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Amino Acid Composition, Molecular Weight Distribution and Antioxidant Stability of Shrimp Processing Byproduct Hydrolysate

J. Zhao, G.R. Huang, M.N. Zhang, W.W. Chen and J.X. Jiang
Department of Food Science, College of Life Sciences, China Jiliang University, Xiasha High Education Area, 310018, Hangzhou, Zhejiang Province, Peoples' Republic of China

Corresponding Author: G.R. Huang, Department of Food Science, College of Life Sciences, China Jiliang University, Xiasha High Education Area, 310018, Hangzhou, Zhejiang Province, Peoples' Republic of China

ABSTRACT

Protein hydrolysate have many practical applications in a various of industries due to the bioactive peptides related to their amino acid composition, sequence and molecular weight. The amino acid composition, molecular weight distribution and antioxidant stability of alcalase hydrolysate were investigated in this study. The hydrolysate was separated into five fractions by ultra filtration system with different molecular weight cutoff with 10, 5, 3 and 1 kDa, respectively. The protein content, 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity and molecular weight of each fraction were determined. In addition, the antioxidant stability of the hydrolysate under several operating conditions was studied. The results showed that the hydrolysate was composed with high amounts of hydrophobic amino acids (40.4%) which might contribute to the high antioxidant activity. The fraction with molecular weight lower than 1 kDa exhibited the highest antioxidative activity among the five fractions. The antioxidant stability experiments showed that the hydrolysate was stable when it was heated up to 100°C and the relative antioxidative activity could be maintained nearly 70% at very low pH of 2.0. Glucose and sucrose had negative effects on the antioxidative activity, in which the relative activity of about 80% was retained. Sodium chloride and sodium benzoate had little or no effects on the antioxidative activity of the hydrolysate. The effects of Zn^{2+} and Cu^{2+} on the antioxidative activity were significant and dependent on metal concentration. The shrimp processing byproduct hydrolysate may be a potential natural food antioxidant in the future.

Key words: Antioxidative peptide, enzymatic hydrolysis, alcalase, ultrafiltration, DPPH

INTRODUCTION

Lipid oxidation in foods is a serious problem to the food industry. The control of lipid oxidation in food products is desirable and lots of the natural antioxidants derived from fruits, plants and animals have been studied by many researchers. For example, the vitamins (Baydasa *et al*., 2002), polyphenols (Gramza-Michalowska and Korczak, 2007; Lelono *et al*., 2009), flavonoids (Bele *et al*., 2010; Gajula *et al*., 2009), astaxanthin (Lorenz and Cysewski, 2000), active peptides (Mendis *et al*., 2005) and many extracts from original or fermented materials (Puangpronpitag *et al*., 2010; Chye and Sim, 2009; Ham *et al*., 2010; Shokrzadeh and Ebadi, 2006) have been reported to replace synthetic antioxidants, such as butylated hydroxyanisole butylated hydroxytoluene and propylgallate. Shrimp processing byproduct is an important source of bioactive

molecules including protein, chitin, carotenoids and astaxanthin which may be recovered for utilization as ingredients in various food applications. Sachindra and Mahendrakar (2005) reported that depending on the species, the solid waste generation in processing of shrimps ranges from 48 to 56% of the total bodyweight, the major components are protein (35-50%), chitin (15-25%), minerals (10-15%) and astaxanthin, a well known natural antioxidant which can be recovered using organic solvents and vegetable oils. The rest after extraction were used as feed, fertilizer or even simply dumped which contained lots of proteins. Many studies had been done in order to make better use of the proteins from shrimp processing byproduct, such as the nutritive proteins (Cordova-Murueta and Garcia-Carreno, 2002), shrimp paste (Mizutani *et al*., 1992), bioactive peptides (Cheung and Li-Chan, 2010; Mendis *et al*., 2005; Babu *et al*., 2008) and so on. Protein hydrolysate had been used in many industries, such as pharmaceuticals, human nutrition, animal nutrition, cosmetics or the microorganisms growth media (Cheung and Li-Chan, 2010; Clemente, 2000; Martone *et al*., 2005). Leal *et al*. (2010) had demonstrated that shrimp protein hydrolysate was a promising protein feedstuff and accounted for as much as 6% of Nile tilapia (*Oreochromis niloticus*, L.) diets with no adverse effects on growth and nutrient utilization.

Peptides present in enzymatically digested protein hydrolysate have exhibited different physicochemical properties and biological activities depending on their molecular weights and amino acid sequences (Kim and Wijesekara, 2010). In recent years, natural products with antioxidative activity, especially antioxidative peptides, have drawn the attention of researchers, due to their low molecular weight, easy absorption and high activity (Sarmadi and Ismail, 2010). Application of enzymatic hydrolysis in combination with ultrafiltration treatment will be an interesting attempt to utilize this protein source.

During the previous study, it was found that Shrimp Processing Byproduct Hydrolysate (SPBH) produced by alcalase exhibited high 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (Huang *et al*., 2011). The SPBH exhibited high antioxidative activity with a dose-dependent manner by several *in vitro* evaluation systems. The shrimp processing byproduct might be a good resource to produce antioxidative peptides and a potential source of natural antioxidants. In this study, amino acid composition , the relationship between antioxidant activity and molecular weight distribution and the antioxidant stability of SPBH were studied for further applications as food additives and diet nutrients.

MATERIALS AND METHODS

Materials and chemicals: Shrimp processing byproduct was purchased at autumn 2010 from local Seafood Market, Zhejiang, China. It was defatted, dried and grounded into fine powder, then stored at -20°C until used. Alcalase (250 U mg^{-1}) was purchased from Shanghai Chemical Reagent Co. Ltd., China. 1,1-diphenyl-2-picrylhydrazyl (DPPH), vitamin B_{12}, aprotinin, cytochrome C and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, USA). All other chemicals used in the experiments were from commercial resource and of analytical grade.

Preparation of the SPBH: Alcalase was chosen to hydrolysis the shrimp processing byproduct to produce antioxidative peptides (Huang *et al*., 2011). The hydrolysis conditions were: temperature of 50.5°C, time duration of 120 min, pH 8.5 and enzyme-to-substrate ratio of 415 U g^{-1}. A 100 g portion of shrimp processing byproduct powder was homogenized in 1 L of distilled water and the homogenate was preheated for 10 min at 50.5°C, then adding 166 mg alcalase to the mixture to begin hydrolysis. During hydrolysis, the mixture was adjusted to pH 8.5 using 1 N NaOH or 1 N

HCl every 15 min. At the end of hydrolysis, the solution was boiled for 10 min to inactivate the enzyme and then centrifuged at 3, 500 g for 10 min at 4°C. The supernatant was collected for further study.

Ultrafiltration of SPBH: The supernatant of SPBH was collected and subjected to ultrafiltration membranes system with Molecular Weight Cutoff (MWCO) of 10, 5, 3 and 1 kDa, respectively. Five peptide fractions with different Molecular Weight (MW) ranges were prepared from SPBH: SPBH-I (>10 kDa), SPBH-II (5-10 kDa), SPBH-III (3-5 kDa), SPBH-IV (1-3 kDa) and SPBH-V (<1 kDa). The peptide fractions were lyophilized and stored at -20°C until use.

Determination of protein content: The protein contents of fractions after ultrafiltration of SPBH were determined by Folin-phenol method (Lowry *et al.*, 1951) using Bovine Serum Albumin (BSA) as standard.

DPPH radical scavenging activity: DPPH Radical Scavenging Activity (DSA) was assayed following the method of Wu *et al.* (2003) with some modifications. The 0.375 mL of each sample solution at different concentrations was added to 2.0 mL 0.1 mmol L^{-1} DPPH dissolved in methanol. The mixture was shaken and left for 30 min in dark at room temperature and the absorbance of the resulting solution was measured at 517 nm. The DSA was calculated as a percentage of DPPH discoloration using the Eq. 1:

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

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

Determination of amino acid composition and molecular weight distribution: The total amino acid composition was determined according to the method of Heu *et al.* (2003). The SPBH sample was placed in an ampoule and mixed with 6 N HCl. After sealing the ampoule under vacuum, the SPBH was hydrolyzed at 110°C for 24 h. The sample was diluted, filtered and loaded on a Model S433D automatic amino acid analyzer (Sykam Corp., Eresing, Germany) for amino acid analysis. The Molecular Weight (MW) distribution of the antioxidant hydrolysate was analyzed by Gel Permeation Chromatography (GPC) on a GPC column (5 Diol-120-II, 7.5×600 mm) following the method of Dong *et al.* (2008) with some modifications. Vitamin B_{12}, aprotinin, cytochrome C and bovine serum albumin were used as MW standards. The mobile phase was 10 mmol L^{-1} phosphate buffer solution including 100 mmol L^{-1} Na_2SO_4 at a flow rate of 1.0 mL min^{-1} and the eluent was monitored at 214 nm.

Antioxidant stability

Thermal and pH stability of antioxidant activity: The thermal and pH stability was analyzed following the method of Binsan *et al.* (2008).

Effect of food additives and metal ions on the antioxidant activity: The effects of food additives and metal ions on the antioxidant activity of SPBH were determined. Food additives (glucose, sucrose, sodium chloride and sodium benzoate) and metal ions solutions ($CuSO_4$, $ZnSO_4$,

Table 1: Concentrations of food additives and metal ions used in the antioxidant stability experiments

Food additives and metal ion	Concentration				
Glucose (%)	2	4	6	8	10
Sucrose (%)	2	4	6	8	10
Sodium chloride (%)	0.5	1.0	1.5	2.5	3.0
Sodium benzoate (mg L^{-1})	40	80	120	160	200
*Metal ions (mg L^{-1})	5	10	50	100	500

*The metal ions mean Cu^{2+}, Zn^{2+}, K^{+}, Ca^{2+} and Mg^{2+}, respectively

KCl, $CaCl_2$ and $MgCl_2$) were used to study the antioxidant stability of SPBH. Sample of SPBH (100 μL) was added to the food additives or metal ions solution (1.9 mL) with various concentrations to the SPBH and the residual DSA was determined. The final concentrations of food additives and metal ions in the stability experiments were listed in Table 1. Distilled water instead of samples was used for control.

Retention activity: The retention activity was calculated as a percentage of DSA using the Eq. 2:

$$\text{Retention activity (\%)} = \frac{DSA_s}{DSA_o} \times 100 \quad (2)$$

where, DSA_s and DSA_0 represented the absorbance of sample and control, respectively.

RESULTS AND DISCUSSION

Amino acid composition: The amino acid composition of SPBH was presented in Table 2. The amino acid compositions of SPBH revealed that they were rich in Glu, Asp, Gly and Leu with values of 16.4, 13.4, 8.2 and 7.5%, respectively. It was in good agreement with the data reported by Synowiecki and Al-Khateeb (2000) using commercially available alcalase to recover the protein and chitin from shrimp processing discards. It was also reported that Glu, Asp and Arg tended to predominate in the hydrolysate during autolysis of shrimp head (Cao *et al*., 2008). The Glu, Asp, Gly and Ala were flavour enhancers which might account for the good taste (Kirimura and Shimizu, 1969). The SPBH also had an extremely high content of flavour amino acids which accounted 44% of the total amino acids. Nevertheless, the sensitive amino acids, such as Met and Try were not detected in SPBH. Shahidi *et al.* (1995) reported that sensitive amino acids, such as Met and Try, presented in smaller amounts after hydrolysis of capelin proteins. The essential amino acids of SPBH made up 32.2% of all amino acids which was a little lower than the reference values of 40% recommended by Food and Agriculture Organization (FAO/WHO, 2001). The content of hydrophobic amino acids (Pro, Gly, Ala, Val, Ile, Leu and Phe) in SPBH was 40.4%. The high antioxidant activity of SPBH might be relationship to the high level of hydrophobic amino acids. Rajapakse *et al.* (2005) reported that for protein hydrolysate and peptides, an increasement in hydrophobicity would increase their solubility in lipid and therefore enhanced their antioxidant activity. Peptides derived from many materials with increased hydrophobicity had been reported to relate with antioxidant activity (Chen *et al*., 1996, 1998; Li *et al*., 2008). The high level of hydrophobic amino acids might contribute to the bitterness of the hydrolysate. FitzGerald and O'Cuinn (2006) reported that bitterness of protein hydrolysates was associated with the release of peptides containing hydrophobic amino acid residues.

Table 2: Amino acids composition of SPBH

Amino acids	Composition (%)	Amino acids	Composition (%)
Asp	13.4	Ile	4.5
Thr	4.5	Leu	7.5
Ser	5.2	Tyr	3.0
Glu	16.4	Phe	4.5
Pro	4.5	His	6.7
Gly	8.2	Lys	6.0
Ala	6.0	Arg	4.5
Cys	<1	Met	<1
Val	5.2	Try	<1

Table 3: The protein distribution and DSA of five fractions by ultrafiltration

	Fractions				
Item	SPBH-I	SPBH-II	SPBH-III	SPBH-IV	SPBH-V
Molecular weight (kDa)	>10	5-10	3-5	1-3	<1
Protein distribution (%)	19.7±0.2^{c}	30.8±0.23^{a}	27.26±0.14^{b}	13.75±0.05^{d}	10.07±0.04^{e}
DSA (%)	39.39±0.89^{e}	57.56±1.27^{c}	49.39±1.35^{d}	62.78±1.44^{b}	65.11±0.95^{a}

The values are Mean±SD of triplicate measurements. Values with different letters in the same row are significantly different ($p<0.05$)

Ultrafiltration of SPBH: The relative protein contents and DSA of five fractions by ultrafiltration membrane were determined as shown in Table 3. The protein was mainly distributed in SPBH-II and SPBH-III fractions and the SPBH-V fraction had the least quantity of protein. The DSA of five fractions (at 500 μg mL^{-1} level) were also detected. From the results (Table 3), it was found that SPBH-IV and SPBH-V showed significantly higher DSA (62.78 and 65.11%, respectively) than the other three fractions at significant level $p<0.05$. SPBH-V with MW lower than 1 kDa exhibited the highest antioxidative activity of all. SPBH-I with the largest molecular weight exhibited the lowest DSA (<40%). However, the high DSA of SPBH might be thought to be due to the low molecular weight, as well as the sequence of peptides. These findings were in agreement with other studies (Dong *et al*., 2008; Xie *et al*., 2008) and supported the fact that functional properties of antioxidant peptides were highly influenced by properties such as molecular mass. Many reports had already shown that the antioxidant activity of hydrolysate was depended on their molecular weight distribution (Moure *et al*., 2006; Zhu *et al*., 2006; Dong *et al*., 2008; Li *et al*., 2008).

All fractions enriched in peptides obtained following the ultrafiltration steps, were analyzed by gel permeation chromatography in order to evaluate their molecular weight distribution. The molecular weight distribution of five fractions by ultrafiltration membrane was determined by gel permeation chromatography. From the HPLC chromatograms (Fig. 1), it was found that ultrafiltraion had the effectiveness of separation by MWCO. The chromatographic data indicated that SPBH-V with the highest antioxidant activity was composed of four major fractions. The molecular weights of four major fractions were around 1.6 kDa, 800, 500 and 300 Da, respectively. In the future studies, the four major fractions should be further purified and characterized.

Antioxidant stability: The thermal stability of antioxidant activity of SPBH was shown in Fig. 2a. The retention activity was decreased with a rise of temperature but activities of all fractions were more than 90% remained. Arcan and Yemenicioglu (2007) also found that antioxidant activities of water-soluble protein extracts from chickpeas indicated a high free radical scavenging

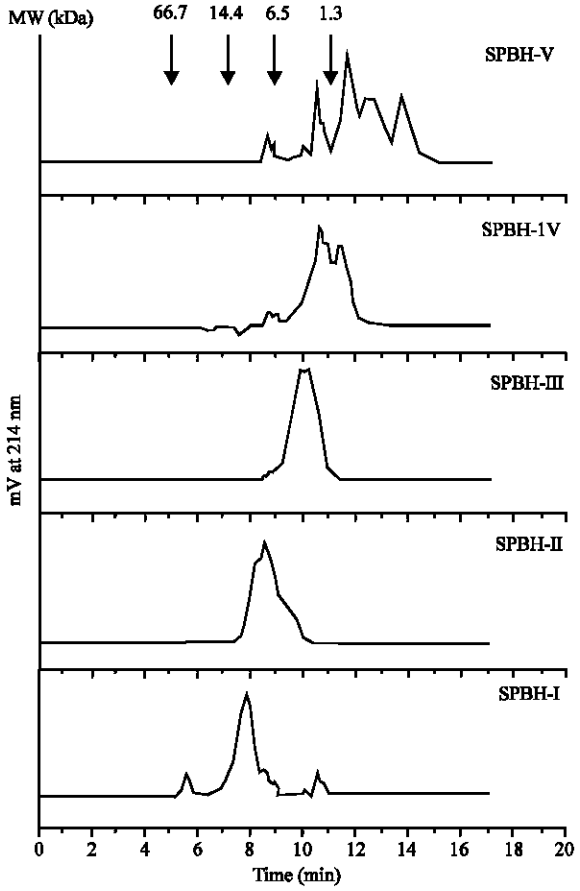

Fig. 1: Molecular weight distribution of SPBH fractions by gel permeation chromatography

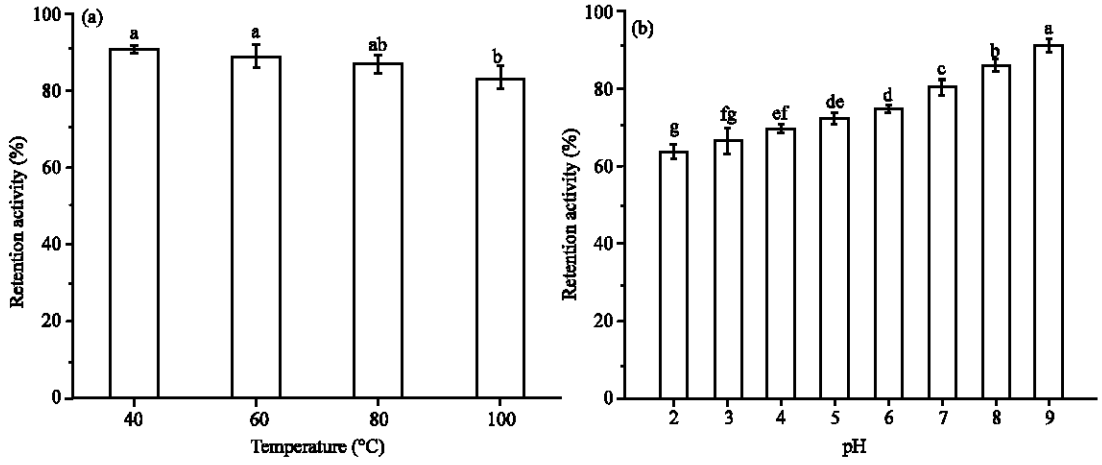

Fig. 2(a-b): Thermal and pH antioxidant stability of the SPBH. The values are Mean±SD of triplicate measurements. Values with different letters are significantly different ($p<0.05$)

capacity and thermostability. The effects of pH on the antioxidant stability of the SPBH were shown in Fig. 2b. The acidic pH mostly affected antioxidative activities of SPBH. It could be found

that the antioxidant stability of SPBH decreased slightly with the increase of acidity. Acidic condition might change the antioxidant property. Though the SPBH was more stable in alkaline and neutral environment, the retention of antioxidant activity maintained nearly 70% of the original activity in pH 2.0. Binsan *et al.* (2008) reported that DPPH radical scavenging activity of water soluble fraction from Mungoong decreased slightly when the pH was above 8. The antioxidant activity of water soluble fraction was more than 80% remained when heated up to 100°C. This study showed that SPBH exhibited good thermal stability and was stable at alkaline conditions.

Slight decreases in DSA at present of glucose and sucrose were observed and no marked changes were observed (Fig. 3a, b). It seemed that glucose caused more changes than that of sucrose at low concentrations. Instead sucrose had a little stronger effect to antioxidant activity of SPBH than that of glucose at high concentrations. At present of glucose or sucrose, the retention activity of about 80% remained. Sodium chloride and sodium benzoate had little or no effect on the DSA (Fig. 3c, d). The effects of metal ions on the antioxidant stability were investigated and showed that the majority metal ions inhibited the DSA to various extents (Fig. 4). The antioxidant activity was inhibited by all metal ions used. The effects of Cu^{2+} and Zn^{2+} were notable while the retention activity was more than 80% in the conditions with high concentration of K^{+}, Ca^{2+} and Mg^{2+}. Nkhili and Brat (2011) reported that the presence of metal ions in the studied matrix will influence antioxidant stability, thereby leading to the under estimation of their antioxidant properties. So far, the SPBH should avoid contacting with the substance including Zn^{2+} and Cu^{2+} during processing.

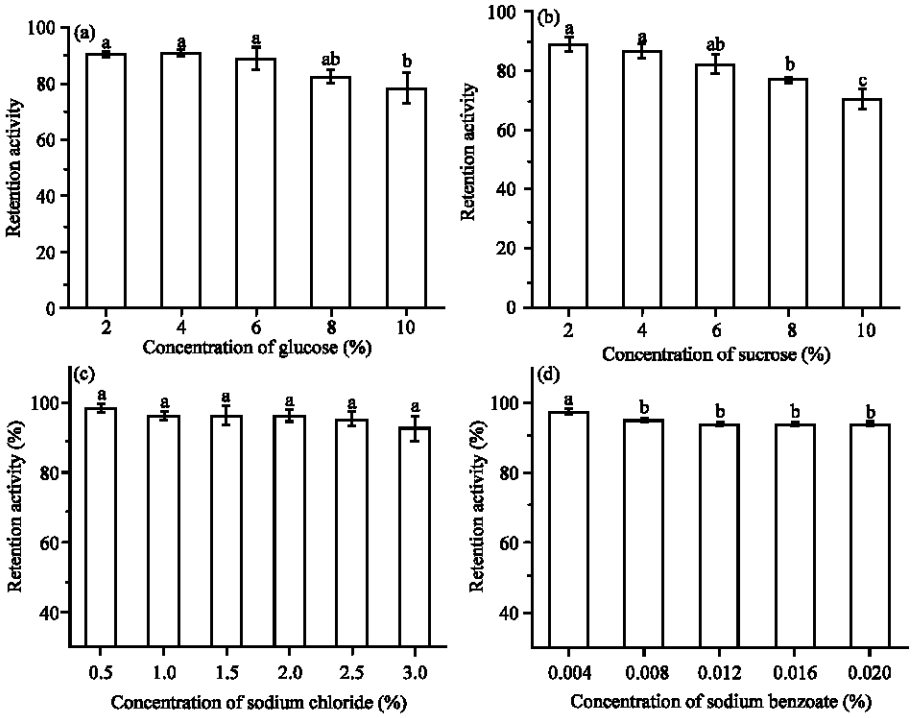

Fig. 3(a-b): Effects of glucose, sucrose, sodium chloride and sodium benzoate on antioxidative activity of SPBH. The values are Mean±SD of triplicate measurements. Values with different letters are significantly different ($p<0.05$)

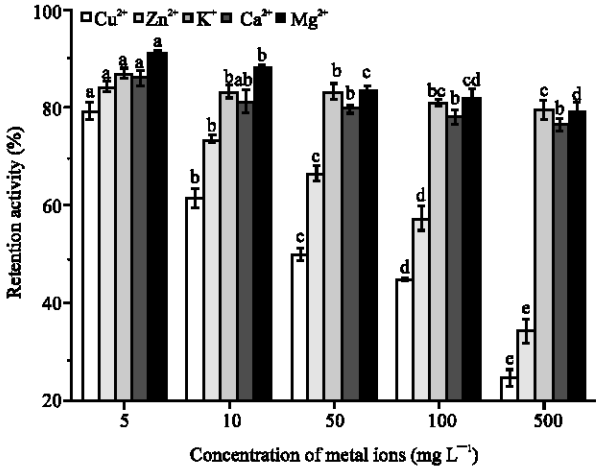

Fig. 4: Effects of metal ions on antioxidative activity of SPBH. The values are Mean±SD of triplicate measurements. Values with different letters in the same metal ion are significantly different ($p<0.05$)

CONCLUSION

The antioxidant activity of SPBH was related to the amino acids, molecular weight and affected by environment conditions. SPBH had high nutritional value and the amino acid compositions were rich in hydrophobic amino acids which might contribute to the high antioxidant activity and bitterness. The SPBH-V fraction with the MW lower than 1 kDa exhibited the highest activity which composed of four major fractions in chromatogram. This fraction could be further purified and characterized to obtain new potential antioxidant peptides. SPBH had good stability in various conditions but the effects of metal ions, such as $Zn^{2+}$and Cu^{2+} on the antioxidative activity were significant. Therefore, the SPBH avoid contacting with the substance including Zn^{2+} and Cu^{2+} during processing. It might be used as food additives and diet nutrients, further detailed studies in specific food systems and debittering should be applied in order to obtain SPBH with the sensorial acceptability for further applications.

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