The Antihypertensive Activity of Angiotensin-Converting Enzyme Inhibitory Peptide Containing in Bovine Lactoferrin

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Abstract

Angiotensin I-converting enzyme (ACE) inhibitory peptide was isolated from the bovine lactoferrin hydrolysate using peptic hydrolysis by 2-step of reverse-phase high-performance liquid chromatography. This peptide was identified as Leu-Arg-Pro-Val-Ala-Ala and it produced a concentration-dependent inhibition of ACE activity *in vitro* with an IC_{50} value of about 4.14 μM. Also, this inhibition was identified as noncompetitive from the Lineweaver-Burk plot. Moreover, the antihypertensive activity of Leu-Arg-Pro-Val-Ala-Ala was investigated by the intravenous injection into spontaneously hypertensive rats (SHRs). A dose-dependent reduction of systolic blood pressure by this peptide was observed at 60 min after injection and it maximally decreased the blood pressure at a rate of 1 nmol/ml/kg. The blood pressure lowering activity of this peptide was calculated as 210% of captopril (10 pmol/ml/kg) that was used as positive control. Otherwise, identification of this peptide in the blood of SHRs was carried out chromatographically. Reduction of blood pressure coincides with the peak peptide concentration in the serum. Thus, we conclude that this peptide inhibits ACE activity *in vitro* and lowers systolic blood pressure in spontaneously hypertensive rat.

Key Words: angiotensin-converting enzyme (ACE) inhibitory peptide, bovine lactoferrin, captopril, spontaneously hypertensive rat

Introduction

Enzymatic hydrolysis of food proteins has produced many active peptides with biologic activities including immunostimulation (11, 27), opioid-like action (29, 36), antithrombotic (30), bactericidal (4) or angiotensin-converting enzyme (ACE) (dipeptidyl carboxypeptidase, EC 3.4.15.1) inhibition (2, 10). Recently, inhibition of ACE has been the focus of research. ACE is a zinc protease which plays a key role in the regulation of blood pressure because it cleaves angiotensin I to angiotensin II and releases C-terminal dipeptide (6). As the well-known vasoconstrictor, angiotensin II causes the constriction of blood vessels in humans and animals in addition to the release of aldosterone from the adrenal cortex, which leads to an accumulation of sodium ions in the human body and increases the systolic blood pressure (9). In addition,

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ACE also hydrolyses and inactivates bradykinin, a vasodilator, to increase the blood pressure (32). Thus, inhibition of this enzyme is believed to lower the blood pressure. Actually, ACE inhibitors exhibit antihypertensive effect in spontaneously hypertensive rats (SHRs) and hypertensive patients (7). The synthetic ACE inhibitors, including captopril, are widely used to treat cardiovascular disorders. However, they caused adverse effects including dry cough, allergic reactions, taste disturbances and skin rashes. Therefore, development of the safer ACE inhibitors has been a main interest in recent. Eventually, studies are focused on the ACE inhibitory peptides derived from casein (18, 25, 28, 31, 33), fish muscle (15, 34), silk fibroin (26), egg yolks (35), gelatin (14), plasma (24) and plant proteins (23). Several ACE inhibitors show antihypertensive effect and the beneficial effect on glucose and lipid metabolism (8), decrease of insulin requirement in diabetic disorders, increase of exercise tolerance and others (12).

Lactoferrin (Lf), an iron-binding glycoprotein of the transferrin family, is present in many biologic fluids including milk, saliva, tears, mucous fluid and blood. Lf consists of a single polypeptide chain having a molecular mass of about 80 kDa and it possesses two iron ion-binding sites per molecule (1). Several physiological functions of Lf including modulation of the inflammatory response, activation of immune system and control of myelopoiesis have been mentioned (5). The existence of antimicrobial domains near the N-terminus of bovine and human Lf was isolated and named as lactoferricin. The antibacterial activity of lactoferricin was 400-fold stronger than that of undigested Lf against to *Escherichia coli* O-111 (3). Effect of Lf on ACE activity remained obscure. Therefore, we employed bovine lactoferrin (bLf) to purify new peptide using the enzymatic hydrolysis method and effects of this new peptide on ACE activity and blood pressure were investigated in the present study.

Materials and Methods

Chemicals

The bLf was kindly obtained from Morinaga Milk Industry Co., Zama City, Japan. Porcine pepsin (EC 3.4.23.1), bovine trypsin (EC 2.4.21.4) and α-chymotrypsin (EC 3.4.21.1) were purchased from Sigma Chemical Co., St. Louis, MO, USA. ACE (EC 3.4.15.1) and hippuryl-histidyl-leucine were obtained from Sigma. Other reagents used were analytical grade.

Proteolytic Digestion of Bovine Lactoferrin

The bLf was dissolved in distilled water at 5% (w/v) and the pH was adjusted to 2.5 or 7.8 for peptic or tryptic and chymotryptic digestion. Each protease was added at the final concentration of 1% (w/w of substrate). The hydrolysis reaction was performed at 37°C for 4 h and terminated by heating at 80°C for 15 min. Reaction mixtures containing pepsin were neutralized by 1N NaOH or 1N HCl. The precipitate of insoluble peptides formed in each reaction mixture was separated by centrifugation (15,000 x g, 15 min). The supernatant was retained and frozen until the experiment.

Isolation and Purification of ACE Inhibitory Peptide

The supernatants obtained from proteolytic digestions were fractionated by reverse-phase HPLC *via* 2-step. In the preliminary experiment, a CAPCELL PAK C18 column (4.6 mm ID × 150 mm, a product of Shiseido, Tokyo, Japen) was applied and a TSKgel ODS 80-Ts column (4.6 mm ID × 150 mm) was used as the secondary step. A mixture of solvent A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in acetonitrile) through a convex gradient of A and B (0 to 45% B) was applied to the column at a flow rate of 1 ml/min for 25 min and the absorbance of eluate at 230 nm was monitored. Fractions were collected and the acetonitrile was removed using a centrifugal evaporator. Then, the samples were dissolved in water and neutralized by adding an alkaline solution for the assay of ACE inhibitory activity. The amino acid sequence of the peptide showing the most potent activity was determined using an Applied Biosystems gas-phase sequencer 492 Protein Sequencer (Applied Biosystems Inc., Foster City, CA, USA). Otherwise, the peptide showed ACE inhibitory activity was ordered to prepare using Fmoc amino acid active derivatives from Sigma-Genosys-Japan (Sapporo, Japan).

Measurement of ACE Inhibitory Activity

The peptide was assayed *in vitro* for the ability to inhibit ACE activity according to the method described by National Food Research Institute, Tsukuba City, Japan. In brief, 100 µl of 4.7 mM hippuryl-L-histidyl-L-leucine/300 mM NaCl/400 mM phosphate buffer solution (pH 8.5) was added with 50 µl of testing peptide or vehicle used to dissolve the testing peptide. Then, 100 µl (2.5 mU) of ACE/distilled water was mixed with the above substrate solution to initiate the reaction that was carried out by incubation in a water bath at 37 ± 1°C under shaking for 60 min. Finally, 1.5 ml of 0.3 M sodium hydroxide was added to terminate the reaction. The formed histidyl-leucine was then labeled by 100 µl of 2% phthaldialdehyde/methanol at room temperature for 10 min and the

reaction was terminated by 200 µl of 3 M HCl. The formed fluorescence compound was diluted with distilled water to 250 times and the fluorescence intensity was then estimated by a spectrofluorometer (EX340, EM455; Hitachi, F-3000). Substrate with distilled water only was used as the blank, while the mixture without testing peptide but the same volume of distilled water was treated as control. The inhibitory ratio (%) of ACE was calculated as (C - A)/(C - B) × 100%, where A is absorbance of samples under the presence of inhibitor or testing peptide, B is absorbance of the blank and C is absorbance of the control. Peptide sample was tested at five concentrations to construct the standard curve for the determination of the IC_{50} value (concentration of inhibitor required to inhibit 50% of the ACE activity). Peptide sample was tested in triplicate.

Then, the pattern for ACE inhibition was further investigated using Lineweaver-Burk plot analysis as described previously (17). ACE inhibitory activity was measured at different concentrations of hippuryl-histidyl-leucine (substrate; S). The reaction rate (V) was defined as the change of formed product in the same reaction time. Lineweaver-Burk plots (1/[S] against 1/V) of three curves (two curves for testing peptide at two effective concentrations and one control curve) were employed to determine the type of this inhibition; competitive or non-competitive.

Antihypertensive Activity of ACE Inhibitory Peptide

Effects of testing peptide on the systolic blood pressure of spontaneous hypertensive rat or Wistar-Kyoto rat (WKY) were determined by intravenous injection into male SHRs or WKYs (obtained form the Animal Center of National Science Council, Taipei, Taiwan) that were maintained in an air-conditioned room (25 ± 1°C) and kept on a 12:12 light-dark cycle (light on at 06:00 h). Food and water were available ad libitum. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. The body weight of rats used was between 410 to 550 g and the solution of testing peptide prepared at desired concentration was injected at a ratio of 1 ml/kg of body weight. The blood pressure was measured by tail-cuff method using the MK-2000 blood pressure meter (Muromachi Kikai, Tokyo, Japan) as described previously (19). Systolic blood pressure was then calculated from four measurements of one animal at desired time.

The testing peptide (Leu-Arg-Pro-Val-Ala-Ala) was dissolved in physiological salt solution for intravenous administration. After injection of testing peptide or saline (control) into tail vein of SHRs, blood pressures were measured at the desired intervals (10, 20, 40, 60, 90 and 120 min) and the time before injection (0 min). Similar injections of captopril [(2S)-N-(3-mercapto-2-methylpropionyl)-L-proline] (Sigma), Leu-Arg-Pro (Bachem) or pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (Sigma) were used as the positive control. The blood pressure lowering activity of this testing peptide (1 nmol/ml/kg) was compared with that from another three ACE inhibitors including captopril (10 pmol/ml/kg), Leu-Arg-Pro (1 nmol/ml/kg) and pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (1 nmol/ml/kg).

Characterization of Testing Peptide in Serum from SHRs

The fraction in serums drew from femoral vein of SHRs under anesthesia with intra-peritoneal injection of pentobarbital (35 mg/kg) at 0 min and 60 min after intravenous injection of Leu-Arg-Pro-Val-Ala-Ala was investigated. The obtained blood sample was then centrifuged at 13,000 x g, 10 min and the supernatant was used to assay in RP-HPLC, using a LiChroCART C18 column (4 mm × 250 mm, a product of Merck, Frankfurter City, Germany) and a mixture of solvent A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in acetonitrile). A linear gradient of A and B (0 to 67% B) was applied to the column for 18 min at a flow rate of 1 ml/min and the absorbance of the eluate at 214 nm was monitored.

Statistical Analysis

Data are expressed as the mean ± SEM for the number (n) of animals in each group indicated in table and figure. Repeated measures analysis of variance (ANOVA) was used to analyze the changes of blood pressure and other parameters. The Dunnett range post-hoc comparisons were used to determine the source of significant differences where appropriate. The obtained *P* value of 0.05 or less was considered statistically significant.

Results

Isolation of ACE Inhibitory Peptide from bLf

As shown in Fig. 1a, a substance (dotted arrow) appeared in one fraction by the HPLC on a CAPCELL PAK C18 column. The active fraction was further fractionated by another HPLC on a TSKgel ODS 80-Ts column to obtain one peptide showing a single peak on HPLC (Fig. 1b). The amino acid sequence of this peptide was identified as Leu-Arg-Pro-Val-Ala-Ala.

ACE Inhibitory Activity of Testing Peptide

In the *in vitro* assay of ACE activity, this pep-

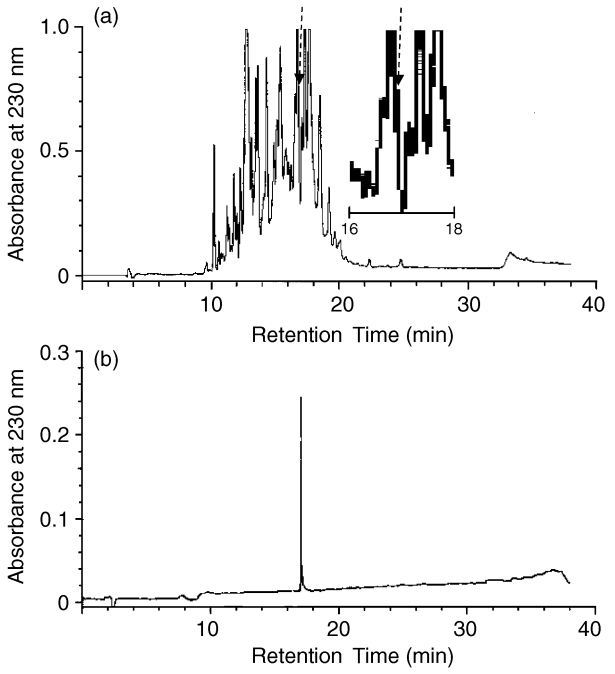

Fig. 1. Purification of ACE inhibitory peptide obtained from peptic hydrolysis of bovine lactoferrin by RP-HPLC on two ODS columns. (a) primary analysis in HPLC and (b) secondary analysis in HPLC. The fraction (dotted arrow) on the first HPLC indicated most potent ACE inhibitory activity was applied to the second HPLC. Gradient condition for elution on both HPLCs was indicated in Materials and Methods.

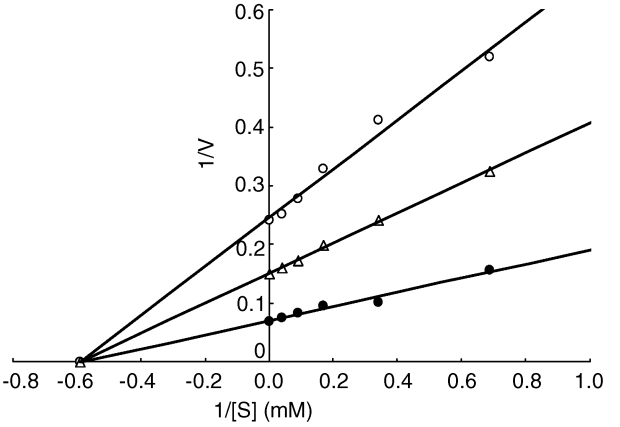

Fig. 2. Lineweaver-Burk plot of ACE activity in the presence of testing peptide at 2.6 μM (○) or 1.3 μM (△) to compare with the vehicle-treated control (●).

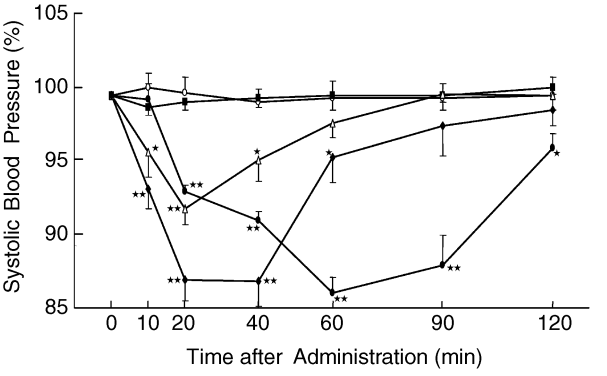

Fig. 3. Antihypertensive activity of synthetic Leu-Arg-Pro-Val-Ala-Ala after intravenous administration into hypertensive rats (SHRs). The testing peptide was administered at doses of 1 pmol/ml/kg(■), 10 pmol/ml/kg (△), 0.1 nmol/ml/kg (◆) or 1 nmol/ml/kg (●) to compare with saline-treated control (○). Changes of systolic blood pressure from time zero were expressed with means ± SEM. Asterisks indicate the statistical difference with control (*$P < 0.05$; **$P < 0.01$ by Student's *t*-test; $n = 8$).

tide produced a concentration-dependent inhibition with an IC_{50} value about 4.14 μM. The synthetic Leu-Arg-Pro-Val-Ala-Ala showed a similar inhibition of ACE activity as the isolated peptide. Moreover, the IC_{50} value of synthetic Leu-Arg-Pro was 0.17 μM.

Pattern of the Inhibition of ACE Activity by Testing Peptide

The pattern of ACE inhibition by this testing peptide, Leu-Arg-Pro-Val-Ala-Ala, was investigated by Lineweaver-Burk plots. Synthetic Leu-Arg-Pro-Val-Ala-Ala at 1.3 or 2.6 μM was used and the plots showed this inhibition of ACE to be a non-competitive (Fig. 2).

Antihypertensive Activity of Testing Peptide after Intravenous Administration in SHR

Leu-Arg-Pro-Val-Ala-Ala dissolved in saline was used to inject into SHR intravenously and the systolic blood pressure of SHR was measured by tail-cuff method at 0, 10, 20, 40, 60, 90 and 120 min after injection. Value of blood pressure was calculated as the percentage of that measured at 0 min. At the dose of 10 pmol/ml/kg, Leu-Arg-Pro-Val-Ala-Ala decreased the blood pressure in SHR at 10 min after injection ($P < 0.05$) and showed a maximum effect at 20 min as compared with the saline-treated control. At a higher dose (0.1 nmol/ml/kg), this peptide produced a more marked antihypertensive effect at 10 min after injection and showed a longer decrease of blood pressure (lowered the systolic blood pressure by 12.4% even at 40 min after injection). Moreover, as shown in Fig. 3, this peptide at the highest dose (1 nmol/ml/kg) exerted a long-lasting effect from 20 to 120 min ($P < 0.05$) but the blood pressure was reduced by 13.2 % in a way similar to level produced at higher dose (0.1 nmol/ml/kg). However, intrave-

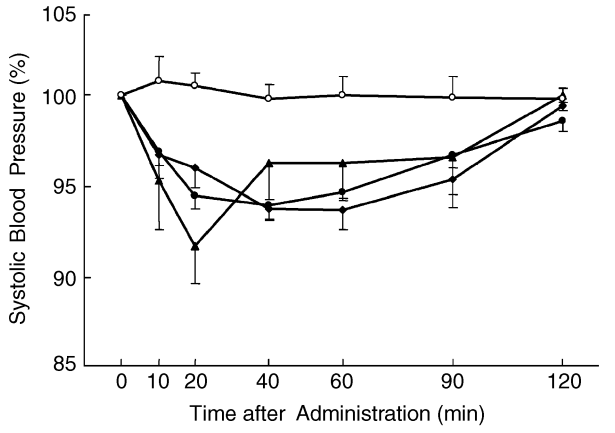

Fig. 4. Changes of blood pressure in SHR receiving an intravenous injection of three ACE inhibitors including captopril (◆), Leu-Arg-Pro (●) and pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (▲). Also, the normotensive Wistar-Kyoto rats (WKY) received injection of Leu-Arg-Pro-Val-Ala-Ala was indicated (○). Percentage changes of the systolic blood pressure from time zero was expressed with means ± SEM (n = 4) in each point.

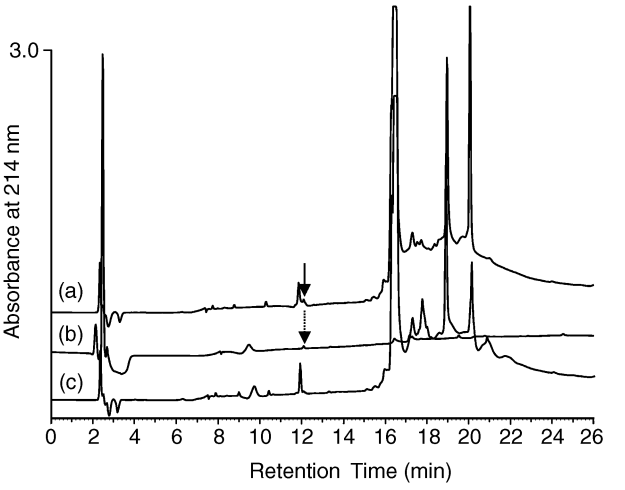

Fig. 5. Fractionation of the serum from femoral vein of SHRs in RP-HPLC; (a) testing peptide (solid arrow) observed in the serum from SHRs receiving peptide administration (1 nmol/ml/kg), (b) pure testing peptide (1 μM, 250 μl) as the positive control (dotted arrow), (c) negative control of the serum from femoral vein of SHRs before peptide injection. Gradient condition for elution on HPLC was indicated in Materials and Methods.

nous injection of Leu-Arg-Pro-Val-Ala-Ala at dose (1 nmol/ml/kg) in SHRs failed to modify the blood pressure of normotensive WKY rats (Fig. 4).

As the positive control, three ACE inhibitors including captopril (10 pmol/ml/kg), Leu-Arg-Pro (1 nmol/ml/kg) and pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (1 nmol/ml/kg) were used. They exhibited a marked effect to lower the systolic blood pressure of SHR after intravenous injection (Fig. 4). The blood pressure lowering activity of this peptide was about 210 % of captopril (10 pmol/ml/kg), 220 % of Leu-Arg-Pro (1 nmol/ml/kg) and 159 % of that induced by pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (1 nmol/ml/kg) in SHRs.

Identification of Active Peptide from the Serum of SHRs

In the analysis of RP-HPLC, peak (solid arrow) of this peptide fractionated from the serum drew from femoral vein of SHRs at the most effective time (60 min after administration of Leu-Arg-Pro-Val-Ala-Ala at 1 nmol/ml/kg) was observed (Fig. 5a). This peak was compared with the pure peptide (dotted arrow) using as the standard (Fig. 5b). The same retention time showed that the bioactive peptide effective to lower the blood pressure was Leu-Arg-Pro-Val-Ala-Ala. However, there was no peak observed at same retention time in serum samples drawn before peptide injection that was taken as blank (Fig. 5c). Also, the peak (arrow) for serum of SHRs was also checked to be a same material as the standard by an approximate spectrum with 0.9961 using RP-HPLC system (L-7450A; Hitachi, Ltd., Tokyo, Japan).

Discussion

In the present study, we purified Leu-Arg-Pro-Val-Ala-Ala from bovine lactoferrin (bLf) and found that this peptide has an inhibitory effect on ACE activity *in vitro*. Also, this inhibition was smaller than that of Leu-Arg-Pro, another peptide showing an IC_{50} value for ACE at 0.17 μM. Moreover, the synthetic Leu-Arg-Pro-Val-Ala-Ala produced a similar inhibition of ACE activity as that isolated from the protein hydrolysate of α-zein (22) or fish (21) although the IC_{50} value was somewhat lower than the previous one. The synthetic Leu-Arg-Pro-Val-Ala-Ala showed a similar inhibition of ACE activity *in vitro* as the purified one. Thus, we employed the synthetic peptide to instead of purified one for investigation in the present study. Lineweaver-Burk plots analysis of this peptide (Leu-Arg-Pro-Val-Ala-Ala) at two effective concentrations indicated this inhibition of ACE to be non-competitive (Fig. 2). The non-competitive inhibition of ACE by this peptide can thus be considered.

The antihypertensive action was investigated in SHR using the intravenous injection of this peptide or positive control. Figure 3 showed the time course of systolic blood pressure change in SHRs induced by this peptide. A dose-dependent decrease of blood pressure by this peptide from 10^{-12} to 10^{-10} mol/ml/kg was observed. Also, the effect was lasted longer in a

dose-related manner. The action of this peptide exhibited throughout the total measurement duration (120 min). It showed that at the dose of 1 nmol/ml/kg this peptide produced a maximum pressure lowering action in a way similar to that caused by 0.1 nmol/ml/kg at 60 min after injection. But the pressure lowering level by this peptide at the dose of 1 nmol/ml/kg was only about half of the level caused by 0.1 nmol/ml/kg at 20 min after injection. This might be due to a large mass was formed in blood circulation by this peptide at high dose (1 nmol/ml/kg) to result in a prolongation of this peptide reaching the active site of ACE. However, intravenous injection of Leu-Arg-Pro-Val-Ala-Ala at the most effective dose in SHRs failed to modify the blood pressure of normotensive WKY rats (Fig. 4). Antihypertensive action of this peptide can thus be considered. This result suggested that the effect of this hexa-peptide was specific to animal at hypertensive state, similar to the actions of Val-Pro-Pro and Ile-Pro-Pro that were also isolated from milk protein (25). Actually, blood pressure lowering activity of this peptide was about 210% of captopril (10 pmol/ml/kg); the well-known ACE inhibitor (13). Also, the activity of this peptide was 220% of Leu- Arg-Pro (1 nmol/ml/kg) and 159 % of that induced by pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro (1 nmol/ml/kg) in SHRs. Both peptides were mentioned to inhibit ACE activity effectively in previous reports (16, 20). However, different to the inhibition of ACE activity, the blood pressure lowering action of Leu-Arg-Pro became less than this peptide. This difference seems related to the pharmacokinetic change of Leu-Arg-Pro in SHRs, probably the rapid metabolism and/or elimination which needs more studies to prove in advance.

In the serum from SHRs, peak of this peptide was observed as the standard on RP-HPLC (Fig. 5) at the effective time for lowering of blood pressure. It seems that this peptide was not hydrolyzed by endogenous enzymes existed in blood, kidney or other organs and it reacted with ACE by the form of hexapeptide. This view was not mentioned before. Actually, a direct analysis of ACE activity in the serum of SHRs by this testing peptide will be helpful. However, the employed method for ACE activity was available for *in vitro* assay only. Thus, more studies are required to clarify the detailed mechanism of this action both in animal and human subjects in the future.

In conclusion, the obtained results indicate that peptic hydrolysis of bLf may release an ACE inhibitory peptide possessing antihypertensive activity in SHR. Thus, the ACE inhibitory peptide derived from lactoferrin hydrolysates would be useful for development of new antihypertensive agent and/or new ACE inhibitor.

Acknowledgments

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