SOYBEAN PROTEIN-DERIVED HYDROLYSATE AFFECTS BLOOD PRESSURE IN SPONTANEOUSLY HYPERTENSIVE RATS

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ABSTRACT

The aim of this study was to investigate the inhibitory activity of angiotensin-converting enzyme (ACE) and antihypertensive effects of soybean protein hydrolysate in spontaneously hypertensive rats (SHRs). The inhibitory activities against ACE with an IC_{50} *of the peptidic fraction were 0.82 (casein), 0.73 (soybean protein isolate), and 0.48 mg/mL (soybean acid-precipitated protein), respectively. Peptic hydrolysate containing 1% NaCl was added to the SHRs' feed (5% of each protein hydrolysate). Systolic blood pressure of the soybean protein hydrolysate-supplemented groups were significantly lower than that of the casein hydrolysate-supplemented group in the study period. These data suggested that soybean protein hydrolysate may retard the development of hypertension in SHRs by its ACE inhibitory effect* in vivo.

INTRODUCTION

Hypertension is the most common public health problem in developed countries (Miura *et al.* 2001). It is often called a silent killer because people with hypertension can be asymptomatic for years and then have a fatal heart attack or stroke. Most people (90% ~ 95%) with high blood pressure have essential hypertension, for which the cause cannot be determined, and as a

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result, treatment is non-specific (Linden *et al.* 2001; Luft 2001). Although no cure is available, prevention and management can decrease the incidence of hypertension and related degenerative diseases. Some of the decline in cardiovascular disease mortality over the last two decades has been attributed to the increased detection and control of hypertension. The emphasis on life-style modifications has given diet a prominent role in both the primary prevention and management of hypertension.

The angiotensin-converting enzyme (ACE, dipeptidyl carboxypeptidase, EC 3.4.15.1) plays an important role in regulating blood pressure. It catalyzes the conversion of the inactive angiotensin I to the potent vasoconstrictor angiotensin II by cleaving the histidyl-leucine group from the C-terminal. ACE inhibitors are now widely used and are considered to be effective antihypertensive agents causing a fall in blood pressure comparable to those produced by thiazides, β-blockers, and calcium antagonists (Pool *et al.* 1989). Recently, many peptides isolated from food proteins were found to have ACE inhibitory activities and to decrease the blood pressure after oral administration (Yamamoto 1997). Daily use of food that has some peptides with potent ACE inhibitory activity may be effective for maintaining blood pressure in a good condition.

Soybeans, which contain 35% protein and are widely available, have been an important dietary protein source in traditional Oriental diets. In recent studies, soybean protein has been found to have cholesterol-lowering effects (Chen *et al.* 2002a) and a soybean-based diet can better attenuate the development of hypertension in comparison to a casein-based diet (Nevala *et al.* 2000). In this study, we prepared hydrolysate from soybean protein and determined the ACE inhibitory activity and blood pressure-lowering effect *in vivo*.

MATERIALS AND METHODS

Materials

Casein and soybean protein isolate (SPI) were purchased from ICN Biochemicals (Aurora, OH). Soybean acid-precipitated protein (APP) was prepared from defatted soybean flour using the method of Iwabuchi and Yamauchi (1987).

Sample Preparation

Fifty grams each of casein, SPI and APP powders were immersed in 1 L of deionized water and then homogenized. The homogenate was adjusted to pH 2 with 1 N HCl. Pepsin at 750 mg was added to the homogenate, and the mixture was incubated at 39C for 12 h. The peptic digest was adjusted to pH 7 with 1 N NaOH, boiled for 10 min, and filtered. The filtrate was dialyzed

against 10 L of deionized water in a cellulose tubular membrane (90 cm, molecular cut off 12-14 kDa, Membrane Filtration Products, San Antonio, TX) in a cool room (4C) for 2 days. The outer solution was applied to a Dowex 50W (H^+ form, Dow Chemical, Midland, MI) column (45 × 200 mm) equilibrated with deionized water. The column was washed sufficiently with deionized water to remove most impurities, and peptides were eluted with 500 mL of a 3.0% ammonia solution. After evaporation under a vacuum, the peptidic fraction was applied to a Sephadex G-25 column (2.6 × 140 cm, medium, Pharmacia, Uppsala, Sweden) equilibrated with deionized water. The eluate was gel-filtered at a flow rate of 30 mL/h, and fractions of 8 mL were collected. The peptide content of each fraction was measured by the Lowry method, using bovine serum albumin as the standard. The peptidic fractions were collected and lyophilized to prepare a peptic hydrolysate of casein (CAH), soybean protein isolate (SPH), and soybean acid-precipitated protein (APH) powder.

ACE Inhibitory Activity

ACE inhibition (n = 3 for each sample) was assayed by a modification of the method of Cheung and Cushman (1973). Fifty microliters of a sample solution and 150 μL of 2.5 mU ACE (rabbit lung lyophilized powder, Sigma) solution were added to 150 μL of a 12.5 mM substrate (Hip-Gly-Gly, Sigma) solution in 1.0 M NaCl-borate buffer at pH 8.3. After incubation at 37C for 1 h, the reaction was stopped by adding 150 μL of 0.5 N HCl. The liberated hippuric acid was extracted with 1.5 mL of ethyl acetate, and the absorbance of the extract was determined at 228 nm to evaluate the ACE inhibitory activity. The inhibition was calculated from the equation [(Ec - Es)/(Ec - Eb)] × 100, where Es is the absorbance of the reaction mixture (positive control), Ec is the absorbance of the buffer (test), and Eb is the absorbance when the stop solution (0.5 N HCl) was added before the reaction occurred (negative control). The inhibitory activity was defined as the amount needed to inhibit 50% of ACE activity (IC_{50}).

Animals, Diets, and Analyses

Twenty-four spontaneously hypertensive rats (SHRs, male, 8 weeks old) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Rats were housed in individual cages which were in a room under controlled lighting 0800-2000 h at 23 ± 2C and a relative humidity of 55% ± 5%. SHRs were randomly divided into three groups (n = 8): a CAH group (with a diet containing 5% casein hydrolysate powder), an SPH group (with 5% SPI hydrolysate powder), and an APH group (with 5% APP hydrolysate powder) group. Diets were modified from the AIN-93M mixture (Reeves *et al.* 1993) and contained 1% sodium chloride, and free access

was allowed to distilled water containing 0.9% sodium chloride for 8 weeks. The systolic blood pressure (SBP) was measured at the beginning of and at 2-week intervals during the study by the tail-cuff method using an electro-sphygmomanometer (Model 179, Blood Pressure Analyzer IITC, Woodland Hills, CA). After 12-h fasting, at least five readings were recorded, the maximum and minimum values were discarded, and two average SBP values were calculated from the remaining three values.

Statistical Analysis

Data were analyzed using one-way ANOVA and Fisher's least significant difference test with the SAS program (the Statistical Analysis System, version 6.12). Results are expressed as the mean $\pm$ SD. A P value of < 0.05 was taken as the level of statistical significance.

RESULTS

Purification of Peptidic Fractions from Soybeans

In this study, ACE-inhibitory peptidic fractions were isolated from the peptic hydrolysate of casein, SPI, and APP by dialysis, ion-exchange, and gel filtration column chromatography as described in "Materials and Methods". The Sephadex G-25 chromatogram of the peptic digest of samples eluted from the Dowex 50W (H^+ form) column is shown in Fig. 1. A major peak with ACE inhibitory activity was obtained from the peptidic fraction (fraction numbers 15 ~ 35, molecular weight range 300 ~ 5000) which was pooled, evaporated, and lyophilized to dryness. The yield of the peptide powder from 50 g each of casein, SPI, and APP was 25.8, 18.6, and 19.8 g, respectively.

ACE Inhibitory Activity

Three peptidic fractions (CAH, SPH, and APH) were obtained from gel filtration chromatography on a Sephadex G-25 column. The ACE inhibitory activity was also measured. The IC_{50} values of the peptidic fractions were 0.82 (CAH), 0.73 (SPH), and 0.48 mg/mL (APH), respectively (Fig. 2). The ACE inhibitory activity of the APH fraction was stronger than that of the SPH or CAH fractions.

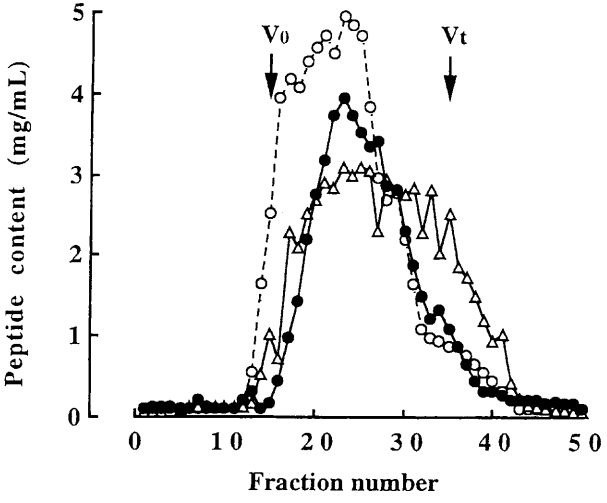

FIG. 1. COLUMN CHROMATOGRAM OF PEPTIDES DERIVED FROM THE ENZYMATIC DIGESTION OF CASEIN, SOYBEAN PROTEIN ISOLATE, AND SOYBEAN ACID-PRECIPITATE PROTEIN ON SEPHADEX G-25

The column was equilibrated and eluted with 0.1 M phosphate buffer. Molecular marker, V_0: blue dextran (MW: 2 000 000), Vt: cobalamin (MW: 1 335). The peptide content was determined by the Lowry method, and the bovine serum albumin was used as a standard. -○-; peptic hydrolysate of casein, -Δ-; peptic hydrolysate of soybean protein isolate, -•-; peptic hydrolysate of soybean acid-precipitate protein.

Animal Study

We confirmed that the peptidic fractions in the peptic digests of each protein produced antihypertensive activity by oral administration in SHRs. The growth curve is shown in Fig. 3. The average initial body weight of rats was 160.3 ± 12.1 g, and the final average body weight was 298.0 ± 15.7 g after an 8-week experimental period. The body weight was significantly lower in the CAH group than the APH group starting from 2 weeks. The food intake of each of the CAH, SPH, and APH groups was 14.2 ± 1.5, 15.2 ± 2.6, and 15.4 ± 2.6 g/day, respectively. Changes in SBP are shown in Fig. 4. SBP values of the SPH and APH groups were lower in comparison with that of the CAH group starting from 4 weeks, whereas the SBP of the CAH group gradually increased during the experimental period. The development of hypertension was attenuated in both SPH and APH groups when compared to the CAH-supplemented diet.

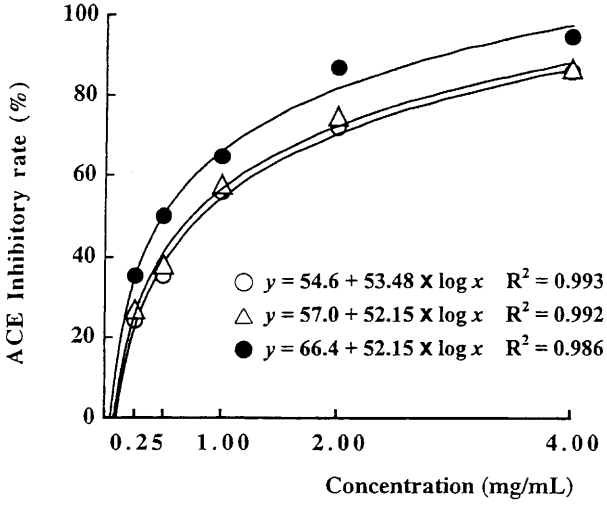

FIG. 2. THE INHIBITORY RATE AGAINST THE ANGIOTENSIN-CONVERTING ENZYME OF THE PEPTIDIC FRACTION PURIFIED WITH SEPHADEX G-25 CHROMATOGRAPHY -○-; peptic hydrolysate of casein, -Δ-; peptic hydrolysate of soybean protein isolate, -•-; peptic hydrolysate of soybean acid-precipitate protein.

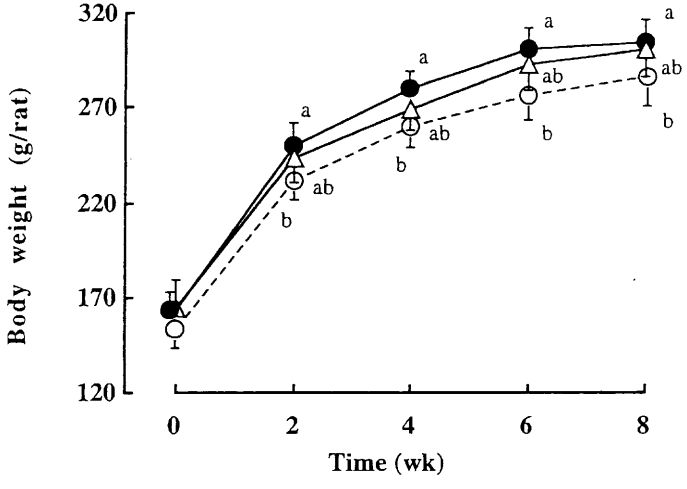

FIG. 3. BODY WEIGHT OF SHRs FED DIFFERENT DIETS

Values are expressed as the mean ± SD. $n = 8$ for each group. Means at the same time that do not share the same letter differ significantly, $P < 0.05$. -○-; CAH group, -Δ-; SPH group, -•-; APH group.

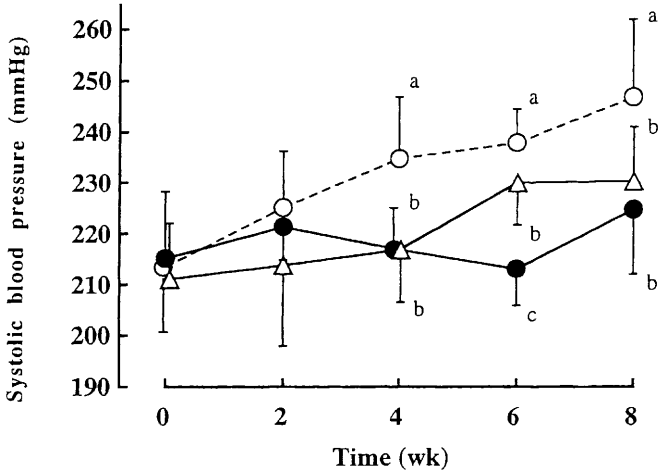

FIG. 4. SYSTOLIC BLOOD PRESSURE OF SHRs FED DIFFERENT DIETS
Each point represents the mean change in systolic blood pressure. Vertical bars represent the mean ± SD, $n = 8$ for each group. Means at the same time that do not share the same letter differ significantly, $P < 0.05$. -o-; CAH group, -Δ-; SPH group, -•-; APH group.

DISCUSSION

In this study, we further determined the effects of different types of soybean protein hydrolysate in the diet. Soybean protein hydrolysate made from the employed purification process contains more than 90% protein, and the molecular weight of soybean protein hydrolysate is less than 12 kDa. Many ACE inhibitory peptides have been isolated from hydrolysates of various food proteins. Additionally, the relation between dietary protein hydrolysate and blood pressure has been discussed in many reports. However, it is known that some peptides with potent ACE inhibitory activity either *in vitro* or intravenously are inactivated by oral administration. These inactive peptides, as only substrates of ACE (Yoshikawa 1993), do not decrease the blood pressure *in vivo*. The presence of ACE-inhibitory peptides in the peptic digests of soybean protein suggests that they could be responsible, at least partially, for the observed blood pressure-lowering effect of soybean protein (Imura *et al.* 1993; Nevala *et al.* 2000).

A study by Nevala *et al.* (2000) in animals showed a significant hypotensive effect of dietary soybean protein. Miyoshi *et al.* (1991) isolated tripeptides from zein by tryptic hydrolysis and found that they were ACE-inhibitory peptides.

Suetsuna *et al.* (1991) and Kawasaki *et al.* (2000) also isolated ACE-inhibitory peptides from an enzymatic hydrolysate of sardine muscle. All of these peptides with ACE inhibitory activity are composed of 2-10 amino acid residues and can be designed and synthesized according to a biochemical model in the laboratory. On the correlation between structure and activity of ACE-inhibitory peptides, Cheung *et al.* (1980) reported that peptides with highly potent inhibitory activity have Pro, Phe, or Tyr at the C-terminal and Val or Ile at the N-terminal. There are three active sites of ACE: the zinc ion, the hydrogen bond, and a positively charged residue binding site. In a previous study, we identified four ACE-inhibitory peptides from the pepsin digests of soybean protein (Chen *et al.* 2002b). In the present study, we found that the amino acid profile of the soybean protein hydrolysate has more Leu, Phe, Arg, and Thr than that of casein, and has less Glu, Ser, and Pro (data not shown). It is thought that the hydrophobic side chain may be the binding site with the active site of ACE. This may partially explain how these inhibitory agents work by their special chemical structure.

Many kinds of short-chain peptides isolated from the hydrolysis of dietary protein, like tuna muscle and fermented milk, were found to have powerful ACE inhibitory activities (Kohama *et al.* 1991; Masuda *et al.* 1996). An *in vitro* study also showed that peptides from pepsin-digested soybean broth could decrease the conversion of angiotensin I into angiotenisin II (Chen *et al.* 1997). In our study, blood pressures of both the SPH and APH groups were lower than that of the CAH group. The results suggest that the peptide fraction might lower blood pressure on account of the physiologically functional peptides it contains. In 1970, Ferreira *et al.* found that a low-molecular-weight fraction from snake venom (*Bothrops jararaca*) potentiates the activity of bradykinin, and they named it the bradykinin-potentiating factor. It inhibits the proteolytic enzymes which inactivate bradykinin and catalyze the conversion of angiotensin I into angiotensin II. ACE inhibitors are important in cardiovascular therapeutics for they can reduce the generation of angiotensin II, a vasoconstrictor that can inhibit increases in blood pressure (Kato and Suzuki 1971; Ondetti 1977). For example, captopril, enalapril, and lisinopril are all widely used in clinical practice today.

Karaki *et al.* (1990) suggested that SHRs fed a diet containing 30% casein hydrolysate caused a decrease in blood pressure after 2 weeks. Another study found that giving SHRs a peptide extract from dried-salted fish significantly decreased the SBP after 16 days (Astawan *et al.* 1995). In a long-term study in rats fed a diet containing 2.5% sour milk, significant decreases in blood pressure were found after 14 weeks (Nakamura *et al.* 1996). In our present study, a significant decrease in blood pressure was found in SHRs fed a diet containing only 5% SPH or APH, and this suggests that soybean protein hydrolysate may have a greater potency than casein hydrolysates to reduce blood pressure. The

in vitro study showed that the inhibitory effect of SPH on ACE activity was similar to that of CAH, but the blood pressure-lowering effect of CAH was equivalent to that of APH *in vivo*. The effect of increasing SBP in the CAH group was similar to that in the control group fed with a normal diet in a previous study. Therefore, 5% CAH did not inhibit the increasing effect on SBP. This indicates that CAH had an inhibitory effect on ACE activity *in vitro*, but may be degraded and lose its activity in the GI tract.

TABLE 1.
COMPOSITION OF THE EXPERIMENTAL DIETS (%)[1]

Group	CAH	SPH	APH
Cornstarch	52	52	52
Casein	15	15	15
CAH *	5	-	-
SPH *	-	5	-
APH *	-	-	5
Sucrose	7	7	7
Soybean oil	6	6	6
Mineral mixture	6	6	6
Cellulose	6	6	6
Vitamin mixture	2	2	2
NaCl	1	1	1

[1] Casein (high nitrogen), sucrose (food grade), soybean oil, mineral mixture (AIN-93 mineral mixture), cellulose (nonnutritive bulk), and vitamin mixture (AIN-93 vitamin mixture) were obtained from ICN Biochemicals (Aurora, OH). Cornstarch was purchased from Samyang Genex (Seoul, Korea). Sodium chloride was obtained from Wako Pure Chemical (Osaka, Japan).

* CAH; peptic hydrolysate of casein, SPH; soybean protein isolate, APH; soybean acid-precipitated protein.

SHRs fed each diet had no significant difference in food intake, but body weights of the CAH group were significantly lower than those of the APH group. The lower feeding efficiency in hypertensive rats may have been caused by excitation of the peripheral sympathetic nervous system. Scheidegger *et al.* (1984) suggested that rats fed with terbutaline sulfate for 2 weeks to stimulate β-receptors caused an elevation in the metabolic rate. Patients with hypertension taking β-blockers had lower resting energy expenditure (Lamont 2000). This suggests that the increase in angiotenisin II levels in hypertensive rats may stimulate the sympathetic nervous system and cause a lower feeding efficiency.

SHRs fed soybean protein hydrolysate with ACE inhibition potency may have lower plasma and tissue angiotenisin II levels and have a lower metabolic rate which could have caused the higher body weight than that of the CAH group in this study.

Soybeans and their products have been a very popular food in the Orient since ancient times, so their safety is well established. Daily use of food that has some peptides with potent ACE inhibitory activity could be effective for maintaining blood pressure at a healthy level. Although our peptide fraction was weaker than medical treatment, soybean protein is eaten as food. Additionally, side effects of drugs which inhibit ACE activity, including coughs, fever, exanthem eruption, and leukopenia, have been reported (Lipworth *et al.* 1989). However, these side effects have not been found in the many studies of ACE inhibitors isolated from dietary protein (Sugiyama *et al.* 1991; Yoshikawa *et al.* 2000).

In conclusion, soybean protein hydrolysate, especially acid-precipitated protein, can lower blood pressure and is one of the important factors which may decreases ACE activity *in vitro*. Modification of daily eating habits can play an important role in the prevention and management of hypertension. The mechanism occurring between ACE and dietary antihypertensive peptides still remains unclear today and further investigation is required to identify how these peptides work to lower blood pressure by inhibiting ACE activity *in vivo*.

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