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Antioxidant Activities of Peptide Fractions Derived from Freshwater Mussel Protein Using Ultrasound-Assisted Enzymatic Hydrolysis

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Abstract

Dong Z., Tian G., Xu Z., Li M., Xu M., Zhou Y., Ren H. (2017): Antioxidant activities of peptide fractions derived from freshwater mussel protein using ultrasound-assisted enzymatic hydrolysis. Czech J. Food Sci., 35: 328–338.

The freshwater mussel protein was hydrolysed using ultrasound-assisted enzymolysis. Ultrasound-assisted freshwater mussel protein hydrolysates (UPH) were divided into four fractions (> 10, 6–10, 3–6, and < 3 kDa) using ultrafiltration, and the fraction with the highest antioxidant activity was further subdivided into four fractions (F_1–F_4) using gel chromatography. The amino acid compositions and antioxidant activities (DPPH, hydroxyl and superoxide radical scavenging activities, reducing power, ferrous ion chelating activity, and inhibition of linoleic acid oxidation) of peptide fractions were investigated. The results showed that the antioxidant activity of the < 3 kDa fraction was significantly higher than that of UPH, > 10, 6–10, and 3–6 kDa fractions. The antioxidant activity of F_2 was again higher compared with the < 3 kDa fraction and higher than that of F_1, F_3, and F_4. Amino acid analysis showed that the antioxidant activities (except for chelating activity) of peptides increased with increasing hydrophobic amino acid content. The < 3 kDa and F_2 fractions exhibited strong inhibition of linoleic acid oxidation, their effects being even better than that of ascorbic acid (Vc) and L-glutathione (GSH). Therefore, these peptide fractions from freshwater mussel may be a potential natural antioxidant that could be added to various foods.

Keywords: freshwater mussel; ultrasound-assisted; purification; peptides; amino acid composition; antioxidant activities

Reactive oxygen species (ROS) and free radicals are generated in the course of normal physiological activities and especially during cellular respiration in humans and other aerobic organisms. ROS and free radicals can play roles in many diseases, such as atherosclerosis, high blood pressure, inflammation, cancer, diabetes, and Alzheimer's disease (Diaz *et al*. 1997; Bougatef *et al*. 2010; Ngo *et al*. 2010; Salim *et al*. 2010; Samadi & Ismail 2010; Chandrasekara & Shahidi 2011). Therefore, increasing attention is being paid to the relationships between free radicals and aging or disease. Antioxidants have been widely used in the food, cosmetics, medical and other industries, in the form of compounds such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). Synthetic antioxidants are most commonly employed, but the use of these must be strictly controlled due to potential health issues (Kim & Wijesekara 2010; Chalamaiah *et al*. 2012; Farvin *et al*. 2014). In recent years, the gradual development of antioxidant peptides by various researchers has revealed that these exhibit higher antioxidant activity and stable structures, as well having low molecular weights and being easy to absorb while not causing harmful immune responses (Qian *et al*. 2008; Liu *et al*. 2010). Compared with other antioxidants, antioxidant peptides have greater potential for development and eventual application.

With the development and utilisation of aquatic products, research on the preparation of bioactive

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peptides from aquatic proteins has gradually expanded. Normally, the bioactive peptide is composed of 3–20 amino acids, and the molecular weight is less than 6 kDa (Kim & Wijesekara 2010; Samadi & Ismail 2010). Many studies have shown that bioactive peptides have many functional properties, such as a lowering of blood pressure (Bougatef *et al*. 2008; Sun *et al*. 2011; Ko *et al*. 2012a), as well as antioxidant (Rajapakse *et al*. 2005a; Ko *et al*. 2012b; Girgih *et al*. 2013), anti-inflammatory (Yang *et al*. 2012), and antibacterial properties (McCann *et al*. 2006).

The freshwater mussel, *Lamellibranchia*: *Unionidae*, has rich nutritional value. The base content of protein is as high as 51.07% in dry meat, and the essential amino acids account for 44.43% of the total amino acid content. In addition, freshwater mussel meat is a high-quality edible resource that is rich in polysaccharides, unsaturated fatty acids, and mineral elements (Zhang *et al*. 2012). There are rich and low-cost resources of wild freshwater mussels in most rivers and lakes in China. In recent years, research into the composition of active ingredients in freshwater mussels has attracted more and more attention, but due to its thick flesh and smell of soil, the farming of mussels has not undergone large-scale industrial development.

Until now, there are only a few studies in the literature concerned with freshwater mussel protein-derived peptides. These studies have mainly evaluated the inhibition of angiotensin I-converting enzyme (ACE) of freshwater mussel hydrolysates. The aim of this study was to identify potential natural antioxidants in freshwater mussel, which could then be used in various kinds of foods. Hydrolysates were obtained using ultrasound-assisted enzymatic hydrolysis, and were purified through a tangential flow filtration system and gel chromatography. The amino acid compositions of the peptide fractions were analysed and the antioxidant activities were determined on the basis of reducing power, DPPH, hydroxyl radical, and superoxide radical scavenging activity, ferrous ion chelating activity, and the inhibition of linoleic acid oxidation.

MATERIAL AND METHODS

Fresh live freshwater mussels were bought from the Songhua River basin in Jilin Province, China, and transported to the laboratory at room temperature. After removing shells and viscera, the freshwater mussel meat was frozen in polyethylene bags at –18°C until use. The raw material was thawed using running cold water (at about 20°C) before use, broken up in a Waring blender for 2 min at high speed and homogenised twice in a colloid mill. Freshwater mussel homogenate was used in the next step of enzymatic hydrolysis.

Neutrase 0.8 L (EC: 3.4.24.28, CAS: 9080-56-2) was purchased from Novozymes Biotechnology (China); 2,2-diphenyl-1-picrylhydrazyl (DPPH), pyrogallol (1,2,3-trihydroxybenzene), reduced L-glutathione (GSH), ascorbic acid (Vc), linoleic acid, and ferrozine were purchased from Sigma Chemicals (China). Sephadex G-25 was purchased from Pharmacia (China); potassium ferricyanide, ammonium thiocyanate, trichloroacetic acid (TCA), ethylene diamine tetra acetic acid (EDTA), and other analytical grade chemical reagents were obtained from GuangFu Technology Development (China).

Preparation of ultrasound-assisted enzymatic hydrolysates from freshwater mussel protein. The content of freshwater mussel protein was assayed using the Kjeldahl method. Freshwater mussel homogenate (92 g) was dispersed into 58 ml of distilled water. The mixture was adjusted to pH 6.0 using 1 M HCl followed by the addition of Neutrase 0.8 L (43.85×10^4 units/g protein) at 4141 units/g freshwater mussel protein. The mixture was subjected to ultrasonication at 40°C and 150 W for 30 minutes. Incubated continued at 51°C with stirring for 3 hours. Then, the mixture was placed in boiling water for 10 min to inactivate proteases, cooled to room temperature and centrifuged at 10 000 r/min for 10 min at 4°C. The supernatant was collected as the ultrasound-assisted freshwater mussel protein enzymatic hydrolysate (UPH). A portion of the hydrolysate was freeze-dried and stored at –18°C until further analysis.

Ultrafiltration separation of enzymatic hydrolysates. Ultrasound-assisted freshwater mussel protein enzymatic hydrolysis solution (without lyophilisation) was passed through a tangential flow filtration system with molecular weight cut-offs of 10, 6, and 3 kDa from Super Yu Membrane Separation Technology (China). Four peptide fractions (> 10, 6–10, 3–6, and < 3 kDa) were collected and freeze-dried separately. The lyophilised powders were solubilised with distilled water to a concentration of 1.0 mg/ml for the determination of antioxidant activities (reducing power and DPPH radical scavenging activity).

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Table 1. Preparation of buffer solutions

Reagents	Buffer solutions				
	B1	B2	B3	B4	B5
Distilled water (ml)	700	700	700	700	700
Sodium citrate (g)	6.19	7.74	13.31	26.67	–
Sodium hydroxide (g)	–	–	–	–	8.00
Sodium chloride (g)	5.66	7.07	3.74	54.35	–
Citric acid (g)	19.80	22.00	12.80	6.10	–
Ethanol (ml)	130.0	20.0	4.0	/	100.0
Benzyl alcohol (ml)	–	–	–	5.0	–
Thio double ethanol (ml)	5.0	5.0	5.0	–	–
Polyethylene glycol monooleyl ether* (ml)	4.0	4.0	4.0	4.0	4.0
Octanoic acid (ml)	0.1	0.1	0.1	0.1	0.1
pH	3.3	3.2	4.0	4.9	–

*25 g was dissolved in 100 ml of distilled water

Sephadex G-25 gel filtration chromatography. The fraction with the highest antioxidant activity was dissolved in distilled water to a concentration of 25 mg/ml, and then the sample (1.0 ml) was separated on a Sephadex G-25 column (2.0 × 60 cm). The column was operated in downward flow at room temperature. Distilled water was used to equilibrate the column and to elute the peptide fractions at a flow rate of 0.7 ml/minute. Fractions of 2.5 ml were collected, and the eluted peaks were detected using ultraviolet absorbance at 215 nm. The four eluted fractions (F_1, F_2, F_3, and F_4) were collected, concentrated, and lyophilised. The eluted powders were dissolved in distilled water to a concentration of 1.0 mg/ml for determination of antioxidant activity (reducing power and DPPH radical scavenging activity).

Amino acid composition analysis. The sample (200 mg) was placed in hydrolysis tubes and digested with 10 ml HCl (6 M) at 110°C for 22 h under nitrogen atmosphere. After acid hydrolysis, the sample was cooled to room temperature and transferred to a 50-ml capacity bottle. After filtration, the filtrate (2 ml) was evaporated in a rotary vacuum evaporator with in a water bath set to 50°C, then dissolved with 0.02 M HCl and transferred to a Hitachi L-8900 automatic amino acid analyser. The post-column derivatisation method was used and the instrument was operated according to the manual. Technical and operating parameters were the following: separation column size 4.6 mm ID × 60 mm, particle size 3 μm;, resin type 2622 Hitachi dedicated ion exchange resin;, reactive column size 4.6 mm ID × 40 mm, filling material emery inert material; flow rate 0.40 ml/min (pump 1) and 0.35 ml/min (pump 2), detection wavelengths 570 and 440 nm., separation column temperature 57°C, reaction column temperature 135°C, injection volume 20 μl. The used reagents were formulated according to Tables 1 and 2. Instrument operation was carried out according to Table 3. The amino acid composition was expressed as g of amino acid per 100 g of protein.

Reducing power. Substances with higher reducing power generally have a high capability of providing electrons, and many studies have shown that the antioxidant activity of some natural antioxidants

Table 2. Preparation of ninhydrin reagents

Preparation steps	Reaction reagents		
	R1	R2	R3
1	dipropylene glycol monomethyl ether, 979 ml	distilled water, 336 ml	distilled water, 900 ml
2	ninhydrin, 39 g	lithium acetate, 204 g	ethanol, 50 ml
3	nitrogen bubbling, decomposition, 5 min	glacial acetic acid, 123 ml	add distilled water to 1000 ml
4	sodium borohydride, 81 mg	dipropylene glycol monomethyl ether, 401 ml	–
5	nitrogen bubbling, 30 min	nitrogen bubbling, 10 min	–

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Table 3. Gradient elution and reaction program (%)

Time (min)	Pump 1					Pump 2		
	B1	B2	B3	B4	B5	R1	R2	R3
0.0	100	0	0	0	0	50	50	0
2.5	100	0	0	0	0	–	–	–
2.6	0	100	0	0	0	–	–	–
4.5	0	100	0	0	0	–	–	–
4.6	0	0	100	0	0	–	–	–
12.8	0	0	100	0	0	–	–	–
12.9	0	0	0	100	0	–	–	–
29.0	0	0	0	100	0	–	–	–
29.1	0	0	0	0	100	–	–	–
32.0	–	–	–	–	–	50	50	0
32.1	–	–	–	–	–	0	0	100
33.0	0	0	0	0	100	–	–	–
33.1	0	100	0	0	0	–	–	–
34.0	0	100	0	0	0	–	–	–
34.1	100	0	0	0	0	–	–	–
37.0	–	–	–	–	–	0	0	100
37.1	–	–	–	–	–	50	50	0
53.0	100	0	0	0	0	–	–	–

has a direct relationship with their reducing power (Duh 1998; Thana *et al*. 2008). Therefore, reducing power can be used as a measure of the antioxidant activity of a sample.

The reducing power of the peptide fractions was measured according to the method of Bougatef *et al*. (2010) with minor modifications. A 1.2-ml aliquot of sample or 1.2 ml distilled water (blank) were mixed with 3.0 ml of 0.2 M phosphate buffer (pH 6.6) and 3.0 ml of 1% (w/v) potassium ferricyanide. The mixture was incubated at 50°C for 30 minutes. After incubation, 3.0 ml of 10% TCA were added to the reaction mixture, followed by centrifugation at 5000 *g* for 10 minutes. Thereafter, 2.0 ml of mixture was combined with 2.0 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride solution and allowed to stand at room temperature for 10 minutes. Finally, the absorbance of the resultant solution was measured at 700 nm. Vc and GSH were used as positive controls. A higher absorbance of the reaction mixture indicated higher reducing power.

DPPH radical scavenging activity. The activity of peptide fractions against the DPPH radical was measured using the modified method of You *et al*. (2011). DPPH radical (2.0 ml, 0.1 mM) dissolved in 95% ethanol was added to 2.0 ml of sample solution and kept for 30 min (at room temperature) in the dark. The absorbance of the resulting mixture was measured at 517 nm. Vc and GSH were used as positive controls. The DPPH radical scavenging activity was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \left(1 - \frac{A_1 - A_2}{A_3}\right) \times 100$$

where: A_1 – absorbance of the sample with DPPH solution; A_2 – absorbance of the sample without DPPH solution; A_3 – absorbance of the distilled water with the DPPH solution

Hydroxyl radical scavenging activity. This assay was performed according to a previously developed method of Huang *et al*. (2012) with some modifications. A mixture of 1.0 ml of sample and 0.5 ml of salicylic acid-ethanol (10 mM) were mixed with 0.5 ml of $FeSO_4$ (10 mM). Then, 0.5 ml of H_2O_2 (8.8 mM) were added. The mixture was incubated at 37°C for 30 min and then absorbance was measured at 510 nm. Vc and GSH were used as positive controls. To calculate hydroxyl radical scavenging, the following equation was used:

$$\text{Hydroxyl radical scavenging activity (\%)} = \left(1 - \frac{A_s - A_c}{A_b}\right) \times 100$$

where: A_s – absorbance of the sample; A_c – absorbance of a control solution lacking H_2O_2; A_b – absorbance of a blank solution containing distilled water instead of sample

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Superoxide radical scavenging activity. The scavenging activity of peptide fractions was determined according to the method reported by Li *et al.* (2008) with some modifications. Briefly, 0.2 ml of sample or 0.2 ml distilled water (blank) were mixed with 5.6 ml of 0.1 M Tris-HCl buffer (pH 8.2). The mixture was incubated at 25°C in the dark for 10 minutes. After incubation, 0.2 ml pyrogallol (3 mM in 10 mM HCl) was added to the mixture. Then, the absorbance of the mixture was measured at 325 nm every 30 s for 4 min, and a slope was calculated as the absorbance/minute. Vc and GSH were used as positive controls. The superoxide radical scavenging activity was calculated as follows:

$$\text{Superoxide radical scavenging activity (\%)} = \left(1 - \frac{A_s}{A_b}\right) \times 100$$

where: A_b – reaction rate (ΔA/min) based on absorbance of the blank group in the superoxide radical anion generation system; A_s – reaction rate (ΔA/min) based on absorbance of the sample

Ferrous ion chelating activity. The ability of the freshwater mussel peptides to chelate iron(II) was evaluated using the method of Gülçin *et al.* (2011) with some modifications. One ml of sample was premixed with 2.0 ml of double-distilled water and 0.05 ml of 2 mM ferrous chloride solution. After 3 min at room temperature, the reaction was initiated by the addition of 0.1 ml of ferrozine (5 mM). The mixture was shaken vigorously and left at room temperature for 10 minutes. The absorbance of the resulting solution was measured at 562 nm using EDTA as a positive control. The chelating capacity was calculated as follows:

$$\text{Chelating ability (\%)} = \left(1 - \frac{A_s}{A_b}\right) \times 100$$

where: A_s – absorbance of the sample at 562 nm; A_b – absorbance of the control (distilled water) at 562 nm

Inhibition of linoleic acid oxidation. The inhibition of linoleic acid oxidation was measured using the method of Tanzadehpanah *et al.* (2012), which was modified as follows. Briefly, 1.0 ml of sample (dissolved in 50 mM sodium phosphate buffer, pH 7.0) was mixed with 0.5 ml of distilled water and 1.0 ml of 50 mM linoleic acid in 95% ethanol. The reaction mixture was incubated in a 5.0 ml conical flask with a screw cap at 60°C in a dark room to accelerate oxidation for 72 hours. After incubation, 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate solution and 0.1 ml of ferrous chloride solution (20 mM in 3.5% HCl) were added to 0.1 ml of reaction mixture. Then, the mixture was stirred for 3 min at room temperature, and the absorbance was measured at 500 nm. Vc and GSH were used as positive controls. The percentage of oxidation inhibition was calculated as follows:

$$\text{Inhibition (\%)} = \left(1 - \frac{A_{s_1} - A_{s_0}}{A_{b_1} - A_{b_0}}\right) \times 100$$

where: A_{s_1}, A_{s_0} – sample absorbance at t = 72 h and t = 0, respectively; A_{b_1}, A_{b_0} – negative control absorbance at t = 72 h and t = 0, respectively

Statistical analysis. All data were reported as the means of three parallel determinations. Statistical comparisons of the mean values were performed using analysis of variance (ANOVA), followed by Duncan's multiple range test in SPSS (17.0) software. Results were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Ultrafiltration separation of enzymatic hydrolysates. The differences in antioxidant activity between UPH and the four fractions at a concentration of 1.0 mg/ml were determined as shown in Figure 1.

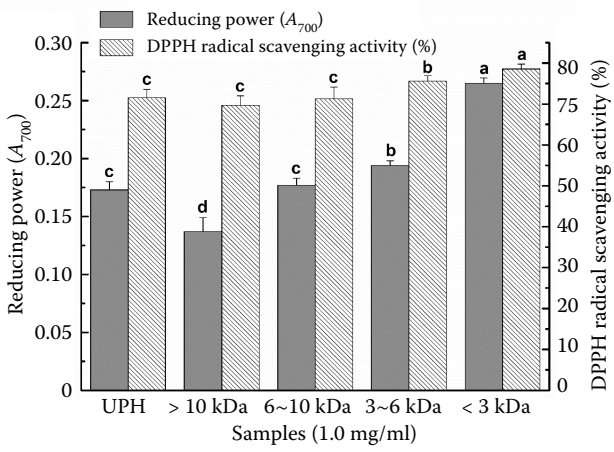

Figure 1. The reducing powers and DPPH radical scavenging activities of UPH and the four peptide fractions (with molecular weights of > 10, 6–10, 3–6, and < 3 kDa)

Variance analysis of each group of data with different evaluation indexes, α = 0.05; values sharing the same letter are not significantly different from each other, while differing letters indicate that the difference is significant)

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With decreasing molecular weight, the reducing power of the peptide fractions gradually increased, especially in the < 3 kDa fraction, whose reducing power was significantly ($P < 0.05$) higher than that of the other three fractions and of UPH, as indicated in Figure 1. The DPPH radical scavenging activity of the < 3 kDa fraction was also significantly ($P < 0.05$) higher than that of the other three fractions and UPH. This indicates that with decreasing molecular weight, the ability of peptide fractions to scavenge DPPH radicals increased, which was consistent with the results for reducing power. Thus, the < 3 kDa molecular weight fraction had the highest antioxidant activity, which is in line with previous reports (Rajapakse *et al*. 2005b; Samadi & Ismail 2010), where it has been demonstrated that the majority of peptides with high antioxidant activity are of small molecular weight. Moosmann and Behl (2002) reported that oxidant-antioxidant systems are more accessibility for small peptides and amino acids than than to large peptides and proteins.

Sephadex G-25 gel filtration chromatography of < 3 kDa peptide fraction. The < 3 kDa fraction was further fractionated using Sephadex G-25 gel filtration chromatography, and F_1, F_2, F_3, and F_4 were obtained as shown in Figure 2. The antioxidant activity of each fraction at a concentration of 1.0 mg/ml was determined as shown in Figure 3.

The highest reducing power showed F_2 (A_{700} = 0.312) in comparison with the other three fractions (F_1 0.194; F_3 0.210; F_4 0.188). The reducing power of F_2 was also significantly higher ($P < 0.05$) than that of UPH (A_{700} = 0.176) and the < 3 kDa fraction (A_{700} = 0.259). The reducing power of F_2 was highest, followed by the < 3 kDa fraction, and the sample with the lowest reducing power was UPH. The reducing powers of F_1, F_3, F_4 were slightly higher than that of UPH, but they were lower than that of the < 3 kDa fraction as indicated in Figure 3. F_2 possessed the highest DPPH radical scavenging capacity (81.3%) out of all groups. However, the DPPH radical scavenging activity of F_1 was not significantly better than that of the < 3 kDa fraction, and the scavenging activity of F_3 and F_4 was even significantly decreased ($P < 0.05$). A comprehensive comparison of the results of the two assays showed that purified F_2 had the best antioxidant capacity, which indicated that the whole separation process is effective in purifying antioxidant peptides. The average molecular weight of F_2 was higher than that of F_3 and F_4 in line with the principle of gel chromatography, indicating that the antioxidant activity of a peptide does not always increase with decreasing molecular weight. This result is in accordance with the study of Xia *et al*. (2012) that demonstrated that large peptides possessed much greater ($P < 0.05$) DPPH scavenging activity than the small ones. We suggest that the antioxidant activity of the peptides isolated from freshwater mussel may be related to amino acid composition as well as the molecular weight. Taheri *et al*. (2014) reported that the higher radical scavenging activity of peptide fractions from salted herring brine may be due to the presence of higher amounts of histidine, both in the free form and incorporated into peptides.

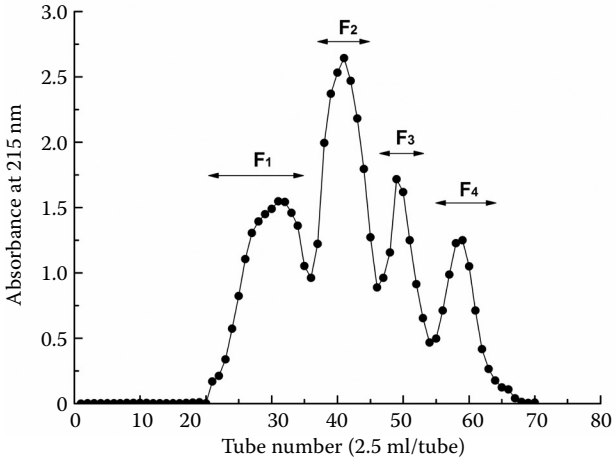

Figure 2. Sephadex G-25 gel filtration chromatograms of the < 3 kDa fraction

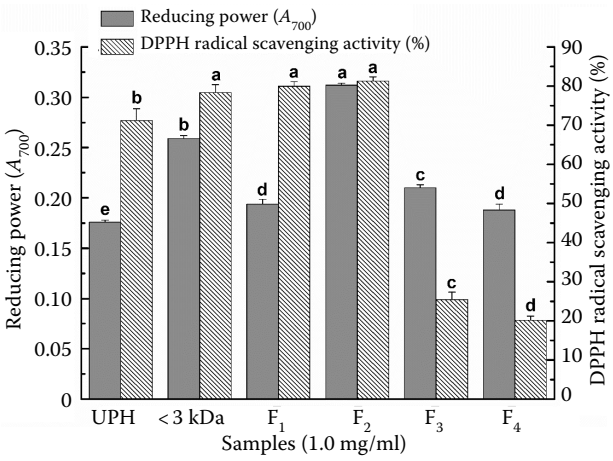

Figure 3. Reducing powers and DPPH radical scavenging activities of UPH, < 3 kDa fraction, F_1, F_2, F_3, and F_4 fractions

Variance analysis of each group of data with different evaluation indexes, α = 0.05; values sharing the same letter are not significantly different from each other, while differing letters indicate that the difference is significant)

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Amino acid composition analysis. Amino acid contents of freshwater mussel protein, and the < 3 kDa and F_2 fractions, were determined as shown in Table 4. The content of cysteine and tryptophan have not been determined in this study, but, according to Zhang *et al*. (2013) and data from the China Food Nutrition Network (2010), the content of these two amino acids in the freshwater mussel are very low (under 2% of all proteinogenic amino acids), and, therefore, their effects on the antioxidant activity of proteins cannot be expected to be significant. It can be seen from Table 4 that the EAAs (Ile, Leu, Lys, Met, Phe, Thr, and Val) in freshwater mussel protein, the < 3 kDa and F_2 peptide fractions were 15.24, 18.27, and 18.62 g/100 g, respectively. The HAA contents were 15.49 g/100 g (freshwater mussel protein), 17.75 g/100 g (< 3 kDa), and 18.46 g/100 g (F_2), respectively. These results show that the process of enzymatic hydrolysis, ultrafiltration, and chromatography increased the contents of HAA, which is similar to what was reported by Girgih *et al*. (2013) and He *et al*. (2013). Samadi and Ismail (2010) reported that high HAA content could promote peptide entry into target organs through hydrophobic interactions with membrane lipid bilayers, which could improve antioxidant activity *in vitro*. In the process of purification, methionine and histidine content increased, and these amino acids usually exhibit a certain degree of antioxidant activity in the free state. Histidine exhibits strong radical scavenging activity due to the decomposition of its imidazole ring (Yong & Karel 1978). The purification process decreases the content of glutamic acid and aspartic acid in NCAA, which may have a negative effect on antioxidant activity. Udenigwe and Aluko (2011) reported that glutamic acid and aspartic acid in NCAA have strong antioxidant effects due to the presence of excess electrons that can be donated during interaction with free radicals. The content of aspartic acid, glutamic acid, alanine, leucine, lysine, and arginine was high in the freshwater mussel protein and peptide fractions. Aspartic acid and glutamic acid impart a fibre taste, while glycine is responsible for sweet flavour. In summary, freshwater mussel peptides with high nutritional value can not only be developed as a functional food with high antioxidant activity, but can also be used as a condiment.

Table 4. Amino acid composition of freshwater mussel protein, < 3 kDa, and F_2 peptide fractions (g/100 g sample)

Amino acid	Mussel protein	< 3 kDa	F_2
Asp	4.10	3.82	2.54
Thr	1.90	2.10	1.86
Ser	2.08	2.13	2.00
Glu	6.66	6.71	3.95
Pro	1.80	1.66	1.31
Gly	2.60	2.31	2.04
Ala	2.44	3.21	3.16
Cys	nd	nd	nd
Val	2.05	2.53	2.28
Met	0.85	1.26	1.54
Ile	1.99	2.40	2.30
Leu	3.34	4.32	4.66
Tyr	1.22	0.16	0.09
Phe	1.80	2.21	3.12
Lys	3.31	3.45	2.86
His	0.84	0.98	1.20
Arg	2.98	3.35	3.43
Trp	nd	nd	nd
EAA	15.24	18.27	18.62
HAA	15.49	17.75	18.46
AAA	3.02	2.37	3.21
PCAA	7.13	7.78	7.49
NCAA	10.76	10.53	6.49

nd – not determined; essential amino acids (EAA) = isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine; hydrophobic amino acids (HAA) = alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, tryptophan, proline, methionine and cysteine; aromatic amino acids (AAA) = phenylalanine, tryptophan and tyrosine; positively charged amino acids (PCAA) = arginine, histidine and lysine; negatively charged amino acids (NCAA) = aspartic acid and glutamic acid

Reducing power. The ability of polypeptides to reduce Fe^{3+} to Fe^{2+} is used to evaluate reducing power (You *et al*. 2009). The reducing power of each group was determined as shown in Figure 4A. The reducing powers of the < 3 kDa and F_2 fractions increased gradually with increasing concentration. Compared to the < 3 kDa fraction, the reducing power of F_2 was slightly greater. The absorbance values were 0.441 (< 3 kDa) and 0.634 (F_2), respectively, at a concentration of 2.0 mg/ml. This indicates that chromatography purification could improve the reducing power of peptide fractions. Taken together with the data in Table 4, it can be concluded that with increasing HAA content, the reducing powers of peptide fractions were also enhanced, which is consistent with previously reported data (Pownall *et al*. 2010). The

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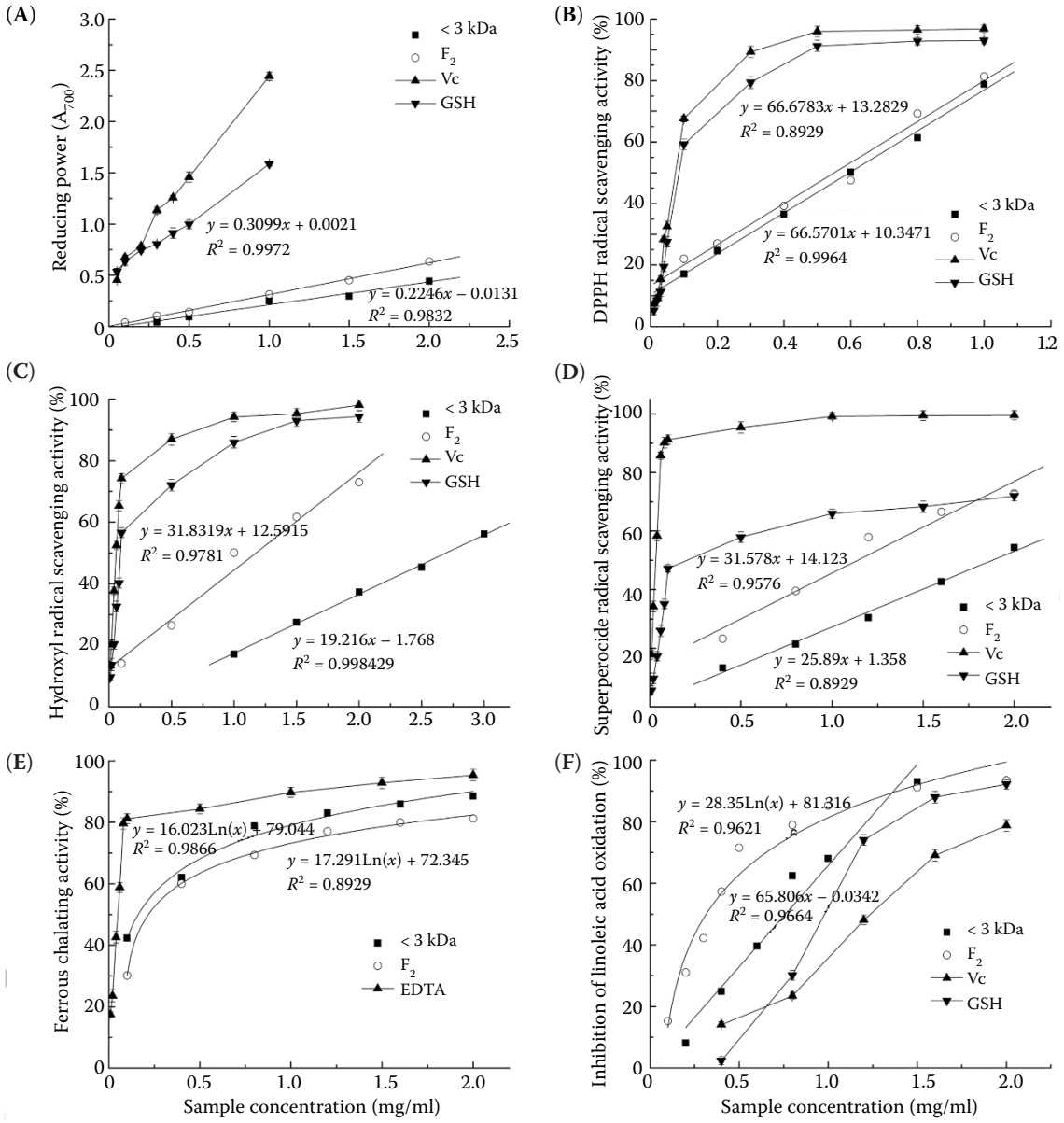

Figure 4. Antioxidant activities of < 3 kDa and F_2 fractions from mussel hydrolysates and positive controls

reducing powers of the < 3 kDa and F_2 fractions were both significantly ($P < 0.05$) lower than those of Vc and GSH, which was to be expected.

Radical scavenging activities. DPPH radical scavenging activity is a common method to evaluate *in vitro* antioxidant activity; the degree of colour change of the mixture depends on the ability of the sample to quench the free radicals (Xie *et al.* 2008). As shown in Figure 4B, the DPPH radical scavenging activities of the < 3 kDa and F_2 fraction increased rapidly with increasing concentrations from 0 to 1.0 mg/ml. At a sample concentration of 1.0 mg/ml, the DPPH radical scavenging activities of the samples were 78.8% (< 3 kDa) and 81.3% (F_2), respectively, which were only slightly lower than those of Vc (96.8%) and GSH (93.0%). These data indicate that the process of chromatography purification contributed critically to obtaining fractions with robust DPPH radical scavenging activity. The scavenging rate of DPPH increased with increasing HAA content (Table 4), a trend which was similar to the results for reducing power.

The hydroxyl radical scavenging activity in all samples increased with increasing concentrations (Figure 4C). The F_2 fraction exhibited a higher hydroxyl radical scavenging activity compared to the < 3 kDa fraction ($P < 0.05$), which was increased by almost 196% at the level of 2.0 mg/ml. It was also observed

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that the content of HAA played an important role in the of scavenging hydroxyl radicals (Table 4). Studies have reported that the type of amino acid, such as His and Met, and specific peptide structures and amino acid sequences are responsible for hydroxyl radical scavenging activity (Hernández-Ledesma *et al*. 2005). Moreover, the scavenging rate of F_2 (50.1%, 1.0 mg/ml) was markedly higher than the same rates in salmon peptides (Girgih *et al*. 2013) and pea peptides (Pownall *et al*. 2010), where the highest scavenging activities were 28 and 17% (at 1.0 mg/ml), respectively. Therefore, the F_2 fraction of freshwater mussel peptides exhibits superior hydroxyl radical scavenging activity.

Figure 4D shows that the superoxide radical scavenging activity of the samples increased with increasing concentration. The F_2 fraction exhibited a higher superoxide radical scavenging activity compared with the < 3 kDa fraction, which even slightly exceeded that of GSH (F_2 72.9%, GSH 72.0%, at the level of 2.0 mg/ml). Previous studies in salmon (Girgih *et al*. 2013) and pea seed (Pownall *et al*. 2010) reported that RP-HPLC fractionation led to an increased ability to scavenge superoxide radicals, although peptide concentrations were at the same level (1.0 mg/ml). In contrast, the RP-HPLC peptide fractions from cod (*Gadus morhua*) had significantly ($P < 0.05$) lower radical scavenging activity (15–40%) than unfractionated CPH (45%), as reported by Girgih *et al*. (2015).

Ferrous ion chelating activity. As shown in Figure 4E, both < 3 kDa and F_2 fractions were highly effective at chelating ferrous ion; chelating activity was 88.6% for the < 3 kDa fraction and 81.2% for the F_2 fraction, respectively, at a concentration of 2.0 mg/ml. These values were only slightly lower than those of the positive control (EDTA 95.4%). In contrast to results for other antioxidant activities, the chelating activity of the < 3 kDa fraction was higher than that of the F_2 fraction. This suggests that increased peptide chain length could promote higher iron chelating activity. The strong metal chelating properties of long-chain peptides may be due to the synergistic effects of a higher number of amino acid residues when compared to the shorter peptides. A potential synergistic effect within the < 3 kDa fraction may have been disrupted when the peptides were separated into F_1–F_4 fractions in chromatography purification. This result is similar to that of Girgih *et al*. (2011). He *et al*. (2013) reported that the membrane fractions of rapeseed protein hydrolysate showed superior metal chelating properties compared to the unfractionated protein hydrolysate.

Inhibition of linoleic acid oxidation. The abilities of the < 3 kDa and F_2 fractions to inhibit linoleic acid oxidation in comparison to Vc and GSH, are shown in Figure 4F. The inhibitory activity of the sample increased rapidly with increasing concentration. In addition, the positive controls (Vc and GSH) in this study were significantly ($P < 0.05$) worse in preventing oxidation compared to the peptide fractions. This result indicates that the freshwater mussel peptides possess a marked ability to inhibit linoleic acid oxidation. This may be due to an increased content of hydrophobic amino acids, and is in agreement with the results for radical scavenging and reducing power described above. The hydrophobicity of amino acids within a peptide lead to more interactions between the peptides and the fatty acids, resulting in protection against oxidation (Mendis *et al*. 2005).

CONCLUSION

Peptide fractions with high antioxidant activities were obtained from UPH by ultrafiltration and gel chromatography. After chromatography purification, the antioxidant activities (reducing power, radical scavenging ability, and ability to inhibit linoleic acid autoxidation) of freshwater mussel peptides were all improved, especially the hydroxyl radical scavenging ability. In addition, both the < 3 kDa and F_2 fractions showed strong chelating abilities for ferrous ion with the chelating ability of the < 3 kDa fraction higher than that of the F_2 fraction. To conclude, peptide fractions derived from wild freshwater mussel protein could potentially be used as functional ingredients in various kinds of foods. Additional research is needed to identify the amino acid sequences of peptides with antioxidant activity in the freshwater mussel protein hydrolysate in order to further study structure-function relationships.

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