme preparations and chemicals

Proteases including Alcalase, Neutrase, Protamex, Flavourzyme and Corolase were obtained from Novozymes (Denmark) and AB enzymes (Germany). Optimal working temperature and pH of these enzymes were shown in Table 1. Chemicals were purchased from Sigma–Aldrich and Merck. All reagents were of analytical

grade. Double–distilled water was used in experiments.

Table 1. *Optimal working pH and temperature of the enzymes used in this study*

Enzyme	Optimal pH	Optimal Temperature
Alcalase	7.5	55°C
Neutrase	8	50°C
Protamex	6.5	50°C
Corolase	7	55°C
Flavourzyme	7	50°C

2.2. Methods

2.2.1. Chemical composition analysis

The contents of moisture, crude protein, crude fat and ash of the WLSH are determined based on the methods of AOAC (2000) [3]. The total crude protein content is determined using Kjeldahl method with Nitrogen conversion factor of 6.25.

2.2.2. Preparation of protein hydrolysates from shrimp head waste

The preparation of hydrolysates is performed according to the procedure of Bhaskar and Mahendrakar [4] with slight modification. Water is added with the desired ratio and the mixture is heated at 90°C for 10 minutes to deactivate endogenous enzymes. Desired enzyme is added after pH value is controlled using 1M NaOH or HCl solution. After the required hydrolysis time, the reaction is terminated by heating the hydrolysate for 10 min at 90°C to deactivate the enzyme. The hydrolysate is then centrifuged to collect the supernatant. The obtained supernatants are freeze-dried using freeze-dryer (Alpha 1-2/Ldplus, UK) and stored at -20°C until used.

2.2.3. Effect of WLSH: water ratio on protein recovery yield

Protein recovery yield is determined by the percentage of protein content in the protein hydrolysate compared to the crude protein content of the WLSH. For this experiment, Alcalase is used for hydrolysis at its recommended pH and temperature (Table 1), E:S ratio of 30 U/g protein, hydrolysis time of 4 hours and WLSH: water ratio in range from 1:1 to 1:5 (w/v).

2.2.4. Effects of hydrolysis condition on the antioxidant activity of protein hydrolysate from shrimp head waste

The effect of enzyme type, pH, temperature, E:S ratio and hydrolysis time on the antioxidant capacity of the protein hydrolysate are examined using single factor test method which is performed by one factor varied with different levels while other factors are fixed [5].

2.2.5. Determination of DPPH radical-scavenging capacity

The DPPH radical scavenging capacity is assayed employing the method of Sharma and Bhat [6] with slight modification. The mixture of sample and DPPH is incubated in the dark at room temperature for 30 minutes. The absorbance at 517 nm is determined by a spectrophotometer. The scavenging activity is calculated with the following formula:

$$DPPH\ scavenging\ activity\ (\%) = \frac{A_0 - (A_1 - A_2)}{A_0} * 100 \quad (1)$$

Where A_0 denotes the absorbance of the blank (distilled water instead of sample), A_1 is the absorbance of the mixture containing sample, and A_2 is the absorbance of the mixture without DPPH.

2.2.6. FRAP assay

A modified method of Benzie and Strain [7] is used to determine the ferric reducing capacity of hydrolysates. According to this method, at low pH, a colorless ferric complex (Fe^{3+}-tripyridyltriazine) is reduced to a blue-colored ferrous complex (Fe^{2+}-tripyridyltriazine) by the action of electron-donating antioxidants. By measuring the change of absorbance at 593 nm, the reduction is monitored.

2.2.7. Optimization of E: S ratio and hydrolysis time for maximizing the antioxidant activity of the protein hydrolysate from shrimp head waste

A randomised, quadratic central composite circumscribe response surface design is used to optimize E:S ratio and hydrolysis time. The dependant variable is antioxidant activity of the hydrolysate. The Modde software (version 5.0) is used to generate experimental planning and to process data. Each factor in the design is investigated at five different levels ($-\sqrt{2}$, -1, 0, +1, $+\sqrt{2}$). The total number of experiments is 13 and the number of central experiments is 5.

2.2.8. Statistical Analysis

Data is presented as means ± standard deviations of triplicate determinations. Analysis of variance (one-way ANOVA) is performed on the data, and the significance is determined using Tukey method ($p<0.05$). These analyses are performed using the Statgraphics Centurion 18 software.

3. Results and discussion

3.1. Proximate composition analysis of the WLSH

Chemical compositions of the WLSH include 81.4 ± 0.3% moisture, 55.9 ± 0.6 % crude protein, 4.3 ± 0.2 % crude lipid and 23.1 ± 0.2 % ash (on dry weight basis). The protein content is consistent with that in the previous study of Guerard et al. [8]. The high protein content of the WLSH exhibits a potential to be a source of bioactive peptides and protein hydrolysates.

3.2. Effect of WLSH:water ratio on protein recovery yield

Enzymatic hydrolysis creates a mixture of free amino acids and oligo-peptides from intact proteins [9], augmenting the protein recovery yield. As seen in Figure 1, the protein recovery yield reaches the peak of 4.25 ± 0.14% at the ratio of WLSH: water of 1:4 (w/v). The more amount of solvent is used, the higher the obtained protein content is. Besides, adequate amount of water could quickly disperse the product of hydrolysis, preventing inhibition of hydrolysis by feedback effect [10]. The protein recovery yield decreases when the WLSH : water ratio is greater than 1:4. It may be due to the fact that higher amount of water could limit the contact between protein and protease, reducing hydrolysis rate. Therefore, to minimize the amount of water used for hydrolysis, the

WLSH: water ratio of 1:4 is used for further experiment.

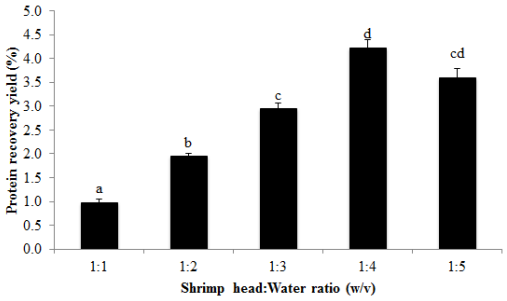

Figure 1. *Effect of WLSH : water ratio on protein recovery yield. Bars with different letters indicate significant differences (P<0.05)*

3.3. Effect of enzyme type on antioxidant activity of protein hydrolysate from shrimp head waste

Protease specificity influences the composition, size, and concentration of peptides as well as their amino acid sequences, impacting the bioactivity of the obtained protein hydrolysates [11]. In this study, Flavourzyme proteolysate shows the highest antioxidant capacity with DPPH scavenging activity of 61.12 ± 0.22% and FRAP value of 314.4 ± 9.6 µM TE, followed by Neutrase, Alcalase, Corolase and Protamex hydrolysate (Figure 2).

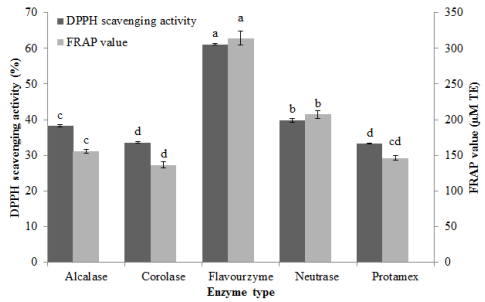

Figure 2. *Effect of enzyme type on the antioxidant activity of WLSH proteolysate. Bars with different letters indicate significant differences (P<0.05)*

It could be due to the fact that Flavourzyme preparation contains both endo- and exo-peptidases which have a broad substrate specificity [12], releasing more antioxidant peptides. Besides, the finding of He et al. [13] indicates that Flavourzyme protein hydrolysate contains high concentration of hydrophobic amino acids which could increase the presence of peptides at the water-lipid interface, as a result, accessing to scavenge free radicals from the lipid phase. Flavourzyme hydrolysate from salmon by-product also exhibits the highest antioxidant capacity [14]. Hence, Flavourzyme is used for further study.

3.4. Effect of pH on antioxidant activity of protein hydrolysate from shrimp head waste

As illustrated in Figure 3, both DPPH scavenging activity and FRAP value reach the highest values of 62.72 ± 2.02% and 312.56 ± 3.73 µM TE, respectively, at pH 7, optimal pH. It may be due to the fact that the environmental pH significantly affects the ionization ability of substrate and enzyme through changing their charge distribution and conformation, affecting catalytic activity of enzyme and antioxidant activity of proteolysate [10]. The antioxidant potential of protein hydrolysate depends on its amino acid composition and sequences of peptides present in it. Non-optimal pH reduces the number of generated antioxidant peptides through decreasing catalytic activity of enzyme. Hence, pH 7 is selected for further experiments.

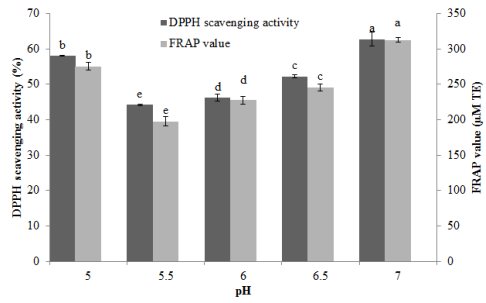

Figure 3. *Effect of pH on antioxidant activity of WLSH proteolysate. Bars with different letters indicate significant differences (P<0.05)*

3.5. Effect of temperature on antioxidant activity of protein hydrolysate from shrimp head waste

The temperature – antioxidant activity profile (Figure 4) shows that both DPPH scavenging activity and FRAP value of the shrimp head proteolysate reaches the peaks of 72.47 ± 1.53% and 358.28 ± 12.58 µM TE, respectively, at hydrolysis temperature of 45°C.

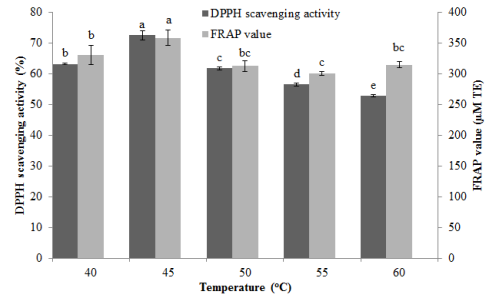

Figure 4. *Effect of temperature on antioxidant activity of WLSH proteolysate. Bars with different letters indicate significant differences (P<0.05)*

Temperature changes the conformation of substrate and enzyme, exposing the hydrophobic or hydrogen-donating amino acid buried inside the protein [15], enhancing the antioxidant activity of the protein hydrolysate. Non-optimal temperature limits the contact between enzyme and substrate molecules through decreasing the movement of these molecules or changing their configuration, lowering the formation of antioxidant peptides. Similar observation is also reported by Ren et al. [15]. Therefore, the hydrolysis temperature of 45°C is employed for further studies.

3.6. Effect of E:S ratio on antioxidant activity of protein hydrolysate from shrimp head waste

As depicted in Figure 5, both DPPH scavenging activity and FRAP value of the proteolysate reach the peaks of 77.50 ± 0.31% and 421.80 ± 13.42 µM TE, respectively, at the E:S ratio of 50 U/g protein. The adequate amount of enzyme for substrate hydrolysis enhances the recovery yield of protein hydrolysate with high antioxidant activity. Lower or higher enzyme amount may cause the excess or

lack of substrate for the hydrolysis reaction, lowering the antioxidant capacity of the proteolysate. Similar finding was also found in previous studies of Gunasekaran et al. [2]. Therefore, the E:S ratio of 50 U/g protein is used for further analysis.

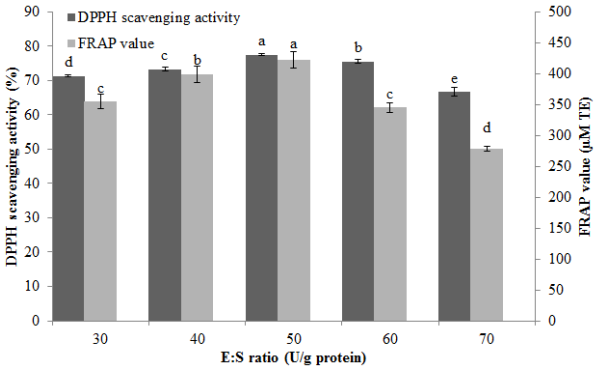

Figure 5. *Effect of E:S ratio on the antioxidant activity of WLSH proteolysate. Bars with different letters indicate significant differences (P<0.05)*

3.7. Effect of hydrolysis time on antioxidant activity of protein hydrolysate from shrimp head waste

Figure 6 demonstrates the effect of hydrolysis time on the antioxidant activity of the WLSH proteolysate.

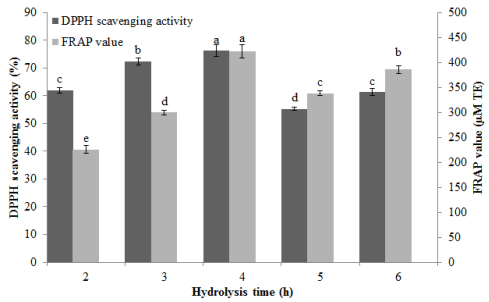

Figure 6. *Effect of hydrolysis time on the antioxidant activity of WLSH proteolysate. Bars with different letters indicate significant differences (P<0.05)*

Antioxidant capacity of the protein hydrolysate increases along with prolonging time of hydrolysis and at 4 hours of hydrolysis, the DPPH scavenging activity and FRAP value of the proteolysate reach the peaks of 76.27±2.04% and 422.00±13.15 µM TE, respectively. However, longer hydrolysis could cause deeper cleavage of the enzyme on generated antioxidant peptides or reduce the enzyme catalytic activity, lowering the antioxidant capacity of proteolysate. This finding is consistent with the result of Bordbar et al. [16]. Hence, the hydrolysis time of 4 hours is picked for further experiments.

3.7.1. Optimization of E:S ratio and hydrolysis time for maximizing the DPPH scavenging activity of the protein hydrolysate from shrimp head waste using RSM

To suggest the proper model, multiple regression analysis is performed on the experimental data and the final predictive function achieved is as follows:

$$Y = 83.63 + 2.14X_1 + 1.14X_2 - 1.69X_1^2 - 1.69X_2^2 \quad (2)$$

where Y, X_1, X_2 are the DPPH scavenging activity (%), E:S ratio (U/g protein) and hydrolysis time (hour), respectively. The E:S ratio is changed from 40 to 60 U/g protein and the hydrolysis time is varied from 3 to 5 hours. The effect of each variable on the response is determined at 95% confidence level. Four terms of X_1^2, X_2^2, X_1 and X_2 are estimated as significant effects whilst the effect of X_1X_2 is insignificant. The regression model is significant ($p<0.05$) with the coefficient of determination (R^2) of 0.986.

In order to determine optimal levels of the variables for the antioxidant power, a three-dimensional surface plot is constructed according to the quadratic function (2) (Figure 7). The optimal condition includes the E/S ratio of 54.87 U/g protein and the hydrolysis time of 4.23 hours with a predictive maximal response of 80.74%.

To evaluate the accuracy of the model, three independent replicates are conducted for measuring antioxidant potential under the optimal condition. The average DPPH scavenging activity is 80.55 ± 0.87%. The experimental value is nearly the same as the predicted value from quadratic function (2).

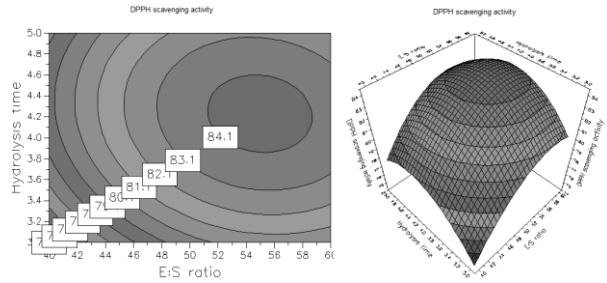

Figure 7. *Response surface plot for antioxidant activity of WLSH proteolysate using DPPH scavenging method*

4. Conclusion

This study reveals that hydrolysis condition including protease type, pH, temperature, E: S ratio and hydrolysis time have a significant effect on the antioxidant activity of the WLSH protein hydrolysate. The hydrolysis condition after optimization includes hydrolysis enzyme of Flavourzyme, pH 7, 45ºC, 4.23 hours and E: S ratio of 54.87 U/g protein, and the DPPH scavenging activity of the proteolysate achieves 80.74%. This study suggests that the WLSH could be considered as a promising source of antioxidant peptides or protein hydrolysates, which would add the value for the underutilized by-product and minimize its negative effect on the environment. However, *in vivo* experiments on antioxidant mechanism and activity should be done for further utilization of this by-product.

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