ELSEVIER

Identification of an antihypertensive peptide from peptic digest of wakame (*Undaria pinnatifida*)

Kunio Suetsuna* and Takahisa Nakano†

**Department of Food Science and Technology, National Fisheries University, Shimonoseki, Japan and †Department of Research and Development, Riken Vitman Co. Ltd., 2-9-18, Misaki-cho Chiyoda-ku, Tokyo 101-8370, Japan*

A peptide fraction having activity against angiotensin I-converting enzyme (ACE) was separated from the peptic digest of protein prepared from wakame (Undaria pinnatifida) *by ion-exchange chromatographies and gel-filtration. Fractions with high ACE inhibitory activity were combined and further chromatographed on a reverse-phase column to yield four tetrapeptides with ACE inhibitory properties. These tetrapeptides were identified by sequence analysis and fast atom bombardment mass spectrometry as Ala-Ile-Tyr-Lys (IC_{50}: 213 μM), Tyr-Lys-Tyr-Tyr (64.2 μM), Lys-Phe-Tyr-Gly (90.5 μM), and Tyr-Asn-Lys-Leu (21 μM). Each tetrapeptide was synthesized and its antihypertensive activity was determined after oral administration in spontaneously hypertensive rats. The blood pressure significantly decreased after tetrapeptide ingestion. The present study demonstrated that dietary wakame may have beneficial effects on hypertension.* (J. Nutr. Biochem. 11: 450−454, 2000)

Keywords: tetrapeptide from wakame; angiotensin I-converting enzyme; inhibitory activity; spontaneously hypertensive rat

Introduction

The angiotensin I-converting enzyme (ACE) participates in regulating blood pressure in the renin–angiotensin system, and inhibitors such as captopril[1–3] and enalapril[4] have been used as antihypertensive drugs. The ACE inhibitory activity of foods has been studied, and some ACE inhibitory peptides were produced by the enzymatic digests of various food materials including casein,[5,6] zein,[7,8] gelatin,[9] sake,[10] sour milk,[11] sardine muscle,[12,13] tuna muscle,[14] dried salted fish,[15] dried bonito,[16] fish sauce,[17] *Porphyra yezoensis*,[18] and *Hijikia fusiformis*.[19] The ACE inhibitory peptides of sour milk have been found to decrease blood pressure in humans.[20]

Several studies suggest that dietary ingestion of wakame has been shown to decrease blood pressure in humans.[21–23] Wakame contains large quantities of alginate and minerals. Alginate has been reported to reduce blood pressure, and calcium and magnesium also reduced blood pressure in intervention trials. However, alginate and minerals in wakame are far lower than the effective dose needed to lower blood pressure.[24–27] Therefore, the blood pressure-lowering effects of wakame may not be attributed to alginate and minerals.

In this article, we describe the identification of ACE inhibitory peptides derived from wakame, their structures, and hypotensive action of orally administered peptides on spontaneously hypertensive rats (SHRs).

Materials and methods

Materials

Wakame was collected from the cultivation ground Kitagami-cyo of Miyagi Prefecture, Japan during February and March 1996. After washing with water and heat air drying, wakame was powdered by using an ultracentrifuge mill. Pepsin was obtained from porcine stomach mucosa (Wako Pure Chemical Industries, Osaka, Japan). Hip-his-leu was obtained from the Peptide Institute (Osaka, Japan), and ACE from rabbit-lung acetone powder was obtained from Sigma Chemical Co. (St. Louis, MO USA).

Assay for ACE inhibitory activity

The extent of ACE inhibition was assayed by a modification of the method of Cheung and Cushman.[28] Fifty microliters of a sample

Address correspondence to Dr. K. Suetsuna, Department of Food Science and Technology, National Fisheries University, 2-7-1 Nagata-Honmachi, Shimonoseki, Yamaguchi 759-6595, Japan.
Received February 15, 1999; accepted July 21, 2000.

655 Avenue of the Americas, New York, NY 10010
0955-2863/00/$–see front matter
PII S0955-2863(00)00110-8

solution was mixed with 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 hr, the reaction was stopped by adding 250 μL of 0.5N HCl. The liberated hippuric acid was extracted with 1.5 mL of ethylacetate, and absorbance at 228 nm was determined to evaluate ACE inhibitory activity. The inhibition was shown as equal to ([Ec − Es]/[Ec − Eb]) × 100, where Es is absorbance with test sample added to the reaction mixture, Ec is absorbance with buffer added (instead of the test sample), and Eb is absorbance when the stop solution was added before the reaction occured. 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.

Purification of the peptides from digest of wakame

Wakame powder (23.6 g) was immersed in 700 mL of distilled water and then homogenized. The homogenate was adjusted to pH 2 with HCl. Seven hundred eight milligrams of pepsin was added to the homogenate at 45°C, and then enzymatic digestion was done for 5 hr. The pepsin digest was adjusted to pH 7 with sodium hydroxide. The digest was boiled for 10 min to inactive the enzyme, and then filtered.

The filtrate of enzymatic digest was dialyzed against 10 L of deionized water in seamless cellulose tubing (36 inches, Wako Pure Chemical Industries, Osaka, Japan) for 2 days. The outer solution was applied to a Dowex 50W column (2.6 × 20 cm, 50–100 mesh, H^+form, Dow Chemical Co., Midland, MI USA) equilibrated with deionized water. The column was washed sufficiently with deionized water to remove some impurities, and then peptides eluted with 300 mL of 3.7% ammonia solution. After being evaporated under vacuum, the concentrate was applied to a SP-Sephvadex C-25 (2 × 50 cm, H^+form, Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with deionized water. The eluate was chromatographed using a linear gradient with 500 mL each of deionized water and 1.5% sodium chloride solution at a flow rate of 70 mL/hr, and each fraction of 10 mL was obtained. The active fractions were collected and lyophilized to prepare a peptide powder (SP fraction).

The peptides in the most potent SP fraction were further purified by reverse-phase high performance liquid chromatography (HPLC) with Develosil octadecylsilano (ODS-5) column (4.6 × 250 cm, Nomura Chemical, Ltd., Nagoya, Japan), using a gradient of acetonitrile (MeCN) from 0% to 25% in 0.05% trifuruoroacetic acid (TFA) for 2 hr at a flow rate of 1.0 mL/min, and the eluate was monitored at 220 nm.

Amino acid sequence analyses and peptide synthesis

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

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

Peptides were synthesized by a solid-phase method using a 433A automated peptide synthesizer (Applied Biosystems, Inc., Foster, CA USA), followed by treatment with hydrogen fluoride to cut off the support resin and to remove all of the protecting groups. The final products were homogeneous on high-resolution reverse-phase (RP-HPLC) with a Develosil ODS column (4.6 × 150 mm), using a gradient of MeCN from 83% to 53% in 0.1% TFA for 40 min at a flow rate of 1.3 mL/min, and the elute was monitored at 215 nm. The results of amino acid analyses and sequence analyses agreed well with expected values.

Antihypertensive effect on SHRs

Male 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 15-week-old SHRs (280–330 g of body weight) was measured. Six SHRs that had been given synthetic peptides (50 mg/kg body weight) and captopril (10 mg/kg body weight) dissolved in 0.9% saline by gastric incubation were kept at 40°C for 10 min, and the SBP was measured by the tailcuff with a UR-5000 programmed electro-sphygmomanometer (Ueda Co. Ltd., Tokyo, Japan). At least five readings were recorded, the maximum and minimum values were discarded, and average 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

Identification of ACE inhibitory peptides

Peptides having potent ACE inhibitory activity were isolated from peptic digestion of Wakame usiong a Dowex 50W(H^+), Sephadex G-25, and SP-Sephadex C-25 column as discribed previously.[29] The fractions having a molecular weight of 300 to 1,000 were collected and concentrated to dryness to give a peptide powder at 0.234 mg/mL of reaction mixture concentration that provided 50% inhibition against ACE activity. The yield of the peptide powder from 23.6 g of wakame powder was 3.7 g.

The peptide fractions were purified further by RP-HPLC (*Figure 1*). Although approximately 100 peaks were detected by this chromatography, potent inhibitory peptides were obtained in four peaks. Retention times were 32.0, 80.7, 96.1, and 99.6 min, and IC_{50} values were 213, 64.2, 90.5, and 21 mM, respectively. Amino acid analysis of the peptide after 6N HCl hydrolysis found the amino acids listed in *Table 1*. The ion peak (MH^+) of each inhibitor appeared at m/z of the theoretical value in the FAB-MS. Using protein sequencing, primary structures of the individual peptides were identified. Thus, the amino acid sequences of the peptides were Ala-Ile-Tyr-Lys, Tyr-Lys-Tyr-Tyr, Lys-Phe-Tyr-Gly, and Tyr-Asn-Lys-Leu. All of the active peptides had a tyrosine and lysine residue in the structure.

Hypotensive effect of the identified peptides on SHRs

Hypotensive activity of each tetrapeptide was evaluated by measuring the change SBP at 1, 2, 3, 4, and 6 hr after oral administration of 50 mg of chemically synthesized tetrapeptides per kg of body weight (*Figure 2*). SBP did not change in control rats during the study period (6 hr). Captopril (10 mg/kg) lowered SBP significantly from 1 to 4 hr after administration of the drug. A single dose (50 mg/kg) of the various tetrapeptides significantly reduced 40–50 mmHg in SBP.

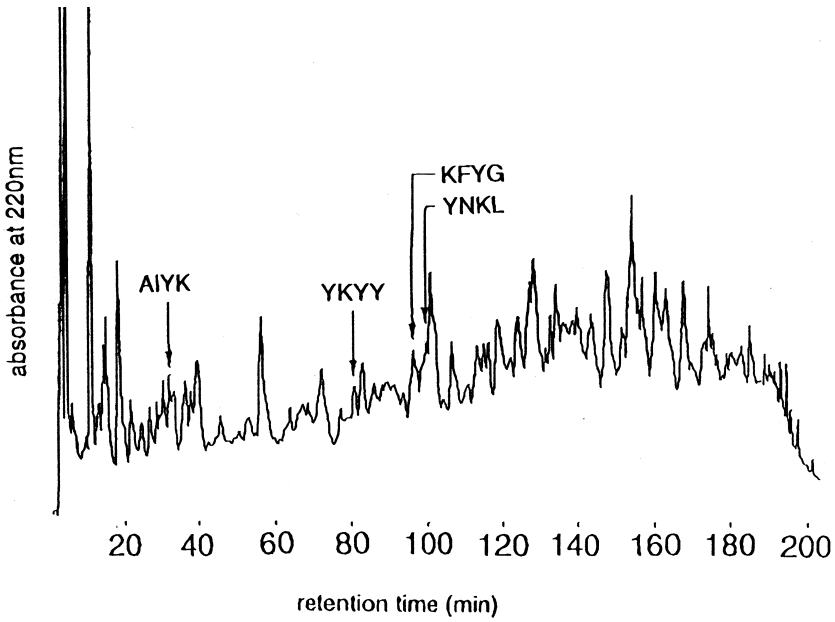

Figure 1 A chromatogram on reverse-phase Develosil octadecylsilano-5 column of the active fraction isolated from the SP Sephadex C-25 column. For column conditions, see the Materials and methods section. The peaks marked ALYK, YKYY, KFYG, and YNKL, representing the tetrapeptides Ala-Ile-Tyr-Lys, Tyr-Lys-Tyr-tyr, Lys-Phe-Tyr-Gly, and Tyr-Asn-Lys-Leu, were found to have ACE inhibitory activity.

Discussion

In this study, we identified four ACE inhibitory tetrapeptides from the peptic digest of wakame. These four tetrapeptides were the first found to have this activity. It is known that some peptides with potent ACE inhibitory activity *in vitro* or intravenously are inactive in oral administration. These inactive peptides are only used as substrates of ACE and do not decrease the blood pressure *in vivo*.[30] Therefore, the ACE inhibitory activity of the peptides did not correlate with antihypertensive activity found in SHRs.[31] We confirmed that four tetrapeptides showed the antihypertensive activity by oral administration in SHRs. Oral administration of the four synthesized tetrapeptides showed blood pressure-reducing activity qualitatively similar to that of captopril. Although each ACE inhibitory activity of tetrapeptides derived from wakame have different IC_{50} values, the blood pressure-lowering effect in SHRs (50 mg/kg) was almost the same level, except for Ala-Ile-Tyr-Lys. It was assumed that these four tetrapeptides themselves might have potent value as ACE inhibitory peptides, or might be digested in the body as shorter fragments; di- or tripeptides are easily absorbed in the intestine. Fujita and Yoshikawa[32] reported that Leu-Lys-Pro-Asn-Met derived from fish protein was found to be hydrolyzed to produce Leu-Lys-Pro with 8-fold higher ACE inhibitory activity relative to the parent peptide, suggesting that a parent peptide can be regarded as a prodrug-type ACE inhibitory peptide. The value of IC_{50} in the fragments of tetrapeptides derived from wakame are listed in *Table 2*. There are high potent ACE inhibitory activity di- or tripeptides to the parent tetrapeptides. An antihypertensive effect in SHRs may attribute to these shorter peptides.

Recently, the antihypertensive effect of wakame was

Table 1 Analytical data (for tetrapeptides isolated from wakame) and ACE inhibitor activity (of synthetic tetrapeptides)

Peptide	Amino acid ratio in HCl hydrolysate[a]	FAB-MS (MH^+)	IC_{50} (μM)[b]
Ala-Ile-Tyr-Lys	Ala 1.11, Ile 1.08, Tyr 0.93, Lys 1.16	494	213
Tyr-Lys-Tyr-Tyr	Tyr 2.94, Lys 1.02	636	64.2
Lys-Phe-Tyr-Gly	Lys 1.05, Phe 0.91, Tyr 0.94, Gly 0.89	514	90.5
Tyr-Asn-Lys-Leu	Tyr 1.01, Asn 0.96, Lys 1.10, Leu 1.07	537	21

All amino acids are of the L-configuration.
[a]Each peptide was hydrolyzed with 6N hydrochloric acid (HCl) at 110°C for 24 hr.
[b]The concentration of peptide needed to inhibit 50% of the ACE activity.
ACE–angiotensin I-converting enzyme. FAB-MS–fast atom bombardment mass spectrum.

Table 2 ACE inhibitory activity of synthetic tetrapeptides

Peptide	IC_{50} (μM)
Ala-Ile-Tyr-Lys	213
Ile-Tyr-Lys	177
Tyr-Lys	610
Ile-Tyr	2.65
Tyr-Lys-Tyr-Tyr	64.2
Tyr-Lys-Tyr	43.5
Lys-Tyr-Tyr	79
Lys-Tyr	13
Lys-Phe-Tyr-Gly	90.5
Lys-Phe-Tyr	45
Lys-Phe	28.3
Phe-Tyr	3.74
Tyr-Asn-Lys-Leu	21
Tyr-Asn-Lys	125
Asn-Lys-Leu	88
Asn-Lys	810
Lys-Leu	50.2

All amino acids are of the L-configuration.
ACE–angiotensin I-converting enzyme.

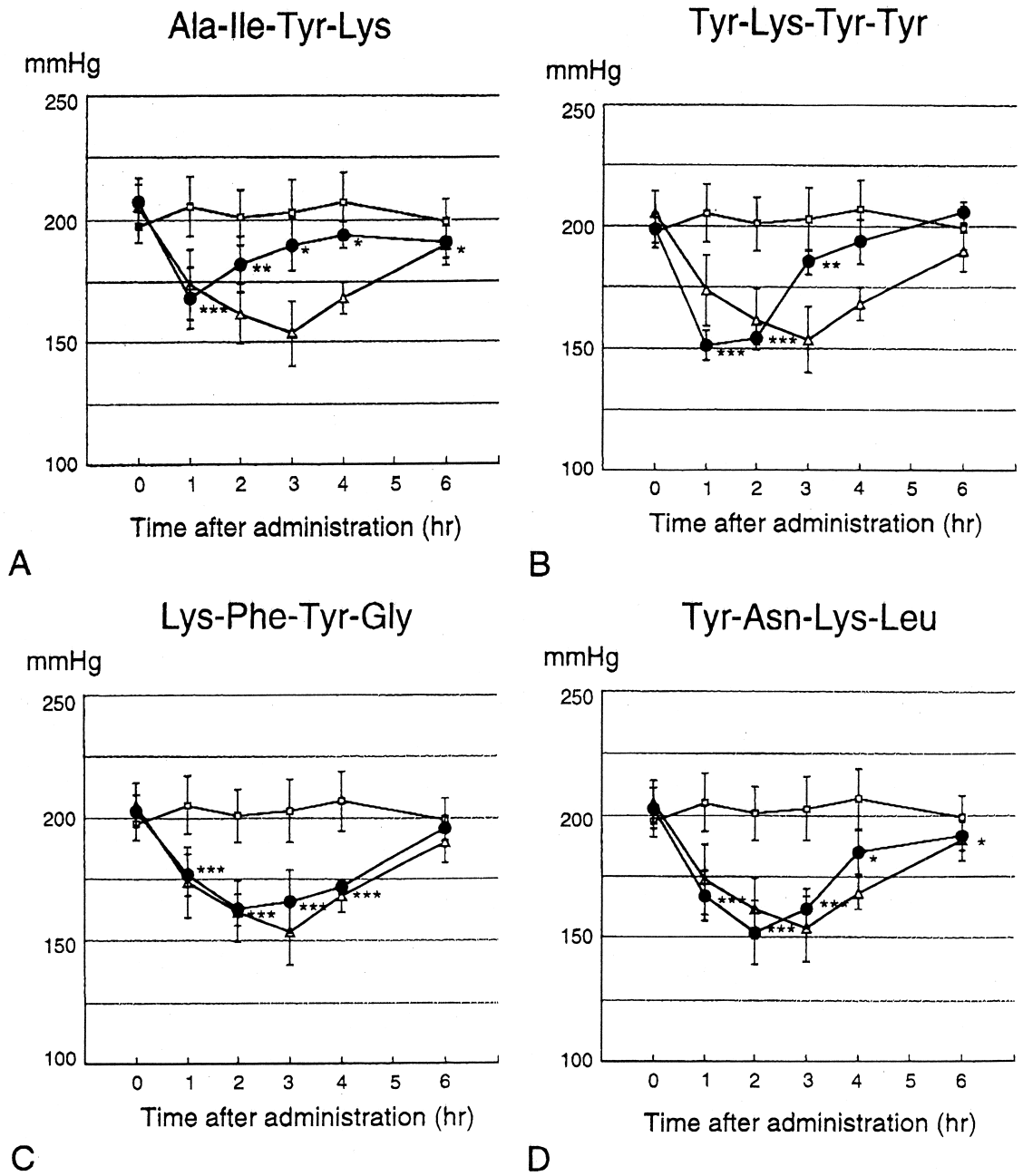

Figure 2 Antihypertensive effect in spontaneously hypertensive rats of single oral doses of the four tetrapeptides (A–D) with angiotensin I-converting enzyme inhibitory activity isolated from wakame. Each point represents the mean change in systolic blood pressure in five rats: □ control (0.9% saline); △ captopril (oral 10 mg/kg); ● each tetrapeptide (oral 50 mg/kg). Different from control at $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

shown in hypertensive patients.[22] The systolic blood pressure of patients decreased significantly, by 14 ± 3 mmHg, after daily oral administration of 3.3 g of dried wakame after 4 weeks. Diastolic pressure also decreased fiber and minerals. However, these fiber and minerals are far lower than the effective dose needed to lower the blood pressure. The presence of these ACE inhibitory tetrapeptides in peptic digest of wakame suggests that they could be responsible, at least partly, for the observed blood pressure-lowering effects of wakame.[22]

Wakame is the most popular edible seaweed in Japan since ancient times, so that its safety is established. Daily use of a food that has some peptides with potent ACE inhibitory activity could be effective in keeping blood pressure in good condition.

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 Ferreira, S.H., Bartelt, D.C., and Greene, L.J. (1970). Isolation of bradykinin-potentiating peptides from *Bothrops jararaca* venom. *Biochemistry* **9,** 2583–2593

3 Kato, H. and Suzuki, T. (1971). Bradykinin-potentiating peptides

from the venom of *Agkistrodon halys blomhoffii*: Isolation of five bradykinin potentiators B and C. *Biochemistry* **10,** 972–980

4 Sawayama, T., Itokawa, A., Shimada, K., Doi, Y., Kimura, K., and Nishimura, H. (1990). Synthesis of 1-[(s)-3-acetylthio-2-methylpropanoyl]-L-propyl-L-phenylalanine (Alacepril) and one of its active metabolites, the desacetyl derivative (DU-1227). *Chem. Pharm. Bull.* **38,** 529–531

5 Maruyama, S., Mitachi, H., Awaya, J., Kurono, M., Tomizuka, N., and Suzuki, H.(1987). Angiotensin I-converting enzyme inhibitory activity of the c-terminal hexapeptide of as1-casen. *Agric. Biol. Chem.* **51,** 2557–2561

6 Kohmura M., Nio, N., Kubo, K., Minoshima, Y., Munekata, E., and Ariyoshi, Y. (1989). Inhibition of angiotensin-converting enzyme by synthetic peptides of human β-casein. *Agric. Biol. Chem.* **53,** 2107–2114

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

8 Yano, S., Suzuki, K., and Funatsu, G. (1996). Isolation from α-zein of thermolysin peptides with angiotensin I-converting enzyme inhibitory activity. *Biosci. Biotech. Biochem.* **60,** 661–663

9 Ohshima, G., Shimabukuro, H., and Nagasama, K. (1979). Peptide inhibitors of angiotensin-converting enzyme in digests of gelatin by bacterial collagenase. *Biochim. Biophys. Acta* **566,** 128–137

10 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

11 Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., and Takano, T. (1995). Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J. Dairy Sci.* **78,** 777–783

12 Suetsuna, K. (1992). *Study on the inhibitory activities against angiotensin I-converting enzyme of the peptides derived from sardine muscle.* Doctoral Thesis, Tohoku University, Miyagi, Sendai, Japan

13 Matsufuji, H., Matsui, T., Ohshige, S., Kawasaki, T., Osajima, K., and Osajima, Y. (1995). Antihypertensive effects of angiotensin fragments in SHR. *Biosci. Biotech. Biochem.* **59,** 1398–1401

14 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

15 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

16 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

17 Okamoto(Kaimura), 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

18 Suetsuna, K. (1998). Purification and identification of angiotensin I-converting enzyme inhibitors from the red algae *Porphyra yezoensis*. *J. Mar. Biotechnol.* **6,** 163–167

19 Suetsuna, K. (1998). Separation and identification of angiotensin I-converting enzyme inhibitors from peptic digest of *Hizikia fusiformis* protein. *Nippon Suisan Gakkaishi* **64,** 862–866

20 Hata, Y., Yamamoto, M., Ohni, M., Nakajima, K., Nakamura, Y., and Takano, T. (1996). A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am. J. Clin. Nutr.* **64,** 767–771

21 Nara, Y., Miura, A., Moriguchi, Y., and Yamori, Y. (1998). Medical treatment of risk factor—Dietary treatment. *Jpn. Circ. J.* **61(Suppl 1),** 66

22 Nakano, T., Hidaka, H., Uchida, J., Nakajima, K., and Hata, Y. (1998). Hypotensive effects of wakame. *J. Jpn. Soc. Clin. Nutr.* **20,** 92

23 Tani, M., Tani, Y., Masuda, K., and Kato, T. (1998). Ingestion of wakame and its urinary components. *Eiyokaizengakkai* Fukui, October 8–9:210

24 Yamori, Y., Nara, Y., Tsubouchi, T., Sogawa, Y., Ikeda, K., and Horie, R. (1986). Dietary presentation of stroke and its mechanisms in stroke-prone spontaneously hypertension rats preventive effects of dietary fibre and palmitoleic acid. *J. Hypertension* **4(Suppl 3),** S449–S452

25 Krotkiewski, M., Aurell, M., Holm, G., Grimby, G., and Szczepanik, J. (1991). Effects of a sodium-potassium ion-exchanging seaweed preparation in mild hypertension. *Am. J. Hypertension* **4,** 483–488

26 Witterman, J.C.M., Grobbee, D.E., Derkx, F.H.M., Bouillon, R., Brujin, A.M., and Hofman, A. (1994). Reduction of blood pressure with oral magnesium supplementation in women with mild to moderate hypertension. *Am. J. Clin. Nutr.* **60,** 129–135

27 McCarron, D.A. and Morris, C.D. (1985). Blood pressure response to oral calcium in persons with mild to moderate hypertension. *Ann. Intern. Med.* **103,** 825–831

28 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

29 Suetsuna, K. (1998). Isolation and characterization of angiotensin I-converting enzyme inhibitor dipeptides derived from *Allium sativum* L. (GARLIC). *Nutri. Biochem.* **9,** 415–419

30 Yoshikawa, M. (1993). Physiological function of foods and bioassay in vitro. *Kagaku to Seibutsu* **31,** 342

31 Yamamoto, N., Maeno, M., and Takano, T. (1999). Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *J. Dairy Sci.* **82,** 1388–1393

32 Fujita, H. and Yoshikawa, M. (1999). LKPNM: A prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology* **44,** 123–127