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Purification of a Novel Angiotensin I-Converting Enzyme (ACE) Inhibitory Peptide with an Antihypertensive Effect from Loach (*Misgurnus anguillicaudatus*)

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ABSTRACT: To isolate and characterize novel angiotensin I-converting enzyme (ACE) inhibitory peptide from loach (*Misgurnus anguillicaudatus*), six proteases, pepsin, α-chymotrypsin, bromelain, papain, alcalase, and Neutrase, were used to hydrolyze loach protein. The hydrolysate (LPH) generated by bromelain [ratio of enzyme to substrate, 3:1000 (w/w)] was found to have the highest ACE inhibitory activity (IC_{50}, 613.2 ± 8.3 μg/mL). Therefore, it was treated by ultrafiltration to afford fraction of LPH-IV (MW < 2.5 kDa) with an IC_{50} of 231.2 ± 3.8 μg/mL, having higher activity than the other fractions. Then, LPH-IV was isolated and purified by consecutive purification steps of gel filtration chromatography and reverse-phase high-performance liquid chromatography to afford a purified peptide with an IC_{50} of 18.2 ± 0.9 μg/mL, an increase of 33.7-fold in ACE inhibitory activity as compared with that of LPH. The purified peptide was identified as Ala-His-Leu-Leu (452 Da) by Q-TOF mass spectrometry and amino acid analyzer. An antihypertensive effect in spontaneously hypertensive rats revealed that oral administration of LPH-IV could decrease systolic blood pressure significantly.

KEYWORDS: *loach (Misgurnus anguillicaudatus), ACE inhibitory peptide, mass spectrometry, antihypertensive effect*

INTRODUCTION

Hypertension, defined as systolic blood pressure (SBP) above 140 mm Hg and/or diastolic blood pressure above 90 mm Hg, is one of the major risk factors for the development of cardiovascular diseases, stroke, and end-stage renal disease.[1] It is estimated that the prevalence of hypertension in the world is about 15−20% of adults. In view of its high prevalence and importance, changes in lifestyle, dietary approaches, and pharmacological treatments are broadly applied to treat hypertension. The synthetic drugs currently used for hypertension treatment, such as captopril, alacepril, lisinopril, and enalapril, have been reported to have undesirable side effects such as cough, skin discomfort, taste disturbances, and excessively low blood pressure.[2] Furthermore, it has been recognized that nutritional factors play a significant role in the prevention and/or treatment of hypertension. Therefore, search and development to find nontoxic and economical antihypertensive substances from food resources has received great attention.

Food intake is being increasingly considered not only as a source of nutrients but also as a source of bioactive compounds, including bioactive peptides. Bioactive peptides usually contain 3−20 amino acid residues per molecule, and they are inactive within the sequence of the parent protein molecule. They can be released by the action of gastrointestinal enzymes in vivo or by using specific enzymes in vitro, and they can also be produced during the manufacture of certain foods. These peptides usually have multifunctional properties and are easily absorbed.[3−5] Of various kinds of bioactive peptides, angiotensin I-converting enzyme (ACE) inhibitory peptides have been extensively studied over the past three decades. ACE, a dipeptidyl carboxypeptidase (EC 3.4.15.1) that belongs to the class of zinc proteases,[6] plays a crucial role in the regulation of blood pressure through its action on two body systems. In the kinin−kallikrein system, ACE inactivates the antihypertensive vasodilator bradykinin, while in the renin−angiotensin system, ACE acts on the inactive decapeptide (angiotensin I) to hydrolyze His-Leu from its C-terminal and produces the potent vasopressor octapeptide (angiotensin II).[7] Therefore, inhibition of ACE is considered to be a useful therapeutic approach in the treatment of hypertension. Accordingly, many ACE inhibitory peptides have been found to date in various food protein sources such as milk, soybean, corn, and rapeseed.[8−11] Various ACE inhibitory peptides have also been isolated from fish proteins such as sardine,[12] tuna,[4] shark,[13] and yellowfin sole.[14]

Loach (*Misgurnus anguillicaudatus*) is a prevalent freshwater fish in East Asia. It serves as a kind of delicious and nutritious food and also has long been employed as a traditional Chinese medicine for the treatment of hepatitis, osteomyelitis, carbuncles, inflammations, and cancers, as well as for recovery from debility.[15] Recently, polysaccharides and antioxidative and antimicrobial peptides purified from loach or its mucus have been investigated for their potential bioactive activities.[16−18] However, its antihypertensive activity of bioactive peptides has not yet been investigated. Therefore, the main purpose of the present study was to isolate and characterize ACE inhibitory peptide derived from loach protein hydrolysate (LPH). Furthermore, the antihypertensive effect in vivo of LPH, to support its potential use as blood pressure-lowering activity in

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an animal study, was determined by using spontaneously hypertensive rats (SHR) whose blood kinetics change and course of diseases are similar to human.

MATERIALS AND METHODS

Materials and Chemicals. Live loach (*M. anguilliacaudatus*) of 8.0 ± 1.5 g body weight (BW) and 9.0 ± 2.5 cm body length were obtained from a local market in Nanjing of China, and they were raised in clean water for 1 week in our laboratory before use. After killing, the meat (without head, tail, skin, bone, and blood) was collected, ground twice by a meat grinder with a 4 mm holes plate (MM 12, Shaoguan Dajin Food Machine Co., Shaoguan, China), kept in a polyethylene bag, and stored at −18.0 °C until use. SHRs were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). *N*-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG) and ACE from porcine kidney were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Bromelain with the nominal activity of 3×10^5 U/g was obtained from Shanghai Kayon Biological Technology Co., Ltd. (Shanghai, China). Pepsin (2.5×10^5 U/g), α-chymotrypsin (5.8×10^5 U/g), and papain (1.5×10^5 U/g) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Alcalase 2.4 L (7.5×10^5 U/g) and Neutrase (2.2×10^5 U/g) were obtained from Novozymes (Beijing, China). All other chemicals and reagents were analytical grade.

Preparation of LPH. One hundred grams of loach meat was mixed with 200 mL of distilled water and homogenized at a speed of 10000 rpm for 1 min by using a T25 basic homogenizer (IKA, Staufen, Germany). Then, enzymatic hydrolysis of the homogenate was carried out by using various commercial enzymes (pepsin, α-chymotrypsin, bromelain, papain, alcalase, and Neutrase) under each optimal condition with an amount of enzyme of 900 U/g loach meat. For bromelain, the ratio of enzyme to substrate was 3:1000 (w/w), and the hydrolysis was conducted in a water bath shaker (SHZ-22, Taicang, China) at a pH of 5.5. After 6 h of incubation, the reaction was terminated by heating in a boiling water bath for 10 min. The digest was then subjected to centrifugation at 10000*g* for 20 min, and the resulting supernatant was lyophilized (FDU-1200, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) to afford LPH. The LPH was stored in a desiccator for further use.

Assay of ACE Inhibitory Activity. The ACE inhibitory activity was measured according to the reported method with slight modifications.[19] Briefly, 10 μL of ACE solution (0.25 U/mL) and 10 μL of LPH solution or demineralized water (blank) were placed in the wells of a 96-well microtiter plate. The reaction was started by the addition of 150 μL of preheated (37.0 °C) substrate of FAPGG (1 mM in 50 mM Tris-HCl containing 0.3 M NaCl, pH 7.5). The absorbance at 340 nm during the incubation at 37.0 °C was recorded every 30 s for 25 min by a Tristar LB-941 Microplate Reader (Berthold Technologies, Bad Wilbad, Germany). The slope of the linear part of the absorbance curve (ΔA_{340} per min), preferentially taken from 10 to 25 min of incubation, was used as a measurement of the ACE inhibitory activity. The ACE inhibition activity was calculated according to the following equation:

$$\text{ACE inhibition activity (\%)} = [1 - (\Delta A_{\text{inhibitor}}/\Delta A_{\text{control}})] \times 100$$

where $\Delta A_{\text{inhibitor}}$ and $\Delta A_{\text{control}}$ are the slopes of the sample with inhibitor and of the control, respectively. All runs were performed in four replicates. The ACE inhibitory activity was quantified by a regression analysis of ACE inhibitory activity (%) versus peptide concentration and defined as an IC_{50} value, that is, the peptide concentration required to produce 50% ACE inhibition under the described conditions.

Purification of ACE Inhibitory Peptide from LPH. *Ultrafiltration.* The lyophilized LPH was dissolved in distilled water and fractionated by ultrafiltration using a bioreactor system (Separa-Tech Membrane Technology Development Co., Ltd., Wuxi, China) with a range of molecular weight cutoff (MWCO) membranes of 10, 5, and 2.5 kDa, respectively. LPH was initially subjected to ultrafiltration using a 10 kDa MWCO membrane to generate a retentate (LPH-I) and permeate. The 10 kDa permeate was further fractionated over a 5 kDa MWCO membrane to produce a 5 kDa retentate (LPH-II). The 5 kDa permeate was then separated into a 2.5 kDa retentate (LPH-III) and permeate (LPH-IV) over a 2.5 kDa MWCO membrane. The resulting LPH-I, LPH-II, LPH-III, and LPH-IV were collected and lyophilized for the assay of ACE inhibitory activity and further purification.

Gel Filtration Chromatography. The fraction with the highest ACE inhibitory activity among LPH-I, LPH-II, LPH-III, and LPH-IV was further purified by gel filtration chromatography. The bioactive fraction dissolved in distilled water at a concentration of 30 mg/mL was loaded onto a Sephadex G-15 column (1.6 cm × 100 cm). The column was eluted with distilled water at a flow of 1.0 mL/min, and the eluate was collected (4 mL/tube) and monitored by measuring the absorbance at 280 nm using an online spectrophotometer. Each fraction was collected, concentrated by a rotary evaporator, and freeze-dried. The fraction with the highest ACE inhibitory activity was subjected to the next purification step.

High-Performance Liquid Chromatography (HPLC). The fraction exhibiting the highest ACE inhibitory activity from the gel filtration chromatography purification was further purified by using preparative reverse-phase HPLC on a Agilent Zorbax SB-C18 column (9.4 mm × 250 mm, Agilent Technologies Co., Ltd., Santa Clara, CA). The elution was performed with a linear gradient of acetonitrile (0−10% for 10 min) containing 0.1% trifluoroacetic acid at a flow rate of 0.8 mL/min, and its absorbance was monitored at 280 nm. The fractions were concentrated and freeze-dried, and their ACE inhibitory activities were determined. The most active fraction was subjected to a second reverse-phase HPLC purification using the same conditions as described above. The resulting purified peptide was used for the analysis of amino acid sequence.

Analysis of Amino Acid Sequence of Purified Peptide. The purified peptide was hydrolyzed by 6 N HCl for 24 h at 110 °C, and its amino acid composition was determined by L-8900 Amino Acid Analyzer (Hitachi High-Technologies Co., Tokyo, Japan). The molecular mass and amino acid sequence of purified peptide from LPH were analyzed by means of Q-TOF tandem mass spectrometry (Micromass Co., Manchester, U.K.) coupled with an electrospray ionization (ESI) source. Briefly, the purified peptide dissolved in methanol/water (1:1, v/v) was infused into an ESI source, and the molecular mass was determined. Following the determination of molecular mass, peptide was automatically selected for fragmentation, and sequence information was obtained by tandem MS analysis.

SHRs and Measurement of SBP. The antihypertensive activity in vivo of the fraction with the highest ACE inhibitory activity by ultrfiltration (LPH-IV) was evaluated. In the present study, male SHRs, 9 weeks old and grade of specific pathogen-free with BW of 220−280 g, were used. They were housed individually in steel cages in a room kept at 24 °C with a 12 h light/dark cycle and free access to both food (standard laboratory diet) and water. Prior to oral administration of LPH-IV, the blood pressures of the rats were measured three times during a 1 week period, and the rats were selected for further test based on their average blood pressures.

The rats were randomly divided into 4 groups ($n = 6$) as control group, captopril (positive control) group and treatment groups (low dosage and high dosage of LPH-IV). The rats in control group were administrated with 0.5% saline solution. The rats in high dosage group (30 mg/day kg BW) and low dosage group (10 mg/day kg BW) were given the LPH-IV solutions dissolved in the same volume of saline solution via gastric intubation, while the rats in positive control group was given 10 mg/day kg BW of captopril in a similar manner. The rats were put in a thermostatic box at 37 °C for 5 min to measure SBP. The SBP of each rat was measured for three times at 2, 4, 6, 8, 14, and 24 h after oral administration by the tail-cuff method with a BESN-II Multichannel Noninvasive Pressure Measurement System (Beijing Success Technology Development Co., Ltd., Beijing, China). Results were expressed as means ± standard errors (SDs).

Determination of ACE Inhibition Pattern. Different concentrations of ACE inhibitory peptide were added to each reaction mixture, and the enzyme activities were measured as described above. The ACE inhibitory pattern in the presence of inhibitor was investigated by the Lineweaver−Burk plots.

Statistical Analysis. Data were expressed as means ± SDs and subjected to one-way analysis of variance (ANOVA). Duncan's new multiple range test was performed to determine the significant difference using SPSS 13.0 software (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Preparation of LPH. Figure 1 shows the flow chart for the preparation of ACE inhibitory peptide from loach. First, a total

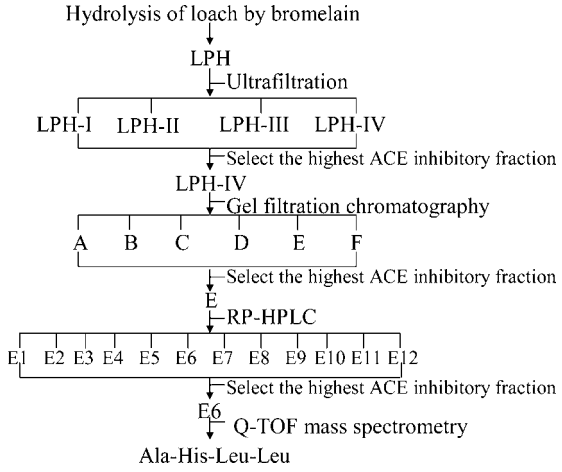

Figure 1. Process flow diagram for the separation of ACE inhibitory peptide from loach.

of six proteolytic enzymes, pepsin, α-chymotrypsin, bromelain, papain, alcalase, and Neutrase, were examined for the production of ACE inhibitory peptides in the present study. It was found that the ACE inhibitory activity of LPH varied greatly due to the different enzyme source (Figure 2), reflecting

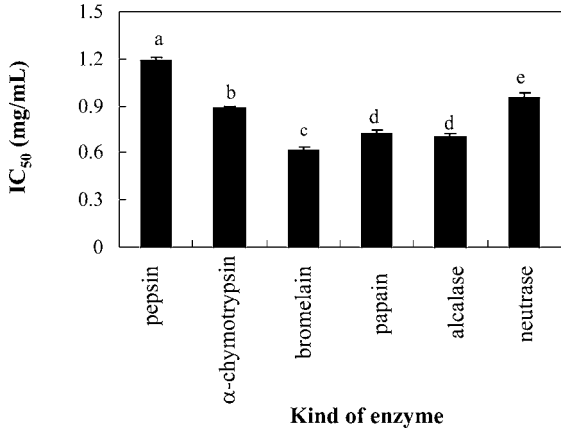

Figure 2. ACE inhibitory activity of enzymatic hydrolysate from loach proteins by pepsin, α-chymotrypsin, bromelain, papain, alcalase, or Neutrase. Data are expressed as means ± SDs (n = 3), and a−e represent significant difference (p < 0.05) between groups.

the variations in protein cleavage sites by proteases. LPH generated by bromelain exhibited the highest ACE inhibitory activity with an IC_{50} value of 613.2 ± 8.3 μg/mL, and there was significant difference (p < 0.05) in IC_{50} value between the treatments with bromelain and other proteases of pepsin, alcalase, Neutrase, papain, and α-chymotrypsin. Therefore, LPH generated by bromelain was used for further study.

The homogenate of loach meat did not show any ACE inhibitory activity (data not shown), while all LPHs generated by proteolytic enzymes displayed ACE inhibitory activities. The results indicated that enzymatic treatment is necessary to breakdown loach proteins to release bioactive peptides.

Purification of ACE Inhibitory Peptide. A common feature of ACE inhibitory peptides being their relative restricted size and relative hydrophobic C-terminal makes fractionation based on size a promising step. Accordingly, LPH generated by bromelain was fractionated by using different MWCO membranes. As a result, four fractions of LPH-I, LPH-II, LPH-III, and LPH-IV with MW of >10, 5−10, 2.5−5, and <2.5 kDa, respectively, were obtained. As shown in Table 1, their

Table 1. Effects of Ultrafiltration Membrane's Molecular Weight Cut-off on ACE Inhibitory Activities and Recoveries of Fractions from LPH Generated by Bromelain

sample	molecular weight (kDa)	recovery (%)	ACE inhibitory activity (IC_{50}, μg/mL)
LPH-I	>10	3.3	653.3 ± 6.3
LPH-II	5−10	2.7	551.2 ± 2.2
LPH-III	2.5−5	16.0	454.0 ± 4.2
LPH-IV	<2.5	78.0	231.2 ± 3.8

ACE inhibitory activities were found to be dependent on their molecular weights. Among the four fractions, LPH-IV (molecular weight <2.5 kDa) showed the highest ACE inhibitory activity with an IC_{50} value of 231.2 ± 3.8 μg/mL, while LPH-I (molecular weight >10 kDa) had the lowest activity (IC_{50}, 653.3 ± 6.3 μg/mL). The results are consistent with other reports of increased ACE inhibitory activity as the molecular weight of hydrolysate fraction decreases.[20−22] It has been reported that short peptides with 3−10 amino acids exhibit higher ACE inhibitory activity and other bioactive properties than their parent native proteins or large polypeptides.[23] Furthermore, LPH-IV accounted for 78% of the LPH generated, indicating that LPH generated by bromelain was mainly made up of low molecular weight peptides. The results demonstrated that ultrafiltration is a useful method for enrichment of ACE inhibitory peptide fractions from enzymatic hydrolysate of protein.[11,21,24] Therefore, LPH-IV was selected as the material in the following study.

Gel filtration is a method that makes possible the separation of substances with different molecular dimensions, and it has been used for desalting protein solutions, protein separation, and other colloids from low molecular weight substances.[25] Therefore, gel filtration chromatography (Sephadex G-15 column, 1.6 cm × 100 cm) was used to fractionate LPH-IV in the present study. As a result, fractions A−E were obtained. In addition, fraction E was found to exhibit the strongest ACE inhibitory activity with an IC_{50} value of 89.6 ± 3.4 μg/mL (Table 2), a 6.8-fold increase of activity as compared with that of LPH.

Fraction E from the gel filtration chromatography was further separated by reverse-phase HPLC using. As shown in Figure 3A, mixture components in fraction E were separated by a column of Zorbax SB-C18 into 12 major fractions. Furthermore, fraction E6 exhibited the highest ACE inhibitory

Table 2. Summary for the Purification of ACE Inhibitory Peptide from Loach Protein Hydrolysate Generated by Bromelain

sample	purification step	purification fold	IC_{50} (μg/mL)
LPH	LPH	1.0	613.2 ± 8.3
LPH-IV	ultrafiltration	2.6	231.2 ± 3.8
fraction E	Sephadex G-15	6.8	89.6 ± 3.4
fraction E6	reverse phase HPLC	33.7	18.2 ± 0.9

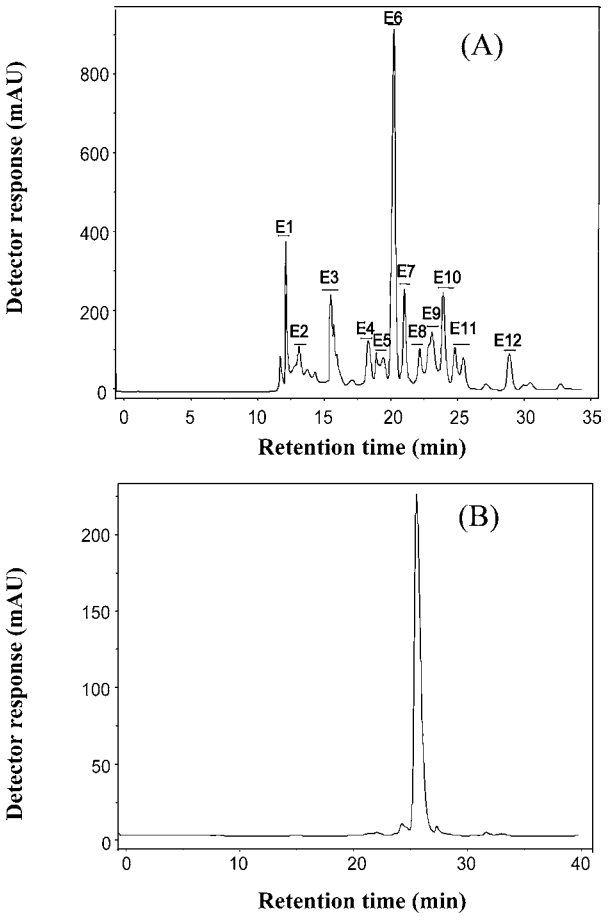

Figure 3. Reverse-phase HPLC chromatogram of fraction E obtained from Sephadex G-15 gel filtration chromatography (A) and reverse-phase HPLC chromatogram of fraction E6 (B).

activity with an IC_{50} value of 18.2 ± 0.9 μg/mL (a 33.7-fold increase of activity as compared with that of LPH). To characterize fraction E6, it was further subjected to a second reverse-phase HPLC fractionation, resulting in a single peak for peptide characterization (Figure 3B). The IC_{50} values and purification folds of these fractions obtained during the purification steps are summarized as shown in Table 2. The ACE inhibitory peptide was purified 33.7-fold from LPH by using four-step purification procedure.

Identification of ACE Inhibitory Peptide by Q-TOF Mass Spectrum. The specific amino acid composition is a critical factor for ACE inhibitory activity. Therefore, the amino acid composition of the ACE inhibitory peptide was determined by an amino acid analyzer to produce an amino acid profile. As a result, it contained Leu, His, and Ala. They are all observed in many other ACE inhibitory peptides from food protein hydrolysates, such as YGL from whey,[26] LVQGS from soybean,[27] LGFPTTKYYFPHF (IC_{50} = 4.92 μM) from porcine hemoglobin,[28] and Trp-Pro-Glu-Ala-Ala-Glu-Leu-Met-Met-Glu-Val-Asp-Pro from bigeye tuna dark muscle (IC_{50} = 21.6 μM).[29] To identify the molecular mass and amino acid sequence of the purified peptide, it was subjected to a mass spectrometer. In peptide identification, the mass spectrometric approaches have become more and more widely used to analyze the amino acid sequences of peptides and proteins due to the less time-consuming, high-throughput, and high sensitivity, as compared with the Edman degradation method.[30] The molecular mass of fraction E6 was determined to be 452 Da, and the amino acid sequence of the purified peptide was identified as Ala-His-Leu-Leu by Q-TOF ESI mass spectroscopy (Figure 4).

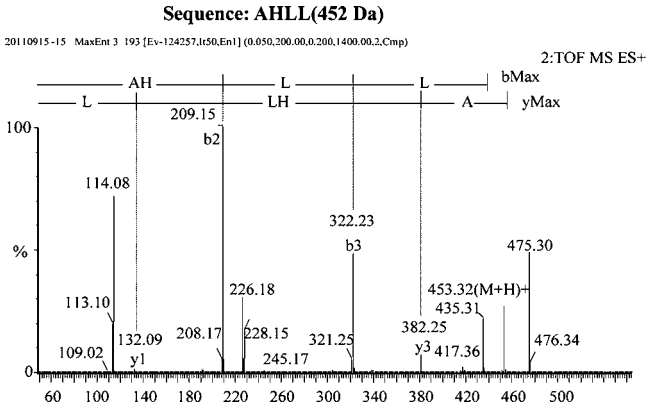

Figure 4. Q-TOF mass spectrum of the fragmentation at m/z 452.3 induced by collision. The sequence of peptide is displayed with the fragment ions observed in the spectrum, and the fragment ions are labeled according to the nomenclature.

Inhibition Pattern of Purified Peptide. The ACE inhibition pattern of the peptide purified from loach was investigated using Lineweaver–Burk plots. As shown in Figure 5, the inhibitory kinetic mechanism of this peptide was competitive, which means the combination between substrate and ACE affects the inhibitory activity of this peptide against ACE.

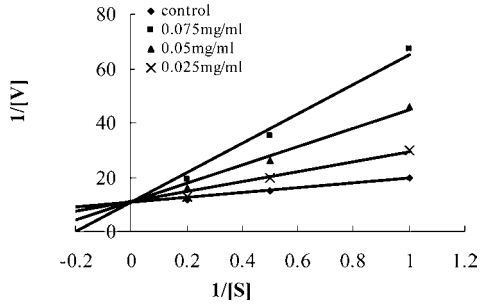

Figure 5. Lineweaver–Burk plots of ACE inhibitor activity in the presence of the purified peptide. [V] and [S] represent reaction velocity and substrate concentration, respectively.

Antihypertensive Effect of LPH-IV on SHRs. The antihypertensive effect of LPH-IV was evaluated by measuring the changes of SBP in SHR at 2, 4, 6, 8, 14, and 24 h after oral administration of LPH-IV. In the present study, captopril was used as a positive control at a dose of 10 mg/kg BW. As shown in Figure 6, there were significant differences in SBP between the treatment of LPH-IV and the control from 2 to 24 h except

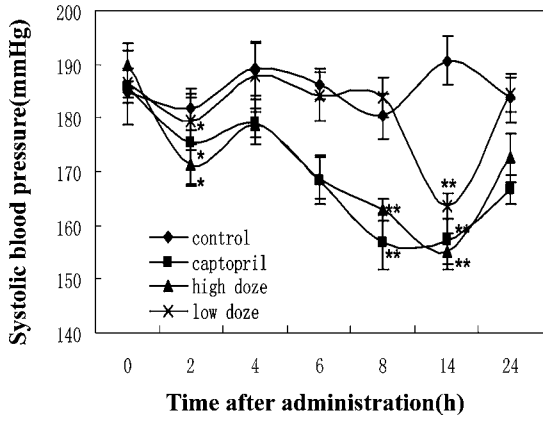

Figure 6. Systolic blood pressure-depressing activity of LPH-IV: control (◆); high dose of LPH-IV (▲); low dose of LPH-IV (×); captopril (■). Data are expressed as mean ± SD; *, $p < 0.05$, and **, $p < 0.01$, indicate significant difference against control.

at 4 h. After 2 h of oral administration, a significant decrease ($p < 0.05$) in SBP value caused by treatment of LPH-IV at a dose of 30 mg/kg BW was observed. The effect was similar to that (from 185 to 175 mmHg) of the commercial antihypertensive drug, captopril. The drug exerted an antihypertensive effect after 8 h postadministration. However, the maximal decreases ($p < 0.01$) in SBP value caused by 30 mg/kg BW of LPH-IV were observed after 14 h postadministration. It might be due to different absorption, metabolism, or transport rates to sites of action. The result suggested that LPH-IV produced a clear antihypertensive effect in SHR at a dose of 30 mg/kg BW. Furthermore, no allergic reaction was noted during the experiment.

In conclusion, the LPH generated by bromelain was treated by ultrafiltration and consecutive purification of gel filtration chromatography and reverse-phase HPLC, resulting in a novel ACE inhibitory peptide of Ala-His-Leu-Leu (452 Da) with an IC_{50} value of 40.3 μM and an increase of 33.7-fold in ACE inhibitory activity as compared with that of crude LPH. The inhibitory kinetics of the peptide was found to be competitive. Finally, we demonstrated that LPH-IV had an antihypertensive effect on SHR after 8 and 14 h of postadministration. Considering its low molecular mass and no allergenic property, it is possible to produce natural antihypertensive peptides from loach proteins through enzymatic hydrolysis and purification for the development of antihypertensive drugs and functional foods. Further work is in progress and will be reported.

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