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Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH)

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Abstract

Chickpea protein hydrolysate (CPH) was fractionated by gel filtration on Sephadex G-25. The antioxidant and free radical-scavenging activities of four CPH fractions (Fra.I, Fra.II, Fra.III, and Fra.IV) were measured using reducing power, inhibition of linoleic acid autoxidation, and 1,1-diphenyl-2- pycrylhydrazyl (DPPH)/superoxide/hydroxyl radical-scavenging assay. The antioxidant activity of Fra.IV (81.13%) was closer to that of α-tocopherol (83.66%) but lower than that of BHT (99.71%) in the linoleic acid oxidation system. Amino acid analyses showed that Fra.IV with the strongest antioxidant activity also had the highest total hydrophobic amino acids content (38.94% THAA) and hydrophobicity (125.62 kcal/mol amino acid residue) compared with the other three fractions. The molecular weight distribution of Fra.IV was found to vary from 200 to 3000 Da.

Keywords: Chickpea protein hydrolysate; Antioxidant activity; Free radical-scavenging activity; Hydrophobicity; Molecular weight distribution

1. Introduction

It is well known that lipid peroxidation is a major course of quality changes which affect the flavor, texture and appearance in foods (Rajapakse, Mendis, Byun, & Kim, 2005a). In addition, it has been recognized that oxidative stress plays a significant role in a number of age specific diseases. The factors involved in these diseases are the lipid peroxides and low molecular weight compounds produced during the late stage of the oxidative reaction. Hence, to prevent foods from undergoing deterioration and to provide protection against various diseases, it is important to inhibit the peroxidation of lipids and formation of free radicals occurring in the living body and foodstuffs (Halliwell, Murcia, Chirico, & Arumoma, 1995). In recent years interest in utilizing natural antioxidants has increased substantially (Shahidi, Liyana-Pathirana, & Wall, 2006). This has led to new investigations into assessing the antioxidant potential of biologically active peptides from protein hydrolysates such as soy protein (Chen, Muramoto, Yamauchi, & Kiyoshi, 1996; Moure, Domınguez, & Parajó, 2006), wheat protein (Peña-Ramos & Xiong, 2001; Zhu, Zhou, & Qian, 2006), milk casein (Kunio, Hiroyuki, & Hirotomo, 2000), porcine myofibrillar protein (Saiga, Tanabe, & Nishimura, 2003), fish protein (Kim et al., 2001; Wu, Chen, & Shiau, 2003). The antioxidant properties of these hydrolysates have been ascribed to the cooperative effect of a number of properties, including their ability to scavenge free radicals, to act as metal–ion chelator, oxygen quencher or hydrogen donor, and to the possibility of preventing the penetration of lipid oxidation initiators by forming a membrane around oil droplets (Moure et al., 2006).

Chickpea (*Cicer arietinum* L.), an annual herbage plant, is the third most important grain legume in the world on the basis of total grain production (FAO, 1994). In 1980s, hundreds of chickpea species were imported from the International Center for Agricultural Research in the Dry Areas (ICARDA) and the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT)

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and have been planted in Gansu, Qinghai and Xinjiang of China (Zhang, Jiang, & Wang, 2007).

Chickpea proteins have been considered a suitable source of dietary protein due to their good balance in essential amino acids composition, high bioavailability, and low level of antinutritional factors (Clemente et al., 1999b). The preparation method, emulsifying capability, solubility, gelation characteristic and antioxidant activity of chickpea proteins have been studied (Arcan & Yemeniciog, 2007; Gil & Nadal, 1996; Kaur & Singh, 2007; Newman, Roth, & Lockerman, 1987; Zhang et al., 2007). Several biological activities, such as angiotensin I-converting enzyme (ACE) inhibition and reduction of antigenic activity, have been reported for CPH (Clemente, Vioque, Sánchez-Vioque, Pedroche, & Millán, 1999a; Yust et al., 2003). However, there is little information concerning the antioxidant and free radicals scavenging properties of CPH. This study, therefore, investigated the antioxidant potential of different CPH fractions using several measurements, including the reducing power, the ability to inhibit the autoxidation of linoleic acid, the scavenging effect on DPPH/superoxide/hydroxyl radicals. Furthermore, amino acid composition and molecular weight distribution were also evaluated to determine their relationship with the antioxidant activity.

2. Materials and methods

2.1. Materials

Chickpea was obtained from Xinjiang Fuyuan Fruit & Vegetable Products Co., Ltd. (Xinjiang, China). Linoleic acid, 1,1-diphenyl-2-pycrylhydrazyl (DPPH) were products of Sigma Chemical Co. (St. Louis, MO, USA). Alcalase 2.4 L FG was purchased from Novo Co. (Novo Nordisk, Bagsvaerd, Denmark). All other chemicals and reagents were of the highest grade commercially available.

2.2. Production of chickpea protein isolate (CPI)

Chickpea protein isolate (CPI) was produced according to the method described by Zhang et al. (2007) with little modification. Chickpea seeds were ground and defatted with hexane. The defatted flour was dried in the ventilator overnight at 20 °C. CPI was obtained by dispersing the defatted chickpea flour in NaOH solution (pH 8.3) at ratio 1:10 (w/v) and extracted by stirring for 1 h. After pH adjustment to the isoelectric point (pH 4.3), the precipitate obtained by centrifugation at 8000*g* for 15 min was lyophilized and stored at −20 °C.

2.3. Preparation of CPH

Chickpea protein isolate (20 g) was suspended in distilled water (1000 ml) and hydrolyzed with alcalase (400 μl) at 50 °C, after adjusting pH of the mixture to 8.0 with 0.5 M NaOH. The degree of hydrolysis (DH) was determined by using the pH-stat method as previously described by Adler-Nissen (1986). After maintaining the pH constant with 0.5 M NaOH during hydrolysis time (40 min), the enzyme was inactivated at 95 °C for 10 min, and the hydrolysate solution was cooled down to room temperature, neutralized and centrifuged at 20,000*g* for 10 min. The supernatants at DH 15 were lyophilized and stored at −20 °C.

2.4. Fractionation of CPH

CPH (20 mg) was loaded onto Sephadex G-25 gel filtration column (1.6 × 150 cm). Separation was obtained with distilled water at a flow rate of 20 ml/h and eluted fractions (5.0 ml) were pooled after spectrophotometric measurements at 280 nm.

2.5. Reducing power

The reducing power of CPH fractions was measured according to Oyaizu (1988). The sample (0, 1.0, 2.0, 3.0, 4.0 and 5.0 mg) was added to 0.2 M phosphate buffer (pH 7.0) and 1% (w/v) potassium ferricyanide. The mixture was first incubated at 50 °C for 20 min, then 10% (w/v) TCA was added and the reaction mixture was centrifuged at 6500*g* for 10 min. The upper layer obtained after centrifugation was mixed with distilled water and 0.1% (w/v) ferric chloride in a test tube. After 10 min, the absorbance of the resulting solution was read at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.6. Inhibition of linoleic acid autoxidation

The antioxidant activities of CPH fractions were measured based on the method of Osawa and Namiki (1985) with some modifications. One milligram of sample was dissolved in 1.5 ml of 0.1 M phosphate buffer (pH 7.0), and added to 1 ml of linoleic acid (50 mM) dissolved with ethanol (99.5%) in a glass test tube which was sealed tightly with silicon rubber cap and kept at 60 °C in the dark for 8 days. The degree of oxidation was evaluated by measuring the ferric thiocyanate values according to the method described by Mitsuta, Yasumoto, and Iwami (1996). The sample solution (100 μl) incubated in the linoleic acid model system described above was mixed with 4.7 ml of 75% ethanol, 0.1 ml of 30% (w/v) ammonium thiocyanate, and 0.1 ml of 0.02 M ferrous chloride dissolved in 1 M HCl. After 3 min, the degree of color development that represents linoleic acid oxidation was measured spectrophotometrically at 500 nm. The antioxidant activities of butylated hydroxytoluene (BHT) and α-tocopherol were also assayed at the same concentration for comparison purposes.

2.7. DPPH radical-scavenging activity

The scavenging effect of CPH fractions on 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was measured

according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992) with little modification. Two millilitres of each sample solution (1.0 mg/ml) were added to 2 ml of 0.1 mM DPPH dissolved in 95% ethanol. The mixture was shaken and left for 30 min at room temperature, and the absorbance of resulting solution was read at 517 nm. A lower absorbance represents a higher DPPH scavenging activity. The scavenging effect was expressed as shown in the following equation:

$$\text{DPPH scavenging activity (\%)} = [(\text{Blank absorbance} - \text{Sample absorbance})/\text{Blank absorbance}] \times 100 \quad (1)$$

2.8. Hydroxyl radical-scavenging activity

The hydroxyl radical-scavenging assay was carried out using the method described by de Avellar et al. (2004) with some modifications. Both 1,10-phenanthroline (0.75 mM) and $FeSO_4$ (0.75 mM) were dissolved in phosphate buffer (pH 7.4) and mixed thoroughly. H_2O_2 (0.01%) and CPH fractions (1.5 mg/ml) were added. The mixture was incubated at 37 °C for 60 min, and the absorbance was measured at 536 nm. Results were determined using the following equation:

$$\text{Hydroxyl radical} - \text{scavenging activity (\%)} = [(A_S - A_1)/(A_0 - A_1)] \times 100 \quad (2)$$

where A_S, absorbance of the sample; A_1, absorbance of control solution containing 1,10-phenanthroline, $FeSO_4$ and H_2O_2; A_0, absorbance of blank solution containing 1,10-phenanthroline and $FeSO_4$.

2.9. Superoxide radical-scavenging activity

The superoxide radical-scavenging activity was estimated at 25 °C using the spectrophotometric monitoring of the inhibition of pyrogallol autoxidation as previously described by Marklund and Marklund (1974) with some modifications. This assay is dependent on the reducing activity of test compound by an $O_2^{-}\cdot$-dependent reaction, which releases chromphoric products. Pyrogallol solution (3 mM) was added into a tube containing CPH fractions (2.0 mg/ml) previously dissolved in Tris–HCl–EDTA buffer (0.1 M, pH 8.0). The optical density (OD) was measured in triplicate at 320 nm using a spectrophotometer. The antioxidant activity was determined as the percentage of inhibiting pyrogallol autoxidation, which was calculated from OD in the presence or absence of pyrogallol and CPH fraction.

2.10. Amino acid composition

The lyophilized hydrolysate fractions were digested with HCl (6 M) at 110 °C for 24 h under nitrogen atmosphere. Reversed phase high performance liquid chromatography (RP-HPLC) analysis was carried out in an Agilent 1100 (Agilent Technologies, Palo Alto, CA, USA) assembly system after precolumn derivatization with *o*-phthaldialdehyde (OPA). Each sample (1 μl) was injected on a Zorbax 80 A C18 column (4.6 i.d. × 180 mm, Agilent Technologies, Palo Alto, CA, USA) at 40 °C with detection at 338 and 262 nm. Mobile phase A was 7.35 mmol/l sodium acetate/triethylamine/tetrahydrofuran (500:0.12:2.5, v/v/v), adjusted to pH 7.2 with acetic acid, while mobile phase B (pH 7.2) was 7.35 mmol/l sodium acetate/methanol/acetonitrile (1:2:2, v/v/v). The amino acid composition was expressed as g of amino acid per 100 g of protein.

2.11. Determination of molecular weight distribution

The CPH fraction with the strongest antioxidant and free radical-scavenging activities was analyzed for molecular weight distribution using a Waters™ 600E Advanced Protein Purification System (Waters Corporation, Milford, MA, USA). The hydrolysates were loaded onto TSK gel G2000 SWXL column (7.8 i.d. × 300 mm, Tosoh, Tokyo, Japan), eluted with 45% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 0.5 ml/min and monitored at 220 nm. A molecular weight calibration curve was obtained from the following standards from Sigma: cytochrome C (12,500 Da), aprotinin (6500 Da), bacitracin (1450 Da), tetrapeptide GGYR (451 Da), and tripeptide GGG (189 Da). Results were processed using Millennium[32] Version 3.05 software (Waters Corporation, Milford, MA 01757, USA).

2.12. Statistical analysis

All the tests were done in triplicate and data were averaged. Standard deviation was also calculated. Student's *t*-test was used to evaluate significant differences ($P < 0.05$) between the means for each sample.

3. Results and discussion

3.1. CPH Fractionation

CPH was fractionated by gel filtration column chromatography on Sephadex G-25, and Fra.I, Fra.II, Fra.III and Fra.IV were obtained, respectively, as shown in Fig. 1. Each fraction was freeze-dried and stored at −20 °C until further use.

3.2. Reducing power

Different studies have indicated that antioxidant activity and reducing power are related (Duh, 1998; Duh, Tu, & Yen, 1999). The reducing power of CPH fractions as a function of their concentration is shown in Fig. 2. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e.

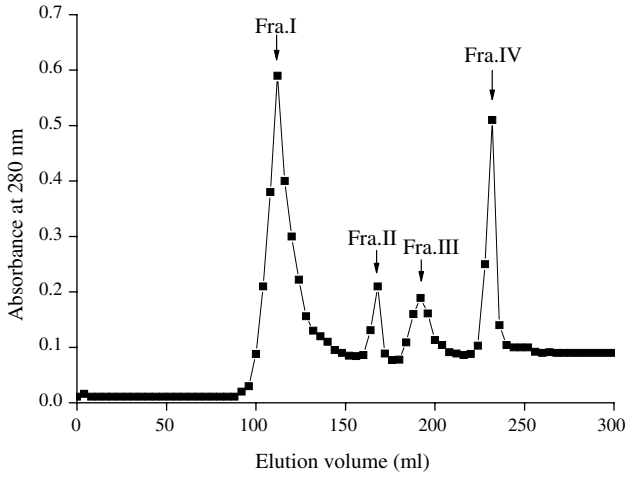

Fig. 1. Elution profile of CPH fractions separated by gel filtration on Sephadex G-25.

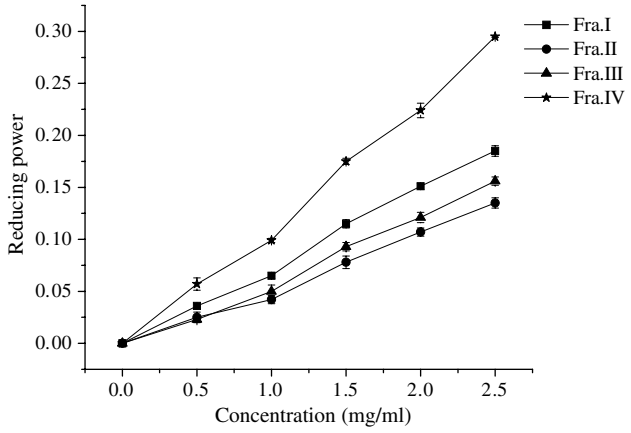

Fig. 2. Reducing power of CPH fractions used at different concentrations.

antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration (Ferreira, Baptista, Vilas-Boas, & Barros, 2007). The highest reducing power was found in Fra.IV, followed by Fra.I, Fra.III and Fra.II, respectively. The reducing power of CPH fractions increased with increasing their concentrations. A similar observation has been reported by Zhu et al. (2006) on wheat germ protein hydrolysate (WGPH).

3.3. Inhibition of linoleic acid autoxidation

Peroxidation of fatty acids can cause deleterious effects in foods by forming complex mixture of secondary breakdown products of lipid peroxides. Further intake of these foods can cause a number of adverse effects including toxicity to mammalian cells. Therefore, the CPH fractions were further characterized for their antioxidant activity by assessing their ability to protect linoleic acid against oxidation. Lipid peroxidation is thought to proceed via radical mediated abstraction of hydrogen atoms from methylene carbons in polyunsaturated fatty acids (Rajapakse et al., 2005a).

The antioxidant properties of CPH fractions, determined using the thiocyanate method, were compared with those of BHT and α-tocopherol. As shown in Fig. 3, the oxidation of linoleic acid was markedly inhibited by the addition of CPH fractions. Among the four fractions, the highest antioxidant activity was found in Fra.IV, which exhibited a significant ($P < 0.05$) inhibition of linoleic acid peroxidation. Fra.IV exhibited an inhibition of linoleic acid peroxidation (81.13%) similar to that of α-tocopherol (83.66%) but lower than that of BHT (99.71%). Many natural antioxidants are less potent than synthetic antioxidants, but they can be used at higher concentrations than the synthetic ones, due to the very restrictive toxicological parameters of these latter. In addition, the incorporation of protein hydrolysate to foods could confer desirable nutritional and functional properties (Kim, Je, & Kim, 2007).

3.4. DPPH radical-scavenging activity

The relatively stable DPPH radical has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and thus to evaluate the antioxidant activity (Jao & Ko, 2002). These antioxidants donate hydrogen to free radicals, leading to non-toxic species and therefore to inhibition of the propagation phase of lipid oxidation. Results shown in Table 1 revealed that Fra.IV at 1.0 mg/ml exhibits an excellent DPPH radical-scavenging activity (85.82%), which is higher than that of *P* fraction isolated from wheat gluten hydrolysate (Wang, Zhao, Zhao, & Jiang, 2007). The other fractions also showed good DPPH radical-scavenging activities in the order of Fra.I > Fra.III > Fra.II. The CPH fractions possibly contained some substrates, which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction.

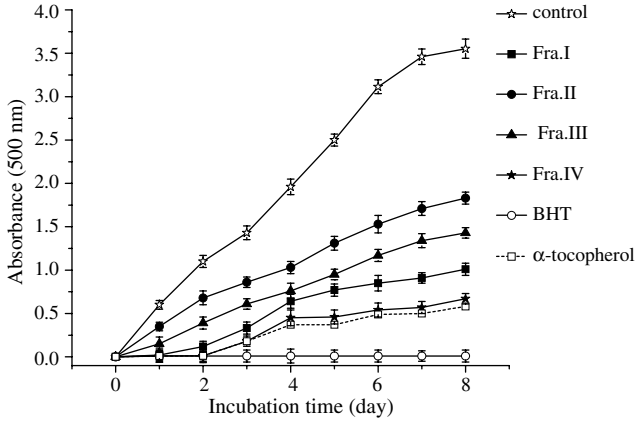

Fig. 3. Lipid peroxidation measured in linoleic acid model system for 8 days in the presence of different CPH fractions. Higher UV absorbance at 500 nm represents higher linoleic acid peroxidation. Values are means of triplicates.

Table 1
Free radicals scavenging effects of fractionated CPH

Fractions	Free radicals scavenging effects (%)		
	DPPH radical[a]	Hydroxyl radical[b]	Superoxide radical[c]
Fra.I	71.26 ± 2.12	64.74 ± 1.06	57.05 ± 1.92
Fra.II	44.31 ± 1.54	38.42 ± 2.01	35.03 ± 1.76
Fra.III	59.28 ± 1.66	56.36 ± 1.54	47.13 ± 1.31
Fra.IV	85.82 ± 1.33	81.39 ± 1.21	69.15 ± 1.23

[a] Scavenging effects were tested at 1.0 mg/ml.
[b] Scavenging effects were tested at 1.5 mg/ml.
[c] Scavenging effects were tested at 2.0 mg/ml.

3.5. Hydroxyl radical-scavenging activity

It is well known that the radical system used for antioxidant evaluation may influence the experimental results, and two or more radical systems are required to investigate the radical-scavenging capacities of a selected antioxidant (Yu et al., 2002). The CPH fractions were analyzed for hydroxyl radical-scavenging activity to better examine their antioxidant properties.

Hydroxyl radical is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metal–ions, such as copper or iron. When a hydroxyl radical reacts with aromatic compounds, it can add on across a double bond, resulting in hydroxycyclohexadienyl radical. The resulting radical can undergo further reactions, such as reaction with oxygen, to give peroxyl radical, or decompose to phenoxyl-type radicals by water elimination (Lee, Koo, & Min, 2004). The hydroxyl radical-scavenging abilities of different CPH fractions are also shown in Table 1. The inhibition of hydroxyl radical exhibited by Fra.IV (81.39%) was closer to that of peptide isolated from hoki (*Johnius belengerii*) frame protein by gastrointestinal digestion (Kim et al., 2007). In addition, Fra.I, Fra.II and Fra.III used at the same concentration exhibited 64.74%, 38.42% and 56.36% hydroxyl radical-scavenging activity, respectively.

3.6. Superoxide radical-scavenging activity

Numerous biological reactions generate superoxide radical ($O_2^{-\cdot}$) which is a highly toxic species. Although they cannot directly initiate lipid oxidation, superoxide radical anions are potential precursors of highly reactive species, such as hydroxyl radical, and thus study of the scavenging of this radical is important (Kanatt, Chander, & Sharma, 2007).

Superoxide anion scavenging of the different CPH fractions is presented also in Table 1. All samples treated in this experiment showed considerable scavenging abilities over superoxide anion. Addition of Fra.IV at 2.0 mg/ml showed the highest superoxide anion scavenging activity (69.15%), which is similar to that of antioxidant peptides isolated from fermented mushroom (Sun, He, & Xie, 2004). Fra.I, Fra.II and Fra.III, used at the same concentration, showed 57.05%, 35.03% and 47.13% superoxide anion scavenging effect, respectively. Results showed that Fra.IV has good antioxidant and free radicals scavenging activities and this fraction can be a potential source of natural antioxidant.

3.7. Amino acid composition

The CPH fractions collected from chromatography on Sephadex G-25 were subjected to amino acid composition analyses in order to determine the possible effect of the amino acid profile on antioxidant activity. The amino acid compositions of these fractions revealed that they are rich in Arg, Phe, Lys, Leu, Ala and Asp (Table 2). In addition, the total hydrophobic amino acids (THAA) content in Fra.IV was found to be higher than those in the other three fractions. For protein hydrolysates and peptides, an increase in hydrophobicity will increase their solubility in lipid and therefore enhance their antioxidant activity (Rajapakse, Mendis, Jung, Je, & Kim, 2005b; Saiga et al., 2003). In fact, peptides derived from many protein sources with increased hydrophobicity have been reported to relate with antioxidative properties (Chen, Muramoto, & Yamauchi, 1995; Chen et al., 1996; Chen, Muramoto, Yamauchi, & Kenshiro, 1998). Calculation of the hydrophobic indices or hydrophobicity (HΦ) according to Bigelow (1967) showed that the hydrophobicity exhibited by Fra.IV (125.61 kcal/mol amino acid residue (AAR)) was higher than that of Fra.I (114.34 kcal/mol AAR), Fra.II (103.77 kcal/mol AAR) and Fra.III (103.64 kcal/mol AAR), respectively. The highest antioxidant and free radicals scavenging activities were presumably due to the highest content in total hydrophobic amino acid (THAA) and consequently the highest hydrophobicity.

Table 2
Amino acid composition of CPH fractions separated by gel filtration on Sephadex G-25

Amino acids	Fra.I	Fra.II	Fra.III	Fra.IV
Aspartic acid	6.02	4.96	5.67	3.32
Glutamic acid	6.12	5.93	9.66	6.09
Serine	5.05	4.14	5.85	5.16
Histidine	4.37	4.06	5.56	4.83
Glycine	3.15	4.75	6.56	5.82
Threonine	1.02	1.92	1.82	1.38
Arginine	25.04	33.13	13.74	16.40
Alanine	4.03	2.64	6.93	4.50
Tyrosine	1.94	4.84	0.99	1.18
Cysteine	0.50	0.42	0.17	0.13
Valine	3.52	2.55	2.96	3.89
Methionine	1.11	0.90	1.85	1.91
Phenylalanine	6.76	3.53	4.62	13.56
Isoleucine	3.63	2.85	2.91	3.80
Leucine	9.50	6.05	7.19	10.10
Lysine	15.45	14.52	15.37	17.77
Proline	3.12	2.83	8.19	4.97
THAA (g/100 g)	30.49	23.36	27.45	38.94
HΦ (kcal/mol AAR)	114.34	103.77	103.64	125.62

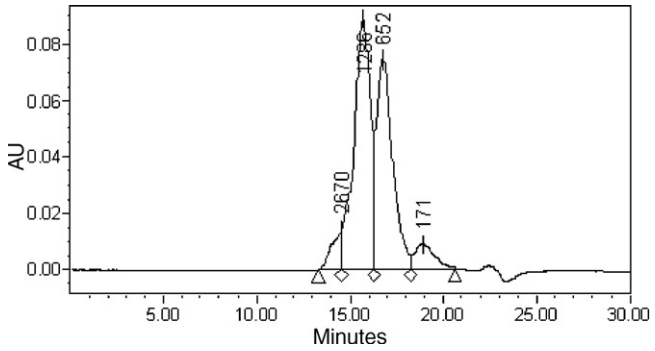

Fig. 4. Molecular weight distribution of CPH fraction having the highest antioxidant activity. Molecular weight range for each peak: 940–2622 Da (1), 220–940 Da (2) and 80–220 Da (3).

3.8. Molecular weight distribution

Considering that Fra.IV was found to possess the highest antioxidant activity, this fraction was therefore analyzed for molecular weight distribution (Fig. 4). The chromatographic data indicated that this fraction was composed of low molecular weight peptides whose major peaks were located at 940–2622 Da (49.21%) and 220–940 Da (40.63%). A number of studies had already shown that the antioxidant activity of hydrolysates is depending on their molecular weight distribution (Moure et al., 2006; Peña-Ramos, Xiong, & Arteaga, 2004; Wang et al., 2007). In this study, results revealed that the peptide fraction with molecular weight ranging from 200 to 3000 Da was probably associated with higher antioxidant activity. These findings are in agreement with observations from other studies and support the fact that functional properties of antioxidative peptides are highly influenced by properties such as molecular mass (Jeon, Byun, & Kim, 2000; Kim et al., 2007).

4. Conclusions

The different CPH fractions obtained by gel filtration exhibited different antioxidant and free radicals scavenging activities. Results revealed that Fra.IV has the highest antioxidant and free radicals scavenging activities. The molecular weight distribution of peptide from Fra.IV showed that its peaks were mainly located at 940–2622 Da (49.21%) and 220–940 Da (40.63%). Moreover, the size (usually lower molecular weight), the amino acid composition and the hydrophobicity (HΦ) of CPH fractions were found to be strongly correlated with their antioxidant activity. A further research on the purification of peptide isolated from the CPH fraction as well as the mechanisms of its antioxidant activity is undergoing.

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