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Food Chemistry 103 (2007) 301–312
Food Chemistry
www.elsevier.com/locate/foodchem

Antioxidant activity of protein extracts from heat-treated or thermally processed chickpeas and white beans

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Received 30 January 2006; received in revised form 28 March 2006; accepted 26 July 2006

Abstract

In this study, antioxidant activities of water-soluble protein extracts from chickpeas and white beans were investigated. The area under the curve (AUC) values of lyophilized crude protein extracts (dialyzed or undialyzed) from thermally processed (121 °C for 20 min) or heat-treated (90 °C for 20 min) chickpeas (73–91 μmol trolox/g) and white beans (39–67 μmol trolox/g) indicated a higher free radical-scavenging capacity and thermostability for chickpea proteins than for white bean proteins. The thermal processing also increased the Fe^{+2}-chelating capacity of lyophilized chickpea crude protein extracts 1.8-fold whereas it caused a 2.3-fold reduction in the Fe^{+2}-chelating capacity of lyophilized white bean crude protein extracts. Dialysis increased the protein content of lyophilized chickpea extracts 1.5–2-fold but it did not affect the protein content of lyophilized white bean extracts significantly. Ammonium sulfate precipitation was not effective for selective precipitation of antioxidant proteins. However, it improved the free radical-scavenging capacity of lyophilized protein extracts from thermally processed chickpeas and white beans by almost 25% and 100%, respectively. DEAE-cellulose chromatography, indicated the presence of five (A_1–A_5) and three (B_1–B_3) antioxidant protein fractions in heat-treated and thermally processed chickpea protein extracts, respectively, and can be used for the partial purification of antioxidant proteins. The results of this study showed the good potential of chickpea proteins as thermostable natural food antioxidants.

Keywords: Legumes; Proteins; Radical scavenging; Antioxidant activity; Thermal stability

1. Introduction

Due to health concerns of consumers, extensive research has been conducted on natural antioxidants, such as protein or phenolic extracts and vitamins E and C, in place of synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Moore et al., 2003; Rajalakshmi & Narasimhan, 1996; Vermeiren, Devlieghere, van Beest, de Kruijf, & Debevere, 1999; Wu, Weller, Hamouz, Cuppett, & Schnepf, 2001). The natural antioxidants are readily accepted by consumers and do not require safety tests if they are components of food that are generally recognized as safe (GRAS) (Rajalakshmi & Narasimhan, 1996).

Recently, the use of natural protein extracts or purified proteins as antioxidants has attracted particular interest. Many food proteins, including milk proteins, such as lactoferrin, β-lactoglobulin and casein, soy proteins, mushroom proteins, egg albumen proteins and egg yolk phosvitin, maize zein, potato patatin and yam dioscorin, were reported to have antioxidant activity (Chiue, Kusano, & Iwami, 1997; Hou et al., 2001; Kouoh et al., 1999; Liu, Han, Lee, Hsu, & Hou, 2003; Maheswari, Ramadoss, & Krishnaswamy, 1997; Rajalakshmi & Narasimhan, 1996; Satue-Gracia, Frankel, Rangavajhyala, & German, 2000; Zhao et al., 2004). The proteins owe their antioxidant activity to their constituent amino acids. For example, the antioxidant activities of aromatic amino acids such as

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0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.07.050

tyrosine, phenylalanine and tryptophan and the sulfur-containing amino acid, cysteine, are due to their ability to donate protons to free radicals (Hu, McClements, & Decker, 2003; Je, Park, & Kim, 2004; Rajapakse, Mendis, Jung, Je, & Kim, 2005) whereas basic amino acids, such as lysine and arginine, and acidic amino acids, such as aspartate and glutamate, exercise antioxidant activity by chelating metal ions (Rajapakse et al., 2005; Saiga, Tanabe, & Nishumura, 2003). It was reported that the basic amino acid, histidine, may behave both as a radical-scavenger and a metal-chelator due to its imidazole ring (Je et al., 2004; Rajapakse et al., 2005). However, the presence of some antioxidant amino acids in sequence is not the only factor that determines the antioxidative properties of proteins. The correct positioning of the amino acids in a protein sequence is also a very important factor, and effective for antioxidant activity (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Rajapakse et al., 2005). For example, peptides having proline at the N-terminus more effectively prevent oxidation of linoleic acid than do peptides having proline at the C-terminus (Chen et al., 1996). Also, peptides having histidine residues at the N-terminus show higher metal-chelating activity than do peptides having histidine at the C-terminus (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). It has also been reported that there is a close relationship between the hydrophobicity and antioxidant activity of proteins and peptides (Chen, Muramoto, & Yamauchi, 1995; Chiue et al., 1997; Rajapakse et al., 2005; Saiga et al., 2003). For example, the antioxidant activity of the hydrophobic protein, zein, has been attributed to its ability to bind and bury unsaturated lipids (Chiue et al., 1997). Moreover, Hu et al. (2003) reported that the cationic characteristics of proteins are also important for their antioxidant activity since positively charged groups help inhibition of lipid oxidation by electrostatic repulsion of transition metals away from the lipid droplets. Thus, it seems that, besides their free radical-scavenging and iron-binding activities, proteins also prevent lipid oxidation by isolating lipids from free radicals and metal ions.

Other advantages of using proteins as antioxidant substances in food systems are their additional functions, such as nutritive value, emulsifying activity, antimicrobial activity, foam and gel formation, flavour binding, increase of viscosity or film formation. For example, besides their antioxidant activity, proteins, such as casein and bovine serum albumin, and protein isolates, such as whey protein isolate and soy protein isolate, are well known for their emulsifying activity (Al-Malah, Azzam, & Omari, 2000; Fukuzawa et al., 2005; Hu et al., 2003; Kouoh et al., 1999; Pearce & Kinsella, 1978). Calcium caseinate and whey proteins can form edible films with antioxidant activity (Le Tien, Vachon, Mateescu, & Lacroix, 2001). Lactoferrin and phosvitin are proteins that show both antioxidant and antimicrobial activity (Huang, Satue-Gracia, Frankel, & German, 1999; Khan et al., 2000; Maheswari et al., 1997; Liceaga-Gesualdo, Li-Chan, & Skura, 2001; Recio & Visser, 2000; Satue-Gracia et al., 2000).

In plants and animals, the major water-soluble protein fraction is formed by albumins (Damodaran, 1996). In the literature, the antioxidant activities of albumins from legumes, such as broad beans, white and brown beans and light peas (Okada & Okada, 1998; Wolosiak & Klepacka, 2002) and bovine and human serum (Aime et al., 2003; Fukuzawa et al., 2005; Kouoh et al., 1999; Wolosiak & Klepacka, 2002), have been well documented. The emulsifying and foaming activity of animal and plant origin albumins has also been reported by different workers (Al-Malah et al., 2000; Burnett et al., 2002; Damodaran, 1996; Ma & Harwalkar, 1984; Pearce & Kinsella, 1978; Tong, Sasaki, McClements, & Decker, 2000). In the literature, no studies have been reported on the antioxidant activity of chickpea albumins. The effect of thermal processing on antioxidant activity of legume albumins has also not been investigated. Chickpea protein quality is higher than that of common bean (Freidman, 1996). Chickpeas also contain lower amounts of toxic and antinutritive factors than do the common beans (Hernandez-Infante, Sousa, Montalvo, & Tena, 1998). Thus, in this study, to evaluate the antioxidant activity and thermostability of chickpea albumins, we have compared the free radical-scavenging and iron-binding capacities of crude or partially purified water-soluble protein extracts from heat-treated or thermally processed chickpeas and white beans. The antioxidant protein fractions of protein extracts from chickpeas were also separated by anion-exchange chromatography and their free radical-scavenging capacities were determined.

2. Materials and methods

2.1. Materials

Dried chickpeas (cv. Koçbaşı) and white beans (cv. Dermason) were purchased from a supermarket in Izmir (Turkey). The dialysis tubing (12,000 MW, prepared as described in the product manual), bovine serum albumin (fraction V), DEAE-cellulose (fast flow column, prepared as described in the product manual), insoluble PVPP (polyvinylpolypyrrolidone), ABTS (2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid)), linoleic acid (99%) and Tween 20 were purchased from Sigma Chem. Co. (St. Louis, Mo., USA). Ammonium sulfate (for biochemistry) was purchased from Merck (Darmstadt). Trolox and Ferrous chloride tetrahydrate were purchased from Fluka (Switzerland). Ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4′,4″-disulfonic acid monosodium salt) was purchased from Fluka (USA). Sericin (Silk Biochemical Co. Ltd, Japan) was kindly donated by Assistant Professor Dr. Oğuz Bayraktar from Izmir Institute of Technology, Izmir, Turkey.

2.2. Heat-treatment of legumes

Heat-treatment was specifically applied for the inactivation of lipoxygenase (LOX), the enzyme responsible for the

oxidation of lipids in food. To prepare the samples, the legumes were first rehydrated in distilled water for 12 h at room temperature. The samples were then put into sacks made from cheesecloth and heated at 90 °C for 5, 10, 15 or 20 min. The suitable heating period was determined to be 20 min, by considering the residual activities of LOX in heated chickpeas (LOX inactivated in 5–10 min at 90 °C) and white beans (LOX inactivated in 15–20 min at 90 °C). The LOX activity was assayed in water extracts of legume homogenates by using linoleic acid dissolved by Tween 20, as described by Yemenicioğlu, Özkan, Velioğlu, and Cemeroğlu (1998).

2.3. Thermal processing of legumes

Thermal processing was applied to compare the thermostabilities of antioxidant proteins in chickpeas and white beans. It was supposed that thermal processing might be needed to obtain microbiologically stable protein extracts with reduced antinutrients and allergens that exist in most legumes (Hernandez-Infante et al., 1998). For the preparation of thermally processed samples, the legumes were put into flasks containing distilled water and thermally processed at 121 °C for 20 min. The treated samples were then cooled and processed immediately to acetone powder.

2.4. Preparation of acetone powders

To remove phenolic compounds and lipids, acetone powders were used as sources of protein extracts. For the preparation of acetone powders, heat-treated or thermally processed chick peas or white beans (prepared initially from 50 g dry sample) were homogenized in a Waring blender for 3 min with 200 ml of cold acetone. The slurry obtained was filtered under vacuum through a Buchner funnel containing a Whatman No:1 filter paper and the residue remaining on the filter paper was collected. The homogenization with 200 ml acetone and filtration were then repeated twice more for the collected residue and the powder, left overnight to evaporate the acetone, was stored at −18 °C until it was used in the extraction.

2.5. Preparation of crude protein extracts

To prepare the water-soluble crude protein extracts from heat-treated or thermally processed chickpeas or white beans, the extraction method given by Genovese and Lajolo (1998) was applied with minor modifications. Briefly, 20 g of acetone powder, 0.5 g of insoluble PVPP (used to absorb possible residual phenolic compounds) and 180 ml of distilled water were mixed and extracted with a magnetic stirrer for 2 h at room temperature. The extract was then filtered through 4-layers of cheesecloth to collect the filtrate and the cake was discharged. The filtrate was centrifuged at 15,000*g* for 30 min at 4 °C and the supernatant was dialyzed for 72 h (48 h against 5×2 l of distilled water and 24 h against 3×2 l of deionized water) at 4 °C. After dialysis, the extract was clarified by centrifugation at 4500*g* for 15 min at 4 °C and lyophilized and stored at −18 °C until it was used for antioxidant activity determination. The lyophilization was conducted by using a freeze-drier (Labconco, FreeZone, 6 l, Kansas City, MO, USA) working between −44 and −47 °C for collector temperature and 50×10^{-3} and 100×10^{-3} mBar vacuum.

2.6. Partial purification of crude protein extracts by ammonium sulfate precipitation

For partial purification, solid ammonium sulfate was added slowly to crude protein extracts, obtained as described in Section 2.5, without applying 72 h of dialysis. The addition of ammonium sulfate was conducted at 4 °C up to 90% saturation. The mixture was then stirred slowly for 1.5 h at 4 °C and the precipitate obtained by centrifugation at 15,000*g* for 30 min at 4 °C was dissolved in 20 ml of distilled water. The extract was then dialyzed for 36 h (against 4×2 l of distilled water) at 4 °C, clarified by centrifugation at 15,000*g* for 30 min at 4 °C and lyophilized and stored at −18 °C.

2.7. Purification of antioxidant protein fractions by DEAE-cellulose chromatography

Crude protein extracts were obtained as described in Section 2.5 with a 72 h dialysis. Following dialysis, the extracts were once more centrifuged at $4500 \times g$ for 15 min at 4 °C and loaded onto a DEAE-cellulose fast-flow column (2.4 cm diameter, 10 cm height), previously equilibrated with 0.01 M Na-phosphate buffer, pH 7.0. The washing of the column was conducted with 300 ml of equilibration buffer and it was then eluted with a continuous linear gradient of 0–1.5 M NaCl prepared in 0.01 M Na-phosphate buffer at pH 7.0. Fractions (5 ml) collected from the column were assayed for their antioxidant activity against ABTS free radical, as described in Section 2.8. However, the inhibition period of tests for collected fractions was shortened to 2 min to complete the measurements of all samples as soon as possible and prevent possible changes in the antioxidant properties of proteins. The protein content of the fractions was also monitored by measuring absorbance value at 280 nm. After determination of the profiles of antioxidant activity and protein, the most active fractions of separated proteins were combined and once more tested for antioxidant activity. However, the test period of these samples was set to 15 min and their total antioxidant activities in purification tables were expressed as trolox equivalents (μmol). The specific antioxidant activity was calculated by dividing total antioxidant activity by protein content, determined by the Lowry method. The obtained protein fractions were then dialyzed for 24 h (against 3×2 l of distilled water) at 4 °C and lyophilized and stored at −18 °C.

2.8. Determination of free radical-scavenging activity

The free radical-scavenging activity was determined, as described in Re et al. (1999), by using ABTS free radical prepared by oxidation with potassium persulfate. The reaction mixtures for the measurements were formed by mixing 0.1 ml of protein solution or extract and 1.9 ml of ABTS free radical solution diluted with PBS (initial absorbance at 734 nm was almost 0.7). The discoloration of dark blue-coloured ABTS free radical by the antioxidant protein was monitored at 734 nm for 15 min. All measurements were performed in triplicate. The free radical-scavenging capacities of samples were determined by dividing the area of their inhibition of ABTS free radical (%)/concentration (μg/reaction mixture) ratio vs. period of inhibition test (1, 6 and 15 min) curves by that area of the same curve of the standard antioxidant, trolox. The area under the curve value (AUC) determined by this method represents the free radical-scavenging capacity as μmol trolox per g of lyophilized protein extract. Bovine serum albumin and sericin were used as standard proteins for comparison of antioxidant activities.

2.9. Determination of Fe^{+2}-chelating capacity

The Fe^{+2}-chelating capacities of protein extracts were determined as described in Rajapakse et al. (2005) with minor modifications. Briefly, 2 ml of protein solution was mixed with 0.1 ml of 1mM $FeCl_2.4\,H_2O$ solution. After 30 min incubation at room temperature, 0.1 ml of 0.5 mM ferrozine was added to the mixture and its absorbance was read at 562 nm after a 10 min incubation. The percent Fe^{+2}-chelating activities of samples were determined by using deionized water in place of sample. The Fe^{+2}-chelating capacities of samples were determined by dividing the slope of the initial linear portion of their Fe^{+2}-chelating capacity (%) vs. concentration (μg/reaction mixture) curves by that slope of the same curve of the chelating agent EDTA. The results were expressed as μmol EDTA per g of lyophilized extract. All measurements were performed in triplicate.

2.10. Determination of protein content

The protein contents of lyophilized samples and protein extracts were determined by the Lowry method, by using bovine serum albumin (BSA) as standard (Harris, 1987). The average of five measurements was used to calculate the protein content.

3. Results and discussion

3.1. Water-soluble protein contents of lyophilized extracts

Since extractions in this work were conducted with water, the proteins discussed in this study are mainly water-soluble albumins. In the literature, due to differences in the efficiency of extraction and separation of albumins and globulins, different reports exist on the albumin contents of legume proteins. For example, Bhatty (1982) reported that albumins form almost 12–13% of proteins in chickpeas and beans, whereas Genovese and Lajolo (1998) reported the albumin content of bean proteins to be 28%.

As seen in Table 1, the lyophilized crude chickpea extracts contained 1.4–2.8-fold more soluble protein than did lyophilized crude white bean extracts obtained by the same procedures. The water-soluble protein content of lyophilized samples, changed between 0.15 and 0.51 g/g, indicated the presence of some impurities in the extracts other than proteins. In chickpeas and white beans, these substances are possibly carbohydrates such as soluble mono-, di- and oligosaccharides and colloidal starch and pectin (Garcia, Filisetti, Udaeta, & Lajolo, 1998; Saini & Knights, 1984; Sanchez-Mata, Penuela-Teruel, Camara-

Table 1
Free radical-scavenging capacity (AUC values) and water-soluble protein contents of different lyophilized protein extracts

Process/sample name	Purification step(s)	Protein content (g/g)	AUC value (μmol trolox/g)
Standard proteins			
BSA	–	–	36
Sericin	–	–	148
Lyophilized chickpea protein extracts			
Heat-treatment	Crude extract	0.26	73
Heat-treatment	Dialyzed crude extract	0.51	91
Thermal processing	Crude extract	0.34	77
Thermal processing	Dialyzed crude extract	0.51	82
Thermal processing	$(NH_4)_2SO_4$ precipitated dialyzed crude extract	0.49	102
Heat-treatment	DEAE-cellulose purified A_2 fraction	–	135
Thermal processing	DEAE-cellulose purified B_2 fraction	–	98
Lyophilized white bean protein extracts			
Heat-treatment	Crude extract	0.19	56
Heat-treatment	Dialyzed crude extract	0.22	67
Thermal processing	Crude extract	0.15	46
Thermal processing	Dialyzed crude extract	0.18	39
Thermal processing	$(NH_4)_2SO_4$-precipitated dialyzed crude extract	0.23	77

Hurtado, Diez-Marques, & Torija-Isasa, 1998). The high affinity of carbohydrates to legume albumins is well known (Genovese & Lajolo, 1998). Thus, it is possible that some of these carbohydrates were bound ionically or covalently to proteins. Thermal processing increased the soluble protein content of lyophilized chickpea extracts. However, a slight reduction was observed in the soluble protein content of lyophilized white bean extracts after thermal processing. The application of dialysis caused 1.5–2.0-fold increase of soluble protein content in lyophilized crude protein extracts of chickpeas. The increase of protein content by the dialysis was related to the precipitation or removal of non-protein substances, such as carbohydrates, during this process. It is also possible than some of the proteins, particularly residual globulins, were also insolubilized and precipitated during dialysis. For this reason, in heat-treated chickpeas, the amount of lyophilized dry powder from dialyzed crude extract was 79% less than that from the undialyzed one. The precipitations also occurred during dialysis of white bean crude protein extracts. For example, in heat-treated white beans, the amount of lyophilized dry powder obtained from dialyzed crude extract was 49% less than that obtained from undialyzed crude extract. However, in white beans, the protein content of lyophilized extracts did not increase markedly after dialysis. It appears that dialysis caused the precipitation and/or removal of both protein and non-protein substances of white bean crude extracts.

3.2. Free radical-scavenging capacity of lyophilized crude protein extracts

The AUC values of lyophilized chickpea and white bean crude protein extracts (dialyzed or undialyzed) changed between 73 and 91 μmol trolox/g and 39 and 67 μmol trolox/g, respectively (Table 1). Thus, it is clear that the lyophilized crude protein extracts obtained from heat-treated or thermally processed chickpeas showed higher free radical-scavenging capacity than did those from heat-treated or thermally processed white beans (Figs. 1 and 2). In lyophilized chickpea crude protein extracts, no significant change occurred in the free radical-scavenging capacity after thermal processing. In contrast, the thermal processing reduced the free radical-scavenging capacity of lyophilized crude white bean protein extracts. In lyophilized crude protein extracts of heat-treated chickpeas and white beans and thermally processed chickpeas, dialysis increased the free radical-scavenging capacity slightly. However, the dialysis caused a reduction in the free radical-scavenging capacity of lyophilized crude extracts form thermally processed white beans. In the literature, it was reported that heating caused aggregation of bean albumins by different mechanisms, including the formation of disulfide bonds (Rocha, Genovese, & Lajolo, 2002). Thus, it is likely that the aggregation by thermal processing reduced or masked some of the antioxidant groups in white bean proteins. It is also

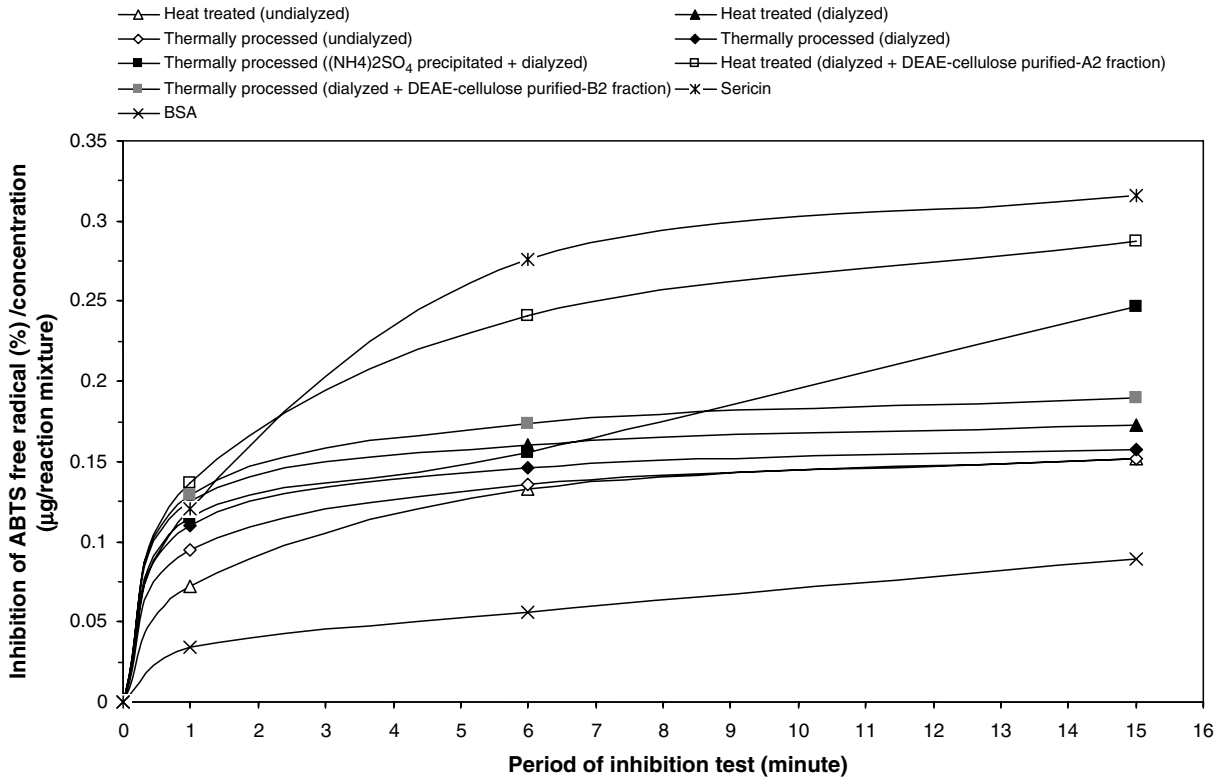

Fig. 1. Free radical-scavenging capacities of different lyophilized protein extracts from heat-treated or thermally processed chickpeas.

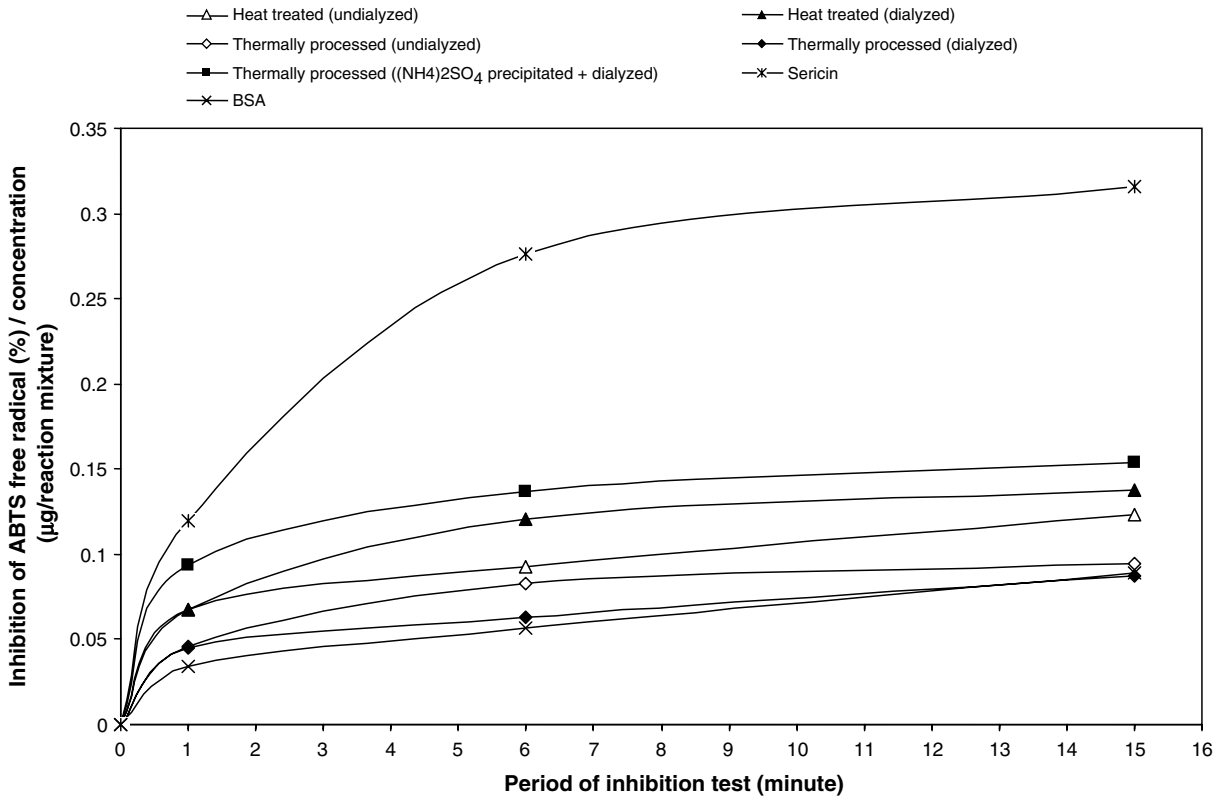

Fig. 2. Free radical-scavenging capacities of different lyophilized protein extracts from heat-treated or thermally processed white beans.

possible that some of the antioxidant proteins lost their solubility and precipitated during extraction and dialysis.

On the other hand, the AUC values also showed that the lyophilized crude chickpea and white bean protein extracts (dialyzed or undialyzed) had 2–2.5- and 1.1–1.9-fold higher antioxidant activity than had BSA, respectively. However, both lyophilized crude chickpea and white bean extracts showed lower antioxidant activity than did the sericin, an antioxidant protein patented for food applications (Yamada, Fuwa, & Nomura, 2000).

3.3. Fe^{+2}-chelating capacity of lyophilized crude protein extracts

As seen in Table 2, all lyophilized preparations obtained from dialyzed crude protein extracts showed Fe^{+2}-chelating activity. In fact, this was expected since 50% and 40% of amino acids in chickpea and dry bean albumins are metal-chelating amino acids, namely lysine, arginine, aspartic acid, glutamic acid and histidine, respectively (Bhatty, 1982). In lyophilized crude protein extracts of chickpeas, the Fe^{+2}-chelating capacity increased almost 1.8-fold by thermal processing. In contrast, thermal processing reduced the Fe^{+2}-chelating capacity of lyophilized crude protein extracts of white beans almost 2.3-fold. This result once more confirmed the significantly different thermostabilities of antioxidant components in chickpeas and white beans. It seems that the conformational changes caused by thermal processing increased accessibility to metal chelating groups of chickpea proteins while it reduced the accessibility of metal-chelating groups in white bean proteins. On the other hand, sericin did not show a marked Fe^{+2}-chelating activity. Also, except for the lyophilized preparations obtained from thermally processed white beans, all lyophilized crude

Table 2
Fe^{+2}-chelating capacities of different lyophilized protein extracts

Process/sample name	Fe^{+2}-chelating capacity (μmol EDTA/g)
BSA	30 (0–3000)[a]
Sericin	3 (0–3000)
Heat-treated chickpeas (dialyzed crude extract)	50 (0–1500)
Thermally processed chickpeas (dialyzed crude extract)	90 (0–600)
Heat-treated white beans (dialyzed crude extract)	70 (0–600)
Thermally processed white beans (dialyzed crude extract)	30 (0–1500)

[a] The concentration range of data used to calculate the Fe^{+2}-chelating capacity.

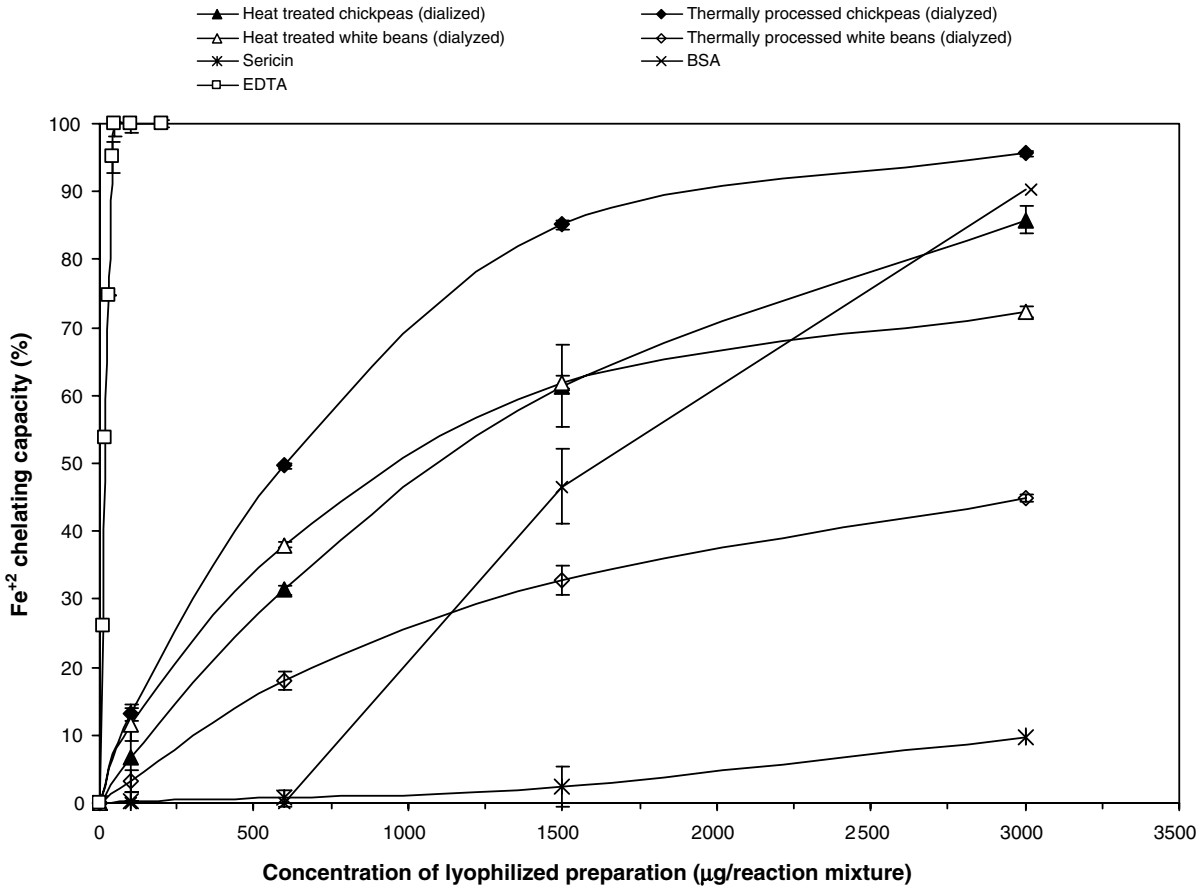

Fig. 3. Fe^{+2}-chelating capacities of different lyophilized protein extracts from heat-treated or thermally processed chickpeas and white beans.

protein extracts showed significantly higher Fe^{+2} chelating capacity than did BSA (Fig. 3).

3.4. Ammonium sulfate precipitation of crude protein extracts

To better understand the effect of thermal processing on antioxidant activity, the crude protein extracts of thermally processed samples were also partially purified by ammonium sulfate precipitation. As seen in Tables 3 and 4, ammonium sulfate precipitation, at 90% saturation, reduced the yield of antioxidant activity for both chickpea and white bean protein extracts by almost 60%. It seems that the ammonium sulfate did not precipitate most of the antioxidants at the studied saturation or it caused the aggregation of proteins and led to masking of the antioxidant activity. The protein yield from ammonium sulfate precipitation was greater than that for antioxidant activity and occurred at the levels of 54% and 50% for chickpea and white bean proteins, respectively. Thus, this caused a drop of specific antioxidant activity in both extracts. In chickpea extracts, the application of dialysis further reduced the yield of antioxidant activity, but the yield of protein did not change significantly. In contrast, in white bean extracts, the application of dialysis reduced yields of both antioxidant activity and protein.

Following dialysis, the ammonium sulfate-precipitated extracts were lyophilized and assayed for protein content.

Table 3
Partial purification of crude protein extracts from thermally processed chickpeas

Step	Volume (ml)	Total antioxidant activity[a] (µmol trolox)	Total protein (mg)	Specific antioxidant activity (µmol trolox/mg protein)	Yield of antioxidant activity (%)	Purity (fold)	Yield of protein (%)
Crude extract							
	52.5	89.5	225	0.40	100	1.0	100
90% $(NH_4)_2$ SO_4 precipitation							
	17.5	34.6	122	0.28	39	0.7	54
36 h dialysis at 4 °C							
	21.0	26.9	113	0.24	30	0.6	50

[a] Trolox equivalents formed in 15 min test period.

Table 4
Partial purification of crude protein extracts from thermally processed white beans

Step	Volume (ml)	Total antioxidant activity[a] (μmol trolox)	Total protein (mg)	Specific antioxidant activity (μmol trolox/mg protein)	Yield of antioxidant activity (%)	Purity (fold)	Yield of protein (%)
Crude extract							
	47	65.8	152	0.43	100	1.0	100
90% $(NH_4)_2$ SO_4 precipitation							
	26	27.8	76	0.37	42	0.86	50
36 h dialysis at 4 °C							
	30	19.8	49	0.40	30	0.93	32

[a] Trolox equivalents formed in 15 min test period.

Table 5
Purification of antioxidant protein fractions from heat-treated chickpeas

Step	Volume (ml)	Total antioxidant activity[a] (μmol trolox)	Total protein (mg)	Specific antioxidant activity (μmol trolox/mg protein)	Yield of antioxidant activity (%)	Purity (fold)	Yield of protein (%)
Crude extract							
	46	59	171	0.35	100	1.0	100
72 h dialysis at 4 °C							
	50	34	126	0.27	58	0.77	74
DEAE-cellulose chromatography							
A_1	35	3.7	7.6	0.49	6.3	1.40	4.4
A_2	35	5.4	5.7	0.95	9.2	2.71	3.3
A_3	20	3.1	9.5	0.33	5.3	0.94	5.6
A_4	25	2.4	12.5	0.19	4.1	0.54	7.3
A_5	25	1.8	8.4	0.21	3.1	0.6	4.9

[a] Trolox equivalents formed in 15 min test period.

Table 6
Purification of antioxidant protein fractions from thermally processed chickpeas

Step	Volume (ml)	Total antioxidant activity[a] (μmol trolox)	Total protein (mg)	Specific antioxidant activity (μmol trolox/mg protein)	Yield of antioxidant activity (%)	Purity (fold)	Yield of protein (%)
Crude extract							
	47	54	155	0.35	100	1.0	100
72 h dialysis at 4 °C							
	37.5	17.5	100	0.18	32	0.51	65
DEAE-cellulose chromatography							
B_1	35	3.2	6.6	0.49	5.9	1.40	4.3
B_2	35	20.0	29.8	0.67	37	1.91	19.2
B_3	30	7.3	16.4	0.45	13.5	1.29	10.6

[a] Trolox equivalents formed in 15 min test period.

By ammonium sulfate precipitation, an increase in the protein content of lyophilized preparations was expected. However, the results of protein assays showed that the water-soluble protein contents of lyophilized partially purified extracts were almost the same as those of dialyzed crude extracts (Table 1). It seems that, besides proteins, other major hydrocolloids, such as starch and pectin, were also precipitated by the ammonium sulfate.

3.5. Free radical-scavenging capacity of lyophilized ammonium sulfate-precipitated protein extracts

The results of free radical inhibition tests for lyophilized dialyzed crude extracts and ammonium sulfate-precipitated and dialyzed crude extracts from thermally processed chickpeas and white beans showed that the free radical-scavenging capacity was increased by almost 25% and 100% by the ammonium sulfate precipitation, respectively (Table 1). Partial purification with ammonium sulfate precipitation and dialysis did not increase the specific antioxidant activity of protein extracts. Thus, increase in the antioxidant activity of lyophilized protein extracts by partial purification may be related to the removal of some oxidation sensitive substances that may reduce the antioxidant groups of proteins during the lyophilization process. The AUC values also indicated the presence of 1.3-fold higher free radical-scavenging capacity of lyophilized partially purified chickpea protein extracts than the lyophilized par-

tially purified white bean protein extracts. This result was in line with the antioxidant activity measurements conducted on crude protein extracts. However, it should be noted that the higher antioxidant activity of lyophilized partially purified extracts from thermally processed chickpeas is related to their higher protein content but not to their greater specific antioxidant activity. In the literature, studies related to the effects of heat-treatment or thermal processing, on antioxidant activity of legume proteins are scarce. However, both water-soluble chickpea and white bean antioxidant proteins are much more thermostable than the high molecular weight whey antioxidant proteins that aggregated and lost almost 90% of their free radical-scavenging activity after mild heating at 70 °C for 15 min (Tong et al., 2000). The lyophilized partially purified protein extracts from thermally processed chickpeas and white beans also showed almost 2.8- and 2.1-fold higher free radical-scavenging capacity than did BSA, respectively. However, similarly to the lyophilized crude protein extracts both lyophilized partially purified protein extracts showed lower antioxidant activity than did the sericin.

3.6. DEAE-cellulose chromatography of dialyzed crude protein extracts

To increase the purity of antioxidant proteins and to determine the effect of thermal processing on free radical-scavenging activity profile of protein fractions, dialyzed crude protein extracts were applied to DEAE-cellulose fast flow columns. The elution profiles of antioxidant activities and protein contents indicated the presence of five antioxidant fractions (A_1–A_5) in dialyzed crude protein extracts of heat-treated chickpeas (Fig. 4). Among these fractions, $A_1$is a neutral or cationic fraction, since it was not bound by the DEAE-cellulose anion-exchanger. The four antioxidant protein fractions, A_2, A_3, A_4 and A_5, constitute the antioxidant proteins retained by the DEAE-cellulose column. On the other hand, the elution profiles of antioxidant activities and protein contents indicated the presence of three antioxidant fractions (B_1–B_3) in dialyzed crude protein extracts of thermally processed chickpeas (Fig. 5). B_1 constitutes the unretained antioxidant protein fraction reduced by the thermal processing and B_2 and B_3 constitute the antioxidant proteins retained by the DEAE-cellulose column. These results clearly showed that the thermal processing reduced the number of antioxidant protein fractions. The thermal processing also slightly reduced the total protein contents of crude protein extracts. Moreover, the increased areas of the protein peaks (almost 20%) retained by the DEAE-cellulose column for thermally processed chickpea extract suggested that the thermal processing also affected the anion-exchange properties of proteins. On the other hand, in both chromatograms obtained in this study, the elution of the major antioxidant protein frac-

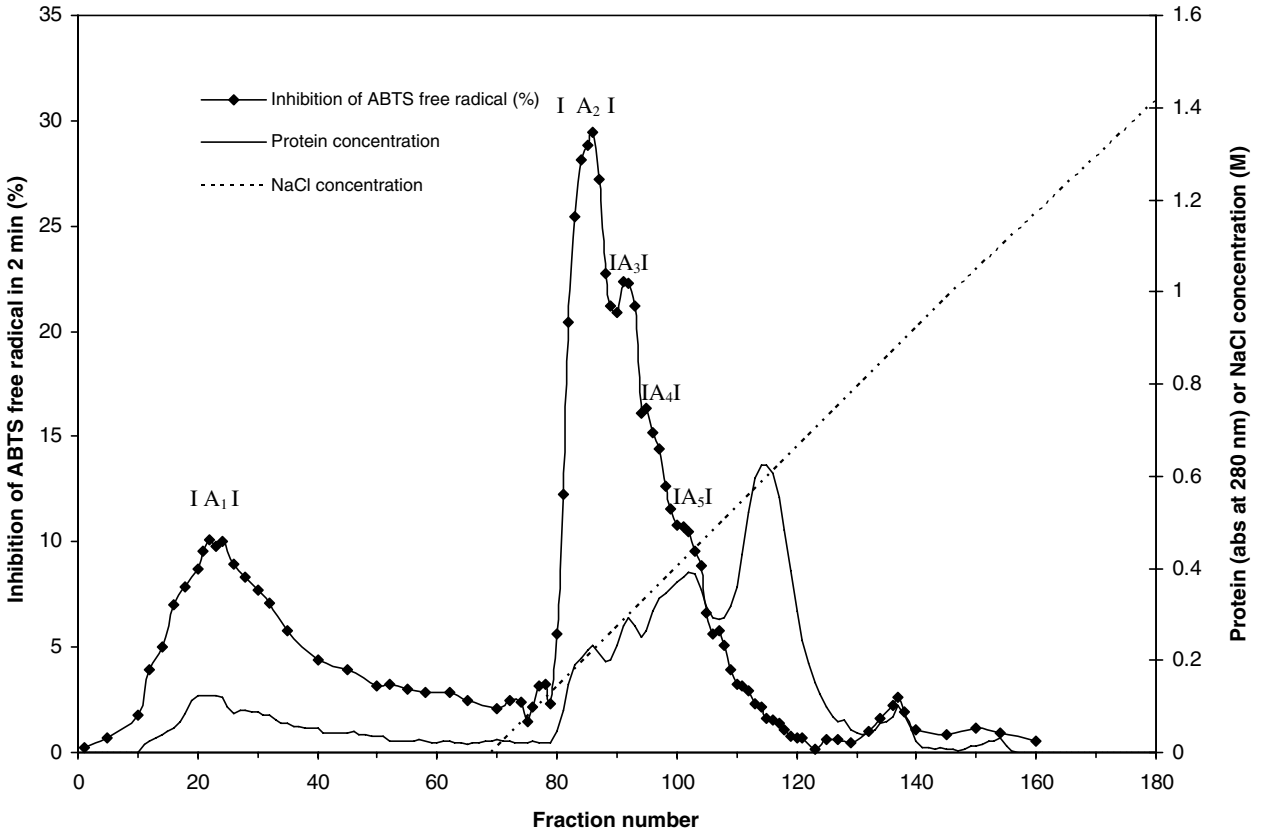

Fig. 4. DEAE-cellulose chromatography of dialyzed protein extract from heat-treated chickpeas.

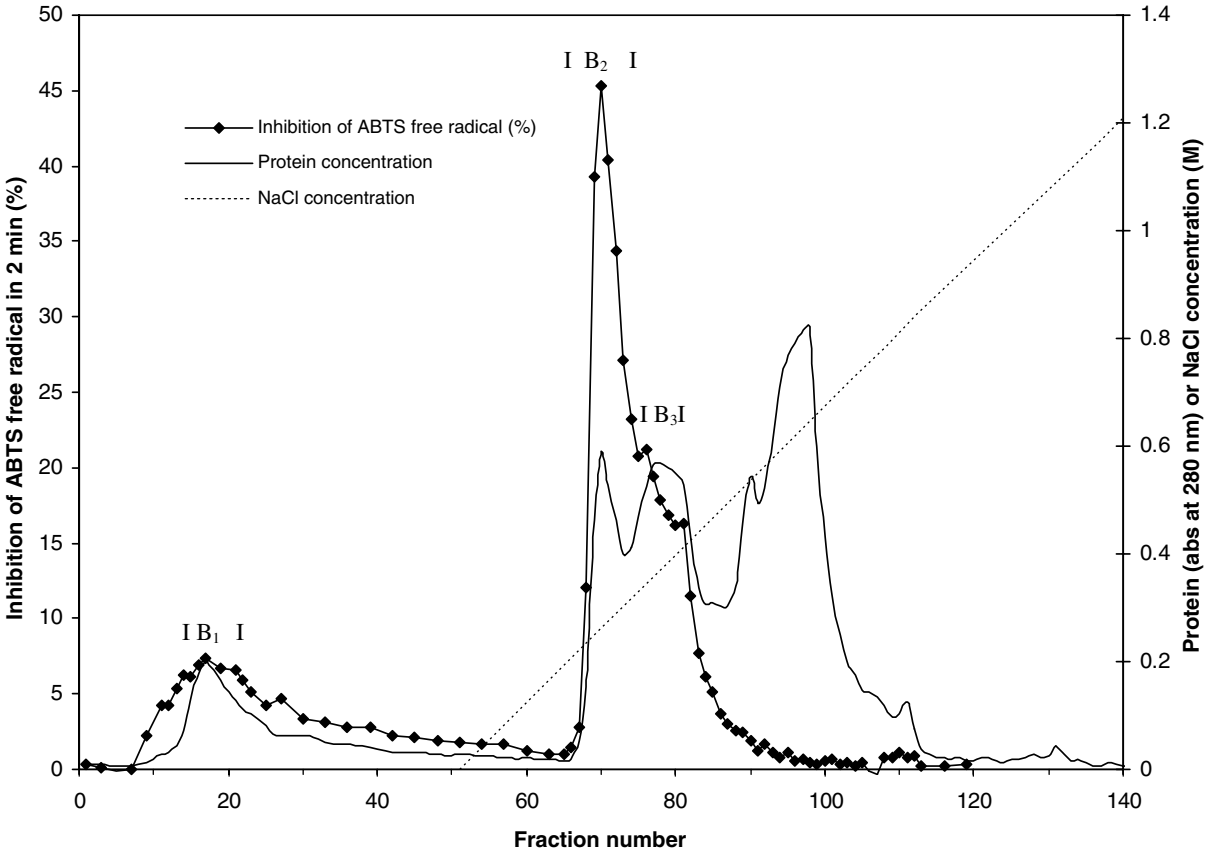

Fig. 5. DEAE-cellulose chromatography of dialyzed protein extract from thermally processed chickpeas.

tions began by the initiation of the linear gradient of NaCl and ended when NaCl concentration reached almost 0.5 M. The protein peaks, eluted above 0.5 M NaCl concentration, did not show any marked free radical-scavenging activity. The area of the inhibition vs. fraction number curve obtained for heat-treated chickpea protein extract was almost 25% greater than the area obtained for the same curve of thermally processed chickpea protein extract. The highest specific antioxidant activities and purification-fold values were obtained for the A_2 and B_2 fractions from heat-treated and thermally processed chickpeas, respectively (Tables 5 and 6). The high antioxidant activity of these protein fractions may be due to the higher frequency and/or proper positioning of antioxidant amino acids in their sequence. It is also possible that some covalently linked phenolic compounds make a contribution to the free radical-scavenging activity of these proteins. Further studies at the molecular level are needed to understand the exact mechanism of action of antioxidant proteins. However, this study clearly showed that there is a specific group of chickpea proteins associated with the antioxidant activity and these proteins may be fractionated and partially purified by anion-exchange chromatography. It is also interesting to note that, for protein extracts of thermally processed chickpeas, the total amount of antioxidant activity eluted from the DEAE-cellulose column (minimum 31 μmol trolox) was higher than the total amount of antioxidant activity loaded onto this column (17.5 μmol trolox). It seems that some prooxidants are removed from the protein extracts by DEAE-cellulose chromatography. It is also possible that the partial purification helped in unmasking of the antioxidant activity of proteins. In the literature, there are very few studies related to fractionation of antioxidant proteins from legumes. In fact, the only study determined was that of Okada and Okada (1998) who demonstrated the presence of two antioxidant albumin fractions in broad beans.

3.7. Free radical-scavenging capacity of lyophilized main antioxidant protein fractions separated by DEAE-cellulose chromatography

The free radical-scavenging activities of the main antioxidant fractions (A_2 and B_2) were also determined following lyophilization. The free radical-scavenging capacity of A_2, obtained from heat-treated chickpeas, was found almost 40% higher than that of B_2, obtained from thermally processed chickpeas (Table 1). However, it should be noted that the amount of protein (almost 29.8 mg before lyophilization) and lyophilized sample (almost 32 mg) of B_2 was almost 5- and 3-fold higher than those of A_2, respectively. The A_2 and B_2 fractions showed almost 3.8-and 2.7-fold higher free radical-scavenging capacity than did BSA, respectively. The A_2 fraction also showed almost the same free radical-

scavenging capacity as sericin, whereas B_2 showed 66% of the free radical-scavenging capacity of sericin.

4. Conclusions

This study clearly showed the higher free radical-scavenging capacities of soluble proteins from chickpeas than those from white beans. The water-soluble chickpea antioxidant proteins are also much more thermostable than the water-soluble white bean antioxidant proteins and their free radical scavenging and Fe^{+2}-chelating capacities were maintained and improved following thermal processing. Dialysis increased the water-soluble protein content of lyophilized chickpea extracts but it did not significantly affect the water-soluble protein content of lyophilized white bean extracts. Ammonium sulfate precipitation is not effective for the selective precipitation of antioxidant protein fractions. However, anion-exchange chromatography may be used for the partial purification of antioxidant proteins from heat-treated or thermally processed chickpea protein extracts. The results of this work show the good potential of soluble chickpea proteins as natural thermostable food antioxidants. Further screening of different chickpea cultivars for soluble protein content, antioxidant activity and thermostability is needed for selection of suitable cultivars.

Acknowledgements

This project was funded mainly by the Research Foundation of the Izmir Institute of Technology (Project # 2004 İYTE 05). Also, partial support was obtained from the related MİSAG # 221 project of The Scientific and Technical Research Council of Turkey (TÜBİTAK).

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