IDENTIFICATION OF ANGIOTENSIN I-CONVERTING ENZYME INHIBITORY PEPTIDES DERIVED FROM THE PEPTIC DIGEST OF SOYBEAN PROTEIN

JIUN-RONG CHEN[1], TAKASHI OKADA[2], KOJI MURAMOTO[2], KUNIO SUETSUNA[3] and SUH-CHING YANG[1,4]

[1]*Department of Nutrition and Health Sciences*
Taipei Medical University
Taipei 110, Taiwan

[2]*Department of Bioresources Chemistry*
Faculty of Agriculture
Tohoku University
Sendai 981-8555, Japan

[3]*Department of Food Science and Technology*
National Fisheries University
Yamaguchi 759-6595, Japan

Accepted for Publication May 2, 2002

ABSTRACT

Peptidic fractions which inhibit angiotensin I-converting enzyme (ACE) were separated from peptic digests of soybean by ion exchange chromatography and gel filtration. Further separation of the peptidic fractions by ODS HPLC afforded active peptides, the amino acid sequences of which were identified by Edman's procedure as: Ile-Ala (inhibitory against ACE with an IC_{50} of 153 μM), Tyr-Leu-Ala-Gly-Asn-Gln (14 μM), Phe-Phe-Leu (37 μM), Ile-Tyr-Leu-Leu (42 μM), and Val-Met-Asp-Lys-Pro-Gln-Gly (39 μM). The antihypertensive activity of the soybean peptides was also investigated. Peptide fractions (2.0 g/kg body weight, oral administration) markedly lowered the blood pressure of spontaneously hypertensive rats (SHRs).

INTRODUCTION

The renin-angiotensin system plays an important role in the regulation of an organisms' water, electrolytes, and blood. The angiotensin I-converting enzyme (ACE, dipeptidyl carboxypeptidase, EC 3.4.15.1) participates in

[4] Corresponding author. FAX: +886-2-2737-3112; E-mail: sokei@tmu.edu.tw

Journal of Food Biochemistry **26** (2003) 543–554. *All Rights Reserved.*
©*Copyright 2003 by Food & Nutrition Press, Inc., Trumbull, Connecticut.*

regulating blood pressure in the renin-angiotensin system; and its inhibitors, such as captopril (Kato and Suzuki 1971; Ondetti *et al.* 1977) and enalapril (Sawayama *et al.* 1990), have been used as antihypertensive drugs. The ACE-inhibitory activity of foods has been studied, and it was found that some ACE-inhibitory peptides are produced by enzymatic digestion of various food proteins, including tuna muscle (Kohama *et al.* 1991), sardine muscle (Suetsuna 1991), dried bonito (Yokoyama *et al.* 1992), dried-salted fish (Astawan *et al.* 1995), ovalbumin (Fujita *et al.* 1995), fish sauce (Okamoto *et al.* 1995), and fish water-soluble protein (Wako *et al.* 1999). Additionally, Masuda *et al.* (1996) reported that sour milk and some ACE-inhibitory hydrolysates of milk protein not only decreased blood pressure but also the risk of stroke in stroke-prone spontaneously hypertensive rats (SHR-SP).

Many human and animal studies have revealed that ingestion of food proteins results in decreased blood pressure, but the mechanism is still unknown. Suetsuna (1991) and Sugiyama *et al.* (1991) suggested that the hydrolysates of food proteins decrease blood pressure by regulating the renin-angiotensin system. Washburn *et al.* (1999) reported that ingestion of soybean protein decreases blood pressure in humans. However, there are few studies about ACE-inhibitory peptides derived from soybean protein and their hypertensive effects. In this study, we describe the isolation of ACE-inhibitory peptides derived from soybean protein and the structures and antihypertensive actions of these orally administered peptides on SHRs.

MATERIALS AND METHODS

Purification of Peptides from Soybeans

Three hundred grams of soybean were put in deionized water overnight and homogenized in 2 L of deionized water. The pH of the homogenate was adjusted to 2.0 with 2 N HCl, and 3 g of pepsin (from porcine gastric mucosa, EC 3.4.23.1, E. Merck) was added. After 24 h of digestion at 37C, the residue from the hydrolysate was removed by filtration (Filter paper No. 2, Toyo Roshi, Tokyo, Japan), and the supernatant was centrifuged (12,000 × *g*, 20 min, 4C). The supernatant was applied to a column (45 × 200 mm) of Dowex 50 W (50 ~ 100 mesh, H^+ form) cation exchange resin. The column was washed sufficiently with deionized water to remove most impurities, and then the desired peptides were eluted with 500 mL of a 5% ammonia solution. The peptides were concentrated to 5 mL under vacuum. The concentrate was applied to a column of Sephadex G-25 (26 × 1400 mm, Pharmacia) equilibrated with deionized water. The eluate was gel-filtered at a flow rate of 30 mL/h, and fractions of 7.8 mL were collected. The peptide fractions were collected and dried using the

method described above to give soybean protein hydrolysate (SPH). About 3 g of the SPH were dissolved in deionized water, and the solution was applied to a column of SP Sephadex C-25 (20 × 500 mm, Pharmacia, H^+ form) equilibrated with deionized water. The eluate was chromatographed by linear gradient using 1 L of deionized water to 1 L 3% NaCl solution at a flow rate of 30 mL/h, and fractions of 7.8 mL each were obtained. The active fractions were collected and freeze-dried to prepare a peptide powder. The amino acid composition of each peptide fraction was determined using an amino acid analysis system (Shimazu, model LC-10AT) after hydrolysis (6 N HCl, 24 h, 110C, under vacuum).

Measurement of ACE Inhibitory Activity

ACE inhibition was assayed by a modification of the method of Cheung and Cushman (1973). Fifty microliters of a sample solution and 100 μL of 2.5 mU angiotensin I-converting enzyme (ACE, from rabbit lung acetone powder, Sigma) solution were added to 100 μL of a 12.5 mM substrate (hippuryl-L-histydyl-L-leucine, 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 250 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 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), Ec is the absorbance of the buffer (test), and Eb is the absorbance when the stop solution was added before the reaction occurred (negative). ACE inhibitory activity was assayed in the presence or absence of inhibitors, and the concentration of ACE inhibitors needed to inhibit 50% of ACE activity was defined as the IC_{50} value.

Animal Study

Spontaneously hypertensive rats (SHRs, male) were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan) and fed laboratory chow (#5010, PMI® Nutrition, MO). All animals were housed individually in an air-conditioned room at 23 ± 2C. The systolic blood pressure (SBP) of 15-week-old SHRs (280-330 g body weight) was measured. A group of six SHRs given the peptidic fraction powder in 0.9% saline (2.0 g/kg body weight, *p.o.*) or captopril (10 mg/kg body weight, *p.o.*) dissolved in 0.9% saline via gastric intubation was kept at 30C, and SBP was measured by the tail-cuff method using a programmed electro-sphygmomanometer (Model 179, IITC Life Science, Woodland Hills, CA). At least five readings were recorded, the maximum and minimum values were discarded, and averaged SBP values were calculated from the remaining three values.

Purification of ACE-inhibitory Peptides

The peptidic fractions were further purified by reverse-phase HPLC on a Develosil ODS-5 column (4.6 × 250 mm, Nomura Chemical, Japan), using a gradient elution of 0% to 16% acetonitrile in 0.05% trifluoroacetic acid (TFA) for 60 min at a flow rate 1.0 mL/min; the eluates were monitored at 220 nm. Peptides were hydrolyzed with 6 N HCl containing 0.1% phenol at 110C for 24 h, and the hydrolysates were analyzed with an amino acid analysis kit (amino-chrome™, Ciba-Corning, UK). Sequence analysis was performed by stepwise Edman degradation using a gas-phase automated sequencer Model 477A (Applied Biosystem) coupled with HPLC identification of the resulting PTH-amino acid.

Statistical Analysis

Data were analyzed by one-way analysis of variance followed by Duncan's multiple range test. A difference of $P < 0.05$ was considered significant.

RESULTS

Purification of Peptide from Soybeans

In this study, ACE-inhibitory peptides were isolated from the peptic hydrolysate of soybean by ion-exchange and gel filtration column chromatography as described in Materials and Methods. The Sephadex G-25 chromatogram of the enzymatic digest of soybean eluted from the Dowex 50W (H^+ form) column is shown in Fig 1. Major peaks with ACE-inhibitory activity were obtained from the peptidic fractions (molecular weight range 300-5000) which were pooled, lyophilized or evaporated to dryness. The yield of the peptide powder from 300 g (dry weight) of soybean was 19.8 g. The peptides were fractionated by ion-exchange chromatography on SP Sephadex C-25 (H^+) to give SP-1 (fraction number, 18 to 42; average chain length, 4.3) and SP-II (43 to 70; 4.9) as shown in Fig. 2.

ACE-inhibitory Activity

Two soybean-peptide fractions (SP-1, SP-II) were obtained from ion-exchange chromatography on SP Sephadex C-25. The ACE-inhibitory activity was also measured. The IC_{50} value of the peptide fractions were 0.24 mg/mL (SP-1) and 1.2 mg/mL (SP-II), respectively. The ACE-inhibitory activity of the SP-1 fraction was stronger than that of the SP-II fraction. The result of amino acid analysis showed that there were more branch chain amino acids (including Val, Ile, and Leu) in the SP-1 than the SP-II fraction. According to the

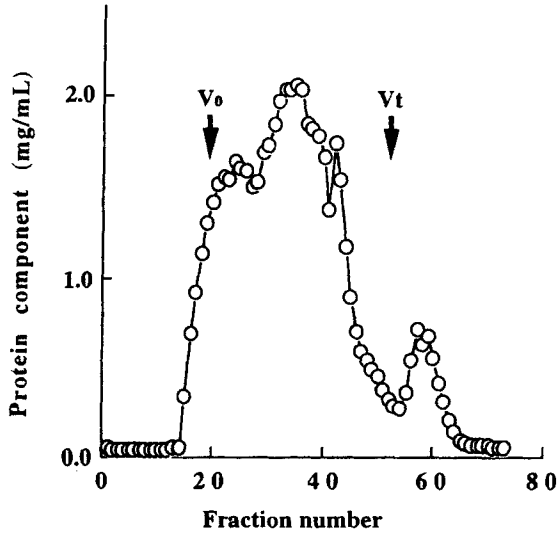

FIG. 1. COLUMN CHROMATOGRAM OF HYDROLYSATE FROM SOYBEAN PROTEIN ON SEPHADEX G-25

The protein component (-o-) was determined using the Lowry method, and bovine serum albumin was used as the standard.

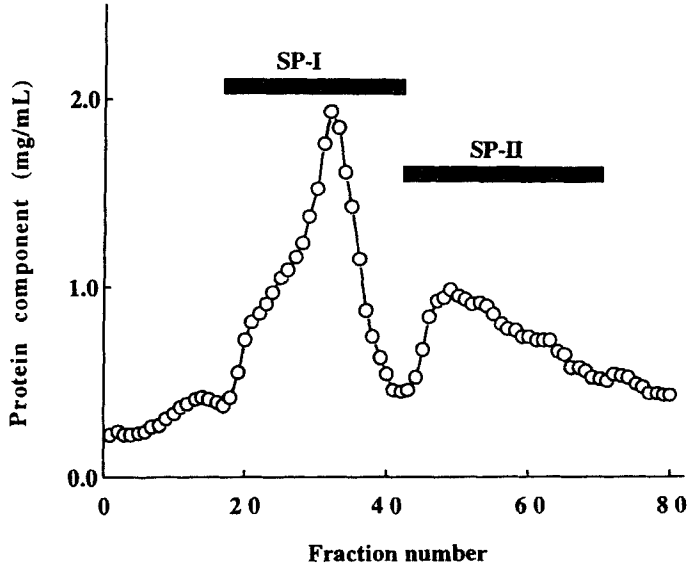

FIG. 2. COLUMN CHROMATOGRAM OF SOYBEAN PEPTIDE ON SP SEPHADEX C-25

The protein component (-o-) was determined using the Lowry method, and bovine serum albumin was used as the standard.

calculation of total amino acid, SP-1 contains 46% hydrophobic amino acids whereas SP-II contains 36%.

Animal Study

We confirmed that the peptidic fractions in the peptic digests of soybean protein produced antihypertensive activity by oral administration into SHRs. Oral administration of the SP-1 fraction reduced blood pressure in SHRs, with an effect comparable to that of captopril. Antihypertensive activity of the SP-1 fraction was weaker than that of captopril. Antihypertensive activity of the peptidic fraction from soybean protein was evaluated by measuring the change in SBP at 2, 4, and 6 h after oral administration of the SP-1 fraction. The SBP did not change in control rats during the study period (6 h). Captopril lowered SBP significantly from 2 to 6 h after administration of the drug. A single dose of the SP-1 fraction from soybean protein significantly reduced SBP to 17.5 mmHg at 2 h, and this antihypertensive effect continued for 6 h after administration (Fig. 3).

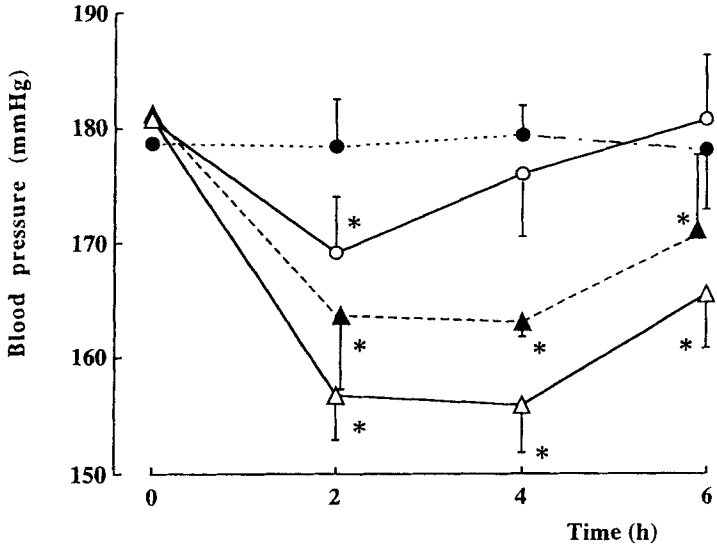

FIG. 3. ANTIHYPERTENSIVE EFFECTS ON SHRs OF THE PEPTIDIC FRACTION FROM THE PEPTIC DIGESTS OF SOYBEAN PROTEIN

Each point represents the mean change in systolic blood pressure in six SHRs. Vertical bars represent the mean ± SD, $n = 6$. Significantly different from the value before administration on the same line at $P < 0.05$ (*). •; control (0.9% saline), Δ; captopril (10 mg/kg, *p.o.*), o; soybean protein hydrolysate (2 g/kg BW, *p.o.*), ●; SP-I fraction (2 g/kg BW, *p.o.*).

ACE-inhibitory Peptides

The SP-I fraction was dissolved in distilled water and applied to an ODS column. Figure 2 shows a preparative HPLC chromatogram of the fraction. Although approximately 100 peaks were detected with chromatography (Fig. 4), four peaks of potent inhibitory peptides were obtained from the peptic digest of soybean protein. Retention times were 19.7, 27.5, 47.5, and 48.2 min, and IC_{50} values were 153, 14, 37, and 42 μM, respectively. Amino acid analysis of each inhibitor after 6 N HCl hydrolysis revealed the amino acids listed in Table 1. Using an amino acid sequencer (Applied Biosystems 477A), primary structures of the individual peptides were identified. The amino acid sequences of these peptides were: Ile-Ala, Tyr-Leu-Ala-Gly-Asn-Gln, Phe-Phe-Leu, and Ile-Tyr-Leu-Leu. Almost all of the active peptides had branch chain amino acid residues in the structure.

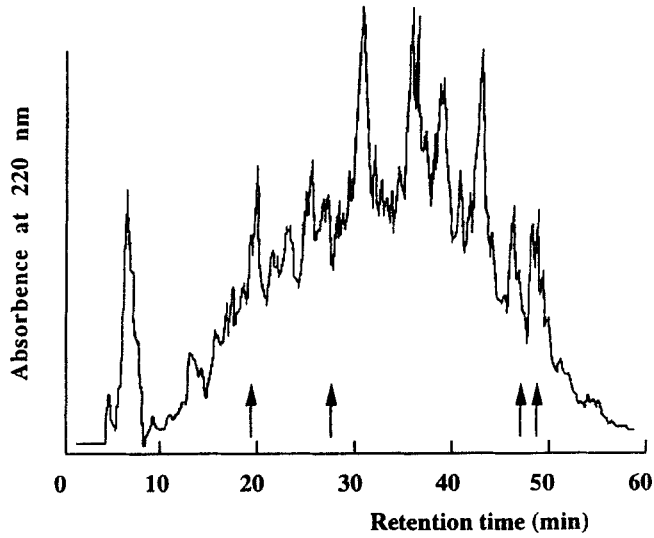

FIG. 4. A CHROMATOGRAM ON A REVERSE-PHASE DEVELOSIL ODS-5 COLUMN OF THE SP-I FRACTION ISOLATED FROM A SP SEPHADEX C-25 COLUMN
The peaks marked Ile-Ala, Tyr-Leu-Ala-Gly-Asn-Gln, Phe-Phe-Leu, and Ile-Tyr-Leu-Leu, were found to have ACE-inhibitory activity.

TABLE 1.
ANALYTICAL DATA AND ACE-INHIBITORY ACTIVITY OF PEPTIDES DERIVED FROM SOYBEAN PROTEIN HYDROLYSATE

Retention Time (min)	Peptide Sequence	Amino Acid Ratio in Acid Hydrolysate[a]	IC_{50}[b](μM)
19.7	Ile-Ala	Ile: 1.20, Ala: 1.00	153
27.5	Tyr-Leu-Ala-Gly-Asn-Gln	Tyr: 1.00, Leu: 0.81, Ala: 1.31, Gly: 1.26, Asx: 1.03, Glx: 0.98	14
47.5	Phe-Phe-Leu	Phe: 1.81, Leu: 1.00	37
48.2	Ile-Tyr-Leu-Leu	Ile: 1.00, Tyr: 0.73, Leu: 1.67	42

[a] Each peptide was hydrolyzed with 6 N HCl at 110C for 24 h.
[b] The concentration of ACE-inhibitory peptide required to inhibit 50% of ACE activity.

DISCUSSION

Angiotensin-converting enzyme plays an important physiological role in the regulation of blood pressure. Many ACE inhibitory peptides have been isolated from hydrolysates of various proteinaceous food materials. 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 *et al.* 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 (Yamori *et al.* 1978; Imura *et al.* 1993; Nevala *et al.* 2000).

Miyoshi *et al.* (1991) isolated tripeptides from zein by tryptic hydrolysis and found that these are ACE-inhibitory peptides. Suetsuna *et al.* (1991) and Kawasaki *et al.* (2000) also isolated ACE-inhibitory peptides from 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. Each of them will be evaluated with animal experiments before clinical uses. On the correlation between structure and activity of the 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. In this study, we identified four ACE-inhibitory peptides from the pepsin digests of soybean protein. There are three active sites of ACE, including the zinc ion, hydrogen bond, and positively charged residue binding site. In this study, three leucine-containing peptides and one isoleucine-containing peptide were found to have ACE-inhibitory activity. All of the active peptides have branch chain amino acid residues in the structure. It is considered that the hydrophobic side chain may

be the binding site with the active site of ACE. This may partially explain that these inhibitory agents work by their special chemical structure.

For the practical purpose of utilizing food materials as physiological modulators, it is necessary to confirm the antihypertensive effect of orally administrated peptic digests of soybean protein peptidic fractions on SHRs. In this study, we identified the ACE-inhibitory peptides from soybean protein which are orally antihypertensive. Further experiments are necessary and will be performed to confirm the antihypertensive effect of these peptides by oral administration. 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 activity (Kohama *et al.* 1989; Masuda *et al.* 1996). An *in vitro* study also showed that peptides from pepsin-digested soybean broth can decrease the conversion of angiotensin I into angiotenisin II (Chen *et al.* 1997). In our study, blood pressures of both experimental groups were lower than that of the control group. The results suggest that the peptide fraction might lower blood pressure on account of the physiologically functional peptides it contains. Ferreira *et al.* (1970) found that a low-molecular-weight fraction from snake venom (*Bothrops jararaca*) potentiates the activity of bradykinin and named it 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 of blood pressure. For example, captopril, enalapril, and lisinopril are all widely used in clinical practice today.

Soybean has been a very popular food in the Orient since ancient times, so its 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 is weaker than captopril which is used as 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 were not found in many studies of ACE inhibitors isolated from dietary protein (Sugiyama *et al.* 1991; Yoshikawa *et al.* 2000).

In conclusion, soybean protein hydrolysate, especially the SP-1 fraction, can lower blood pressure and one of the important factors may be a decrease in ACE activity *in vitro*. Regarding the prevention and management of hypertension, modification of daily eating habits plays an important role. The mechanism between the 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*.

REFERENCES

ASTAWAN, M., WAHYUNI, M., YASUHARA, T., YAMADA, K., TADOKORO, T. and MAEKAWA, A. 1995. Effects of angiotensin I-converting enzyme inhibitory substances derived from Indonesian dried-salted fish on blood pressure of rats. Biosci. Biotechnol. Biochem. *59*, 524–529.

CHEN, J.R., YANG, S.C., LIU, Y.L., SUETSUNA, K. and SHIEH, M.J. 1997. Peptides with angiotensin I converting enzyme inhibitory activity in pepsin-digests of soybean broth. Nutr. Sci. J. *22*, 435–444.

CHEUNG, H.S. and CUSHMAN, D.W. 1973. Inhibition of homogeneous angiotensin converting enzyme of rabbit lung by synthetic venom peptides of *Bothrops jararaca*. Biochim. Biophys. Acta. *293*, 451–463.

CHEUNG, H.S., WANG, F.L., ONDETTI, M.A., SABO, E.F. and CUSHMAN, D.W. 1980. Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. J. Biol. Chem. *255*, 401–407.

FERREIRA, S.H., BARTELT, D.C. and GREENE, L.J. 1970. Isolation of bradykinin-potentiating peptides from *Bothrops jararaca* venom. Biochemistry *9*, 2583–2593.

FUJITA, H., SASAKI, R. and YOSHIKAWA, M. 1995. Potentiation of the antihypertensive activity of orally administered ovokinin, a vasorelaxing peptide derived from ovalbumin, by emulsification in egg phosphatidylcholine. Biosci. Biotechnol. Biochem. *59*, 2344–2345.

IMURA, T., KANAZAWA, T., WATANABE, T., FUKUSHI, Y., KUDOU, S., UCHIDA, T., OSANAI, T. and ONODERA, K. 1993. Hypotensive effect of soy protein and its hydrolysate. Ann. NY Acad. Sci. *676*, 327–330.

KATO, H. and SUZUKI, T. 1971. Bradykinin-potentiating peptides from the venom of *Agkistrodon halys blomhoffi*. Isolation of five bradykinin potentiators and the amino acid sequences of two of them, potentiators B and C. Biochemistry *10*, 972–980.

KAWASAKI, T., SEKI, E., OSAJIMA, K., YOSIDA, M., ASADA, K., MATSUI, T. and OSAJIMA, Y. 2000. Antihypertensive effect of valyl-tyrosine, a short chain peptide derived from sardine muscle hydrolyzate, on mild hypertensive subjects. J. Hum. Hypertens. *14*, 519–523.

KOHAMA, Y., OKA, H., KAYAMORI, Y., TSUJIKAWA, K., MIMURA, T., NAGASE, Y. and SATAKE, M. 1991. Potent synthetic analogues of angiotensin-converting enzyme inhibitor derived from tuna muscle. Agric. Biol. Chem. *55*, 2169–2170.

LIPWORTH, B.J., McMURRAY, J.J., CLARK, R.A. and STRUTHERS, A.D. 1989. Development of persistent late onset asthma following treatment with captopril. Eur. Respir. J. *2*, 586–588.

MASUDA, O., NAKAMURA, Y. and TAKANO, T. 1996. Antihypertensive peptides are present in aorta after oral administration of sour milk containing these peptides of spontaneously hypertensive rats. J. Nutr. *126*, 3063–3068.

MIYOSHI, S., ISHIKAWA, H., KANEKO, T., FUKUI, F., TANAKA, H. and MARUYAMA, S. 1991. Structures and activity of angiotensin-converting enzyme inhibitors in an alpha-zein hydrolysate. Agric. Biol. Chem. *5*, 1313–1318.

NEVALA, R., VASKONEN, T., VEHNIAINEN, J., KORPELA, R. and VAPAATALO, H. 2000. Soy based diet attenuates the development of hypertension when compared to casein based diet in spontaneously hypertensive rat. Life Sci. *66*, 115–124.

OKAMOTO, A., MATSUMOTO, E., IWASHITA, A., YASUHARA, T., KAWAMURA, Y., KOIZUMI, Y. and YANAGIDA, F. 1995. Angiotensin I-converting enzyme inhibitory action of fish sauce. Food Sci. Technol. Intern. *1*, 101–106.

ONDETTI, M.A., RUBIN, B. and CUSHMAN, D.W. 1977. Design of specific inhibitors of angiotensin-converting enzyme: New class of orally active antihypertensive agents. Science *196*, 441–444.

SAWAYAMA, T., TSUKAMOTO, M., SASAGAWA, T., NISHIMURA, K., DEGUCHI, T., TAKEYAMA, K. and HOSOKI, K. 1990. Angiotensin-converting enzyme inhibitors: synthesis and biological activity of N-substituted tripeptide inhibitors. Chem. Pharm. Bull. *38*, 110–105

SUETSUNA, K. 1991. Effects of an oral administration of tripeptides derived from sardine muscle, soybean, and pig plasma on blood pressures in hypertension rats, and angiotensin I converting enzyme inhibitors. Clin. Rep. *25*, 2245–2261.

SUETSUNA, K., YAMAUCHI, F., DOI, K., HIROTA, T. and IKUTA, N. 1991. Biological properties of angiotensin I-converting enzyme inhibitor, an octapeptide derived from sardine muscle. Clin. Rep. *25*, 3422–3428.

SUGIYAMA, K., TAKADA, K., EGAWA, M. and YAMAMOTO, I. 1991. Hypotensive effect of fish protein hydrolysate. Nippon Nogeikagaku Kaishi *65*, 35–43.

WAKO, Y., ABE, Y., HANDA, T. and ISHIKAWA, S. 1999. Angiotensin I-converting enzyme inhibitors in fish water soluble protein hydrolyzates prepared by bioreactor. Food Sci. Technol. Res. *5*, 378–380.

WASHBURN, S., BURKE, G.L., MORGAN, T. and ANTHONY, M. 1999. Effect of soy protein supplementation on serum lipoproteins, blood pressure, and menopausal symptoms in perimenopausal women. Menopause *6*, 7–13.

YAMORI, Y., HORIE, R., OHTAKA, M., NARA, Y. and IKEDA, K. 1978. Prophylactic trials for stroke in stroke-prone SHR. Amino acid analysis of various diets and their prophylactic effect. Jpn. Heart *19*, 624–626.

YOKOYAMA, K., CHIBA, H. and YOSHIKAWA, M. 1992. Peptide inhibitors for angiotensin I-converting enzyme from thermolysin digest of dried bonito. Biosci. Biotech. Biochem. *56*, 1541–1545.

YOSHIKAWA, M. 1993. Physiological function of foods and bioassay in vitro. Kagaku to Seibutsu *31*, 342–346.

YOSHIKAWA, M., FUJITA, H., MATOBA, N., TAKENAKA, Y., YAMAMOTO, T., YAMAUCHI, R., TSURUKI, H. and TAKAHATA, K. 2000. Bioactive peptides derived from food proteins preventing lifestyle-related diseases. Biofactors *12*, 143–146.