Scavenging Effect of Water Soluble Proteins in Broad Beans on Free Radicals and Active Oxygen Species

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Water soluble proteins (WSP) in broad beans, *Vicia faba*, were purified, and their scavenging effects on free radicals and active oxygen species were investigated. The purification steps included ammonium sulfate precipitation followed by sequential chromatography on Sephadex G-75. The final gel filtration step yielded two peaks of scavenging activity, each containing M_r values of 70 kDa (peak I) and 28 kDa (peak II). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis of peak II fraction gave a single band with M_r of 14 kDa, indicating that peak II protein is dimeric. WSP exhibited a marked scavenging effect on superoxide, and also an effect on hydrogen peroxide, but not so much on 1,1-diphenyl-2-picrylhydrazyl radical. WSP had only a small amount of sulfhydryl groups. Thus, the sulfhydryl groups are not responsible for the scavenging activity of WSP.

Keywords: *Broad beans (Vicia faba); antioxidant protein; free radicals; antioxidants*

INTRODUCTION

All aerobic organisms are at risk of being damaged by activated oxygen species such as superoxide, hydrogen peroxide, hydroxyl radical, and singlet oxygen. Because of the high reactivity, these agents cause various injuries such as carcinogenesis, inflammation, and aging (Ames, 1993). The aerobes have natural antioxidant activities due to enzymes and/or some metabolites. These agents are known to be important for the prevention of pollution damage (Bandiani et al., 1993) in plants as well as various diseases in both plants and animals. Antioxidants are found in many kinds of fruits and vegetables and include ascorbic acid, α-tocopherol, glutathione, β-carotene, chlorogenic acid, quercetin, and other phenolic compounds (Jones et al., 1992; Larson, 1988). More importantly, some of the proteins and amino acids have known antioxidant activities. Each of the amino acids of protein has been reported to be either anti-, pro-, or non-oxidant. Cysteine, methionine, histidine, tryptophan, and lysine are of antioxidant activity (Taylor and Richardson, 1980a). Among them, cysteine is the only one being active in a linoleate emulsion. It is more potent at 10^{-4} M in a linoleate emulsion than *tert*-butylhydroxyanisole (BHA), *tert*-butylated hydroxytoluene, or α-tocopherol and than BHA at 10^{-3} M. The antioxidant activity of cysteine is due to its sulfhydryl group, acting as the free radical scavenger, and thus as the antioxidants, in biological and other systems (Taylor and Richardson, 1980a). The net effect of thiols in many chemical systems as the antioxidants results from the ability of sulfhydryls to donate the labile hydrogen to free radicals. Various kinds of proteins are also found to be antioxidants or free radical scavengers in many living organisms. These enzymes, superoxide dismutase (SOD), catalase, and glutathione peroxidase, have already been isolated from many kinds of organisms. Some of them were tested also as antioxidants with the proper enzymic activity for skim milk (Taylor and Richardson, 1980b), soybean (Pratt, 1972), yeast (Kim et al., 1988), and human red blood cells (Lim et al., 1994). Antioxidant proteins (PRP) besides those enzymes have also been found in these biological specimens. Among them PRP from yeast or human red blood cells has been well investigated. It is shown that they functioned also as sulfur radical scavengers. Moreover, the yeast PRP (YPRP) did not show any known antioxidant enzyme activity, which was the first one not to (Kim et al., 1988). Sulfhydryl groups on PRP contributed essentially to the thiol-specific antioxidant activities (Lim et al., 1993). In the case of human blood cells, PRP (HPRP) had known enzymic activities such as SOD, catalase, and glutathione peroxidase (Lim et al., 1994). HPRP showed also the antioxidant activity: it directly scavenged reactive oxygen species, presumably hydroxyl radical-like species.

Broad beans (*Vicia faba*) contain 5% protein, 4% carbohydrates, and 15−16% solid residue per 100 g of fresh weight. Thus, the beans are a good source of protein and amino acids in the diet as commonly used. It is interesting and important to clarify the PRP activity of broad beans. The aims of this study were (1) to determine what type of oxidants WSP can scavenge; (2) to test the ability of the WSP to protect the *tert*-butylhydroperoxide (BHP)-induced peroxidation of bovine liver homogenate; (3) to evaluate the ability of WSP to chelate metal ions; and (4) to identify proteins responsible for the antioxidant activities.

MATERIALS AND METHODS

Chemicals. Catechin, BHA, and cytochrome *c* were obtained from Sigma Chemical Co., Ltd. (St. Louis, MO). Hydrogen peroxide was purchased from E. Merck (Darmstadt, Germany). DPPH was obtained from Fluka Chemie AG (Buchs, Switzerland). Sephadex G-75 was the product of

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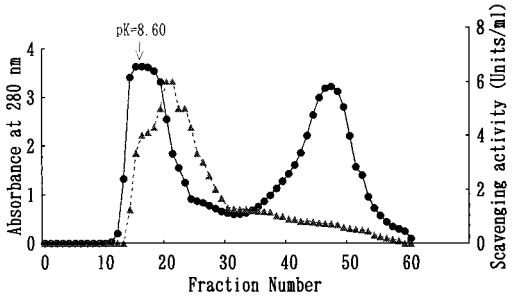

Figure 1. Sephadex G-75 column chromatography I of WSP from broad beans: ●, absorbance at 280 nm; ▲, superoxide-scavenging activity. Absorbance at 280 nm and superoxide-scavenging activity were monitored in eluted 3-mL fractions.

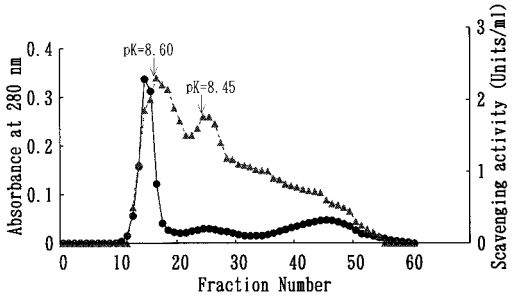

Figure 2. Sephadex G-75 column chromatography II of WSP from broad beans: ●, absorbance at 280 nm; ▲, superoxide-scavenging activity. Absorbance at 280 nm and superoxide-scavenging activity were assayed in every fractionated eluate of 3 mL.

Pharmacia (Uppsala, Sweden). All other chemicals were analytical grade products from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Protein Concentration. Protein was assayed spectrophotometrically either directly on protein solutions at A_{280} or according to the method of Bradford (1976) using bovine serum albumin as the standard.

Purification. *Step 1: Extraction.* Broad beans were obtained from a market and immediately shelled by hand, being stored at −20 °C until used. Frozen specimens (150 g) were suspended in a buffer (50 mM potassium phosphate, pH 7.8) and homogenized for 5 min at 10 000 rpm in a chilled (0 °C) Waring blender. The homogenates were centrifuged at 4 °C for 10 min at 10000*g* to give the water soluble extracts.

Step 2: Ammonium Sulfate Precipitation. The extracts obtained in step 1 were brought to 40% as to ammonium sulfate by slow addition of 100% ammonium sulfate solution. After being kept for 2 h at 4 °C (all of the following procedures were carried out at 4 °C), the suspension was centrifuged for 10 min at 10000*g*. The supernatant obtained was brought to 90% as to ammonium sulfate by the gradual addition of 100% ammonium sulfate solution. After the same centrifugation procedure, the resulting precipitates were suspended in 50 mM Tris-HCl buffer, pH 7.4 (gel filtration buffer).

Step 3: Gel Filtration I. The suspension (∼5 mL) obtained in step 2 was applied at a flow rate of 0.5 mL/min on a Sephadex G-75 column (2.5 × 30 cm), having previously been equilibrated with the gel filtration buffer. Three milliliters of each eluate was collected. Each of the fractions was subjected to A_{280} measurement and to the superoxide-scavenging assay, as described later. The fractions including the peak (fractions 14−16) were reserved (Figure 1).

Step 4: Gel Filtration II. The reserved fractions were subjected to the same chromatographic procedure for purification of WSP. Each of the fractions (fractions 24 and 25) gave the results that the activity peak coincided with that of the protein (Figure 2). The fractions eluted at the retention volume of 48 mL (fraction 16: peak I) and 72−75 mL (fractions 24 and 25: peak II) from gel filtration II were pooled, divided into aliquots, and stored at −20 °C. The protein in these is WSP, which has been used for the appraisal of its various activities as an antioxidant.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS−PAGE). Peaks I and II were subjected to SDS−PAGE. The resolving gel cotained 15% acrylamide (2.6% Bis) and 0.1% SDS in 1.5 M Tris-HCl buffer at pH 8.8, and the stacking gel contained 4% acrylamide and 0.1% SDS in 0.5 M Tris-HCl buffer at pH 6.8. The sample proteins were dispersed in 0.5 M Tris-HCl buffer at pH 6.8 containing 2% SDS, 0.05% β-mercaptoethanol, and 0.01% bromophenol blue and heated at 100 °C for 2 min. Electrophoresis was performed usually at 40 mA for 4 h, at room temperature. Following electrophoresis, the gels were stained with 0.1% Coomassie Brilliant Blue (R) in 50% (v/v) methanol and 7% acetic acid solution for 20 min. After staining, the gels were destained with five changes of a 7% acetic acid solution for a total period of 24 h and photographed.

Determination of the Effects on Superoxide. This activity of the WSP to inhibit the superoxide-dependent reduction of cytochrome *c* was monitored by measuring the rate of the increase in absorbance at 550 nm (Nihonbunko UV-460). The superoxide was generated enzymatically. The reaction mixture (3 mL) contained in 50 mM potassium phosphate, pH 7.8, and 0.1 mM ethylenediaminetetraacetic acid (EDTA): 10 μM ferricytochrome *c*, 50 μM xanthine, 0.01 μM xanthine oxidase, and an appropriate volume of the test WSP-fraction (McCord and Fridovich, 1969). The reaction at 25 °C was initiated by the addition of xanthine oxidase to the mixture. The superoxide- scavenging activity was expressed as units/ml sample or units/mg protein, where one unit of the activity was the amount of enzyme needed to cause half-maximal inhibition of cytochrome *c* reduction. All the tests were replicated three times, and the results were averaged.

Determination of the Effects on Hydrogen Peroxide. The ability of WSP to scavenge hydrogen peroxide was measured with a spectrophotometer (Ruch et al., 1989). The reaction at 20 °C was started by mixing an appropriate volume of the WSP fraction with 2 mL of 2 mM hydrogen peroxide solution to make the total volume 3 mL with phosphate-buffered saline. After 10 min reaction, the change in A_{230} of the reaction mixture was measured against the reference solution containing only the WSP. The molar absorption coefficient of hydrogen peroxide was 81 M^{-1} cm^{-1} (Beers and Sizer, 1952). All the tests were replicated three times, and the results were averaged.

Determination of the Effects on DPPH Radical. Two milliliters of WSP solution and 1 mL of DPPH radical solution were mixed (the final concentration of DPPH was 2.0×10^{-4} M) and shaken vigorously. The mixture was left to stand for 30 min, and the change in A_{517} was measured (Blois, 1958). The same assay was replicated three times, and the results were averaged.

Determination of the Chelating Activity on Metal Ions. The chelating activity of WSP on Fe^{2+} and Cu^{2+} was measured according to the method of Shimada et al. (1992). The frozen WSP solution was thawed, and a buffer containing hexamine and KCl was added to make the final concentration 10 mM and the pH 5.0. Two milliliters of the WSP solution was added to 2 mL of 10 mM hexamine buffer, pH 5.0, containing 10 mM KCl and 3 mM $FeSO_4$ or 3 mM $CuSO_4$. After the addition of 0.2 mL of 1 mM tetramethyl murexide to the final solution, absorbance at 480 nm was measured with a spectrophotometer. To prevent Fe^{2+} or Cu^{2+} oxidation, the measurement was carried out at 20 °C. All of the tests were replicated three times, and the results were averaged.

Antioxidant Activity. Bovine liver was homogenized in 19 volumes of 50 mM phosphate buffer, pH 7.4, and 0.5 mL of homogenate was added to a mixture of 25 μL of 1.0% aqueous solution of BHP, 0.5 mL of a sample solution, and 2.225 mL of H_2O. This reaction mixture was incubated at 37 °C for 15 min, and the content of thiobarbituric acid reactive substances (TBARS) in the mixture was determined according to the method of Yoshino et al. (1994) (TBARS 1). As the control, the homogenate was peroxidized by BHP without the antioxidants (TBARS 2). The reactions without BHP were carried

Table 1. Purification of WSP from Broad Beans[a]

fraction	protein (mg)	scavenging activity (units)	specific activity (units/mg)	purification (-fold)	recovery (%)
homogenate	1160	23700	20.4	1.00	100
40−90% (w/v) ammonium sulfate fraction	228	5270	23.1	1.13	22.2
chromatography I					
sum of fractions 14−16	0.801	51.0	63.7	3.12	0.215
chromatography II					
sum of fractions 24−25	0.050	5.35	107	5.25	0.0226

[a] The starting material was 150 g of broad beans. The superoxide-scavenging activity of WSP was measured at 25 °C by inhibition of the superoxide-dependent reduction of cytochrome *c*, measured at 550 nm. Each value is the mean ± standard deviation of three replicate analyses.

Table 2. Effect of Dialysis on Superoxide-Scavenging Activity of WSP[a]

dialysis	activity (units/mL)
before	4.49 ± 0.10
after	0.506 ± 0.05

[a] WSP concentration was 16.7 μg/mL. The activities given were measured as described under Materials and Methods. The dialysis against 50 mM Tris-HCl buffer, pH 7.4, was carried out at 4 °C for 24 h. Each value is the mean ± standard deviation of three replicate analyses.

out for each of the test substance as the blank (TBARS 3 and TBARS 4, respectively). The antioxidant potential of the sample was calculated by using the following equation:

$$\text{antioxidant activity (\%)} = [1 - (\text{TBARS 1} - \text{TBARS 3})/(\text{TBARS 2} - \text{TBARS 4})] \times 100$$

All of the tests were replicated three times, and the results were averaged.

Sulfhydryl Assay. Sulfhydryl groups were measured with 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB) (Ellman, 1959). Typically, 0.1 mL of the sample solution and 3 mL of a buffer (50 mM Tris-HCl, pH 8.0) were mixed, and the pH of the mixture was readjusted to pH 8.0. To this was added 0.025 mL of DTNB solution (10 mM, in 50 mM potassium phosphate buffer, pH 7.0). The reaction mixture was incubated at 25 °C for 5 min, and A_{414} was measured against the blank solution (water in place of sample). To correct the turbidity, the absorbance of samples without DTNB was measured against water, and a standard curve was constructed. All of the tests were replicated three times, and the results were averaged.

RESULTS

Purification of WSP. Procedures for the purification of WSP from broad beans are summarized in Table 1. Application of dialysis irreversibly reduced the superoxide-scavenging activity of WSP (Table 2). The last purification step (Figure 2) yielded two superoxide-scavenging activity peaks I (fraction 16) and II (fractions 24 and 25), at retention volumes of 48 and 72−75 mL, respectively. The molecular weights of these proteins determined from a plot of the logarithm of molecular weight versus mobility (figure not shown) are approximately 70 000 and 28 000, respectively. SDS−PAGE of peak II showed a single band with a molecular weight of 14 000 (Figure 3, lane II). The peak II protein may appear to contain two small, identical subunits. Peak I yielded a band of 18 000 and several impurity bands of higher molecular weight (Figure 3, lane I). Although peak I (WSP) was not completely homogeneous, it was possible to identify the 14-kDa band as the polypeptide of WSP in several trials of the same procedure.

The heat inactivation of the superoxide-scavenging activity of the purified WSP is shown in Table 3. The activity of the WSP was little changed in the incubation for 2 min at 100 °C. The half-life of the protein at 100 °C was calculated to be 12.9 min, the decay being assumed to be exponential.

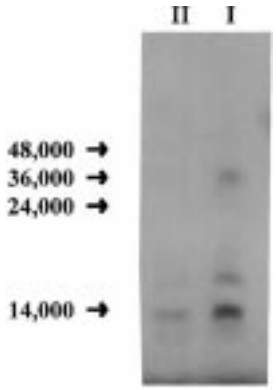

Figure 3. SDS−PAGE of WSP. The WSP eluted at the retention volume of 48 mL (lane I = peak I) and 72−75 mL (lane II = peak II) were subjected to SDS−polyacrylamide gel (15%). Arrows indicate M_r standards (14 000; 24 000; 36 000; 48 000).

Table 3. Effect of Heat Treatment on Superoxide-Scavenging Activity of WSP[a]

heat treatment	activity (units/mL)	inhibition (%)
before	5.59 ± 0.10	0
after	5.02 ± 0.15	11.2

[a] The scavenging capacity of the WSP was investigated by incubation for 2 min at 100 °C. The activities given were measured as described under Materials and Methods. Each value is the mean ± standard deviation of three replicate analyses.

Table 4. Superoxide-Scavenging Activity of Various Substances[a]

substance	concentration	activity (units/mL)
WSP	16.7 μg/mL	4.29 ± 0.15
	1.67 μg/mL	1.58 ± 0.08
	0.167 μg/mL	0.556 ± 0.05
BHA	75.0 μM	5.00 ± 0.10
catechin	2.50 μM	4.29 ± 0.08
L-cysteine	100 μM	2.73 ± 0.12
	200 μM	3.00 ± 0.10

[a] The activities given were measured as described under Materials and Methods. Each value is the mean ± standard deviation of three replicate analyses.

Scavenging of Superoxide by WSP. The ability of WSP to scavenge superoxide generated by the xanthin/xanthine oxidase system (X-XOD) is shown in Table 4. The addition of WSP (16.7 μg/mL) to the X-XOD system significantly ($p < 0.05$) diminished the production of superoxide. Catechin and BHA at concentrations of 2.5 and 75 μM, respectively, also exhibited marked superoxide-scavenging activities. Cysteine showed less scavenging activity than WSP. To rule out the inhibition of xanthine oxidase activity by the WSP solution,

Table 5. Scavenging Effects of Various Substances on Hydrogen Peroxide[a]

substance	concentration	H_2O_2 (mM)	inhibition (%)
WSP	0 μg/mL	2.00	0
	0.167 μg/mL	1.98 ± 0.05	1.00
	1.67 μg/mL	1.86 ± 0.04	4.00
	16.7 μg/mL	1.08 ± 0.06	46.0
BHA	43.0 μM	1.93 ± 0.02	3.50
catechin	14.0 μM	1.70 ± 0.06	15.0
L-cysteine	100 μM	1.99 ± 0.05	0.500
	200 μM	1.99 ± 0.08	0.500

[a] The values given were measured as described under Materials and Methods. Each value is the mean ± standard deviation of three replicate analyses.

Table 6. Scavenging Effects of Various Substances on DPPH Radical[a]

substance	concentration	$A_{517\ nm}$	inhibition (%)
WSP	0 μg/mL	0.300 ± 0.010	0
	0.167 μg/mL	0.296 ± 0.005	1.33
	1.67 μg/mL	0.280 ± 0.004	6.67
	16.7 μg/mL	0.300 ± 0.006	0
BHA	240 μM	0.030 ± 0.005	90.0
catechin	8.00 μM	0.048 ± 0.006	84.0
L-cysteine	100 μM	0.222 ± 0.005	26.0
	200 μM	0.211 ± 0.008	29.7

[a] The values given were measured as described under Materials and Methods. Each value is the mean ± standard deviation of three replicate analyses.

we carried out the nonenzymatic reduction of alloxan by reduced nicotinamide nucleotide (Miwa et al., 1982). The results showed that WSP worked as the superoxide scavenger in this system as well (data not shown).

Scavenging of Hydrogen Peroxide by WSP. The scavenging activity of WSP on hydrogen peroxide is shown in Table 5. Though WSP had scavenging activity on hydrogen peroxide, the effect was less sharp than that on superoxide. The 46% inhibition (near D_{50}) at 16.7 μg/mL of WSP gives a specific activity of 55.1 μmol of hydrogen peroxide reduction/mg of WSP. Catechin possessed a slight hydrogen peroxide scavenging activity (15%). Cysteine had negligible scavenging activity.

Measurement of DPPH Radical-Scavenging Activity. The scavenging activity of WSP on DPPH radical is shown in Table 6. It was strange that no inhibition was observed at 16.7 μg/mL. This could be due to some of the contaminants protecting DPPH, because of the so much higher protein content of the WSP specimen used. Catechin and BHA at the concentrations tested, respectively, exhibited marked scavenging activities (>80%). Cysteine also inhibited the DPPH radical.

Properties of WSP. The ultraviolet−visible spectrum of the purified scavenging protein is similar to that of most other proteins. At a concentration yielding A_{280} = 0.10, no detectable absorption was observed in the range of 320−600 nm, showing that this protein does not contain prosthetic groups such as heme and flavin. An absorbance intensity of 0.001 could have been detected in the range, easily (data not shown).

The number of cysteine residues in WSP was measured using Ellman's reagent, DTNB (Table 7). WSP (16.7 μg/mL ≈ 1.2 μM) had a small amount of sulfhydryl groups (12 μM). Thus, 10 sulfhydryl groups per 14 kDa polypeptide were detected.

Comparison of the Antioxidant Activities of WSP and Several Known Antioxidants. Table 8 shows the antioxidant activity of WSP and two known antioxidants. The three substances were effective, and WSP was the lowest. BHA had the strongest protective effect on the BHP-induced peroxidation of bovine liver homogenates. The antioxidant activity was observed to increase in the following order: WSP < catechin < BHA.

Table 7. Sulfhydryl Content of Broad Bean Fractions[a]

fraction	sulfhydryl content (μM)
homogenate	731 ± 10.0
40−90% (w/v) ammonium sulfate fraction	446 ± 15.0
chromatography I	
sum of fractions 14−16	12.9 ± 2.00
chromatography II	
peak I (fraction 16)	5.72 ± 1.00
peak II (fractions 24 and 25)	12.6 ± 1.20

[a] The values given were measured as described under Materials and Methods. Each value is the mean ± standard deviation of three replicate analyses.

Table 8. Antioxidant Activity of Various Substances[a]

substance	concentration	antioxidant activity (%)
WSP	16.7 μg/mL	66.0 ± 1.60
BHA	240 μM	91.3 ± 1.50
catechin	8.00 μM	81.0 ± 2.50

[a] The activities given were measured as described under Materials and Methods. Each value is the mean ± standard deviation of three replicate analyses.

Table 9. Chelating Effects of Various Substances on Fe^{2+} and Cu^{2+}[a]

		$A_{480\ nm}$	
substance	concentration	Fe^{2+}	Cu^{2+}
control	0	0.944 ± 0.002	1.211 ± 0.004
WSP	16.7 μg/mL	0.695 ± 0.004	1.111 ± 0.004
	1.67 μg/mL	0.907 ± 0.003	1.189 ± 0.003
	0.167 μg/mL	0.935 ± 0.005	1.207 ± 0.006
EDTA	0.5 M	0.379 ± 0.004	0.388 ± 0.002
citric acid	0.5 M	0.568 ± 0.003	0.611 ± 0.003

[a] The values given were measured as described under Materials and Methods. Each value is the mean ± standard deviation of three replicate analyses.

Measurements of Chelating Activity on Metal Ions. The ability of WSP to form complexes with metal ions is shown in Table 9. Citric acid and EDTA showed chelating effects, but WSP showed no chelating activity except the case of Fe^{2+} at the highest concentration.

DISCUSSION

The purification procedures for WSP from broad beans described in this paper involve ammonium sulfate fractionation and gel filtration chromatography, but the final enzyme preparation may still contain trace amounts of other proteins as seen in the electrophoresis photograms. The subunit structure of WSP, therefore, was not clear. In the present study, gel filtration chromatography yielded two superoxide-scavenging activity peaks I and II. SDS−PAGE of peak II showed a single band with a molecular weight of 14 000 as far as WSP amount used in the experiments. Sephadex column chromatography is a powerful tool for the isolation of WSP. Several investigators (Somers, 1966; Woof et al., 1967) have reported successful separations of flavonoid compounds on Sephadex columns, even though Sephadex tends to absorb aromatic compounds, particularly phenols. Sephadex column chromatography was successfully used for separating WSP in the broad beans.

A DEAE-cellulose column retained HPRP (Lim et al., 1994), while WSP of broad beans was not adsorbed onto the column (data not shown). Thus, the properties of WSP are somewhat different from those of HPRP. Application of dialysis against a buffer irreversibly reduced the superoxide-scavenging activity of WSP (Table 2). This fact may principally be due to the removal of low molecular weight substance(s) necessary for the WSP activity. The details, however, are not clear. For the purpose of separating of the scavenging activity peak, a different condition may be more effective. However, when the fractions (fractions 14−16) from gel filtration I were subjected to the dialysis procedure, the WSP sample lost the superoxide-scavenging activity. Therefore, we did not use a different medium or even different conditions for the same medium.

The molecular weight of WSP was 14 000, while those of PRP obtained from human red blood cells and from yeast was 25 000 and 27 000, respectively, having been determined by SDS−PAGE. This indicates that the size of WSP is almost half that of PRPs.

Ilan et al. (1976) showed that superoxide acts as either an oxidizing or a reducing agent, depending on the substrate oxidation potential. Superoxide indirectly initiates lipid oxidation as a result of superoxide and the emerging hydrogen peroxide acting as precursors of hydroxyl radicals (Kellogg and Fridovich, 1975; Aurand et al., 1977). Superoxide also decomposes to form stronger oxidative species such as hydroxyl radical and hydrogen peroxide, which initiate the peroxidation of lipid (Dahl and Richardson, 1978). The antioxidant activity of WSP is considered to affect its scavenging of superoxide. Hydrogen peroxide has only a weak activity on the initiation of lipid peroxidation (Namiki, 1990). However, its activity as an active oxygen species comes from its potential to produce a highly reactive hydroxyl radical through the Fenton reaction (Cohen and Heikkila, 1974). Therefore, the ability of WSP to scavenge hydrogen peroxide may contribute to inhibition of the peroxidation of lipids.

By using the method of Shimada et al. (1992), we could not show that WSP was capable of chelating Fe^{2+} and Cu^{2+} (Table 9). This implies that transferrin and lactoferrin can afford some degree of chelation, but WSP may not be one of those structures. This leads to the conclusion that, in vivo, WSP would be unlikely to chelate sufficiently the endogenous metal ions.

The fact that YPRP and HPRP possessed neither superoxide dismutase nor catalase is based on the direct measurement of these enzyme activities by using the protein preparations, while WSP from broad beans possessed antioxidant activity toward superoxide and hydrogen peroxide. These results clearly show the structural differences between PRP and WSP.

We do not yet know exactly how WSP works as the antioxidant agent. A plausible mechanism may be due to the existence of sulfhydryl groups in a small amount, the content of which was calculated to be 10 sulfhydryl groups per one 14-kDa WSP molecule. WSP showed significant activity to scavenge superoxide upon being compared with cysteine: ~17 (= 200 μM/12 μM) times more active than cysteine, being compared at nearly the same activity observed. The concentration dependence of the cysteine-scavenging activity was much different from that of WSP. Cysteine does not scavenge hydrogen peroxide, but WSP did. Therefore, sulfhydryl groups apparently do not contribute to the scavenging activity of WSP. Our results suggest that sulfhydryl groups are not the principal antioxidant agents in broad beans. The hydroxyl groups and/or carboxyl groups in amino acids may be responsible for the scavenging activity of WSP.

We thus conclude that the WSP of broad beans has a strong hydrogen-donating ability and is a good scavenger of the active oxygen species, including superoxide and hydrogen peroxide. This property seems to be important to explain how the antioxidant activity of WSP arises.

ABBREVIATIONS USED

WSP, water soluble protein; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DTNB, 5,5′-dithiobis(2-nitrobenzoic) acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; BHA, *tert*-butylhydroxyanisole; BHP, *tert*-butylhydroperoxide; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; TBARS, thiobarbituric acid reactive substances; X-XOD, xanthin/xanthine oxidase; PRP, thiol-specific antioxidant protein; HPRP, thiol-specific antioxidant protein in human red blood cells; YPRP, thiol-specific antioxidant protein in yeast; SOD, superoxide dismutase.

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