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Antihypertensive Effect of Peptide-Enriched Soy Sauce-Like Seasoning and Identification of Its Angiotensin I-Converting Enzyme Inhibitory Substances

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We have developed a peptide-enriched soy sauce-like seasoning termed Fermented Soybean Seasoning (FSS), by modifying the process of soy sauce brewing. The FSS has a 2.7-fold higher concentration of total peptides than regular soy sauce. The angiotensin I-converting enzyme (ACE) inhibitory activity of FSS (IC_{50} = 454 μg/mL) was greater than that of regular soy sauce (IC_{50} = 1620 μg/mL). The FSS demonstrated antihypertensive effects both in spontaneously hypertensive rats and in Dahl salt-sensitive rats during continuous feeding. The ACE inhibitory substances were purified from FSS by reversed-phase chromatography. Ala-Trp (IC_{50} = 10 μg/mL), Gly-Trp (30 μg/mL), Ala-Tyr (48 μg/mL), Ser-Tyr (67 μg/mL), Gly-Tyr (97 μg/mL), Ala-Phe (190 μg/mL), Val-Pro (480 μg/mL), Ala-Ile (690 μg/mL), Val-Gly (1100 μg/mL), and a nonprotein amino acid, nicotianamine (0.26 μg/mL), were identified. The concentrations of these substances in the FSS were revealed to be higher than that of regular soy sauce through quantitative LC-MS/MS analysis.

KEYWORDS: Angiotensin I-converting enzyme; peptide; antihypertensive effect; spontaneously hypertensive rat; Dahl salt-sensitive rat; soy sauce; fermentation

INTRODUCTION

Many epidemiological studies have demonstrated that there is a relationship between increases in blood pressure and cardiovascular diseases (*1*). Worldwide prevalence estimates for hypertension may be as much as 1 billion individuals, and approximately 7.1 million deaths per year may be attributable to hypertension (*2*). The prevention and management of hypertension have been major public health challenges.

Recently, a number of foodstuffs with potential for risk reduction of cardiovascular diseases have been investigated, for example, alkaline protease hydrolysate derived from sardine muscles (*3*), protease hydrolysate derived from wakame (*Undaria pinnatifida*) (*4*), and protease hydrolysate derived from soybean protein isolates (*5*). Their physiological functions of in vitro and in vivo hypotensive effects were induced via inhibition of angiotensin I-converting enzyme by low molecular weight peptides derived from various food protein resources.

Angiotensin I-converting enzyme (ACE, EC 3.4.15.1) plays an important role in the renin−angiotensin−aldosterone system (RAAS), which regulates the arterial blood pressure and the salt/water balance of body fluids (*6*). Some synthetic inhibitors of ACE, such as captopril and enalapril, have been established and are widely used for hypertension therapy (*7*). Many kinds of peptides also have been reported to inhibit ACE because ACE is a dipeptidyl carboxypeptidase with broad substrate specificity (*8*).

Soy sauce is an Asian traditional fermented sauce, which is widely used to season foods. It has been reported to have ACE inhibitory activity with an IC_{50} value of 1400 μg/mL (*9*). Oka et al. identified 13 types of neutral peptides and 13 types of acidic peptides from soy sauce (*10*, *11*); however, it was unclear what the ACE inhibitory activity and hypotensive effects of these peptides were.

Soy sauce is made from soybean and wheat and contains proteins which are hydrolyzed by proteolytic enzymes produced from the filamentous fungus *Aspergillus oryzae* or *Aspergillus sojae*. During the process of fermentation, the proteins are degraded into peptides by endoproteases, and then they are further degraded into free amino acids by exoproteases (**Figure 1A**). In regular soy sauce fermentation, 70−90% of the total proteins are degraded into free amino acids in the course of 6−24 months (*12*). Some peptidase-resistant peptides remain in soy sauce (*13*); therefore, soy sauce with insufficient peptide degradation was expected to have an increased peptide content and ACE inhibitory activity.

In the present study, we have developed a peptide-enriched soy sauce-like seasoning termed Fermented Soybean Seasoning (FSS) by modifying the process of soy sauce brewing. From the standpoint of fermentation engineering, peptides contained in the brewing soy sauce could be considered as "intermediate" in the degradation of protein to amino acid. We combined two approaches for increasing the peptide yield (**Figure 1B**). First, we increased the protein content, which represents the "initial substrate concentration" in the primary material of FSS, by increasing soybean usage with a reduction in wheat usage, because soybean has higher protein content than that of wheat. Second, we shortened the fermentation time, which represents the "reaction time of peptidase", to restrain the degradation of peptides into amino acids.

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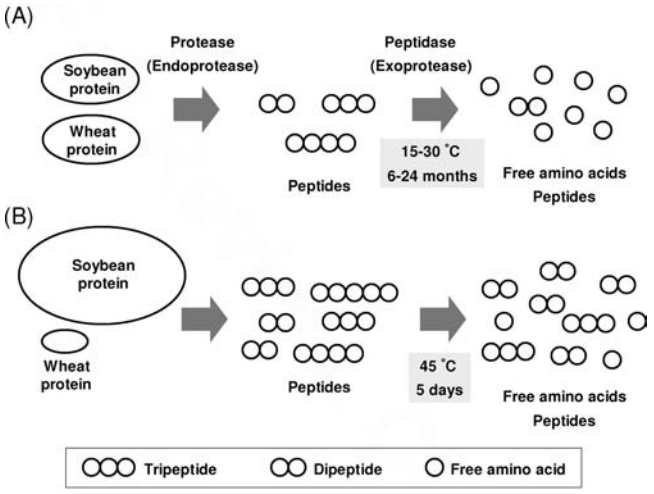

Figure 1. Protein degradation model in soy sauce fermentation and strategy for increasing peptide yield. Most of the total proteins are degraded into free amino acids in regular soy sauce fermentation (**A**). Combined approaches for increasing the peptide yield (**B**). First, we increased protein content. Second, we shortened the fermentation time to restrain the peptide degradation into amino acids.

We subsequently investigated the antihypertensive effects of the FSS in two types of hypertensive model rats. One was spontaneously hypertensive rats (SHR) as a model of essential hypertension, and the other was Dahl salt-sensitive rats (Dahl-S) as a model of salt-sensitive hypertension. We also purified and identified ACE inhibitory substances from the FSS.

MATERIALS AND METHODS

Materials. Soybean, wheat, and seed starter of *A. sojae* were obtained from the Production division, Kikkoman Corp. (Chiba, Japan). Synthetic peptides were purchased from Kokusan Chemical Co., Ltd. (Tokyo, Japan). A colorimetric assay kit for ACE activity was purchased from Fujirebio Corp. (Tokyo, Japan). ACE from rabbit lung was purchased from Sigma Chemical Co. (St. Louis, USA). Regular soy sauce (*Koikuchi* type) used in this study was a commercially available product of Kikkoman Corp. SHR were purchased from Japan SLC, Inc. (Shizuoka, Japan). Dahl-S rats were purchased from CLEA Japan, Inc. (Tokyo, Japan). The standard diet for rats was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). IPCC-MS7 was purchased from GL Sciences, Inc. (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of FSS. Equal quantities of steamed soybean and roasted wheat were mixed. Seed starter called *Tane koji*, rich in conidia of *A. sojae*, was inoculated and cultivated for 3 days under controlled temperatures from 25 to 40 °C and humidity of 95%. The resulting culture formed dry mash called *Shoyu koji*. Following this, 5 times quantity of puffed soybean was added and suspended into 10 times quantity of brine to form *Moromi*. The *Moromi* was then fermented for 5 days at 45 °C and agitated with a rotor blade at 60 rpm. After the fermentation was achieved, the *Moromi* was poured onto cloths, folded, and pressed, and raw sauce was squeezed. Then it was heated for 10 s at 115 °C to sterilize and inactivate the enzymes.

Yield of Peptides in FSS. Peptide content was determined by the acid hydrolysis method (*14*). Samples (4 mL) and 12 M HCl (4 mL) were mixed in a glass vial and incubated for 20 h at 110 °C to hydrolyze peptides. The hydrolysate was filtered using a filter unit (DISMIC-25CS 0.45 μm, ADVANTEC, Tokyo, Japan). The amino acid compositions of the samples and their hydrolysates were determined using an amino acid analyzer (L-8500, Hitachi Ltd., Tokyo, Japan). The difference in total amino acid content before and after hydrolysis was calculated to be the peptide content.

Analytical Reversed-Phase High-Performance Liquid Chromatography (HPLC). Analytical reversed-phase HPLC was conducted using a LC-10A HPLC system (Shimadzu Corp., Kyoto, Japan), equipped with a C18 column (CAPCELL PAK C18 MGIII 4.6 mm i.d. × 250 mm, 5 μm, Shiseido Co. Ltd., Tokyo, Japan) at 40 °C. Chromatography was carried out using a linear binary gradient of solvent A (distilled water containing 0.1% TFA) to solvent B (30% acetonitrile in water containing 0.1% TFA) for 60 min at a flow rate of 0.9 mL/min. The samples (10 μL) were injected after dilution 1:9 with solvent A.

Assay for ACE Inhibitory Activity. ACE inhibitory activity was measured using an ACE color kit (Fujirebio Corp., Tokyo, Japan) (*15*). The assay was conducted according to the manufacturer's instructions with some modifications. Purified rabbit lung ACE solution (67 mU/mL in 50 mM sodium borate buffer pH 8.3, 15 μL, Sigma Chemical Co., St. Louis, USA), sample solution (15 μL), and substrate solution containing *p*-hydroxybenzoylglycyl-L-histidyl-L-leucine (60 μL, provided in the kit) were mixed and incubated for 40 min at 37 °C, resulting in the hydrolysis to *p*-hydroxybenzoyl-glycine and L-histidyl-L-leucine. "Substrate for blank" solution was used for the blank tube. Developer solution (150 μL, provided in the kit) containing hippuricase was added to the sample and blank tubes to cleave *p*-hydroxybenzoyl-glycine and form *p*-hydroxybenzoic acid. Quinoneimine dye was produced by oxidation and condensation of the *p*-hydroxybenzoic acid with 4-aminoantipyrine and sodium metaperiodate. After 5 min incubation, 200 μL of the respective mixture was transferred to a 96-well microplate, and then the absorbance was measured at 505 nm using a microplate reader (SH-9000, Corona Electric Co.,Ltd., Ibaraki, Japan). The concentration of the samples required to inhibit 50% of the ACE activity was defined as the IC_{50} value.

Antihypertensive Effect of FSS During Continuous Feeding in SHR. All the animal experiments were conducted in compliance with the guidelines of the Japanese association for laboratory animal science and the guideline for animal experiments of the research and development division of Kikkoman Corporation. Spontaneously hypertensive rats (SHR/Izm, 8 weeks old, male, Japan SLC Inc., Shizuoka, Japan) were housed individually in steel cages in a room kept at 23 ± 1 °C with a 12 h light and dark cycle. SHR were fed a standard diet (MF, Oriental Yeast Co. Ltd., Tokyo, Japan), and water was given ad libitum. Systolic blood pressure (SBP) and heart rate were measured by the tail-cuff method using a sphygmomanometer (MK-2000, Muromachi Kikai Ltd., Tokyo, Japan). Before measurements, conscious rats were placed in a restraining chamber for 3 min at room temperature.

After 2 weeks of maintenance, a long-term feeding experiment began. SHR were divided into two groups (8 rats/group). Rats in the control group were fed a standard diet containing brine. Rats in the test group were fed a standard diet containing the FSS (10% v/w). The salt concentration in each chow was equalized to 3.3% (w/w) with sodium chloride. Water was given ad libitum. The body weight and blood pressure of the rats were measured every week. The food and water intakes were measured every 3 days. The results are expressed as mean and standard deviation (SD). The significance of the differences between the control and FSS group was analyzed using the Student's paired *t*-test.

Dose-Dependent Effect of FSS During Continuous Feeding in Dahl Salt-Sensitive Rats. Dahl salt-sensitive rats (Dahl-S/Sea, 8 weeks old, male, CLEA Japan, Inc., Tokyo, Japan) were used. After 2 weeks of maintenance, a continuous feeding experiment began. Dahl-S rats were divided into three groups (6 rats/group). Rats in the control group were fed a standard diet containing regular soy sauce (25% v/w). Rats in the low-dose group were fed a standard diet containing the FSS (5% v/w) and regular soy sauce (20% v/w). Rats in the high-dose group were fed a standard diet containing the FSS (10% v/w) and regular soy sauce (15% v/w). The salt concentration in each chow was equalized to 3.3% (w/w) with sodium chloride. The body weight and blood pressure of the rats were measured every other week. The significance differences among each group were analyzed using the one-way analysis of variance (ANOVA) with posthoc analysis by Tukey's test.

Purification of ACE Inhibitory Substances from FSS. FSS (5 L) was desalted with electrodialyzer (ASTOM Corp., Tokyo, Japan). After the pH was adjusted to 3.5 with trifluoroacetic acid (TFA), it was applied onto a reversed-phase column (SP-120-40/60-ODS-B, 150 mm i.d. × 1000 mm, 5 μm, Daiso Co., Ltd. Osaka, Japan). Chromatography was carried out using a linear gradient of solvent A (distilled water containing 0.1% TFA) to solvent B (acetonitrile containing 0.1% TFA) for 25 h at a flow

rate of 45 mL/min. The fractions were evaporated and lyophilized. Then they were, respectively, applied onto a reversed-phase HPLC column (Cosmosil-5C18-ARII, 20 mm i.d. × 250 mm, 5 μm, Nacalai Tesque, Kyoto, Japan). Chromatography was carried out using a linear gradient of solvent A (distilled water containing 0.1% TFA) to solvent B (70% acetonitrile in water containing 0.1% TFA) for 90 min at a flow rate of 5 mL/min. The fractions displaying ACE inhibition were evaporated and subjected to further purification. A reversed-phase C30 HPLC column (Develosil RPAQUEOUS-AR, 20 mm i.d. × 250 mm, 5 μm, Nomura Chemical Co., Ltd., Aichi, Japan) was used with a linear gradient of solvent A (distilled water) to solvent B (70% acetonitrile in water) for 90 min at a flow rate of 5 mL/min.

Characterization of the ACE Inhibitory Substances. ^{1}H-nuclear magnetic resonance (NMR) spectra were recorded at 500.1 MHz, and ^{13}C NMR spectra were recorded at 125.7 MHz with a NMR spectrometer (AVANCE 500, Bruker BioSpin GmbH, Rheinstetten, Germany) using sodium 3-(trimethylsilyl) propionate as an internal standard and deuterium oxide as a solvent. The candidate structure was confirmed with a corresponding synthetic molecule.

The molecular formula was confirmed using liquid chromatography/mass spectrometry (LC-MS, QSTAR Elite, Applied Biosystems Inc., Foster City, USA) consisting of an Agilent 1100 separation module (Agilent Technologies, Inc., Santa Clara, USA). Chromatographic separation was achieved using a C30 column (Develosil RPAQUEOUS 2.0 mm i.d. × 150 mm, 5 μm, Nomura Chemical Co. Ltd., Aichi, Japan) at 20 °C at a flow rate of 0.2 mL/min. The injection volume was 10 μL. Binary mobile phases (A, distilled water containing 0.1% formic acid; B, acetonitrile containing 0.1% formic acid) were used as a gradient system. Mass analysis by electrospray ionization (ESI) was used in the positive mode, with a capillary voltage at 5.5 kV and source temperature at 450 °C. The LC-MS parameters and data analysis were performed through Analyst QS 2.0 software (Applied Biosystems/MDS SCIEX).

The amino acid sequences of purified peptides were determined with a protein sequencer (492HT, Applied Biosystems, Inc.). The analysis was conducted according to the manufacturer's instructions using pulsed-liquid mode.

Quantitative Determination of the Identified ACE Inhibitory Substances. The samples were quantitatively analyzed using a LC-MS/MS (Quattro Micro API, Waters Co., Milford, USA) consisting of an Alliance 2695 separation module (Waters Co.). Chromatographic separation using a C18 column (2.1 mm i.d. × 150 mm, 5 μm, SunFire, Waters Co.) at 28 °C was performed at a flow rate of 0.2 mL/min. The samples (5 μL) were injected after adequate dilution with distilled water. Binary mobile phases (A, 20% acetonitrile in water containing 5 mM IPCC-MS7 (GL Sciences Inc., Tokyo, Japan) as an ion pair reagent; B, acetonitrile) were used as the gradient system. Mass analysis using ESI was employed in positive mode, with a capillary voltage at 2.2 kV, a cone voltage at 20 V, collision energy at 15 eV, source temperature at 120 °C, desolvation (N_2) gas temperature at 350 °C, and argon as the collision gas. MassLynx software ver. 4.0 was used for data analysis. Multiple reaction monitoring (MRM) of the peptides was applied for peak selectivity, based on the transition from the precursor ion to the fragment ion. For example, quantification of a peptide Gly-Tyr was performed as follows. The precursor ion of Gly-Tyr was detected at m/z 238.43, and the fragment ion was selected at m/z 164.95 by means of a standard reagent. These specific ions were applied to highly selective detection of Gly-Tyr in complicated samples used in this study. The levels of Gly-Tyr in test samples were determined from a standard curve containing 5 points of 0, 0.2, 0.5, 1.0, and 2.0 μg/mL. Quantitative determination of other peptides was performed in the same manner.

RESULTS AND DISCUSSION

Yield of Peptides and ACE Inhibitory Activity of FSS. FSS was prepared as described in **Figure 1B**. We determined the total peptide content and ACE inhibitory activity of FSS compared with regular soy sauce (*Koikuchi* type) (**Table 1**). FSS had a 2.7-fold higher concentration of total peptides than that of regular soy sauce. The ACE inhibitory activity of FSS (IC_{50} = 454 μg/mL) was greater than that of regular soy sauce (IC_{50} = 1620 μg/mL). The reversed-phase chromatograms of FSS and regular soy sauce are shown in **Figure 2**. The peak profiles were pronouncedly different. Various large peaks appeared in the chromatogram of FSS, indicating that FSS has a wide variety and large amounts of peptides.

Table 1. Yield of Peptides and ACE Inhibitory Activity of Fermented Soybean Seasoning and Regular Soy Sauce

samples	yield of peptides (mg/mL)	ACE inhibitory activity (IC_{50}, μg/mL)
fermented soybean seasoning	79	454
regular soy sauce	29	1620

These results indicated that FSS had higher peptide content and a greater ACE inhibitory activity than regular soy sauce. The difference was attributed to the modification of soy sauce fermentation. First, the protein content in the primary material of FSS was initially higher to generate more peptide. The compounding ratio (soybean:wheat = 92:8) was higher than that of regular soy sauce (50:50). Soybean has higher protein content accounting for ca. 35% of the total composition compared with that of wheat (ca. 10%). Second, the fermentation time of FSS was shortened to restrain peptide degradation. The fermentation time of FSS was 5 days, whereas that of regular soy sauce was 6−24 months. Zhu et al. also reported that ACE inhibitory peptides were derived from modified ethanol-added soy sauce with short-term fermentation (*16*). We have taken a different strategy from Zhu et al. and successfully developed a peptide-enriched soy sauce-like seasoning through these combined approaches

Antihypertensive Effect of FSS During Continuous Feeding in SHR. Since FSS showed a potent in vitro ACE inhibitory activity, the antihypertensive effect in SHR was investigated using a continuous feeding experiment for 11 weeks. Changes in SBP are shown in **Figure 3**. The SBP gradually increased throughout the course of the experiment. The SBP in the FSS group tended to be lower than that of the control group and statistically significant after 7 weeks ($p < 0.05$). The SBP in the control group reached 234 mmHg, while that of the FSS group was 214 mmHg at the end of feeding. There were no significant differences in food intake and body weight between the control and the test groups. After the experiment, all the rats were sacrificed, and the internal organs were examined. No abnormalities were found in the organs.

Yamakoshi et al. have reported the effect of the chronic oral administration of NaCl solution or the same salt concentration of regular soy sauce on SBP in SHR (*17*). There was no significant difference in SBP between both groups throughout the experimental period. These results suggested that FSS has more potent antihypertensive effects in SHR than regular soy sauce.

Dose-Dependent Effect of FSS During Continuous Feeding in Dahl-S Rats. Another continuous feeding experiment was conducted using Dahl-S rats to investigate the antihypertensive effect of FSS for salt-sensitive hypertension. In this experiment, regular soy sauce (25% v/w) was added to the standard diet in the control group. FSS was partially substituted for regular soy sauce in the low and high dose groups. As shown in **Figure 4**, the mean SBP of the low dose group was significantly lower than that of the control group at 6 weeks, and that of the high dose group was significantly lower at 4 and 6 weeks. FSS suppressed the elevation of SBP in a dose-dependent manner after 4 weeks. The result indicated that FSS has a more potent antihypertensive effect than regular soy sauce.

We have confirmed the in vivo antihypertensive effect of FSS both in SHR and Dahl-S rats. These results suggested that FSS is effective for essential hypertension and salt-sensitive hypertension. This is probably because of RAAS suppression induced by

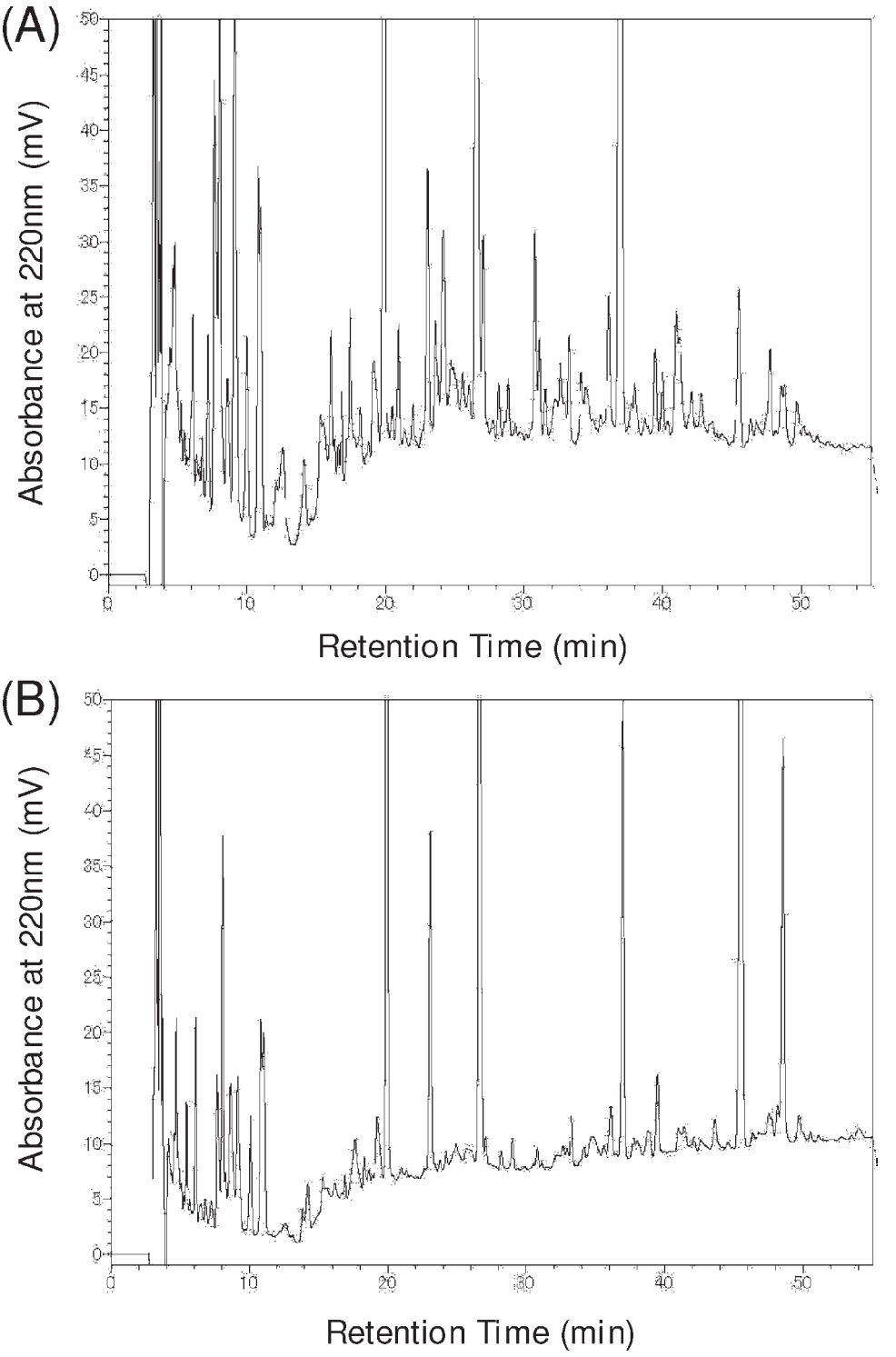

Figure 2. Chromatograms of the fermented soybean seasoning (**A**) and regular soy sauce (**B**) on a reversed-phase CAPCELL PAK C18 MGIII column. Chromatography was carried out using a linear binary gradient of solvent A (distilled water containing 0.1% TFA) to solvent B (30% acetonitrile in water containing 0.1% TFA) for 60 min at a flow rate of 0.9 mL/min.

ACE inhibitory substances in the FSS. A large number of food protein hydrolysates were also found to exert antihypertensive effects. Most of them contained ACE inhibitory peptides (*3*−*5*). As mentioned above, FSS also had higher peptide content and ACE inhibitory activity, indicating that potent ACE inhibitory peptides were contained in the FSS.

Purification and Characterization of ACE Inhibitory Substances from FSS. At the first step of purification, desalted FSS was divided into seven fractions following the order of elution time by reversed-phase chromatography (**Table 2**). All fractions showed ACE inhibitory activity (IC_{50} = 172−772 μg/mL), and Fr.4 exerted the most potent inhibition (IC_{50} = 132 μg/mL). Each fraction was subjected to further purification. The purity of active fractions was verified by analytical reversed-phase HPLC and thin-layer chromatography (TLC). If the HPLC chromatogram showed a single peak and the TLC also showed a single spot, the fraction was subjected to structure analysis; otherwise, it was subjected to rechromatography.

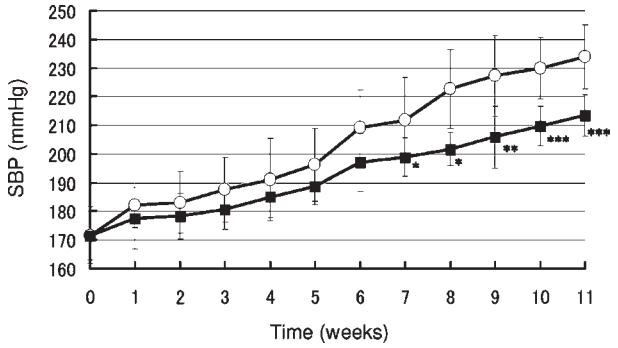

Figure 3. Antihypertensive effect of Fermented Soybean Seasoning (FSS) during continuous feeding in spontaneously hypertensive rats. Each point represents the mean systolic blood pressure in eight rats. The vertical bars indicate the standard deviation. Rats in the control group were fed a standard diet containing brine (○), and rats in the test group were fed a standard diet containing 10% (v/w) FSS (■). The salt concentration in each chow was equalized to 3.3% (w/w) with sodium chloride. Significant differences from the control group were evaluated by Student's paired *t*-test: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

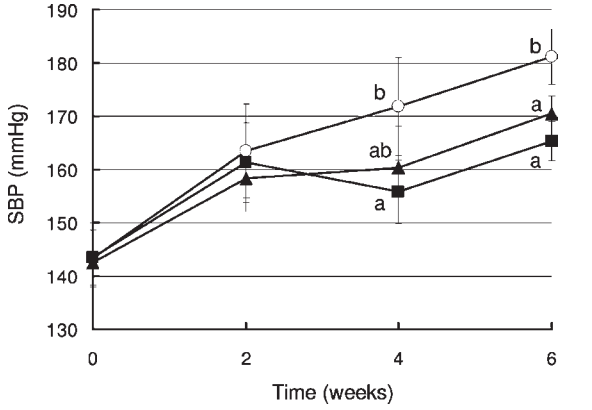

Figure 4. Dose-dependent effect of FSS during continuous feeding in Dahl salt-sensitive rats. Each point represents the mean systolic blood pressure in six rats. The vertical bars indicate the standard deviation. Rats in the control group (○) were fed a standard diet containing regular soy sauce (25% v/w). Rats in the low-dose group (▲) were fed a standard diet containing the FSS (5% v/w) and regular soy sauce (20% v/w). Rats in the high-dose group (■) were fed a standard diet containing the FSS (10% v/w) and regular soy sauce (15% v/w). The salt concentration in each chow was equalized to 3.3% (w/w) with sodium chloride. Significant differences among each group were evaluated by one-way analysis of variance (ANOVA) with posthoc analysis by Tukey's test. Data points with the same letter are not significantly different ($p > 0.05$).

The molecular structure was determined from ^{1}H NMR and ^{13}C NMR spectra, and the molecular formula was confirmed using LC-MS. The amino acid sequences of peptides were determined with a protein sequencer. In each analysis, the candidate structure was confirmed using the corresponding synthetic molecule. As an example for the identified peptides, **Figure 5** shows the mass spectrum of one of the purified fractions and a corresponding synthetic molecule, Gly-Tyr. Each spectrum demonstrated a completely identical pattern.

We identified nine kinds of ACE inhibitory peptides: Ala-Trp (IC_{50} = 10 μg/mL), Gly-Trp (30 μg/mL), Ala-Tyr (48 μg/mL), Ser-Tyr (67 μg/mL), Gly-Tyr (97 μg/mL), Ala-Phe (190 μg/mL), Val-Pro (480 μg/mL), Ala-Ile (690 μg/mL), Val-Gly (1100 μg/mL), and a nonprotein amino acid, nicotianamine (0.26 μg/mL)

Table 2. ACE inhibitory activity of the first step fraction of Fermented Soybean Seasoning and identified ACE inhibitory substances.

fraction number	ACE inhibitory activity (IC50, μg/ml)	identified substances
1	772	–
2	442	Nicotianamine
3	172	Val-Gly
4	132	Gly-Tyr, Ser-Tyr, Ala-Tyr, Ala-Ile
5	172	Val-Pro
6	224	Ala-Phe
7	199	Gly-Trp, Ala-Trp

(**Table 2**). Ala-Trp, Gly-Trp, Ala-Tyr, Gly-Tyr, Ala-Phe, Val-Pro, and Val-Gly were previously reported to have ACE inhibitory activity by Cheung et al. (*18*). Ser-Tyr was also reported to have ACE inhibitory activity by Suetsuna (*19*). Nicotianamine has been isolated from many plants including soybeans, and its wide distribution in the plant kingdom has been confirmed. Kinoshita et al. have isolated nicotianamine as an ACE inhibitory substance from soy sauce (*20*).

Quantitative Determination of the Identified ACE Inhibitory Substances. Several foods fermented with soybean, for example, modified soy sauce (*16*), miso (Japanese soybean paste) (*21*), Korean soybean paste (*22*), tofuyo (*23*), and natto (*24*), are known to contain ACE inhibitory peptides. However, no information is available about the quantification of their identified peptides.

In this study, the concentration of identified ACE inhibitory substances in FSS and regular soy sauce was analyzed through quantitative analysis using LC-MS/MS (**Table 3**). It was revealed that the concentration of identified ACE inhibitory substances in FSS was significantly higher than that of regular soy sauce. The content of Trp-including peptides, Ala-Trp and Gly-Trp, and Tyr-including peptides, Ala-Tyr, Gly-Tyr, and Ser-Tyr, was particularly enriched. For example, the Ser-Tyr concentration was 33-fold higher than regular soy sauce. These ACE inhibitory peptides are likely to remain under the fermenting conditions of FSS, while most of them are degraded into free amino acids in regular soy sauce.

Nicotianamine was also enriched in FSS. This likely resulted from the higher soybean usage in FSS because soybean contains a large quantity of nicotianamine. Kinoshita et al. suggested that the nicotianamine in soy sauce is derived from soybeans (*20*).

In conclusion, in this study, it was proven that modified soy sauce termed fermented soybean seasoning employing higher soybean usage and short-term fermentation has potent ACE inhibitory activity and antihypertensive effects both in essential hypertension and in salt-sensitive hypertension model rats. The ACE inhibitory substances were identified from FSS. The concentrations of these substances were quantified and found to be contained in higher than normal levels found in regular soy sauce. We clarified that the antihypertensive effects of FSS resulted mainly from ACE inhibitory substances, peptides, and nicotianamine. To the best of our knowledge, this is the first report that describes not only the identification but also quantification of ACE inhibitory substances from soy sauce or soy sauce-like seasoning.

To date, hundreds of ACE inhibitory peptides have been identified from various foodstuffs. However, most of them were derived by protein hydrolysis with industrial protease preparations. Our study is a unique approach to generate peptides by modifying the process of a traditional fermented food. Some proteins, especially whey, casein, and soy protein,

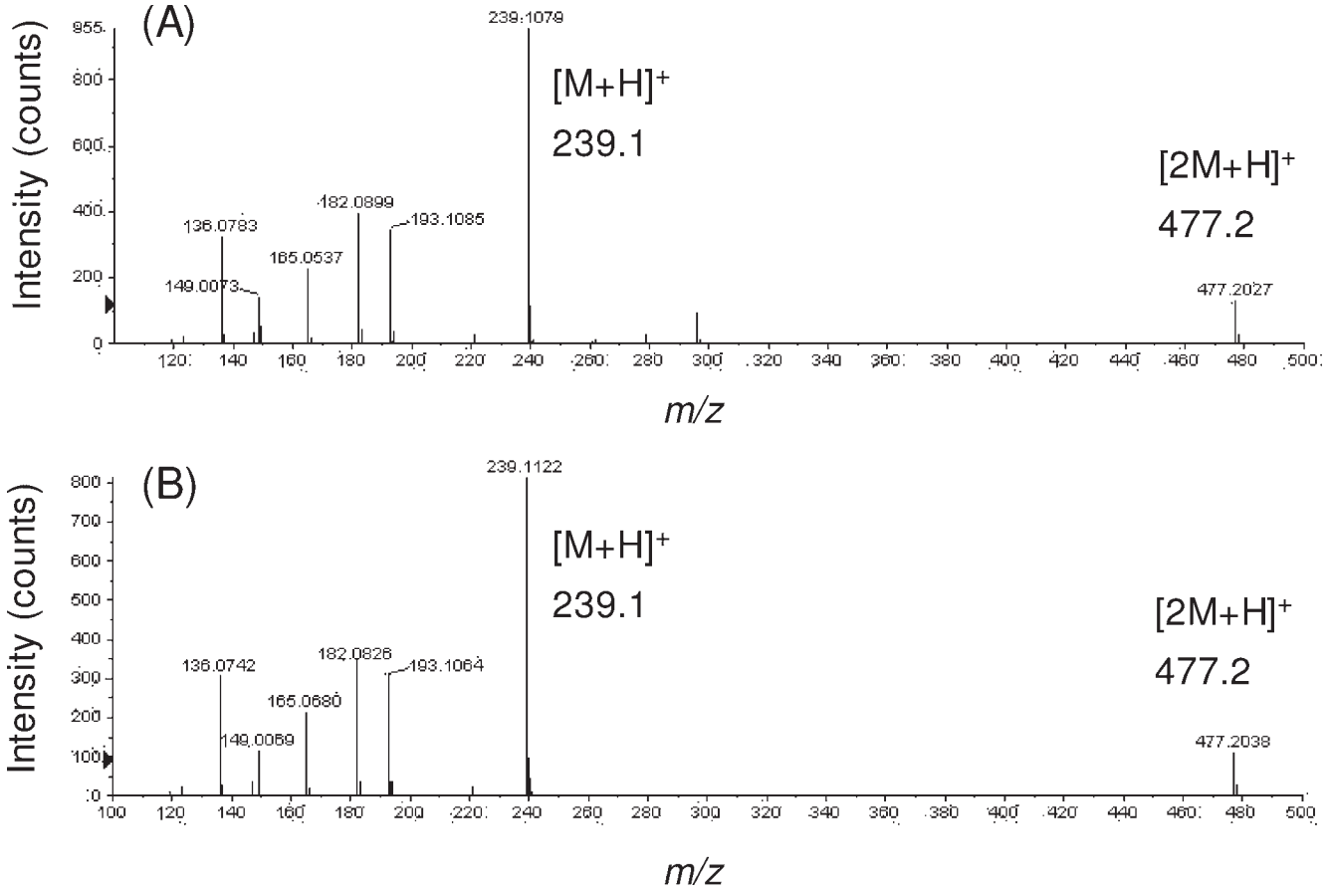

Figure 5. Mass spectrum of purified ACE inhibitory substances from FSS (**A**) and a corresponding synthetic molecule, Gly-Tyr (**B**). LC-MS analysis was carried out with the QSTAR Elite system. The electrospray ionization (ESI) was used in positive mode.

Table 3. ACE Inhibitory Activity and Content of Identified ACE Inhibitory Substances in Fermented Soybean Seasoning and Regular Soy Sauce

		content (μg/mL)	
ACE inhibitory substances	ACE inhibitory activity (IC_{50}, μg/mL)	fermented soybean seasoning	regular soy sauce
Ala-Trp	10	9	1
Gly-Trp	30	25	1
Ala-Tyr	48	43	4
Ser-Tyr	67	100	3
Gly-Tyr	97	136	19
Ala-Phe	190	45	4
Val-Pro	480	491	194
Ala-Ile	690	101	12
Val-Gly	1100	141	70
Nicotianamine	0.26	133	13

have a bitter taste after enzymatic hydrolysis (*25*). Although FSS contains a large amount of soy protein derived peptides, it does not taste that bitter. It has a preferable taste similar to regular soy sauce, thus this can render it possible to be utilized in a usual diet as a seasoning agent. Therefore, FSS may prove very useful for preventing hypertension and related cardiovascular diseases.

ABBREVIATIONS USED

ACE, angiotensin I-converting enzyme; RAAS, renin–angiotensin–aldosterone system; FSS, Fermented Soybean Seasoning; SHR, spontaneously hypertensive rat; SBP, systolic blood pressure; Dahl-S, Dahl salt-sensitive rat; ANOVA, analysis of variance; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; LC-MS, liquid chromatography/mass spectrometry; TLC, thin layer chromatography; ESI, electrospray ionization.

LITERATURE CITED

(1) Miura, K.; Daviglus, M. L.; Dyer, A. R.; Liu, K.; Garside, D. B.; Stamler, J.; Greenland, P. Relationship of blood pressure to 25-year mortality due to coronary heart disease, cardiovascular diseases, and all causes in young adult men: the Chicago Heart Association Detection Project in Industry. *Arch. Intern. Med.* **2001**, *161*, 1501–1508.

(2) Chobanian, A. V.; Bakris, G. L.; Black, H. R.; Cushman, W. C.; Green, L. A.; Izzo, J. L., Jr.; Jones, D. W.; Materson, B. J.; Oparil, S.; Wright, J. T., Jr.; Roccella, E. J. Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. National Heart, Lung, and Blood Institute; National High Blood Pressure Education Program Coordinating Committee. Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension* **2003**, *42*, 1206–1252.

(3) Matsufuji, H.; Matsui, T.; Seki, E.; Osajima, K.; Nakashima, M.; Osajima, Y. Angiotensin I-converting enzyme inhibitory peptides in an alkaline protease hydrolyzate derived from sardine muscle. *Biosci. Biotechnol. Biochem.* **1994**, *58*, 2244–2245.

(4) Sato, M.; Hosokawa, T.; Yamaguchi, T.; Nakano, T.; Muramoto, K.; Kahara, T.; Funayama, K.; Kobayashi, A.; Nakano, T. Angiotensin I-converting enzyme inhibitory peptides derived from wakame (*Undaria pinnatifida*) and their antihypertensive effect in spontaneously hypertensive rats. *J. Agric. Food Chem.* **2002**, *50*, 6245–6252.

(5) Kodera, T.; Nio, N. Identification of an angiotensin I-converting enzyme inhibitory peptides from protein hydrolysates by a soybean protease and the antihypertensive effects of hydrolysates in spontaneously hypertensive model rats. *J. Food Sci.* **2006**, *71*, C164–173.
(6) Laragh, J. H.; Bear, L.; Brunner, H. R.; Buhler, F. G.; Ealey, J. E.; Vaughan, E. D. Renin, angiotensin and aldosterone system in pathogenesis and management of hypertensive vascular disease. *Am. J. Med.* **1972**, *52*, 633–652.
(7) Ondetti, M. A.; Cushman, D. W. Inhibition of the renin-angiotensin system. A new approach to the therapy of hypertension. *J. Med. Chem.* **1981**, *24*, 355–361.
(8) Skidgel, R. A.; Erdös, E. G. The broad substrate specificity of human angiotensin I converting enzyme. *Clin. Exp. Hypertens. A* **1987**, *9*, 243–259.
(9) Okamoto, A; Hanagata, H; Matsumoto, E; Kawamura, Y; Koizumi, Y; Yanagida, F.; Angiotensin, I converting enzyme inhibitory activities of various fermented foods. *Biosci. Biotechnol. Biochem.* **1995**, *59*, 1147–1149.
(10) Oka, S.; Nagata, K. Isolation and characterization of neutral peptides in soy sauce. *Agric. Biol. Chem.* **1974**, *38*, 1185–1194.
(11) Oka, S.; Nagata, K. Isolation and characterization of acidic peptides in soy sauce. *Agric. Biol. Chem.* **1974**, *38*, 1195–1202.
(12) Fukushima, D. Industrialization of fermented soy sauce production centering around Japanese shoyu. In *Industrialization of indigenous fermented foods*, 2nd ed.; Steinkraus, K. H., Ed.; Marcel Dekker: New York, 2004; pp 1−88.
(13) Tachi, H. X-prolyl-dipeptidyl-aminopeptidase activity during soy sauce fermentation. *J. Brew. Soc. Japan* **1996**, *91*, 138–140.
(14) Iemura, Y.; Yamada, T.; Takahashi, T.; Furukawa, K.; Hara, S. Properties of the peptides liberated from rice protein in *sokujo-moto*. *J. Biosci. Bioeng.* **1999**, *88*, 276–280.
(15) Kasahara, Y.; Ashihara, Y. Colorimetry of angiotensin-I converting enzyme activity in serum. *Clin. Chem.* **1981**, *27*, 1922–1925.
(16) Zhu, X. L.; Watanabe, K.; Shiraishi, K.; Ueki, T.; Noda, Y.; Matsui, T.; Matsumoto, K. Identification of ACE-inhibitory peptides in salt-free soy sauce that are transportable across caco-2 cell monolayers. *Peptides* **2008**, *29*, 338–344.
(17) Yamakoshi, J.; Fukuda, S.; Satoh, T.; Tsuji, R.; Saito, M.; Obata, A.; Matsuyama, A.; Kikuchi, M.; Kawasaki, T. Antihypertensive and natriuretic effects of less-sodium soy sauce containing γ-aminobutyric acid in spontaneously hypertensive rats. *Biosci. Biotechnol. Biochem.* **2007**, *71*, 165–173.
(18) Cheung, H. S.; Wang, F. L.; Ondetti, M. A.; Sabo, E. F.; Cushman, D. W. Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. *J. Biol. Chem.* **1980**, *255*, 401–407.
(19) Suetsuna, K. Isolation and characterization of angiotensin I-converting enzyme inhibitor dipeptides derived from *Allium sativum L* (garlic). *J. Nutr. Biochem.* **1998**, *9*, 415–419.
(20) Kinoshita, E.; Yamakoshi, J.; Kikuchi, M. Purification and identification of an angiotensin I-converting enzyme inhibitor from soy sauce. *Biosci. Biotechnol. Biochem.* **1993**, *57*, 1107–1110.
(21) Takahama, A.; Iwashita, A.; Matsuzawa, M.; Takahashi, H.; Nakatsuka, M.; Yahata, S. Anti-hypertensive peptides derived from fermented soybean paste-miso. *Int. News Fats, Oils Relat. Mater.* **1993**, *4*, 525.
(22) Shin, Z. I.; Yu, R.; Park, S. A.; Chung, D. K.; Ahn, C. W.; Nam, H. S.; Kim, K. S.; Lee, H. J. His-His-Leu, an angiotensin I converting enzyme inhibitory peptide derived from Korean soybean paste, exerts antihypertensive activity in vivo. *J. Agric. Food Chem.* **2001**, *49*, 3004–3009.
(23) Kuba, M.; Tanaka, K.; Tawata, S.; Takeda, Y.; Yasuda, M. Angiotensin I-converting enzyme inhibitory peptides isolated from tofuyo fermented soybean food. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 1278–1283.
(24) Okamoto, A.; Hanagata, H.; Kawamura, Y.; Yanagida, F. Antihypertensive substances in fermented soybean, natto. *Plant Foods Hum. Nutr.* **1995**, *47*, 39–47.
(25) Seo, W. H.; Lee, H. G.; Baek, H. H. Evaluation of bitterness in enzymatic hydrolysates of soy protein isolate by taste dilution analysis. *J. Food Sci.* **2008**, *73*, S416.

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