*Food Sci. Biotechnol.* 20(3): 651-657 (2011)
DOI 10.1007/s10068-011-0092-8

RESEARCH ARTICLE

# Effect of Defatting and Enzyme Type on Antioxidative Activity of Shrimp Processing Byproducts Hydrolysate

**Guang-Rong Huang, Jing Zhao, and Jia-Xin Jiang**

Received: 29 October 2010 / Revised: 10 December 2010 / Accepted: 3 March 2011 / Published Online: 30 June 2011


**Abstract** Shrimp processing byproducts (SPB) was digested by 6 proteases (trypsin, pepsin, neutrase, Protamex, Flavourzyme, and Alcalase) to produce antioxidative peptides. Both degree of hydrolysis (DH) and DPPH radical scavenging activity (DSA) of the Alcalase hydrolysate were the highest of all. The effect of defatting on DH and DSA of the Alcalase hydrolysate was significant. The DH decreased while the DSA increased after defatting of the byproducts. The antioxidative activity of Alcalase hydrolysate was also investigated using several *in vitro* assays, including DPPH, ABTS radical scavenging assays (ASA), reducing power assay, and chelating activity. The antioxidative activity of the hydrolysate was obviously concentration dependent. The SPB Alcalase hydrolysate exhibited notable DSA and ASA with the $IC_{50}$ values of 500 and 7.4 μg/mL, respectively. And the hydrolysate showed 38.9% chelating activity at 120 μg/mL level. The SPB Alcalase hydrolysate was a potential source of natural antioxidants.

**Keywords:** shrimp processing byproduct, antioxidative activity, hydrolysis, defatting, chelating activity

## Introduction

The importance of oxidation in the body and foodstuffs has been widely recognized. Free radicals could be formed as a result of oxidative metabolism which is essential for the survival of cells in body. They can overwhelm protective enzymes such as superoxide dismutase, catalase, and peroxidase, causing destructive and lethal cellular effects by oxidizing membrane lipids, cellular proteins, DNA, and enzymes as a result of the occurrence of diseases, such as cardiovascular diseases, diabetes mellitus, neurological disorders, and even the Alzheimer's disease (1,2). Currently, synthetic additives such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and *tert*-butylhydroquinone (TBHQ) are widely used because of their high antioxidant activity. However, the potential adverse effects of these synthetic additives have stimulated their replacement by natural antioxidants derived from dietary sources (3).

A larger number of studies have showed that protein hydrolysates may possess antioxidant activity. Many antioxidative petitides have been and are being isolated from various marine bioresources such as squid muscle (4), algae (5), yellow stripe trevally (6,7), tuna backbone protein (8), and cod (9). Shrimp processing byproducts (SPB) is important sources of bioactive molecules. The protein content of SPB ranges from 48 to 56% of the total body weight (10). If the protein in the byproducts is recovered and utilized, it can represent signicant economic and social benefits. The SPB was generally used as fertilizer to produce shrimp sauce by salt-fermentation (11). Recent studies have focused on the production of bioactive peptides with antimicrobial (12,13), antioxidative (14), and angiotensin I-converting enzyme (ACE) inhibitory activity (15). To date and the best of our knowledge, many researches of SPB on antioxidative activity have been undertaken about carotenoid-protein complexes (16), extractions of fermented shrimp biowastes (17), shrimp head autolysis products (14), and hydrolysis optimization (18), but little has been known about the effect of defatting and enzyme type on antioxidative activity of SPB hydrolysate. Proteases including endo-peptidase and exopeptidase have different action sites to produce peptides with various antioxidant activity. The enzymatic effect of protease was different due to different

Guang-Rong Huang (✉), Jing Zhao, Jia-Xin Jiang
College of Life Sciences, China Jiliang University, Hangzhou, Zhejiang Province 310018, China
Tel: +86-571-8687-5628; Fax: +86-571-8691-4449
E-mail: grhuang@126.com

materials. It was necessary to find out the optimal protease and pre-treatment method of raw materials in order to obtain high antioxidant peptides.

Therefore, this study aimed to choose the optimum enzyme for producing the antioxidative peptides from SPB and study the effect of defatting on the antioxidative activities. Different methods including the scavenging abilities on free radicals, the reducing capacity, and the ferrous-chelating activity were used to evaluate the antioxidant activities.

## Materials and Methods

**Materials** SPB used in this study was purchased from local Seafood Market, Zhejiang, China. It was dried and grounded into fine powder, and then stored at −20°C until used. Trypsin (2,500 U/mg), Flavourzyme (500 U/mg), neutrase (500 U/mg), Alcalase (250 U/mg), Protamex (50 U/mg), and pepsin (3,000 U/mg) were purchased from Shanghai Kayyon Biological Technology Co., Ltd. (Shanghai, China). DPPH, ABTS, BHT, and trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used in the experiments were from commercial resource and of analytical grade.

**Preparation of the shrimp processing byproducts hydrolysates** SPB was defatted with petroleum ether at 50°C for 12 h by reflux extraction. Then the residue was dried under reduced pressure. To prepare SPB hydrolysates, enzymatic hydrolysis was performed using various enzymes at 4,000 U/g-protein level for 5 h, and the optimal conditions of the 6 enzymes were shown in Table 1. The reaction was terminated by boiling at 100°C for 10 min, and then the hydrolysate was centrifuged at 3,500×g for 10 min at 4°C. The supernatant was lyophilized and stored at −20°C before further analysis.

**Determination of the degree of hydrolysis** The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken ($N_1$–$N_3$) to the total number of bonds/unit weight ($N_2$), was calculated according to the TNBS method by Izco *et al.* (19). The DH was calculated by Eq. 1:

$$\mathrm{DH}(\%)=\frac{N_1-N_3}{N_2}\times 100 \quad (1)$$

where, $N_1$ is free amino nitrogen of enzymatic hydrolysate, mmol/g; $N_2$ is total amino nitrogen of the SPB, mmol/g; and $N_3$ is free amino nitrogen of SPB, mmol/g. Total amino nitrogen of SPB was measured after microwave digestion by 6 mol/L HCl.

**Table 1. Optimum conditions of enzymatic hydrolysis for tested enzymes**

| Enzyme | Buffer | Time (h) | pH | Temperature (°C) |
|---|---|---|---|---|
| Blank | 50 mmol/L Sodium phosphate | 5 | 7.0 | 50 |
| Trypsin | | | 8.0 | 37 |
| Pepsin | | | 2.0 | 37 |
| Protamex | | | 8.0 | 45 |
| Flavourzyme | | | 7.0 | 50 |
| Neutrase | | | 7.0 | 50 |
| Alcalase | | | 8.0 | 50 |

**Proximate analysis** Moisture, protein, fat, and ash of samples were determined according to the method of AOAC (20). The soluble protein in SPB solution was determined after precipitation by 10% trichoroacetic acid.

**Determination of the antioxidative activities**

***DPPH radical scavenging activity*:** The DPPH radical scavenging activity (DSA) was assayed following the method of Wu *et al.* (21) with some modifications. A 0.375 mL of sample was added to 2.0 mL of 0.1 mmol/L DPPH dissolved in methanol. The mixture was shaken and left for 30 min in dark at room temperature, and the absorbance was measured at 517 nm. BHT was used as a positive control to compare the scavenging effect. The DSA was calculated as a percentage of DPPH discoloration using the Eq. 2:

$$\mathrm{DSA}(\%)=\left(1-\frac{A_s-A_0}{A_{DPPH}}\right)\times 100 \quad (2)$$

where, $A_s$, $A_0$, and $A_{DPPH}$ represent the absorbance of the sample, the sample blank, and the control, respectively.

***ABTS radical scavenging activity*:** The ABTS radical-scavenging activity (ASA) was determined according to the method of Re *et al.* (22). The working solution was prepared by mixing 20 mL of 7 mmol/L ABTS stock solution and 352 μL of 140 mmol/L potassium persulphate stock solution, and left for 12 h at room temperature in dark. The working solution was then diluted by water in order to obtain an absorbance of 1.1±0.02 units at 734 nm. Fresh ABTS solution was prepared for each day. A 0.5 mL of sample solution at different concentrations was added to the fresh ABTS working solution, and the mixture was left at room temperature for 6 min in dark. The absorbance was then measured at 734 nm. The ASA was calculated as a percentage of ABTS discoloration using the Eq. 3:

$$\mathrm{ASA}(\%)=\left(1-\frac{A_s-A_0}{A_{ABTS}}\right)\times 100 \quad (3)$$

where, $A_s$, $A_0$, and $A_{ABTS}$ represent the absorbance of the sample, the sample blank, and the control, respectively.

***Reducing power*:** Reducing power was determined by the method of Oyaiza (23). The sample solution was mixed with 2.5 mL of 0.2 mol/L phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. A 2.5 mL of 10% trichoroacetic acid was added to the mixture, followed by centrifugation at 3,500×g for 10 min. The upper layer of solution (2.5 mL) was mixed with 2.5 mL of distilled water and 2.5 mL of 0.1% $FeCl_3$. The absorbance was read at 700 nm after keeping the mixture for 10 min. Increased absorbance of the reaction mixture indicates stronger reducing power. BHT was prepared for the positive control.

***Chelating activity*:** Ferrous ion-chelating activity was determined according to the method of Dinis *et al.* (24). A 1.0 mL of samples was added to 0.05 mL of 2 mmol/L $FeCl_2$ solution. The reaction was initiated by the addition of 0.1 mL of 5 mmol/L ferrozine, and then the mixture was shaken vigorously and left at room temperature for 10 min. The absorbance was then measured at 562 nm. EDTA was used as a positive control. The chelating ability of ferrozine–$Fe^{2+}$ complex formation was given in Eq. 4:

$$\text{Ferrous ion-chelating activity (\%)} = \left(1 - \frac{A_s - A_0}{A_{Fe}}\right) \times 100 \quad (4)$$

where, $A_{Fe}$ and $A_0$ were the absorbance of the control and the sample blank, respectively. $A_s$ was the absorbance in the presence of samples.

**Determination of the molecular weight distribution** The molecular weight distribution of the antioxidant hydrolysate was analyzed by gel permeation chromatography (GPC) on a GPC column (5Diol-120-II, 7.5×600 mm). Vitamin $B_{12}$ (1.3 kDa), aprotinin (6.5 kDa), cytochrome C (12.4 kDa), and bovine serum albumin (66.7 kDa) were used as molecular standards. The mobile phase was 10 mmol/L phosphate buffer (pH 7.0) including 100 mmol/L $Na_2SO_4$ at a flow rate of 1.0 mL/min and the eluent was monitored at 214 nm.

**Statistical analysis** All samples were prepared and their activities were measured in triplicate. Standard deviation was also calculated. Statistical analysis of the data was carried out by Origin 8.0 (OriginLab Corporation, Northampton, MA, USA).

## Results and Discussion

**Proximate chemical compositions of the raw material** Chemical compositions of non-defatted and defatted SPB are shown in Table 2. SPB has a fat content of 1.49%. SPB has a high nutrition value not only due to the amino composition but also the unsaturated fatty acids. Both of the protein and fat content in our study were lower than those of snow crab (25) and shrimp byproducts reported by Bueno-Solano *et al.* (26). If the fat content was more than 1%, it should be removed using organic solvent or adding antioxidant to prevent the oxidation. The lipid oxidation during the hydrolysis can produce many compounds containing carbonyl groups, which can cause condensation reaction with alkaline groups in protein. They may generate brown pigment and the stench of hydrolyzed protein due to the reaction with other factors including heavy metals, oxygen, and so on. Marine products, as they contain more unsaturated fatty acids, are more vulnerable to these changes. The presence of fat can shorten the shelf life and may affect the antioxidant properties during the hydrolysis because of its auto-oxidation. The total protein increased from 14.7 to 19.4%, while the content of soluble protein decreased.

**Table 2. Chemical composition of shrimp processing byproducts**

| Composition (%) | Shrimp processing byproduct | Defatted shrimp processing byproduct |
|---|---|---|
| Total protein | 14.7±1.98[1)] | 19.4±4.24 |
| Soluble protein[2)] | 11.0±0.40 | 10.3±0.08 |
| Fat | 1.49±0.01 | <0.1 |
| Ash | 39.1±0.40 | 40.2±0.70 |
| Moisture | 5.54±0.17 | 7.51±0.37 |

[1)]Values are mean±SD ($n$=3); Content was expressed as dry basis except for moisture.
[2)]Soluble protein was expressed as a % of total protein.

**Effect of defatting and enzyme type on antioxidative activity** In order to select the best protease to hydrolyze the SPB for antioxidant peptides, the material was independently hydrolyzed by trypsin, neutrase, pepsin, Protamex, Flavourzyme, and Alcalase, respectively. The Alcalase hydrolysate gave the highest DH of all (Fig. 1). The antioxidant activities of different hydrolysates were evaluated by the DSA test system. All of the hydrolysates were capable of scavenging DPPH radicals. There was no obvious relationship between the DSA and hydrolysis time, which may be due to the sufficient amount of enzyme added. In general, Alcalase hydrolysate also exhibited the highest DSA, as shown in Fig. 2. The antioxidative activity of Protamex hydrolysate was first increased and then decreased with time, while Flavourzyme hydrolysate was in contrary. Comparison with DH and DSA, to some extent, it was shown that DSA was related to the DH and enzyme type. Klompong *et al.* (7) reported that yellow stripe trevally (*Selaroides leptolepis*) hydrolysate by Alcalase had higher DSA than Flavourzyme, which had lower DH. Liu *et al.* (27) reported that the antioxidant activity of porcine plasma protein Alcalase hydrolysate increased with DH. You *et al.* (28) indicated that loach

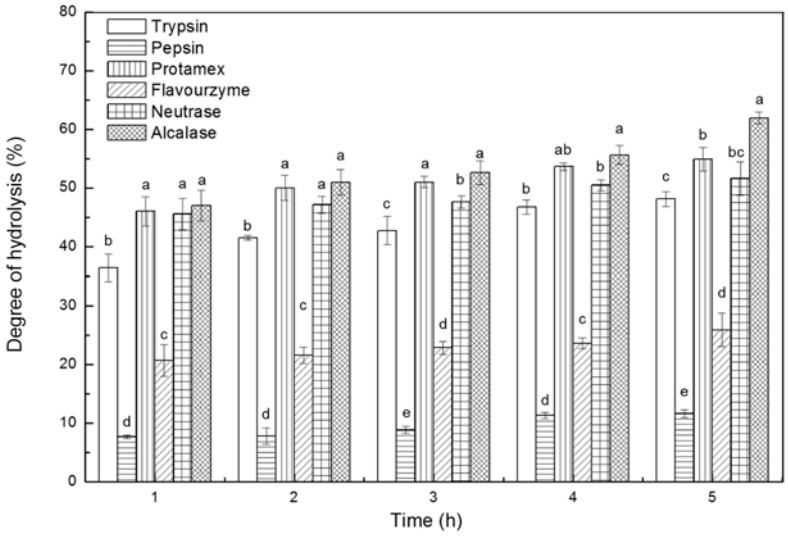


**Fig. 1. Degree of hydrolysis of shrimp processing byproducts by 6 enzymes.** Each value is expressed as mean±SD; Means in the same hydrolysis time with different letters are significant difference at $p<0.05$.

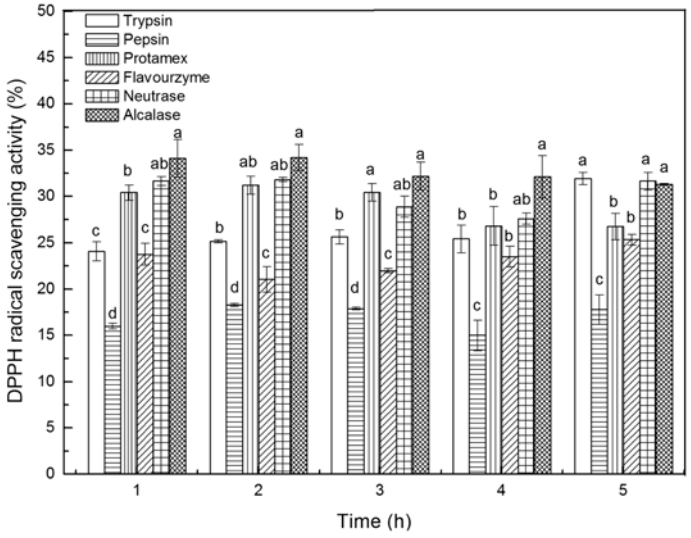


**Fig. 2. DPPH radical scavenging activity of shrimp processing byproducts.** Each value is expressed as mean±SD; Means in the same hydrolysis time with different letters are significant difference at $p<0.05$.

protein hydrolysate was potent antioxidant which was signicantly affected by DH. Extensive hydrolysis could adversely affect functional properties of peptides (29). The DH of the rice endosperm protein hydrolysate by chymotrypsin was higher than that by neutrase, however, the DSA by chymotrypsin was lower (30). This phenomenon indicated that peptides with reactive groups were gradually exposed during hydrolysis. The antioxidative activity of hydrolyzed peptides might be not only related to DH, but also related to other factors, such as the increase or decrease of the hydrophobic, aromatic, acidic, or basic amino acids and different composition and structure. Therefore, Alcalase was chosen as the best enzyme to hydrolyze SPB for further study.

The Alcalase (1,000 U/g protein) was added to hydrolyze SPB and the DH and DSA were detected every 2 h. Results showed that the defatted SPB hydrolysate has lower DH, but higher DSA than the original product hydrolysate (Table 2). This result was in accordance with Hoyle and Merrltt (31) who found that defatted herring (*Clupea harengus*) protein hydrolysate had lower DH than the original product hydrolysate. Klompong *et al.* (7), Sakanaka and Tachibana (32) also reported that the DH decreased after defatting. They explained that protein was vulnerable to solvent, and that the reaction between protein and protease was weakened after defatting. It was said that the solubility of defatted protein was decreased because isopropanol and other alcohols had been known to compete

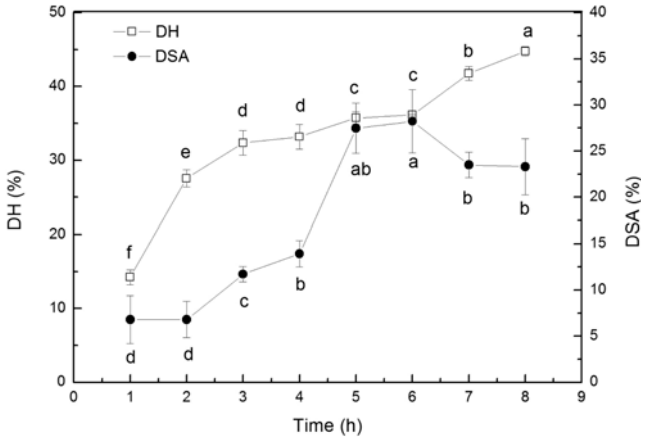


**Fig. 3. Degree of hydrolysis (DH), protein content, and DPPH radical scavenging acidity (DSA) of defatted shrimp processing byproducts during Alcalase hydrolysis.**

for binding water with protein (7). The higher DSA of the defatted SPB hydrolysate proved that the hydrolysate had some oxidation factors, and weakened the antioxidant activity of hydrolyzed peptides. So it is necessary to remove fat from the raw materials in further studies in order to obtain hydrolysate with high antioxidative activity. After a series of experiments, the optimal amount of enzyme (1,000 U/g protein) and the optimal hydrolysis time were chosen to produce antioxidant peptides. The result was shown in Fig. 3. It could be seen that at the time of 6 h, the DSA was the highest of all, and the DH had leveled off.

**Antioxidative activity of Alcalase hydrolysate** The DSA increased with an increment of sample content (Fig. 4). The $IC_{50}$ (the amount of antioxidant needed to decrease the radical concentration by 50%) values of BHT and defatted SPB hydrolysate were 0.29 and 0.50 mg/mL, respectively. Zhang *et al.* (33) reported that the rapeseed peptide fraction possessed DSA, and the $IC_{50}$ value was 0.49 mg/mL. When the protein content in defatted SPB hydrolysate was more than 0.5 mg/mL, a little amount of water-soluble pigment in the hydrolysate may interfere with the determination, which can not be extracted by petroleum. Some reports indicated that these pigments might be the product of Maillard reaction (17) and fragments from carotenoid-protein (16), which also had antioxidant activity. It needed further purification to evaluate its activity. But, these results revealed that defatted SPB hydrolysate had higher antioxidative activity and was more suitable for the further research on the purification and application.

The pre-formed radical monocation of ABTS is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants (22). The scavenging activity of both defatted

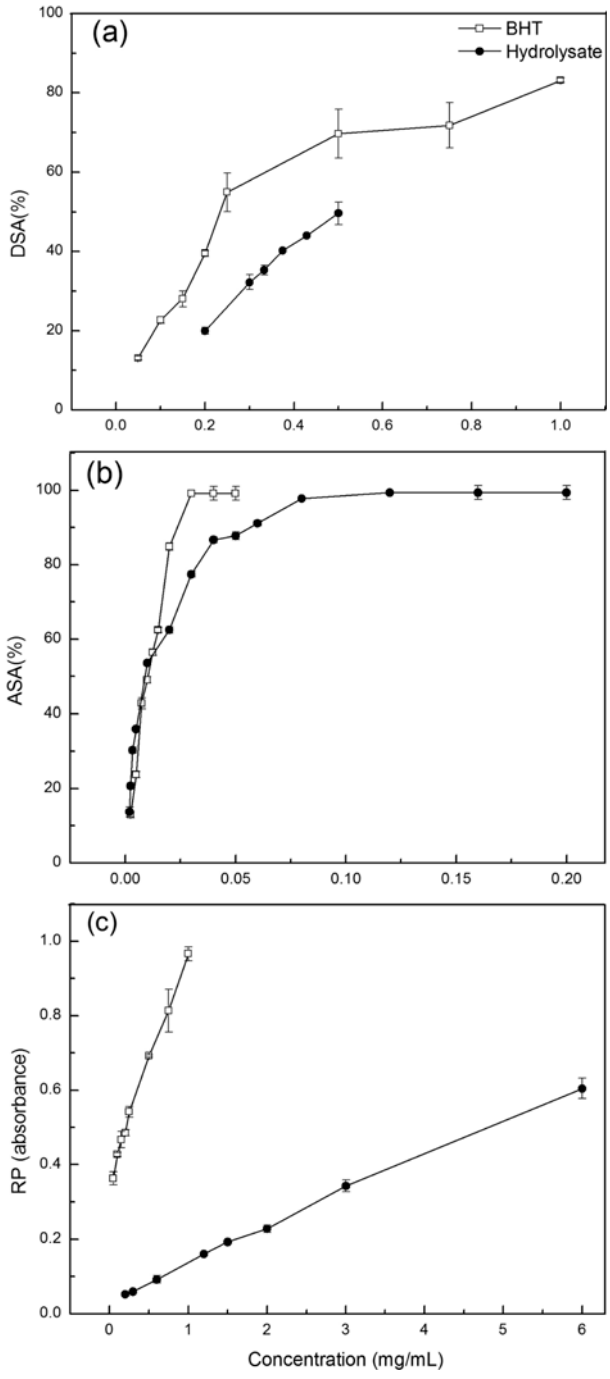


**Fig. 4. Antioxidative activity of Alacase hydrolysate: DPPH (a), ABTS (b), and reduced power (c).**

SPB hydrolysate and BHT increased steadily and reached a maximum plateau. The $IC_{50}$ values of BHT and defatted SPB hydrolysate were 7.2 and 7.4 μg/mL, respectively. The ASA of BHT was a little higher than defatted SPB hydrolysate. They all had high activity at low concentrations. When the concentration of defatted SPB hydrolysate protein reached 80 μg/mL, the ASA was approaching to 100%. Both the DSA and ASA of SPB Alcalase hydrolysate are much higher than fermented shrimp biowaste (34).

**Table 3. Degree of hydrolysis and DPPH radical scavenging activity of non-defatted and defatted shrimp processing byproducts (SPB) Alacase hydrolysate**

| Hydrolysis time (h) | Degree of hydrolysis (%) | | DPPH radical scavenging activity (%) | |
|---|---|---|---|---|
| | SPB | Defatted SPB | SPB | Defatted SPB |
| 2 | 30.7±0.68a[1)] | 23.6±0.44a | 8.36±0.66a | 28.4±2.08a |
| 4 | 38.0±2.06b | 34.2±0.94b | 14.6±2.11ab | 27.3±0.97ab |
| 6 | 49.4±1.04c | 34.8±1.04c | 14.7±1.74ab | 28.0±0.39b |
| 8 | 67.2±0.80d | 55.9±1.82d | 20.6±1.23b | 31.4±1.83c |
| 10 | 68.2±0.26d | 55.1±2.33d | 25.3±2.27b | 34.8±2.38c |

[1)]Values are mean±SD ($n$=3); Means in the same columns with different lowercase (a-d) are signicant difference at $p<0.05$.

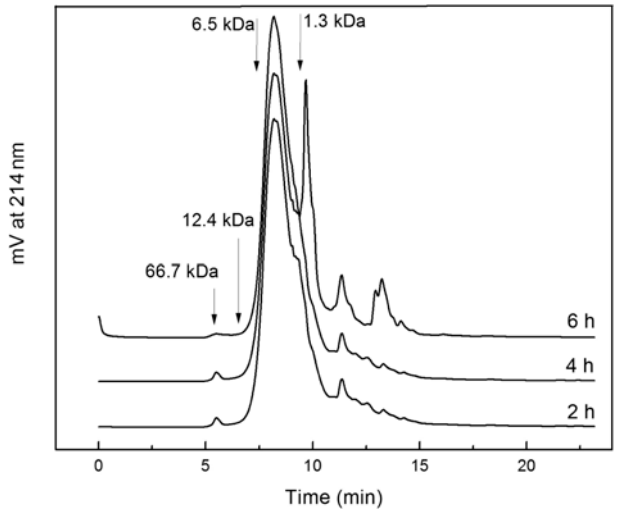


**Fig. 5. Molecular weight distribution of defatted shrimp processing byproducts hydrolysate.**

Compared to the reported papers, both of the $IC_{50}$ values of DSA and ASA were lower. It was related with many factors including the antioxidative peptides, products of Maillard reaction, water-soluble pigment fragments from pigment-protein complex, and even the interaction between them, which were all worthy of further study.

Free radicals form stable substances by accepting donated electrons, and the free radical chain reactions are thus interrupted (35). So, samples with higher reducing power have better abilities to donate electron, hydrogen, and free radicals to form stable substances, interrupting the free radical chain reactions. It has been widely accepted that the higher the absorbance at 700 nm, the greater the reducing power. The reducing power of defatted SPB and BHT are shown in Fig. 4. The reducing power was inuenced clearly by the concentration, and the reducing power of SPB hydrolysate was lower than that of BHT. At the absorbance of 0.5, the concentration of BHT was 0.22 mg/mL, while the concentration of hydrolysate was 4.8 mg/mL.

It has been recognized that transition metal ions are involved in many oxidation reaction *in vivo*. Ferrous ions ($Fe^{2+}$) can catalyze Haber-Weiss reaction and induce superoxide anion to form more hazardous hydroxyl radicals. Hydroxyl radicals react rapidly with the adjacent biomolecules and induce severe damage. It has been reported that the scavenging of hydroxyl radicals by antioxidant was effective mainly via chelating of metal ions (36). The ferrous chelating activity of SPB hydrolysate was 38.9% at 0.12 mg/mL while the chelating activity of EDTA reached 73.4% at 0.1 mg/mL.

**Molecular weight distribution** Many studies showed that the function of protein hydrolysate was related to the molecular weight distribution. Roberts *et al.* (37) suggested that small molecular weight peptides could permeate the cell membrane easier and had better bioactivity. The molecular weight distribution of defatted SPB hydrolysate for 2, 4, and 6 h centered on 5 kDa (Fig. 5). The lower molecular weight of peptides was increased with hydrolysis time increased, and the quantity of peptides smaller than 1.3kDa was significantly increased at hydrolysis time of 6 h.

**Acknowledgments** This research was supported in part by Zhejiang Provincial Natural Science Foundation of China under Grant No. Y 3110278.

## References

1. Stadtman ER. Protein oxidation and aging. Free Radical Res. 40: 1250-1258 (2006)
2. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell B. 39: 44-84 (2007)
3. Chen C, Pearson AM, Gray JI. Effects of synthetic antioxidants (BHA, BHT, and PG) on the mutagenicity of IQ-like compounds. Food Chem. 43: 177-183 (1992)
4. Rajapakse N, Mendis E, Byun HG, Kim SK. Purification and *in vitro* antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. J. Nutr. Biochem. 16: 562-569 (2005)
5. Sheih IC, Wu TK, Fang TJ. Antioxidant properties of a new antioxidative peptide from algae protein waste hydrolysate in different oxidation systems. Bioresource Technol. 100: 3419-3425 (2009)
6. Klompong V, Benjakul S, Kantachote D, Shahidi F. Comparative

study on oxidative activity of yellow stripe trevally protein hydrolysate produced from Alcalase and Flavourzyme. Int. J. Food Sci. Tech. 43: 1019-1026 (2008)
7. Klompong V, Benjakul S, Kantachote D, Shahidi F. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (*Selaroides leptolepis*) as influenced by the degree of hydrolysis and enzyme type. Food Chem. 102: 1317-1327 (2007)
8. Je JY, Qian ZJ, Byun HG, Kim SK. Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis. Process Biochem. 42: 840-846 (2007)
9. Slizyte R, Mozuraityte R, Martez-Alvarez O, Falch E, Fouchereau-Peron M, Rustad T. Functional, bioactive and antioxidative properties of hydrolysates obtained from cod (*Gadus morhua*) backbones. Process Biochem. 44: 668-677 (2009)
10. Sachindra NM, Bhaskar N, Mahendrakar NS. Carotenoids in different body components of Indian shrimps. J. Sci. Food Agr. 85: 167-172 (2005)
11. Kim JS, Shahidi F, Heu MS. Tenderization of meat by salt-fermented sauce from shrimp processing byproducts. Food Chem. 93: 243-249 (2005)
12. Destoumieux D, Munoz M, Bulet P, Bachère E. Penaeidins, a family of antimicrobial peptides from penaeid shrimp (*Crustacea, Decapoda*). Cell Mol. Life Sci. 57: 1260-1271 (2000)
13. Li L, Wang JX, Zhao XF, Kang CJ, Liu N, Xiang JH, Li FH, Sueda S, Kondo H. High level expression, purification, and characterization of the shrimp antimicrobial peptide, Ch-penaeidin, in *Pichia pastoris*. Protein Expres. Purif. 39: 144-151 (2005)
14. Cao W, Zhang C, Hong P, Ji H. Response surface methodology for autolysis parameters optimization of shrimp head and amino acids released during autolysis. Food Chem. 109: 176-183 (2008)
15. Balti R, Nedjar-Arroume N, Bougatef A, Guillochon D, Nasri M. Three novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) using digestive proteases. Food Res. Int. 43: 1136-1143 (2010)
16. Babu CM, Chakrabarti R, Surya KR. Enzymatic isolation of carotenoid-protein complex from shrimp head waste and its use as a source of carotenoids. LWT-Food Sci. Technol. 41: 227-235 (2008)
17. Binsan W, Benjakul S, Visessanguan W, Roytrakul S, Tanaka M, Kishimura H. Antioxidative activity of *mungoong*, an extract paste, from the cephalothorax of white shrimp (*Litopenaeus vannamei*). Food Chem. 106: 185-193 (2008)
18. Guerard F, Sumaya-Martinez MT, Laroque D, Chabeaud A, Dufossé L. Optimization of free radical scavenging activity by response surface methodology in the hydrolysis of shrimp processing discards. Process Biochem. 42: 1486-1491 (2007)
19. Izco JM, Torre P, Barcina Y. Ripening of Ossau-Iraty cheese: Determination of free amino acids by RP-HPLC and of total free amino acids by the TNBS method. Food Control 11: 7-11 (2000)
20. AOAC. Official Method of Analysis. of AOAC Int. 16th ed. Method 991.19. Association of Official Analytical Chemistis, Arlington, VA, USA (1995)
21. Wu HC, Chen HM, Shiau CY. Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (*Scomber austriasicus*). Food Res. Int. 36: 949-957 (2003)
22. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Bio. Med. 26: 1231-1237 (1999)
23. Oyaiza M. Studies on products of browning reaction: Antioxidative activity of products of browning reaction prepared from glucosamine. J. Nutr. 44: 307-315 (1986)
24. Dinis TC, Almeida LM, Madeira VM. Lipid peroxidation in sarcoplasmic reticulum membranes: Effect on functional and biophysical properties. Arch. Biochem. Biophys. 301: 256-264 (1993)
25. Beaulieu L, Thibodeau J, Bryl P, Carbonneau M. Characterization of enzymatic hydrolyzed snow crab (*Chionoecetes opilio*) by-product fractions: A source of high-valued biomolecules. Bioresource Technol. 100: 3332-3342 (2009)
26. Bueno-Solano C, López-Cervantes J, Campas-Baypoli ON, Lauterio-García R, Adan-Bante NP, Sánchez-Machado DI. Chemical and biological characteristics of protein hydrolysates from fermented shrimp byproducts. Food Chem. 112: 671-675 (2009)
27. Liu Q, Kong B, Xiong YL, Xia X. Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. Food Chem. 118: 403-410 (2010)
28. You L, Zhao M, Cui C, Zhao H, Yang B. Effect of degree of hydrolysis on the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates. Innov. Food Sci. Emerg. 10: 235-240 (2009)
29. Kristinsson HG, Rasco BA. Biochemical and functional properties of Atlantic salmon (*Salmo salar*) muscle proteins hydrolyzed with various alkaline proteases. J. Agr. Food Chem. 48: 657-666 (2000)
30. Zhang J, Zhang H, Wang L, Guo X, Wang X, Yao H. Antioxidant activities of the rice endosperm protein hydrolysate: Identification of the active peptide. Eur. Food Res. Technol. 229: 709-719 (2009)
31. Hoyle NT, Merrltt JH. Quality of fish protein hydrolysates from herring (*Clupea harengus*). J. Food Sci. 59: 76-79 (1994)
32. Sakanaka S, Tachibana Y. Active oxygen scavenging activity of egg-yolk protein hydrolysates and their effects on lipid oxidation in beef and tuna homogenates. Food Chem. 95: 243-249 (2006)
33. Zhang SB, Wang Z, Xu SY. Antioxidant and antithrombotic activities of rapeseed peptides. J. Am. Oil Chem. Soc. 85: 521-527 (2008)
34. Sachindra NM, Bhaskar N. *In vitro* antioxidant activity of liquor from fermented shrimp biowaste. Bioresource Technol. 99: 9013-9016 (2008)
35. Yamashoji S, Kajimoto G. Antioxidant effect of Gly-Gly-His on Cu (II)-catalyzed autoxidation and photosensitized oxidation of lipids. Agr. Biol. Chem. Tokyo 44: 2735-2736 (1980)
36. Gordon MH. The mechanism of antioxidant action *in vitro*. pp. 1-18. In: Food Antioxidants. Hudson BJ (ed). Elsevier Applied Science, London, UK (1990)
37. Roberts PR, Burney JD, Black KW, Zaloga GP. Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. Digestion 60: 332-337 (2000)