Hypotensive and Physiological Effect of Angiotensin Converting Enzyme Inhibitory Peptides Derived from Soy Protein on Spontaneously Hypertensive Rats

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Angiotensin converting enzyme (ACE) inhibitory peptides prepared from soy protein by the action of alcalase enzyme was tested for its hypotensive effect on spontaneously hypertensive rats (SHR). Captopril, an ACE inhibitor used widely for hypertension treatment, was also applied in comparison. A significant ($p < 0.05$) decrease in systolic blood pressure of SHR was observed when soy ACE inhibitory peptides were orally administrated at three different dose levels (100, 500, and 1000 mg/kg of body weight/day), whereas little change occurred in the blood pressure of normotensive rats even at the highest dose. After a month-long feeding, blood pressure readings of SHR fell by ~38 mmHg from the original level at the lowest dose; a steadily and progressively hypotensive effect existed for these soy ACE inhibitory peptides administration groups. An obvious fluctuation was observed at the third week, although Captopril had a stronger hypotensive effect. The ACE activity of serum, aorta and lung, and lipid content of serum of SHR upon administration of soy ACE inhibitory peptides did not show a significant difference from that of the control group, whereas the serum ACE activity increased and the aorta ACE activity decreased significantly ($p < 0.05$) for the Captopril group. Serum Na^+ concentration decreased significantly in both the peptides-treated groups and the Captopril-treated group in comparison with the control group, whereas no lowering effect was observed for serum K^+ and serum Ca^{2+} concentrations. These results suggested that the hypotensive effect of ACE inhibitory peptides derived from soy protein could be at least partly attributed to the action on salt/water balance.

Keywords: *Soy angiotensin converting enzyme (ACE) inhibitory peptides; spontaneously hypertensive rats (SHR); hypotensive effect; Na^+*

INTRODUCTION

Recently, the new relationship between food and health has drawn considerable attention; of interest are the physiological functions of some food components against certain ailments. Hypertension is a worldwide problem of epidemic proportions, which presents in 15−20% of all adults. It is the most common serious chronic health problem because it carries with it a high risk of cardiovascular complication (*1*). As an insidious and ubiquitous disease, hypertension and its complications may contribute to the deaths of 1.5 million and the disability of another 1.5 million Americans each year (*1*). Now, it is suggested that hypertension is closely related to food components, especially sodium chloride and protein intake, and the antihypertensive peptides may be associated with the presence of an antihypertensive peptide motif.

Angiotensin converting enzyme (ACE, EC 3.4.15.1) plays an important role in the renin-angiotensin system (RAS), which regulates both arterial blood pressure and the salt/water balance (*2*, *3*). Since the first discovery of ACE inhibitors from snakes, inhibitors of ACE have been shown to lower blood pressure in hypertensive animals and human beings (*4*−*8*). Some specific synthetic inhibitors of ACE, such as Caporal and Enalapril, have established themselves in the therapy of hypertension and congestive heart failure (*9*). Successive dietary ingestion of moderate ACE inhibitors derived from food proteins has been expected to prevent hypertensive diseases, and for this purpose, ACE inhibitory peptides from food proteins were isolated; some of them have proved to be effective against spontaneously hypertensive rats (SHR) and hypertensive patients (*10*, *11*). Much research has been concentrated on gelatin (*12*), casein (*13*), zein (*14*, *15*), sake (*16*), sardine muscle (*17*), tuna muscle (*18*), and other food proteins. Few studies were available on soy protein derived ACE inhibitory peptides.

As the major protein resource in most developing countries soybean is widely consumed as processed foods (tofu, natto, tempe, miso, shoyu, etc.) in China and Southeast Asian countries. ACE inhibitory activity was found in fermented soybean products such as soy sauce (*19*) and natto (*20*); some of them show relatively strong antihypertensive activity against SHR. Previous studies have shown that the soy protein hydrolysate had a high ACE inhibitory activity and stable gastrointestinal protease resistance in vitro (*21*), but their in vivo activity needs to be explored at the same time. The objectives of this study were to investigate the blood-lowering and physiological effect of ACE inhibitory

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Table 1. Basal Compositions of the Diets[a]

diet	g/100 g of diet	ingredient	g/100 g of diet
wheat	40	protein	21
maize	25	fat	3.2
soybean meal	17	carbohydrate	57
wheat bran	8	crude fiber	2.5
fish flour	7	ash	5.6
bone flour	1	water	10.7
yeast powder	1		
vitamins, microminerals, and lysine, etc.	1		

[a] Results provided by Nantong Medical institute.

peptides derived from soy protein on SHR in vivo and discuss its plausible effective mechanism.

MATERIALS AND METHODS

Preparation of Soy ACE Inhibitory Peptides. Test sample was prepared in a 15 L reactor, the conditions as described previously (*21*): A 15 L solution of defatted soy meal at 7.5% (w/w) protein was digested by Alcalase 2.4 L (provided by Novo Nordisk Biochem, China, Inc., as a free gift; the enzyme-to-substrate ratio used was 0.04, v/w, on the basis of protein weight) at stable pH (9.0) and temperature (50 °C) for 12 h. The pH was maintained at pH 9.0 by continuous addition of 4 N NaOH. The digestion was adjusted to pH 4.0 by 6 N HCl after reaction to inactivate the enzyme, and the hydrolysate was ultrafiltrated through an ultrafiltration membrane (cutoff molecular weight = 10000, Sartorius Co.) after centrifugation (3000g, 25 min). Supernatant was further fractionated on the strong acid cationic exchange resin (732 cation resin, Shanghai Resin Factory, Shanghai, China); the active fraction was then collected and lyophilized. The total recovery of peptide was 14.55% (w/w) in this procedure.

Molecular Weight Measurement. The molecular weight of peptides was determined by HPLC (model HP1050) with a TSK gel 2000 SW_{XL} (7.5 × 300 mm) column (Supelco, Bellefonte, PA), accompanied by a TSK gel guard column (75 × 50 mm), eluted by 45% acetonitrile (55:45, v/v, acetonitrile/water) containing 0.1% trifluoroacetic acid (TFA). The flow rate was 0.5 mL/min, monitored by UV detector at an absorbance of 210 nm.

Standard molecular weights included cytochrome *c* (MW 12500), aprotinin (MW 6500), bacitracin (MW 1450), glycyl-glycyl-tyrosyl-arginine (MW 451), and glycyl-glycyl-glycine (MW 189) purchased from Sigma Chemical Co. (St. Louis, MO). The linear relationship between standard molecular weight and retention time was set up as $\log MW = 7.1915 - 0.22998t$ ($r = 0.9926$; MW, molecular weight of peptide; *t*, retention time); the molecular weight of soy protein ACE inhibitory peptides was calculated according to this equation.

Experimental Animals and Diets. Female SHR, 6 weeks old [systolic blood pressure (SBP) > 160 mmHg], and age-matched normotensive rats were purchased from Shanghai Hypertension Institute (Shanghai, People's Republic of China). The rats were fed a commercial nonpurified chow diet (Table 1) to help them to acclimate to their new environment. After a week's feeding, the rats were weighed and housed in individual metabolism cages with wire bottoms in a room maintained at 25 °C and a relative humidity of 55%, with lights for 12 h a day (lighting from 7:00 a.m. to 7:00 p.m.). The diet and drinking water available ad libitum.

Experimental Methods. SHR were randomly divided into five groups of four rats each. Oral administration was performed as follows: control group (no peptide or drug administration); soy ACE inhibitory peptides at three different levels [100, 500, and 1000 mg/kg of body weight of rat (BW)/day, indicated as low dose, intermediate dose, and high dose, respectively]; Captropril administrated as a positive control at a dose of 50 mg/kg of BW/day; and another normotensive rat group fed with the highest dose of ACE peptides to determine its effect on the blood pressure of normotensive rats.

Table 2. Soy ACE Inhibitory Peptides Composition (Percent, Dry Weight)

peptide (N × 6.25)	91.79	ash	0.54
total glycoside	4.18	moisture	2.92

The dose range was set by our pretest result (unpublished data). When soy ACE inhibitory peptides were administered, the corresponding amount of protein was removed from the rat chow to achieve the constant protein intake daily for all experimental rats. Captopril was purchased from Huarui Medicine Co. (Wuxi, People's Republic of China).

Blood Pressure Measurement. SBP was measured every 6 days by the tail-cuff method according to Fujita et al. (*8*); the rats were warmed by gastric incubation for 10 min in a 40 °C thermostat box before their blood pressure was measured with a programmed electrosphygmomanometer (model UR-1000; Ueda Co., Ltd., Tokyo, Japan).

Analytical Methods. At the end of the feeding period, rats were sacrificed by exsanguination from the abdominal aorta; blood was collected and placed for 1 h at room temperature (25 °C) and then was centrifuged at 1000*g* for 15 min to obtain serum, which was used for serum ACE activity, lipid content, hemoglobin content, and mineral content determinations. Triglycerides, total cholesterol, high-density lipoprotein, and β-lipoprotein in serum were measured by commercial diagnostic kits (Wako Pure Chemicals Inc., Osaka, Japan), hemoglobin was measured using another commercial hemoglobin test kit (Wako Pure Chemicals Inc.), and mineral content was determined with a Perkin-Elmer model 2380 atomic absorption spectrophotometer with a standard air−acetylene flame. ACE was determined according to the method of Cushman and Cheung (*22*) with some modifications: 150 μL of 5 mM hippuryl-L-histidyl-L-leucine (HHL; Sigma Chemical Co.,) borate buffer (containing 100 mM borate and 300 mM NaCl, pH 8.3) was preincubated at 37 °C for 5 min; 100 μL of prepared ACE crude solution was added, and the mixture was incubated at 37 °C for 45 min. Borate buffer (100 mM, pH 8.3) was used instead of ACE crude solution for bland determination. One unit of ACE activity was defined as the amount of enzyme that cleaves 1 mol of substrate per minute.

Preparation of ACE Crude Solution. Serum ACE crude solution was prepared as described above. Lung and thoracic aorta were washed with 0.9% saline, after chopping and homogenization with pre-cooled 100 mM borate buffer (pH 8.3, at the ratio of 5, v/w); the homogenate was centrifuged at 3000*g* for 20 min, and the supernatant was used as crude ACE extract of those tissues for ACE activity determination.

Statistical Analysis. All of the data were subjected to Statistical Analysis System software, version 6.10 (SAS Institute, Cary, NC), analysis of variance (ANOVA) procedures to test for significance among the different dose levels; α was taken as 0.05, and a *p* value <0.05 was taken as indication of a significant difference.

RESULT AND DISCUSSION

Proximate Analysis. Table 2 shows the composition of soy ACE inhibitory peptides after lyophilization; peptides accounted for as much as 91.79% and belonged mainly to oligopeptides as demonstrated by gel HPLC (Figure 1); soy ACE inhibitory peptides had a molecular weight below 954. If we considered the average amino acid residue as 120, the peptide lengths were between 2 and 8, which suggested that soy ACE inhibitory peptides consisted mainly of low molecular weight peptides. Previous work indicated that oligopeptides would be less susceptible to proteolytic action and thus could be easily absorbed and utilized by the gut after oral administration (*23*, *24*).

Effect of Soy ACE Inhibitory Peptides on SBP. Results of SBP readings after different treatments are shown in Table 3. SBP of SHR decreased significantly ($p < 0.05$) after soy ACE inhibitory peptides adminis-

Table 3. Effect of Oral Administration of Soybean Protein ACE Inhibitory Peptides on Blood Pressure (Mean ± SD)[a]

dose (mg/kg of BW/day)	time (day) 0	6	12	18	24	30
0	185.3 ± 3.21a	183.3 ± 9.87a	180.3 ± 4.51a	178.3 ± 7.64a	183.3 ± 6.43a	174.00 ± 11.53a
100	193.25 ± 28.14a	183.75 ± 24.88ab	176.75 ± 17.27bc	173.50 ± 18.36cd	170.00 ± 17.35d	155.50 ± 15.63e
500	195.00 ± 22.89a	177.00 ± 20.03b	171.25 ± 11.00bc	162.75 ± 26.17c	163.50 ± 33.52c	*b*
1000	190.50 ± 18.65a	169.00 ± 2.58b	171.50 ± 8.39b	165.25 ± 6.65bc	156.50 ± 11.39c	151.50 ± 4.95c
50 (Captopril)	194.00 ± 12.12a	164.00 ± 3.46b	151.67 ± 3.51c	158.00 ± 2.65bc	140.33 ± 15.63d	148.00 ± 15.59cd
1000 (normotensive rat)	126.20 ± 14.69n	127.60 ± 12.38n	129.40 ± 16.71n	135.20 ± 10.35n	128.00 ± 9.62n	136.50 ± 9.61n

[a] Means within the same row or column with different characters are significantly different ($p < 0.05$). [b] Data missing.

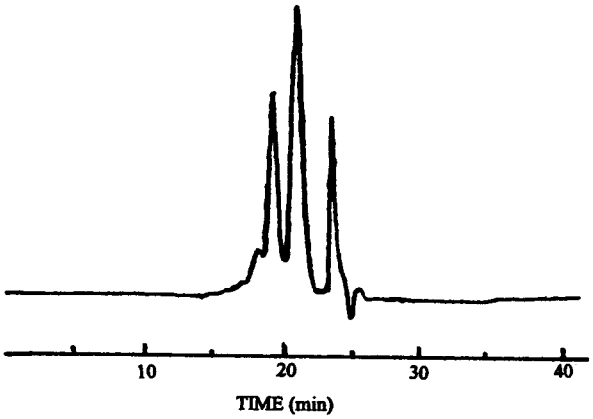

Figure 1. Molecular weight distribution of soy ACE inhibitory peptides as determined by TSK gel 2000 SW_{XL} (7.5 × 300 mm) column. Experimental method and conditions are described under Materials and Methods.

tration for 6 days at 500 and 1000 mg/kg of BW/day levels; at the 12th day the lowest administered level decreased SBP significantly also. A decrease in the SBP of SHR from 193.25 ± 28.14 to 155.50 ± 15.63 mmHg was found for a 1-month-long feeding at the dose of 100 mg/kg of BW/day. Marayama's group (*25*, *26*, *41*) have taken a systemic approach work to the development of ACE inhibitory peptides from casein; a significant blood-pressure reduction ($p < 0.05$) was observed at the second week after a continuous oral feeding of a feed containing 30% casein hydrolysate. Astawan et al. (*27*) reported that the SBP of SHR was significantly decreased when as much as of 5 g of crude peptide from Indonesian dried-salted fish/kg of BW/day was fed orally for 16 days. It seemed that soy ACE inhibitory peptides had a stronger hypotensive effect than those previously reported for casein or fish hydrolysates.

Captopril showed a stronger SBP lowering effect than that of soy ACE inhibitory peptides; SBP decreased up to 30 mmHg in the first week, whereas an obvious fluctuation existed at the third week and continued until the end of the experimental period (Table 3). It is well-known that fluctuation during a blood pressure lowering period is associated with dangers such as cerebral hemorrhage, stroke, etc. A steadily and progressively lowering effect such as that found with the feeding of soy ACE inhibitory peptides to SHR during a 1-month-long period was appreciated, and indicated the advantage of protein-derived ACE inhibitory peptides. No significant decrease was discovered in the blood pressure of normotensive rats fed even at the highest dose, which suggested that ACE inhibitory peptides derived from soy protein had no adverse effect on the blood pressure of normontensive rats.

The relationship between the dose of soy ACE inhibitory peptides and blood pressure reduction of SHR is shown in Figure 2, which indicates that of blood pressure was reduced exponentially with regard to increasing dose. A regression equation [$y = -17.0193 - 2.4657 \ln(x + 0.01)$ ($r = 0.9841$, $p = 0.008$; y = SBP reduction value; x = dose] was obtained after the dose

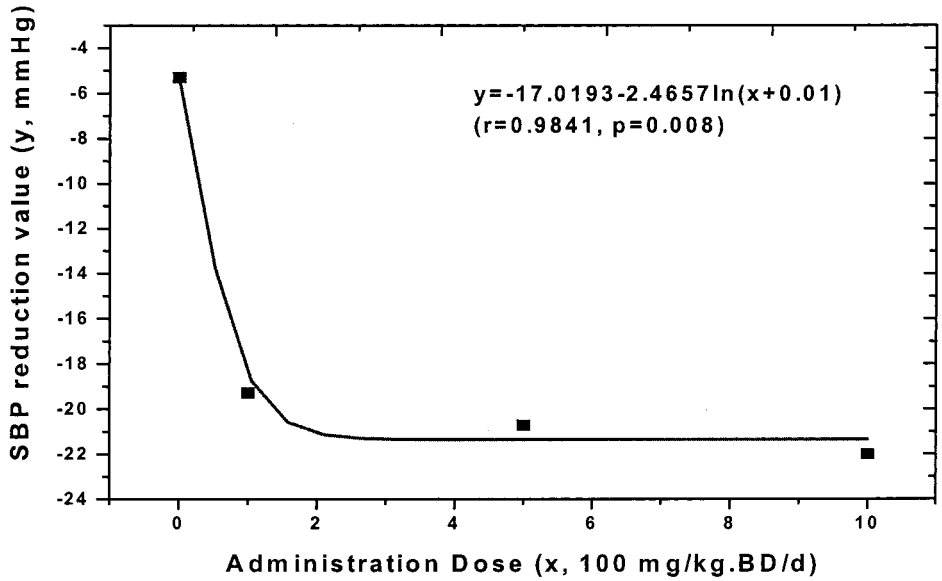

Figure 2. Relationship between administration dose of soy ACE inhibitory peptides and blood pressure reduction of SHR. y = the value of blood pressure reduction; x = soy ACE inhibitory peptides administration dose to SHR. SBP reduction values were calculated as SBP values at day 6 (or 12, 18, 24, or 30) minus the SBP value at the start day, expressed as the mean value (n = 5). SBP reduction tendency was obtained after sigmoidal fit analysis (Origin 5.0).

Table 4. Effect of Oral Administration of Soybean Protein ACE Inhibitory Peptides on Serum Lipids and Hemoglobin Content[a]

	Tch (mmol/L)	TG (mmol/L)	HDL (mmol/L)	LDL (mmol/L)	HDL/LDL	apoA1 (mg/dL)	apoB (mg/dL)	apoA1/apoB	Hb (g/L)
control	1.66 ± 0.03	0.91 ± 0.05	0.97 ± 0.06	0.51 ± 0.04	1.92 ± 0.25	7.67 ± 1.53	9.67 ± 3.05	0.82 ± 0.19	151.00 ± 7.36
peptides	1.94 ± 0.20	0.84 ± 0.35	1.11 ± 0.11	0.64 ± 0.13	1.73 ± 0.36	10.78 ± 4.76	13.33 ± 5.15	0.83 ± 0.27	150.49 ± 7.16
Captopril	1.84 ± 0.10	1.09 ± 0.46	1.07 ± 0.11	0.55 ± 0.08	1.95 ± 0.21	11.00 ± 1.73	16.67 ± 4.51	0.68 ± 0.13	143.07 ± 8.03

[a] No significance difference existed from the control ($p > 0.05$). Values are mean ± SD. n for the control and Captopril group = 4; for the peptides group $n = 12$. Tch, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triacyglycerols; Hb, hemoglobin.

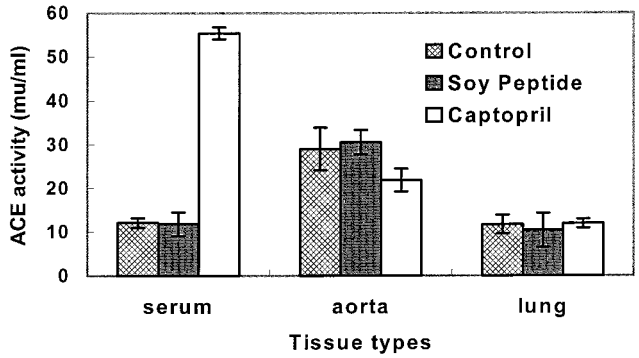

Figure 3. ACE in vivo activity of different tissues of SHR after different treatments. Values are mean ± SD. n for the control and Captopril groups was 4 and for the peptides group, 12.

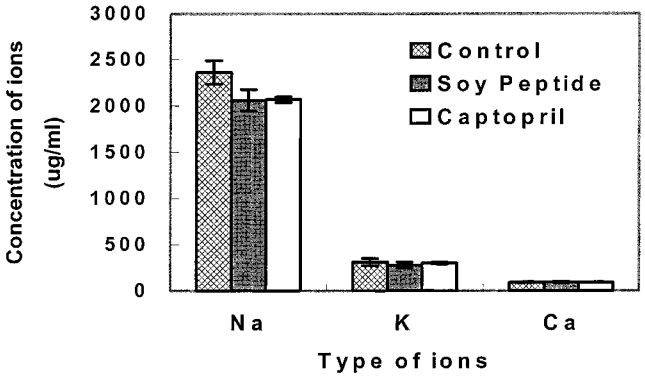

Figure 4. Concentration of serum cation of SHR after different treatments. Values are mean ± SD. n for the control group and the Captopril group was 4 and for the peptides group, 12.

values were subjected to exponent transformation. This equation indicated that a sharp decrease in blood pressure would not occur even though a higher dose of soy ACE inhibitory peptides was fed to SHR.

Serum and Histology ACE Activity. Because ACE inhibitors act via the RAS system, it was interesting to examine its effect in vivo on the ACE activity of SHR after administration of soy ACE inhibitory peptides. After a 1-month feeding experiment, soy ACE inhibitory peptides did not show a significant effect ($p < 0.05$) on the ACE activity of serum, aorta, and lung as compared with that of the control group, whereas the activity of serum was greatly enhanced ($p < 0.05$) and that of aorta was reduced ($p < 0.05$) after a 1-month-long Captopril administration (Figure 3). According to the theory of the RAS system, the activity of ACE should be lowered after administration of ACE inhibitors, but we found this phenomenon only in aorta after Captopril adminstration, whereas the group fed soy ACE inhibitory peptides showed no significant effect on the ACE activity of those organs.

This finding coincided with the results of Murakami et al. (*28*), although they (*29*) also reported that the fish diet showed a distinctly lower level ($p < 0.05$) of serum ACE activity than the control group fed by a commercial stock chow; ACE activity of serum was also enhanced when Captopril was administered. Previously, researchers have suggested that administration of ACE inhibitors has been shown to induce ACE and lead to a marked increase in plasma ACE level (*30*−*32*). The real cause of the increased serum ACE activity during continuous administration of Captopril is not yet known. The different responses on serum ACE activity of protein ACE inhibitory peptides and Captopril indicated they might take effect via different antihypertensive mechanisms. Further work on this related area should be explored.

Effect on the Content of Serum Lipids. No significant changes occurred in the content of serum lipids of SHR after soy ACE inhibitory peptides and Captopril administration (Table 4), which was coincident with the results of Weidmann (*33*), who reported that ACE inhibitors had no significant effect on lipid metabolism.

Effect on the Content of Minerals. The content of serum potassium and calcium remained stable, whereas that of sodium reduced significantly ($p < 0.05$), after a 1-month feeding of both the soy ACE inhibitory peptides and Captropril as compared with the control group (Figure 4), which suggested a sodium excretion effect of ACE inhibitors. This is the first report on the function of sodium excretion for these antihypertensive peptides derived from food proteins.

Despite considerable skepticism remaining concerning the sodium−blood pressure relationship on the part of some scientists, compelling epidemiological evidence exists to support a relationship between dietary sodium intake and blood pressure and its cardiovascular consequences (*34*). Interventional studies have provided further confirmation of this relationship (*35*) and have led to the recommendation that dietary sodium intake be restricted in hypertension patients (*36*). Independent of the effects of potassium on blood pressure, this ion is known to affect blood vessels (*37*) and the occurrence of vascular disease (*37*, *38*). Calcium intake has also emerged as a confounding element in some studies of the effects of sodium on blood pressure, similar to that previously described for potassium (*39*). The effect of soybean ACE inhibitory peptides on the serum content of these mineral elements content reflected the plausible mechanism of blood-pressure lowering. On the basis of the theory of RAS, an increase in the level of Ang II would increase the concentration of Na^+, whereas ACE inhibitors intake would decrease the formation of Ang II; thus, the concentration of Na^+ decreased. This result suggested that the antihypertensive effect of peptides from food proteins on the blood pressure of SHR was probably due to the improvement or regulation of the salt balance in addition to the simplicity of inhibition of ACE in this view. Because isoflavones have the same

range of molecular weights as the peptides used in this study, their (isoflavones') contributions to the observed blood pressure lowering effect of the peptide preparation cannot be totally excluded. This is because isoflavones have been shown to inhibit cotransport of sodium, potassium, and chloride (*40*), which also have impacts on hypertension. Further work on isoflavone-free soy ACE inhibitory peptides should be performed for comparison.

Conclusion. ACE inhibitory peptides derived from soy protein had a significant hypotensive effect on SHR at the 100 mg/kg of BW/day administration level; their effect was stable and progressive. Although soy ACE inhibitory peptides were less active than synthetic drugs such as Captopril, their significance, however, lies in the fact that they were contained in food taken daily and met the need for naturalness and safety. As the number of hypertensive people in the world is increasing, ACE inhibitory peptides derived from food proteins may play an indispensable role in prophylaxis and prevention of this ailments in the long term. As soy ACE inhibitory peptides have a low molecular weight, they could be dissolved easily in different solutions or added to other food types for functional food components.

It was interesting to note an Na^+ excretion function after soy ACE inhibitory peptides administration; further work on wash-out experiments should be performed to study reversibility.

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