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Antihypertensive effects of *Undaria pinnatifida* (wakame) peptide on blood pressure in spontaneously hypertensive rats

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Abstract

We examined the angiotensin I-converting enzyme (ACE) inhibitory activity and antihypertensive effect of the hot water extract of wakame, *Undaria pinnatifida*. Ten dipeptides were isolated from the extract by several steps of chromatography, and their amino acid sequences were Tyr-His, Lys-Trp, Lys-Tyr, Lys-Phe, Phe-Tyr, Val-Trp, Val-Phe, Ile-Tyr, Ile-Trp, and Val-Tyr. Both single administration and repeated oral administration of synthetic Tyr-His, Lys-Tyr, Phe-Tyr, and Ile-Tyr significantly decreased blood pressure in spontaneously hypertensive rats.

Keywords: Wakame; Dipeptide; Hot water extract; Angiotensin converting enzyme; blood pressure

1. Introduction

Hypertension was identified as a cardiovascular risk factor in the late 1950s and still remains a public health issue [1]. It is often called a "silent killer" because persons with hypertension can be asymptomatic for years and then have a fatal heart attack or stroke. Although there has been a recent increase in hypertension awareness and treatment, only a small percentage of affected individuals are being treated to goal [2]. Most persons (90–95%) with high blood pressure have essential hypertension, for which the cause cannot be determined; and as a result, treatment is nonspecific. The renin-angiotensin system plays an important role in the regulation of an organism's water, electrolytes, and blood [3]. The angiotensin I-converting enzyme (ACE, dipeptidyl carboxypeptidase, EC 3.4.15.1) participates in regulating blood pressure in the renin-angiotensin system; and its inhibitors, such as captopril [4, 5] and enalapril [6], have been used as antihypertensive drugs.

There are many physiological functions of food ingredients. 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 casein, [7, 8], zein [9, 10], soybean protein [11], dried-salted fish [12], ovalbumin [13], fish sauce [14], and fish water-soluble protein [15]. Additionally, Hata et al. [16] reported that sour milk decreased blood pressure in hypertensive subjects.

Wakame containing almost 15% protein has been a very popular food in the oriental countries [17]. Daily use of food that contains some peptides with potent ACE-inhibitory activity could be effective for maintaining blood pressure at a healthy level. Yamori et al. [18] reported that the alginate (water-soluble fiber of wakame) had a hypotensive effect in spontaneously hypertensive rats (SHRs). In addition, several studies suggested that dietary ingestion of wakame decreases blood pressure in humans [19, 20]. In our previous study, we isolated four ACE-inhibitory tetrapeptides from wakame enzymatic hydrolysates. In this study, we isolated 10 dipeptides from wakame by hot water extraction and treated SHRs with the four dipeptides containing the greatest ACE-inhibitory potential to investigate their hypotensive effects in vivo.

2. Materials and methods

2.1. Materials

Undaria pinnatifida (wakame) was cultured and harvested in Tokushima County, Japan, during January and

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June 1998. After washing with deionized water, wakame sample was dried and powdered using an ultracentrifugal mill. The composition of wakame powder was as follows: carbohydrate 41.5%, protein 36.5%, fat 3.3%, and ash 15.2%. Hippuryl-L-histydyl-L-leucine (HHL) was obtained from the Peptide Institute (Osaka, Japan), and angiotensin I–converting enzyme (ACE) from rabbit lung acetone powder was obtained from Sigma (St. Louis, MO).

2.2. Purification of dipeptides from wakame

Powdered wakame (60 g) was placed in 2 L of deionized water and heated to 93°C for 20 minutes. The hot water extract was filtered to remove the residue and centrifuged at 20,000 × *g* for 20 minutes at 4°C. The supernatant was dialyzed against 10 L of deionized water in a cellulose tubular membrane (90 cm, Wako Chemicals, Osaka, Japan) for 2 days. The outer solution was applied to a Dowex 50W (H^+ form, Dow Chemical, Midland, MI) column (45 × 450 mm) equilibrated with deionized water. The column was washed thoroughly with deionized water, and the retained peptides were then eluted with 500 mL of 2 N NH_4OH. The peptide fraction was concentrated to 40 mL under a vacuum. Five milliliters of concentrate was applied to a Sephadex G-25 column (2.6 × 140 cm, Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with phosphate buffer (0.1 mol/L, pH 7.0), and gel-filtered at a flow rate of 30 mL/h. Each fraction of 8.6 mL was collected. The peptide content of each fraction was measured by the method of Lowry et al. [21] using bovine serum albumin as the standard. The peptide fractions were concentrated to dryness to produce peptide powder.

The peptide powder from wakame was further isolated and purified by reverse-phase high-performance liquid chromatography (HPLC) with a Develosil ODS-5 column (4.6 × 250 mm, Nomura Chemical, Japan) using a linear gradient of acetonitrile from 0% to 25% in 0.05% trifluoroacetic acid for 180 minutes at a flow rate of 1.0 mL/min; the elute was monitored at 220 nm. The active fractions were concentrated to 0.5 mL using a centrifugal evaporator. The ACE-inhibitory peptides were rechromatographed on a Asahipack CG-320HQ column (7.6 × 300 mm, Showa Denko, Japan) using 25% acetonitrile in ammonium-acetate buffer (50 mmol/L, pH 6.8) at a flow rate of 0.5 mL/min.

2.3. ACE inhibitory activity

ACE inhibition was assayed by a modification of the method of Cheung and Cushman [22]. A quantity of 50 μL of a sample solution and 100 μL of a 2.5 mU ACE (rabbit lung lyophilized powder, Sigma) solution were added to 100 μL of a 12.5 mmol/L substrate (HHL) solution in 1.0 mol/L NaCl-borate buffer at pH 8.3. After incubation at 37°C for 20 minutes, 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 the ACE-inhibitory activity. The inhibition was calculated from the equation $[(Ec - Es)/(Ec - Eb)] \times 100$, 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). The inhibitory activity was defined as the amount needed to inhibit 50% of ACE activity (IC_{50}).

2.4. Peptide identification and synthesis

Peptides were hydrolyzed in 6 N hydrochloric acid containing 0.1% phenol at 110°C for 24 h, and the hydrolysate was analyzed using a PICO-TAG™ amino acid analyzer (Waters, Milford, MA). Sequence analysis was done by stepwise Edman's degradation using a 477A gas-phase automatic sequencer (Applied Biosystems, Foster City, CA) coupled to HPLC, for identification of the resulting PTH-amino acid compounds. The molecular mass of each peptide was confirmed from its fast atom bombardment mass spectrum (FAB-MS) obtained with a JEOL DX-300 spectrometer (Nippon Denshi, Japan). Peptides were synthesized by a solid-phase method using a 433A automated peptide synthesizer (Applied Biosystems) 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 Capcell Pak SG-120 column (10 × 250 mm, Shiseido, Japan) with a gradient of acetonitrile of from 5% to 25% in 0.05% trifluoroacetic acid for 30 minutes at a flow rate of 1.5 mL/min.

2.5. Animal study

2.5.1. Single administration

SHRs were purchased from Saitama Animal Facility Center (Saitama, Japan) and fed laboratory chow (CE-2, Clea Japan, Tokyo, Japan). After a 2-week adaptation period, SHRs with systolic blood pressure (SBP) > 160 mm Hg and body weights of 280–320 g were randomly divided into four groups. A group of six SHRs was given the synthetic dipeptide (Tyr-His, Lys-Tyr, Phe-Tyr, and Ile-Tyr; 50 mg/kg body weight) dissolved in 0.9% saline via gastric intubation at 8 AM. SBP, mean blood pressure (MBP), and diastolic blood pressure (DBP) were measured at the beginning and at 3-h intervals using a tail-cuff with a UR-5000 programmed electro-sphygmomamometer (Ueda Co., Tokyo, Japan). The dosage was according to the previous study [17, 23]. At least five readings were recorded, the maximal and minimal values were discarded, and blood pressure values were calculated from the remaining three values.

2.5.2. Continuous administration

The source and feeding-type of the experimental animals were the same as those in mentioned above in the "Single administration" section. SHRs were randomly divided into four groups ($n = 6$). In the first week of the experimental

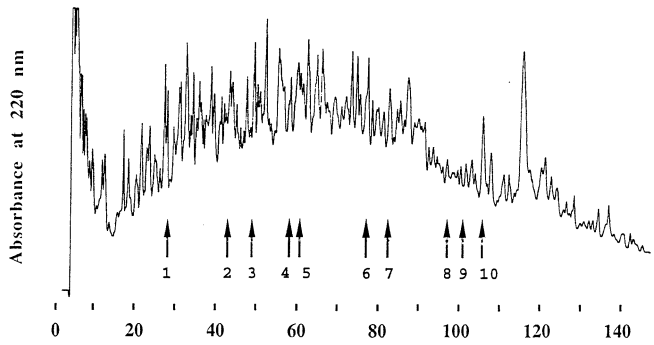

Fig. 1. A chromatogram on a reversed-phase ODS-5 column of the peptidic fraction from the hot water extract of wakame. The peaks marked 1–10 (representing the dipeptides Tyr-His, Lys-Trp, Lys-Tyr, Lys-Phe, Phe-Tyr, Val-Trp, Val-Phe, Ile-Tyr, Ile-Trp, and Val-Tyr) were found to have ACE-inhibitory activity.

period, SHRs were daily given the synthetic dipeptide (Tyr-His, Lys-Tyr, Phe-Tyr, and Ile-Tyr; 10 mg/day/kg body weight) dissolved in 0.9% saline via gastric intubation for 7 days. The blood pressure was measured weekly during the study period by the tail-cuff method as described above.

Statistical analysis

Results are expressed as the mean ± SEM. The significance of differences of blood pressure before and after administration was analyzed using the Student *t* test. A *P* value of < 0.05 was taken as the level of statistical significance.

3. Results

3.1. Purification and identification of ACE inhibitory peptides derived from wakame

In this study, ACE-inhibitory peptides were isolated from the hot water extract of wakame by dialysis and ion-exchange column chromatography (as described in "Methods and materials" section). The yield of the peptide powder from 60 g (dry weight) of wakame was 7.2 g. The peptidic fraction was dissolved in distilled water and applied to an ODS-5 column. Figure 1 shows a preparative HPLC chromatogram of the fraction. Although approximately 100 peaks were detected with chromatography, 10 peaks of potent inhibitory peptides were obtained from the peptidic fraction of wakame (Table 1). The IC_{50} values of Tyr-His, Lys-Tyr, Phe-Tyr, and Ile-Tyr were lower than those of the other dipeptides. These four active peptides had tyrosine residues in the structure.

3.2. Hypotensive effect of wakame peptides

We confirmed that four dipeptides (Tyr-His, Lys-Tyr, Phe-Tyr, and Ile-Tyr) derived from the hot water extract of wakame produced antihypertensive activity by oral administration into SHRs.

Table 1
Analytical data and ACE inhibitory activity of the isolated dipeptides by HPLC

Retention time (min)*	Dipeptide	Amino acid ratio†	IC_{50} (μmol/L)‡
27.5	Tyr-His	Tyr 1.03, His 0.81	5.1
42.6	Lys-Trp	Lys 0.87, Trp 0.99	10.8
48.6	Lys-Tyr	Lys 0.79, Tyr 1.08	7.7
57.5	Lys-Phe	Lys 0.85, Phe 1.12	28.3
59.9	Phe-Tyr	Phe 1.08, Tyr 1.16	3.7
76.6	Val-Trp	Val 1.04, Trp 0.92	10.8
81.8	Val-Phe	Val 1.02, Phe 1.04	43.7
96.1	Ile-Tyr	Ile 1.26, Tyr 0.97	2.7
100.6	Ile-Trp	Ile 1.33, Trp 0.94	12.4
104.8	Val-Tyr	Val 1.27, Tyr 1.14	11.3

All amino acids had an L-configuration.
* Expressed from the results of HPLC (Fig. 1).
† Each peptide was hydrolyzed with 6 N hydrochloric acid at 110°C for 24 h.
‡ The concentration of peptide needed to inhibit 50% of the ACE activity.

3.2.1. Single administration

Antihypertensive activity of each peptide from the hot water extract of wakame was evaluated by measuring changes in SBP, MBP, and DBP at 1-hour intervals after oral administration at a dose of 50 mg/kg of body weight. After administration of Tyr-His, Lys-Tyr, Phe-Tyr, and Ile-Tyr, SBP was significantly lowered by 50 mm Hg at 3 hours (Fig. 2a), 45 mm Hg at 6 hours (Fig. 2b), 46 mm Hg at 3 hours (Fig. 2c), and 33 mm Hg at 3 hours (Fig. 2d), respectively. The hypotensive effect lasted for another 3–24 hours (Fig. 2). After the administration of Tyr-His, Ile-Tyr, and Phe-Tyr, blood pressure showed the lowest value at 3 hours. Resumption of the hypertensive blood pressure occurred faster for Tyr-His than for the other two dipeptides. The tendency for MBP and DBP versus time was similar to that for SBP. On the other hand, after the oral administration of Lys-Tyr, the blood pressure showed the lowest value at 6 hours, and the duration of its blood pressure-lowering activity was continued for 4 hours.

3.2.2. Continuous administration

After 1 week of oral administration of Tyr-His, Lys-Tyr, Phe-Tyr, and Ile-Tyr (10 mg/day/kg body weight), SBP was significantly lowered by 34, 26, 34, and 25 mm Hg in week 1, respectively. The hypotensive effect lasted for another 3–8 weeks (Fig. 3). Additionally, after administration of Lys-Tyr, the lowest MBP and DBP were shown in week 2 by a reduction of 20–22 mm Hg, and this was consistent with results for SBP.

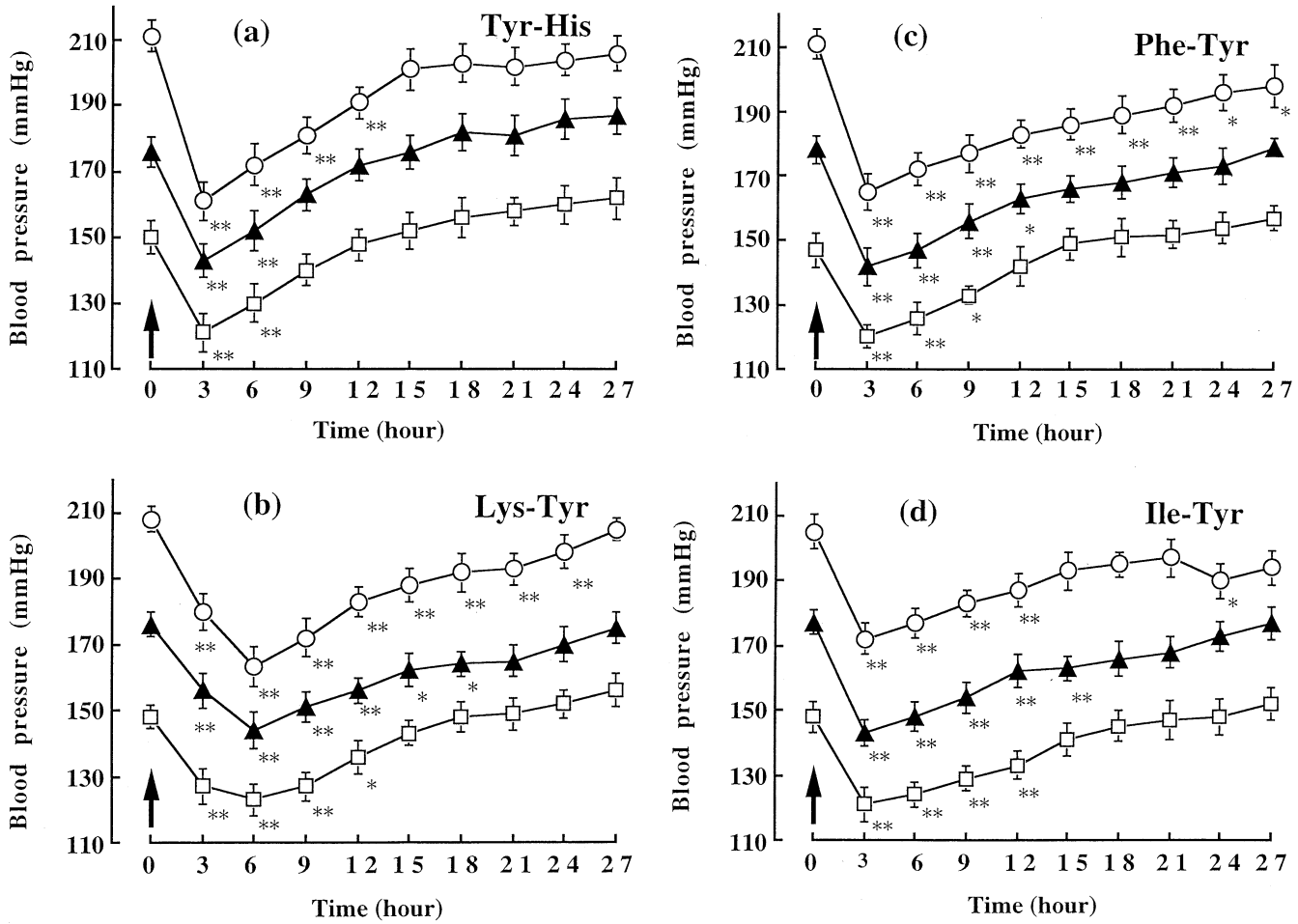

Fig. 2. Effect of single administration of the synthetic peptides, Ile-Tyr, Phe-Tyr, Tyr-His, and Lys-Tyr on the blood pressure of SHRs.. Vertical bars represent the mean ± SEM ($n = 6$). Significantly different from the blood pressure before administration: $^{*}P < 0.05$, $^{**}P < 0.01$). Right arrow indicates single dose administration (50 mg/kg body weight). Open circles connected by lines represent systolic blood pressure (SBP); filled triangles connected by lines indicate mean blood pressure (MBP); open squares connected by lines indicate diastolic blood pressure (DBP).

4. Discussion

A previous study showed that the recovery rate of ACE-inhibitory peptides from wakame hydrolyzed by a protease was low [17]. Thus, it is important to find a new preparation process to increase the recovery rate. Compared with enzymatic hydrolysis, we obtained a greater number of dipeptides from wakame by hot water extraction in the present study. Seaweed contains various kinds of polysaccharides, and therefore it may be easier to extract nitrogen compounds using hot water or organic solvents.

In this study, four tyrosine-containing peptides were found to have ACE-inhibitory activity. There are three active sites of ACE: the zinc ion, hydrogen bond, and positively charged residue binding site. It is thought that the aromatic side chain may be the binding site with the active site of ACE. Their special chemical structures may partially explain why these inhibitory agents work.

Recently, several studies observed that the peptic digests of the red alga *Porphyra yezoensis* [24] and the brown algae *Undaria pinnatifida* [17] and *Hijikia fusiformis* [25] produced lowered blood pressure in SHRs. These data suggested that certain peptides might possess potent antihypertensive effects comparable to these of therapeutic drugs. Suetsune and Nakano [17] isolated peptidic fractions from wakame by ion exchange chromatography using an SP Sephadex C-25 (H^+) column. The peptidic fraction was further chramatographed on a reversed-phase column to yield four tetrapeptides with ACE-inhibitory properties. These peptides were Ala-Ile-Tyr-Lys (IC_{50} 213 μmol/L), Tyr-Lys-Tyr-Tyr (64.2 μmol/L), Lys-Phe-Tyr-Gly (90.5 μmol/L), and Tyr-Asn-Lys-Leu (21 μmol/L). For the practical purpose of using food materials as physiological modulators, it is necessary to confirm the antihypertensive effect of orally administrated peptidic fractions of wakame on SHRs. In this study, we identified the ACE-inhibitory peptides from the wakame hot water extract that are orally antihypertensive. Many kinds of short-chain peptides isolated from the hydrolysis of dietary protein, such as tuna muscle and fermented milk, were found to have powerful ACE-inhibitory activity [26, 27]. An in vitro study also showed that peptides from pepsin-digested chlorella and

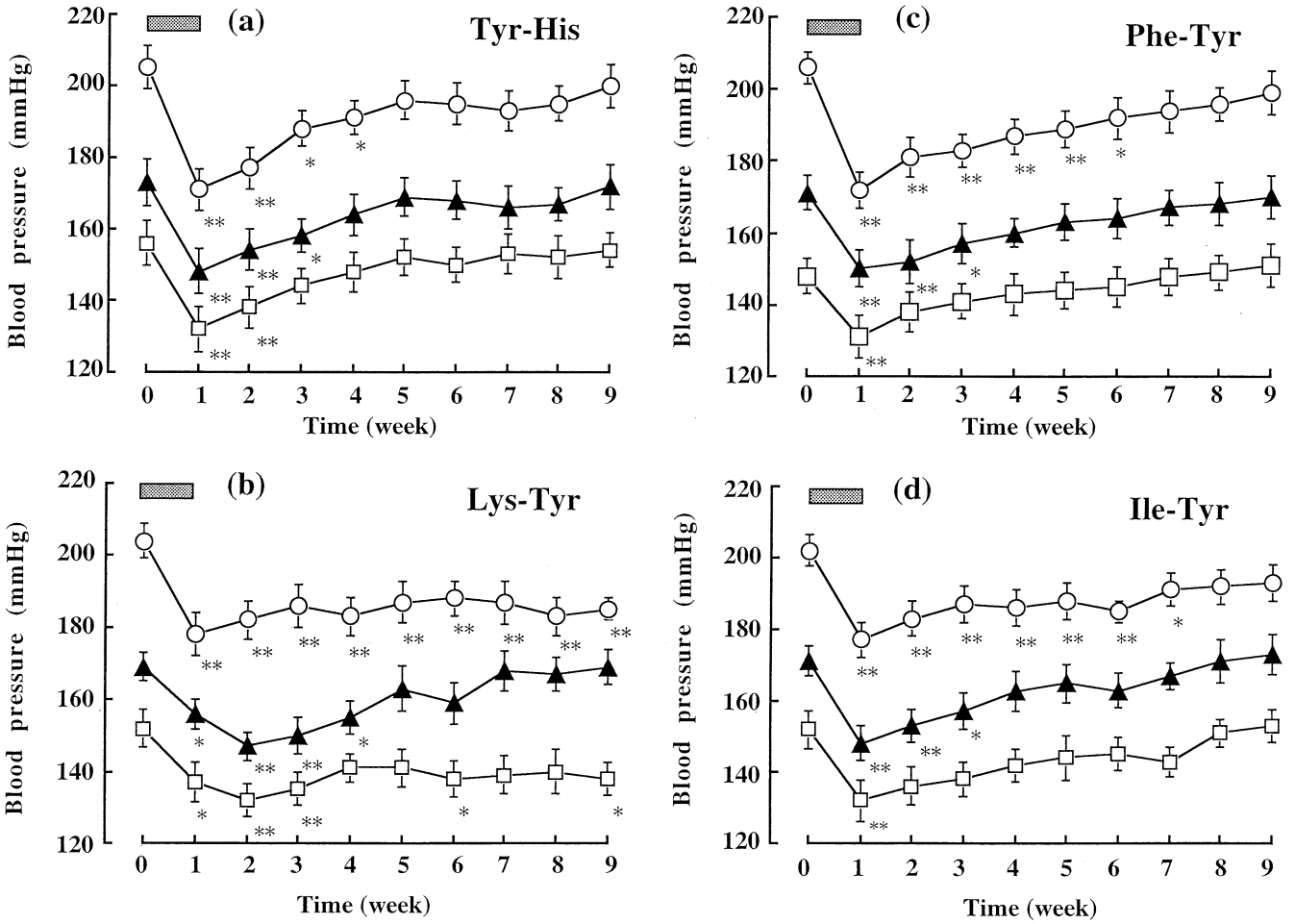

Fig. 3. Effect of continuous administration of the synthetic peptides, Ile-Tyr, Phe-Tyr, Tyr-His, and Lys-Tyr, on the blood pressure of SHRs. Vertical bars represent the mean ± SEM ($n = 6$). Significantly different from the blood pressure before administration: * $p < 0.05$, ** $p < 0.01$). Shaded bars indicate oral administration period (10 mg/day/kg body weight). Open circles connected by lines indicate systolic blood pressure (SBP); filled triangles connected by lines indicate mean blood pressure (MBP); open squares connected by lines indicate diastolic blood pressure (DBP).

spirulina can decrease the conversion of angiotensin I into angiotenisin II [28].

Although each ACE-inhibitory activity of dipeptides derived from wakame has different IC_{50} values, the blood pressure–lowering effect in SHRs occurred at almost the same level, but the period for which the effect lasted differed. It is suspected that variations in the blood pressure–lowering effect result from different absorption and degradation rates for these dipeptides in the GI tract and plasma, respectively. ACE-inhibitory peptides should have resistance to gastrointestinal enzymes and can be absorbed in their intact active form to lower blood pressure. In this study, SHRs, the primary hypertension animal model, were treated with four ACE-inhibitory dipeptides, and changes in blood pressure were determined. With single administration, the lowest blood pressure was found 3 hours after treatment with Tyr-His, Phe-Tyr, or Ile-Tyr, and then the blood pressure gradually recovered. Rats administered Lys-Tyr showed the lowest blood pressure after 6 hours, and then it also gradually recovered. A consistent result was found in the long-term study in that rats fed Tyr-His, Phe-Tyr, or Ile-Tyr showed the lowest blood pressure in the first week, and rats fed Lys-Tyr showed the lowest MBP and DBP in the second week, whereas SBP remained low until week 9. Lys-Tyr had lower ACE-inhibitory activity compared with the other three dipeptides (Ile-Tyr, Phe-Tyr, and Tyr-His) but showed a greater hypotensive effect in vivo. This suggests that Lys-Tyr may have greater resistance against protease than the other dipeptides, and thus it may be absorbed in an intact form and showed its physiological effect in vivo. On the correlation between the structure and activity of ACE-inhibitory peptides, Cheung et al. [29] 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 10 ACE-inhibitory peptides from the hot water extract of wakame, *Undaria pinnatifida*, that had the same properties. These four active peptides had tyrosine residues in their structures. In 1970, Ferreira et al. [30] 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 proteolytic enzymes that in-

activate bradykinin and catalyze the conversion of angiotensin I into angiotensin II. ACE inhibitors are important in cardiovascular therapeutics because they can reduce the generation of angiotensin II, a vasoconstrictor that can inhibit increases in blood pressure. For example, captopril, enalapril, and lisinopril are all widely used in clinical practice today.

The Ministry of Health, Labour, and Welfare of Japan has identified four kinds of functional foods with hypotensive effects, including casein hydrolysate, sour milk, dried bonito, and sardine hydrolysate. All of them contain special peptides and may therefore have ACE-inhibitory activity. We have demonstrated that derivatives of wakame have antihypertensive properties in the animal model. Further studies are needed to determine the effects of these ACE-inhibitory peptides on blood pressure in hypertensive patients.

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