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Porcine Skeletal Muscle Troponin Is a Good Source of Peptides with Angiotensin-I Converting Enzyme Inhibitory Activity and Antihypertensive Effects in Spontaneously Hypertensive Rats

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In the search for novel peptides that inhibit the angiotensin I-converting enzyme (ACE), porcine skeletal troponin was hydrolyzed with pepsin, and the products were subjected to various types of chromatography to isolate active peptides. Glu-Lys-Glu-Arg-Glu-Arg-Gln (EKERERQ) and Lys-Arg-Gln-Lys-Tyr-Asp-Ile (KRQKYDI) were identified as active peptides, and their 50% inhibitory concentrations were found to be 552.5 and 26.2 μM, respectively. These are novel ACE inhibitory peptides, and the activity of KRQKYDI was the strongest among previously reported troponin-originated peptides. KRQKYDI was slowly hydrolyzed by treatment with ACE, and kinetic studies indicated that this peptide was a competitive inhibitor of the enzyme. When KRQKYDI was administered orally to spontaneously hypertensive rats (SHR) at a dose of 10 mg/kg, a temporary antihypertensive activity was observed at 3 and 6 h after administration.

KEYWORDS: Angiotensin I-converting enzyme inhibitory peptide; antihypertensive effect; spontaneously hypertensive rat; protease digestion; porcine skeletal troponin

INTRODUCTION

Lifestyle-related diseases, such as obesity, diabetes mellitus, hypertension (blood pressure >140/90 mmHg), and cardiovascular disease, have recently been increasing. Heart disease and vascular disease, respectively, are the second and third most common causes of death in Japan (*1*). The relationship between food and health has attracted considerable attention; of interest are the physiological effects of some food components in counteracting certain ailments (*2*). In particular, hypertension is a worldwide problem of epidemic proportions, which presents in 15–20% of all adults. It is the most common serious chronic health problem because it carries a high risk of cardiovascular complications (*3*). In Japan, more than 20 million people suffer from hypertensive disease, 90% or more of whom suffer from essential hypertension (*4*). Essential hypertension is believed to be induced by various factors relating to lifestyle and heredity (*4*).

Angiotensin I-converting enzyme (ACE), which is a dipeptidylcarboxypeptidase, plays an important physiological role in regulating blood pressure (*5*). ACE converts an inactive form of decapeptide, angiotensin I, to a potent vasoconstrictor, octapeptide angiotensin II, and inactivates bradykinin, which has a depressor action. For these reasons, specific inhibitors of ACE are useful for regulating physiological activities associated with ACE in the human body (*6*). ACE is of great interest to those studying hypertensive disease because it catalyzes the production of a hypertensive peptide, angiotensin II, as well as the destruction of a hypotensive peptide, bradykinin.

It has been elucidated that certain food constituents have beneficial physiological effects, and it is important to use such food constituents for the maintenance and promotion of health. For instance, some peptides have recently been reported to play an important role in controlling the development of hypertension by regulating the rennin−angiotensin system (*7, 8*).

ACE inhibitory peptides derived from foods, especially milk proteins (casein and whey proteins), have been reported to show antihypertensive effects in spontaneously hypertensive rats (SHR) when administered orally (*9–12*). However, there is little available information regarding the derivation of ACE inhibitory activity from muscle proteins of domestic meat animals (*13*).

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The ACE inhibitory peptides have been studied in foods from animal muscle, such as sardine muscle (*14*), dried bonito (*15*), krill (*16*), Alaska pollack (*17*), and pork (*13*). Several researchers reported on ACE inhibitory peptides from myosin, actin, and some water-soluble proteins (*13, 15*). Katayama et al. (*18*) suggested that ACE inhibitory peptides were generated not only from these proteins but also from regulatory proteins such as tropomyosin and troponin. Although an ACE inhibitory peptide, Arg-Met-Leu-Gly-Gln-Thr-Pro-Thr-Lys (RMLGQTPTK), has been isolated from troponin (*19*), the hydrolysate of troponin was expected to include other peptides with ACE inhibitory activity because of its variability as demonstrated in some chromatographic analyses. Moreover, these ACE inhibitory peptides are also expected to have antihypertensive effects on SHR.

In the present study, we describe the isolation of some novel ACE inhibitory peptides from the hydrolysate of porcine troponin. Among them, the peptide with the greatest ACE inhibitory activity was investigated for its digestibility by ACE and for the nature of its inhibitory effect. The peptide was administered to SHR to determine whether it played a vital role as an antihypertensive substance in vivo.

MATERIALS AND METHODS

Troponin Hydrolysate. Crude troponin from Japanese domestic pork was prepared as described by Katayama et al. (*18*). It was then hydrolyzed with pepsin (Sigma Chemical Co., St. Louis, MO), and after centrifugation, the supernatant of the hydrolysate was used in the experiments (*18*).

ACE Inhibitory Activity Assay. ACE inhibitory activity was measured according to the method of Cushman and Cheung (*20*), with slight modifications (*21*). This assay is based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL) catalyzed by ACE.

A sample solution of peptide (6 μL) was mixed with 50 μL of 7.6 mM HHL (Nacalai Tesque Inc., Kyoto, Japan) as substrate containing 100 mM sodium borate buffer (pH 8.3) and 608 mM NaCl and then preincubated at 37 °C for 5 min. The reaction was initiated by the addition 20 μL of 60 milliunits/mL rabbit lung ACE (Sigma Chemical Co.) in a buffer containing 0.25 M sodium borate buffer (pH 8.3) followed by incubation of the mixture at 37 °C for 30 min. The reaction was stopped by adding 554 μL of 0.1 N HCl except in the case of the blank, which was treated with 554 μL of 0.1 N HCl before preincubation. The hippuric acid liberated by ACE was extracted by adding 1.5 mL of ethyl acetate followed by vigorous shaking of the mixture for 2 min. After centrifugation at 3000 rpm for 20 min, 1 mL of the ethyl acetate layer was collected. Aside from that, 1 mL of the ethyl acetate solution was evaporated at 100 °C for 10 min. The hippuric acid was then dissolved in 1 mL of 1 M NaCl and its concentration determined photometrically at 228 nm. The concentration of ACE inhibitors required to inhibit 50% of ACE activity was defined as the IC_{50} value. The IC_{50} of the reaction mixture was determined without any fractionation.

Purification of ACE Inhibitory Peptide from Troponin Hydrolysate. Anion exchange chromatography was used to separate the troponin hydrolysate. The hydrolysate was applied to a DE53 (Whatman International Ltd., Kent, U.K.) column (16 × 150 mm) and eluted with a gradient of 0–300 mM NaCl in 20 mM Tris−acetate (pH 7.5) at a flow rate of 1.13 mL/min. Every 7 min (7.91 mL), eluted fractions were collected and desalted using a Sep-Pak Plus C_{18} cartridge (Waters Co., Milford, MA) and 50% CH_3CN. Using reverse phase (RP) high-performance liquid chromatography (HPLC) and a Cosmosil $5C_{18}$ AR-II column (4.5 × 150 mm, Nacalai Tesque), the active fraction was then separated by gradient elution with 1–80% CH_3CN in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 mL/min. The active fractions were then applied to the same column and eluted with 12 or 16% CH_3CN in 0.1% TFA at a flow rate of 0.5 mL/min. The active fractions obtained by RP-HPLC were then separated using gel filtration HPLC and a TSK-gel $G2000SW_{XL}$ column (7.8 × 300 mm, Tosoh Co., Tokyo, Japan) and were eluted with 20 mM sodium phosphate (pH 7.0) at a flow rate of 0.5 mL/min. Finally, the active fractions were separated by three iterations of RP-HPLC using a Cosmosil 5PE-MS column (4.6 × 250 mm, Nacalai Tesque) and 12–15% CH_3CN in 0.1% TFA at a flow rate of 1 mL/min. The amino acid sequences of the finally obtained active fractions were analyzed with a protein sequencer Procise 492 (Applied Biosystems, Foster City, CA).

Synthetic Peptides. The synthesized ACE inhibitory peptides from troponin hydrolysate were sourced from Sigma Genosys Japan Co. (Ishikari, Hokkaido, Japan) for the analysis of their properties as antihypertensive peptides.

Digestibility of Peptide to ACE. A 10 μL aliquot of the ACE inhibitory peptide (final concentration was 1 mg/mL) was mixed with 80 μL of buffer solution (*19*); the final concentrations of the components were 400 mM in NaCl and 100 mM in borate buffer (pH 8.5). Then the mixture was preincubated at 37 °C for 5 min and the reaction was initiated by the addition of 10 μL of 200 milliunits/mL rabbit lung ACE for various incubation times at 37 °C. The reaction was stopped by heating at 95 °C for 20 min, and the solution was cooled in ice at 0 °C. The reaction mixture was analyzed by RP-HPLC to determine how efficiently the peptide had been digested. A 20 μL aliquot of solution was injected onto an Inertsil ODS-2 column (6.0 × 150 mm, GL Sciences Inc., Tokyo, Japan) and eluted with a gradient of 1–80% CH_3CN in 0.1% TFA at a flow rate of 1 mL/min. The concentration of peptide in the fractions was calculated from the peak area of the HPLC trace.

Kinetic Evaluation of Type of Inhibition. Initial velocities of ACE in the presence or absence of inhibitory peptide (50 μM) were determined at various concentrations of HHL. The resultant data were used to produce a reciprocal plot (*22*), with velocity being shown on the vertical axis and the concentration of HHL on the horizontal axis. The type of inhibition exhibited by the peptide was confirmed graphically.

Antihypertensive Activity after Oral Administration in SHR. Animals used in this study were maintained in accordance with the guidelines of the Institutional Animal Care and Utilization Committee in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals. The animals were purchased from Charles River Japan, Inc. (Yokohama, Japan); four SHRs (Izm) were used as the controls and two as test animals (aged 8 weeks). They were kept in a room with a 12 h light/dark cycle (lights on between 7:00 a.m. and 7:00 p.m.). Temperature and humidity were controlled at 23 ± 1 °C and 50 ± 10%, respectively. Diet (CRF-1, Charles River Japan) and tap water (0.2 μm filtered) were available ad libitum. After 4 weeks of maintenance, the SHRs were used for the experiment, and their initial systolic blood pressures (SBP) were 205–225 mmHg. The peptide was dissolved in distilled water (10 mg/mL) and was administered orally using a flexible sonde to SHRs at a dose of 10 mg/kg of body weight (10 mL/kg). The same volume of distilled water was administered to the control group. The SBP of the rats was measured at 0, 3, 6, 9, and 24 h after administration by the tail-cuff method with a programmable electro-sphygmomanometer (BP-98A; Softron Co., Ltd., Tokyo, Japan) after the rat had been warmed in a chamber maintained at 38.7 °C for 15 min. The differences between SBP values of rats from the different experimental groups were analyzed using Student's *t* test.

RESULTS AND DISCUSSION

ACE Inhibitory Activity of Troponin Hydrolysate and Its Fragment Fractions. In a previous publication we demonstrated the ACE inhibitory activity of a troponin hydrolysate produced by pepsin digestion and calculated its IC_{50} to be 130 μg/mL (*18*). We also identified a novel ACE inhibitory peptide, RMLGQTPTK; other active peptides were also detected in the hydrolysate (*19*). Consequently, we attempted to isolate other more active peptides from the above-mentioned hydrolysate.

The most active fraction separated by anion exchange chromatography (which was the first to be eluted from the column) was then subjected to RP-HPLC using a Cosmosil $5C_{18}$

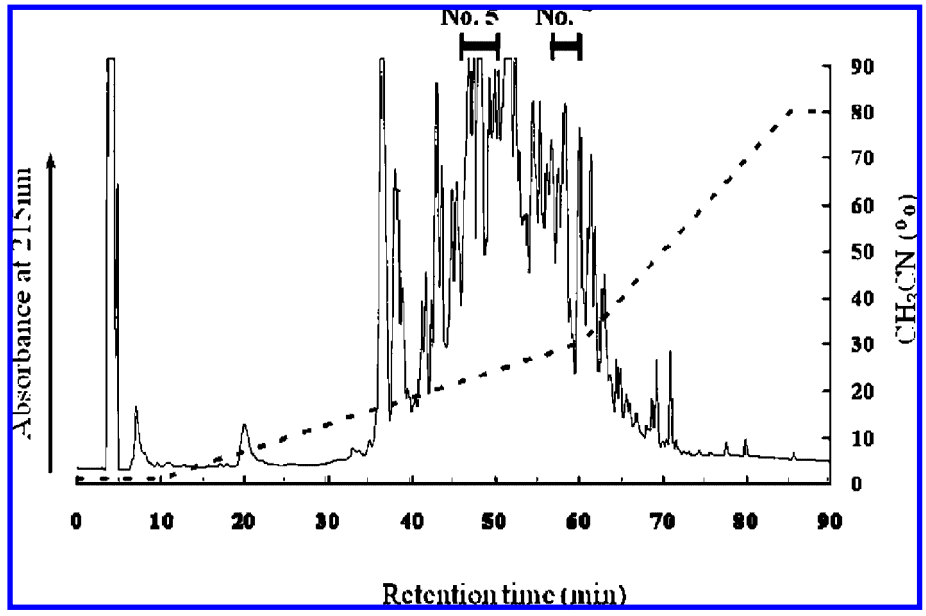

Figure 1. RP-HPLC chromatogram of the most active fraction from ion exchange separation. The most active fraction was applied to a Cosmosil $5C_{18}$ AR-II (4.5 × 150 mm, Nacalai Tesque) and eluted with a gradient of 1–80% CH_3CN in 0.1% TFA at a flow rate of 0.5 mL/min. Eluted fractions were collected and measured for their ACE inhibitory activities. Fractions 5 and 7 are active fractions.

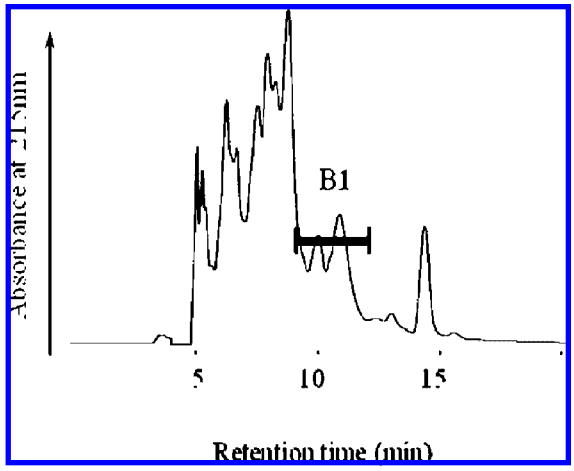

Figure 2. Second chromatogram of fraction 5 from the first RP-HPLC. Fraction 5 was reseparated with the same RP-HPLC system. It was eluted with 12% CH_3CN in 0.1% TFA at a flow rate of 0.5 mL/min. Fractions were collected and their ACE inhibitory activities measured. A highly active fraction (B1) indicated by a black bar was separated on the next step.

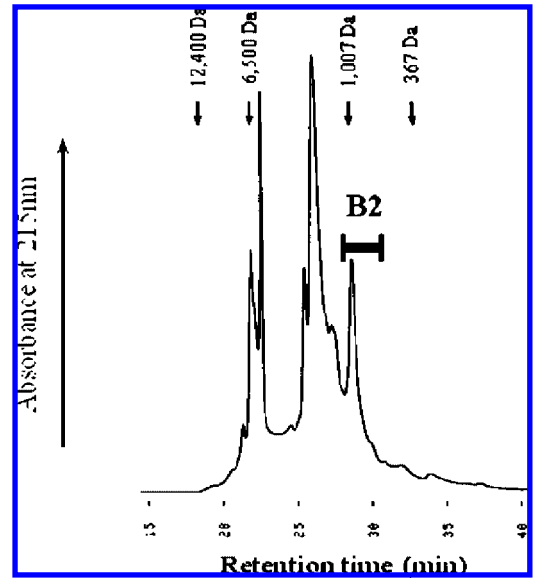

Figure 3. Gel filtration chromatogram of fraction B1. Fraction B1 was applied to a TSK-gel G2000SWXL (7.8 × 300 mm, Tosoh) and eluted with a 20 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.5 mL/min. A highly active fraction (B2) is indicated by a black bar. Cytochrome *c* (12400 Da), aprotinin (6500 Da), oxytocin (1007 Da), and riboflavin (367 Da) were used as molecular mass standards.

AR-II column, and two active fractions (5 and 7) were obtained (**Figure 1**). The retention times of fractions 5 and 7 were 46–50 and 57–60 min, respectively and their IC_{50} values were, respectively, 138 and 114 μg/mL. Fraction 5 was applied to the same column and eluted with 12% CH_3CN, and an active fraction (B1) was obtained (**Figure 2**). Fraction B1 was separated by gel filtration HPLC, and a second fraction (B2), which had inhibitory activity (IC_{50} = 27 μg/mL), was also detected. Furthermore, the retention time of fraction B2 indicated that its molecular mass was in the region of 1000 Da (**Figure 3**). Finally, by three iterations of RP-HPLC using a Cosmosil 5PE-MS column, a single peak fraction (B) was separated (**Figure 4**). In contrast, when fraction 7 was applied to the Cosmosil $5C_{18}$ AR-II column and eluted with 16% CH_3CN, a single active fraction (L1) was obtained (**Figure 5**). Fraction L1 was subjected to the gel filtration HPLC, and an active fraction (L2, IC_{50} = 57 μg/mL) was found with a molecular mass estimated to be about 1000 Da (**Figure 6**). As described above, fraction L2 was separated by three iterations of RP-HPLC, and a single peak fraction (L) was obtained (**Figure 7**).

In addition, all of the separation steps produced several active fractions. Similar to the case of myosin as described by Katayama et al. (*23*), troponin was expected to be a superior source of ACE inhibitory peptides and to be effective as a food component with antihypertension properties in vivo.

Amino Acid Sequences and Activities of ACE Inhibitory Peptides. Analysis using a protein sequencer showed the composition of fractions B and L to be Glu-Lys-Glu-Arg-Glu-Arg-Gln (EKERERQ, molecular mass = 974) and Lys-Arg-Gln-Lys-Tyr-Asp-Ile (KRQKYDI, molecular mass = 950), respectively. They were recognized as novel ACE inhibitory

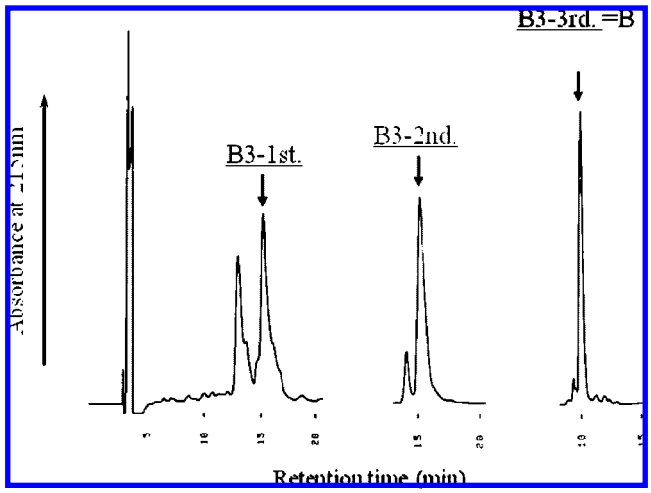

Figure 4. Chromatogram of RP-HPLC using PE-MS column. Fraction B2 was separated with three iterations of RP-HPLC using Cosmosil 5PE-MS (4.6 × 250 mm, Nacalai Tesque). It was eluted with 12% (for first and second) or 14% (for third) CH_3CN in 0.1% TFA at a flow rate of 1 mL/min. Active peaks are indicated with arrows. The amino acid sequence of the active peptide (B) was determined.

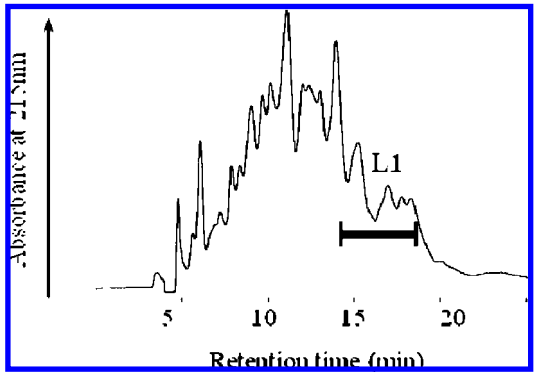

Figure 5. Second chromatogram of fraction 7 from the first RP-HPLC. Fraction 7 was reseparated with the same RP-HPLC system. It was eluted with 16% CH_3CN in 0.1% TFA at a flow rate of 0.5 mL/min. Fractions were collected and their ACE inhibitory activities measured. A highly active fraction (L1) indicated by a black bar was separated on the next step.

peptides. A search for sequence homology in databases revealed that these peptides respectively showed sequence homology with amino acids at positions 65–71 and 198–204 in troponin T of rabbit. Although the sequence of porcine troponin T has not been completely identified as yet, troponin T of porcine was considered to be the same as the troponin T of rabbit. These peptides were synthesized, and their ACE inhibitory activities (IC_{50}) were found to be 552.5 and 26.2 μM, respectively, and the activity of KRQKYDI was greater than that of troponin-originated peptides previously reported (*19*). These peptide inhibitors are secondary products derived from a regulatory protein, whereas the peptide inhibitors of muscle origin were produced from myofibrillar structural proteins (myosin and actin) or water-soluble proteins (*13, 15*).

Reactivity of ACE Inhibitory Peptide to ACE. The hydrolysis of synthesized KRQKYDI, which is the strongest ACE inhibitory peptide among those derived from troponin, by ACE and its ability to inhibit this enzyme were investigated. RP-HPLC analysis of a mixture of KRQKYDI and ACE showed that ACE slowly digested the peptide (**Figure 8**) over a long period of time. The IC_{50} (micrograms per milliliter) of the mixture decreased after 20 min and did not change substantially

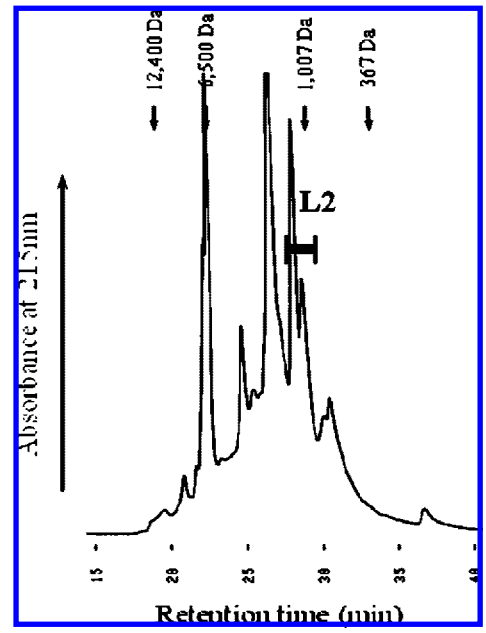

Figure 6. Gel filtration chromatogram of fraction L1. Fraction L1 was applied to a TSK-gel G2000SWXL (7.8 × 300 mm) and eluted with a 20 mM sodium phosphate buffer (pH 7.0) at a flow rate of 0.5 mL/min. A highly active fraction (L2) is indicated by a black bar. Molecular mass standards were the same in **Figure 3**.

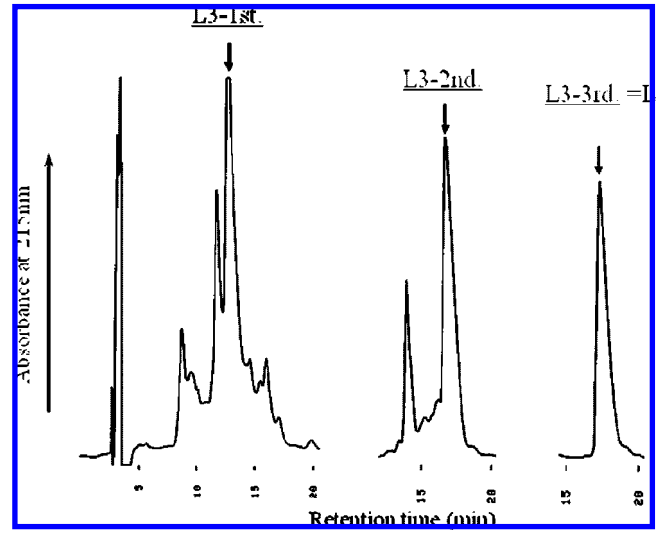

Figure 7. Chromatogram of RP-HPLC using PE-MS column. Fraction L2 was separated with three iterations of RP-HPLC using Cosmosil 5PE-MS (4.6 × 250 mm). It was eluted with 15% (for first) or 12% (for second and third) CH_3CN in 0.1% TFA at a flow rate of 1 mL/min. Active peaks are indicated with arrows. The amino acid sequence of the active peptide (L) was determined.

after this time. This result indicated that the ACE inhibitory activity of products from digestion might be stronger than that of the original peptide, KRQKYDI. ACE inhibitory oligopeptides were classified by Fujita et al. (*24*) into three types (real inhibitor, pro-drug, and competitive substrate) by evaluating their patterns of hydrolysis by ACE and the activities of their hydrolysates. According to this classification, KRQKYDI was considered to be a substrate-type inhibitor. **Figure 9** shows the Lineweaver–Burk plots that represent typical cases of competitive inhibition. The simplest explanation for such competitive inhibition is that the inhibitors bind to the active site of the

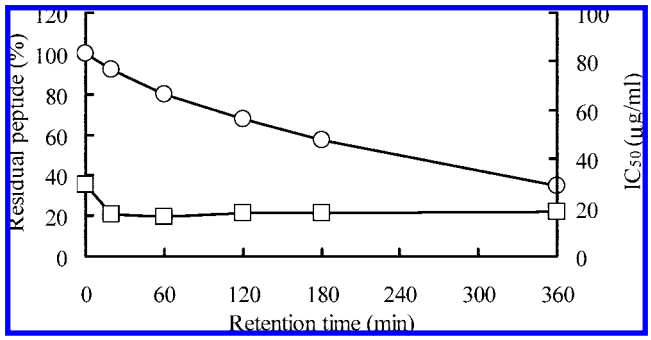

Figure 8. Reduction of KRQKYDI by ACE and its ACE inhibitory activity. VKKVLGNP was digested by ACE for various durations. The amount of residual peptide (circles) was calculated from peak area of RP-HPLC. ACE inhibitory activity (squares) of the reaction mixture was also measured.

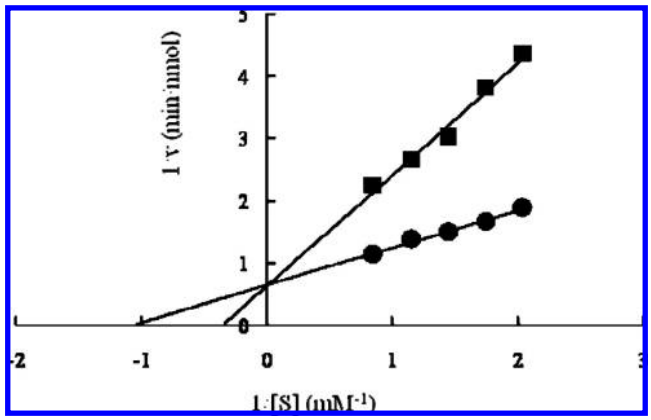

Figure 9. Kinetic evaluation of ACE inhibitory peptide. KRQKYDI was kinetically analyzed for its inhibition type at the concentration of 50 μM (squares). Circles show data in the absence of peptide. [S] = concentration of HHL; [v] = initial velocity of ACE.

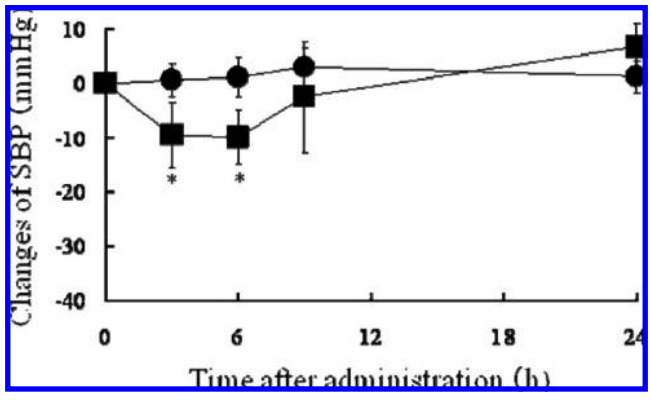

Figure 10. Effect of a single oral administration of KRQKYDI on SHR. Changes of systolic blood pressure (SBP) from zero time are expressed as means, and the vertical bars represent the standard deviations. Treatments were control (circles; distilled water) and 10 mg/kg of KRQKYDI (squares). Significant difference from the control: $*$, $p < 0.05$.

enzyme in a manner similar to that of the enzyme substrates. We speculated that KRQKYDI worked in vivo as one of the factors contributing to suppression of blood pressure.

Antihypertensive Action in SHR. As the synthetic KRQKYDI showed the strongest ACE inhibitory activity, it was used for oral administration to SHR. The SBP decreased by 9.3 mmHg 3 h after administration, and after 6 h it had decreased further by up to 9.9 mmHg (**Figure 10**). By 9 h postadministration SBP had risen; however, it was slightly lower than the value of control sample measured at the same time. The values of SBP 3 and 6 h after administration of the peptide had decreased significantly ($p < 0.05$) when compared to the values of the control sample. This result showed that the peptide was effective and was able to maintain low levels of SBP in the long term; overall, the peptide is considered to be a safe hypotensor. We therefore hypothesize that the orally administered peptide might temporarily inhibit ACE and may then be hydrolyzed by ACE. Consequently, the blood pressure system treated with ACE might be restored to almost the preadministration state of the peptide within 24 h after administration. Although many ACE inhibitory peptides were isolated from many foods, each of them must act as a hyptensor to some extent in vivo. Troponin is one of the minor components in meat and makes up about 5% of the muscle protein. Myosin, the major protein in muscle, has been shown to contain hypotensive peptides (*25*). In addition to the considerable function of myosin, troponin is considered to be a good source of hypotensor activity as we isolated another ACE inhibitory peptide from troponin (*19*), and it played a functional role in reduce blood pressure in vivo.

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