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Isoflavone Content and Its Potential Contribution to the Antihypertensive Activity in Soybean Angiotensin I Converting Enzyme Inhibitory Peptides

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A soybean angiotensin I converting enzyme (ACE) inhibitory peptide fraction was reported to have antihypertensive activity in a rat study. The purpose of the present study was to examine if the presence of isoflavones in the soybean ACE inhibitory peptide fraction may contribute to the blood-pressure-lowering property. The isoflavone concentration in soybean samples was analyzed on a C_{18} reverse-phase column using a two-step gradient solvent system. Producing soybean hydrolysate led to a nearly 40% loss of isoflavones compared with the original soybean flour, but the isoflavone composition did not change and the dominant isoflavone chemicals remained as 6″-*O*-malonylgenistin and 6″-*O*-malonyldaidzin. Ion exchange chromatography affected significantly both the content and the composition of the isoflavones. The dominant isoflavones in the ion-exchanged fraction were aglycones and nonacylated isoflavones, accounting for 95.8% of the total amount of 987.7 μg/g. It was calculated that the isoflavone content in the soybean ACE inhibitory peptide fraction was 25 times less than the minimal effective isoflavone dose reported. In vitro study also showed that adding isoflavones into both soybean flour hydrolysate and soybean ACE inhibitory peptide samples to a concentration of as high as 31.5% (w/w) did not affect ACE inhibitory activity (IC_{50} values). The findings along with previously published results indicated that the contribution of isoflavones in soybean ACE inhibitory peptide fraction to the blood-pressure-lowering property in a short-term feeding study might be negligible.

KEYWORDS: Soybean; angiotensin I converting enzyme; peptides; isoflavones; antihypertensive activity

INTRODUCTION

Soybean is an economically important crop in many countries. In 1999, the U.S. Food and Drug Administration (FDA) issued a health claim stating "diets low in saturated fat and cholesterol that include 25 g of soy protein per day may reduce the risk of heart disease" (*1*). The presence of isoflavones in many soybean products prompted researchers to suggest a role for isoflavones; unfortunately, it is still unclear to what extent the isoflavones are responsible for the benefit. Isoflavones are a subclass of the large and more ubiquitous flavonoid group, recognized to possess a wide range of healthy benefits such as prevention of certain cancers, cardiovascular diseases, and osteoporosis and alleviation of menopausal symptoms (*2*). However, the FDA stated that "the evidence did not support a significant role for soybean isoflavones in cholesterol-lowering effects of soy protein" (*1*).

Heart disease is the number one leading cause of death and represents a major health concern in many developed countries. High blood cholesterol and high blood pressure are two of the identified major risk factors for heart disease. In addition to the cholesterol-lowering effect, a blood-pressure-lowering effect of soybean and soybean products was reported in rat studies and clinical trials. For example, Rivas et al. (*3*) reported that consumption of soybean milk (500 mL twice daily) had modest, but significant hypotensive action in essential hypertensive patients; Welty et al. (*4*) surprisingly found that eating roasted soybean nuts (half a cup) daily lowered systolic blood pressure by 10% and diastolic blood pressure by 7% for hypertensive women. However, a role of isoflavones in the blood-pressure-lowering effect has not been established. Hodgson et al. (*5*) showed that purified isoflavones did not affect either systolic or diastolic blood pressure (*5*). Moreover, a hypertensive case was reported after consumption of isoflavone supplement (*6*).

Recent research studies have shown that peptides (fragments of soybean proteins) may play a role in soybean physiological activities, particularly those related to the prevention of chronic diseases (*7*). Peptides with inhibitory activity against angiotensin I converting enzyme (ACE), an enzyme responsible for blood pressure regulation, have been well documented. These peptides were most commonly produced by fermentation or enzymatic hydrolysis of soybean proteins (*7, 8*); however, the presence of

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the isoflavones and the potential contribution of isoflavones to the antihypertensive activity in the soybean ACE inhibitory peptides have not been examined. The objectives of this study were to determine the isoflavone concentrations in the soybean ACE inhibitory peptides and their possible contributions to the antihypertensive activity.

MATERIALS AND METHODS

Materials and Reagents. Nutrisoy 7B flour (lot 991201C) and isolated soybean protein (lot 02041922) were gifts from Archer Daniels Midland Co. (Decatur, IL). Isoflavone standards (daidzin, 6″-*O*-acetyldaidzin, daidzein, genistein, 6″-*O*-acetylgenistin, 6″-*O*-malonylgenistin, genistin, glycitin, 6″-*O*-acetylglycitin, glycitein) were purchased from LC Laboratories (Woburn, MA). HPLC-grade acetonitrile, acetic acid, and spectra-analyzed-grade dimethyl sulfoxide (DMSO) were obtained from Fisher Scientific (Pittsburgh, PA). Angiotensin converting enzyme (from rabbit lung) and hippuryl-histidyl-leucine (HHL) were obtained from Sigma Chemical Co. (St. Louis, MO). Hippuric acid (HA) and trifluoroacetic acid (TFA) were obtained from Acros Organics (Fair Lawn, NJ). HPLC-grade water generated by a Milli-Q system (Millipore, Bedford, MA) was used for the preparation of the mobile phase.

Preparation of Soybean ACE Inhibitory Peptides. Soybean hydrolysate and soybean ACE inhibitory peptides were prepared according to the method of Wu and Ding (*8*). Nutrisoy 7B flour or soybean protein isolate was mixed thoroughly using a magnetic stirrer with distilled water into a 5% (w/w, protein/water) slurry. The pH of the slurry was adjusted to 8.0 and the temperature was raised to 50 °C prior to enzymatic hydrolysis by Acalase 2.4 L. Enzyme was added at the ratio of 4% (w/w, on the basis of protein content of slurry). During the reaction, the temperature and the pH of the slurry were maintained constant. After 3 h of reaction, the enzyme was inactivated by adjusting the pH to 4.0 with 6 N HCl. Unhydrolyzed proteins and large peptides as well as other polymers were removed by centrifugation at 6000*g* for 25 min. The residue was suspended again with 300 mL of water and centrifuged at the same conditions as above. The resulting clear supernatants were then combined, freeze-dried, and stored at −20 °C until further analysis.

Dowex HCR-W2 ion-exchange resin (2 L, Dowex hydrogen, strongly acidic cation, 8% cross-linking, 16−40 mesh; Aldrich Chemical Co., Inc.) was packed into a Spectra/Chroma LC column (i.d. = 7.5 cm, volume = 44.2 mL/cm, Fisher Scientific, Pittsburgh, PA). The column was equilibrated by 4 column volumes (CV) of purified water (8 L of water) before 300 mL of soybean protein hydrolysate (∼15.6 g of hydrolysate powder) was loaded. After the unbound material had been washed out using 6 L of purified water, the bound peptides were eluted by 6 L of 2 N ammonium hydroxide. Eluted fraction was collected, pooled, concentrated by evaporation under reduced pressure to eliminate the ammonium, and then freeze-dried. The resin was regenerated by washing with 8 L of purified water, 4 L of 2 N HCl, and following 8 L of purified water prior to reuse.

Isoflavone Extraction. Approximately 0.5 g of soy flour was accurately weighed into a screw-cap test tube (125 mm × 20 mm) and was extracted at room temperature for 2 h on a rotary mixer with 10 mL of methanol/acetonitrile/water (4:3:2, v/v/v). After a brief centrifugation (2000*g* for 10 min), a portion of the supernatant was removed with a syringe, filtered through a 0.45 μm PVDF filter into a sample vial, and analyzed by HPLC. The isoflavone contents in soybean hydrolysates and soybean ACE inhibitory peptide fractions were prepared by dissolving the samples in 0.1% acetic acid water solution. These samples were then filtered through a 0.45 μm PVDF filter prior to HPLC analysis.

Isoflavone Analysis. Separation and quantification of isoflavones were performed on a symmetry C_{18} reverse-phase column (4.6 × 250 mm, 5 μm particle size, Waters, Milford, MA) according to the method of Griffith and Collison (*9*) with slight modifications. All samples were run automatically via sample management system at a fixed injection volume of 10 μL on a HPLC Waters 2690 Separation Module System. UV absorbance was scanned from 200 to 350 nm using a Waters model 996 photodiode array detector, and peak area was integrated automatically with the supplied software at 254 nm. The sample compartment temperature and column temperature were kept at 15 and 34 °C, respectively. The column was eluted by a two-solvent system: (A) 0.1% acetic acid in water and (B) 0.1% acetic acid in acetonitrile. The column was held constant at 15% B for the first 10 min, increased to 35% B over 45 min and then to 55% B over 10 min. The concentration of B was brought back to 15% over 5 min and held for another 5 min before the next run.

Individual isoflavone standards were used for peak identification according to elution time, UV spectra, and spiking tests. Isoflavone quantification was based on calibration curves for each of the 10 isoflavone standards; quantification of 6″-*O*-malonyldaidzin and 6″-*O*-malonylglycitin, however, was based on their corresponding glucosides adjusted for the molecular weight difference (*10*). Each sample was analyzed in duplicate, and mean values are reported.

Determination of ACE Inhibitory Activity. ACE inhibitory activity was determined by the direct HPLC injection method (*11*). A total reaction volume of 70 μL was composed of 50 μL of 2.17 mM HHL, 10 μL of 2 mU of ACE, and 10 μL of various concentrations of protein hydrolysates (all prepared with 100 mM borate buffer, containing 300 mM NaCl, pH 8.3). For the control test, 10 μL of buffer solution was used instead of the sample solution. After 30 min of incubation at 37 °C, the reaction was terminated by adding 85 μL of 1 N HCl. The reaction solution was then filtered through a 0.45 μm nylon syringe filter, and 10 μL of sample was injected directly on a Symmetry C18 column (3.0 × 150 mm, 5 μm, Waters Inc.) for HA quantification. HPLC analysis was performed on a Waters 2690 Separation Module equipped with a 996 photodiode array detector. Instrument control and data collection and analysis were undertaken using Millennium Chromatography Manager Software version 2.15 (Waters Inc.). The column was eluted by a two-solvent system: (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile as reported (*11*).

To study the effects of adding isoflavones on the ACE inhibitory activity of soybean hydrolysates, isoflavone standards containing genistein (0.57 mg), glycitin (0.80 mg), genistin (1.05 mg), 6^2-*O*-acetyldaidzin (0.63 mg), 6^2-*O*-acetylglycitin (0.48 mg), daidzein (0.75 mg), glycitein (0.46 mg), 6^2-*O*-malonygenistin (0.54 mg), 6^2-*O*-acetylgenistin (0.40 mg), and daidzin (0.62 mg) were dissolved by an equal volume of DMSO and a mixed solvent [methanol/acetonitrile/water = 4:3:2 (v/v/v)] to a concentration of 1.26 mg/mL. This solution was further diluted by borate buffer (used in ACE determination) to a concentration of 0.63 mg/mL. The diluted isoflavone standard solution was then mixed with the soybean hydrolysate sample (2 mg/mL, prepared by borate buffer) at the same volume, accounting for 31.5% of isoflavones in the hydrolysate sample and used for ACE analysis. To evaluate the influence of the solvent, a blank solvent was in parallel prepared and analyzed.

The IC_{50} value was defined as the amount of inhibitory substance that resulted in 50% inhibition of ACE activity in the reaction system. The percent inhibition versus peptide concentrations (mg/mL) curves were constructed using at least five separate determinations (duplicate determinations). Regression was conducted with the Microsoft Excel 97 SR-1 software (Microsoft Corp., Redmond, WA).

Statistics Analysis. The General Linear Model, PROC GLM, in SAS system (version 8.2, SAS Institute, Inc., Cary, NC) was used to compare means at $p < 0.05$.

RESULTS

Detection and Quantification of Isoflavones in the Presence of Peptides. Extraction of isoflavones from soybean products is the essential prerequisite for their determinations; 80% methanol or 53−60% acetonitrile aqueous are the most widely used extraction solvents (*9, 12*). Despite recent advances in extraction technology, developing an efficient method of isoflavone extraction from soybean products remains a challenge. Unlike many soybean products, soybean enzymatic hydrolysate samples are water-soluble samples. This provides an alternative option to eliminate the common organic solvent extraction step by dissolving the samples in water solution. However, it should

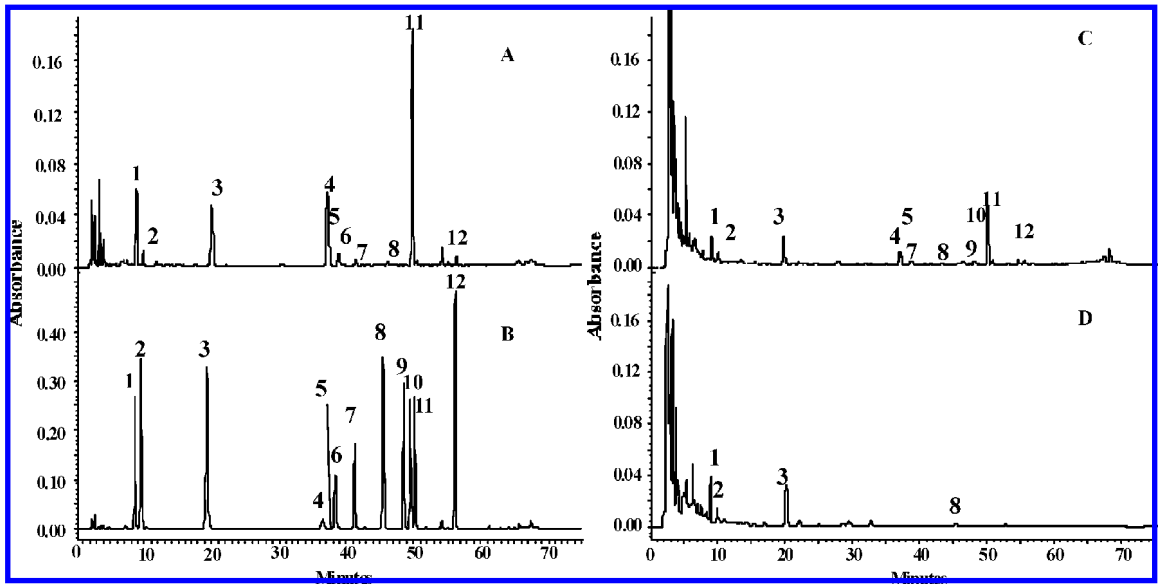

Figure 1. HPLC chromatograms of soybean flour sample extracted with methanol/acetonitrile/water (4:3:2, v/v/v) (**A**) and its spiked sample with isoflavone standards (**B**), soybean flour hydrolysate (**C**), and soybean protein isolate hydrolysate (**D**) prepared by dissolving the samples in 0.1% acetic acid water solution. The protein concentrations were zero in soybean flour extract, 55.3% in soybean flour hydrolysate, and 97% in soybean protein isolate hydrolysate. Peak identification: 1, daidzin; 2, glycitin; 3, genistin; 4, 6″-*O*-malonyldaidzin; 5, 6″-*O*-malonylglycitin; 6, 6″-*O*-acetyldaidzin; 7, 6″-*O*-acetylglycitin; 8, daidzein; 9, glycitein; 10, 6″-*O*-acetylgenistin; 11, 6″-*O*-malonylgenistin; 12, genistein.

Table 1. Content of Isoflavones[a] (Micrograms of Aglycone per Gram of Dry Weight) in Soybean ACE Inhibitory Peptides and Soybean Flour

isoflavone compound	soybean flour hydrolysate (SFH)	percentage of chemicals in SFH	soybean ACE inhibitory peptides	soybean isolate protein hydrolysate	soybean flour extract (SFE)	percentage of chemicals in SFE
daidzein	67.7	4.8	182.1	24.7	37.5	2.1
daidzin	180.4	12.9	251.2	52.0	252.4	14.0
6″-*O*-acetyldaidzin	—[b]	—	—	4.1	12.5	0.7
6″-*O*-malonyldaidzin	412.8	29.5	0.0	51.7	482.5	26.8
genistein	49.4	3.5	104.7	28.3	47.5	2.6
genistin	186.7	13.3	352.4	110.3	288.4	16.0
6″-*O*-acetylgenistin	5.1	0.4	—	8.6	11.4	0.6
6″-*O*-malonylgenistin	395.5	28.3	41.3	125.8	557.1	31.0
glycitein	17.8	1.3	—	—	—	0.0
glycitin	31.7	2.3	56.0	12.0	36.7	2.0
6″-*O*-acetylglycitin	12.4	0.9	—	5.0	23.3	1.3
6″-*O*-malonylglycitin	40.1	2.9	—	5.5	48.6	2.7
total aglycones	1399.5		987.7	428.1	1797.9	
total aglycone	1380.2		946.9	417.0		

[a] Values are the mean of duplicate determinations, expressed in the form of aglycones. [b] Not detected.

be noted that isoflavones coexist with peptides in these hydrolysates, possessing similar UV spectra and molecular weight ranges (*8*). Therefore, it is critical to discriminate these peptides from isoflavones during analysis. **Figure 1** shows the chromatograms of soybean flour extract (**Figure 1A**) and its spiked sample (**Figure 1B**), soybean flour hydrolysate (**Figure 1C**), and soybean protein isolate hydrolysate (**Figure 1D**). Individual isoflavone peaks were confirmed by individual authentic standards based on their elution time, spiked test, UV spectra, and HPLC-MS analysis (data not shown). Although the protein contents ranged widely from zero in soybean flour extract to 55.3% in soybean flour hydrolysate and 97% in soybean protein isolate hydrolysate, our results showed that peptides did not coelute with isoflavone peaks under the current condition.

Soybean ACE inhibitory peptides are composed mainly of short-chain peptides (*8*), so their hydrophobicities were probably weaker than those of the isoflavones. Therefore, these peptides could be eluted earlier than isoflavones on a reverse-phase column. Although the protein concentration increased significantly from soybean flour solvent extract to soybean flour hydrolysate and soybean isolated protein hydrolysate, the separation efficiencies of individual isoflavone compounds are constant for the samples tested. The total elution time of the current condition is 75 min, which is close to the elution time (73 min) under the standard method (*13*).

Isoflavone Content in Soybean ACE Inhibitory Peptides. The isoflavone content and composition in soybean ACE inhibitory peptide samples and soybean flour are shown in **Table 1**. The total isoflavone concentration in Nutrisoy 7B flour was 1797.9 μg/g (dw) in the form of aglycones. This value was significantly higher than the mean amount of 1311.9 μg/g in the USDA−Iowa State University Database (*14*) but lower than the reported value of 2071 μg/g (*15*). The major isoflavones in Nutrisoy 7B flour, which was minimally heat processed, were 6″-*O*-malonylgenistin and 6″-*O*-malonyldaidzin. These two compounds accounted for 57.8% of the total aglycone content in soybean flour, compared with 70% in soybean protein (*9*) or 80.5% in the whole soybean (*16*). However, Wang and Murphy (*13*) reported that 6″-*O*-malonylgenistin and genistin were the major isoflavones in soybean flour, accounting for 71% of the

total isoflavones. Soybean varieties and processing conditions may be responsible for the differences.

On the basis of aglycones, the recovery of isoflavones in the hydrolysate was 61.1% of the initial isoflavone content of the flour (**Table 1**). The isoflavone composition of the soybean flour hydrolysate (SFH) was similar to that of soybean flour extract (SFE). 6″-*O*-Malonylgenistin and 6″-O-malonyldaidzin remained as the principal compounds in the hydrolyzed sample (**Table 1**), accounting for 57.8% of the total isoflavones. These results indicated that isoflavones were not degraded or interconverted during the enzymatic production of protein hydrolysates. It is generally believed that malonylglucoside conjugates are the most fragile compounds among these isoflavone chemicals, and their stability was greatly affected by environmental factors and processing conditions (*9, 17*). Now, it has become a common practice to extract isoflavones from soybean-containing foods at relatively lower temperatures (<30 °C) to prevent degradation of malonyl conjugates (*13*). Theoretically, the relatively high temperature (50 °C) and alkaline pH (8.0) conditions applied during hydrolysis could have caused degradation of the malonyl conjugates. In the preparation of isolated soybean protein under pH of 8.5 at room temperature, Wang and Murphy (*18*) reported a significant increase in the content of aglycones and a reduction in malonyl conjugates in soybean processing. In this study, however, the ratio of aglycone chemicals was increased slightly from 4.7% in the soybean flour to 9.6% in the soybean hydrolysate, which suggested the relatively high stability of these conjugates during enzymatic hydrolysis. Factors such as the formation of peptides in the enzymatic hydrolysis and the high water content, in addition to temperature and pH, might contribute to the increased stability of malonyl conjugates. Barnes et al. (*19*) reported the amount of malonylglucoside conjugates did not decrease whether extracted at room temperature or at 60 °C from 1 h to extended 4 h in the form of soy milk. In the form of soy protein isolate, however, the amount of malonylglucoside conjugates was significantly decreased at 60 °C in the first hour. We speculated that the high water content and the buffering environment formed during enzymatic hydrolysis might be partly responsible for the enhanced stability of conjugated chemicals.

Ion exchange chromatography of soybean hydrolysate, on the other hand, significantly affected the content and composition of isoflavones as shown in **Table 1**. The total isoflavone content in soybean ACE inhibitory peptide fraction was reduced to 987.7 μg/g, nearly half of the amount measured in soybean flour. The most noticeable change was that the content of daidzein, which was increased to 182.1 μg/g, was nearly 5 times higher than the amount in the soybean flour. Because there were no more 6″-*O*-malonyldaidzin and 6″-*O*-acetyldaidzin left in the ion-exchanged fraction, the increase in daidzein content was attributed largely to the degradation of these two conjugates. The observed higher daidzein and lower malonygenistin levels in the ACE inhibitory peptide fraction when compared to those in SFE are due to the alkali condition (ammonium hydroxide was applied to wash out the ACE inhibitory peptide fraction). The glucosidal ester linkages would be broken down in an alkali environment. The only existing conjugated isoflavone in the ion-exchanged fraction was 6″-*O*-malonylgenistin. The most dominant forms were the aglycone and nonacylated glycosides, accounting for 95.8% of the total amount. As expected, soybean protein isolate hydrolysate contained the lowest amount of isoflavones as a result of isolate preparation (*18*), accounting for 23.8% of the total isoflavones in soybean flour.

Table 2. Influence of Exogenous Isoflavone Standards on the in Vitro ACE Inhibitory Activity

sample	protein concn (%)	calcd isoflavones (μg/mL)	IC_{50}[a] (μg of powder/mL)
soybean flour hydrolysate	55.3	2.5	109.6 ± 4.1 a
soybean flour hydrolysate + isoflavones	55.3	317.5	101.6 ± 1.2 a
soybean ACE inhibitory peptides	97.0	1.4	55.0 ± 0.9 b
soybean ACE inhibitory peptides + isoflavones	97.0	316.4	55.2 ± 0.0 b

[a] Mean of two determinations. Values with different letters were significantly different at $\alpha = 0.05$.

Effects of Isoflavones on in Vitro ACE Inhibitory Activity. The effect of isoflavones on in vitro ACE inhibitory activity was evaluated by the HPLC method (*11*). A blank sample containing the solvents used to solubilize isoflavones did not show inhibitory activity against ACE. When isoflavones were added at a concentration of as high as 31.5% (w/w) into both soybean flour hydrolysate and soybean ACE inhibitory peptides, the IC_{50} values were not affected compared with their hydrolysate samples (**Table 2**). These results indicated that isoflavones are unlikely to affect blood pressure via the inhibition of ACE activity. Although structurally different, flavonoids with ACE inhibitory activity have been identified from the leaves of the persimmon Diospyros Kaki (*20*).

There are reports that isoflavones such as genistein and equol (a metabolite of daidzein) could possess natriuretic and vasorelaxant properties in rats (*21, 22*), reduce vascular smooth muscle contractions after angiotensin II administration (*23*), inhibit ACE expression via estrogen receptor and subsequently ERK1/2 signaling pathway in aortic endothelial cells (*24*), or improve endothelial function via activation of nitric oxide synthase in an estrogen-independent manner (*25*), thus exerting blood-pressure-lowering effect. Direct injection of genistein also showed lower mean arterial pressure (MAP) in male and ovariectomized (OVX) female spontaneously hypertensive rats (SHR) (*26*) as well as in intact female borderline hypertensive rats (*27*). Yamori et al. (*28*) demonstrated that daidzein could attenuate postovariectomy blood pressure elevation by accelerating vascular nitric oxide production in stroke-prone SHR. On the contrary, Martin et al. (*29*) verified that soy diet showed selectively significant blood-pressure-lowering effect in OVX female SHR groups relating to an as yet undefined interaction with the autonomic nervous system instead of the nitric oxide response.

Although the blood-pressure-lowering effect of soybean and soybean products has also been reported in some clinical trials (*3, 30*), the extent of the contribution of isoflavones remains unclear. Most researchers tended to link the antihypertensive activity of soybean products to the isoflavones; however, results are controversial. A strong and significant correlation was observed between the urinary genstein excretion and the decrease in diastolic blood pressure at the consumption of a large amount of soybean milk (*3*). However, no relationship has been reported between the dose of isoflavones and the decrease of blood pressure in the literature (*3*). Interestingly, a 7% decrease in systolic and diastolic blood pressure was observed in healthy postmenopausal women who consumed a

preparation containing 100% unconjugated isoflavones, but not a preparation containing 70% unconjugated isoflavones (*30*). Significant diastolic blood-pressure-lowering effect was seen in the twice-daily soy diet but not in the single soybean diet, even though subjects received the same total dose (20 g of soybean protein containing 34 mg of isoflavones) (*31*). There was a slight tendency to decreasing blood pressure whether using high (73 mg daily) or low (10 mg daily) isoflavone-containing diets (*32*). Han et al. (*33*) reported that daily administration of 100 mg of soybean isoflavone for 4 months in postmenopausal women did not affect blood pressure. Similar results were also reported by other research groups (*5*). The fact that a higher soybean protein diet had a higher tendency toward lowering blood pressure than purified isoflavones indicated a role of soybean proteins. Another plausible mechanism may involve the formation of ACE inhibitory peptides during the digestion of soybean proteins in the gastrointestinal tract (*8, 34*).

After comparison with the results of Hodgson et al. (*5*), Rivas et al. (*3*) suggested that a soybean diet containing a large amount of isoflavones is essential to obtain a blood-pressure-lowering effect. The isoflavone amount in Rivas's work was a daily intake of 63 mg of equivalent daidzein and 80 mg of equivalent genistein (*3*), which is higher than Hodgson's dose of 55 mg of isoflavones (*5*) and other doses reported (*6, 32*). Provided the average body weight of subjects is 60 kg, the highest ever reported dose of 960 mg of genistein and 134.4 mg of daidzein in formulation B of Bloedon's trial did not show significant blood-pressure-lowering activity in healthy postmenopasusal women (*30*). A significant blood-pressure-lowering effect of the soybean ACE inhibitory peptides was observed at a dose of 100 mg/kg of body weight in our study, where the content of isoflavones was >25 times lower than the minimal effective dose of isoflavones reported (*3, 8*). Therefore, the contribution of isoflavones in the soybean ACE inhibitory peptides to the observed blood-pressure-lowering effect might be negligible. However, we could not exclude the potential (or indirect) contribution to improved blood pressure at elevated levels of isoflavones in the long-term, because isoflavones are thought to possess a favorable effect in reducing cardiovascular risk factors as well as vascular function (*2*).

Soybean proteins are a rich source of bioactive peptides. Compared with animal-derived bioactive peptides, the inherently associated isoflavones in soybean-derived products may affect uniquely the peptide bioactivities because isoflavones are reported to have various bioactivities. However, the residual isoflavone content in the peptide products is decided largely by the processing conditions. On the basis of in vitro results and literature review, we have indicated that the contribution of isoflavones to a blood-pressure-lowering effect in soybean ACE inhibitory peptides may be negligible. Nevertheless, future in vivo study may be helpful in providing further insight on the extent of contribution of isoflavones on the blood-pressure-lowering property in the peptide fraction.

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