Antioxidant and free radical-scavenging activities of black soybean peptides (BSP)

Ren Haiwei

(*College of Life Science and Engineering, Lanzhou University of Technology, Lanzhou* 730050, *China*)

Abstract: The antioxidant and free radical-scavenging activities of black soybean peptides (BSP) fractions (Fra-Ⅰ, Fra-Ⅱ, Fra-Ⅲ) were investigated using reducing power and DPPH·/superoxide/hydroxyl radical-scavenging assay. The reducing power activity of Fra-Ⅲ was closer to that of BHT but lower than that of ascorbic acid. Fra-Ⅲ showed the strongest scavenging activity against free radicals. The radical-scavenging effect was in a dose-dependent manner and the IC_{50} values for DPPH·, superoxide and hydroxyl radicals were found to be 1.873, 1.684 and 1.735 mg/mL, respectively. Amino acid analysis showed that Fra-Ⅲ had high hydrophobic amino acids (HAA) content and hydrophobicity. The molecular weight distribution of Fra-Ⅲ was found to vary from 100 to 1,000 Da mainly. The antioxidant activity of Fra-Ⅲ is clearly related to the amino acid composition, the content of HAA and the molecular mass. The present study suggests that BSP with low molecular weight are useful nutritional antioxidant and potential functional factor for anti-aging.

Key words: black soybean protein peptides (BSP), antioxidant activity, free radical-scavenging activity, amino acid composition, molecular weight distribution

DOI: 10.3965/j.issn.1934-6344.2010.02.064-069

Citation: Ren Haiwei. Antioxidant and free radical-scavenging activities of black soybean peptides (BSP). Int J Agric & Biol Eng, 2010, 3(2): 64－69.

1 Introduction

In recent years research interest in utilizing natural antioxidants has increased substantially. This has led to new investigations into assessing the antioxidant potential of bioactive peptides from protein such as soybean protein, peanut protein, corn protein, chickpea protein[1-4]. Among different types of bioactive peptides, soybean peptides with free radical-scavenging activity have been extensively studied as the cell or tissue injury caused by the free radicals chain reaction of lipid peroxidation. The antioxidant activities of soybean peptides have been ascribed to the cooperative effect of a number of properties, including their ability to scavenge free radicals, to oxygen quencher and hydrogen donor.

Black soybean (*Glycine max* L.), an annual herbage plant, a variety of soybean with a black seed coat that belongs to the family Leguminosae, has been widely utilized as a tonic food and material in oriental medicine for hundreds of years. According to grain size, black soybean is divided into small black soybean (100 grains weight≤13 g), medium black soybean (100 grains weight 13-25 g) and big black soybean (100 grains weight≥ 25 g). Black soybean can also be classified into yellow and green cotyledon on the basis of cotyledon color. The traditional Chinese medicine theory believes that black soybean has been used as a component in ancient medicines to treat diabetes, hypertension, anti-aging, cosmetology, black-hair and so on. Black soybean containing herbal prescriptions can increase the number of circulating white blood cells in leukopenic patients. Present studies have proved that the anthocyanins of seed coat which is a kind of polyphenol has free radical-

Received date: 2009-02-19 **Accepted date:** 2010-03-09

Biography: Ren Haiwei (1983－), male, Research direction: development on functional foods. College of Life Science and Engineering, Lanzhou University of Technology, Lanzhou 730050, China. Email: rhw5257@sohu.com

scavenging activities and the characteristics of adipogenesis inhibitory peptides of black soybean have been definite[5,6]. Therefore, the intake of black soybean may be more useful for preventing oxidation-related diseases such as atherosclerotic diseases and various types of cancers.

In spite of the physiological importance and potentially greater health benefits of black soybean, there is little information concerning the antioxidant activity of BSP. This study, therefore, investigated free radical-scavenging activities and the antioxidant potential of different BSP fractions using several measurements, including the reducing power and the scavenging effect on 1,1-diphenyl-2-pycrylhydrazyl (DPPH·) /superoxide / hydroxyl radicals. Furthermore, amino acid composition and molecular weight distribution were also evaluated to determine their relationship with the antioxidant activity.

2 Materials and methods

2.1 Materials

Black soybean was obtained from Agricultural Bureau of Jingle County in Shanxi Province. 1,1-diphenyl-2-pycrylhydrazyl (DPPH·), l,10-phenanthroline, hydrogen peroxide(H_2O_2) and potassium ferricyanide were purchased from Sigma Chemical Company. 2709 Alkali protease and AS 1.398 neutral protease were purchased from PANGBO Biotechnology Limited Company of Guangxi. SephadexG-50 was purchased from Pharmacia Company. All other chemicals used in the experiments were the highest grade commercially available products.

2.2 Production of black soybean protein isolate (BSPI)

BSPI was produced according to the method described by Wang et al. with little modification[7]. Black soybean seeds were grinded and defatted with hexane. The defatted flour was dried in the ventilator overnight at 30℃. BSPI was obtained by dispersing the defatted black soybean flour in NaOH solution (pH 8.0) at ratio 1:10 (w/v) and extracted by stirring for 60 min. After pH value adjustment to the isoelectric point (pH 4.5), the precipitate obtained by centrifugation at 10,000 g was lyophilized.

2.3 Preparation of BSP

BSPI was suspended in distilled water at 8.0% (w/v) and heated at 90℃ for 15 min. BSPI solution was hydrolyzed by 2709 Alkali protease (pH 9.0) and AS1.398 neutral protease (pH 7.0) at 50℃ for 240 min, respectively. The pH value of the mixture was adjusted to keep constant with 0.1 mol/L NaOH during the hydrolysis. The degree of hydrolysis was determined by using the pH-stat method. After hydrolyzing for 480 min, the enzyme was inactivated at 90℃ for 10 min, and the hydrolysate solution was centrifuged at 20,000×*g* for 15 min after cooling down to room temperature. The supernatants were stored at low temperature for separation.

2.4 Fractionation of BSP

The hydrolysate solution was loaded onto SephadexG-50 gel filtration column (1.6 cm ×70 cm) to separate and purify. Separation was obtained with Tris-HCl buffer (0.05 mol/L, pH 7.2) at a flow rate of 2 mL/min and eluted fractions were collected with automatic collector detector and measured by UV at 220 nm. After gel filtration, they were separated to three peaks such as Fra-Ⅰ, Fra-Ⅱ and Fra-Ⅲ according to the molecular size. Each fraction was freeze-dried respectively and stored at 4℃.

2.5 Reducing power activity

Different concentrations of BSP fractions were mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of 1.0% potassium ferricyanide. The mixtures were incubated at 50℃ for 20 min. After incubation, 2.5 mL of 10% TCA was added to the reaction mixture, followed by centrifugation at 6,000×*g* for 10 min. The upper layer (5 mL) was mixed with 5 mL of distilled water and 1mL of 0.1% $FeCl_3$ and the absorbance of the resultant solution was measured at 700 nm. The antioxidant activities of BHA and ascorbic acid were also assayed for comparison purposes. Increased absorbance of the reaction mixture indicated increased reducing power. According to the curve from results, the median effective dose (RP_{50}) was calculated. The RP_{50} is the concentration which is required to 50% reducing power activity[8].

2.6 Measurement of DPPH radical-scavenging activity

DPPH· radical-scavenging activity of BSP fractions was measured according to the method of Ferreira et al. with little modification[9]. Test samples in 4 mL of water were mixed with 1 mL of 99.5% ethanol containing 0.02% DPPH·. This mixture was shaken and left for 30 min at room temperature, and the absorbance of the mixture was measured at 515 nm. The ascorbic acid was positive control. A lower absorbance represents a higher DPPH· scavenging activity. The scavenging effect was expressed by the following equation as,

DPPH· scavenging activity(%)=100−

$$\frac{(\text{DPPH}_{\text{blank}} - \text{control sample}) - \text{DPPH}_{\text{sample}}}{\text{DPPH}_{\text{blank}}} \times 100\% \quad (1)$$

Where: DPPH· blank is the value of 4 mL of water/1 mL of ethanol including 0.02% DPPH·; DPPH· sample is the value of 4 mL of sample solution/1 mL of ethanol including 0.02% DPPH·; and the control sample is the value of 4 mL of sample solution/1 mL of ethanol.

2.7 Hydroxyl (•OH) radical-scavenging activity

•OH radical scavenging assay was carried out using the method described by de Halliwell et al. with some modifications[10]. Both 1,10-phenanthroline (5 mmol/L) and $FeSO_4$ (7.5 mmol/L) were dissolved in phosphate buffer (pH 7.4) and mixed thoroughly. H_2O_2 (0.01 mmol/L) and BSP fractions were added. The mixture was incubated at 37℃ for 60 min, and the absorbance was measured at 536 nm. The scavenging activity on hydroxyl radicals was expressed by the following equation as，

$$\text{•OH scavenging activity}(\%) = \frac{A_s - A_1}{A_0 - A_1} \times 100\% \quad (2)$$

Where: A_S is absorbance of the sample; A_1 is absorbance of control solution containing 1,10-phenanth-roline, $FeSO_4$ and H_2O_2; A_0 is absorbance of blank solution containing 1,10-phenanthroline and $FeSO_4$.

2.8 Superoxide anion(O_2^-•) radical-scavenging activity

O_2^-• radical scavenging activity of BSP fractions was determined using the nitro-blue tetrazolium (NBT) reduction method. In this method, O_2^-• generated in vitro by the xanthine oxidase (XOD) reduced the yellow dye(NBT^{2+}) to produce the blue formazan, which was measured at 550 nm. The reaction mixture contained 0.5 mL of 0.8 mmol/L xanthine in 0.1 mmol/L phosphate buffer (pH 8.0), 0.51 mmol/L NBT in 0.1 mmol/L phosphate buffer and 0.1 mL sample solution. After incubating at 37℃ for 10 min, the reaction was initiated by adding 1.0 mL of XOD (0.049 U/mL) and incubated at 37℃ for 20 min, the reaction was stopped by adding 2.0 mL of 69 mmol/L SDS. The absorbance of the reaction mixture was measured at 550 nm. The scavenging effect was calculated according to the following formula:

$$O_2^-\text{• scavenging effect}(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\% \quad (3)$$

2.9 Amino acid composition analysis

Amino acid composition analysis was performed according to the method described by Kunio et al[11]. Fra-Ⅲ was hydrolyzed in 6 mol/L HCl under vacuum at 110℃ for 24 h and analyzed by using an automatic amino acid auto-analyzer. Especially, tryptophan was analyzed by the glyoxylic acid method at 570 nm. The amino acid composition was expressed as g of amino acid per 100 g protein.

2.10 Determination of molecular weight distribution

Fra-Ⅲ was analyzed for molecular weight distribution using a Waters^{TM}600E Advanced Protein Purification System. Fra-Ⅲ was loaded onto TSK gel G2000 SW_{XL} column (7.8 mm×300 mm), eluted with 45% (V/V) acetonitrile containing 0.1% (V/V) trifluoroacetic acid (TFA) at a flow rate of 0.5 mL/min and monitored at 220 nm. A molecular weight calibration curve was obtained from the following standards: cytochrome C (12500), bacitracin (1450), tetrapeptide GGYR (451), tripeptide GGG (189).

3 Results and analysis

3.1 Reducing power

The result of the study shows that antioxidant activity and reducing power are related. The relationship between reducing power of BSP fractions and their concentration is shown in Figure 1. The highest

reducing power was found in Fra-Ⅲ (RP_{50}1.172 mg/mL), which was higher than that of BHT (RP_{50}2.526 mg/mL) but lower than that of ascorbic acid (RP_{50} 0.836 mg/mL), followed by Fra-Ⅱ and Fra-Ⅰ successively. The reducing power of BSP fractions increased with increasing their concentrations and decreasing molecular weight.

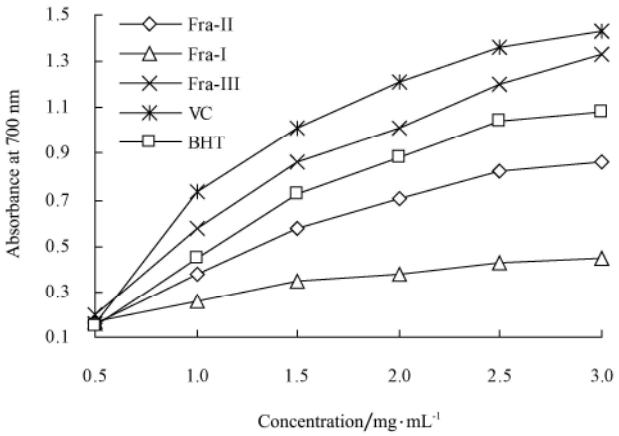

Figure 1 Reducing power of BSP fractions at different concentrations

3.2 DPPH· radical-scavenging activity

DPPH· is a relatively stable free radical which has commonly been used in antioxidant activity analysis. Results shown in Figure 2 revealed that Fra-Ⅰ, Fra-Ⅱ, Fra-Ⅲ of BSP had the ability to quench the DPPH· free radical. The IC_{50} values of Fra-Ⅰ, Fra-Ⅱ, and Fra-Ⅲ were found to be 6.938, 4.557 and 1.873 mg/mL, respectively. Especially Fra-Ⅲ at 3.0 mg/mL exhibits an excellent DPPH· radical-scavenging activity (73.78%), which is closer to that of ascorbic acid (86.75%), and higher than that of Fra-Ⅱ and Fra-Ⅰ. Obviously, the scavenging effect of BSP fractions increased with increasing concentrations of the samples. The results revealed that Fra-Ⅲ 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.

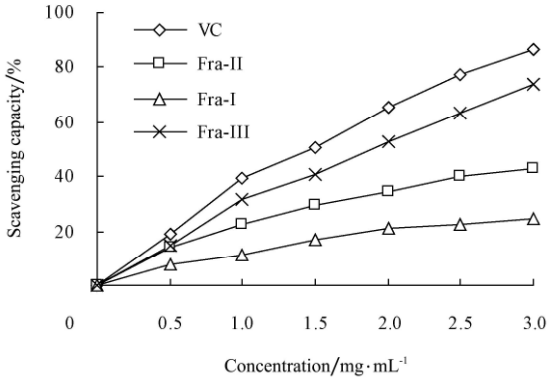

Figure 2 DPPH· scavenging effect of BSP fractions at different concentrations

3.3 •OH radical-scavenging activity

BSP fractions were analyzed for •OH scavenging activity to better examine their antioxidant properties, because the different radical systems used for antioxidant evaluation may influence the experimental results. •OH is the most reactive free radical and can be formed from superoxide anion and hydrogen peroxide, in the presence of metalions, such as copper or iron. The •OH scavenging abilities of BSP fractions were also showed in Figure 3. The IC_{50} values for hydroxyl radicals of Fra-Ⅰ, Fra-Ⅱ and Fra-Ⅲ were found to be 10.561, 7.486 and 1.735 mg/mL, respectively. The inhibition of hydroxyl radical exhibited by Fra-Ⅲ (78.23%) was closer to that of ascorbic acid (87.42%). In addition, Fra-Ⅱ and Fra-Ⅰ used at the same concentration of 3 mg/mL exhibited 41.47% and 19.55% hydroxyl radical-scavenging activity, respectively.

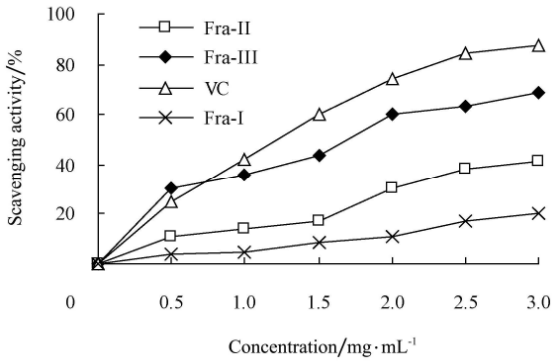

Figure 3 •OH scavenging effects of BSP fractions at different concentrations

3.4 Superoxide anion(O_2^{-}•) radical scavenging

Numerous biological reactions generate O_2^{-}• which is a highly toxic species. Although they cannot directly initiate lipid oxidation, O_2^{-}• are potential precursors of highly reactive species, such as hydroxyl radical, and thus study of the scavenging of this radical is important[12].

O_2^{-}• scavenging of BSP fractions was presented in Figure 4. All samples treated in this experiment showed considerable scavenging abilities over superoxide anion. The IC_{50} values of Fra-Ⅰ, Fra-Ⅱ and Fra-Ⅲ were found to be 8.361, 4.753 and 1.684 mg/mL, respectively. Results showed that Fra-Ⅲ has good antioxidant and free radical scavenging activity and this fraction can be a potential source of natural antioxidant.

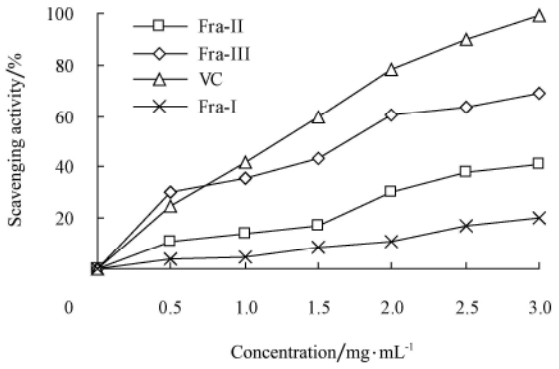

Figure 4 O_2 scavenging effects of BSP fractions at different concentrations

3.5 Amino acid composition

To confirm the possible effect of the amino acid profile on antioxidant activity, the amino acids composition of Fra-Ⅲ was analyzed and results are presented in Table 1. Glu, Arg, Phe, Lys, Leu, Ala and Asp were the major constituent amino acids of Fra-Ⅲ. It is commonly believed that His, Met and Cys are very important to the radical scavenging activity of peptides due to their special structures of characteristics: the imidazole group in His has the proton-donation ability; Met is prone to oxidation of the Met sulfoxide; Cys donates the sulfur hydrogen. These amino acids could favor the radical scavenging properties of Fra-Ⅲ. Therefore, the amino acids composition might play an important role on its activity. The same conclusions were also found by Suetsuna et al[13].

In addition, the results indicated the total hydrophobic amino acids (HAA) content in Fra-Ⅲ was found to be 30.03%. It was reported that an increase in hydrophobicity (Hφ) of peptides will increase their solubility in lipid and therefore enhance their antioxidant activity. Calculation of the hydrophobic indices showed that Hφ was 438.48 kJ/mol Amino Acid Rresidue (AAR). Thus, the conclusion could be made that the highest antioxidant activities of Fra-Ⅲ were presumably due to the highest content of THAA and the amino acids sequence of the peptides as well as the higher Hφ.

Table 1 Amino acid composition of Fra-Ⅲ (g/100 g)

Amino acid	percentage content	Amino acid	percentage content
Asp	8.12	Val	3.02
Thr	2.38	Leu	5.18
Glu	17.89	Met	0.73
Ser	3.36	Ile	3.18
Pro	2.36	Tyr	1.62
Gly	2.68	Phe	3.38
Ala	2.67	His	1.78
Cys	1.06	Arg	5.12
Lys	3.82	Trp	1.12
HAA(g/100 g)	30.03	Hφ (kJ/mol AAR)	438.48

3.6 Molecular weight distribution

Considering that the Fra-Ⅲ was found to possess the highest antioxidant activity, this fraction was therefore analyzed for molecular weight distribution. The HPLC indicated that this fraction was composed of low molecular weight peptides whose major peaks were located at 1006-400 Da (22.65%), 400-200 Da (58.37%), 200-50 Da (18.95%). A number of studies had already shown that the antioxidant activity of peptides was depending on their molecular weight distribution. In this study, results revealed that the peptide fraction with molecular weight ranging from 100 to 1,000 Da was probably associated with higher antioxidant activity. These findings were in agreement with observations from other studies and supported the fact that functional properties of antioxidative peptides were significantly influenced by properties such as molecular mass[14].

4 Conclusions

Oxidation is an essential reaction in all living organisms. The formation of free radicals and other reactive oxygen species are unavoidable during the oxidative metabolic process. These reactive radicals play an important role in signal transduction[15]. However, excessive free radicals can cause oxidative stress, which could lead to cell injury and tissue damage and play an important role in the etiology of many pathological conditions in human body. Free radical scavenger is a

preventive antioxidant and the scavenging activity can be used as the indication of prevention from increased oxidative stress. The activity assessment for fractions separated by gel filtration showed that low-molecular-weight peptides (Mw<1,000 Da) were important for their antioxidant abilities. Moreover, the molecular size, the amino acids composition and the Hφ value appeared to collectively contribute to the strong antioxidant bioactivity. However, the complexity of human physiological structure suggests that Fra-Ⅲ must be used for the in vivo experiment to estimate the antioxidant activities. A further research on organism antioxidation and the antioxidant mechanism is undergoing.

[References]

[1] Chen H M, Muramoto K, Yamauchi F, Fujimoto K, Nokihara K. Antioxidant activity of design peptides based on the antioxidant peptide isolated from digests of a soybean protein. Journal of Agricultural and Food Chemistry, 1996; 44: 2619－2623.

[2] Hwang J Y, Shue Y S, Chang H M. Antioxidative activity of roasted and deffated peanut kernels. Food Research International, 2001; 34: 639－647.

[3] He H, Xie B J, Yang Z. Studies on hydrolysed peptide of corn and soybean protein and its activity. Cereals, Oils and Foodstuffs Technology, 2002; 10(1): 14－16.

[4] Clemente A, Vioque J, Sánchez-Vioque R, Pedroche J, Bautista J, Millán F. Protein quality of chickpea (*Cicer arietinum* L.) protein hydrolysates. Food Chemistry, 1999; (67): 269－274.

[5] Choung M G, Baek I Y, Kang S T, Han W Y, Shine D C, Moon H P, et al. Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.). Agric. Food Chem, 2001; (49): 5848－5851.

[6] Kim H J, Bae I Y, Ahn C W, Lee S, Lee H G. Purification and identification of adipogenesis inhibitory peptide from black soybean protein hydrolysate. PEPTIDES, 2007; (28): 2098－2103.

[7] Wang C Q, Ren H W. Study on Preparation Technology of Small Black-soybean Peptide. Food Science, 2008; 29(5): 231－233.

[8] Li Y H, Liu J, Zhang T, Jiang B, Mu W M. Enzymolysis technology optimization for production of antioxidant peptides from chickpea protein (in Chinese). Transactions of the CSAE, 2008; 24(1): 268－273.

[9] Ren Y, Ren G X, Ma T J, Ping H, Niu X. Extraction and antioxidant activity of avenanthramides from oat bran (in Chinese). Transactions of the CSAE, 2008; 24(5): 265－269.

[10] Jia J Q, Ma H L, Qu W J, Ding Q Z, Cao H, Luo L, et al. Ultrasonic pretreatment for preparation of antioxidant peptides from rice protein (in Chinese). Transactions of the CSAE, 2008; 24(8): 288－293.

[11] Kunio, S, Hiroyuki, U, Hirotomo, O. Isolation and characterization of free radical-scavenging activities peptides derived from casein. Journal of Nutritional Biochemistry, 2000; (11): 128－131.

[12] Li Y H, Jiang B, Zhang T, Mu W M, Liu J. Antioxidant and free radical-scavenging activities of chickpea protein hydrolysate (CPH). Food Chemistry, 2008; 106: 444－450.

[13] Suetsuna K, Ukeda H, Ochi H. Isolation and characterization of free radical scavenging activity peptides derived from casein. Journal of Nutrition and Biochemistry, 2000; 11: 128－131.

[14] Kim S Y, Je J Y, Kim S K. Purification and characterization of antioxidant peptide from hoki (Johnius belengerii) frame protein by gastrointestinal digestion. Journal of Nutritional Biochemistry, 2007; 18: 31－38.

[15] Hancock J T, Desikan R, Neill S J. Role of reactive oxygen species in cell signaling pathways. Biochemical Society Transactions, 2001; 29: 345－350.