INVESTIGATION OF ANTIOXIDANT ACTIVITY OF PROTEOLYSATE DERIVED FROM *ACETES JAPONICUS*

KHẢO SÁT HOẠT TÍNH KHÁNG OXY HÓA CỦA DỊCH THỦY PHÂN PROTEIN TỪ CON RUỐC (*ACETES JAPONICUS*)

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Abstract - This research investigated the antioxidant activity of proteolysate from *Acetes japonicus*. Firstly, chemical composition of the *Acetes* was analyzed. Then, the effect of *Acetes* : water ratio on protein recovery yield and the effect of the enzyme type, pH, temperature, enzyme to substrate (E:S) ratio and hydrolysis time on the antioxidant potential of the proteolysate were examined. Next, the response surface methodology (RSM) was employed to optimize hydrolysis through the E:S ratio and hydrolysis time. The results showed that the *Acetes* contained 12.3±0.1% moisture, 72.8±0.7% protein, 4.3±0.2% lipid and 16.8±0.2% ash (on a dry weight basis). The protein recovery yield achieved 13.4 ± 0.6% with the *Acetes* : water ratio of 1:8 (w/v). The optimal hydrolysis condition included Flavourzyme, pH 7, 55°C, E:S ratio of 59.39 U/g protein, hydrolysis time of 3.05 hours and the DPPH scavenging activity of the proteolysate reached 67.82%. This study suggested a new way of utilizing *Acetes* as an antioxidant proteolysate which could be applied as a food or an natural antioxidant additive substituting for synthetic compounds.

Key words - *Acetes japonicus*; antioxidant activity; proteolysate; bioactivities; enzymatic hydrolysis.

Tóm tắt - Nghiên cứu này khảo sát hoạt tính kháng oxy hóa của dịch thủy phân protein từ con ruốc khô (*Acetes japonicus*). Trước tiên, thành phần hóa học của con ruốc khô được xác định. Tiếp theo, ảnh hưởng của tỷ lệ ruốc:nước đến hiệu suất thu hồi protein, 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 con ruốc khô chứa 12,3±0,1% ẩm, 72,8±0,7% protein, 4,3±0,2% béo và 16,8±0,2% tro (theo hàm lượng chất khô). Hiệu suất thu hồi protein đạt 13,4±0,6% với tỷ lệ ruốc : nước 1:8 (w/v). Với điều kiện thủy phân tối ưu, hoạt tính nhốt DPPH đạt 67,82%. Nghiên cứu này đề xuất hướng sử dụng mới cho con ruốc như dịch thủy phân có hoạt tính kháng oxy hóa, có thể sử dụng như thực phẩm chức năng hoặc phụ gia kháng oxy hóa tự nhiên thay thế cho các hợp chất tổng hợp.

Từ khóa - Con ruốc; kháng oxy hóa; dịch thủy phân; hoạt tính sinh học; thủy phân enzyme.

1. Introduction

Synthetic antioxidants such as Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT), t-Butyl HydroQuinone (TBHQ) and Propyl Gallate (PG) are known as popular antioxidant compounds utilized in the food industry to prevent the spoilage caused by oxidation. However, these agents show potential hazards, and their usage is limited in several countries. Therefore, it is necessary to find and develop new natural antioxidants to substitute for synthetic agents [1]. Previous researches revealed that peptides from aquatic sources and by-products owned antioxidant capacity [2, 3].

Acetes japonicus has low economic value and has been exploited inefficiently in Vietnam. However, it contains essential amino acids, accounting for 31.1% of total amount of amino acids in this species [4]. Moreover, its high protein content (72.8%) shows that the small shrimp can be considered as a natural source of peptides or proteolysates which may own bioactivities. Until now, there have been no reports on antioxidant activity of proteolysate derived from the *Acetes*.

The objectives of this study are to (i) analyse chemical composition of the *Acetes*; (ii) investigate the effect of *Acetes*:water ratio on protein recovery yield and the effect of hydrolysis conditions on antioxidant activity of *Acetes* protein hydrolysate; (iii) optimize proteolysis conditions for maximizing the antioxidant activity of the proteolysate.

2. Materials and methods

2.1. Materials

2.1.1. Acetes japonicus

Acetes used in this study was bought from a company in Ninh Thuan province, Vietnam with its moisture of 12.3 ± 0.12%.

2.1.2. Enzyme preparations and chemicals

Proteases including Alcalase, Neutrase, Protamex, Flavourzyme and Corolase were obtained from Novozymes (Denmark) and AB enzymes (Germany). Chemicals were purchased from Sigma–Aldrich and Merck. All reagents were of analytical grade. Double–distilled water was used in experiments.

2.2. Methods

2.2.1. Chemical composition analysis

The contents of moisture, crude protein, crude fat and ash of the *Acetes* were determined based on the methods of AOAC (2000) [5]. The total crude protein content was determined using Kjeldahl method with Nitrogen conversion factor of 6.25.

2.2.2. Preparation of Acetes japonicus hydrolysates

The preparation of hydrolysates was performed according to the procedure of Bhaskar and Mahendrakar [6] with slight modification. Water was added with the desired ratio and the mixture was heated at 90°C for 10 minutes to deactivate endogenous enzymes. Desired

enzyme was added after pH value was controlled using 1M NaOH or HCl solution. After the required hydrolysis time, the reaction was terminated by heating the hydrolysate for 10 min at 90°C to deactivate the enzyme. The hydrolysate was then centrifuged to collect the supernatant. The obtained supernatants were freeze-dried using freeze-dryer (Alpha 1-2/Ldplus, UK) and stored at -20°C until used.

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

Protein recovery yield was determined by the percentage of protein content in the proteolysate comparing to the crude protein content of the *Acetes*. For this experiment, Alcalase was used for hydrolysis at its recommended pH and temperature, E:S ratio of 30 U/g protein, hydrolysis time of 4 hours and *Acetes*: water ratio in range from 1:3 to 1:10 (w/v).

2.2.4. Effect of hydrolysis conditions on the antioxidant activity of Acetes proteolysate

The effect of enzyme type, pH, temperature, E:S ratio and hydrolysis time on the antioxidant capacity of the proteolysate were examined using a single factor test method which was performed by one factor varied with different levels while other factors fixed.

2.2.5. Determination of DPPH radical-scavenging capacity

The DPPH radical scavenging capacity was assayed employing the method of Gunasekaran et al. [3] with slight modification. The mixture of sample and DPPH was incubated in the dark at room temperature of 30 minutes. The absorbance at 517 nm was determined by a spectrophotometer. The scavenging activity was 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 Gunasekaran et al. [3] was 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 Acetes proteolysate

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

2.2.8. Statistical Analysis

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

3. Results and discussion

3.1. Proximate composition analysis of the Acetes japonicus

The chemical composition of the *Acetes* consisted of 12.3 ± 0.1 % of moisture, 72.8 ± 0.7 % of protein, 4.3 ± 0.2 % of fat and 16.8 ± 0.2 % of ash (on a dry weight basis). The protein content was in agreement with that of different shrimp species in the research of Savage and Foulds [7]. According to previous studies, the protein content of 40.06% - 91.2% (on a dry weight basis) was appropriate to produce antioxidant proteolysates or peptides [2, 3]. Thus, the small shrimp could be considered as a source of bioactive peptides and proteolysates.

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

Theoretically, the more the amount of solvent was used, the higher the obtained protein content in the hydrolysate was. A mixture of free amino acids and oligo-peptides from intact proteins was generated during enzymatic hydrolysis [8], enhancing the protein recovery yield. In this study, the protein recovery yield reached the peak of 13.4 ± 0.6% with the ratio of *Acetes*:water of 1:8 (w/v) (Figure 1).

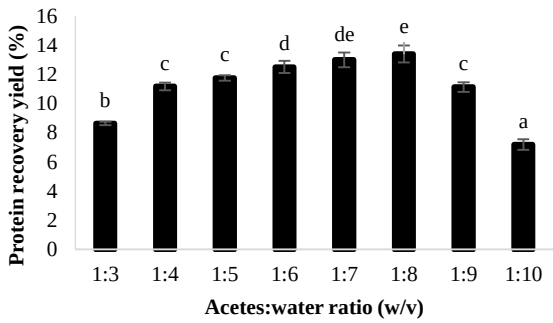

Figure 1. *Effect of Acetes:water ratio on protein recovery yield. Bars with different letters indicate significant differences (P<0.05)*

In addition, a sufficient amount of water could quickly disperse the product of hydrolysis, preventing inhibition of hydrolysis by feedback effect [9]. The protein recovery yield decreased when the *Acetes*:water ratio was greater than 1:8. A higher amount of water could limit the contact between protein and enzyme, reducing hydrolysis rate. Thus, the *Acetes* : water ratio of 1:8 was used for further analysis.

3.3. Effect of enzyme type on antioxidant activity of Acetes proteolysate

In this study, Flavourzyme proteolysate showed the highest antioxidant capacity with DPPH scavenging activity of 46.40 ± 0.38% and FRAP value of 112.10 ± 6.8 µM TE, followed by Neutrase, Alcalase, Corolase and Protamex hydrolysate (Figure 2). It could be due to the fact

that Flavourzyme preparation contains both endo- and exo-peptidases which had a broad substrate specificity [10], releasing more antioxidant peptides. Flavourzyme was also proven to be the best candidate to obtain the proteolysate possessing the highest antioxidant capacity from round scad muscle [11]. Hence, Flavourzyme was used for further experiments.

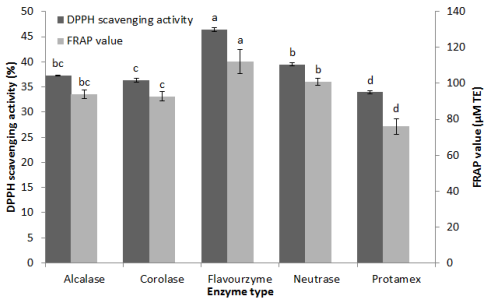

Figure 2. *The effect of enzyme type on the antioxidant activity of Acetes proteolysate. Bars with different letters indicate significant differences (P<0.05)*

3.4. Effect of pH on antioxidant activity of Acetes proteolysate

In this study, both DPPH scavenging activity and FRAP value reached the highest values of 44.38 ± 0.14% and 105.77 ± 3.4 µM TE, respectively, at pH 7, optimal pH (Figure 3).

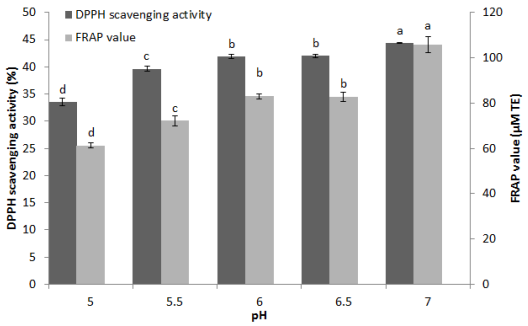

Figure 3. *Effect of pH on antioxidant activity of Acetes proteolysate. Bars with different letters indicate significant differences (P<0.05)*

It may be due to the fact that the environmental pH significantly affects the ionization ability of the substrate and the enzyme through changing their charge distribution and conformation, affecting the catalytic activity of the enzyme and the antioxidant activity of the proteolysate [9]. The antioxidant potential of the proteolysate depends on its amino acid composition and the sequence of peptides present in it. Non-optimal pH reduced the amount of generated antioxidant peptides through decreasing catalytic activity of enzyme. Hence, pH 7 was selected for further experiments.

3.5. Effect of temperature on antioxidant activity of Acetes proteolysate

As described in Figure 4, both DPPH scavenging activity and FRAP value of the *Acetes* proteolysate augmented along with the increase of temperature and reached the peaks of 51.00 ± 1.86% and 168.47 ± 1.60 µM TE, respectively, at hydrolysis temperature of 55ºC. Temperature changed the conformation of the substrate and the enzyme, exposing the hydrophobic or hydrogen-donating amino acid buried inside the protein [12], enhancing the antioxidant activity of the proteolysate. Non-optimal temperature limited the contact between enzyme and substrate molecules through decreasing the movement of these molecules or changing the configuration of the enzyme and the substrate, lowering the formation of antioxidant peptides. Similar finding was also reported by Ren et al. [12]. Therefore, the hydrolysis temperature of 55ºC was employed for further studies.

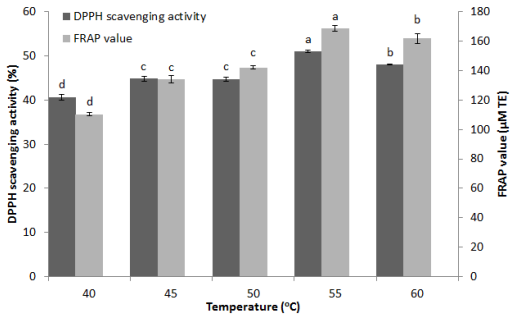

Figure 4. *Effect of temperature on antioxidant activity of Acetes proteolysate. Bars with different letters indicate significant differences (P<0.05)*

3.6. Effect of E:S ratio on antioxidant activity of Acetes proteolysate

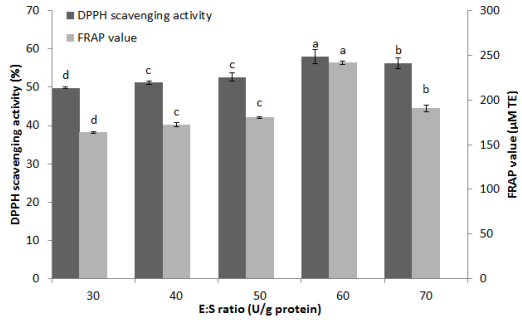

Figure 5. *The effect of E:S ratio on the antioxidant activity of Acetes proteolysate. Bars with different letters indicate significant differences (P<0.05)*

The E:S ratio – antioxidant activity profile (Figure 5) showed that both the DPPH scavenging activity and FRAP value of the proteolysate reached the peaks of 58.07 ± 0.02% and 241.95 ± 3.40 µM TE, respectively, at the E:S ratio of 60 U/g protein. The adequate amount of enzyme for substrate enhanced the recovery yield of proteolysate with high antioxidant activity. A lower or higher enzyme amount may cause excess or lack of substrate for the hydrolysis reaction, lowering the antioxidant capacity of the proteolysate. Similar observation was also found in previous studies of Gunasekaran et al. [3]. Therefore, the E:S ratio of 60 U/g protein was used for further analysis.

3.7. Effect of hydrolysis time on antioxidant activity of Acetes proteolysate

As seen in the Figure 6, the antioxidant capacity of the proteolysate increased along with the prolonged time of hydrolysis. At the hydrolysis time of 3 hours, the DPPH scavenging activity and FRAP value of the proteolysate reached the peaks of 67.32 ± 0.28% and 322.14 ± 11.00 µM TE, respectively.

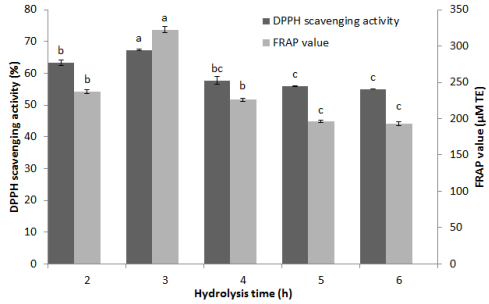

Figure 6. *The effect of hydrolysis time on the antioxidant activity of Acetes proteolysate. Bars with different letters indicate significant differences (P<0.05)*

However, longer hydrolysis could cause deeper cleavage of the enzyme on generated peptides or reduce the enzyme catalytic activity, lowering the antioxidant capacity of proteolysate. This observation was in agreement with the finding of Bordbar et al. [13]. Hence, the hydrolysis time of 3 hours was picked for further experiments.

3.8. Optimization of E:S ratio and hydrolysis time for maximizing the DPPH scavenging activity of the Acetes proteolysate using RSM

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

$$Y = 67.82 - 1.70X_1^2 - 2.15X_2^2 - 2.66X_1X_2 \qquad (2)$$

Where Y, X_1, X_2 were the DPPH scavenging activity (%), E:S ratio (U/g protein) and hydrolysis time (hour), respectively. The E:S ratio was changed from 50 to 70 U/g protein and the hydrolysis time was varied from 2 to 4 hours. The effect of each variable on the response was determined at 95% confidence level. Three terms of X_1^2, X_2^2 and X_1X_2 were estimated as significant effects whilst the effect of X_1 and X_2 were insignificant. The regression model was significant ($p<0.05$) with the coefficient of determination (R^2) of 0.95.

In order to determine optimal levels of the variables for the antioxidant power, a three-dimensional surface plot was constructed according to the quadratic function (2) (Figure 7). The optimal condition included the E/S ratio of 59.39 U/g protein and the hydrolysis time of 3.05 hours with a predictive maximal response of 67.82%. The DPPH scavenging activity of the *Acetes* proteolysate was remarkably higher than that of shrimp head proteolysate with hydrolysis condition including Alcalase, pH 8.2, temperature of 45.4°C, E/S ratio of 1.8% and hydrolysis time of 3 hours [3]. It also showed a higher antioxidant potential than that of *Sphyrna lewini* muscle proteolysate when hydrolysing the muscle at pH 6, 50◦C, enzyme dose of 1.2% and hydrolysis time of 2 hours using papain [14]. Besides, the antioxidant capacity of the small shrimp proteolysate was higher than that of mackerel proteolysate as well when the mackerel was hydrolysed at 50ºC, a protease amount of 0.5% and hydrolysis time of 10 hours [15].

To evaluate the accuracy of the model, three independent replicates were conducted for measuring antioxidant potential under the optimal condition. The DPPH scavenging activity was 68.01 ± 1.45%. The experimental value was nearly the same as the predicted value from quadratic function (2).

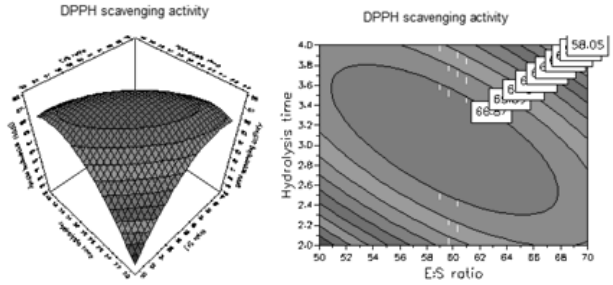

Figure 7. *Response surface plot for antioxidant activity of Acetes proteolysate using DPPH scavenging method*

4. Conclusion

This study reported that the hydrolysis condition including the protease type, pH, temperature, E:S ratio and hydrolysis time significantly affected the antioxidant activity of the proteolysate. The hydrolysis condition after optimization included a hydrolysis enzyme of Flavourzyme, pH 7, 55ºC, 3.05 hours and E:S ratio of 59.39 U/g protein, and the DPPH scavenging activity of the proteolysate reached 67.82%. It could be concluded that the small shrimp was a promising source of antioxidant peptides or proteolysates which can be used as a functional food or an antioxidant additive. However, further researches *in vivo* should be done to use the small shrimp source more effectively.

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REFERENCES

[1] G.S. Centenarm, M.S. Mellado, C. Prentice-Hernández, "Antioxidant Activity of Protein Hydrolysates of Fish and Chicken Bones", Advance *Journal of Food Science and Technology*, 3 (4), 2010, 280-288.

[2] J. Jia, Y. Zhou, J. Lu et al., "Enzymatic hydrolysis of Alaska pollack (Theragra chalcogramma) skin and antioxidant activity of the resulting hydrolysate", *Journal of the Science of Food and Agriculture*, 90, 2010, 635–640.

[3] J. Gunasekaran, N. Kannuchamy, S. Kannaiyan et al., "Protein Hydrolysates from Shrimp (Metapenaeus dobsoni) Head Waste: Optimization of Extraction Conditions by Response Surface Methodology", *Journal of Aquatic Food Product Technology*, 24 (5), 2015, 429-442.

[4] S.H. Choi, A. Kobayashi, T. Yamanishi, "Odor of cooked small shrimp, Acetes japonicus Kishinouye: Difference between raw material and fermented product", *Agricultural and Biological Chemistry 47 (2)*, 1983, 337-342

[5] AOAC, AOAC-Methods of Analysis, 2000.

[6] N. Bhaskar, N.S. Mahendrakar, "Protein hydrolysate from visceral waste proteins of Catla (Catla catla): Optimization of hydrolysis conditions for a commercial neutral protease", *Bioresource Technology*, 99, 2008, 4105–4111.

[7] G.P. Savage, M.J. Foulds, "Chemical composition and nutritive value of antarctic krill (Euphausia superba) and southern blue whiting (Micromesistius austral is)", *New Zealand Journal of Marine and Freshwater Research*, 21, 1987, 599-604.

[8] S.D.A.d. Santos, V.G. Martins, M. Salas-Mellado et al., "Evaluation of Functional Properties in Protein Hydrolysates from Bluewing Searobin (Prionotus punctatus) Obtained with Different Microbial Enzymes", *Food and Bioprocess Technology*, 4, 2011, 1399–1406.

[9] G. Shu, B. Zhang, Q. Zhang et al., "Effect of Temperature, pH, Enzyme to Substrate Ratio, Substrate Concentration and Time on the Antioxidative Activity of Hydrolysates from Goat Milk Casein by Alcalase", *Acta Universitatis Cibiniensis. Series E: Food Technology*, 20 (2), 2017, 29-38.

[10] R.J.S.d. Castro, H.H. Sato, "A response surface approach on optimization of hydrolysis parameters for the production of egg white protein hydrolysates with antioxidant activities", *Biocatalysis and Agricultural Biotechnology*, 4, 2015, 55–62.

[11] Y. Thiansilakul, S. Benjakul, F. Shahidi, "Antioxidative Activity of Protein Hydrolysate from Round Scad Muscle Using Alcalase and Flavourzyme", *Journal of Food Biochemistry*, 31 (2), 2006, 266-287.

[12] J. Ren, M. Zhao, J. Shi et al., "Optimization of antioxidant peptide production from grass carp sarcoplasmic protein using response surface methodology", *LWT - Food Science and Technology*, 41, 2008, 1624-1632.

[13] S. Bordbar, A. Ebrahimpour, A.A. Hamid et al., "The Improvement of The Endogenous Antioxidant Property of Stone Fish (Actinopyga lecanora) Tissue Using Enzymatic Proteolysis", *BioMed Research International*, 2013, 2013, 1-9.

[14] B. Wang, Z.-R. Li, C.-F. Chi et al., "Preparation and evaluation of antioxidant peptides from ethanol-soluble proteins hydrolysate of Sphyrna lewini muscle", Peptides 36, 2012, 240–250.

[15] H.C. Wu, H.M. Chen, C.Y. Shiau, "Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (Scomber austriacius)", *Food Research International*, 36, 2003, 949-957.

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