Antioxidative Peptides and Angiotensin I-converting Enzyme Inhibitory Peptides Obtained from Soybean by Protease Treatment at Pilot Scale

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Abstract: A pilot-scale production of soybean oligopeptides (SOPs) was developed in this study, and the SOPs had a high protein content (91.45%) and low molecular weight (84.37% of the product were less than 1000 u). The *in vitro* antioxidant activity and angiotensin I-converting enzyme (ACE) inhibitory activity of SOPs were analyzed. SOPs were then separated by reversed-phase high performance liquid chromatography, and six major fractions (numbered from 1–6) were collected and subjected to mass spectrometry to identify the active peptides. The sequences of 41 peptide fragments were identified, and 15 peptide fragments were selected to evaluate the antioxidative and ACE inhibitory activities using the aforementioned methods. The results showed that SOPs contained two novel potent antioxidative peptide fragments (Tyr-Glu, 8.61 ± 0.42 mmol Trolox equivalents/g sample; Asp-Tyr-Arg, 6.53 ± 0.34 mmol Trolox equivalents/g sample) and four potent ACE inhibitory peptide fragments (Leu-Val-Arg, IC_{50} = 51.75 μM; Leu-Tyr, IC_{50} = 305.76 μM; Asp-Tyr-Arg, IC_{50} = 1082.95 μM; Asp-Phe, IC_{50} = 1106.04 μM). Most of them were novel antioxidant or ACE inhibitory peptides derived from soybeans. This study suggested that SOPs and their active peptides might be used as antioxidative or hypotensive substances in food additives, dietary nutrients, and pharmaceutical agents.

Key words: angiotensin-converting-enzyme inhibitor (ACE) inhibitory activity; antioxidant activity; enzymatic hydrolysis; low-molecular-weight peptides; soybean

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中试规模酶解制备的大豆低聚肽中抗氧化肽和 ACE 抑制肽的分离和鉴定

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摘要：本研究开发了一种通过中试规模制备大豆低聚肽的方法，制备出的大豆低聚肽具有较高的蛋白含量（91.45%）和较低的分子量（84.37%的分子量小于 1000 u）。测定了大豆低聚肽的抗氧化和血管紧张素转化酶（ACE）抑制活性，然后利用反相高效液相色谱对其进行分离纯化，收集六个主要的液相组分，分别命名为组分 1~6，利用质谱仪对这些组分进行肽段结构鉴定。共鉴定出 41 个肽段结构，选择 15 个肽段进行抗氧化和 ACE 抑制活性评价。结果表明，大豆低聚肽中有两个新型抗氧化肽段和四个 ACE 抑制肽段。抗氧化肽段分别为：Tyr-Glu，8.61±0.42 mmol Trolox 等同物/g 样品；Asp-Tyr-Arg，6.53±0.34 mmol Trolox 等同物/g 样品；ACE 抑制肽段分别为：Leu-Val-Arg，IC_{50}=51.75 μM；Leu-Tyr，IC_{50}=305.76 μM；Asp-Tyr-Arg，IC_{50}=1082.95 μM；Asp-Phe，IC_{50}=1106.04 μM。这些肽段大多数是从大豆中新发现的抗氧化肽段和 ACE 抑制肽段。大豆低聚肽和这些活性肽段可作为抗氧化或降血压物质用于食品添加剂、营养物及医药制品中。

关键词：ACE 抑制活性；抗氧化活性；酶解；低聚肽；大豆

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Recently, peptides from enzymatic hydrolysates of food proteins have received greater attention from food scientists than ever before. Many bioactive peptides, with health benefits such as antioxidant, antihypertensive, antimicrobial, opioid, immunomodulatory, or hypocholesterolemic activity, have been identified from food protein hydrolysates[1~6]. Among bioactive components, those with antioxidant activity or antihypertensive effect have been the most extensively studied, due to their potential beneficial effects related to many diseases. Interesting phenomena is that some of the peptides have multifunctional properties, such as antioxidant capacity and antihypertensive effect.

Reactive oxygen species (ROS) have been shown to be associated with the aetiology and pathogenesis of human physiological and disease conditions such as cardiovascular diseases, cancer, aging and diabetes mellitus, due to their roles in the oxidative degradation of biological macromolecules[7~9]. Meanwhile, lipid oxidation occurring in food products causes deteriorations of food quality and shortening of shelf life[7]. Artificial antioxidants exhibit strong antioxidant activity, but their use is strictly regulated because of potential health hazards. Therefore, research and development to find safer, innovative and economical antioxidants is necessary for the enhancement of the body's antioxidant defenses through dietary supplement and lipid oxidation inhibition in foods. A variety of antioxidant peptides have been purified and identified in hydrolysates from food proteins such as egg[10], ham[11] and mushroom[12].

Hypertension is the most common serious chronic health problem throughout the world, affecting 15~20% of adults. It has been considered as a risk factor for developing cardiovascular diseases (arteriosclerosis, stroke and myocardial infarction) and end-stage renal disease[13,14]. Angiotensin I-converting enzyme (EC3.4.15.1; ACE) plays a key role in the rennin-angiotensin system, which regulates human blood pressure. Inhibition of ACE prevents conversion of decapeptide angiotensin I to the potent vasoconstrictor octapeptide angiotensin II, making it one of the most effective methods for suppressing increase in blood pressure[15]. ACE inhibitory drugs have demonstrated their usefulness, however, they are not entirely without side effects, such as coughing, allergic reactions and skin rashes[16]. Many reports have been published on ACE inhibitory peptides from various food proteins, such as corn[15], chicken[17] and walnut[18].

Soybean, from the most cultivated plant in the world, is rich in proteins (40~50%), lipid (20~30%) and carbohydrates (26~30%), and consequently the subjected of extensive scientific research[19]. As a protein source, soybean protein has a good balance of essential amino acids, and is reported to have the nutritional value that is equal to that of animal protein[20]. Many researchers have tried to utilized soybean protein as a source of bioactive peptides to enhance the value of the soybean hydrolesates. For example, Dia et al.[21] reported the anti-inflammatory activity of cancer preventive peptide lunasin derived from soybean. Wu et al. [22]used alcalase enzyme to produce ACE inhibitory peptides from soybean protein. Nevertheless, to the authors' knowledge, 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.

We have reported 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[22]. However, separation and purification, structure identification and active peptides of SOP were not studies. In the present study, a new practical, pilot-scale production of SOP having high protein content and low molecular weights was developed. The antioxidant activity and ACE inhibitory activity of SOP obtained by enzymatic treatment were investigated. Furthermore, antioxidant peptides and ACE inhibitory peptides were isolated from SOP and characterized.

1 Materials and methods

1.1 Materials and reagents

Isolated soy protein (ISP) was supplied by CF Haishi Biotechnology Co. Ltd. (Beijing, China). Acalase 2.4 L and Protex 13 FL were purchased from Novozymes Biological Co. (Tianjin, China) and Genencor Division of Danisco (Wuxi, China), respectively. Hippuryl-histidyl-leucine (HHL), hippuric acid (HA), ACE (from rabbit

lung) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Acetonitrile and methanol were bought from Fisher Scientific (Pittsburgh, PA). Trifluoroacetic acid (TFA) was purchased from Alfa Aesar. The synthesized peptides were obtained from Scilight Biotechnology Co., Ltd (Beijing, China). The purity of peptide synthesized was more than 98% by high performance liquid chromatography (HPLC) analysis. All other reagents were of analytical or guaranteed reagent grade.

1.2 Enzymatic hydrolysis and preparation of SOP

ISP (150 kg) was dissolved in distilled water at a ratio of 8:100 and stirred with a homogenizer (Donghua Homogenizer Factory, Shanghai, China) at 25,000 g for 10 min (SYGQ105 tube centrifuge, Shanghai Shiyuan Bioengneering Equipment Co., Shanghai, China). The protein slurry was first digested by Alcalase 2.4 L using enzyme to substrate protein ratio of 1:100 (*m/m*) at pH 8.5 and a temperature of 60 ℃ for 2 h. The resulting mixture was further digested by Protex 13 FL (1:100, *m/m*) at pH 8.5 and 50 ℃ for 2 h. Hydrolysis was carried out in a thermostatically stirred-batch reactor (Dongding Machinery Co., Wenzhou, China). The pH was maintained constant during hydrolysis by continuous addition of 1 M NaOH. At the end of the reaction, the hydrolysis was stopped by heating at 95 ℃ for 10 min in a waterbath. The hydrolysate was centrifuged (LG10-2.4A, Beijing LAB Centrifuge Co. Ltd, China) at 3,000 g for 25 min. The supernatant was passed through 10 and 1 ku molecular weight cut-off ceramic membranes (Filter and Membrane Technology Co. Ltd, Fujian, China) successively to obtain the permeate liquid with low-molecular-weight peptides. Afterwards, the permeate liquid was dialysed through a 100 u MWCO nanofilter membrane (Filter and Membrane Technology Co. Ltd, Fujian, China) at pH 7.0±0.5 to remove residual salt. Then, the retentate was evaporated by an R-151 rotavapor (BUCHI Co. Ltd, Switzerland) until the solid content of the concentrated liquid reached 30~40%. Finally, the concentrated liquid was spray dried using an L-217 Lab spray dryer (Beijing Laiheng Lab-Equipments Co. Ltd, China). The SOP powder (37.5 kg) obtained was stored in a desiccator for further analysis. The preparation process of SOP was undertaken repeatedly and three independent batches of ISP were analysed.

1.3 Chemical composition and amino acid composition assay

Chemical composition of SOP was analyzed according to the methods of the Association of Official Analytical Chemists[23]. Amino acid composition was determined by an 835-50 automatic amino acid analyzer (Hitach, Ltd., Tokyo, Japan) according to the method of Yang et al.[24].

1.4 Determination of molecular weight distribution

Molecular weight distribution was evaluated using a LC-20A high performance liquid chromatography (HPLC) system (Shmadzu, Kyoto, Japan) equipped with a TSK gel G2000 SWXL 300×7.8 mm column (Tosoh, Tokyo, Japan). The mobile phase used was acetonitrile/water (45:55, *V/V*) containing 0.1 % (*V/V*) trifluoroacetic acid. Samples were eluted at a flow rate of 0.5 mL/min and monitored at 220 nm at 30 ℃. Tripeptide GGG (Mr 189), tetrapeptide GGYR (Mr 451), bacitracin (Mr 1450), aprotinin (Mr 6500) and cytochrome C (Mr 12500) (Sigma Chemical Co., St. Louis, USA) were used as molecular weight standards.

1.5 Assay of DPPH radical scavenging activity

DPPH radical scavenging effect was measured according to the method of Wu, Chen and Shiau[25]. A volume of 1.5 mL of each sample with various solid concentrations was added to 1.5 mL of 0.1 mM DPPH in 95% ethanol. The mixture was shaken and left to stand for 30 min in the dark , and then the absorbance was measured at 517 nm (A_S) using an UV22100 spectrophotometer (UNICO Instrument Co., Ltd., Shanghai, China). Ethanol was used instead of the samples in control experiment (A_C), and the blank was prepared as described above, but without DPPH (A_B). The scavenging activity was calculated as $[1-(A_S-A_B)/(A_C-A_B)]\times100\%$.

1.6 Hydroxyl radical scavenging activity assay

The hydroxyl radical scavenging effect was measured using the method of Smirnoff and Cumbes[26] with some modifications. The reaction system containing 1 mL of 2.3 mM $FeSO_4$, 1 mL of 2.3 mM sodium salicylate in ethanol, 0.5 mL of samples with different concentrations, and 1 mL of 2.2 mM H_2O_2 was incubated for 1 h at 37 ℃ in a water bath. After the incubation, the absorbance of the resulting solution was measured spectrophotometrically at 510 nm (A_S). Distilled water was used instead of samples in control experiment (A_C). The scavenging activity of samples was calculated using $[(A_C-A_S)/A_C]\times100\%$.

1.7 Determination of superoxide radical scavenging activity

The scavenging activity was evaluated by pyrogallol autoxidation systems with some modification[8,27]. The reaction mixture containing 4.5 mL of 50 mM Tris-HCl buffer (pH 8.2) and 4.1 mL of distilled water was pre-incubated at 25 ℃ for 20 min. Then 0.1 mL of sample solution and 0.3 mL of 3 mM pyrogallol in 10 mM HCl were added. The rate of superoxide radical-induced polymerisation of pyrogallol ($\Delta A/min_S$) was measured as increased in absorbance at 420 nm for 3 min at room temperature. The control was prepared in the same manner except that distilled water used instead of sample ($\Delta A/min_C$). The abilities to scavenge the superoxide radical was calculated using $(\Delta A/min_C-\Delta A/min_S)/\Delta A/min_C\times100\%$.

1.8 Assay of ABTS radical scavenging activity

The ABTS radical scavenging activity was determined according to a modified version of the method described by Re et al.[28]. The stock solution of ABTS radical consisted of 7 mM ABTS in 2.45 mM potassium persulfate, kept in the dark at room temperature for 16 h. An aliquot of stock solution was diluted with distilled water in order to prepare the working solution of ABTS radical with absorbance at 734 nm of 0.70 ± 0.05. A volume of 10 μL of samples or distilled water (in case of the control) was mixed with 200 μL of ABTS radical working solution and the absorbance was recorded at 734 nm after 6 min. A standard curve using Trolox ranging from 0.15 to 1.5 mM was performed. The scavenging activity was expressed as mmol Trolox equivalents (TE)/g sample.

1.9 Determination of reducing power

The reducing power was assayed by the method of Oyaizu[29]. Two mL of samples (5 mg/mL) were added to 2 mL of 0.2 M phosphate buffer (pH 6.6) and 2 mL of 1% potassium ferricyanide. The mixture was incubated at 50 ℃ for 20 min, and then 2 mL of 10% TCA was added to the reaction mixture. A volume of 2 mL from each incubated mixture was mixed with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride in test tube. After a 10 min reaction, the absorbance of the resulting solution was measured at 700 nm. A high absorbance was indicative of strong reducing power.

1.10 ACE inhibitory activity measurement

The ACE inhibitory activity was analyzed using the method of Cushman and Cheung[30] with some modifications[31]. Briefly, 20 μL of sample and 30μL of ACE solution (60 mU/mL) were pre-incubated at 37 ℃ for 5 min. Afterwards, 50 μL of substrate (7.6 mM HHL in 50 mM sodium borate buffer containing 300 mM NaCl at pH 8.3) was added and the mixture incubated for 30 min at the same temperature. The reaction was stopped with 100 μL of 1 M HCl. HA liberated by ACE was determined by reversed high performance liquid chromatography (RP-HPLC) on an Inertsil ODS-SP C18 column (4.6×150 mm, Shimadzu, Kyoto, Japan). ACE inhibitory activity was calculated as the peak area.

1.11 Separation of SOP with RP-HPLC

SOP was separated with a RP-HPLC system (Shmadzu, Kyoto, Japan) equipped with an XBridge BEH130 C18 column (4.6×250 mm, Waters, USA). Gradient elution was eluent A (Milli-Q water containing 0.1% (*V/V*) TFA) and eluent B (80% (*V/V*) acetonitrile containing 0.1% (*V/V*) TFA). The separation was performed at a flow rate of 0.6 mL/min with a nonlinear gradient as follows: 0~50 min, 0~15% B; 50~120 min, 15~40% B; 120~140 min, 40~80% B. The effluent was monitored at 220 nm. Sample concentration was 10 mg/mL and the injection volume was 50 μL. The

separation procedure was repeated for six times until enough samples were collected. The fractions from the RP-HPLC system were freeze-dried for further analysis.

1.12 Identification of active peptides

Molecular mass and amino acid sequence of peptides were analyzed using a quadrupole time-of-flight mass spectrometer (Q-TOF2; Micromass Co., Manchester, UK) equipped with an electrospray ionisation (ESI) source.

1.13 Statistical analysis

All assays were carried out in triplicate. Data were expressed as means ± standard deviations.

2 Results and discussion

2.1 Chemical composition and amino acid composition of SOP

The chemical composition (on a dry basis) was shown in Table 1. It was observed that the major component of SOP was protein (91.45%), indicating that SOP could be a good source of proteins. The high protein content was a result of the solubilization of protein during hydrolysis, the removal of insoluble undigested non-protein substances after hydrolysis[32]. After enzymatic hydrolysis and purification, lower contents of lipid and sugar were found in SOP than its substrate SPI. The higher ash content of SOP was possibly due to the sodium hydroxide added 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.

As presented in Table 1, SOP was rich in glutamine acid (glutamine acid+glutamine) (24.38%), aspartic acid (12.24%), arginine (8.70%), lysine (7.41%) and leucine (6.03%). The overall amino acid composition was similar to that reported in the literature[33]. A similar percentage of all amino acid residues in both SOP and SPI were discovered. The result suggested that enzymatic hydrolysis did not alter significantly the amino acid composition of soy protein and has the advantage of preserving the nutritive quality of the protein without destruction of amino acids. This finding agreed with a study of Wu and Ding[33].

Table 1 Chemical composition of SOP [a] and SPI [b,c]

	SOP	SPI
Protein (%) [d]	91.45 ± 1.18	90.32 ± 1.24
Lipid (%) [d]	0.19 ± 0.02	1.28 ± 0.13
Sugar (%) [d]	0.48 ± 0.05	1.61 ± 0.12
Ash (%) [d]	3.86 ± 0.29	1.09 ± 0.10
Moisture (%) [d]	5.13 ± 0.38	4.87 ± 0.32
Amino acid composition/%		
Aspartic acid [e]	12.24 ± 0.42	11.96 ± 0.29
Glutamine acid [f]	24.38 ± 0.87	24.19 ± 0.79
Serine	4.89 ± 0.09	4.65 ± 0.10
Glycine	4.10 ± 0.08	4.21 ± 0.09
Alanine	3.66 ± 0.07	3.48 ± 0.08
Histidine	2.59 ± 0.06	2.87 ± 0.05
Arginine	8.70 ± 0.17	8.09 ± 0.15
Proline	5.87 ± 0.10	5.92 ± 0.11
Tryptophan	0.26 ± 0.01	0.70 ± 0.01
Isoleucine	3.42 ± 0.07	3.35 ± 0.08
Leucine	6.03 ± 0.15	6.48 ± 0.14
Valine	3.55 ± 0.09	3.84 ± 0.11
Threonine	3.29 ± 0.08	3.75 ± 0.09
Lysine	7.41 ± 0.12	7.25 ± 0.10
Methionine	1.10 ± 0.01	1.23 ± 0.02
Cysteine	1.65 ± 0.02	1.81 ± 0.03
Tyrosine	3.26 ± 0.07	3.18 ± 0.06
Phenylalanine	3.60 ± 0.08	3.04 ± 0.05
Total	100	100

Note: [a] SOP, soybean oligopeptides; [b] SPI, isolated soy protein; [c] Reported values are means ± SD (n = 3); [d] On a dry basis; [e] Aspartic acid+asparagine; [f] Glutamine acid+glutamine.

The amino acids reportedly have relation to antioxidant properties either in their free forms or as residues in proteins and peptides. The major constituent amino acids in SOP, glutamine acid, aspartic acid, and lysine, may interact with metal iron through their charged residues and inactivate oxidant activity of metal ions. In terms of ACE inhibitory activity, peptides containing proline, tyrosine and alanine exhibited greater ACE inhibitory activity[34]. Thus, SOP is thought to be a good resource to obtain antioxidant peptides and ACE inhibitory peptides.

2.2 Molecular weight distribution of SOP

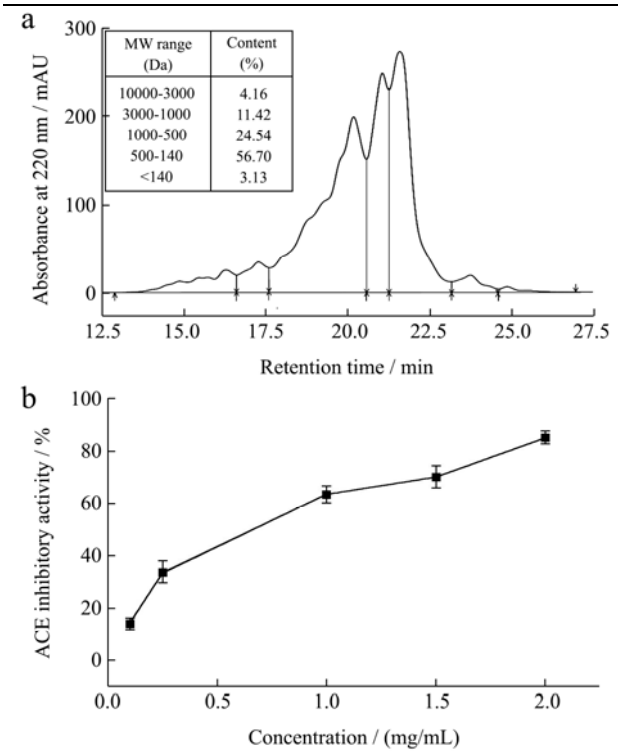

Fig.1 a, Size exclusion chromatography showing the molecular weight (MW) distribution of soybean oligopeptides (SOP),(Inset) Content of peptides with different classes of MW range in SOP. b, Angiotensin I-converting enzyme (ACE) inhibitory activity of SOP at different concentrations

Note: The data were average of triplicate experiments. Error bars show the standard deviations.

The molecular weight distribution of SOP was analyzed by size exclusion chromatography with an HPLC system. The result (Fig.1a) showed that SOP was dominated by peptides below 1000 u (84.37%). The range of 500~140 u was the main molecular weight interval for SOP, which accounted for 56.70%. These peptides were mainly di- and tri-peptides. It has been reported that di- and tri-peptides are actively transported via a specific peptide transporter in the intestinal epithelial cells, and amino acid residues are absorbed more rapidly from di- and tri-peptides than from free amino acids[35]. The results obtained indicated that two-step enzymatic hydrolysis followed by multistage separation was useful to remove large peptides or undigested proteins and to produce oligopeptides. This kind of enzymatic hydrolysis method has also been conducted in previous studies[13,36].

The functional properties of peptides are highly influenced by their molecular structure and weight, which are greatly affected by processing conditions. It is well known that most ACE inhibitory peptides derived from food protein have relatively low molecular weight. Moreover, Previous studies had demonstrated that smaller peptides had a higher level of antioxidative activity than larger peptides and several di- and tri-peptides have shown higher antioxidative activities than the constituent amino acids[37,38]. The accessibility to the oxidant-antioxidant test systems has been shown greater for small peptides than for larger peptides and protein[39].

2.3 Antioxidant properties of SOP

As the antioxidant property depends on several factors such as the type of radical species involved in the reaction and functionality of the antioxidant, it is appropriate to evaluate the antioxidant activity of a sample using various assays. Radical quenching is a primary mechanism of antioxidants to inhibit oxidative processes, and the reducing power is also commonly used to indicate the potential antioxidant activity of a compound. Therefore, SOP was tested for antioxidant activities using radical scavenging assays against DPPH, hydroxyl, superoxide and ABTS, as well as reducing power. Table 2 lists the antioxidant activities of SOP using the four types of tests. As a kind of enzymatic hydrolysate, SOP exhibited a strong scavenging activity against ABTS, DPPH and hydroxyl radicals, and moderate superoxide scavenging activity and reducing power. All these antioxidant activities of SOP increased

Table 2 Antioxidant activities of SOP using four types of assay systems [a]

Sample	Radical scavenging activity				Reducing power [d]
	DPPH [b]	Hydroxyl [b]	Superoxide [b]	ABTS [c]	
SOP	3.06 ± 0.02	5.25 ± 0.19	16.35 ± 0.28	1.29 ± 0.03	0.21 ± 0.01

Note: [a] SOP, soybean oligopeptides; [b] Results are shown as IC_{50} value (mg/mL), defined as the concentration of samples needed to

scavenge 50% of the free radical; [c] Results are shown as mmol Trolox equivalents (TE)/g sample; [d] Results are shown as the absorbance at 700 nm, at the concentration of 5.0 mg/mL.

with increasing concentrations. The results revealed that two-step enzymatic hydrolysis was effective means of obtaining the antioxidant hydrolysate from soybean. With antioxidative property, SOP possibly contained amino acids or peptides which could act as electron donors, react with free radicals to give rise to more stable products and terminate radical chain reactions. Among the four types of assay systems, the ABTS radical scavenging activity of SOP was the strongest. In addition, the ABTS assay is very useful for the assessment of total antioxidant capacity. Therefore, the scavenging potency of ABTS radicals was considered to be the selection criteria of antioxidant peptides isolated from SOP in further experiments.

2.4 ACE inhibitory activity of SOP

As shown in Fig.1b, SOP exhibited ACE inhibitory activity with an IC_{50} value of 0.55 mg/mL. From the results obtained, it was clear that the inhibitory activity of SOP was dose dependent. Motoi prepared wheat gliadin hydrolysate with acid protease, and indicated that its IC_{50} value of ACE inhibitory activity was 1.0 mg/mL[40]. which was comparable to soy protein hydrolysate studied by Wu and Ding (0.34 mg/mL)[33]. Bougatef et al.[41] studied the enzymatic hydrolysates of sardinelle using various proteases which had IC_{50} value varying between 1.24 and 7.4 mg/mL. Compared to these data, SOP showed a high ACE inhibitory activity. The ACE inhibitory activity has been shown greater for small peptides than for large peptides and protein. During processing, the inhibitors are liberated and induce ACE inhibition. SOP was dominated by small peptides, which may be associated with its high ACE inhibitory activity. Besides, Previous study has shown that the ACE inhibitory activity might be associated with the number of hydroxyl groups available to establish hydrogen bonds with ACE[42]. Therefore, it was necessary to purification and identification of antioxidative peptides from SOP, in order to investigate the structure-actvity relationship of SOP.

2.5 Purification and identification of antioxidative peptides from SOP

In order to understand the possible effect of peptide composition on the antioxidant activity and ACE inhibitory activity, SOP was subjected to fractionation with a RP-HPLC column. The elution profiles of SOP are displayed in Fig.2. As shown in this figure, many peaks were detected. Six major fractions, designated as fractions 1-6, were selected for further identification of active sequences. The separation procedure was repeated for six times until enough samples were collected. These fractions were pooled respectively, and then freeze-dried and subjected to a Q-TOF2 mass spectrometer.

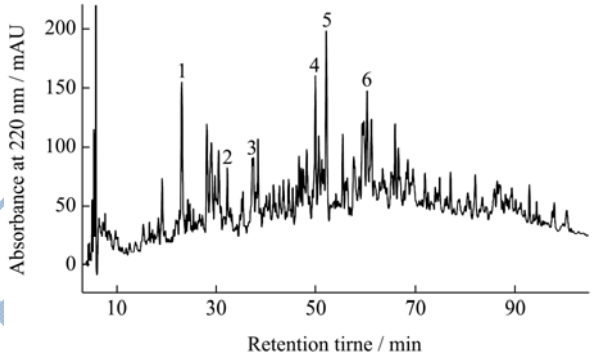

Fig.2 Elution profile of double enzyme (Alcalase 2.4 L and Protex 13 FL) digested soybean oligopeptides (SOP) by reversed-phase high-performance liquid chromatography (RP-HPLC) C18 column

Note: Separation was carried out using a nonlinear gradient of eluent A (0.1% trifluoroacetic acid in Milli-Q water, *V/V*) and eluent B (80% acetonitrile with 0.1% trifluoroacetic acid, *V/V*) at a flow rate of 0.6 mL/min. Collected fractions are designated fractions 1-6. Each fraction was collected according to the elution time, condensed, and free-dried.

Overall, 41 peptides were identified from SOP and the amino acid sequences and molecular masses of the peptides are summarized in Table 3. All of them were peptides with two to six amino acid residues, which was consistent with the molecular weight distribution analysis. Referring to the previous reports, the amino acid compositions of antioxidative peptides and angiotensin I-converting enzyme inhibitory peptides were preliminarily analyzed. For example, previous studies indicated that some di- and tri-peptides containing aromatic amino acid residues (Tyr or Trp), as well as peptides containing Tyr, Pro, or His, show strong

antioxidant activity[37,43]. It was reported that peptides with Tyr, Phe, Trp, Pro or hydrophobic amino acids at the C-terminal had a strong ACE inhibitory activity[44].

Table 3 Identification of peptides by quadrupole time-of-flight mass spectrometer (Q-TOF2) included in the reversed-phase HPLC fractions of SOP

RP-HPLC fraction [b]	amino acid sequence [c]	observed mass	calculated mass [d]
1	Gln-Asp-Pro-Gln (QDPQ)	486.18	486.48
	Glu-Arg-Gln (ERQ)	431.15	431.45
	Ser-Thr-Pro-His (STPH)	440.14	440.46
	Glu-Gly-Gly-Ala-His (EGGAH)	469.16	469.45
	Val-Gly-Pro-Gln (VGPQ)	399.11	399.45
	Asp-Val-Arg (DVR)	388.15	388.42
	Tyr-Glu (YE)	310.07	310.31
2	Asp-Tyr-Ala-Gln (DYAQ)	495.14	495.49
	Asp-Val-Thr-Arg (DVTR)	489.15	489.53
	Leu-Asp-Thr-Asn (LDTN)	461.14	461.47
	Asp-Tyr-Arg (DYR)	452.13	452.47
	Lys-Tyr-Glu (KYE)	438.13	438.48
	Leu-Ser-Thr-Arg (LSTR)	475.18	475.55
3	Val-Pro-Pro-Thr-Arg (VPPTR)	568.29	568.67
	Pro-Gln-Gly-Ala-Arg (PQGAR)	527.29	527.58
	Ser-Pro-Thr-His (SPTH)	440.22	440.46
	Glu-Leu-Arg (ELR)	416.25	416.48
	Asp-Leu-Arg (DLR)	402.23	402.45
	Leu-Glu-Glu (LEE)	389.18	389.41
	Leu-Val-Arg (LVR)	386.27	386.49
	Ala-Asn-Gly-Gly-Lys (ANGGK)	445.23	445.48
4	Pro-Gln-Leu-Arg (PQLR)	512.27	512.61
	Ser-Gly-Asp-Ala-Leu (SGDAL)	461.19	461.47
	Val-Asn-Leu-Ser (VNLS)	431.21	431.49
	Gln-Pro-Gly-Gly-Lys (QPGGK)	485.24	485.54
	Gly-Phe (GF)	222.09	222.24
	Asp-Phe (DF)	280.09	280.28
	Glu-Gly-Leu (EGL)	317.14	317.34
5	Arg-Glu-Phe (REF)	449.19	450.49
	Phe-Ser-Arg (FSR)	408.18	408.46
	Lys-Gly-Phe (KGF)	349.91	350.42
	Leu-Tyr (LY)	294.12	294.35
	Leu-Ala-Gly-Arg (LAGR)	415.22	415.49
	Asp-Ser-Ser-Glu (DSSE)	436.15	436.38
6	Asn-Lys-Pro-Phe (NKPF)	504.22	504.59
	Ala-Ala-Gly-Gly-Ser-Asn (AAGGSN)	475.18	475.46
	Val-Leu-Val-Glu (VLVE)	458.23	458.56
	Pro-Pro-Gln-Glu (PPQE)	469.18	469.49
	Gln-Pro-Gly-Gly-His (QPGGH)	494.15	494.51
	Ala-Ala-Gly-Phe (AAGF)	363.92	364.40

Thr-Pro-Glu (TPE)	344.99	345.35

Note: [a] SOP, soybean oligopeptides; [b] Fractions are indicated in Figure 2; [c] Letters in the parentheses indicate a single-letter representation of amino-acid sequence; [d] Average mass.

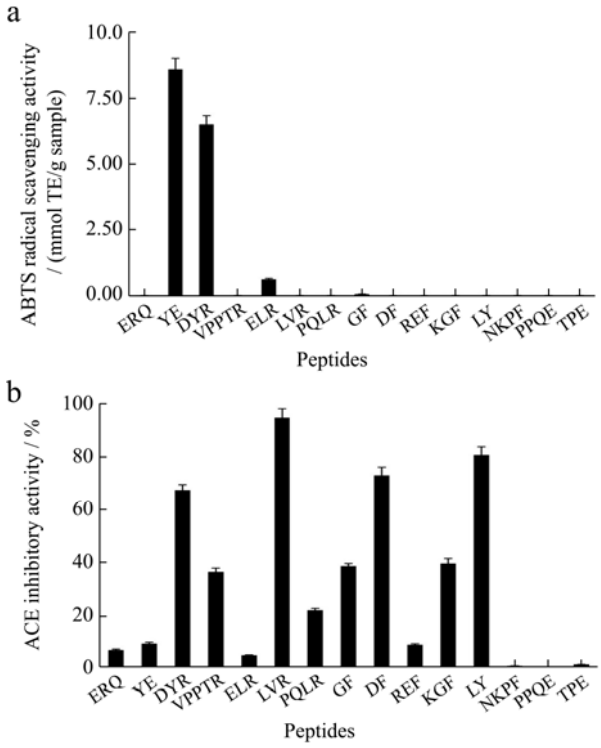

Fig.3 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging activity (A) and angiotensin I-converting enzyme (ACE) inhibitory activity (B) of 15 peptides identified from fractions 1-6 by a quadrupole time-of-flight (Q-TOF2) mass spectrometer

Note: The concentration of the peptides used for ACE inhibitory activity analysis was 1.0 mg/mL. Error bars indicate the standard deviation (n=3). TE = Trolox equivalents.

According to the previous studies, 15 peptides which had these preferable structures and may possess antioxidant activity or ACE inhibitory activity were selected and synthesized for further tests in which their ABTS radical scavenging and ACE inhibitory activities were determined.

The ABTS radical scavenging activities of the peptides were displayed in Fig.3a. Four peptides (Tyr-Glu, Asp-Tyr-Arg, Glu-Leu-Arg and Gly-Phe) exhibit ABTS radical scavenging activities and the former two peptides showed potent effects. Other peptides exhibited considerably lower scavenging activities which were all below 0.02 mmol TE/g sample. The activities of Tyr-Glu (8.61±0.42 mmol TE/g sample) and Asp-Tyr-Arg (6.53±0.34 mmol TE/g sample), respectively, was 6.7 times and 5.1 times higher than that of SOP (1.29 ± 0.03 mmol TE/g sample). The results suggested that Tyr-Glu and Asp-Tyr-Arg could very probably act as electron donors, transforming ABTS radical cation into the non-radical ABTS. To the authors' knowledge, the two peptides are novel peptides with antioxidant activity that have not been reported.

Aromatic amino acid Tyr was commonly detected in both peptides. It is reported that some di- and tri-peptides containing aromatic amino acid residues (Tyr or Trp), as well as peptides containing Tyr, Pro, or His, show strong antioxidant activity[37,43]. This has been reported to be the case for Tyr-Asp, Arg-Tyr and Lys-Asn-Tyr-Pro, derived from royal jelly protein[38]. However, the di-peptides, Leu-Tyr, containing Tyr residue at the C-terminus could not scavenge the radical cations. It is apparent that the higher radical scavenging activity of Tyr-Glu and Asp-Tyr-Arg cannot be only due to the presence of Tyr in the sequences. The antioxidative activity of Tyr may be explained by the special capability of indolic groups to serve as hydrogen donors. The indoyl radicals are much more stable and have longer lifetimes than simple peroxy radicals, so any reverse action or the propagation of the radical-mediated peroxidizing chain reaction are inhibited[45].

Additionally, acidic amino acids were reported to contribute for antioxidant activity. The antioxidative peptides, Tyr-Glu and Asp-Tyr-Arg, consisted of acidic amino acid residue (Glu and Asp). Therefore, the presence of Glu and Asp may have contributed to the higher radical scavenging potential of the peptides. Although the precise mechanism of the synergistic effect is not clear, these antioxidative amino acids were supposed to play an important role in ABTS radical scavenging ability of the peptides.

Saito et al.[45] investigated the antioxidative properties of the combinatorial tripeptide libraries, based on an antioxidative peptide isolated from a soybean protein hydrolysate. Their study showed that each tripeptide had a distinct activity in different assay systems. In the present study, the antioxidant activity of the synthetic peptides was examined by ABTS assay. The mechanism of antioxidant activity in one system may or

may not predict activity in other system. The activities evaluated using other methods are worth investigating further, to understand the antioxidative properties of these peptides more throughly.

2.6 Properties of ACE inhibitory peptides from SOP

The ACE inhibitory activities of the 15 peptides were depicted in Fig.3b. All synthesized peptides possessed activities varying from 0.12% to 94.62% at 1.0 mg/mL. Among them, four peptides exhibited higher activities than the parent protein SOP at the same concentration. The amino acid sequences of the four potent ACE inhibitors were Leu-Val-Arg, Leu-Tyr, Asp-Tyr-Arg and Asp-Phe, and their IC_{50} values were 51.75, 305.76, 1082.95, and 1106.04 μM, respectively. Their ACE inhibitions were approximately 27.5, 6.1, 1.8, and 1.1 times that of SOP. To the best of our knowledge, Asp-Phe and Asp-Tyr-Arg are novel peptides with ACE inhibitory activity that had never been reported. Concerning peptides Leu-Val-Arg and Leu-Tyr, ACE inhibitory activities have been found in ficus carica by Maruyama et al.[46] and in sardine muscle by Matsufuji et al.[47], respectively. Although these two peptides has been reported to be ACE inhibitory peptides in other litterateur, to our knowledge, they were found in salmon skin collagen hydrolysate for the first time.

As for the structure-activity correlations between ACE and ACE inhibitory peptides, it is known that binding to ACE is strongly influenced by the C-terminal amino acid residues. Gobbetti et al.[44] indicated that peptides with Tyr, Phe, Trp, Pro or hydrophobic amino acids at the C-terminal possessed a strong ACE inhibitory activity. In this study, two of the four potent ACE inhibitory peptides identified had Tyr or Phe at the C-terminal. Among the 15 identified peptides, 6 peptides had Tyr or Phe at the C-terminal. On the other hand, previous researches have shown that hydrophobic amino acid residues were preferred at the N- terminal position[14]. In this study, the two most potent inhibitory peptides had Leu at the N-terminal. Interestingly, among the 15 identified peptides, 5 peptides with Arg at the C-terminal showed ACE inhibitory activity. Therefore, the Arg residue may play an important role in these peptides as ACE inhibitors.

It was reported that short chains of peptides are more rapidly absorbed by the human body, and easily cross the intestinal barrier and exert biological effects than single amino acids and longer peptides[48]. In addition, di- and tri-peptides are known to be effective inhibitors, and the antihypertensive drug captopril has been synthesized on the basis of di-peptide configuration[49]. In the present research, the four potent inhibitory peptides were all di- or tri-peptides. Therefore, the inhibitory peptides isolated from SOP are expected to be easily absorbed and contribute to the antihypertensive effects via an in vivo transport system. These peptides may potentially be used as a functional food useful in prevention and treatment of hypertension.

3 Conclusions

According to the results shown above, it could be concluded that the pilot-scale production of SOP is a practical way to utilize soybean. Two potent antioxidative peptide and four potent ACE inhibitory peptides were identified from SOP. Most of them are novel antioxidant or ACE inhibitory peptides derived from soybean. With antioxidant/ACE inhibitory activity, SOP and the active peptides may be beneficial for developing physiologically functional foods. Further studies should be carried out to investigate the *in vivo* effects of each peptide and the mechanism of the active peptides.

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