

Soyabean protein hydrolysate prevents the development of hypertension in spontaneously hypertensive rats

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The aim of the present study was to investigate the anti-hypertensive and angiotensin-converting enzyme (ACE) inhibition effects of soyabean protein hydrolysate in spontaneously hypertensive rats (SHR). Soyabean protein hydrolysate was prepared by peptic hydrolysis and was added into the feed of SHR (0% for the S0 group, 0.5% for the S1 group, and 1% for the S2 group) for 12 weeks. Systolic blood pressure and mean blood pressure of the S1 (164.3 (SEM 4.7); 128.0 (SEM 5.0) mmHg) and S2 (156.8 (SEM 1.6); 120.8 (SEM 3.4) mmHg) groups were significantly lower than those of the S0 group (199.4 (SEM 5.2); 158.3 (SEM 7.0) mmHg) at the end of the study. In the analysis of ACE activity, plasma and heart ACE activities of the S1 and S2 groups were significantly lower than those of the S0 group, and there were no significant differences in aorta, kidney, and lung ACE activities among all SHR. Soyabean protein hydrolysate had no significant effect on plasma lipids, electrolytes, or on left ventricular wall or aorta wall thickness. The results suggest that the long-term administration of soyabean protein hydrolysate might retard the development of hypertension in SHR by its inhibitory effect on ACE *in vivo*.

Hypertension: Soyabean protein: Angiotensin-converting enzyme: Spontaneously hypertensive rats: Blood pressure

Many epidemiological studies have demonstrated that there is a relationship between increases in blood pressure and cardiovascular diseases (Miura *et al.* 2001). Angiotensin-converting enzyme (ACE; EC 3.4.15.1) can catalyse the conversion of inactive angiotensin I to angiotensin II, a potent vasoconstrictor. Therefore, the inhibition of ACE may cause a reduction in blood pressure (Cleland, 1993). The Heart Outcomes Prevention Evaluation Study also indicated that the use of ACE inhibitors may lower the incidence of hypertension and heart failure in the high-risk group of cardiovascular disease (Yusuf *et al.* 2000). Recently, many *in vitro* and *in vivo* studies have shown that dietary protein hydrolysates have ACE inhibitory activity (Yamamoto, 1997). For example, protein hydrolysates of sardine muscle (Kawasaki *et al.* 2000), sour milk (Masuda *et al.* 1996), dried bonito (Yokoyama *et al.* 1992), wheat bran (Matsui *et al.* 2000), and α -zein (Miyoshi *et al.* 1991) have been proposed and used as functional foods for their ACE inhibitory activities after oral administration.

Soya products are an important dietary protein source in Asia. An epidemiological study reported that subjects consuming a traditional Japanese diet had lower blood pressure and plasma lipids than those on a Western diet (Kagan *et al.* 1974). An animal study also demonstrated that a soya-based diet attenuated the development of hypertension in

spontaneously hypertensive rats (SHR) when compared with a casein-based diet (Nevala *et al.* 2000). Studies with 4-week-old female SHR also indicated that dietary soya exerts an anti-hypertensive effect without changing heart rate (Martin *et al.* 2001). An *in vitro* study showed that soyabean protein hydrolysates (SPH) could inhibit ACE activity (Chen *et al.* 2002). In the present study, we prepared SPH by peptic digestion and investigated whether it had effects on blood pressure or on systemic and local ACE activities in SHR after long-term administration.

Materials and methods

Preparation of soyabean protein hydrolysate

Defatted soyabean flour (50 g) was dissolved in tri(hydroxymethyl)-aminomethane-HCl buffer and centrifuged (18 000 g for 20 min at 25°C). The supernatant fractions were adjusted to pH 4.6 by 1 M-HCl and centrifuged (18 000 g for 20 min at 4°C). The precipitate (acid-precipitated protein) was collected and digested by 3% pepsin at 37°C for 24 h. A hydrolysate solution from pepsin digestion was adjusted to pH 7.0, and the precipitate was discarded after centrifugation. The supernatant fraction was then dried, ground to a powder, and stored at 4°C as SPH.

Abbreviations: ACE, angiotensin-converting enzyme; RAS, renin–angiotensin system; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; SPH, soyabean protein hydrolysate; WKY, Wistar–Kyoto.

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Animal experiment

Twenty-four male SHR (8 weeks old) and eight male Wistar–Kyoto (WKY) rats (8 weeks old) were purchased from the National Laboratory Animal Breeding and Research Centre (Taipei, Taiwan). Rats were housed in individual cages which were in a room under controlled lighting 08.00–20.00 hours at $24 \pm 1^\circ\text{C}$ and a relative humidity of $55 \pm 5\%$. All rats had a high-Na diet (containing 1% NaCl) and free access to distilled water containing 0.9% NaCl. After a 1-week adaptation, the SHR were randomly divided into three groups (S0, S1, and S2 groups), and the WKY rats served as the control group. All rats were fed a high-Na diet containing different percentages of SPH (0% in the S0 and control groups, 0.5% in the S1 group, and 1% in the S2 group; Table 1). During the experimental period, food intake and body weight were recorded daily.

Data collection

Blood sampling. At 4-week intervals, overnight fasting tail venous blood was collected and analysed for plasma total cholesterol, triacylglycerol, Na, K, and Cl. After 12 weeks, the rats were killed, and blood samples were collected from the abdominal aorta into heparinised tubes for the measurement of blood lipids and ACE activity. The blood was immediately centrifuged (2000g for 10 min at 4°C), and then plasma samples were stored at -20°C until analysis. All samples were determined using a Hitachi 7170 auto-analyzer (Tokyo, Japan).

Blood pressure. The systolic blood pressure (SBP) and mean blood pressure were measured at 4-week intervals during the study by the tail-cuff method using an electro-sphygmomanometer (model 179; Blood Pressure Analyser IITC, Woodland Hills, CA, USA). After 12 h fasting, at least five readings were recorded, the maximum and minimum values were discarded, and the average blood pressure values were calculated from the remaining three values.

Angiotensin-converting enzyme activity. Enzyme extracts of the aorta, lung, heart, and kidney were prepared by the method of Das & Soffers (1975) with some modifications (Masuda *et al.* 1996). All organs were chopped into

small pieces and homogenised in tri(hydroxymethyl)-aminomethane-HCl (50 mmol/l; pH 7.9) containing 0.3 mol NaCl/l using an ultra-disperser. The suspension was filtered through a nylon mesh. Filtrates were centrifuged at 44 000g for 90 min, and the supernatant fractions were discarded. Pellets were suspended in the above buffer with 0.5% Triton X-100. After 1 h, the suspensions were centrifuged at 1000g for 10 min, and the supernatant fractions were designated the detergent-solubilised ACE fraction. The ACE activities were determined using hippuryl-glycyl-glycine (Hip-Gly-Gly; Sigma, St Louis, MO, USA) as a substrate according to the method of Yamamoto *et al.* (1980), and glycyl-glycine (Gly-Gly; Sigma) was used as the standard. The Gly-Gly liberated by ACE reacts with 2,4,6-trinitrobenzenesulfonic acid and can be measured spectrophotometrically at 420 nm. The enzyme-specific activity was expressed as IU/mg protein. The protein was quantified using the Lowry *et al.* (1951) method.

Pathological analysis. The heart and aorta of rats were fixed in 5% formaldehyde. Samples were stained with haematoxylin and eosin red, and the thicknesses of the left ventricular wall and aortic wall were measured by a pathologist.

Statistical analysis

Data were analysed by two-way ANOVA and Fisher's least significant difference test using the SAS program (the Statistical Analysis System, version 8.0; Cary, NC, USA). Results are expressed as the mean and standard error of the mean. A value of $P < 0.05$ was taken as the level of statistical significance.

Results

Body weight and feeding efficiency

Weights of the three SHR groups were significantly lower than the control group from week 2 ($P < 0.05$; Table 2). At the end of the experiment, the weight of the S0 group was significantly lower than those of the S1 and S2 groups ($P < 0.05$; Table 2). Feeding efficiencies of the S0, S1, and S2 groups were also significantly lower than that of the control group, and there was no difference among the S0, S1, and S2 groups (Table 2).

Blood lipids and electrolytes

No significant differences were found in blood lipids or electrolyte analyses among the SHR groups (Table 3). In the blood analysis, both total cholesterol and triacylglycerols in the three SHR groups were lower than the control group. Plasma Na and Cl levels of the SHR were higher than those of the control group, while the K level was lower.

Blood pressure

In measurement of blood pressure, the SBP and mean blood pressure of the S2 group were lower than those of the S0 group from the second week, whereas those of the S1 group were lower than those of the S0 group from

Table 1. Composition of the experimental diets (g/kg)

Ingredient*	Diet group			
	Control	S0	S1	S2
Maize starch	520	520	520	520
Casein	200	200	195	190
Soyabean protein hydrolysate	0	0	5	10
Sucrose	70	70	70	70
Soyabean oil	60	60	60	60
Cellulose	60	60	60	60
Mineral mixture	60	60	60	60
Vitamin mixture	20	20	20	20
NaCl	10	10	10	10

* Casein (high-N), sucrose (food-grade), soyabean oil, cellulose (non-nutritive bulk), mineral mixture (AIN-93M mineral mixture), and vitamin mixture (AIN-93M vitamin mixture) were obtained from ICN Biochemicals (Aurora, OH, USA). Maize starch was purchased from Samyang Genex (Seoul, Korea). NaCl was obtained from Nacalai Tesque (Kyoto, Japan).

Table 2. Changes in body weight, food intake, and feeding efficiency of rats fed different diets (eight rats per group)†

(Mean values and standard errors of the mean)

	Diet group							
	Control		S0		S1		S2	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Initial body weight (g/rat)	220.9	1.9	210.4	2.8	217.8	2.6	212.8	5.2
Final body weight (g/rat)	458.1	23.0	306.8*	15.3	340.2*†	10.2	355.8*†	7.7
Weight gain (g/rat per d)	2.8	0.3	1.2*	0.2	1.5*†	0.1	1.7*†	0.1
Food intake (g/rat per d)	20.0	0.2	20.0	0.1	19.3	0.3	19.3	0.3
Feeding efficiency§ (%)	14.2	1.4	5.7*	0.8	7.6*	0.6	8.8*	0.3

* Mean value was significantly different from that of the control group at the same time ($P < 0.05$).† Mean value was significantly different from that of the S0 group at the same time ($P < 0.05$).

‡ For details of diets, see Table 1 and p. 508.

§ Feeding efficiency = (daily weight gain/daily food intake) $\times$ 100 %.

week 4 (Fig. 1 (a) and (b)). SBP in the S0, S1, and S2 groups showed no significant changes after the sixth week. This showed that the addition of a soyabean protein supplement in the diet significantly retarded the development of hypertension. However, there were no differences between the SBP and mean blood pressure of the S1 and S2 groups. At the end of the experiment, only the S0 group had higher blood pressure than the control group.

Angiotensin-converting enzyme activities

In the analysis of plasma ACE activity, the control group had the highest value among all groups at the end of the study, and those of the S1 and S2 groups were significantly lower than those of the S0 group ($P < 0.05$; Fig. 2). ACE activities of tissue enzyme extracts are shown in Fig. 3; ACE activities of the aorta, heart, and lung in WKY rats were lower than those in SHR ($P < 0.05$), and there were no significant differences in kidney ACE activities ($P > 0.05$). Only heart ACE activity in SHR fed SPH was lower than that of the S0 group ($P < 0.05$).

Pathological analysis

At the end of the study, there was a significant difference in aorta wall thickness between WKY and SHR, but there were no significant differences among the three SHR

groups. No significant difference was found in left ventricular wall thickness among all groups ($P > 0.05$; Fig. 4).

Discussion

The SHR and WKY rats fed the same diet showed no differences in food intake, but body weights of the SHR were significantly lower than those of the WKY rats. It has been shown that a lower feeding efficiency in hypertensive rats may be caused by excitation of the peripheral sympathetic nervous system (Lamont, 1995; Cabassi *et al.* 2002). The increasing angiotensin II levels in hypertensive rats might stimulate the sympathetic nervous system and cause a lower feeding efficiency. SHR fed SPH with ACE inhibition potency may have lower plasma and tissue angiotensin II levels and have a lower metabolic rate which resulted in a higher body weight compared with the control group at the end of the present study.

Studies have shown that peptides first digested by peptidase in the gastrointestinal tract might be more resistant to the digestive enzymes and can well exert their ACE inhibitory activity *in vivo*. Pepsin is an enzyme responsible for protein digestion in the stomach and its preferred substrate includes peptide bonds adjacent to aromatic amino acids, leucine or methionine. An *in vitro* study demonstrated that peptides with tryptophan, phenylalanine, proline, or tyrosine residues in the C-terminal and valine or isoleucine

Table 3. Plasma total cholesterol, plasma triacylglycerol, and electrolytes of rats fed different diets (eight rats per group)†

(Mean values and standard errors of the mean)

Concentration (mmol/l)	Diet group							
	Control		S0		S1		S2	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Total cholesterol	1.68	0.03	1.39*	0.03	1.24*	0.06	1.19*	0.06
Triacylglycerol	0.47	0.02	0.13*	0.01	0.10*	0.02	0.13*	0.08
Na	141.33	0.45	142.25*	0.37	143.00*	0.27	143.38*	0.26
K	5.95	0.29	5.20*	0.16	5.05*	0.11	4.75*	0.07
Cl	100.17	0.69	103.50*	0.73	103.75*	0.41	104.63*	0.73

* Mean value was significantly different from that of the control group at the end of the experiment ($P < 0.05$).

† For details of diets, see Table 1 and p. 508.

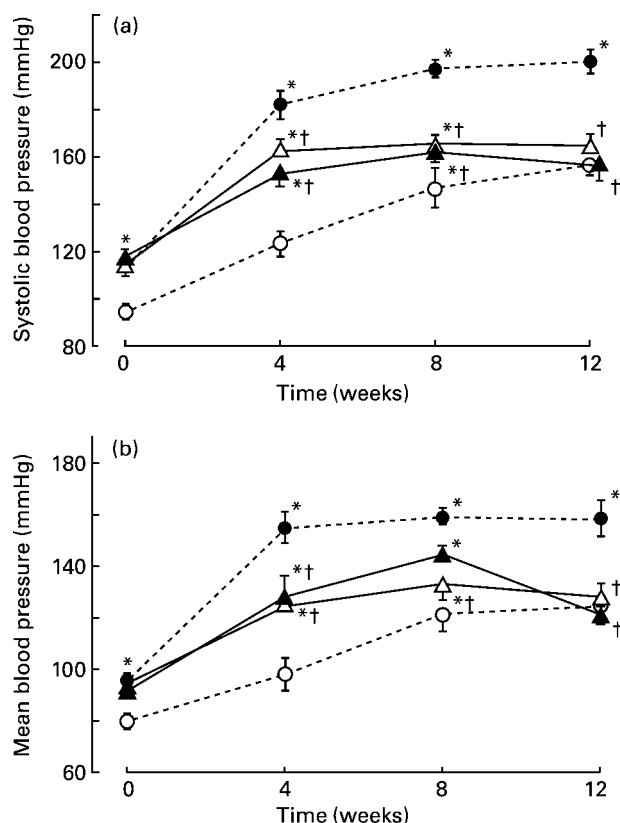


Fig. 1. Systolic blood pressure (a) and mean blood pressure (b) of spontaneously hypertensive rats and Wistar-Kyoto rats fed different diets (eight rats per group). Each point represents the mean change in systolic or mean blood pressure, with vertical bars representing the standard errors of the mean. (○), Control group; (●), S0 group; (Δ), S1 group; (▲), S2 group. For details of the diets, see Table 1 and p. 508. * Mean value was significantly different from that of the control group at the same week ($P < 0.05$). † Mean value was significantly different from that of the S0 group at the same week ($P < 0.05$).

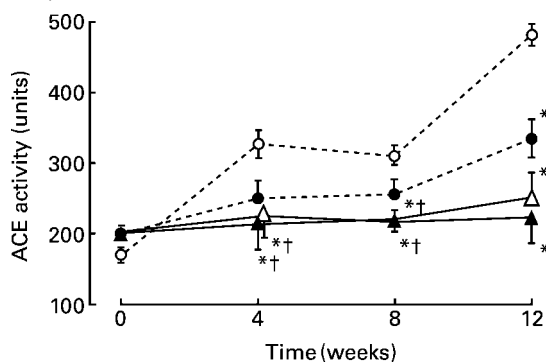


Fig. 2. Plasma angiotensin-converting enzyme (ACE) activity of rats fed different diets (units/l; eight rats per group). Values are means, with vertical bars representing the standard errors of the mean. (○), Control group; (●), S0 group; (Δ), S1 group; (▲), S2 group. For details of the diets, see Table 1 and p. 508. * Mean value was significantly different from that of the control group at the same week ($P < 0.05$). † Mean value was significantly different from that of the S0 group at the same week ($P < 0.05$).

residues in the N-terminal have higher ACE inhibitory activity (Cheung *et al.* 1980). In a study performed by Saito *et al.* (1994), it was found that IYPRY (Ile-Tyr-Pro-Arg-Tyr, a pentapeptide), which is resistant against

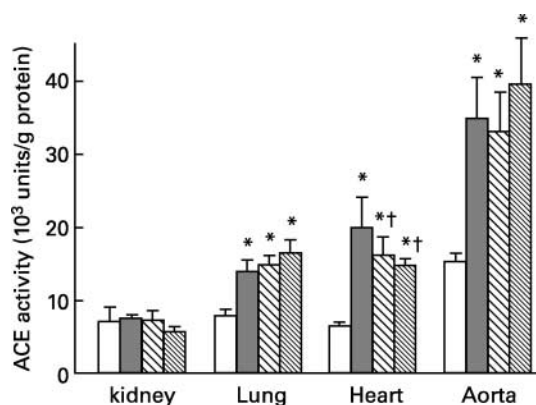


Fig. 3. Tissue angiotensin-converting enzyme (ACE) activity in rats fed different diets (eight rats per group). Values are means, with vertical bars representing the standard errors of the mean. (□), Control group; (■), S0 group; (▨), S1 group; (▩), S2 group. For details of the diets, see Table 1 and p. 508. * Mean value was significantly different from that of the control group at the end of the experiment ($P < 0.05$). † Mean value was significantly different from that of the S0 group at the end of the experiment ($P < 0.05$).

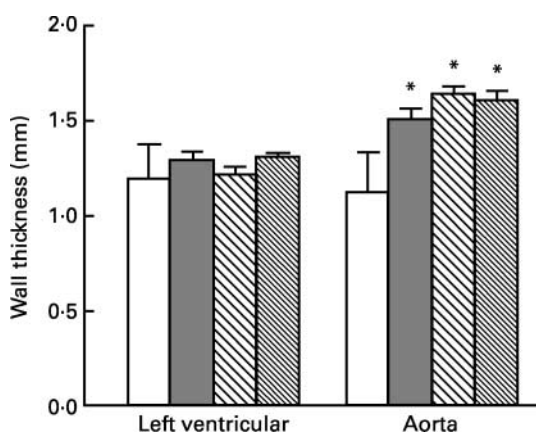


Fig. 4. Left ventricular and aorta wall thickness of rats fed different diets (eight rats per group). Values are means, with vertical bars representing the standard errors of the mean. (□), Control group; (■), S0 group; (▨), S1 group; (▩), S2 group. For details of the diets, see Table 1 and p. 508. * Mean value was significantly different from that of the control group at the end of the experiment ($P < 0.05$).

pepsin and pancreatin, from sake lee presented ACE inhibitory activity in SHR after oral administration. In a previous study, peptides from pepsin-digested soyabean acid-precipitated protein decreased the conversion of angiotensin I into angiotensin II *in vitro* (Chen *et al.* 2002). In the present study, blood pressures in both the S1 and S2 groups were lower than those of the S0 group. These results suggest that SPH may lower blood pressure on account of the physiologically functional peptides it contains, and SPH may retard the development of hypertension. On the other hand, the SBP of the S1 and S2 group remained unchanged since the fourth week of the experiment, while the SBP of the S0 group was increasing. Therefore, SPH may help the hypertensive rats to maintain their blood pressure at a lower level when hypertension is established. Although SPH showed its ACE inhibitory activity in a previous study (Chen *et al.* 2004) and in the present study, it is still not a purified pharmaceutical product but a kind

of food. The difference between 0.5 % and 1 % in the diet may be too little to show a dose-dependent response.

The relationship between dietary protein hydrolysate and blood pressure has been discussed in many previous reports. Feeding SHR a diet containing 30 % casein hydrolysate caused a decrease in blood pressure after 2 weeks (Karaki *et al.* 1990). Another study gave SHR peptides extracted from dried-salted fish, and a significant decrease in the SBP was found after 16 d (Astawan *et al.* 1995). In a long-term study in rats fed a diet with 2.5 % sour milk, a significant decrease in blood pressure was found after 14 weeks (Nakamura *et al.* 1996). In the present study, SHR fed a diet containing only 0.5 % SPH showed retarded development of hypertension after 12 weeks of oral administration, which suggests that SPH may have a greater anti-hypertensive potency than other dietary protein hydrolysates.

Peripheral renin-angiotensin system (RAS) activity has been found to be related to long-term blood pressure management, especially its effects on cardiovascular remodelling (Mancia *et al.* 2002). Previous reports showed that rats fed a high-Na diet could activate peripheral RAS and develop left ventricular and aorta hypertrophy more easily (Takeda *et al.* 2001). In all SHR groups, tissue ACE activity was highest in the aorta, but significant differences between rats fed with or without SPH were only found in the heart. This suggests that peptides with ACE inhibition potency in SPH may have different affinities for tissues. A study on sour milk with two anti-hypertensive tripeptides, Val-Pro-Pro and Ile-Pro-Pro, showed an ACE inhibitory effect mainly in the aorta (Masuda *et al.* 1996). On the other hand, SHR orally administered captopril not only showed an ACE inhibitory effect mainly in the aorta but also elevated plasma ACE activity (Wu & Ding, 2001). The present results which are in contrast to expectations may have been caused by site-specific regulation of tissue RAS to the cardiovascular system (Katoh *et al.* 2000). However, in the study of Martin *et al.* (2002) it was concluded that the soyabean protein may not exert its anti-hypertensive effect by ACE inhibition. The inconsistency may be caused by the model animals being at different stages of hypertension and by the difference between soyabean protein and SPH. Moreover, studies have shown that hypertensive rats may activate the local RAS without affecting the systemic RAS during the feeding of a high-Na diet (Takeda *et al.* 2001). Therefore, ACE inhibitors may produce their anti-hypertensive effects via different mechanisms, and further investigations need to be conducted on the mechanism of SPH's actions on tissue RAS.

In the present study, SPH had no effect on blood lipids or electrolytes. Although there is a report showing that captopril may elevate the blood Na level, the IC₅₀ of the drug was much lower than the SPH used in the present study. Soya isoflavones have been reported to be a phyto-oestrogen, and genistein with natriuretic properties in rats may reduce hypertension by inhibiting Na-K-Cl co-transporters (Martinez *et al.* 1998). However, SPH in the present study was prepared by acid precipitation and peptic hydrolysis of soyabean protein, and no effect of plasma electrolyte concentrations was found. This suggests that the anti-hypertensive effect of SPH in the present study

might not have been caused by isoflavones. Soyabean protein and soya isoflavones have been shown to have hypolipidaemic effects (Pelsuo *et al.* 2000), and the lowering of body weight and blood lipids may cause a decrease in blood pressure (Ferrier *et al.* 2002). However, we found that SPH had no significant effects on SHR lipid metabolism. This may have been because (1) the SPH used in the hypolipidaemic research before was the high-molecular-weight and undigested fractions (Wang *et al.* 1995), and (2) the hypolipidaemic effects of isoflavones are usually shown in post-menopausal women (Demonty *et al.* 2003). Therefore, the blood pressure-lowering effects of SPH in the present study may not be related to the effect on lipid metabolism.

In conclusion, SPH can lower blood pressure in hypertensive model animals. One of the important factors may be the decrease of ACE activity *in vivo*, mainly in the plasma and heart. Further studies are still required on the mechanisms of how soyabean protein affects blood pressure, and the results of those studies may be used in dietary management to prevent the development of hypertension.

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