Processing Stability of Antioxidant Protein Hydrolysates Extracted from Degreased Walnut Meal

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***Abstract*—Degreased walnut meal was hydrolyzed by trypsin and the optimal hydrolysis conditions were at the enzyme to substrate of 9.2×10^5 U/g with the hydrolysis time of 30h. The effects of acidic/alkaline treatments on the antioxidant stability of WPH (liquid walnut meal protein hydrolysate), WPHP-s (spray-dried WPH sample) and WPHP-f (freeze-dried sample) were assessed. These samples were quite stable under acidic conditions, remaining approximately 90% of its original reducing power and 85% of its OH• scavenging activity. However, they were susceptible to alkaline treatments (at pH>10.0) and lost>50% of their antioxidant activities. In terms of heat processing, common pasteurization (65℃×30 min) or autoclaving sterilization (121℃×20 min) treatment did not show notably effects on the antioxidant activities of these three samples. Different drying methods (spray-drying or freeze-drying) demonstrated very slight influence on their structure and activities, as further confirmed by molecular weight distribution assay.**

***Index Terms*—walnut meal, protein hydrolysates, drying methods, antioxidant activity, stability, molecular weigh**

I. Introduction

Protein hydrolysates containing various peptides can be produced by enzymatic hydrolysis, which is an effective way to decompose large protein molecules. Bioactive peptides obtained from food-derived protein hydrolysates have been defined as specific protein fragments which have a positive effect on body functions and may ultimately influence human health [1]. As we all know, excessive amount of reactive radicals may result in cellular damage which initiates diseases including atherosclerosis, arthritis, diabetes and caner [2]-[6]. Also, the reactive species can cause damage in proteins, mutations in DNA and oxidation of membrane phospholipids [7]-[9]. Food-derived proteins hydrolysates or peptides released by enzymatic hydrolysis have been reported to exhibit antioxidative activities [10]-[13]. However, protein hydrolysates will invariably undergo thermal sterilization in food processing before eventually entering the market as final commercial products. Hence, different heat treatment such as pasteurization or high temperature sterilization might be applied for the product processing. In addition, for some applications in the form of oral liquid, beverage or granules, protein hydrolysates may also undergo different pH conditions. Besides, different drying techniques can also influence the physiochemical and biological activity of the products. Freeze drying is based on the dehydration by sublimation of a frozen product. Most of deterioration and microbiological reactions are stopped giving a final product of excellent quality on account of the absence of liquid water and the low temperatures required for the process [14]. Spray drying is one of the most effective drying methods to convert fluid materials into solid or semi-solid particles in a single step and it's widely applied in food, pharmaceutical and chemical industries [15]. Spray drying is more generally used in food industry due to its potential of saving energy as compared with freeze drying. However, freeze drying is especially suitable for thermo-sensitive products, although it needs much energy and long process time [16]. The food-derived protein hydrolysates are mostly mixtures of poly-/oligo-peptides, proteins, free amino acids and even some lipoprotein or glycoprotein. Hence, high temperature process (i.e. spray drying) and low temperature process (i.e. freeze drying) might give rise to quite different physiological properties of the final products.

Degreased walnut meal is the by-product of walnut processing. There are over 40% proteins left which enriches Essential Amino Acid (EAA). So far, the utilization of walnut by-product hasn't been fully studied. Most researches about walnut protein hydrolysates focused on their purification and identification of the peptides, and their biological activities. For instance, the peptides extracted from walnut protein hydrolysates had been proved to exhibit high antioxidant activity and the sequence of the peptide with the highest antioxidant activity was identified to be Ala-Asp-Ala-Phe [17]. Li *et al.* (2008) demonstrated that the walnut residue protein hydrolysates hydrolyzed by papain had high antioxidant activity [18]. To date, there is little research regarding the

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influence of food processing conditions (i.e. pH, temperature or drying parameters, etc.) on the physiological properties of protein hydrolysates.

In the present study, the protein hydrolysates were extracted from walnut meal by enzymatic hydrolysis. Their stabilities during processing, the influence of different sterilization processing and different drying methods on the antioxidant activity of walnut meal protein hydrolysates were studied. Also, the molecular weight distribution of walnut meal protein hydrolysates was analyzed after different drying processes to discover the aggregation and de-aggregation changes of peptides structure under extra-low or extra-high temperature. The results of this study may give fundamental information on maintaining the bioactive property of protein hydrolysates during food processing.

II. Materials and Methods

A. Materials

Walnut meal was purchased from Huizhi Resource Co., Ltd. (Yunnan, China). Tyrisin (46 million U/g protein) was obtained from Shisheng Technology Co., Ltd. (Hangzhou, Zhejiang, China). Acetonitrile, trifluoroacetic acid (TFA), trichloroacetic acid (TCA), potassium ferricyanide and 1,10-phenanthroline were all acquired from Guangzhou Congyuan Instrument Co., Ltd. (Guangzhou, Guangdong, China). Cytochrome C, trasylol, oxidized glutathione (GSSG) and triglycine were products of Sigma Chemical Co. (St. Louis, MO, USA). The ultrapure water was prepared by a Milli-Q water purification system of Millipore (Millipore, Bedford, MA, USA) in our laboratory. All the other chemical reagents used in this study were of analytical grade.

B. Preparation of Protein Hydrolysates

Degreased walnut meal was mixed with distilled water in a ratio of 1:7 (w/w). The mixture was divided into four equal parts and preheated to 55 ℃ before different dosage of trypsin ([E/S] ratio 2.3×10^5U/g, 4.6×10^5U/g, 6.9×10^5U/g and 9.2×10^5U/g, respectively) were added, respectively. The hydrolysis was performed at the optimal condition for trypsin (pH 8.0, 55 ℃) in a thermostat water bath. Samples were taken out for analysis at different time interval (12h, 20h, 30h and 40h, respectively). At the end of hydrolysis, the mixtures were heated in boiling water for 10 min to inactivate the proteases. Then, the hydrolysate was centrifuged using a GL-21M refrigerated high-speed centrifuge (Xiangyi Instrument Co. Ltd., Changsha, China) at 8000*g* for 20 min. The supernatant was then filtered and collected for further processing.

C. Determination of Protein Recovery

Protein recovery was used as an index of nitrogen solubilization to describe the hydrolysis efficiency. For enzymatic hydrolysis, only the soluble protein, peptides or amino acids in supernatant after centrifugation were considered as usable fractions, while the non-soluble fraction would be discarded as waste. Therefore, protein recovery was defined as the usable portion in total protein materials. The weight of soluble fraction was recorded and total nitrogen in supernatant was determined using Kjeldahl method [19]. Protein recovery was calculated according to the following equation:

$$\text{Protein recovery (\%)} = [\text{total protein in supernatant (mg)} / \text{total protein in material (mg)}] \times 100 \quad (1)$$

D. Determination of the Degree of Hydrolysis (DH)

The Degree of Hydrolysis (DH) means that the percentage of free N-terminal amino groups which is cleaved from the protein molecule, which is counted as the ratio of α-amino nitrogen to total nitrogen. The α-amino nitrogen content was measured by a formaldehyde titration method according to the method of Nilsang *et al.* (2005) [20]. The total nitrogen content was determined by Kjeldahl method. DH was calculated according to the following equation.

$$\text{Degree of hydrolysis (DH, \%)} = (N_1/N_2)\times 100 \quad (2)$$

where N_1 is the α-amino nitrogen content; N_2 is the total nitrogen content.

E. Preparation of the Protein Hydrolysate Powder

The protein hydrolysates were dried by two different processing techniques, spray drying and freeze drying, respectively.

For spray drying, the RIKAKIKA SD-1000 spray dryer (RIKAKIKA Joint-stock Company, Tokyo, Japan) was used in the present experiment. The outlet spray drying temperatures were varied between 90 ℃ and 95 ℃ with inlet temperature fixed at 140 ℃. The air quantity of drying was 0.7m/min and the air pressure was 110kPa. The finally obtained dry powder was referred as WPHP-s.

For freeze drying, a laboratory scale freeze drying device (Virtis, the Virtis Company, Gardiner, NY) was used. The walnut protein hydrolysate was first pre-frozen at -80 ℃ on Petri dishes for 3h. Then, the samples were freeze-dried without heating under 0.07mPa vacuum (condenser temperature of 25 ℃) for 24h. The dried mixture was obtained and referred as WPHP-f.

Therefore, totally three samples including one liquid sample (WPH) and two dried sample (WPHP-s and WPHP-f) were obtained for further analysis.

F. Methods of Antioxidant Activity Assays

1) Reducing power assay

The reducing power was measured according to the method of Gu *et al.* [21] with some modification. Two milliliter of sample was mixed with 2mL of phosphate buffer (pH 6.6) and 2 mL of 1% potassium ferricyanide to reach final concentration of 0.5mM. The mixture was incubated at 50 ℃ for 20 min. After that, 2mL of 10% trichloroacetic acid (TCA) was added to the mixture and was then centrifuged at 3000*g* for 10 min. The supernatant (2mL) was mixed with 2mL of distilled water and 0.4mL of 0.1% ferric chloride and was allowed to stand for 10 min. Then, the absorbance was measured at 700nm by a spectrophotometer (UV2550, SHIMADZU, Kyoto, Japan). All determinations were performed in triplicate. The reducing power was calculated as follows.

$$\text{Reducing power assay (A)} = A_i - A_0 \quad (3)$$

where A_i is the absorbance of the sample; A_0 is the absorbance of control solution containing phosphate buffer, potassium ferricyanide, TCA and ferric chloride.

2) Hydroxyl radical scavenging activity assay

The hydroxyl radical scavenging activity was measured according to the method of de Avellar *et al.* (2004) [22] with some modifications. Both 1,10-phenanthroline (0.75mM) and $FeSO_4$ (0.75mM) were dissolved in phosphate buffer (pH 7.4) and mixed thoroughly. Hydrogen peroxide (H_2O_2, 0.01%) and the sample (1mg/ml) were added. The mixture was then incubated at 37℃ for 60min and the absorbance was measured at 536nm. Results were determined using the following equation:

$$\text{Hydroxyl radical scavenging activity (\%)} = [(A_S - A_1)/(A_2 - A_1)] \times 100 \quad (4)$$

where A_S, absorbance of the sample; A_1, absorbance of the control solution containing 1,10-phenanthroline, $FeSO_4$ and H_2O_2; A_2, absorbance of the blank solution containing 1,10-phenanthroline and $FeSO_4$.

G. Effect of Processing Conditions on the Antioxidant Activity of Walnut Protein Hydrolysates (WPH)

The effects of two important food processing (acid treatment, alkali treatment and heat treatment) on the antioxidant activities of WPHs were studied. Three samples, WPH, WPHP-s and WPHP-f, were all made to solutions with final concentration of 1mg/mL using distilled water. For different pH treatment, the pH of each solution was adjusted to 2.0, 4.0, 6.0, 8.0, 10.0 and 12.0, respectively, using 0.1M HCl or 0.1M NaOH. The mixture was then kept at 25℃ for 2h. After that, the pH value was then adjusted to 7.0 with 1, 0.1M HCl or 1, 0.1M NaOH. The samples with their native pH values were regarded as control. The samples were subjected to 25℃×30 min, 65℃×30 min and 121℃×20 min for different heat treatments, respectively, and then rapidly cooled to room temperature in cold water. After pH adjustments or heat treatments, the antioxidant activities of reducing power and hydroxyl radical scavenging activity of all the samples were determined. All determinations were performed with three replications.

H. Determination of Molecular Weight Distribution

The molecular weight distribution of the samples was determined using the method of Li, Jiang, Zhang, Mu, & Liu (2008) [23] with some modification. The WPHP-s and WPHP-f samples with the strongest antioxidant and free radical scavenging activities were analyzed for molecular weight distribution using a High Performance Liquid Chromatography (HPLC) system (LC-20A, Shimadzu Company). The samples were loaded onto a TSK gel SWXL G2000 column (7.8i.d.×300mm, Tosoh, Tokyo, Japan), eluted with 45% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5ml/min at 214nm. A molecular weight calibration curve was obtained from the following standards, cytochrome C (12,384 Da), aprotinin (6500 Da), oxidized glutathione (612 Da) and tripeptide Gly-Gly-Gly (189 Da). Results were processed using Millennium[32] Version 3.05 software (Waters Corporation, Milford, MA 01757, USA).

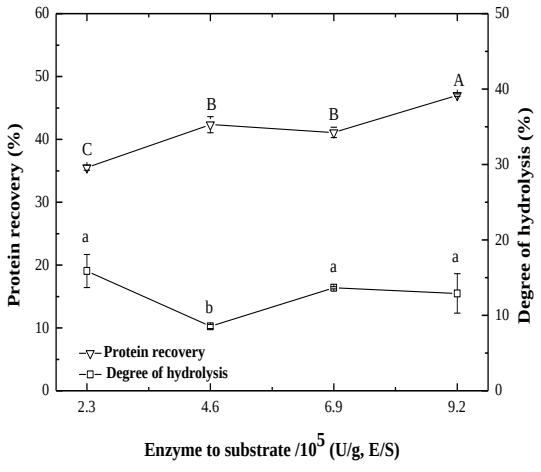
I. Statistical Analysis

All experiments were carried out at least in triplicate and the results were expressed as means ± Standard Deviation (SD). The statistical analysis was processed using Origin Pro Version 8.5 software (Origin Lab Corporation, Northampton, USA). The difference between the control and the experimental groups was analyzed by two-tailed Student's t-test. Difference with $p<0.05$ (*) was considered statistically significant.

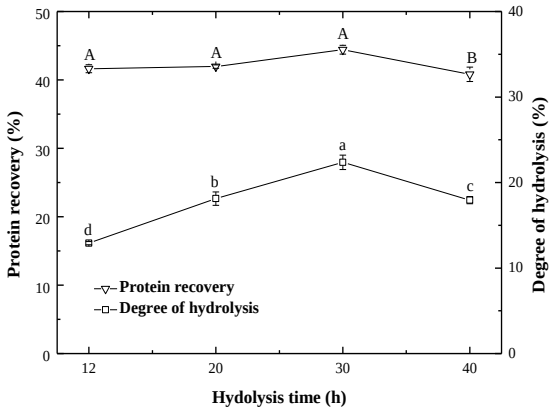
III. Results and Discussion

A Optimization of Enzymatic Hydrolysis Condition

The effects of different protease concentration on protein recovery and the Degree of Hydrolysis (DH) are shown in Fig. 1A. With the increase of protease concentration from 2.3×10^5 to 9.2×10^5U/g, the protein recovery continuously increased from 35.78% to 47.17%. Although there was slight decrease for the protein recovery at the dosage of 6.9×10^5U/g, the difference was not significant ($p>0.05$). It indicated that the dose of substrates surpassed that of the proteases and therefore, the increase of protease dosage could cause the increase of the protein recovery. As for DH, it achieved the maximum value (17.44%) at the protease concentration of 2.3×10^5U/g, However, further increase of protease concentration up to 9.2×10^5U/g did not significantly change DH ($p>0.05$). DH was calculated by the ratio of α-amino nitrogen to total nitrogen in the supernatant after centrifugation at the end of hydrolysis. When more proteases were added, the content of α-amino nitrogen might increase, but at the same time, the total nitrogen calculated in the final supernatant might also increase. Therefore, the reason why DH did not change significantly might be due to the offset of both α-amino nitrogen increasement and total nitrogen increasement. Based on the above results, the protease concentration 9.2×10^5U/g was finally chosen for further analysis.



(A)



(B)

Figure 1. Effect of different enzymatic hydrolysis conditions on protein recovery and degree of hydrolysis. (A) Enzyme dosage; (B) Hydrolysis time. Values with different lower and upper letters for degree of hydrolysis and protein recovery, respectively, are significantly different (p<0.05). Vertical bars indicate mean values ± SD.

The effect of different enzymatic hydrolysis time on protein recovery and DH are shown in Fig. 1B. It was found that the protein recovery slightly increased from 42.04% to 43.97% with hydrolysis time increasing from 10 h to 30 h, but the increase was not significant (p>0.05). Further increase of hydrolysis time to 40 h did not show obvious effect on the protein recovery (p>0.05). In the case of DH, it significantly increased from 13.05% (12h) to 22.96% (30h) (p<0.05), which might be due to the predominate increase of α-amino nitrogen level. However, further increasement of hydrolysis time to 40 h induced the decrease of DH (p<0.05), because the α-amino nitrogen increased at a more moderate pace as compared with the total nitrogen increasement. Therefore, the hydrolysis time of 30h was considered as the optimal one.

B Effects of pH on the Antioxidant Activities of WPH, WPHP-s, WPHP-f

The antioxidant activities of WPH under different pH values (from 2.0 to 12.0) are shown in Fig. 2A. The native pH for WPH was at around 6.4 and it was found that WPH showed the strongest reducing power and OH• scavenging activity at near-natural pH 6.0, which were close to their native antioxidant activities. There was no significant decrease (p>0.05) under acidic conditions when pH was adjusted to 4.0, which was in agreement with the report of Zhu *et al.* [24]. Even when pH was reduced to 2.0, the reducing power of WPH kept at 0.567 ± 0.003 that was approximately 90% of its original value and the OH• scavenging activity was 9.55±1.06%, which maintained about 85% of its original activity. However, when it came to alkaline treatment, the reducing power and the OH• scavenging activity of the WPH were greatly decreased. As pH was adjusted from 8.0 to 12.0, the reducing power declined sharply to 0.243±0.001, which was only 38% of its activity under natural pH condition. Similarly, the OH• scavenging activity of the WPH reduced 50% as pH regulated to 10.0.

The effects of acid or alkali treatments on spray dried sample (WPHP-s) and freeze dried sample (WPHP-f) are shown in Fig. 2B and Fig. 2C, respectively. It was found that for WPHP-s or WPHP-f, the variation tendency of their reducing power was quite similar with that of the WPH with pH value changing from 2.0 to 12.0. Also, the same rule happened to the OH• scavenging activity of WPHP-s and WPHP-f. Therefore, it's safe to get two conclusions so far. The first one was, high temperature treatment in spray drying processing (up to 140 ℃) or low temperature treatment in freeze dry processing (low to -80 ℃) did not show much effect on the antioxidant activities (i.e. reducing power and OH• scavenging activity) of walnut protein hydrolysates. And the second one was, the antioxidant activities of walnut protein hdyrolysates were more sensitive to alkali treatment than acid treatment. Under alkaline condition (pH 10.0 or 12.0), the reducing power and OH• radical scavenging activity was remarkably decreased up to 50% of its original value or even less.

There are many reasons that could explain the loss of antioxidant activity under alkaline condition. Firstly, it was the occurrence of racemization. Under alkaline conditions, racemization reactions would probably occur, forming a mixture of D- and L-isomers. It was known that there were differences of biological activities existed between isomers [25]. Secondly, it could be a deamination reaction. Deamination can be promoted at higher pH values resulting in changes with structure and conformation and loss of antioxidant activity. Thirdly, it might be the activation energy of peptide degradation varies with the change of pH. As Bell & Labuza (1991) [26] reported that the actual degradation of peptides might be affected by different pH values. Alkaline treatment could hydrolyze the side chains of some small peptides and thus affect their antioxidant activities. Also, the hydrogen on the hydrogen donor to scavenging free radicals could be consumed under alkaline condition and thus cause the reducing of antioxidant activities. Therefore, WPH, WPHP-s and WPHP-f were better to be applied in either neutral or acidic food system rather than in alkaline conditions. These results were in accordance with the finding of Faithong *et al.* (2010) [27], who reported that antioxidant activities of Koong-Som (Thai traditional fermented shrimp) tested by DPPH, 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) diammonium salt (ABTS) and Ferric reducing antioxidant potential (FRAP) assays were stable at pH 5.0 to 9.0. Similarly, Nalinanon *et al.* (2011) [28] also found that the ABTS• scavenging activity of ornate threadfin bream muscle protein hydrolysate remained constant over pH range of 1.0 to 10.0 but significantly decreased (p < 0.05) at pH 11.0.

C Effect of Different Heat Treatments on Antioxidant Activity of WPH, WPHP-s and WPHP-f

Thermal stability of the protein hdyrolysates as monitored by reducing power assay and the OH• scavenging activity is shown in Fig. 3. It was found that the antioxidant activities of WPH were quite stable and no significant changes (p>0.05) were found after heat treatment at 65 ℃×30 min (pasteurization) or at 121 ℃×20 min (autoclaving sterilization) (Fig. 3A). As for spray dried sample WPHP-s (Fig. 3B) or freeze dried

sample WPHP-f (Fig. 3C), their antioxidant activities were also stable and the OH• radical scavenging activity of WPHP-s was even increased after pasteurization (p<0.05), which might be due to the exposure of some hydrophobic domain that contributed to the antioxidative properties. The above results proved that WPH, WPHP-s and WPHP-f showed very high thermal stability under the tested temperatures and could be used in thermally processed food without reducing antioxidant activity.

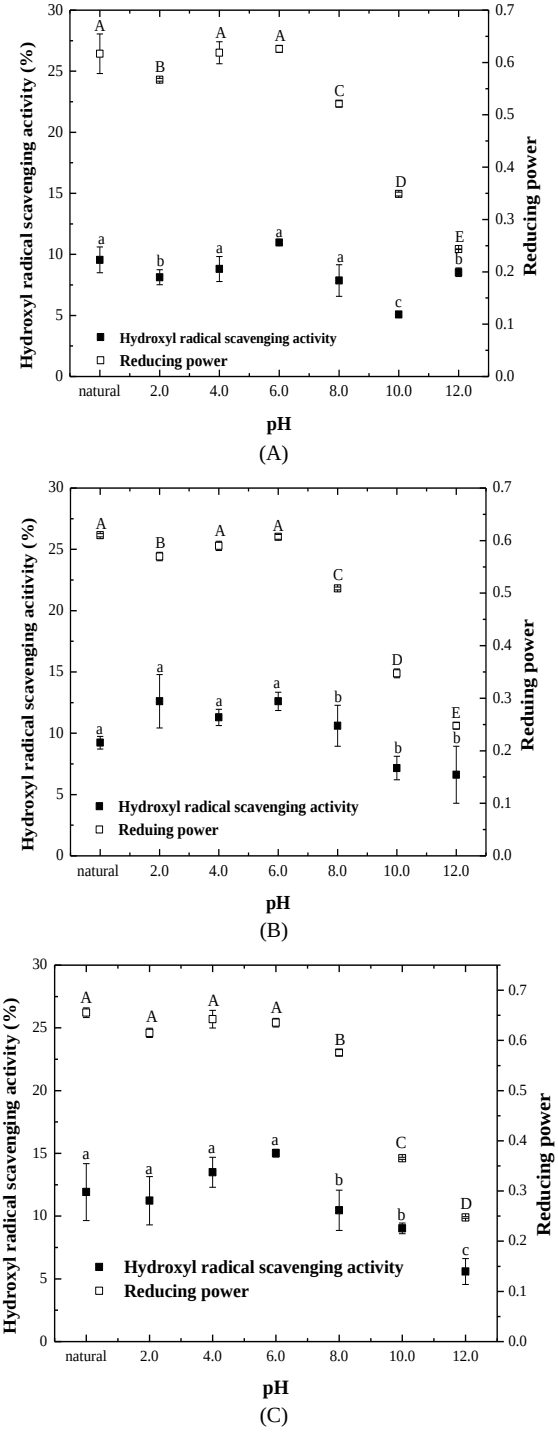


Figure 2. Changes on antioxidant activities of protein hydrolysates extracted from walnut meal in different pH. (A) WPH; (B) WPHP-s; (C) WPHP-f. Values with different lower and upper letters for hydroxyl radical scavenging activity and reducing power, respectively, are significantly different (p<0.05). Vertical bars indicate mean values ± SD.

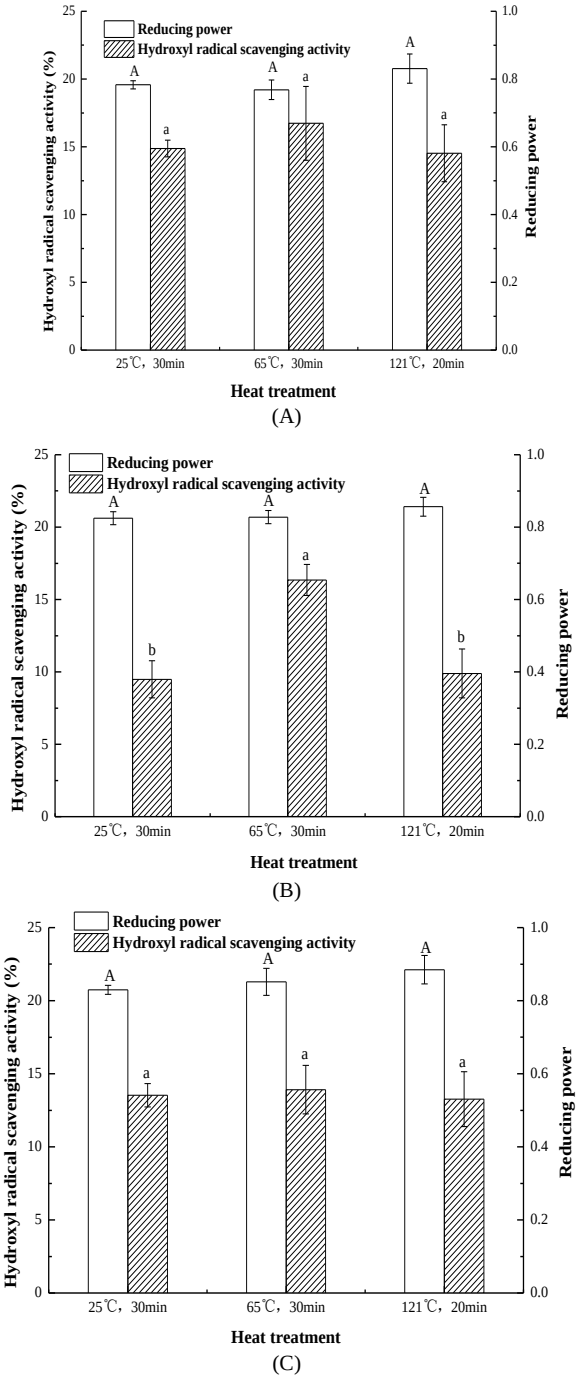


Figure 3. Changes on antioxidant activities of protein hydrolysates extracted from walnut meal in different sterilization methods. (A) WPH; (B) WPHP-s; (C) WPHP-f. Values with different lower and upper letters for hydroxyl radical scavenging activity and reducing power, respectively, are significantly different (p<0.05). Vertical bars indicate mean values ± SD.

D Molecular Weight Distribution of WPHP-s and WPHP-f

Two samples that possessed the highest OH• scavenging activities, one from WPHP-s and the other from WPHP-f, were picked out for molecular weight distribution analysis (Table I). Both of the two selected samples underwent heat treatment at 65 ℃×30 min and had no significant difference (p>0.05) on OH• scavenging activities (Fig. 3). The chromatograms of WPHP-s and

WPHP-f analyzed by TSK gel SWXL G2000 column were shown in Fig. 4. The chromatogram of WPHP-s was found quite similar to that of the WPHP-f, but WPHP-s had earlier retention time than WPHP-f. As shown in Table I, WPHP-s and WPHP-f all had very high percentage of <1kDa protein fraction, 78.84% and 78.95%, respectively. The percentages of the 1-3 kDa fractions were also very close to each other (18.41% and 18.71%, respectively). But the percentages of their 3–5kDa, 5–10kDa and >10 kDa fractions were very low, which totally consists of 2.72% and 2.43% of the total fractions, respectively.

Molecular weight is an important parameter that correlates with the bioactivity of protein hydrolysates. Many researches had found that the antioxidant activity of hydrolysates depends on their molecular weight distribution. For example, Li *et al.* (2008) reported that peptide fraction with molecular weight ranging from 200 to 3,000Da was probably associated with higher antioxidant activity [29]. Nalinanon *et al.* (2008) also reported that low molecular weight peptides contributed to the antioxidant activity [30]. In this study, the samples of WPHP-s and WPHP-f all had very high percentages of low molecular weight (<1kDa) and thus demonstrated similar antioxidant activity. Since these two samples, WPHP-s and WPHP-f, had undergone different dry processing, the above results proved that heat treatment in spray drying up to 140 ℃ or pre-freezing treatment in freeze dry processing (around -80 ℃) did not show much effect on molecular weight distribution of protein hydrolysates. It was in accordance with the tendency shown for antioxidant activities (i.e. reducing power and OH• scavenging activity) of walnut protein hydrolysates.

IV. Conclusions

The antioxidant activities of the walnut meal protein hydrolysates were stable even after pasteurization (65 ℃×30 min) and autoclaving sterilization (121 ℃×20 min) treatments. However, they were susceptible to alkaline pH (>10.0) and might loss >50% of their native antioxidant activities, e.g. reducing power or OH• scavenging activity, under alkaline conditions. Results of different drying methods of the protein hydrolysates showed that there were slight changes of antioxidant activities between WPHP-s and WPHP-f, signifying that they were not sensitive to high- (140 ℃) or low- (-80 ℃) temperature processing. And the molecular weight distribution of WPHP-s and WPHP-f results further confirmed this point.

TABLE I. Molecular Weight Distribution of Walnut Meal Protein Hydrolysates

Drying method	Molecular weight distribution of walnut meal protein hydrolysates (%)				
	>10 kDa	5~10kDa	3~5 kDa	1~3kDa	<1kDa
Spray drying	0.063	0.918	1.744	18.406	78.839
Freeze drying	0.015	0.673	1.654	18.712	78.947

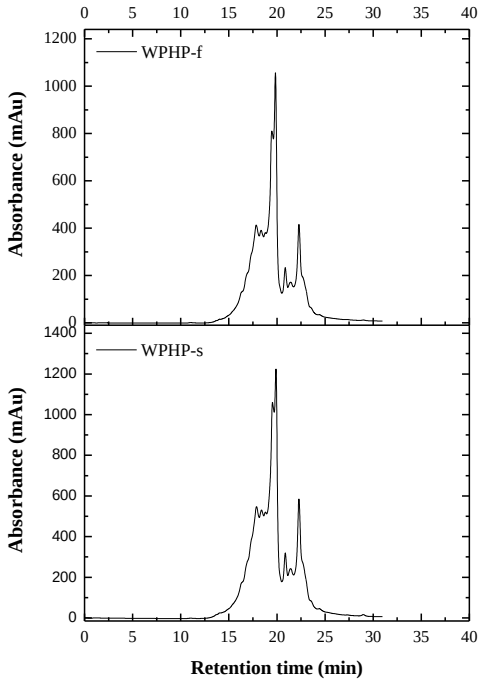


Figure 4. Molecular weight distribution of WPHP-s (A) and WPHP-f (B)

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References

[1] D. D. Kitts and K. Weiler, "Bioactive proteins and peptides from food sources applications of bioprocesses used in isolation and recovery," *Curr. Pharm. Des.*, vol. 9, no. 16, pp. 1309-1323, Jun. 2003.

[2] J. T. Hancock, R. Desikan, and S. J. Neill, "Role of reactive oxygen species in cell signalling pathways," *Biochem. Soc. Trans.*, vol. 29, no. 2, pp. 345-349, 2001.

[3] J. S. Johansen, A. K. Harris, D. J. Rychly, and A. Ergul, "Oxidative stress and the use of antioxidants in diabetes: Linking basic science to clinical practice," *Cardiovasc. Diabetol.*, vol. 4, no. 1, pp. 5, Apr. 2005.

[4] M. Valko, D. Leibfritz, J. Moncol, M. T. Cronin, M. Mazur, and J. Telser, "Free radicals and antioxidants in normal physiological functions and human disease," *Int. J. Biochem. Cell. Biol.*, vol. 39, no. 1, pp. 44-84, Jan. 2007.

[5] B. Halliwell, "Free radicals, antioxidants, and human disease: Curiosity, cause, or consequence," *The Lancet*, vol. 344, no. 8924, pp. 721-724, Sep. 1994.

[6] S. J. Jadhav, S. S. Nimbalkar, A. D. Kulkarni, and D. Madhavi, "Lipid oxidation in biological and food systems," in *Food Antioxidants*, New York, USA: Marcel Dekker Inc., 1996.

[7] J. Lee, N. Koo, and D. B. Min, "Reactive oxygen species, aging, and antioxidative nutraceuticals," *Compr. Rev. Food Sci. Food Saf.*, vol. 3, no. 1, pp. 21-33, Jan. 2004.

[8] M. Alaiz, M. Beppu, K. Ohishi, and K. Kikugawa, "Modification of delipidated apoprotein b of low density lipoprotein by lipid oxidation products in relation to macrophage scavenger receptor binding," *Biol. Pharm. Bull.*, vol. 17, no. 1, pp. 51-57, Jan. 1994.

[9] B. H. Sarmadi and A. Ismail, "Antioxidative peptides from food proteins: A review," *Peptides*, vol. 31, no. 10, pp. 1949-1956, Oct. 2010.
[10] H. P. Jiang, T. Z. Tong, J. H. Sun, Y. J. Xu, Z. X. Zhao, and D. K. Liao, "Purification and characterization of antioxidative peptides from round scad *Decapterus maruadsi* muscle protein hydrolysate," *Food Chem.*, vol. 154, no. 1, pp. 158-163, Jul. 2014.
[11] Y. C. Xia, B. Bamdad, M. Gänzle, and L. Y. Chen, "Fractionation and characterization of antioxidant peptides derived from barley glutelin by enzymatic hydrolysis," *Food Chem.*, vol. 134, no. 3, pp. 1509-1518, Oct. 2012.
[12] C. H. Tang, J. Peng, D. W. Zhen, and Z. Chen, "Physicochemical and antioxidant properties of buckwheat *Fagopyrum Esculentum* moench protein hydrolysates," *Food Chem.*, vol. 115, no. 2, pp. 672-678, Jul. 2009.
[13] W. Chen, Y. Guo, J. Zhang, X. Zhang, and Y. Meng, "Effect of different drying processes on the physicochemical and antioxidant properties of thinned young apple," *Inter. J. Food Eng.*, vol. 11, no. 2, pp. 207-219, Apr. 2015.
[14] C. Ratti, "Hot air and freeze-drying of high-value foods: A review," *J. Food Eng*., vol. 49, no. 4, pp. 311-319, Sep. 2001.
[15] R. Murugesan and V. Orsat, "Spray drying for the production of nutraceutical ingredients: A review," *Food Bio. Process. Tech.*, vol. 5, no. 1, pp. 3-14, Jan. 2012.
[16] Y. F. Maa, P. A. Nguyen, T. Sweeney, S. J. Shire, and C. C. Hsu, "Protein inhalation powders: Spray drying vs spray freeze drying," *Pharm. Res.*, vol. 16, no. 2, pp. 249-254, Feb. 1999.
[17] N. Chen, H. Yang, Y. Sun, J. Niu, and S. Liu, "Purification and identification of antioxidant peptides from walnut *Juglans regia* L. protein hydrolysates," *Peptides*, vol. 38, no. 2, pp. 344-349, Dec. 2012.
[18] Y. F. Li, H. D. Xu, J. H. Chen, Y. P. Li, and H. P. Li, "Studies on antioxdative activity of walnut residue protein hydrolysate by papain," *J. Chin. Inst. Eng.*, vol. 5, no. 2, p. 004, May 2008.
[19] A. G. Asuero, "An overview of the Kjeldahl method of nitrogen determination," *Crit. Rev. Anal. Chem.*, vol. 43, no. 4, pp. 178-223, Jul. 2013.
[20] S. Nilsang, S. Lertsiri, M. Suphantharika, and A. Assavanig, "Optimization of enzymatic hydrolysis of fish soluble concentrate by commercial proteases," *J. Food Eng.*, vol. 70, no. 4, pp. 571-578, Oct. 2005.
[21] L. J. Gu, *et al.*, "Chemical and cellular antioxidant activity of ttwo novel peptides designed based on glutathione structure," *Food Chem. Toxicol.*, vol. 50, no. 11, pp. 4085-4091, Nov. 2012.
[22] I. G. D. Avellar, M. M. Magalhães, A. B. Silva, L. L. Souza, A. C. Leitão, and M. Hermes-Lima, "Reevaluating the role of 1, 10-phenanthroline in oxidative reactions involving ferrous ions and dna damage," *Biochim. Biophys. Acta*, vol. 1675, no. 1-3, pp. 46-53, Nov. 2004.
[23] Y. H. Li, B. Jiang, T. Zhang, W. M. Mu, and J. Liu, "Antioxidant and free radical-scavenging activities of Chickpea Protein Hydrolysate (CPH)," *Food Chem.*, vol. 106, no. 2, pp. 444-450, Jan. 2008.
[24] C. Z. Zhu, W. G. Zhang, Z. L. Kang, G. H. Zhou, and X. L. Xu, "Stability of an antioxidant peptide extracted from Jinhua ham," *Meat. Sci.*, vol. 2, pp. 783-789, 2014.
[25] A. Moure, H. Domínguez, and J. C. Parajó, "Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates," *Process Biochem.*, vol. 41, no. 2, pp. 447-456, Feb. 2006.
[26] L. N. Bell and T. P. Labuza, "Potential pH implications in the freeze-dried state," *Cryo Letters*, vol. 12, no. 4, pp. 235-244, Feb. 1991.
[27] N. Faithong, S. Benjakul, S. Phatcharat, and W. Binsan, "Chemical composition and antioxidative activity of thai traditional fermented shrimp and krill products," *Food Chem.*, vol. 119, no. 1, pp. 133-140, Mar. 2010.
[28] S. Nalinanon, S. Benjakul, H. Kishimura, and F. Shahidi, "Functionalities and antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna," *Food Chem.*, vol. 124, no. 4, pp. 133-140, Feb. 2011.
[29] X. X. Li, L. J. Han, and L. J. Chen, "*In vitro* antioxidant activity of protein hydrolysates prepared from corn gluten meal," *J. Sci. Food Agric.*, vol. 88, no. 9, pp. 1660-1666, Jul. 2008.
[30] S. Nalinanon, S. Benjakul, W. Visessanguan, and H. Kishimura, "Improvement of gelatin extraction from bigeye snapper skin using pepsin-aided process in combination with protease inhibitor," *Food Hydrocoll.*, vol. 22, no. 4, pp. 615-622, Jun. 2008.

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