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Antihypertensive Effect of Angiotensin I Converting Enzyme-Inhibitory Peptide from Hydrolysates of Bigeye Tuna Dark Muscle, *Thunnus obesus*

ZHONG-JI QIAN,† JAE-YOUNG JE,‡ AND SE-KWON KIM*,†

Department of Chemistry, Pukyong National University, Busan 608-737, Korea, and Division of Food Science and Aqualife Medicine, Chonnam National University, Yeosu 550-749, Korea

Angiotensin I converting enzyme (ACE) inhibitory peptide was isolated from tuna dark muscle hydrolysate prepared by alcalase, neutrase, pepsin, papain, α-chymotrypsin, and trypsin, respectively. Among hydrolysates, the pepsin-derived hydrolysate exhibited the highest ACE I inhibitory activity versus those of other enzyme hydrolysates. The structure of the peptide was identified to be Trp-Pro-Glu-Ala-Ala-Glu-Leu-Met-Met-Glu-Val-Asp-Pro (molecular weight 1581 Da) by time of flight mass spectrometry/mass spectrometry analysis, and the IC_{50} value of the peptide was 21.6 μM. The Lineweaver–Burk plots revealed that the peptide acts as a noncompetitive inhibitor, and the inhibitor constant (K_i) was calculated as 26.6 μM using the secondary plots. The peptide had an antihypertensive effect according to the time-course measurement after oral administration to spontaneously hypertensive rats. Maximal reduction was detected 3 h after oral administration at a dose of 10 mg/kg of body weight. These results suggest that the peptide derived from tuna dark muscle would be a beneficial ingredient for functional food or pharmaceuticals against hypertension and its related diseases.

KEYWORDS: ACE inhibitory peptide; antihypertensive effect; kinetics; tuna dark muscle

INTRODUCTION

The renin-angiotensin system plays a pivotal role in blood pressure, and in the pathophysiology of cardiovascular diseases such as congestive heart failure and hypertension (*1*). Renin produces angiotensin I from angiotensinogen, then further, angiotensin I is cleaved by angiotensin I converting enzyme (ACE) to release angiotensin II, a potent vasoconstrictor. ACE also inactivates bradykinin, which has a depressor action. Therefore, the inhibition of ACE activity is a major target for antihypertension.

Since the discovery of ACE inhibitors in snake venom, many studies have been attempted in the synthesis of ACE inhibitors such as captopril, enalapril, alacepril, and lisinopril, which are currently used in the treatment of essential hypertension and heart failure in humans (*2, 3*). However, these synthetic drugs are believed to have certain side effects such as cough, taste disturbances, skin rashes, or angioneurotic edema, all of which might be intrinsically linked to synthetic ACE inhibitors (*4*). Therefore, the search for natural ACE inhibitors as alternatives to synthetic ones is of great interest among researchers for safe and economical use. It has been reported that many ACE inhibitory peptides were obtained by enzymatic digestion from food proteins such as cheese whey (*5*), casein (*6*), sea bream (*7*), sardines (*8*), corn gluten (*9*), soy beans (*10*) and mung beans (*11*). According to the literature (*5–11*), naturally occurring peptides from food proteins may be less potent than synthetic drugs; however, these peptides have not shown adverse effects. Therefore, food-derived ACE inhibitory peptides are believed to be safer and cheaper than synthetic drugs.

The dark muscle of tuna can be easily obtained from byproducts during the processing of fresh products. Dark muscle contains a lot of useful proteins, which can be converted to value-added products by enzymatic hydrolysis. As part of our ongoing investigation of bioactive peptides from fishery byproducts derived from enzymatic hydrolysis, we have focused our attention on the production of antihypertensive peptides from tuna dark muscle protein. Therefore, the aim of this study was to isolate and characterize ACE inhibitory peptides derived from an enzymatic hydrolysate, and we also investigated the antihypertensive action of the purified peptide by oral administration in spontaneously hypertensive rats (SHR).

MATERIALS AND METHODS

Materials. Bigeye tuna dark muscle was donated by Dongwon Fisheries Co. (Busan, Korea). ACE (from rabbit lung), the substrate (hippuryl-histidyl-leucine, HHL) of ACE, and proteases (pepsin, α-chymotrypsin, trypsin, and papain) were products of Sigma Chemical Co. (St. Louis, MO). Other digestive proteases (alcalase and neutrase)

* Author to whom correspondence should be addressed. Tel.: +82-51-620-6375. Fax: +82-51-620-8147. E-mail: sknkim@pknu.ac.kr.
† Pukyong National University.
‡ Chonnam National University.

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Table 1. Conditions for the Hydrolysis of Tuna Dark Muscle

enzyme	buffer	pH	temp. (°C)
alcalase	0.1 M Na_2HPO_4–NaH_2PO_4	7.0	50
α-chymotrypsin	0.1 M Na_2HPO_4–NaH_2PO_4	8.0	37
papain	0.1 M Na_2HPO_4–NaH_2PO_4	6.0	37
pepsin	0.1 M Glycine–HCl	2.0	37
neutrase	0.1 M Na_2HPO_4–NaH_2PO_4	8.0	50
trypsin	0.1 M Na_2HPO_4–NaH_2PO_4	8.0	37

were purchased from Novozymes (Copenhagen, Denmark). Other chemicals and reagents used were of analytical grade and are commercially available.

Enzymactic Hydrolysis of Tuna Dark Muscle. To produce bioactive peptides from dark muscle, enzymatic hydrolysis was performed using various enzymes (alcalase, α-chymotrypsin, neutrase, papain, pepsin, and trypsin) under optimal conditions (**Table 1**). At an enzyme/substrate ratio of 1:100 (w/w), 1% substrate and enzymes were mixed. The mixture was incubated for 8 h at each optimal temperature with stirring and then heated in a boiling water bath for 10 min to inactivate the enzyme. The degree of hydrolysis (DH) was determined by measuring the nitrogen content soluble in 10% trichloroacetic acid, as discussed by Kim et al. (*12*). The hydrolysates were passed through an ultrafiltration (UF) membrane with a 3 kDa molecular weight cutoff (MWCO). The <3 kDa permeate obtained was lyophilized and stored below –80 °C until use.

Purification of ACE Inhibitory Peptide. The hydrolysate (10 mg/mL) showing the highest ACE inhibition was loaded (2 mL) onto a HiPrep 16/10 DEAE FF ion-exchange column equilibrated with a 20 mM sodium acetate buffer (pH 4.0) and eluted with a linear gradient of NaCl (0–2 M) in the same buffer at a flow rate of 2.0 mL/min. Each fraction monitored at 280 nm was collected and concentrated using a rotary evaporator, and an ACE inhibition assay was performed. The major active fractions were combined and lyophilized, dissolved in distilled water, and subjected (100 μL) to reverse-phase HPLC (RP-HPLC) on an ODS C_{18} column (Primesphere 10, 20 mm × 250 mm) with a linear gradient of acetonitrile (0–50% in 55 min) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 2.0 mL/min. The elution peaks were detected at 215 nm, and the active peak was concentrated using a rotary evaporator. For further purification, the active peak from RP-HPLC was loaded onto a Synchropak RPP-100 RP-HPLC analytical column (4.6 mm × 250 mm) using isocratic elution with 20% acetonitrile containing 0.1% TFA at flow rate of 1.0 mL/min.

Determination of Amino Acid Sequence. An accurate molecular mass and amino acid sequence of the purified peptide was determined using a Q-TOF mass spectrometer (Micromass, Altrincham, U.K.) coupled with an electrospray ionization (ESI) source. The purified peptide was separately infused into the electrospray source after being dissolved in methanol/water (1:1, v/v), and the molecular mass was determined by the doubly charged $(M + 2H)^{+2}$ state in the mass spectrum. Following molecular mass determination, the peptide was automatically selected for fragmentation, and sequence information was obtained by tandem MS analysis.

Assay of ACE Inhibitory Activity. The ACE inhibitory activity was measured by the method of Cushman and Cheung (*13*) with slight modifications. A sample solution (2 mg/mL, 50 μL) with 50 μL of ACE solution (25 units/mL) was preincubated at 37 °C for 10 min, and the mixture was incubated with 150 μL of substrate (8.3 mM HHL in a 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) for 30 min at the same temperature. The reaction was terminated by the addition of 250 μL of 1.0 M HCl. The resulting hippuric acid was extracted with 0.5 mL of ethylacetate. After centrifugation (800*g*, 15 min), 0.2 mL of the upper layer was transferred into a test tube and evaporated at room temperature for 2 h in a vacuum. The hippuric acid was dissolved in 1.0 mL of distilled water, and the absorbance was measured at 228 nm using an UV spectrophotometer (Cary 1C, Varian Inc., Australia). The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

Table 2. Degree of Hydrolysis of Tuna Dark Muscle and Their ACE Inhibition by Using the UF Membrane with 3 kDa MWCO (2 mg/mL)

treated enzymes	degree of hydrolysis (%)	ACE inhibition (%)
alcalase	67.22 ± 3.8	47.84 ± 3.4
α-chymotrypsin	77.71 ± 2.5	57.49 ± 2.6
neutrase	80.93 ± 2.8	63.81 ± 3.2
papain	74.45 ± 2.8	20.39 ± 2.8
pepsin	78.72 ± 3.2	80.69 ± 3.4
trypsin	46.28 ± 2.0	36.30 ± 2.6

The inhibitor constant (K_i) value and inhibition mode of the purified peptide were determined by Lineweaver–Burk plots from experiments conducted at four substrate concentrations.

Antihypertensive Effect of Purified Peptide on SHRs. SHRs (each group containing six rats, 10-weeks old, male, 180–240 g of body weight, BW) with tail systolic blood pressures (SBPs) over 180 mmHg were obtained from the Korea Research Institute of Bioscience and Biotechnology (DaeJeon, Korea). SHRs were housed individually in steel cages in a room kept at 24 °C with a 12 h light–dark cycle and fed a standard laboratory diet. Tap water was freely available. Peptide was dissolved in saline at a dose of 10 mg/kg of BW and injected orally (2 mL) using a metal gastric device zoned in SHR. The SBP-lowering efficacy of the inhibitor was compared with that of captopril. Captopril was injected using the same method employed for peptide introduction. Control rats were administered the same volume of saline solution. Following oral administration of the sample, SBP was measured by the tail cuff method using a programmed electrosphygmomanometer (model UR-5000, Ueda Co. Ltd., Tokyo, Japan) after warming up rats in a 40 °C chamber for 10 min.

Data Analysis. All results were expressed as means ± standard error of the mean ($n = 3$). The significance of the differences between SBPs before and after administration was analyzed using Student's *t*-test.

RESULTS

Production of ACE Inhibitory Peptides. To produce ACE inhibitory peptides, tuna dark muscle was hydrolyzed with various enzymes such as alcalase, α-chymotrypsin, pepsin, papain, neutrase, and trypsin. In this experiment, six commercial enzymes were selected to evaluate their effectiveness and ACE inhibition activity for the degradation of tuna dark muscle. After reaction, the extent of protein degradation by proteolytic enzymes was estimated by assessing the DH, and the results are shown in **Table 2**. All proteolytic enzymes except trypsin effectively hydrolyzed tuna dark muscle, and the DH values ranged from 67.22 to 80.93%. Due to low-MW peptides being more potent as bioactive peptides than high-MW peptides (*14*), hydrolysates were further separated using an UF membrane with 3 kDa MWCO, and permeate was collected, lyophilized, and then assayed for ACE inhibition. As shown in **Table 2**, most of the hydrolysates show ACE inhibition activity with a broad spectrum of inhibition ratios. Overall, peptic hydrolysate possessed the highest ACE inhibition activity. To identify ACE inhibitory peptide, we selected peptic hydrolysate for further studies.

Identification of ACE Inhibitory Peptide. Peptic hydrolysate, which shows the dominant ACE inhibitory activity among hydrolysates, was dissolved in a running buffer (20 mM sodium acetate, pH 4.0) and loaded on a HiPrep 16/10 DEAE FF ion-exchange column using fast protein liquid chromatography (FPLC) with a linear gradient of NaCl (0–2 M) at a flow rate of 2.0 mL/min. The elution peaks were monitored at 280 nm, and peptide fractions were divided into three peaks from the adsorption portion. Each peak was collected, lyophilized, and evaluated for ACE inhibitory activity. As shown in **Figure 1**, a second peak in the adsorption portion revealed dominant ACE inhibitory activity, and the inhibition activity was determined

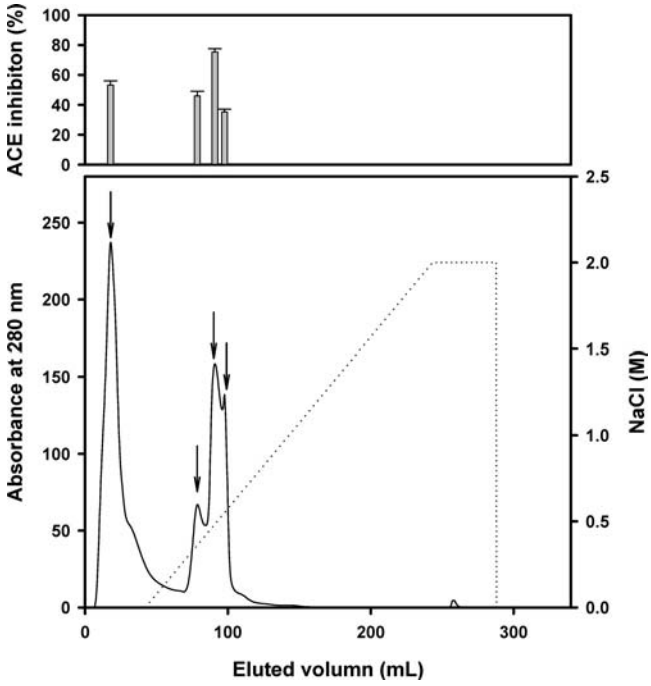

Figure 1. A FPLC chromatogram of peptic hydrolysate loaded (2 mL) on a HiPrep 16/10 DEAE FF ion-exchange column (lower panel) and the ACE inhibitory activity (0.5 mg/mL) (upper panel). Peptides were eluted with a NaCl linear gradient (0–2 M) at a flow rate of 2.0 mL/min. Value means ± SE of three determinations.

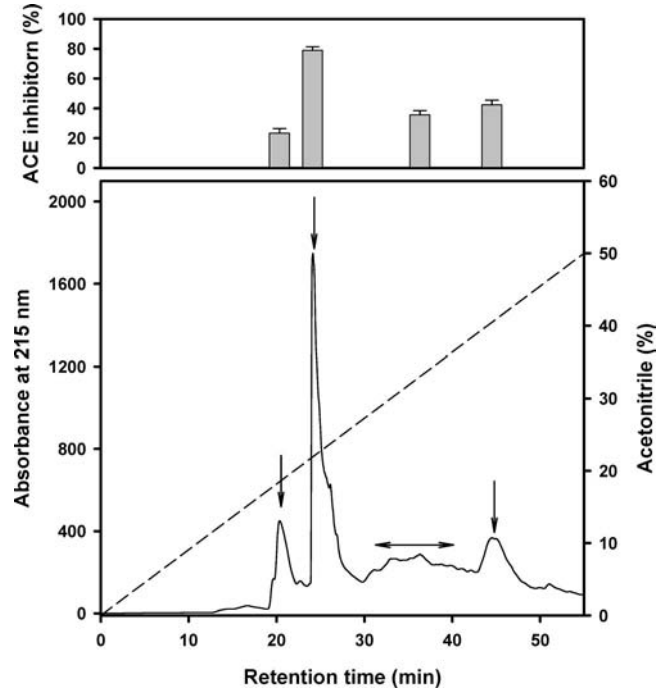

Figure 2. A reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram of the active fraction from FPLC loaded (100 uL) on an ODS C_{18} column (lower panel) and the ACE inhibitory activity (0.5 mg/mL; upper panel). The active fraction was eluted with an acetonitrile linear gradient (0–50% for 55 min) containing 0.1% TFA at a flow rate of 2.0 mL/min. Value means ± SE of three determinations.

to reach 78%. The lyophilized active peak was further purified using RP-HPLC on an ODS C_{18} column with a linear gradient of acetonitrile (0–50%, 55 min) containing 0.1% TFA. Peptide peaks were separated into four fractions; each peak was pooled, lyophilized, and assayed for ACE inhibition. As shown in **Figure 2**, the strongest ACE inhibition peptides were monitored at 25 min of retention time; the ACE inhibition fraction was collected and lyophilized. This fraction was further purified using an analytical column, and the purified peptide was analyzed for its molecular weight and primary structure using time of flight mass spectrometry/mass spectrometry (TOF-MS/MS). As shown in **Figure 3**, the completely purified peptide was obtained, and its amino acid sequence was identified to be WPEAAELMMEVDP (1581 Da). The IC_{50} value of the purified peptide was 21.6 μM.

Inhibitory Kinetics. Lineweaver–Burk plots of ACE without and with the purified peptide (at two concentrations, 15.8 and 31.6 μM) are shown in **Figure 4a**. The pattern of inhibition was noncompetitive, which means that the peptide can combine with an enzyme molecule to produce a dead-end complex, regardless of whether a substrate molecule is bound or not. Hence, the peptide must bind at a different site from the substrate. Therefore, the peptide acts as an ACE inhibitor by forming enzyme–substrate–inhibitor and enzyme–inhibitor complexes during the reaction to reduce the efficiency of catalysis. K_i was calculated as the *x*-axis intercept from a plot of the slopes of the Lineweaver–Burk lines against inhibitor concentrations, and the calculated K_i was 26.6 μM (**Figure 4b**).

Antihypertensive Effect on SHR. The antihypertensive effect of the purified peptide was investigated by measuring the change of SBP at 1, 2, 3, 6, and 9 h after oral administration of 10 mg/kg of the peptide solution. Captopril was used as a positive control, and the control group was injected with the same volume of saline without the peptide. The SBP in the quiescent state of SHR was 182 ± 7.5 mmHg. After oral administration of the peptide and captopril, SBP was clearly decreased, and the maximal decreases in SBP were observed at 3 and 6 h, respectively (**Figure 5**).

DISCUSSION

Several peptides derived from food proteins such as cheese whey (*5*), casein (*6*), sardines (*8*), sour milk (*15*), wheat germ (*16*), zein (*17*), tuna muscle, (*18*) and bonito (*19*) are well known to have ACE inhibitory activities and to exhibit antihypertensive activities in experimental animals. The identified ACE inhibitory peptides are Val-Tyr (IC_{50} of 5.2 μM) from sardine muscle hydrolysate; Val-Pro-Pro (IC_{50} of 9 μM) and Ile-Pro-Pro (IC_{50} of 5 μM) from sour milk; Ile-Val-Tyr (IC_{50} of 0.48 μM) from wheat germ hydrolysate; Leu-Gln-Pro (IC_{50} of 9.6 μM) from zein; and Ile-Val-Gly-Arg-Pro-Arg-His-Glu-Glu (IC_{50} of 6.2 μM), Ala-Leu-Pro-His-Ala (IC_{50} of 10 μM), Phe-Gln-Pro (IC_{50} of 12 μM), Leu-Lys-Pro-Asn-Met (IC_{50} of 17 μM), Asp-Tyr-Gly-Leu-Tyr-Pro- (IC_{50} of 62 μM), and Ile-Lys-Pro-Leu-Asn-Tyr (IC_{50} of 43 μM) isolated from the thermolysin digest of dried bonito. The ACE inhibitory peptide derived from tuna dark muscle in the present study is Trp-Pro-Glu-Ala-Ala-Glu-Leu-Met-Met-Glu-Val-Asp-Pro (IC_{50} of 21.6 μM), and the IC_{50} value reflects moderate activity compared to that of the ACE inhibitory peptides derived from various sources. A kinetic study revealed that ACE inhibitor acts as a noncompetitive inhibitor, which means that it can bind not at active site but at the other sites exclusive of the active site of ACE.

Generally, ACE inhibitors contain one or more of the following molecular functionalities: a zinc binding ligand (usually either a phosphate or carboxylate oxygen, or thiol sulfur), a hydrogen-bond donor, and carboxyl-terminal group (*20, 21*). In the case of peptides, Ondetti and Cushman proposed the binding model for interactions between the substrate and the active site of ACE (*22*). The tripeptides with tryptophan,

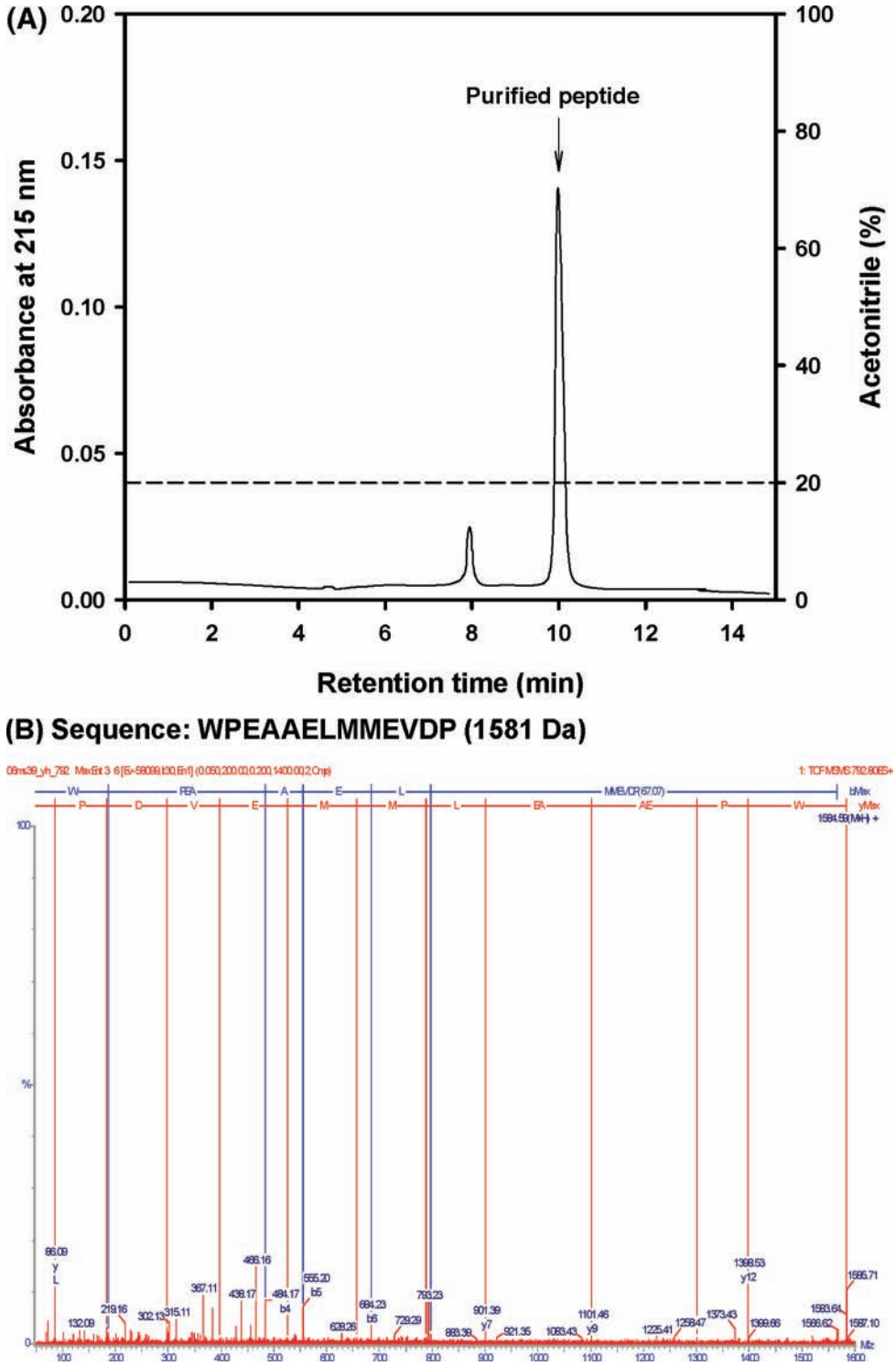

Figure 3. (A) A rechromatogram of the active fraction from RP-HPLC loaded on an analytical column using isocratic elution with 20% acetonitrile containing 0.1% TFA at a flow rate of 1.0 mL/min. (B) Identification of the molecular mass and amino acid sequence of the purified peptide using TOF-MS/MS with an ESI source. Sequencing of the active peptide was acquired over the *m*/*z* range 50–2500 and sequenced by using the PepSeq de nove sequencing algorithm.

tyrosine, phenylalanine, proline, and a hydrophobic amino acid at the C-terminal end were effective for ACE inhibitory activity because of the interaction between three subsites at the active site of ACE (*13*). Furthermore, the positive charge as in the guanidine group of the C-terminal arginine contributes to the ACE inhibitory potency of several peptides, indicating that the binding site may be different from the catalytic site in ACE. The amino acids at the C-terminal end of the ACE inhibitory peptide in this study were valine (hydrophobic) and proline, which may contribute to ACE inhibitory activity.

A single oral administration of the peptide showed a strong suppressive effect on SBP. It seems likely that the peptide mainly contributed to the antihypertensive effect induced by an ACE inhibitory activity. Fujita and Yoshikawa (*23*) classified the ACE inhibitory peptides into three groups based on the fates observed after preincubation with ACE. IC_{50} values of the

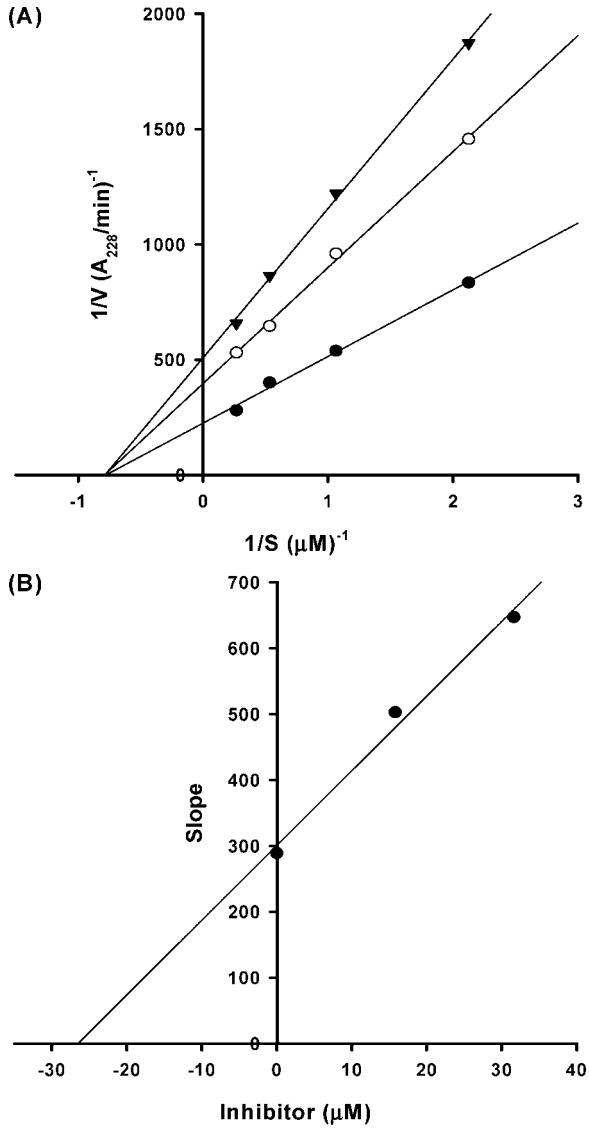

Figure 4. (A) Lineweaver–Burk plots for the inhibition of ACE by the purified peptide. The reactions were performed in the absence of inhibitor (•) or in the presence of 15.8 μM (○) or 31.6 μM (▼) of the purified peptide. (B) Secondary plot of Lineweaver–Burk plots. Each point in the graph represents the means of three determinations.

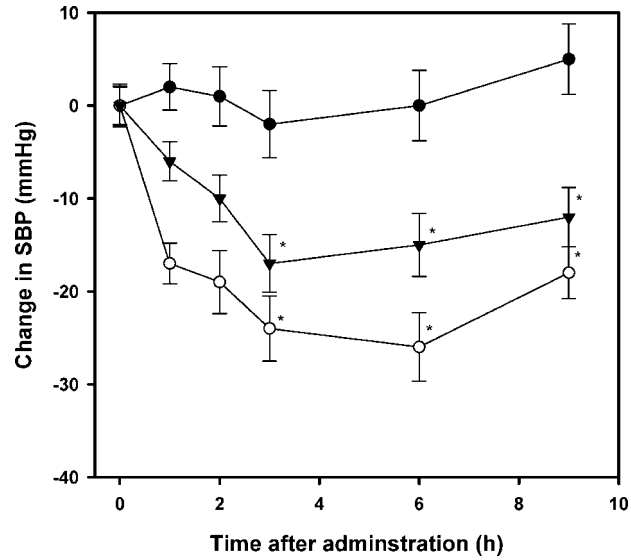

Figure 5. Change in SBP after a single oral administration of the purified peptide in SHRs (each group; $n = 6$). (•) control; (▼) peptide; (○) captopril. Captopril was used as a positive control. Single oral administration was performed with a dose of 10 mg/kg of BW, and SBP was measured 0, 1, 2, 3, 6, and 9 h after the administration. The significance of the difference from the control at $^*P < 0.01$.

inhibitor-type (di- or tripeptides) peptides are not affected despite the preincubation with ACE. Prodrug-type peptides were converted to their true inhibitors with smaller IC_{50} values by ACE, and substrate-type peptides exhibit an elevation of IC_{50} values after preincubation with ACE. Due to the degradation of the substrate type by ACE, this type did not show an antihypertensive effect on SHR. According to these types, our peptide would be a prodrug-type inhibitor. It is well known that small peptides, such as di- or tripeptides (inhibitor type), are easily absorbed in their intact forms in the intestines (*24*), suggesting that our peptide would be digested by gastric juice or protease in the small intestine, and then digested small peptides would be absorbed in the intestines. In addition, a small dietary peptide such as carnosine (β-alanyl-L-histidine) reduced blood pressure readings by a vasodilator effect induced by a relaxant response in an isolated rat aorta (*25*). Takai et al. (*26*) have reported a significant correlation between SBP and ACE activity in the aorta, and Murakami et al. (*27*) have also demonstrated that captopril lowered both SBP and ACE activity in the aorta in stroke-prone SHR. These results suggest that inhibition of the aorta ACE activity may be important for inducing antihypertensive effects in SHR. Further studies, therefore, we will be conducted to identify the mechanism of the peptide for the potent antihypertensive effect.

In conclusion, here, we have purified and characterized the antihypertensive peptide from tuna dark muscle hydrolysate and have demonstrated an antihypertensive effect in SHR. It seems likely that the antihypertensive peptide from tuna dark muscle hydrolysate would be a beneficial ingredient in functional foods or pharmaceuticals against hypertension and its related diseases.

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