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Antioxidant activity of peptides isolated from alfalfa leaf protein hydrolysate

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ABSTRACT

Alfalfa leaf protein, a potential source of high quality protein for human consumption, was hydrolyzed with protease. Alfalfa leaf protein hydrolysate was fractionated by ultrafiltration and the obtained peptides were purified by dynamical adsorption. The antioxidant activity of alfalfa leaf peptides (ALPs) was investigated and compared with that of a native antioxidant, reduced glutathione (GSH), which was used as a reference. The reducing power of ALPs was 0.69 at 2.00 mg/mL. ALPs at 1.60 mg/mL and 0.90 mg/mL exhibited 79.71% and 67.00% of scavenging activities on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide radical, respectively. In addition, ALPs showed 65.15% chelating effect on ferrous ion at 0.50 mg/mL. The molecular weight of the peptides was determined and 67.86% of the total amount was below 1000 Da. Combined with the result of the amino acid profiles, ALPs was believed to have high nutritive value in addition to antioxidant activity.

1. Introduction

In recent years, biomaterials with antioxidative activity, especially antioxidative peptides, have drawn the attention of researchers, due to their low-molecular-weight, easy absorption and high activity. Antioxidative peptides have many advantages over superoxide dismutase (SOD) and other antioxidant enzymes, with simpler structure, more stable under different conditions and without dangerous immunoreaction. Normally, the amount of SOD biosynthesized in vivo can meet the requirement of scavenging superoxide anion radicals. Under certain circumstances, such as in old age or unhealthy state, the generating and scavenging of superoxide anion are imbalance, supplement of antioxidant compounds is of special significance to reduce the damage (Bottino et al., 2002; Nam, Choi, Kang, Koh, Kozukue, & Friedman, 2006; Serbecic & Beutelspacher, 2005; Shibata & Tomita, 2005).

Peptides (2 to less than 100 amino acid residues) with bioactivities have been isolated from different protein hydrolysates obtained by enzymatic hydrolysis with protease. The peptides exhibited several bioactivities, including inhibition of bio-macromolecules peroxidation and elimination of free radicals produced in vivo (Hook, Burton, Yasothornsrikul, Hastings, & Deftos, 2001). It has been reported that some amino acids and their derivatives, such as cysteine, histidine, tryptophan, lysine, arginine, leucine, valine and β-hydroxyl tryptophan, etc., had antioxidant activities (Uchida & Kawakishi, 1992). Furthermore, some low-molecular-weight polypeptides containing histidine, tryptophan, and tyrosine also showed distinct antioxidant activities (Muramoto, Chen, & Yamauchi, 1996).

Leaf protein has been recognized by FAO as a potential and effective source of high quality protein for human consumption due to their abundance of source, nutritive value, and free of animal cholesterol (Chen & Qiu, 2003). The results of mouse breeding showed that leaf protein increased the activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD), as well as decreased the concentration of maloaldehyde (MDA) which was formed in oxidative reaction, therefore, enhanced the function of antioxidant enzyme system (Fu, 2003).

Alfalfa leaf protein, a good source for producing nutritious and functional food, is extracted from alfalfa leaf, a traditional Chinese medicine. Researches have been conducted on preparation procedures, application, property and nutritional value of alfalfa leaf protein. Proteins were isolated from dry powders of six one-year-old and two more than one-year-old Australian alfalfa herbages with a sequential extraction procedure, where albumin was found to be the main protein, glutenin and globin were in small amounts, and alco-protein was not detected (Huang, 2000).

Lamsala, Koegelb, and Gunasekaranc (2007) reported some important physicochemical and functional properties of soluble leaf proteins from alfalfa herbage, such as subunits molecular weight distribution, denaturation temperature, and functional properties like, emulsification, foaming, and solubility. Despite its high nutritive value, the application of alfalfa leaf protein in food is limited since its poor solubility and negative sensory properties in colour, taste and texture (Chen & Qiu, 2003). Enzymatic hydrolysis has been considered as an attractive means to overcome these disadvantages. In our previous work, the method for separation

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and purification of alfalfa leaf soluble protein hydrolysates was established, and the effects of ultrafiltration on the molecular weight distribution and amino acids composition of alfalfa soluble protein hydrolysates were investigated. The reducing power was found to be increased after ultrafiltration (Xie & Jin, in press). Until now, little work has been carried out on the antioxidative peptides isolated from alfalfa leaf protein hydrolysate. This study was undertaken to evaluate the antioxidant activities of the peptides obtained from alfalfa leaf protein by protease hydrolysis. Five in vitro test models were employed and reduced glutathione (GSH) was used as a reference material.

2. Materials and methods

2.1. Materials

Sinkiang alfalfa leaves (containing 25.75% protein, 6.10% fat, 31.26% crude fiber, 13.14% ash, and 23.75% other substances on dry basis, and 41.26% moisture), second cutting of large leaf variety from the ramification period, were supplied by Inner Mongolia Academy of Agriculture (China). Alcalase FG 2.4 L (9.88×10^4 U/g) was purchased from Novo (Denmark) and Luminol was from Fluka (Switzerland). 1,1-diphenyl-2-picrylhydrazy (DPPH) and reduced glutathione (GSH) were purchased from Sigma (USA). All other chemicals used were of analytical grade.

2.2. Preparation of alfalfa leaf peptides (ALPs)

2.2.1. Extraction of soluble alfalfa leaf protein

The comminuted alfalfa leaves (100 g) were mixed with water at a ratio of 1:7 (w/w). After adjusting pH to 9.0 with 0.1 mol/L NaOH, the mixture was incubated at 45 °C for 5 h. The supernatant, collected after centrifugation of the mixture at 3500 r/min for 30 min, was adjusted to pH 4.0 with 0.1 mol/L HCl and stood for 30 min. Soluble alfalfa leaf protein was obtained by centrifugation of the solution at 3500 r/min for 30 min, and by freeze drying the sediment.

2.2.2. Protease hydrolysis of alfalfa leaf protein

In our preliminary experiments, the extent of alfalfa leaf protein degradation by different commercial proteases (Neutrase, Protamex and Flavorzyme, Alcalase) was estimated by assessing the degree of hydrolysis (DH). It was found that the DH of the hydrolysate obtained through hydrolysis by Alcalase was higher than those obtained by Neutrase, Protamex and Flavorzyme, respectively. Therefore the antioxidant activity was investigated on ALPs obtained from Alcalase hydrolysis.

The pH of the 5% (w/v) alfalfa leaf protein suspension was adjusted to 8.0 with 0.1 mol/L NaOH. The suspension was incubated at 50 °C for 30 min, then incubated with Alcalase FG 2.4 L (5%, w/w) at 60 °C for 4 h (pH of the system was kept stable with pH-stat method). After inactivation by keeping at 85 °C for 10 min, the liquid was centrifuged at 3500 r/min for 15 min. The supernatant was desalted by using macroporous resin, and then concentrated under vacuum. At last, the alfalfa leaf protein hydrolysate was obtained by freeze drying.

2.2.3. Purification and desalting of antioxidative peptides from alfalfa leaf protein

At a concentration of 35 mg/mL, the alfalfa leaf protein hydrolysate was fractionated through an ultrafiltration membrane system with a 3000 Da molecular weight cut-off polysulphone (PS) membrane. The ultrafiltration was performed at 0.30 MPa, 20 °C. The filtrate was collected, vacuum-concentrated and freeze-dried. Solution of the obtained crude alfalfa leaf peptides (50 mg/mL) was injected into the chromatography column (DA201-C macroporous resin, Organic Chemicals Co. Ltd., Jiangyin, China) at a flow rate of 0.5 BV/h. Elution was at room temperature using deionized water at a flow rate of 1 BV/h. The eluent was collected (15 mL/tube) until the conductivity of the eluent was equivalent to that of the deionized water. Seventy-five percent (v/v) ethanol was employed for dynamic desorption. The eluent with peak at 220 nm was collected and concentrated under vacuum condition to remove ethanol. The desalted alfalfa leaf peptides were then obtained by freeze drying.

2.3. Antioxidant activities of alfalfa leaf peptides (ALPs)

Since the reducing power of the alfalfa leaf protein hydrolysate was found to be increased after ultrafiltration with a 3000 Da molecular weight cut-off membrane (Xie & Jin, in press). Only the filtrate was used for the following study on antioxidant activities.

2.3.1. Determination of reducing power

The reducing power was measured according to Yen and Chen (1995) with minor modification. An aliquot of 2.5 mL alfalfa leaf peptides (ALPs) and GSH solutions at different concentrations (0.0–2.5 mg/mL) was mixed with 2.5 mL, 1% potassium ferricyanide solution. The mixture was incubated at 50 °C for 20 min. Afterwards 2.5 mL of 10% trichloroacetic acid (TCA) was added to the mixture, which was then centrifuged at 3000 r/min for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm by using a spectrophotometer (721, Shanghai, China) after the solution stood for 10 min. Increasing absorbance of the reaction mixture indicate the increasing reducing power. All determinations were performed in triplicate.

2.3.2. Scavenging activity of DPPH radical

The scavenging activity of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured according to the method of Shimada, Fujikawa, Yahara, and Nakamura (1992) with some modification. An aliquot of 0.5 mL of sample solution at different concentrations (0.0–1.6 mg/mL) was mixed with 2.5 mL of DPPH (25 μg/mL). The reaction mixture was incubated for 30 min in the darkness at room temperature. The absorbance of the resulting solution was measured at 517 nm with a spectrophotometer (721, Shanghai, China). The methanol was used as a control. The radical scavenging capacity of the tested samples was measured as a decrease in the absorbance of DPPH radical and was calculated by using the following equation (Shimada et al., 1992):

$$\text{Inhibition}(\%) = [(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$$

All determinations were performed in triplicate.

2.3.3. Superoxide anion radical scavenging assay

Superoxide anion radical scavenging activity was determined according to Yu, Zhao, Xue, Jin, and Wang (2001) with minor modification. The reaction mixture, with the total volume of 1 mL, contained 100 μL ALPs sample (0.0–0.9 mg/mL), 100 μL pyrogallol (1 mmol/L) and 800 μL mixture of luminal in carbonate buffer (pH 10.2). A sample cell loaded with the mixture was first placed in a bio-chemical luminescence-measuring instrument (BCM) (SHG-C, Shanghai analytical instrument factory, Shanghai, China). When the cell crossed the monitor, a known concentration of the sample was injected into the cell in situ. The chemiluminescence intensity was simultaneously recorded with R program in the processor once every 6 s. Double distilled water was used as a control. The determination was performed with high pressure 3.55 V and discriminating pressure 0.2 V, at 25 °C. The chemiluminescence intensity at the sixth 6 s (CP6s) was used as the standard for the calculation. GSH solution at the same concentration was used as

a reference. Analyses of all samples were run in triplicate and averaged. The superoxide anion radical scavenging activity was calculated by using the following equation:

$$\text{Inhibition}(\%) = [(\text{CP6s}_{\text{control}} - \text{CP6s}_{\text{sample}})/\text{CP6s}_{\text{control}}] \times 100$$

2.3.4. Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was determined according to Fan, Li, and Sha (1998) with some modification. Fifty microliter of sample (0.0–1.2 mg/mL), with double distilled water used as a control, was premixed with 50 μL of *o*-phenanthroline solution (1.0×10^{-3} mol/L), 50 μL of copper sulphate solution (1.0×10^{-3} mol/L), 20 μL of ascorbic acid solution (1.0×10^{-3} mol/L), and 780 μL of borax-boracic acid buffer (pH 9.0). Then 50 μL of hydrogen peroxide solution (4.4×10^{-5} mol/L) was added to start the bio-chemical luminescence reaction. The chemiluminescence intensity was simultaneously recorded with *R* program in the processor once every 6 s. The chemiluminescence intensity at the sixth 6 s (CP6s) was used as the standard for the calculation. GSH solution with the same concentration was used as a reference. Analyses of all samples were run in triplicate and averaged. The measuring conditions and calculation were the same as described in Section 2.3.3.

2.3.5. Metal ions chelating activity

Metal ions chelating activity was measured according to the method described by Decker and Welch (1990), with minor modification. 1 mL of sample solution (0.0–0.6 mg/mL) was premixed with 0.05 mL of iron dichloride solution (2 mmol/L) and 1.85 mL of double distilled water. Afterwards, 0.1 mL of ferrozine solution (5 mmol/L) was added and mixed vigorously. The absorbance was determined at 562 nm after the mixture stood for 10 min at the room temperature. Double distilled water was used as a control. Analyses of all samples were run in triplicate and averaged. The chelating effect was calculated by using the following equation:

$$\text{Chelating effect}(\%) = [(\text{A}_{\text{control}} - \text{A}_{\text{sample}})/\text{A}_{\text{control}}] \times 100$$

2.4. Determination of amino acid and molecular weight distribution

Amino acids contained in the alfalfa leaf peptides were determined with automatic amino acid analyzer (Agilent Technologies Inc., USA). Molecular weight distribution was determined by using a Waters 600 HPLC system, with TSK gel column (2000 SWXL, 300 mm × 7.8 mm), in combination with 2487 UV detector and M32 work station. Elution was at 30 °C using acetonitrile/water/trifluoroacetic acid (45/55/0.1, v/v) at a flow rate of 0.5 mL/min.

2.5. Statistic analysis

The statistical analysis was carried out by using the Statistical Package for Social Sciences (SPSS 11.0, 2001) software. The data were expressed as mean ± SD (standard deviation). A two-tailed Pearson Correlation test was conducted to determine correlations among means.

3. Results and discussion

3.1. Antioxidant activities of alfalfa leaf peptides (ALPs)

Since the response of antioxidants depends on many factors in a given test, the antioxidant activity of a system can be better characterized by using different assays based on different mechanisms (Moure, Dominguez, & Parajo, 2006). We have employed one method for reducing power; three methods based on the radical scavenging capacity: DPPH (1,1-diphenyl-2-picrylhydrazyl) radical, superoxide anion radical and hydroxyl radical; one test for chelating effects on ferrous ions to evaluate the antioxidant activities of alfalfa leaf peptides (ALPs).

3.1.1. Reducing power

Samples with higher reducing power have better abilities to donate electron. Reducing power assay is often used to evaluate the ability of natural antioxidant to donate electron or hydrogen (Dorman, Peltoketo, Hiltunen, & Tikkanen, 2003). It has been widely accepted that the higher the absorbance at 700 nm, the greater the reducing power (Duh, 1998). The reducing power of alfalfa leaf peptides (ALPs) determined at 700 nm is shown in Fig. 1. The reducing power of ALPs was concentration dependent: the value increased with increasing concentration and reached 0.69 at 2.00 mg/mL. The reducing power showed a clear influence of both protein size and concentration, this latter effect being more marked for fractions of reduced molecular weight (Moure et al., 2006). Many researches have revealed that there was a direct correlation between antioxidant activity and reducing power (Duh, 1998; Duh, Du, & Yen, 1999). Free radicals form stable substances by accepting donated electron, the free radical chain reactions are thus interrupted (Yamamoto & Kajimoto, 1980). Within the concentration range of 0.50–2.50 mg/mL, the reducing power of ALPs was lower than that of GSH and was close to that of GSH while the concentration of ALPs was 3 times that of GSH. For the peptides obtained from the soy protein hydrolyzates with different degree of hydrolysis, samples with maximal reducing power contained peptides of 0.7 kDa as the major fraction (Moure et al., 2006). Wu, Chen, and Shiau (2003) reported that the enzymatic hydrolysates derived from mackerel protein possessed reducing power may be associated with carnosine and anserine. The reducing power results revealed that ALPs had good abilities to donate electron which was involved in the antioxidant activity.

3.1.2. DPPH radical scavenging activity

Most free radicals are of high activities and exist only in short time. DPPH is one of the small amounts of free radicals which can keep stable even at room temperature. DPPH radical has a single electron and shows strong absorbance at 517 nm. Its ethanolic solution exhibits modena. The absorbance of ethanolic DPPH solution at 517 nm reduces gradually while the free radicals are scavenged and the color of the solution changes from modena to

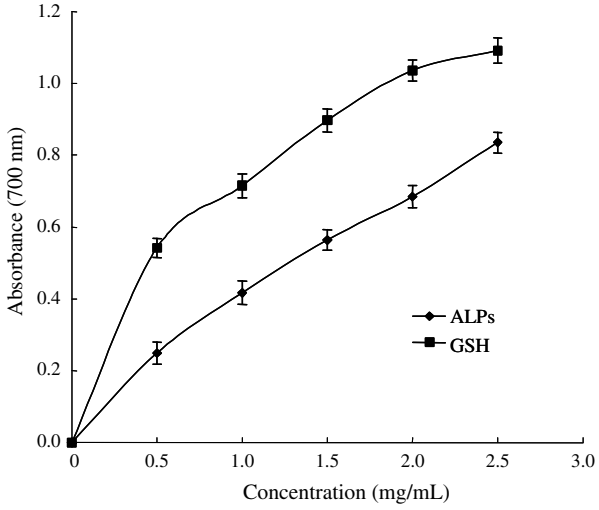

Fig. 1. Reducing power of ALPs. Each value is the mean ± SD of triplicate measurements.

yellow (Yamaguchi, Takamura, Matoba, & Terao, 1998). The method is based on the reduction of the absorbance of methanolic DPPH solution at 517 nm in the presence of a proton-donating substance, due to the formation of the diamagnetic molecule by accepting an electron or hydrogen radical (Cheng, Wang, & Xu, 2006). The higher the concentration the higher level of the DPPH radical scavenging activity was found for ALPs in the samples used in the test (Fig. 2). Similar result has been reported for wheat germ protein hydrolysates (Cheng et al., 2006). The DPPH radical scavenging of ALPs reached 80% at 1.6 mg/mL, which was similar to that of the GSH at 0.4 mg/mL. The scavenging activity increased steadily at the concentration rang of 0.0–1.6 mg/mL for ALPs, while the scavenging reached a maximum plateau from 0.4 to 1.6 mg/mL for GSH.

The DPPH test for scavenging capacity was employed in this study since DPPH has commonly been used in antioxidant activity analysis, and the test system can be used for the primary characterization of the scavenging potential of compounds (Wang, Zhao, Zhao, & Jiang, 2007). Obviously, ALPs had the ability to quench the DPPH radical, which indicated that the ALPs were good antioxidant compounds with radical scavenging activity. ALPs 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.

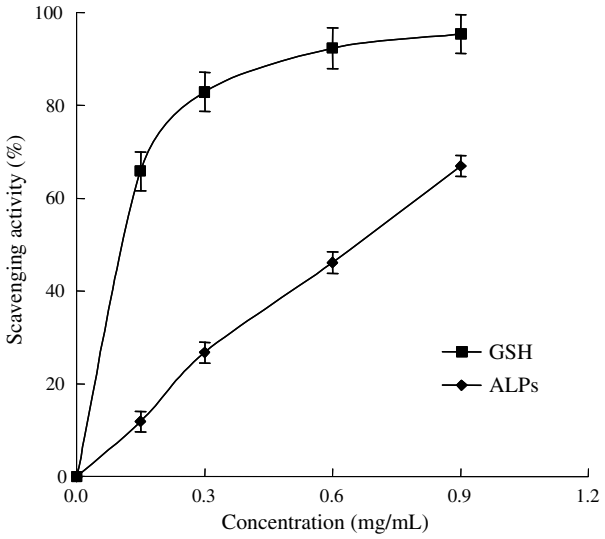

Fig. 3. Scavenging activity of ALPs on superoxide anion radical. Each value is the mean ± SD of triplicate measurements.

3.1.3. Superoxide anion scavenging activity

Superoxide anion radical is normally formed first in cellular oxidation reactions. Although it is not highly reactive, it can produce hydrogen peroxide and hydroxyl radical through dismutation and other types of reaction and is the source of free radicals formed in vivo. Not only superoxide anion radical but also its derivatives are cell-damaging, which can cause damage to DNA and membrane of cell. Therefore, it is of great important to scavenge superoxide anion radical (Macdonald, Galley, & Webster, 2003).

ALPs could scavenge the superoxide anion radical as found for GSH, thus inhibited the chemical luminescence reaction and decreased the chemiluminescence intensity. Fig. 3 shows the percentage inhibition of superoxide anion radicals by ALPs and GSH at different concentrations (0.0–0.9 mg/mL). The superoxide anion scavenging activities and concentrations were almost linear correlated for ALPs ($r = 0.998$, $P < 0.01$). For GSH, the increase of superoxide anion scavenging activity was rapid at low concentrations, while slow at high concentrations. The superoxide anion scavenging of ALPs reached 66.96% at 0.9 mg/mL, which was similar to that of the GSH at 0.15 mg/mL.

The superoxide anion radical scavenging capacities of soy protein hydrolyzates from the fraction with different molecular weights were between 24.7% and 85.6% and were increased with decreasing molecular weight (Moure et al., 2006). An isolated 1 kDa peptide from a peptic hydrolysate of casein showed superoxide anion as well as DPPH radical scavenging activity (Suetsuna, Ukeda, & Ochi, 2000). The scavenging activity against superoxide radical for wheat germ protein hydrolysates (0–0.60 g/L) ranges from 0% to 75.40% (Cheng et al., 2006). The observation that ALPs effectively lowered the cascade of oxidation reactions induced by superoxide anion radical, indicated effective chain breaking antioxidant of ALPs.

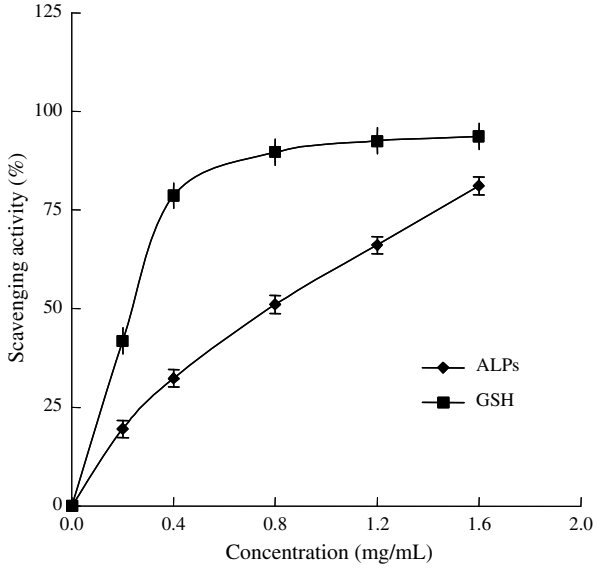

Fig. 2. Scavenging activity of ALPs on DPPH radical. Each value is the mean ± SD of triplicate measurements.

3.1.4. Hydroxyl radical scavenging activity

Among the oxygen radicals, hydroxyl radicals are the most reactive, which can react with almost all the substances in the cell and induce severe damage to cells (Dreher & Junod, 1996). Hydrogen peroxide can also change to hydroxyl radicals in vivo. Hydroxyl radical generation systems include Fenton reaction system, GSH/H_2O_2 system, niacinamide adenine dinucleotide phosphate/H_2O_2 system, *o*-phenanthroline/ascorbic acid/H_2O_2 system, and ascorbic acid/Cu^{2+} system etc. In this study, *o*-phenanthroline/ascorbic acid/H_2O_2 system was used to determine the hydroxyl radicals scavenging capacity of ALPs.

The hydroxyl radicals scavenging activity of ALPs at different concentrations was compared with that of GSH (Fig. 4). Both ALPs and GSH scavenged hydroxyl radicals in a concentration dependent way. At a concentration of 1.2 mg/mL, ALPs exhibited 80% of scavenging activity on hydroxyl radicals, while GSH exhibited nearly 100% of scavenging activity on hydroxyl radicals.

Hydroxyl radical scavenging capacity has been confirmed for peptides from other sources. Je, Park, and Kim (2005) reported that the purified peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate scavenged 35% on hydroxyl radical at 53.6 M using electron spin resonance (ESR) spectroscopy. Antioxidative peptide obtained from hoki (*Johnius belengerii*) frame protein hydrolysate (APHPH) was effectively quenched of hydroxyl radical, and the IC50 value was 17.77 μM (Kim, Je, & Kim, 2007). Soy protein hydrolyzates from the fraction with molecular weight

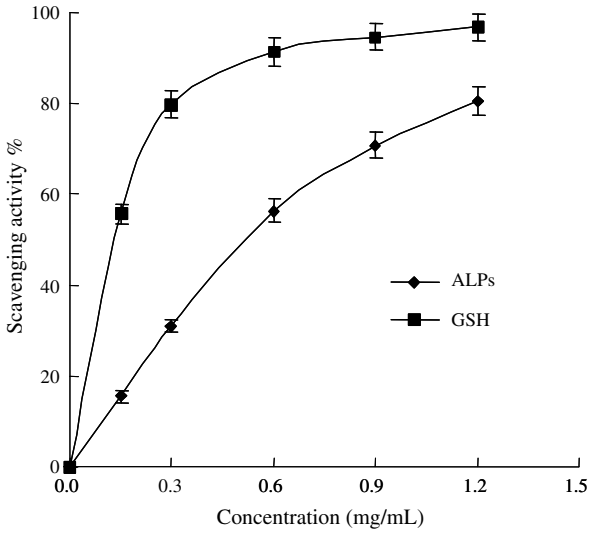

Fig. 4. Scavenging activity of ALPs on hydroxyl radical. Each value is the mean ± SD of triplicate measurements.

between 30 and 50 kDa showed the highest hydroxyl radical scavenging capacity (69.75%, Moure et al., 2006).

The chemical activity of hydroxyl radical is very strong and it easily reacts with biomolecules such as amino acids, proteins, and DNA (Je, Qian, Byun, & Kim, 2007). Therefore, the removal of hydroxyl radical is probably one of the most effective defence of a living body against various diseases. The hydroxyl radical is also effective at initiating lipid peroxidation reactions. Determination of hydroxyl radical scavenging activity provides useful information on antioxidant activities (Kitts, 2005). The high hydroxyl radical scavenging activity shown by ALPs strengthened its antioxidant activity and the ability to protect hydroxyl radical-induced damage.

3.1.5. Chelating activity

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. Ferrous ion is one of the products formed in Fenton reaction, where hydrogen superoxide produces hydroxyl radical (Afans, Dcrozhko, & Brodskii, 1989). It has been reported that the scavenging of hydroxyl radicals by antioxidant was effective mainly via chelating of metal ions. Since compound interfering with the catalytic activity of metal ions could affect the peroxidative process, the measurement of chelating ability is important for evaluating free radical scavenging activity of the compound (Gordon, 1990; Halliwell & Gutteridge, 1990).

In this study, the metal chelating activities were determined by measurement of the reduction rate of red color, which was quantitatively formed by ferrozine with ferrous ions. Fig. 5 shows the chelating activities of ALPs, GSH and EDTA on ferrous ions. The metal chelating activity of ALPs increased with increasing concentrations used in the test, and the correlation coefficient was 0.995 ($P < 0.01$). ALPs exhibited much stronger metal chelating activity (65.15% at 0.5 mg/mL) than that of GSH, which was found to have negligible chelating ability for ferrous ions at the range of 0.1–0.50 mg/mL. However, its metal chelating activity was significantly lower than that of EDTA, which had the strongest chelating capacity and reached 94.10% at 0.1 mg/mL. The metal chelating ability may be involved in antioxidant activity and affects other functions

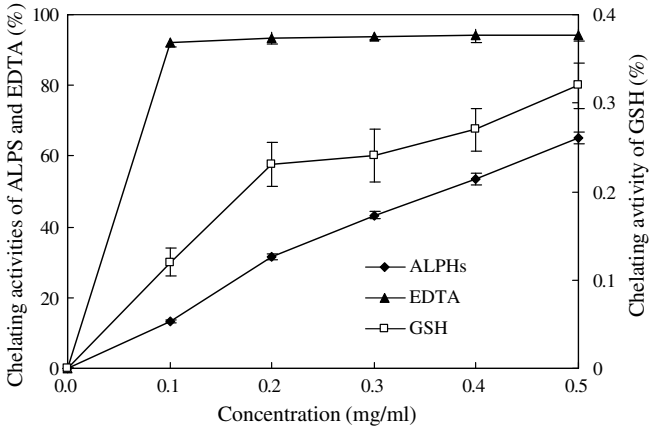

Fig. 5. Chelating effect of ALPs on ferrous ion. Each value is the mean ± SD of triplicate measurements.

that contribute to the antioxidant activity (Moure et al., 2006; Wu et al., 2003). Accordingly, it is suggested that the chelating effect of ALPs on ferrous ions might be the mechanism of the free radical scavenging of ALPs and will be somewhat beneficial to exert protection against oxidative damage.

3.2. Amino acid and molecular weight distribution

Alfalfa leaf peptides (ALPs) were prepared by means of ultrafiltration after enzymatic hydrolysis of soluble alfalfa leaf protein. The total amount of amino acid was 91.56 g per 100 g of sample. Table 1 presents the amino acid profiles of ALPs. Some amino acids, such as His, Tyr, Met, and Cys, had been reported to show antioxidant activity. Especially, histidine exhibited strong radical scavenging activity due to the decomposition of its imidazole ring (Wang et al., 2007). Therefore, the antioxidant activities of the ALPs seemed to be caused by these amino acids in the peptides. Moreover, the antioxidant activity of the ALPs depended upon the amino acid sequence of the peptides. Furthermore, the ratio of amino acids in the ALPs was close to that of protein quality for adult recommended by the FAO. Therefore, ALPs were not only with antioxidant property but also with high nutritive value.

Molecular weight distribution profile of ALPs was analyzed by high performance size exclusion chromatography (HPSEC). Five major populations were displayed in HPSEC chromatogram (Fig. 6). A similar HPSEC elution profile was observed for wheat germ protein hydrolysates with antioxidant properties (Cheng et al., 2006). The molecular weight distributions of ALPs were concentrated in <1000 Da (67.86%, Table 2), indicating that ALPs contained a large proportion of low-molecular-weight peptides with 2–6 amino acid residues. The absorption of peptides with 2–6 amino acids was eas-

Table 1
Amino acid composition of ALPs (g/100 g)

Amino acid	Amount	Amino acid	Amount
Aspartic acid	8.98	Glutamic acid	11.80
Serine	3.67	Histidine	2.61
Glycin	4.81	Threonine	3.90
Arginine	6.25	Alanine	5.51
Tyrosine	4.14	Cysteine	1.53
Valine	5.76	Methionine	1.63
Phenylalanine	5.39	Isoleucine	4.94
Leucine	7.95	Lysine	5.99
Proline	3.82	Tryptophan	2.88
Total (g/100 g sample)	91.56		

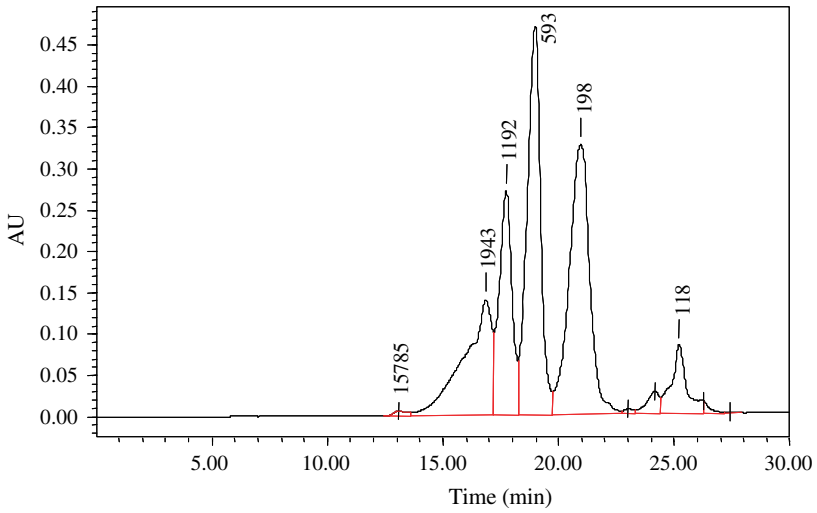

Fig. 6. The molecular weight distribution of ALPs.

Table 2
The molecular weight distribution of ALPs

molecular weight	ALPs (%)
>5000	2.41
5000–3000	4.56
3000–2000	8.07
2000–1000	17.10
1000–500	28.48
500–130	31.27
<130	8.11
Total (<1000)	67.86

ier than that of protein and free amino acids. In addition, the function of protein hydrolysate was related to the molecular weight distribution as well as the amino acids contained (Grimble, 1994; Wang et al., 2007). The molecular weight of the most active peptide from Alaska Pollack frame protein hydrolyzate was 672 Da (Moure et al., 2006). Free radical scavenging activity, superoxide anion scavenging activity, as well as hydroxyl and DPPH radical quenching have also been reported for an isolated 1 kDa peptide from a peptic hydrolysate of casein (Suetsuna et al., 2000). It was postulated that for ALPs, the antioxidant activity of peptide fraction of <1000 Da was greater than that of peptide fraction of higher molecular weights. The antioxidant activity of the peptides isolated from protein hydrolysates depended upon the amino acid sequence of the peptides in the hydrolysates (Wang et al., 2007). Three low-molecular-weight peptides with high antioxidant activities have been isolated from alfalfa leaf protein hydrolysates and purified their amino acid sequence and the relationship between structure and antioxidant activities will be reported elsewhere.

4. Conclusions

Alfalfa leaf peptides (ALPs) obtained in this study exhibited antioxidant function in five different test models in vitro and contained a large proportion of low-molecular-weight (<1000 Da) peptides. ALPs showed good ability to donate electron or hydrogen and were able to scavenge superoxide, hydroxyl and DPPH radicals. The free radical scavenging activities of ALPs were concentration dependent and were close to those of GSH while the concentration of ALPs was 3–5 times that of GSH. Chelating with the transition metal ions of the peptides might be the free radical scavenging mechanism of ALPs. To illustrate the property–structure relationship of the antioxidative peptide, further studies on isolation and purification of individual peptide are needed.

ALPs, stable and low cost, in addition to the new functional characteristic of antioxidant activity investigated in this study, have the potential to replace superoxide dismutase (SOD) and other antioxidant enzymes and will have a new application in food products.

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