ELSEVIER

Rapid Communication

# Isolation and characterization of angiotensin I-converting enzyme inhibitor dipeptides derived from *Allium sativum* L (garlic)

**Kunio Suetsuna**

*Department of Food Science and Technology, National Fisheries University, Shimonoseki, Japan*

*A concentrate of an aqueous extract of* Allium sativum *L. (garlic) was fractionated using ion exchange and gel filtration to isolate fractions with angiotensin I-converting enzyme (ACE) inhibitory activity. Fractions with high ACE inhibitory activity were combined and further chromatographed on a reverse-phase column to yield seven dipeptides with ACE inhibitory properties. These dipeptides were identified by sequence analysis and fast atom bombardment mass spectrometry as Ser-Tyr, Gly-Tyr, Phe-Tyr, Asn-Tyr, Ser-Phe, Gly-Phe, and Asn-Phe, with* $IC_{50}$ *(the amount of peptide needed to inhibit ACE activity) values of 66.3, 72.1, 3.74, 32.6, 130.2, 277.9, and 46.3* $\mu M$*, respectively. Each dipeptide was synthesized and its antihypertensive activity was determined after oral administration in spontaneously hypertensive rats. The blood pressure lowering activity of the dipeptides was lower than that of captopril. However, the presence of these dipeptides in garlic suggests that these compounds may, at least in part, be responsible for the observed antihypertensive effect of garlic (or garlic extracts) in animals and humans. Further, long-term use of dietary garlic may have a protective effect against rise in blood pressure.* (J. Nutr. Biochem. 9:415–419, 1998) © *Elsevier Science Inc. 1998*

**Keywords:** dipeptide from *Allium sativum* L. (garlic); angiotensin I-converting enzyme; inhibitory activity; spontaneously hypertensive rat

## Introduction

Angiotensin I-converting enzyme (ACE) is an important enzyme involved in blood pressure regulation and in electrolyte and fluid balance. Following the discovery of captopril,[1,2] hundreds of potential ACE inhibitors have been synthesized and at least three dozen have been tested clinically. More than a dozen ACE inhibitors have been used extensively in the treatment of essential hypertension and heart failure in humans; these include alacepril, benazepril, captopril, cilazapril, enalapril, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, tandolapril, and zofenopril. Recently, many ACE inhibitory peptides have been isolated from enzymatic digests of various food materials, including casein,[3] zein,[4] gelatin,[5] sake,[6] Masai fermented milk,[7] sardine muscle,[8] tuna muscle,[9] dried salted fish,[10] dried bonito,[11] and fish sauce.[12]

Several studies suggest that garlic contains blood pressure lowering compounds. In spontaneously hypertensive rats, oral doses of garlic extract caused significant sustained reduction in blood pressure after 24 hours.[13] In anesthetized dogs, blood pressure stayed depressed for more than 4 hours after oral administration of garlic powder.[14] Moderate ACE inhibitory activity was found in extracts of wild garlic in vitro and in vivo (in rats).[15] The use of garlic (fresh, powder, or extract) has been shown to decrease blood pressure in humans.[16–18]

In this study, we describe the isolation, purification, and identification of ACE inhibitory dipeptides derived from garlic and the antihypertensive effect of the orally administered garlic dipeptides in spontaneously hypertensive rats.

Address correspondence and reprint requests to Dr. K. Suetsuna at the Department of Food Science and Technology, National Fisheries University, 2-7-1 Nagata-Honmachi, Shimonoseki, Yamaguchi 759-65, Japan.
Received September 10, 1997; accepted April 30, 1998.



655 Avenue of the Americas, New York, NY 10010

0955-2863/98/$19.00
PII S0955-2863(98)00036-9

## Materials and methods

### Materials

Garlic was purchased from the market in Shimonoseki City, Japan. Hippuryl-L-histydyl-L-leucine (Hip-His-Leu) was obtained from the Peptide Institute Inc. (Osaka, Japan), and ACE from rabbit lung acetone powder was obtained from Sigma Chemical Co. (St. Louis, MO). Captopril (as granules) was purchased from Sankyo Co., Ltd. (Tokyo, Japan).

### Purification of the peptides from garlic extract

Two hundred grams of garlic was peeled and homogenized in 500 mL of deionized water. The homogenate was dialyzed in 5 L of deionized water for 20 hours at 4°C. The outer phase was concentrated in vacuo to 200 mL, and 800 mL of cold methanol was added to the concentrate. The precipitate, obtained by filtration (3G-1, glass filter), was dissolved in deionized water (10 mL) and applied to a column (2.5 × 30 cm) of Dowex 50WX4 (50–100 mesh, $H^+$ form, Dow Chemical Co., Midland, Michigan) cation exchange resin. After the column was washed sufficiently with deionized water to remove impurities, the desired peptides were eluted with 1 L of 2N $NH_4OH$. After being concentrated under vacuo to 30 mL, the concentrate (3 mL) was applied to a column (2.5 × 150 cm) of Sephadex G-25 (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with 0.1 M phosphate buffer (pH 7.0). The elute was gel-filtrated with 0.1 M phosphate buffer at a flow rate of 30 mL/hr and 5 mL fractions were collected. Protein content of each fraction was measured by the Lowry method,[19] using bovine serum albumin as the standard. The molecular weight of the peptides was estimated from the retention time relative to standard peptides.[20] The molecular size markers used were as follows: insulin (porcine pancreas), MW 6,000; insulin B chain, 3,500; insulin A chain, 2,250; bradykinin, 1,052; and glycine, 75.

### Purification of the ACE inhibitory peptide by reverse-phase HPLC and peptide identification and synthesis

A solution (7 mg/25 μL) of the most potent fractions eluted from Sephadex G-25 was further isolated and purified by reverse-phase high performance liquid chromatography (HPLC), with a column (4.6 × 150 cm) of Develosil ODS-5 (Nomura Chemical, Ltd., Nagoya, Japan) using a linear gradient of acetonitrile (MeCN) from 0 to 8% in 0.05% trifluoroacetic acid (TFA) for 1 hour at a flow rate of 1.0 mL/min, and the elute was monitored at 220 nm.

Amino acid analysis of peptide was carried out in 6N hydrochloric acid containing 0.1% phenol at 110°C for 24 hours using a PICO-TAG™ amino acid analyzer (Waters Ltd., Milford, MA). Sequence analysis was done by stepwise Edman degradation using a 477A gas-phase automated sequencer (Applied Biosystems, Inc., Foster, California) coupled with HPLC identification of the resulting phenylthiohydantoin (PTH)-amino acid.

The molecular formula of each peptide was confirmed by fast atom bombardment mass spectometry (FAB-MS) obtained with a JEOL DX-300 spectrometer (Nihandenshi Co., Tokyo, Japan).

Peptides were synthesized by a solid-phase method using a 433A automated peptide synthesizer (Applied Biosystems, Inc.), followed by treatment with hydrogen fluoride to cut off the support resin and to remove all of the protecting groups. The synthesized peptides were purified by HPLC on a column of Aquapore TM Prep-10 (Applied Biosystems, Inc.) with a gradient of MeCN from 3.5 to 67% in 0.05% TFA for 30 minutes at a flow rate of 0.5 mL/min, and the elute was monitored at 215 nm.

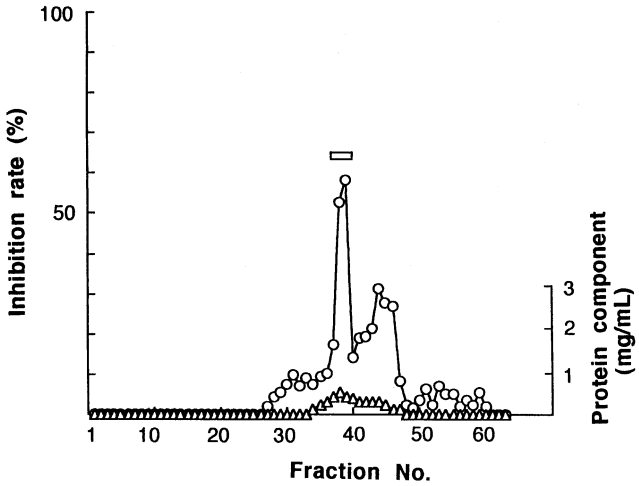


**Figure 1** A chromatogram of garlic extract on Sephadex G-25. -O- represents angiotensin I-converting enzyme inhibitory activity, and -D- represents protein content in each fraction. The bar at the top of the first peak indicates the fractions combined and rechromatographed on octadecylsilano column (see text).

### Assay of ACE inhibitory activity

ACE inhibitory activity was determined by a modification of the method of Cushman and Cheung.[21] Fifty microliters of a sample solution with 100 μL of 2.5 mU ACE solution was added to 100 μL of a 12.5 mM Hip-His-Leu solution in 1.0 M NaCl-borate buffer at pH 8.3. After incubation at 37°C for 1 hour, the reaction was stopped by adding 250 μL of 0.5N HCl. The liberated hippuric acid was extracted with 1.5 mL of ethyl acetate, and absorbence at 228 nm was determined to evaluate ACE inhibitory activity. The inhibition rate (%) was shown as equal to {(Ec-Es)/(Ec-Eb)} × 100, where Es is absorbence with test sample added to the reaction mixture, Ec is absorbence with buffer added (instead of the test sample), and Eb is absorbence when the stop solution was added before the reaction to occur. The activity of an ACE inhibitory peptide was defined as the amount needed to inhibit 50% of the ACE activity ($IC_{50}$) under these conditions.

### Antihypertensive effect in spontaneously hypertensive rats

Female spontaneously hypertensive rats (SHRs) were purchased from Saitama Animal Facility Center (Saitama, Japan) and fed laboratory chow (CE-2, Clea Japan, Tokyo, Japan). The systolic blood pressure (SBP) of 12-week-old SHRs (280–330 g of body weight) was measured. Five SHRs that had been given synthetic dipeptide and captopril dissolved in 0.9% saline by gastric intubation were kept at 40°C for 10 minutes, and the SBP was measured by the tailcuff with a UR-5000 programmed electrosphygmomanometer (Ueda Co., Ltd., Tokyo, Japan). At least five readings were recorded, the maximum and minimum values were discarded, and averaged SBP was calculated from the remaining three values. The significance of differences of SBP before and after administration was analyzed using the Student's *t*-test.

## Results

### Isolation of ACE inhibitory peptides

Peptides having potent ACE inhibitory activity were isolated from garlic homogenates by chromatography on

**Table 1** Analytical data (for dipeptides isolated from garlic) and ACE inhibitor activity (of synthetic dipeptides)

| Peptide | Amino acid ratio in HCl hydrolysate[1] | FAB-MS ($MH^+$) | $IC_{50}$ (μM) |
|---|---|---|---|
| Ser-Tyr | Ser 0.83, Tyr 1.27 | 269 | 66.3 |
| Gly-Tyr | Gly 1.12, Tyr 1.08 | 239 | 72.1 |
| Phe-Tyr | Phe 0.98, Tyr 1.10 | 329 | 3.74 |
| Asn-Tyr | Asn 1.05, Tyr 1.04 | 296 | 32.6 |
| Ser-Phe | Ser 0.91, Tyr 0.97 | 253 | 130.2 |
| Gly-Phe | Gly 1.07, Phe 0.95 | 223 | 277.9 |
| Asn-Phe | Asn 1.12, Phe 0.91 | 280 | 46.3 |

Note: All amino acids are of the L-configuration.
[1]Each peptide was hydrolyzed with 6N hydrochloric acid (HCl) at 110°C for 24 hours.

Dowex 50W and Sephadex G-25. An elution profile on Sephadex G-25 column is shown in *Figure 1*. Fractions with high ACE inhibitory activity were collected and concentrated to dryness to give the peptide powder at 7.8 μg/mL of reaction mixture concentration provided 50% inhibition against ACE activity. The yield of the peptide powder from 200 g of garlic was approximately 400 mg.

### *Analyses and ACE inhibitory peptides*

Although approximately 100 peaks were detected by this chromatography, potent inhibitory peptides were obtained in seven peaks. Retention times were 25.8, 28.0, 31.5, 32.3, 46.2, 51.0, and 52.9 minutes, and $IC_{50}$ were 66.3, 72.1, 3.74, 32.6, 130.2, 277.9, and 46.3 μM, respectively. In the assay system used for measuring inhibitory activity of rabbit lung ACE, the $IC_{50}$ value of captopril was 2.5 nM. Amino acid analysis of each inhibitor after 6N HCl hydrolysis found the amino acids listed in *Table 1*. The ion peak ($MH^+$) of each purified inhibitor appeared at m/z of the theoretical value in the FAB-MS. Using protein sequencing, primary structures of the individual dipeptides were found to be Ser-Tyr, Gly-Tyr, Phe-Tyr, Asn-Tyr, Ser-Phe, Gly-Phe, and Asn-Phe. All of the dipeptides contained a tyrosine or phenylalanine residue at the C terminus (*Figure 2*). Among them, Phe-Tyr was a most potent ACE inhibitor.

### *Antihypertensive effects of the identified peptides on spontaneously hypertensive rats*

Antihypertensive activity of each dipeptide was evaluated by measuring the change in SBP at 0, 1, 2, 3, 4, and 6 hours after oral administration of 200 mg of chemically synthesized dipeptide per kilogram of body weight (*Figure 3, A–G*). SBP did not change in control rats during the study period (6 hr). Captopril (10 mg/kg) lowered SBP significantly from 1 to 4 hours after administration of the drug.

A single dose (200 mg/kg) of the various dipeptides caused maximal decrease in SBP at different times after administration: 1 hour for Asn-Phe, Gly-Phe, and Ser-Phe; 3 hours for Asn-Tyr; and 4 hours for Ser-Tyr, Gly-Tyr, and Phe-Tyr.

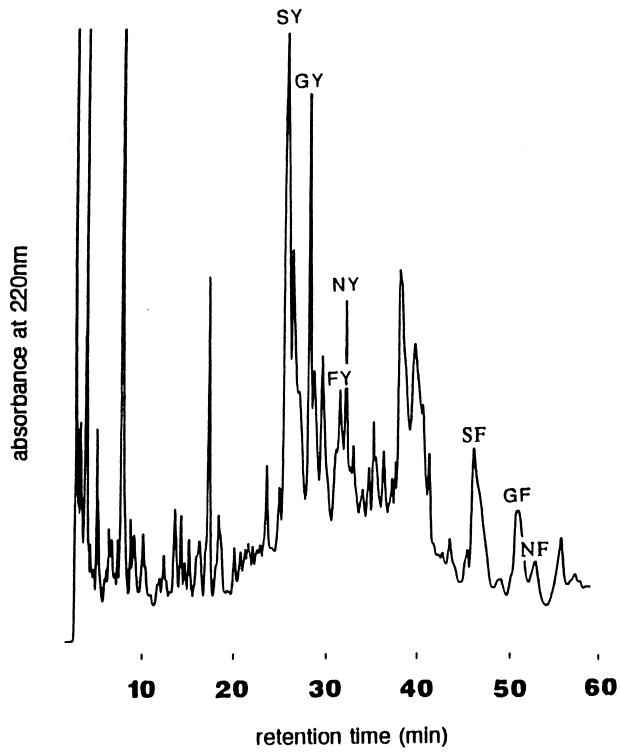


**Figure 2** A chromatogram on reverse-phase Develosil ODS-5 column of the active fraction isolated from the Sephadex column (see *Figure 1*). See the text for column conditions. The peaks marked SY, GY, FY, NY, SF, GF, and NF, representing the dipeptides Ser-Tyr, Gly-Tyr, Phe-Tyr, Asn-Tyr, Ser-Phe, Gly-Phe, and Asn-Phe, respectively, were found to have angiotensin-converting enzyme inhibitor activity.

## Discussion

ACE plays an important physiologic role in the regulation of blood pressure and electrolyte homeostasis. It cleaves angiotensin I to angiotensin II, which is a powerful vasoconstrictor and salt-retaining octapeptide. Moreover, it catalyzes the inactivation of bradykinin, which is a vasodilator and natriuretic nonapeptide. After the successful development of captopril as the first orally active ACE inhibitor, a variety of potent inhibitors have been discovered or have been synthesized. In the present study, the isolated ACE inhibitory dipeptides had a Tyr or Phe at the C terminal; dipeptides with Tyr were more potent than those with Phe, but less potent than the dipeptides with proline at the C terminal.[22] In their structure-activity study, Cheung et al.[22] showed that among the N-terminal amino acids of dipeptides, the branched-chain aliphatic amino acids valine and isoleucine were most effective in increasing peptide binding to the active site of ACE. In our study, ACE inhibitory activity followed the order, with the N-terminal amino acid being Phe>Asn>Ser>Gly at the N terminal; the dipeptide Phe-Tyr was the most potent inhibitor of ACE. Because the concentrations of the dipeptides required to inhibit ACE activity ($IC_{50}$) are rather high, it is possible that these peptides cause ACE inhibition by chelating zinc, which is required for ACE activity.

In the in vivo studies, oral administration of the seven

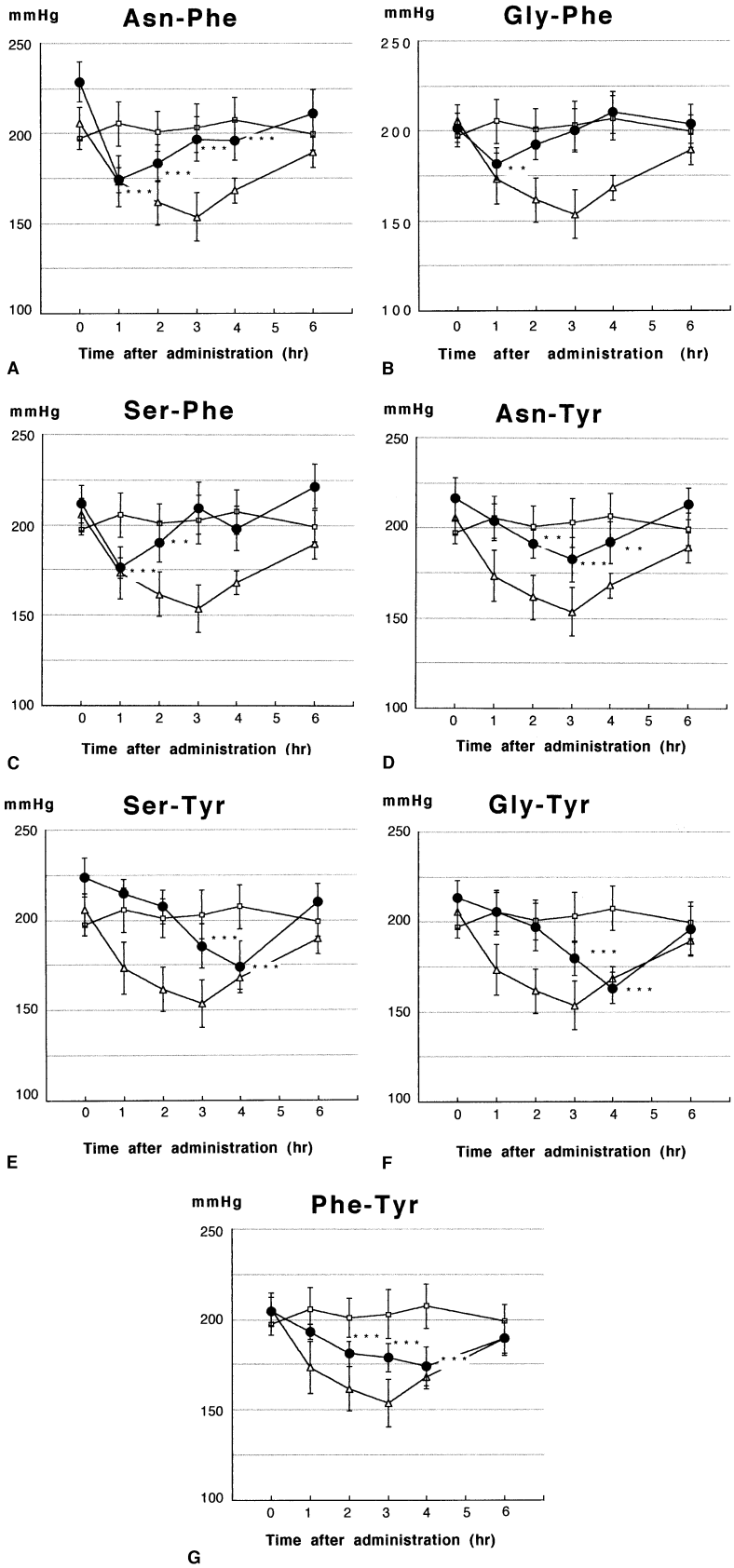


**Figure 3** Antihypertensive effect in spontaneously hypertensive rats of single oral doses of the seven dipeptides (A—G) with angiotensin I-converting enzyme inhibitory activity isolated from garlic. Each point represents the mean change in systolic blood pressure in five rats: -- control (0.9% saline); -Δ- captopril (oral 10 mg/kg); -•- each dipeptide (oral 200 mg/kg). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ from the Sephadex column (see *Figure 1*).

isolated dipeptides showed blood pressure reducing activity qualitatively similar to that of captopril. Dipeptides with Tyr at the C terminal caused slow but prolonged reduction of SBP compared with dipeptides with Phe at the C terminal, which produced rapid decrease in SBP and shorter duration of action. A decrease in blood pressure after oral administration suggests that these dipeptides are small enough to cross the intestinal wall into the blood without significant hydrolysis (to single amino acids) by the digestive proteases.

The presence of these dipeptides in garlic suggests that these simple dipeptides may be responsible, at least in part, for the observed blood pressure lowering effect of garlic (or garlic extracts) in animals[13–15] and humans.[16–18] Although the dipeptides isolated from garlic are weaker than captopril as ACE inhibitors, our finding may have pharmacologic relevance because garlic is used in the diet almost daily in many cultures. Daily use of food that contains antihypertensive ingredients, such as garlic, may keep blood pressure from rising in some individuals. Additional studies must be carried out to determine if the dipeptides (or garlic) added to the regular rat chow would prevent a rise in blood pressure if fed to spontaneously hypertensive rats at an early age.

## References


1 Ondetti, M.A. (1977). Design of specific inhibitors of angiotensin-converting enzyme: New class of orally active antihypertensive agents. *Science* **196,** 441–444

2 Patchett, A.A., Harris, E., Tristram, E.W., Wyvratt, M.J., Wu M.T., Taub D., Peterson E.R., Ikeler, T.J., ten Broeke, J., Payne, L.G., Ondeyka, D.L., Thorsett, E.D., Greenlee, W.J., Lohr, N.S., Hoffsommer, R.D., Joshua, H., Ruyle, H.V., Rothrock, J.W., Aster, S.D., Maycock, A.L., Robinson F.M., Hirschmann, R., Sweet, C.S., Ulm, E.H., Gross, D.M., Vassil, T.C., and Stone, C.A. (1980). A new class of angiotensin-converting enzyme inhibitors. *Nature* **288,** 280–283

3 Maeno, M., Yamamoto, N., and Takano, T. (1996). Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from Lactobacillus helveticus CP790. *J. Dairy Sci.* **79,** 1316–1321

4 Miyoshi, S., Ishikawa, H., Kaneko, T., Fukui, F., Tanaka, H., and Maruyama, S. (1991). Structures and activity of angiotensin-converting enzyme inhibitors in an a-zein hydrolysate. *Agric. Biol. Chem.* **55,** 1313–1318

5 Oshima, G., Shimabukuro, H., and Nagasawa, K. (1979). Peptide inhibitors of angiotensin-converting enzyme in digests of gelatin by bacterial collagenase. *Biochemica et Biophysica Acta* **566,** 128–137

6 Saito, S., Wanezaki (Nakamura), K., Kawato, A., and Imayasu, S. (1994). Structure and activity of angiotensin I converting enzyme inhibitory peptides from sake and sake lees. *Biosci. Biotech. Biochem.* **58,** 1767–1771

7 Isono, Y. (1996). Peptide inhibitors for angiotensin I-converting enzyme from Masai fermented milk. *Food Sci. Technol. Int.* **2,** 213–216

8 Suetsuna, K. (1992). Study on the inhibitory activities against angiotensin I-converting enzyme of the peptides derived from sardine muscle. Doctor's thesis of Tokoku University, Miyagi, Sendai, Japan. 1–92

9 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

10 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. Biotech. Biochem.* **59,** 425–429

11 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

12 Okamoto (Kaimuma), 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. Int.* **1,** 101–106

13 Foushee, D.B., Ruffin, J., and Banerjee, U. (1982). Garlic as a natural agent for the treatment of hypertension: A preliminary report. *Cytobios* **34,** 145–152

14 Pantoja, C.V., Chiang, L.CH., Norris, B.C., and Concha, J.B. (1991). Diuretic, natriuretic and hypotensive effects produced by Allium sativum (garlic) in anaesthetized dogs. *J. Ethnopharmacol.* **31,** 325–331

15 Rietz, B., Isensee, H., Strobach, H., Makdessi, S., and Jacob, R. (1993). Cardioprotective actions of wild garlic (Allium ursinum) in ischemia and reperfusion. *Mol. Cell. Biochem.* **119,** 143–150

16 Ernst, E. (1987). Cardiovascular effects of garlic (Allium sativum): a review. *Pharmatherapeutica* **5,** 83–89

17 Harenbarg, J., Giese, C., and Zimmermann, R. (1988). Effects of dried garlic on blood coagulation, fibrinolysis, platelet aggregation and serum cholesterol levels in patients with hyperlipoproteinemia. *Atherosclerosis* **74,** 247–249

18 Steiner, M., Khan, A.H., and Lin, R.I-S. (1996). A double-blind crossover study in moderately hypercholesterolemic men that compared the effect of aged garlic extract and placebo administration on blood lipids. *Am. J. Clin. Nutr.* **64,** 866–870

19 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275

20 Chen, J.R., Suetsuna, K., and Yamauchi, F. (1995). Isolation and characterization of immunostimulative peptides from soybean. *Nutri. Biochem.* **6,** 310–313

21 Cushman, D.W. and Cheung, H.S. (1971). Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* **20,** 1637–1648

22 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

23 Jaeger, P., Ferguson, R.K., Brunner, H.R., Kirchertz, E.J., and Gavras, H. (1978). Mechanism of blood pressure reduction by teprotide (SQ 20881) in rats. *Kidney Int.* **13,** 289–296