Pilot-scale production of soybean oligopeptides and antioxidant and antihypertensive effects *in vitro* and *in vivo*

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Revised: 23 March 2012 / Accepted: 9 April 2012 / Published online: 25 April 2012
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Abstract Soybean oligopeptides (SOP) with low molecular weights were prepared by two-step enzymatic hydrolysis on a pilot-scale. Peptide and free amino acid contents of SOP were 82.5 ± 1.13 % and 3.7 ± 0.28 % respectively. The molecular weight distribution of SOP was mainly below 1,000 Da (85.4 %), 56.7 % of which were 140–500 Da. SOP showed strong stability to proteolytic digestion by pepsin and trypsin. The antioxidant activities and *in vitro* and *in vivo* antihypertensive effects of SOP were evaluated. Results showed that SOP exhibited 1,1-diphenyl-2-picrylhydrazyl radical scavenging effect ($IC_{50} = 4.5 \pm 0.13$ mg/mL), and significantly inhibited lipid peroxidation in linoleic acid oxidation system ($IC_{50} = 1.2 \pm 0.09$ mg/mL). SOP had potent angiotensin I-converting enzyme inhibitory activity ($IC_{50} = 1.1 \pm 0.06$ mg/mL), and antihypertensive effect in spontaneously hypertensive rats at a dose of 200 mg/kg. This study indicated that SOP could be a natural antioxidative or antihypertensive compound in the medicine and food industries.

Keywords Soybean · Hydrolysates · Antioxidant activity · Antihypertensive activity

Introduction

Digests from protein contained in foods have once been considered a sole supply of essential and nonessential amino acids and of the nitrogen indispensable to the biosynthesis of the proteins and nucleic acids in organisms. For the past two or three decades, however, attention has been increasingly paid to other functions of protein digests, especially to those of biologically active peptides derived from dietary proteins. By virtue of low side-effect profiles and beneficial bioactivities, many bioactive peptides have been isolated from various food materials, including casein (Xu et al. 2011), bovine blood (Kagawa et al. 1998), gelatin (Oshima et al. 1979), meat (Arihara et al. 2001), eggs (Yu et al. 2011) and various fish species like shrimp (Dey and Dora 2011), pink perch (Naqash and Nazeer 2011), tuna (Je et al. 2008), sardine (Khaled et al. 2011; Osajima et al. 2009), bonito (Fujita and Yoshikawa 1999) and salmon (Yang et al. 2010) as well as wheat (Koo et al. 2011; Matsui et al. 1999), corn (Lin et al. 2011), rice (Kannan et al. 2010) and microalgae (Lu et al. 2010). *In vitro* and *in vivo* studies have demonstrated that such peptides can play important biological roles in animals and in human, including antihypertension (Lin et al. 2011; Lu et al. 2011), antioxidation (Je et al. 2008; Liu et al. 2010), cancer prevention (Kannan et al. 2010), antihyperlipidemia (Kagawa et al. 1998), immunoregulation (Yang et al. 2010), and other physiological effects.

Soybean, an important and widely-used source of food proteins, has received increasing interests from the public because of its reported health benefits and health claims approved in many countries. Representatively, the United States' Food and Drug Administration authorized that "25 g of soybean protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease" (U.S.

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Food and Drug Administration 1999) and the United Kingdom's Joint Health Claim Initiative approved that "the inclusion of at least 25 g of soybean protein per day, as part of a diet low in saturated fat, can help reduce blood cholesterol levels" (Xiao 2008). Moreover, soybean is also a potential source of bioactive peptides. By the process of enzymatic hydrolysis or microbial fermentation, many bioactive peptides have been isolated and/or sequenced from soybean protein and traditional fermented soybean products, such as tofuyo (Kuba et al. 2003), soybean paste (Inoue et al. 2009), and soybean sauce (Nakahara et al. 2010), with various functional properties mentioned above.

However, many existing findings are only based on researches carried out under a laboratory condition and few studies were reported regarding the production and bioactive properties of soybean peptide on a pilot-scale. The aims of the present study were therefore to develop a pilot-scale production process of soybean oligopeptides (SOP) and to investigate the composition, antioxidant activity and in vitro and in vivo antihypertensive effects of SOP.

Materials and methods

Materials

Soybean protein isolates (SPI) were obtained from JILIN FUJI Protein Co. LTD (Jilin, China). Alcalase 2.4 L and Protex 13 FL were purchased from Novozymes Biological Co. (Tianjin, China) and Genencor Division of Danisco (Wuxi, China), respectively. The ACCQ-Fluor Reagent kit and amino acid standards were from Waters (Milford, MA, USA). Tertiary-butylhydroquinone (TBHQ) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of reagent grade or better.

Pilot-scale production of SOP

SPI (100 kg) was dispersed in water at a ratio of 7:100 (w/w) in a feed tank and stirred with a homogenizer (Donghua Homogenizer Factory, Shanghai, China) at 26,000 g for 10 min, then the solution was pumped into a 1,000 L thermostatically stirred-batch reactor (Dongding Machinery Co., Wenzhou, China). The solution was adjusted to pH 8.5 with 20 % NaOH and heated to 60 °C. Alcalase 2.4 L was added into the solution at a ratio of 600,000 U/kg (enzyme/protein substrate) and the hydrolysis was kept at pH 8.5 by continuous addition of 20 % NaOH. The degree of hydrolysis (DH) of soybean protein was calculated by using the pH stat method (Adler-Nissen 1986). After the DH reached around 10–15 %, the suspension was cooled down to 50 °C and added with Protex 13 FL at a ratio of

200,000 U/kg (enzyme/protein substrate). Then, the mixture was incubated at 50 °C until the DH reached 20–25 %. The reaction was stopped by heating the mixture to 90 °C for 15 min to inactivate the enzyme, and the resulting hydrolysate was centrifuged at 15,000 g for 10 min (SYGQ105 tube centrifuge, Shanghai Shiyuan Bioengineering Equipment Co., Shanghai, China).

The supernatant was filtered with a UF-5000 ultrafiltration equipment (molecular weight cut-off 5,000 Da, Xinda Membrane Tech. Co. Hefei, China) and then evaporated with a double-effect falling film evaporator (OE2, OECH Machinery Equipment Co., Ltd, Hefei, China) at 0.10 ± 0.02 MPa and 60 ± 5 °C, until the solid content of the concentrated liquid reached 30–40 %. The concentrated solution containing peptides was dried with a spray drier (YG30, Wuxi City Sunlight Drier Factory, Wuxi, China) at a 15 kg/h flow rate with inlet temperature of 160–180 °C and outlet temperature of 80–90 °C. The SOP powder (65 kg) was packaged and stored at room temperature for further analysis.

Composition of SOP

The moisture and the ash content were determined by standard methods (AOAC 2000). Crude protein content is expressed as total nitrogen (N) $\times 6.25$, of which the nitrogen content was determined by the Kjeldal method (AOAC 2001).

Since the macromolecular SPI would not be easily dissolved under acidic condition whereas the oligopeptides would, we calculated the peptide content as the total content of trichloroacetic acid (TCA) soluble proteins minus the content of free amino acids. For determination of the total content of TCA soluble proteins, 2.00 g of the sample was dissolved in 10 mL of 15 % TCA. The solution was allowed to stand for 5 min and then centrifuged at 4,000 rpm for 4 min using a LG10-2.4A centrifuge (Beijing LAB Centrifuge Co., Beijing, China). The total content of TCA soluble proteins was then measured and calculated as 6.25 folds of the nitrogen content in the supernatant.

The amino acid composition of SOP was determined with a Hitach 835–50 amino acid analyzer (Hitach Co., Tokyo, Japan) by using the method described by Yang et al. (2007). For determination of free amino acids content, 2.00 g of SOP was dissolved in 10 mL of 5 % TCA. After filtration and centrifugation, the supernatant was taken for derivatization with an ACCQ-Fluor Reagent kit (Waters, Milford, MA). The derivatives were then separated and analyzed by high-performance liquid chromatography (HPLC) with a C₁₈ column (Inertsil ODS-SP, 4.6 $\times$ 250 mm, 5 μ m, GL). A nonlinear gradient with a proportion of triethylamine buffer (A) versus 60 % acetonitrile (B) was employed as follows: 0–0.5 min, 2 % B; 0.5–4.0 min, 2–5 % B; 4.0–17 min, 5–10 % B; 17–24 min, 10–13 % B; 24–

35 min, 13–18 % B; 35–45, 18–21 % B; 45–90 min, 21–36 % B. The flow rate was 1 mL/min and the effluent was monitored continuously by a fluorescence detector at excitation wavelength of 250 nm and emission wavelength of 395 nm.

Molecular weight (MW) distribution

The MW distribution of SOP was determined with gel permeation chromatography (GPC) on a LC-20A HPLC system. A TSK-GEL G2000SWXL column (7.8×300 mm, Tosoh, Tokyo, Japan) was equilibrated with a mobile phase containing 45 % acetonitrile with 0.1 % trifluoroacetic acid (TFA). Samples were dissolved in the mobile phase at a concentration of 2 mg/mL, and then filtered through a 0.22 µm polyvinylidene fluoride PVDF membrane. Elution was performed isocratically with the same mobile phase at a flow rate of 0.5 mL/min and monitored at 220 nm. A molecular weight calibration curve was prepared from the average retention time of the following standards: cytochrome C (12.5 kDa), aprotinin (6.5 kDa), bacitracin (1,450 Da), tetrapeptide GGYR (451 Da), and tripeptide GGG (189 Da).

Stability of SOP after digestion in vitro

For determination of stability to pepsin digestion, 100 mg of pepsin was dissolved in 10 mL of 0.05 M HCl-KCl buffer (pH 1.7) and then added with 1.00 g of SOP. Afterwards, 5 mL of the solution was immediately transferred into another tube as control and inactivated in boiling water for 10 min. The control and another 5 mL of the solution was incubated at 37 °C for 3 h, and filtered through a 0.22 µm PVDF membrane after diluted 20 times. The filtrates were then subjected to reversed phase HPLC (RP-HPLC) on an XBridge BEH130 C₁₈ column (5 µm, 4.6×250 mm), and detected at 220 nm to determine the change of the eluting chromatogram. The samples were eluted at a flow rate of 0.6 mL/min with a nonlinear gradient with solvent A (0.1 % TFA in Milli-Q water, v/v) and solvent B (80 % acetonitrile with 0.1 % TFA, v/v) as follows: 0–10 min, 0–5 % B; 10–25 min, 5–9 % B; 25–30 min, 9–11 % B; 30–50 min, 11 % B; 50–120 min, 11–40 % B; 120–135 min, 40–50 % B. For stability to trypsin digestion, 200 mg of trypsin was dissolved in 10 mL 0.05 M KH₂PO₄-NaOH buffer (pH=6.8). Other procedures were the same as in the pepsin digestion.

DPPH radical scavenging assay

The DPPH radical scavenging activity of SOP was measured by the method described by Wu et al. (2003) with slight modification. A volume of 2 mL of sample solution and 2 mL of DPPH solution (2×10^{-4} mol/mL in 95 % ethanol)

were mixed and incubated in dark at 25 °C for 30 min. TBHQ was used as the control. The absorbance was measured at 517 nm and the inhibition rate (namely DPPH radical scavenging activity) was calculated as follows: Inhibition rate(%) = $[1 - (A_i - A_j)/A_0] \times 100$, where A_i was the absorbance of the mixture of SOP solution and DPPH solution, A_j was the absorbance of the mixture of SOP solution and 95 % ethanol, and A_0 was the absorbance of the mixture of DPPH solution and 95 % ethanol. The IC₅₀ value was defined as the concentration of SOP solution with an inhibition rate of 50 %.

Lipid peroxidation inhibition assay

A modified method based on Chen et al. (1995) was used to measure the antiperoxidation activity of SOP. Briefly, 1.5 mL of 0.05 M sodium phosphate buffer (pH 7.0) and 100 µL of 20 mM linoleic acid in 99.5 % ethanol were mixed and then added with 100 µL of SOP solution (or TBHQ as the control) and 25 µL of 20 mM FeCl₂-EDTA. The mixed solution was incubated in dark at 50 °C for 2 h, and a 100 µL aliquot of the solution was mixed in sequence with 2 mL of 75 % ethanol, 100 µL of 30 % ammonium thiocyanate (v/v) and 50 µL of 20 mM ferrous chloride solution in 3.5 % HCl (v/v). After vortexed vigorously, the mixture was allowed to react for 3 min. The absorbance was measured at 480 nm and the antiperoxidation activity was calculated as follows: Lipid peroxidation inhibition activity (%) = $[(A_C - A_S)/A_C] \times 100\%$, where A_C and A_S were the absorbance of the reaction system in the absence and presence of SOP or the control, respectively. The IC₅₀ value was defined as the concentration of SOP solution with an inhibition rate of 50 %.

Angiotensin I-converting enzyme (ACE) inhibition assay

ACE was prepared according to Megías et al. (2004) with slight modifications. Fresh porcine lungs (200 g) were washed with 0.2 M boric acid buffer (1:5, pH 8.2) at 4 °C to remove blood vessels and connective tissues, and then diced and homogenized by a Waring blender with the same buffer at 4 °C for about 2 min. The homogenate was kept still for 3 h and centrifuged at 9,000 rpm for 20 min. Then, the resulting supernatant (about 825 mL) was mixed with (NH₄)₂SO₄ to 35 % of saturation in an ice-cold water bath. After standing at 4 °C for 3 h, the mixture was centrifuged at 9,000 rpm for 20 min, and the resulting supernatant was mixed with (NH₄)₂SO₄ to 55 % of saturation. The mixture was kept still at 4 °C overnight and centrifuged at 9,000 rpm for 20 min on the next day. The precipitate dissolved in 0.2 M boric acid buffer (1:5, pH 8.2) was dialyzed in dialysis bags with the cut-off molecular weight of 14,000±

2,000 Da for 3 days, and finally the ACE enzyme solution was obtained.

ACE activity was determined as described by Vermeirssen et al. (2002) with some modifications. The substrate solution (0.001 M) which simulated angiotensin I was prepared by dissolving a furanacryloyl tripeptide (FAPGG) in 0.2 M boric acid buffer (pH 8.2). Then 150 μ L of substrate solution was mixed with 200 μ L of inhibitor solution (SOP or the control captopril), followed by addition of 150 μ L of ACE solution containing 0.3 M NaCl to initiate the reaction. The initial velocities (v_0) were calculated from the decrease in absorbance at 340 nm for 10 min, and the inhibitory activity was calculated as follows: ACE inhibitory activity(%) = $[1 - (v_0 \text{ with inhibitory peptide}) / (v_0 \text{ without inhibitory peptide})] \times 100\%$. The IC_{50} value was defined as the concentration of SOP solution with an inhibition rate of 50 %.

Antihypertensive effect in vivo

Twenty male spontaneously hypertensive rats (SHR) weighing 280–300 g (12 weeks old), were randomly divided into two groups ($n=10$), namely SHR-SOP group and SHR control group. Meanwhile, 21 male Wistar rats weighing 300–320 g were randomly divided into two groups, namely Wistar-SOP group ($n=11$) and Wistar control group ($n=10$). All the rats were provided by Institute of Laboratory Animal Science, Chinese Academy of Sciences (certificate NO. SCXK11-00-0006). The care and treatment of the rats were in accordance with international guidelines for laboratory animals, and the experiment was approved by the local ethics committee.

The animals were acclimatized in rat cages (five rats in each) with free access to food and water in an air-conditioned room (21–24 °C) with a 12 h light and dark cycle for 1 week before beginning the experiments. The SOP solution (0.02 g/mL) was prepared by dissolving SOP in distilled water. Two experimental groups were orally administrated with the SOP solution at a dose of 200 mg/kg daily. The SHR and Wistar control groups were administrated with 0.9 % saline instead of SOP solution in the same manner. The systolic blood pressure (SBP) was measured by a tail-cuff method (RBP-I blood pressure gauge, China-Japan Friendship Hospital, Beijing, China) every week during 6 weeks of treatment.

Statistical analysis

All measurements were taken in triplicates for each sample. Data were statistically analyzed (SPSS 14.0; SPSS, Cary, NC, USA) by independence t tests and paired sample t tests. Differences were considered statistically significant at $P<0.05$.

Results and discussion

Composition of SOP

The composition analysis showed that SOP contained 92.2 ± 1.23 % protein (on a dry basis, $N \times 6.25$), 86.2 ± 1.02 % TCA soluble protein, 3.7 ± 0.28 % free amino acids, namely 82.5 ± 1.13 % peptides, 0.24 ± 0.04 % fat, 0.53 ± 0.06 % carbohydrate, 3.8 ± 0.23 % ash and 4.2 ± 0.31 % moisture. The substrate SPI was composed of 92.6 ± 1.28 % protein, 0.23 ± 0.02 % TCA soluble protein, 1.6 ± 0.12 % fat, 2.0 ± 0.19 % carbohydrate, 1.0 ± 0.08 % ash and 3.2 ± 0.26 % moisture. The results indicated that pilot-scale production was effective in removing most of fat and carbohydrate. However, the higher ash content of SOP possibly was due to the sodium hydroxide added to the reactor to keep the pH constant at a special value during the enzymatic hydrolysis process. This problem might be resolved by adding a desalting step such as nanofiltration in the production. The amino acid profile of SOP was not significantly different from that of SPI (Table 1), indicating that enzymatic hydrolysis did not significantly alter the amino acid composition of soybean proteins. Amino acid analysis

Table 1 Amino acid (AA) composition and free amino acid (FAA) composition of SOP, as well as AA composition of SPI (% w/w)^{a, b}

Amino acid	SOP		SPI
	AA	FAA	AA
Aspartic acid ^c	10.6 $\pm$ 0.25	0.16 $\pm$ 0.03	10.9 $\pm$ 0.28
Glutamine acid ^d	19.3 $\pm$ 0.42	0.37 $\pm$ 0.07	19.6 $\pm$ 0.43
Serine	6.6 $\pm$ 0.17	0.08 $\pm$ 0.01	6.1 $\pm$ 0.15
Histidine	1.8 $\pm$ 0.03	0.14 $\pm$ 0.02	2.1 $\pm$ 0.04
Glycine	4.3 $\pm$ 0.08	0.16 $\pm$ 0.03	4.5 $\pm$ 0.09
Threonine	2.8 $\pm$ 0.05	0.12 $\pm$ 0.02	3.4 $\pm$ 0.07
Arginine	6.0 $\pm$ 0.13	0.03 $\pm$ 0.00	6.4 $\pm$ 0.14
Alanine	3.7 $\pm$ 0.07	0.28 $\pm$ 0.05	4.4 $\pm$ 0.08
Tyrosine	2.4 $\pm$ 0.05	0.44 $\pm$ 0.08	3.0 $\pm$ 0.06
Cysteine	1.1 $\pm$ 0.02	0.01 $\pm$ 0.00	1.6 $\pm$ 0.04
Valine	4.2 $\pm$ 0.08	0.47 $\pm$ 0.09	5.4 $\pm$ 0.11
Methionine	0.79 $\pm$ 0.01	0.07 $\pm$ 0.01	0.81 $\pm$ 0.02
Phenylalanine	3.7 $\pm$ 0.08	0.49 $\pm$ 0.10	4.3 $\pm$ 0.09
Isoleucine	3.2 $\pm$ 0.07	0.15 $\pm$ 0.03	3.7 $\pm$ 0.08
Leucine	4.5 $\pm$ 0.09	0.60 $\pm$ 0.11	4.7 $\pm$ 0.10
Lysine	4.6 $\pm$ 0.09	0.09 $\pm$ 0.01	4.9 $\pm$ 0.10
Proline	4.9 $\pm$ 0.10	0.08 $\pm$ 0.01	5.1 $\pm$ 0.11
Tryptophan	0.25 $\pm$ 0.01	0.00 $\pm$ 0.00	0.79 $\pm$ 0.01
Total	84.7 $\pm$ 0.11	3.7 $\pm$ 0.05	91.7 $\pm$ 0.12

^a SOP soybean oligopeptides; SPI soybean protein isolates

^b $n=3$

^c Aspartic acid+asparagine

^d Glutamine acid+glutamine

revealed that SOP was rich in glutamic acid, aspartic acid, serine, arginine and proline. Free amino acid content of SOP was only 3.7 %, which indicated that SOP was of high quality.

Molecular weight distribution of SOP

The gel permeation chromatographic data of SOP are shown in Fig. 1 and Table 2, and the retention time of molecular weight standards is listed in Table 3. As shown in Fig. 1, seven peaks were occurred in the chromatogram which represented seven main fractions in SOP. The results (Table 2) showed that much of the MW distribution of SOP fell bellow 1,000 Da (85.4 %), and particularly 56.7 % of the peptides were 140–500 Da, suggesting that SOP was mainly composed of short-chain peptides (2–6 amino acid residues). This indicated that pilot-scale production was effective in producing low-molecular-weight oligopeptides and in removing large peptides or undigested proteins. Short-chain peptides, especially di- and tripeptides, were reported to be absorbed more rapidly than free amino acids, and the absorption intensity of peptide amino acids was 70 to 80 % higher than for amino acids provided in a corresponding amino acid mixture (Webb 1990). Therefore, the molecular weight distribution characteristic of SOP indicated that it might be used as a great supplemental dietary protein resource.

Stability of SOP

The chromatogram of SOP before and after in vitro digestion with pepsin and trypsin was showed in Fig. 2, and neither distinct new peak nor distinct change of peak height was observed. SOP showed strong stability to proteolytic digestion by pepsin and trypsin. The similarity of the chromatogram of SOP before and after pepsin and trypsin

Table 2 Molecular weight distribution of soybean oligopeptides (SOP) by size exclusion chromatography

Molecular weight (Da)	Start time (min)	End time (min)	Percentage of peak area (%)
>10000	8.8	13.7	0.05
3000–10000	13.7	16.3	3.6
1000–3000	16.3	18.7	10.9
500–1000	18.7	20.2	25.1
140–500	20.2	22.9	56.7
<140	22.9	28.4	3.6

digestion was 99.1 % and 94.9 % respectively as analyzed by the evaluation software, indicating that no distinct degradation of SOP happened during the in vitro digestion. Our results corresponded with those of previous reports (Chiang et al. 2006; Zhong et al. 2007), which reported that gastrointestinal proteases had very little effect on the ACE inhibitory activity of 10 KDa permeates of soybean protein hydrolysate in vitro or on the cholesterol micellar solubility inhibitory rate of soybean protein Alcalase hydrolysate. These results suggested that SOP might not be hydrolyzed to free amino acids in the gastrointestinal tract and hence reserved its bioactive form after administered as a food ingredient. These findings are in accordance with that reported by Yu et al. (2006) showing the stability of oligopeptides-enriched globin hydrolysate against gastrointestinal proteases.

Antioxidant activities of SOP

The DPPH free radical scavenging activity and the lipid peroxidation inhibitory activity of SOP were investigated as compared to the control TBHQ by using UV spectrophotometry, respectively. SOP exerted dose-dependent effects to suppress DPPH radical and inhibited lipid peroxidation, with IC_{50} values of 4.5 ± 0.13 mg/mL and 1.2 ± 0.09 mg/mL respectively (Table 4). Compared to other studies, SOP exhibited comparable DPPH free radical scavenging activity to wheat germ protein hydrolysate studied by Zhu et al. (2006). The lipid peroxidation inhibitory activity of SOP was higher than that of hydrolysates from soybean and corn gluten meal studied by Chen et al. (1996) and Li et al. (2008), respectively. The results revealed that SOP potentially contained substances which could act as free radical scavengers or hydrogen donors to terminate the radical chain reaction (Kedare and Singh 2011). It was not surprising that TBHQ had much lower IC_{50} values than those of SOP in both assays (Table 4), since TBHQ is a highly effective synthetic antioxidant widely used for stabilizing various oils, fats and foods against oxidative deterioration.

Fig. 1 Size exclusion chromatography of soybean oligopeptides (SOP) on a TSK-GEL G2000SWXL column (7.8×300 mm) eluted in 45 % acetonitrile with 0.1 % trifluoroacetic acid at a flow rate of 0.5 mL/min. Values above the peaks indicate molecular weights

Table 3 Molecular weight and retention time of molecular weight standards

	Cytochrome C	Aprotinin	Bacitracin	Tetrapeptide	Tripeptide GGG
Molecular weight (Da)	12500	6500	1450	451	189
Logarithm of molecular weight	4.1	3.8	3.2	2.7	2.3
Retention time (min)	13.2	14.6	18.0	20.2	22.4

However, there are safety concerns over the use of synthetic antioxidants as food additives (Byun et al. 2009). Protein hydrolysates or peptides have been regarded as non-hazardous natural antioxidants.

The antioxidant activities of peptides were highly influenced by molecular mass and molecular structure properties (Sheih et al. 2009). It has been reported that bioactive peptides usually contain 2–20 amino acid residues and the lower the molecular weight the higher their chance to cross the intestinal barrier and exert biological effects (Byun et al. 2009; Roberts et al. 1999). In our study, much of the MW

distribution of SOP fell below 1,000 Da, possibly accounting for its high antioxidative activity. Moreover, SOP was rich in aromatic amino acid, proline, alanine, and leucine, which possess antioxidative activities against free radicals (Xu et al. 2009).

ACE inhibitory activity in vitro

Table 4 also showed the ACE inhibitory activities of SOP and the control captopril. Captopril is a typical ACE inhibitor used widely to treat hypertension in clinical practice, with an IC_{50} value reported to range from 0.0004×10^{-3} to 0.0050×10^{-3} mg/mL. Our result was close to the reported range, though a little higher possibly due to the detection precision of the measurement system. Although the IC_{50} value of SOP (1.1 ± 0.06 mg/mL) was much higher than that of captopril, which suggested a much lower ACE inhibitory activity in vitro, this value was in accordance with other food protein hydrolysates such as sardinelle by-product protein hydrolysates (1.2 mg/mL) and corn oligopeptides (1.020 mg/mL) (Bougatef et al. 2008; Lin et al. 2011). Regarding to the relationship between structure and activity of ACE inhibitory peptides, Fang et al. (2008) have reported that those peptides contained proline, leucine, alanine and phenylalanine showed highly potent inhibitory activity. SOP was rich in these amino acids, which revealed that these amino acids were likely responsible for a considerable fraction of the ACE inhibitory action of SOP. Zhang et al. (2009) have showed that short-chain peptides exhibit the most potent ACE inhibitory activity. These studies indicated that the potent ACE inhibitory activity of SOP might be related to its high content of certain amino acids and the low-molecular-weight oligopeptides.

Researchers have reported that compared with the synthetic inhibitor captopril, ACE inhibitory peptides obtained from enzymatic hydrolysates of food proteins presented much higher activities in vivo than expected by in vitro activities. Therefore, the ACE inhibitory activity of SOP suggested a possible antihypertension effect in vivo and made the animal experiment necessary.

Antihypertensive effect in vivo

The antihypertensive effect of orally administrated SOP in vivo was evaluated by measuring changes in the SBP of SHR

Fig. 2 Reversed phase high-performance liquid chromatography (RP-HPLC) chromatogram of soybean oligopeptides (SOP) before and after (a) pepsin and (b) trypsin digestion. Separation was carried out on an XBridge BEH130 C18 column (5 μ m, 4.6 $\times$ 250 mm) using a nonlinear gradient of solvent A (0.1 % trifluoroacetic acid in Milli-Q water, v/v) and solvent B (80 % acetonitrile with 0.1 % trifluoroacetic acid, v/v) at a flow rate of 0.6 mL/min

Table 4 The IC_{50} (mg/mL) of SOP and the control in DPPH radical scavenging activity, lipid peroxidation inhibitory activity, and in vitro ACE inhibitory activity^{a, b}

Sample	Antioxidant activity (IC_{50} °)		ACE inhibitory activity (IC_{50} °)
	DPPH radical scavenging activity	lipid peroxidation inhibitory activity	
SOP	4.5 ± 0.13	1.2 ± 0.09	1.1 ± 0.06
TBHQ	$(7.7 \pm 0.24) \times 10^{-3}$	0.14 ± 0.02	—
Captopril	—	—	$(0.013 \pm 0.001) \times 10^{-3}$

^a SOP soybean oligopeptides; SPI soybean protein isolates; DPPH 1,1-diphenyl-2-picrylhydrazyl; ACE angiotensin I-converting enzyme inhibitory activity; TBHQ tertiary-butylhydroquinone

^b $n=3$

^c IC_{50} values were defined as the concentration of an inhibitor with an inhibition rate of 50 %

and Wistar rats. As shown in Fig. 3, oral administration of SOP significantly reduced the SBP of SHR rats after administration for 1 weeks compared to that before administration. As the extension of the experimental period, the SBP of the SHR control group gradually increased, and after 4 weeks, the SBP of the SHR control group was significantly higher than that before administration. Meanwhile, the SBP of the SHR-SOP group was significantly lower than that of the SHR control group on the 1st, 2nd, 4th, 6th weeks, indicating that SOP supplementation could effectively prevent the increase of SBP in SHR and possessed a clear antihypertensive effect. The results were in agreement with previous studies (Lin et al. 2011; Miguel et al. 2009), which also showed the antihypertensive effect of protein hydrolysates in SHR. The antihypertensive effect on SHR is probably due to the ACE inhibitory peptides from protein hydrolysates, which was demonstrated

in the study of Wu and Ding (2002). They characterized the soybean protein-derived ACE inhibitory peptides and concluded that the most economic way to market the peptides was as contained in the crude protein hydrolysates for consumption on a long-term basis for desired therapeutic effects.

As for the Wistar-SOP group, the SBP maintained an approximately stable tendency and had a slight decrease but no significant differences in the treatment period except the last week. No significant differences were observed for the SBP of the Wistar-control group compared to that before administration, except that the SBP was significantly lower on 3rd week than that before administration. Meanwhile, there was no significant difference between the SBP of the Wistar-SOP group and the Wistar control group. Our results suggested SOP supplementation had no remarkable effect on the SBP of Wistar rats, but showed a slight SBP-lowering trend as the extension of treatment. The results indicated that SOP might not influence the blood pressure of normal persons. However, the exact effect of SOP supplementation on Wistar rats needed further study.

Conclusions

Our study demonstrated that SOP produced at pilot-scale was mainly composed of short-chain peptides and maintained a high stability against digestion with pepsin and trypsin in vitro. SOP possessed DPPH radical scavenging capacity, and significantly inhibited lipid peroxidation in linoleic acid oxidation system. In vitro and in vivo tests showed that the SOP had potent antihypertensive effect. Based on these results, it could be concluded that the pilot-scale production of SOP is a practice way to utilise soybean protein. This study suggested that SOP might be useful as food additives, dietary nutrients and pharmaceutical agents. Further works should be done to isolate and characterize potent antioxidative peptides and antihypertensive peptides from SOP.

Fig. 3 Change in systolic blood pressure of spontaneously hypertensive rats and Wistar rats by administering soybean oligopeptides (SOP). Oral administration of SOP solution (0.02 g/mL) or 0.9 % saline was performed at a dose of 200 mg/kg body weight. Systolic blood pressure was measured every week during 6 weeks of treatment. $n=10$ rats. Asterisks indicate significant differences compared to corresponding control (* $P<0.05$, ** $P<0.01$)

Acknowledgments This study has received financial support from the Special Foundation for Research Institute Technical Development Research Program of China (No. 2011EG111236) and the Introduction of Foreign Technical and Managerial Talents Program from State Administration of Foreign Experts Affairs of China (20110440003). We also thank the staff of Institute of Cardiovascular Sciences of Peking University for technology assistance in antihypertensive effect determination.

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