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Preparation and antioxidant properties of crayfish (*Procambarus clarkii*) By-products protein hydrolysates and ultra filtration fractions

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Abstract: Protein isolate from crayfish by-products (CBPI) were hydrolyzed using Alcalase, neutrase, pancreatin and bromelain. Hydrolysis by Alcalase had more remarkable digesting efficiency on crayfish by-products protein than that by the other enzymes. Therefore, protein hydrolysate from Alcalase digestion (CBPHa) was selected to be fractionated by ultrafiltration according to molecular weight into three fractions F1 (MW <1kDa), F2 (MW 1-3kDa) and F3 (MW 3-10kDa). The amino acid determination revealed that CBPI had essential amino acid (EAA) close to that required for human protein synthesis. In vitro activity experiments showed that CBPHa and its fractions possessed considerable antioxidant activity. F1 exhibited the highest DPPH, superoxide radicals scavenging activities and Fe^{2+} chelating ability, whereas F2 showed the best hydroxyl radicals scavenging capacity and reducing power. In addition, all the fractions showed higher super oxide radical scavenging activity than the crude hydrolysates. Our findings suggest that CBPHa and their ultra filtration fractions have the potential for use in nutraceutical and functional food industries to maximize the use of crayfish processing by-products.

Keywords: Crayfish by-products, hydrolysis, ultrafiltration, antioxidant activity

INTRODUCTION

In the last decades, the global production of crayfish (*Procambarus clarkii*) has rapidly increased. In China alone, the production of crayfish is exceeding 5.48×10^6 tons annually. Crayfish processing by-products contain shells and heads accounting for 70–80% of body weight. These wastes are discarded in nature, which will lead to environmental problems. Meanwhile, the by-products contain many useful chemical constituents, such as astaxanthin, chitin, calcium carbonate and protein (Gao *et al*., 2015; Cremades *et al*., 2001; Baek *et al*., 1995).

Therefore, utilization of crayfish by-products (such as the recoveries of proteins from the by-products) to produce value added products is highly desirable. In recent years, natural antioxidants from food protein-derived hydrolysate has attracted increasing attention because of their effective antioxidant properties. Many studies have shown that hydrolysates from various protein sources, such as surimi processing by-products (Liu *et al*., 2014), mung bean (Guerra *et al*., 2017), fish by-products (Gehring *et al*., 2011), salmon byproduct (Gbogouri *et al*., 2004) and Pigeon pea (Olagunju *et al*., 2018) display remarkable antioxidant capacity. Much focus has been placed on researching the use of agro-industrial by-products or waste as a source of antioxidant peptides in order to maximize economic returns and contribute to environmental sustainability. However, studies on the antioxidant activities of hydrolysates from crayfish (*Procambarus clarkii*) by-products protein are rarely reported. In this study, proteins isolate from crayfish by-products (CBPI) were hydrolyzed using Alcalase, neutrase, pancreatin and bromelain to generate CBP hydrolysates (CBPH). Protein hydrolysate from Alcalase digestion (CBPHa) was further fractionated by membrane ultrafiltration into three peptide fractions. Moreover, the potential antioxidant activities of CBPHa and its fractions were evaluated using different in vitro systems.

MATERIALS AND METHODS

Materials and chemicals

Crayfish by-products (including shells and heads) were collected from a crayfish market in Qianjiang city (Hubei, China). Alcalase, neutrase, pancreatin and bromelain were obtained from Solarbio Life Sciences (Beijing, China). 1, 3 and 10 kDa molecular weight cut-off (MWCO) membranes were bought from Millipore Corp. (Billerica, MA, USA). Ferrous chloride, potassium ferricyanide, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 99% acrylamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other reagents used in the experiment were of analytical grade.

Protein isolate preparation

The supplied by-products were washed, ground into uniformity and lyophilized. Then they were defatted with

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isopropanol (1:10, w/v) based on the method of Liu *et al.* (2014). The slurry was stirred at 50°C for 120 min and then centrifuged (3000 rpm, 10 min). The precipitate was collected and reextracted twice to obtain the defatted by-products.

Protein extraction was conducted according to the method of Jeurink *et al.* (2008). The defatted samples were dried at room temperature, suspended in 0.1M NaOH (1:10, w/v) and then extracted by stirring at room temperature 40°C for 1h, followed by centrifuging (10000rpm, 10 min). The supernatants were added ammonium sulphate (70% saturation) to precipitate the solubilized proteins. After that, the mixture was centrifuged (10000rpm, 20 min) and the precipitate was redissolved, followed by dialyzing against distilled water for 48h. The dialysate was lyophilized to produce crayfish by-products protein isolate (CBPI) powder. Protein content was measured according to the method of Markwell *et al.* (1978).

Preparation of hydrolysates

CBPI were hydrolyzed separately with Alcalase, neutrase, pancreatin and bromelain under optimum hydrolysis conditions (table 1). The pH of reaction mixture was maintained using either HCl (0.2M) or NaOH (0.2M). After 5 h digestion period, the pH of the sample solution was adjusted to 7.0, and then the solution was heated at 90°C for 10 min to terminate the hydrolysis. The hydrolysates were centrifuged (10,000 rpm, 20 min). The supernatant lyophilized as crayfish by-products protein hydrolysates (CBPH) powders (CBPHa, CBPHn, CBPHp and CBPHb for Alcalase, neutrase, pancreatin and bromelain hydrolysates, respectively). CBPH percent yields and protein recovery were assayed using the following equation:

$$\text{CBPH yield(\%)}\frac{\text{Weight of hydrolysate}}{\text{Weight of CBPI used for hydrolysis}}\times 100$$

$$\text{CBPH protein recovery(\%)}\frac{\text{Weight of protein in hydrolysate}}{\text{Weight of protein in CBPI used for hydrolysis}}\times 100$$

Determination of Degree of hydrolysis (DH)

DH was assayed based on the method described by *Coscueta ER et al.* (2019).

Fractionation of the protein hydrolysates

Lyophilized CBPHa was dissolved in distilled water with a concentration of 100mg/ml and further separated by using1, 3 and 10kDa molecular weight cutoff (MWCO) membranes. Three fractions were prepared and freeze-dried.

Amino acid analysis

The amino acid composition of CBPI was determined based on the method of Girgih *et al.* (2010) using high performance liquid chromatography (Agilent 1200 HPLC, USA) system after hydrolysis with 6 M HCl. The content of Tyr was assayed after alkaline hydrolysis while methionine and the content of Cys was measured after performic acid oxidation.

Determination of antioxidant activities

Assay of superoxide radical scavenging activity

The activity of superoxide radical scavenging was assayed according to Xie *et al.* (2008) with some modification. In brief, reaction mixtures in a final volume of 3.0ml included the following reagents: 16mM Tris-HCl buffer (pH 8.0), 338μM NADH (reduced form), 30μM PMS, 72μM NBT, and varying concentrations of CBPHa and its fractions. The absorbance was read at 560 nm. GSH was used as positive control and the ability of superoxide radical scavenging was determined as follows: Scavenging activity (%) = $(1\text{-}A\text{sample}_{560}/A\text{control}_{560})\times 100$

Assay of DPPH free radicals scavenging activity

The DPPH radical scavenging capacity was analyzed based on the method of Zhu *et al.* (2006). Briefly, varying concentrations of the samples (2ml) was mixed with 0.002% DPPH solution in ethanol (2ml). The mixture was incubated in the dark for 30 min, and the absorbance was recorded at 517 nm. GSH was served as positive control. The scavenging activity was determined as follows: Scavenging activity (%) = $(1–A\text{sample}_{517}/A\text{control}_{517})\times 100$.)

Assay of Hydroxyl radical scavenging activity

The scavenging activity of the samples was assayed according to Ghiselli *et al.* (1998). In brief, the sample solutions (1 ml) were mixed with 2ml reaction buffer [deoxyribose (2.67 mM), phosphate buffer (pH 7.4, 0.2 M) and EDTA (0.13 mM)] and ferrous ammonium sulfate (1ml, 0.4 mM), followed by adding 1 ml of H_2O_2 (10 mM). The mixture was incubated (37°C, 15min), followed by the addition of 1% thiobarbituaric acid (1ml) and 1ml of 2.0% trichloroacetic acid. The solution was boiled for 15 min and the absorbance was tested at 532 nm. GSH was used as positive control. The activity of scavenging hydroxyl radical was determined as follows: Scavenging activity (%) = $(1–A\text{sample}_{532}/A\text{control}_{532})\times 100$.

Assay of metal chelating activity

The metal chelating ability of the samples was determined according to Zhang, *et al.* (2008). The reaction mixture, containing 1.0ml samples, ferrous chloride (0.05ml, 2 mM), ferrozine solution (0.2ml, 5mM) and water (2.75 ml), was incubated for 10min at room temperature. The absorbance was recorded at 562 nm. GSH was used as positive control. The activity was determined as follows: Chelating ability (%) = $(1–A\text{sample}_{562}/A\text{control}_{562})\times 100$.

Assay of reducing power

The reducing power was assayed according to Phongthai *et al.* (2016). Briefly, reaction solution (4.0 ml) contained phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (1%, w/v), were incubated (50°C) with the samples for 20 min. The reaction was terminated by TCA (10%, w/v). Followed by the addition of distilled water and ferric chloride (0.1%, w/v) solution. The absorbance was read at 700 nm and GSH was used as positive control.

STATISTICAL ANALYSIS

Data were expressed as mean ± standard deviation values from three parallel measurements. The statistical significance of difference between groups was evaluated by ANOVA, followed by Duncan's multiple range test. A significant difference was considered at $P<0.05$.

RESULTS

Amino acid composition of crayfish by-products protein isolate (CBPI)

The amino acid composition of CBPI is presented in table 2. CBPI presented high contents of hydrophobic amino acids (HAA) at 36.58%, such as valine, leucine, isoleucine, methionine, phenylalanine, tyrosine and proline). CBPI contained aromatic amino acids (AAA) at 9.51%. The total essential amino acid content of CBPI close to that recommended standard by FAO/WHO (1991), suggests that CBPI and its hydrolysates are of high nutritional value, in addition to their potential antioxidant capability.

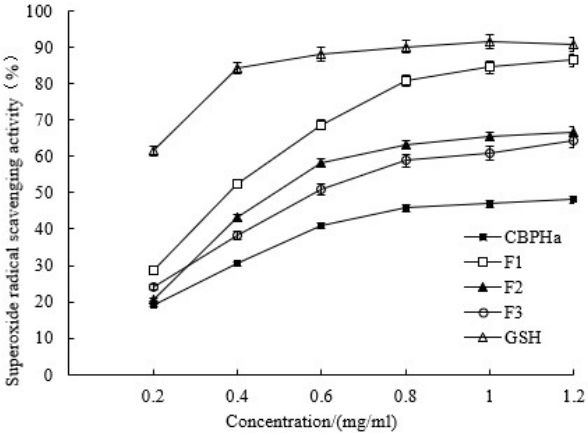

Fig. 1: Superoxide radical scavenging activity of the CBPHa and their ultrafiltration fractions (F1, F2 and F3); Each value represents the mean ± SD, n=3.

Preparation of protein hydrolysates from crayfish by-products (CBPH)

The percentage yield, protein recovery and DH of CBPH are shown in table 3. It was found that the yields of CBPHs were found differed significantly between the enzymes employed ($P<0.05$). The yields obtained for all the enzymatic hydrolyses ranged between 46.74 and 78.19%, with CBPHa had the highest yield. In all the enzymatic hydrolyses, CBPHa had significantly ($P<0.05$) higher protein recovery (69.54%) than that of other enzymes, followed by CBPHn, CBPHp and CBPHb with protein recovery of 58.72, 47.63 and 42.69%, respectively. The results mentioned above suggest that Alcalase is more effective at hydrolyzing the crayfish by-products proteins compared to the other three proteolytic enzymes. In the study, Alcalase achieved a significantly higher DH (29.76%, $P < 0.05$) of crayfish by-products compared to the other enzymes. The DH of pancreatin and neutrase was comparable; whereas it was significantly ($P<0.05$) lower that of bromelain. DH is affected by the cleavage specificities and patterns of the enzyme used, substrate/enzyme ratio, the source of substrate as well as the hydrolysis time (Ketnawa *et al*., 2017). specification during hydrolysis (Humiski & Aluko, 2007).

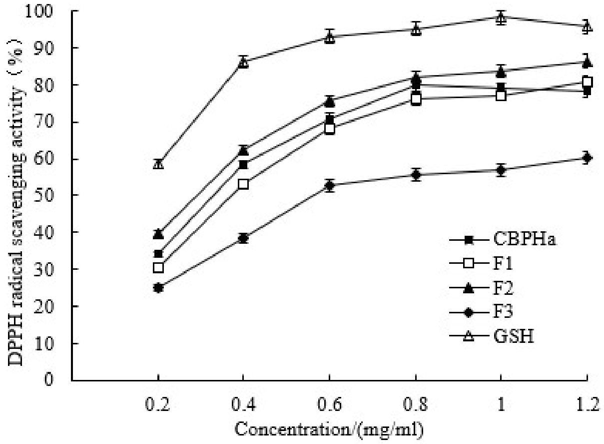

Fig. 2: DPPH radical scavenging activity of the CBPHa and their ultra filtration fractions (F1, F2 and F3); Each value represents the mean ± SD, n=3.

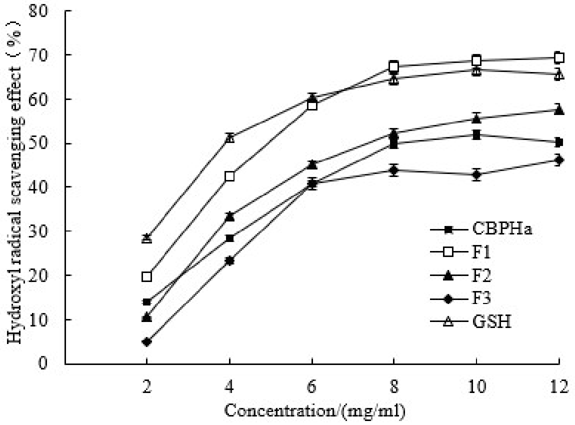

Fig. 3: Hydroxyl radical scavenging activity of the CBPHa and their ultra filtration fractions (F1, F2 and F3); Each value represents the mean ± SD, n=3.

CBPHa was ultrafiltered to get three different fractions namely F1 (MW <1 kDa), F2 (MW 1–3kDa) and F3 (MW 3–10kDa). The yields of the F1, F2 and F3 fractions were 56.3%, 25.2% and 10.9%, respectively. The yield of low molecular weight fraction (F1)>50% indicates Alkaline protease can efficiently hydrolyze CBPI into small molecular peptide, which may bring about improved bioactivity.

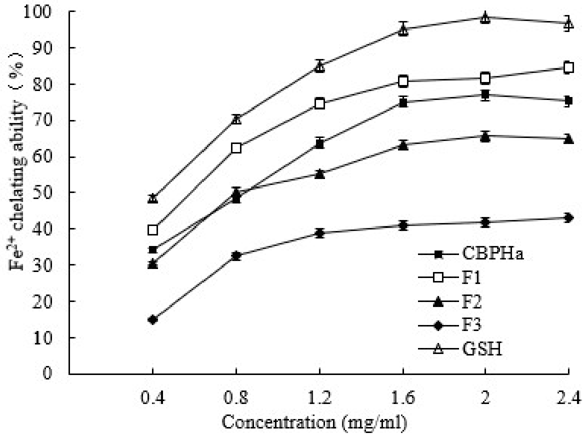

Fig. 4: Fe^{2+} chelating ability of the CBPHa and their ultrafiltration fractions (F1, F2 and F3); Each value represents the mean ± SD, n=3.

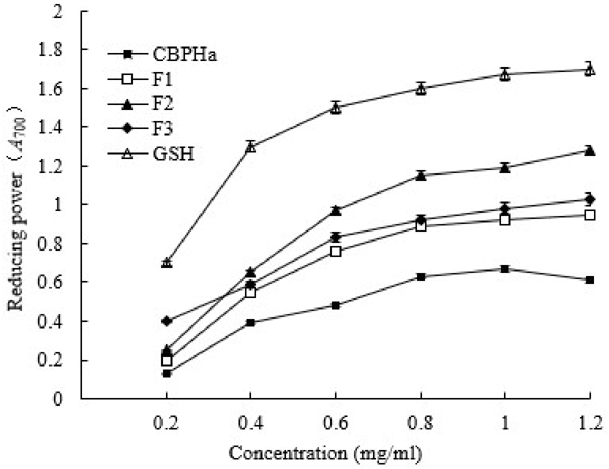

Fig. 5: Reducing power of the CBPHa and their ultrafiltration fractions (F1, F2 and F3); Each value represents the mean ± SD, n=3.

Antioxidant activity analysis

Scavenging activity of Superoxide radical

The superoxide radicals scavenging effects of CBPHa and its fractions were shown in fig. 1. The scavenging effect of all the samples were concentration-dependent and F1 exbihited much higher scavenging effect on superoxide radical than CBPHa and the other fractions, and the inhibition rate is close to that of GSH at the concentration of 1.2 mg/ml. It can be seen that the scavenging ability of the fractions decrease with increasing peptide size, indicating that the superoxide radicals were scavenged more effectively by low molecular weight peptides than by high molecular weight peptides.

Scavenging activity of DPPH

Fig. 2 showed the scavenging activities of CBPHa and its fractions on DPPH free radical were in a concentration-dependent fashion. The scavenging activity of CBPHa, F1, F2, and F3 on DPPH were 78.9%, 77.5%, 83.7% and 56.9% 52.7% at the dose of 1mg /ml, respectively. F2 showed the highest scavenging effect on DPPH, and the inhibition rate was slightly lower than that of GSH.

Scavenging activity of hydroxyl radicals

The scavenging effects on hydroxyl radical of CBPHa and its fractions were shown in fig. 3. The activity of all samples showed a concentration-dependent manner. At the concentration of 8 mg/ml, the scavenging ability was 49.8%, 67.3%, 58.2% and 43.8% for CBPHa, F1, F2 and F3, respectively. It can be seen that membrane ultrafiltration exert a considerable influence on the scavenging effect of hydroxyl radical, with F1 showing the highest activity among all components and the inhibition rate was a little higher than that of GSH (8 mg/ml).

Metal chelating activity

Fig. 4 displayed the chelating capability of CBPHa and its fractions on Fe^{2+} ion were in a concentration-dependent fashion. At the concentration of 1.6 mg/ml, the chelating ability was 74.1%, 80.9%, 63.2% and 40.8% for CBPHa, F1, F2 and F3, respectively. F1 exhibited stronger chelating ability than CBPHa and other fractions, and the inhibition rate was slightly lower than that of GSH (1.6 mg/ml).

Reducing capacity

The reduction ability of compounds can be used as a marker of their potential antioxidant effect (Nabavi *et al.*, 2009). fig. 5 exhibited the reductive activities of CBPHa and its fractions. There was concentration-dependent reducing capacity at all samples. At a concentration of 1.0 mg/ml, all samples showed effective reducing capacity and the absorbance (at 700 nm) was 0.92, 0.98, 1.19 and 0.67 for CBPHa, F1, F2 and F3, respectively. F2 had the highest reducing capacity comparing with CBPHa and the other fractions, significantly lower ($P > 0.05$) than that of GSH. No significant difference ($P > 0.05$) of reducing power was observed between F1 and CBPHa. However, F3 exhibited the lowest reducing ability value regardless of the low or high concentration. The reducing power values of F2 and F1(1 mg/mL) were higher than those obtained from mushroom (*Agaricus bisporus*) protein hydrolysates with similar proteases (Kimatu *et al.*, 2017). MPBHs

DISCUSSION

In this study, CBPI was rich in negatively charged amino acids (NCAA), such as glutamic acid and aspartic acid, exhibiting strong antioxidant activities because of their abundance electrons that can act as electron donors to scavenge free radicals (He *et al.*, 2013). It has been

Table 1: Hydrolysis conditions for the various enzymatic hydrolyses

Protease	Alcalase	Neutrase	Pancreatin	Bromelain
Temp (_C)	55	50	37	50
pH	9	7	7	8
Time (h)	5	5	5	5
E/S ratio (w/w)	2/100	2/100	2/100	2/100
Substrate concentration (w/v)	1/15	1/15	1/15	1/15

Table 2: Amino acid composition of CBPI[a]

Amino acid	g/100g CBPI dry basis	Amino acid	g/100g CBPI dry basis
Asx	8.24±0.29	Leucine	6.38±0.27
Threonine	3.54±0.12	Phenylalanine	2.86±0.11
Serine	3.62±0.15	Lysine	4.25±0.18
Proline	4.59±0.19	Histidine	3.89±0.17
Glx	13.58±0.37	Arginine	4.73±0.25
Glycine	2.03±0.08	Tryptophan	3.56±0.18
Alanine	5.57±0.24	Tyrosine	3.09±0.15
Cysteine	nt	NCAA	21.82
Valine	6.29±0.21	HAA	36.58
Methionine	1.08±0.07	AAA	9.51
Isoleucine	3.15±0.13	EAA	32.76

Negatively charged amino acids (NCAA) –Asx (asparagine + aspartic acid) and Glx (glutamine + glutamic acid).
Hydrophobic amino acids (HAA) –alanine, valine, phenylalanine, tyrosine, tryptophan, isoleucine, leucine, proline, methionine and cysteine.
Aromatic amino acids (AAA) –phenylalanine, tyrosine and tryptophan.
Essential amino acids (EAA) –valine, tryptophan, threonine, leucine, isoleucine, methionine, phenylalanine and lysine.
[a] Data are expressed as means ± standard deviation (n=3).

Table 3: Percentage yield and protein recovery and degree of hydrolysis (DH) of hydrolysates from crayfish by-products Proteins isolate (CBPH)

Enzymes	Alcalase	Pancreatin	Neutrase	Bromelain
Percentage yield	78.19±1.72^{a}	67.83±1.49^{b}	54.68±1.28^{c}	46.74±1.16^{d}
Protein recovery	69.54±1.51^{a}	58.72±1.37^{b}	47.63±1.24^{c}	42.69±1.21^{c}
DH	29.76±0.43^{a}	17.58±0.39^{c}	16.15±0.37^{c}	20.94±0.27^{b}

Each value represents the mean ± SD, n=3. Different letters within each row indicate statistically significant differences at $P<0.05$ using Duncan's Multiple Range Test (DMRT)

reported that HAA can increase the solubility of peptides in lipids thus enhancing their entry into target organs or interaction with free radicals by hydrophobic associations (Kimatu, 2017). AAA in protein hydrolysates has been shown to possess strong free radical scavenging ability, stabilizing electron-deficient free radicals through donating protons, while maintaining its own stability by resonance structure (Sarmadi & Ismail, 2010). Our results are also suggested that CBPI and its hydrolysates are of high nutritional value.

CBPI was hydrolyzed by Alcalase, neutrase, pancreatin and bromelain to produce bioactive hydrolysates. These enzymes have been widely employed to improve the nutritional, bioactive and functional properties of natural proteins (Mune Mune , Minka & Henle). Our study indicates that susceptibility of CBPI to Alcalase hydrolysis to produce higher amount of hydrolysate. The findings of this work were in accordance with results reported by other researchers (Chalamaiah *et al*., 2011; Nchienzia *et al*., 2010). Protein recovery is an important parameter of proteolytic reaction and indicates the efficiency of enzymatic hydrolysis process (Girgih *et al*., 2010). DH is a useful parameter commonly used to monitor the extent of proteolysis and compare different protein hydrolysates (Giménez *et al*., 2009).

CBPHa was selected for follow-up studies based on the above results. Similar to the results of this study, fraction of MW< 1 kDa from Pigeon pea to have the highest scavenging capacity of hydroxyl radical (Olagunju *et al*., 2018).

It is well known that superoxide free radicals are very harmful to cell components the ability to scavenge superoxide free radicals is the key to antioxidant work (Moure *et al*., 2006). The results agree with the findings that <1 kDa molecular weight hydrolysates obtained from pigeon pea and mung bean were the most effective in scavenging superoxide radicals (Olagunju *et al*., 2018; Kimatu *et al*., 2017).

DPPH is a stable free radical, which is an evaluation tool of antioxidative properties of compounds as hydrogen atom donors or free radical scavengers (Jamdar *et al*., 2012). The result was in agreement with findings that protein hydrolysate (MW1-3 kDa) from pea Seed (*Pisum sativum* L.) showed strongest scavenging activity of DPPH (Pownall *et al*., 2010). However, it was inconsistent with the results of previous study which showed that <1 kDa fraction had the highest DPPH scavenging activity (Arise *et al*., 2016; He *et al*., 2013). It would be reasonable to suggest that besides molecular weight, other factors (amino acid composition, peptide conformation, etc) might also influence their DPPH scavenging activity (Jin *et al*., 2016)

The hydroxyl radical is one of the most reactive chemical substances and the scavenging of hydroxyl radical plays an important role in antioxidant defense of in cell for food systems (Aruoma, 1998; Jamdar, *et al*., 2012). Metal chelating activity is considered to be one of the antioxidant mechanisms because it reduces the concentration of catalytic transition metals in lipid peroxidation (Liu *et al*., 2007; Girgih *et al*., 2010). The result is consistent with the findings reported by Xie *et al*. (2019), indicating F1 can reduce the damage to cell components caused by catalytic oxidation of metal ions, making them potential candidates for preservation of lipid foods.

CONCLUSION

In the present study, CBPHa and its fractions exhibited powerful antioxidant ability a to various oxidation systems in vitro. However, the antioxidant activity of CBPHa and its fractions showed different levels of antioxidant effect. F1 (MW <1 kDa) exhibited high scavenging activity of superoxide radical, DPPH and Fe^{2+} chelating ability. In the meanwhile, F2 (MW 1–3kDa) the showed good potential for reducing power and scavenging hydrogen free radical. All the fractions exhibited higher superoxide radical scavenging activity than the crude hydrolysates. Based on the results, the CBPHa and its fractions may be potential candidates for use as antioxidants in functional foods and pharmaceuticals. Overall, the low molecular weight <3 kDa fraction (F1 and F2) exhibits the highest antioxidant ability and its characterization and purification, as well as in vivo activity, needs further investigation. Alcalase produced the highest DH of CBPH which may be owing to the fact that Alcalase has broad catalytic.

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