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Journal of Nutritional Biochemistry 13 (2002) 80–86

THE JOURNAL OF Nutritional Biochemistry

Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR

Toshiro Matsui[a,*], Akiko Yukiyoshi[a], Shima Doi[b], Hiroyuki Sugimoto[b], Hideo Yamada[b], Kiyoshi Matsumoto[a]

[a]*Division of Bioresources and Biosciences, Faculty of Agriculture, Graduate School, Kyushu University, 6–10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan*

[b]*Yamada Apiculture Center, Inc., Ichiba 194, Kagamino-Cho, Tomada-Gun, Okayama 708-0393, Japan*

Received 16 February 2001; received in revised form 23 August 2001; accepted 10 September 2001

Abstract

In order to clarify the potential physiological function of royal jelly (RJ), we report here the gastrointestinal enzyme production of antihypertensive peptides from RJ. Intact RJ and its protein fraction did not retard the action of angiotensin I-converting enzyme (ACE) activity at all. However, development of ACE inhibition power of RJ was newly observed by pepsin hydrolysis (IC_{50}=0.358 mg protein/mL), and the subsequent trypsin and chymotrypsin hydrolyses (IC_{50}=0.099 mg protein/mL). Single oral administration of this gastrointestinal RJ hydrolysate (1 g/kg dose) in 10-week spontaneously hypertensive rat resulted in a significant reduction of systolic blood pressure of 22.7 ± 3.6 mmHg at 2 hr ($P<0.05$ vs. 0 hr by one-way ANOVA, $n=7$). Then, the RJ hydrolysate was fractionated with gel permeation chromatography to obtain the di- and tri-peptides (DTP) fraction. As a result of isolation from the DTP fraction by reversed phase-high performance liquid chromatography, eleven ACE inhibitory peptides were isolated from the DTP-RJ hydrolysate. Some of the ACE inhibitors were derived from the RJ-glycoprotein; eight peptides with the IC_{50} value of <10 μM were identified from natural resources for the first time. Consequently, RJ protein was thought to be a good resource of ACE inhibitory peptides produced by the gastrointestinal enzyme hydrolyses.

Keywords: ACE inhibition; Peptide; Hypertension; Royal jelly; Hydrolysis

1. Introduction

Royal jelly (RJ) of honeybee *Apis mellifera* L. is a yogurt-like bee milk secreted by young bee workers, and is traditionally known to have some diverse nutritional and/or pharmacological functions such as hypotensive activity, antitumor activity, insulin-like action, disinfectant action, and so on [1,2]. As for the disinfectant action by RJ, antibacterial protein has been clarified as Royalisin, which consists of 51 amino acid residues with three disulfide bonds [3]. On the other hand, few researches on hypotensive activity of RJ have been performed. In vitro antihypertensive ability of RJ was investigated by Okuda et al [4], in which they reported that trans-2-octenoic acid and trans-10-hydroxy-2-decenoic acid were involved in the blood pressure (BP) regulation. However, in vivo hypotensive effect of these unsaturated fatty acids would be in doubt due to their gastrointestinal instability or poor in vitro angiotensin I-converting enzyme (ACE, EC 3.4.15.1) (inhibitory ratio; <50% at >1 mM of trans-10-hydroxy-2-decenoic acid) [4]. Thus, in the present study, we have tried to clarify which RJ constituents elicit a physiological function on hypotensive effect.

At the BP regulation system in the body, renin-angiotensin (RA) system is well known to play an important physiological role in the circulatory and/or localized organs. Therefore, pharmatherapeutic examinations to suppress the BP promotion in hypertensives have been attempted by inhibiting the production of pressor active angiotensin (Ang) II or by retarding the catalytic action of ACE [5]. A lot of hypotensive food components have been isolated for achieving the prophylaxis of hypertension [6–8]. In a series of our studies [9–12], we have successfully isolated many natural ACE inhibitory peptides from food resources such as sardine muscle [9] and wheat germ [10]. As for the sardine muscle hydrolysate [11,12], it was demonstrated

* Corresponding author. Tel.: +81 92 642 3012; fax: +81 92 642 3012.

0955-2863/02/$ – see front matter
PII: S0955-2863(01)00198-X

that the intake for 4-week protocol induced potent antihypertensive effect in mild hypertensives as well as casein hydrolysate [13]. This BP lowering in the human study was proved to be due to the suppression of Ang II and aldosterone productions in the circulating RA system [12] by one of the prominent ACE inhibitors, Val-Tyr, in the hydrolysate [14,15]. Thus, the intake of bioactive peptides with ACE inhibitory activity seems to be great for regulating BP system.

From the point of this view, the present study tried to identify ACE inhibitory peptides from RJ protein in order to clarify the potential depressor action of RJ sample. In vivo depressor action of RJ sample was also investigated by using spontaneously hypertensive rat (SHR).

2. Materials and methods

2.1. Materials

Intact Thai royal jelly (RJ) was supplied from Yamada Apiculture Center Inc. (Okayama, Japan). Enzymes used in this study were *porcine gastric mucosa* pepsin, *bovine pancreas* chymotrypsin, and *bovine pancreas* trypsin from Boehringer Mannheim (Tokyo, Japan). Purified rabbit lung ACE was purchased from Sigma (MO, USA). Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) as a synthetic ACE substrate was from Peptide Institute (Osaka, Japan). Superdex Peptide HR 10/30 column (10 × 300 mm) was purchased from Pharmacia Biotech (Uppsala, Sweden), and Cosmosil 5C18-ARII and Cosmosil 5Ph columns (each 4.6 × 250 mm) were from Nacalai Tesque (Kyoto, Japan), respectively. All other reagents used in this study were purchased from Nacalai Tesque.

2.2. Assay for ACE inhibitory activity

ACE inhibitory activity was measured by a modified Lieberman's method as described by Yamamoto et al [16]. Briefly, 25 μL of ACE inhibitor and 50 μL of 12.5 mM Hip-His-Leu in a borate buffer (pH 8.3) containing 200 mM NaCl were incubated with 50 μL of ACE (25 mU/mL) at 37°C for 1 hr. The reaction was stopped by adding 125 μL of 0.5 M HCl, followed by the addition of 750 μL of ethyl acetate (AcOEt). After the extraction of hippuric acid with AcOEt, 250 μL of AcOEt layer was dried under reduced pressure, and redissolved in 1.5 mL of 300 mM NaCl solution. After mixing, the absorbance of the produced hippuric acid at 228 nm was measured with a Shimadzu UV-1200 spectrophotometer (Kyoto, Japan). The experimentally obtained ACE inhibitory ratios (%) at different concentrations of inhibitor (average value from three determinations at each concentration) were used to calculate the IC_{50} value. The ACE inhibitor concentration required to inhibit 50% of the ACE activity under the assayed conditions was defined as the IC_{50} value. Total ACE inhibitory activity was defined as the quotient of the weight of inhibitor or hydrolysate divided by its IC_{50} value [10].

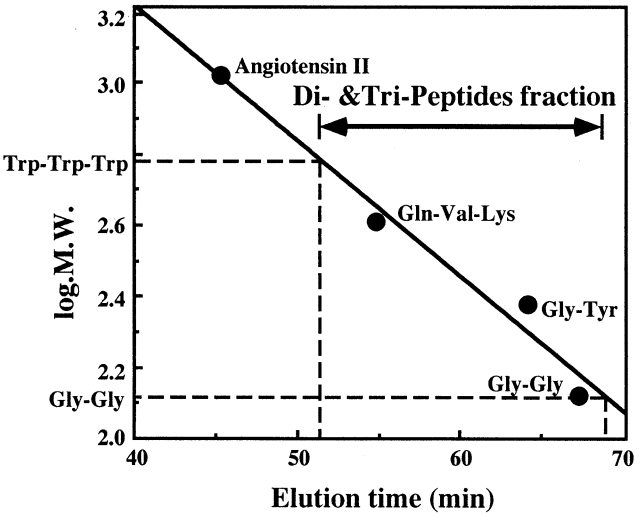

Fig. 1. Calibration of Superdex Peptide HR 10/30 GPC column by using standard peptides. The elution was performed with 30% CH_3CN in 0.1% TFA at a flow rate of 0.3 mL/min (35°C). Di- and tri-peptide fraction eluted between the time of 52 and 69 min was collected.

2.3. Enzymatic hydrolysis of RJ sample

Three grams of intact RJ sample denoted as I-RJ was dissolved in 30 mL of deionized water. Firstly, the I-RJ solution adjusted to pH 1.2 with 20% HCl was subjected to the peptic hydrolysis (P-RJ, 0.4 mg/mL of pepsin for 4 hr at 37°C). Secondly, the prepared P-RJ solution was readjusted to pH 6.8 by adding 20% NaOH and then hydrolyzed with chymotrypsin (0.2 mg/mL) and trypsin (0.2 mg/mL) (PCT-RJ) for 4 hr at 37°C. After heating for 15 min in a boiling-water bath, and centrifuged at 8,500 *g* for 15 min, the supernatant was filtered with Toyo filter paper (No.1, Toyo Roshi). The filtrate was subjected to the lyophilization for ACE inhibitory study. The yield of PCT-RJ from 3 g of I-RJ was 1.2 g.

2.4. Preparation of di- and tri-peptides fraction from RJ hydrolysate

The PCT-RJ (10 mg/mL) was applied with high performance liquid chromatography (HPLC, Shimadzu LC-9A instrument, Kyoto, Japan) on a Superdex Peptide HR 10/30 column (10 × 300 mm) to obtain the fraction rich in di- and tri-peptides (DTP). The elution was performed with 30% acetonitrile (CH_3CN) in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.3 mL/min (35°C), while monitoring the absorbance at 220 nm. The DTP fraction was obtained by using the calibration curve of logarithmic molecular weight against elution time (Figure 1), in which the fraction between the time of 52 and 69 min corresponding to the elution time of Trp-Trp-Trp and Gly-Gly, respectively, was collected.

2.5. Purification of ACE inhibitory peptides

ACE inhibitors were purified from the DTP fraction of PCT-RJ sample by two-step reversed HPLCs. In the first

step, the fraction was applied on a Cosmosil 5Ph column (4.6 × 250 mm) and eluted with a linear CH_3CN gradient (1–40%, 60 min) at a flow rate of 0.3 mL/min, while monitoring the absorbance at 220 nm. The fractions with ACE inhibitory activity were combined and concentrated, and then subjected to the final HPLC separation step (Cosmosil 5C18-ARII, 4.6 × 250 mm). The elution was done in the linear gradient mode of CH_3CN (5–35%, 60 min) in 0.1% TFA at a flow rate of 0.3 mL/min.

2.6. Amino acid analysis

The amino acid composition was analyzed with a Shimadzu LC-10A amino acid analyzer after hydrolysis of 6 M HCl for 24 hr at 110°C. The amino acid sequence was analyzed by automated Edman degradation using a Shimadzu PPSQ-21 protein sequencer.

2.7. Single oral administration of RJ hydrolysate in SHR

Seven SHRs (8-week-old male SHR/NCrj, Charles River Japan, Kanagawa) in each control (saline) and PCT-RJ group were fed a laboratory diet (CE-2, Clea Japan, Tokyo) and given water *ad libitum*. All rats were individually housed for 2 weeks at 21 ± 1°C and 55 ± 5% humidity under controlled lighting from 8:30 to 20:30. Single oral administration of PCT-RJ sample was done in 10-week SHR (269.0 ± 2.6 g), in which the dosage of 1.0 g/kg dissolved in 1 mL of saline solution was injected by intubation with nutritional catheter. Control rats were administered with the same volume of saline solution. SBP and heart rate (HR, beats/min) were measured 0, 2, 4, 6 and 8 hrs after the administration. The BP measurement was performed by the tail-pulse pick up method with a Softron BP system (Softron BP-98A, Tokyo, Japan) after warming the rat in a warm holder kept at 39.0 ± 0.5°C for 10 min.

2.8. Data analysis

Each result for administration study in SHR is expressed as the mean of SBP (mmHg)± SEM (%) of three successive BP measurements. Statistical differences of SBP in control and PCT-RJ groups at each administration time were evaluated by the repeated measures one-way ANOVA. P values <0.05 were considered to be significant.

3. Results

3.1. ACE inhibitory action of RJ sample

I-RJ sample was tested for ACE inhibitory study. As shown in Table 1, I-RJ sample did not inhibit ACE activity at all, although a specific unsaturated fatty acid in it brought about the in vitro ACE inhibition [4]. This indicated that there present no or sufficient active compounds to inhibit ACE in I-RJ within our in vitro experimental conditions. The protein fraction of I-RJ prepared by the precipitation over 10% trichloroacetic acid was also found to be entirely inactive toward the ACE inhibition. On the other hand, successful activation of inactive I-RJ sample was achieved by gastrointestinal enzyme treatments. After the peptic hydrolysis, ACE inhibitory action was newly observed with the IC_{50} value of 1.235 mg/mL. Subsequent chymotryptic and tryptic hydrolyses allowed the ACE inhibitory power to increase by a factor of 4 (IC_{50} value; 0.353 mg/mL) (Table 1). These findings strongly suggested that some newly ACE inhibitors were produced from I-RJ proteins; the IC_{50} values for P-RJ and PCT-RJ samples were 0.358 and 0.099 mg protein/mL, respectively.

Table 1
Change in ACE inhibitory activity of royal jelly sample with various protease treatments

Treatment	IC_{50} value	
	(mg/mL)	(mg protein/mL)
Intact (I-RJ)	NI	NI
TCA	NI	NI
Pepsin (P-RJ)	1.235	0.358
Pepsin → Chymotrypsin & Trypsin (PCT-RJ)	0.353	0.099

Intact RJ sample was hydrolyzed with 0.4 weight % of pepsin for 4 hr, and subsequently with 0.2 weight % of chymotrypsin and trypsin for 4 hr at 37°C. Ten % of trichloroacetic acid (TCA) treatment was done to obtain the protein fraction of RJ sample. NI-no inhibition.

3.2. Isolation and identification of ACE inhibitors

To clarify any active peptides concerning the suppression of Ang II production, partial purification of PCT-RJ hydrolysate on a Superdex Peptide HR 10/30 column was performed according to peptide length. Under the elution condition of 30% CH3CN in 0.1% TFA the DTP fraction that would be preferably absorbed at the small intestine rather than the longer peptides was successfully collected as shown in Figure 2. The yield of the DTP fraction from PCT-RJ hydrolysate was 3.3%, and the IC_{50} value was 0.104 mg/mL. In addition, the magnitude of the ACE inhibitory contribution of DPT fraction to the overall inhibition of PCT-RJ hydrolysate was estimated to be 11.3%.

The DTP fraction was then subjected to the purification on a Cosmosil 5Ph column. As shown in Figure 3, 45 active fractions with ACE inhibitory activity between the elution time of 20 and 80 min were collected individually, followed by the final reversed HPLC purification. Each active fraction was then applied to the reversed HPLC with Cosmosil 5C18-ARII column. Figure 4 shows the example of HPLC purification of active peak eluted at 43 min on the above column. Consequently, 11 peaks were isolated from the DTP fraction. As a result of amino acid and sequence analyses, 8 peaks except for Phe-Tyr,6 Ile-Phe,21 and Ile-Val-Tyr10 were identified as a natural ACE inhibitory peptide for the first time (Table 2). The

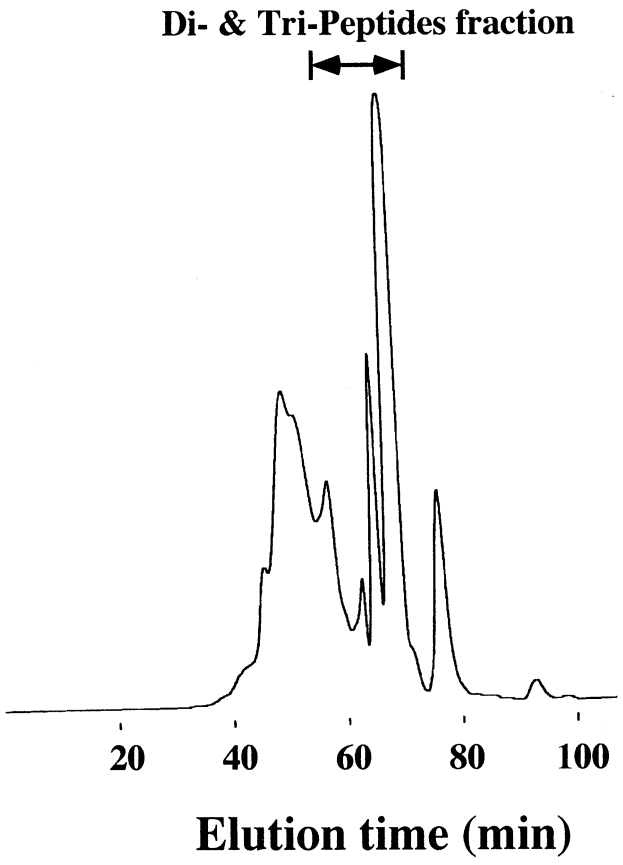

Fig. 2. Elution profile of RJ hydrolysate by gastrointestinal proteases on Superdex Peptide HR 10/30 GPC column. The elution conditions were the same as in Figure 1.

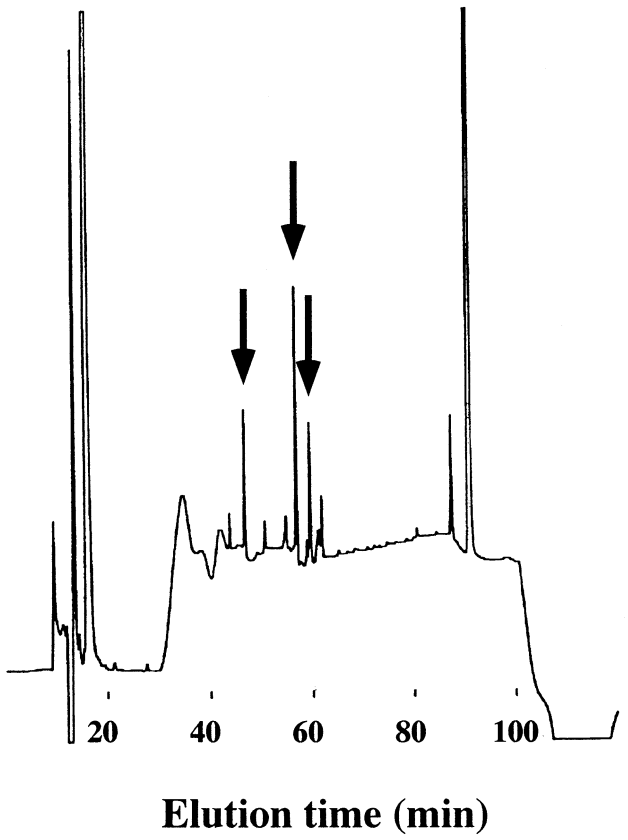

Fig. 4. Final purification of active fraction eluted at 43 min on Cosmosil 5Ph column with Cosmosil 5C18-ARII column. Arrowed peaks with ACE inhibitory activity were isolated. The elution was done in the linear gradient mode of CH_3CN (5–35%, 60 min) in 0.1% TFA at a flow rate of 0.3 mL/min.

retention time of each identified peptide on the final HPLC condition was also listed in Table 2. As predicted in the elution profile on a Superdex Peptide HR 10/30 column, all of the identified peptides were di- and tri-peptides. Most peptides possessed potent ACE inhibitory activity of less than 10 μM of IC_{50} value. The most powerful ACE inhibition was observed for Ile-Val-Tyr (IC_{50}; 0.48 μM), which has been already identified from the wheat germ hydrolysate [10]. According to the magnitude of the ACE inhibitory contribution of each peptide to the overall inhibition of DTP fraction in PCT-RJ, Ile-Val-Tyr (16.9%), Asp-Gly-Leu (10.4%) and Leu-Thr-Phe (6.29%) would be the main contributors in the DTP fraction.

3.3. Antihypertensive effect of PCT-RJ sample on SHR

The prepared PCT-RJ sample was subjected to the single oral administration study in 10-week SHR. As shown in Figure 5, a marked SBP reduction of 22.7 mmHg at 2 hr after administration was observed (SBP_{0hr}; 176.4 $\pm$ 2.7 mmHg, SBP_{2hr}; 153.7 $\pm$ 4.0 mmHg, $P<0.05$, $n=7$), and the effect was maintained for 6 hr. Thereafter, the SBP (SBP_{8hr}; 162.9 $\pm$ 6.5 mmHg, $P>0.05$) tended to recover to the SBP_{0hr}. These depressor actions at 4 and 6 hr after the PCT-RJ infusion were also significant against control (saline) group. During this protocol, HR did not change significantly as shown in Figure 5 (e.g., HR_{0hr}; 397.8 $\pm$ 10.8 beats/min, HR_{2hr}; 373.1 $\pm$ 20.8 beats/min).

4. Discussion

Some peptides, in particular di- and/or tri- peptides, have found to reveal a practical in vivo antihypertensive effect in human [12,13]. Hata et al [13] reported the efficacy of Val-Pro-Pro and Ile-Pro-Pro on BP regulation, in which they speculated that the effect would be caused by the circulatory ACE inhibition as well as their stimulation in aortas [17]. We have also provided the evidence that the intake of bioactive di-peptide, Val-Tyr, derived from sardine muscle hydrolysate resulted in a significant SBP reduction of 9.7 mmHg after 1 week on mild hypertensive subjects [12]. These findings strongly suggested that some bioactive smaller peptides may play an important role in regulating BP via suppression of the RA system.

In this study, I-RJ did not retard the action of ACE activity at all, as shown in Table 1. On the other hand, gastrointestinal protease hydrolysis of RJ by pepsin, fol-

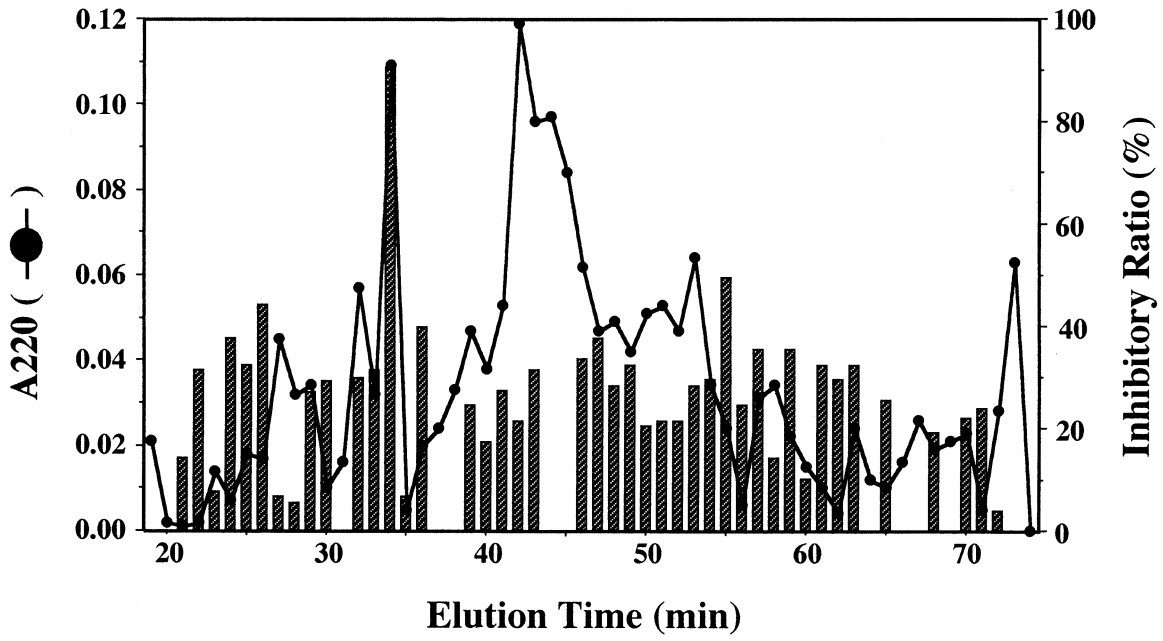

Fig. 3. Separation of ACE inhibitory fractions on Cosmosil 5Ph column. The elution was done in the linear gradient mode of CH_3CN (1–40%, 60 min) at a flow rate of 0.3 mL/min. Each fraction with ACE inhibition was collected individually.

lowed by chymotrypsin, and trypsin resulted in a potent ACE inhibition power of IC_{50} value of 0.099 mg protein/mL (Table 1), being comparable to sardine muscle hydrolysate with in vivo depressor effect (IC_{50}=0.083 mg protein/mL [11]). These findings revealed that inactive I-RJ proteins might be a good ACE inhibitor resource responsible for regulating BP, in which peptidic inhibitors newly produced by gastrointestinal proteases in the gut would be predominant. According to the report by Uno et al. [18], the intake of RJ hydrolysate by pepsin and trypsin decreased high cholesterol levels and increased hemoglobin levels in human. Thus, the RJ hydrolysate would be benefit for improving our homeostasis.

As a result of HPLC separation of PCT-RJ hydrolysate, eleven ACE inhibitory peptides were isolated, and eight peptides with IC_{50} value of <10 μM were newly identified (Table 2). RJ proteins were reported to be mainly composed of glycoproteins [19–21] except for Royalisin [3] with molecular mass of 5,523 Da. Ohashi et al [21] proposed the deduced amino acid sequence of the 56 kDa protein by the

Table 2
Identification of ACE inhibitory peptides from di- and tri-peptide fractions of digested royal jelly

Amino acid sequence	Amino acid ratio in peptide	Retention time** (min)	IC_{50} (μM)	Yield (%)	Contributing ratio to the whole ACE inhibition (%)
Phe-Tyr*	Phe 1.00, Tyr 1.03	59.7	1.67	1.16×10^{-2}	2.20
Lys-Phe	Lys 1.00, Phe 1.01	48.3	116	3.14×10^{-2}	0.10
Ile-Phe*	Ile 1.54, Phe 1.00	60.1	930	0.104	0.04
Ile-Val-Tyr*	Ile 1.00, Val 1.02, Tyr 1.69	66.7	0.48	3.08×10^{-2}	16.9
Ile-Met-Tyr	Ile 1.25, Met 1.00, Tyr 1.12	56.7	1.80	2.05×10^{-2}	3.01
Asp-Gly-Leu	Asp 1.00, Gly 1.02, Leu 0.65	66.7	2.16	6.52×10^{-2}	10.4
Thr-Lys-Tyr	Thr 0.75, Lys 1.00, Tyr 0.87	46.7	2.31	9.13×10^{-3}	1.01
Leu-Thr-Phe	Leu 1.10, Thr 0.47, Phe 1.00	70.1	2.73	6.23×10^{-2}	6.29
Phe-Asn-Phe	Phe 2.00, Asn 1.32	76.1	6.91	5.54×10^{-2}	1.96
Ala-Val-Leu	Ala 1.00, Val 1.01, Leu 1.00	60.5	7.11	8.12×10^{-2}	4.20
Gly-Leu-Tyr	Gly 1.42, Leu 1.02, Tyr 1.00	59.7	8.84	2.16×10^{-2}	0.72
Total				0.493	46.8

Yield of each isolated peptide against the di- and tri-peptide fraction of PCT-RJ sample was calculated. The ratio was calculated from the magnitude of the ACE inhibition of each peptide to the overall inhibition of di- and tri-peptide fraction of PCT-RJ sample.

* Reported peptides; see references in No. 6, 21, and 10 for Phe-Tyr, Ile-Phe, and Ile-Val-Tyr, respectively.

** The HPLC retention time of each peptide was obtained on the Cosmosil 5C18-ARII column with the linear gradient mode of CH_3CN (5–35%, 60 min) in 0.1 % TFA at 0.3 mL/min.

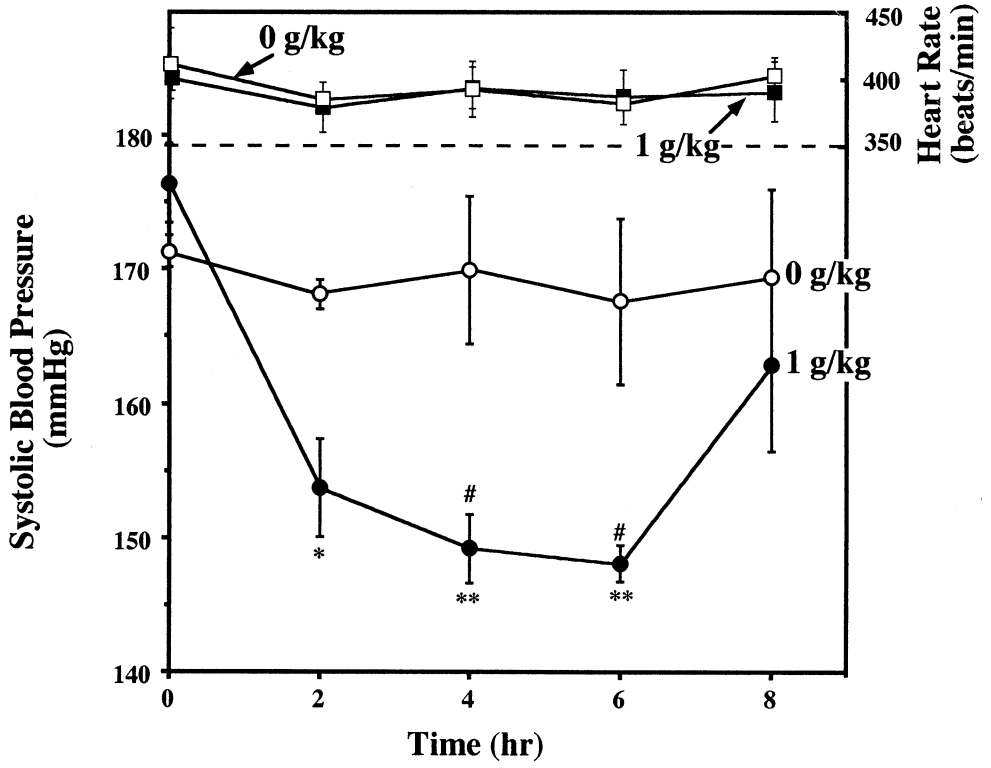

Fig. 5. Changes in systolic blood pressure and heart rate of 10-week SHR by administering PCT-RJ hydrolysate. Single oral administration was performed with the dose of 1.0 g/kg, and the SBP was measured 0, 2, 4, 6 and 8 hrs after the administration. Statistical differences of SBP in PCT-RJ group against before administration (*$P<0.05$ or **$P<0.01$), and against control (#$P<0.05$) were evaluated by the repeated measures one-way ANOVA. Data indicate mean ± SEM ($n=7$).

encoding cDNA. As a result of homology investigation of the isolated peptides with the sequence of 56 kDa protein, the amino acid sequences of Lys-Phe, Ile-Phe, Leu-Thr-Phe, Ile-Val-Tyr, and Phe-Asn-Phe were certainly found in the structure of the 56 kDa protein (38–39), (76–77), (155–157), (214–216), and (393–395), respectively. Although neither the susceptibility of RJ glycoproteins to gastrointestinal enzyme hydrolysis nor the homologies of other isolated peptides with glycoproteins were elucidated, the PCT-RJ ACE inhibitory peptides must be derived from the RJ glycoproteins. By considering that the total contribution ratio of all the identified peptides toward the DTP fraction in the PCT-RJ sample was 46.8%, these identified peptides may be involved in the half ACE inhibitions of the overall DTP fraction. Other ACE inhibitory peptides remained unclear due to low yield. Most of the isolated peptides had aromatic amino acid residues such as Tyr and Phe at the C-terminus, and strongly inhibited ACE activity. The inhibition behavior by these aromatic peptides was in agreement with the results by Cheung et al. [22], in which more favorable ACE inhibition was occurred for peptides with Trp>Tyr>Phe at the C-terminus. Interestingly, Ile-Val-Tyr with the IC_{50} value of 0.48 μM was isolated from the PCT-RJ hydrolysate with the highest ACE inhibitory contribution ratio of 16.9% as well as from the wheat germ hydrolysate [10]. It has been revealed that Ile-Val-Tyr with antihypertensive effect in SHR after intravenous administration would serve in the BP lowering owing to the combined depressor effect of itself and its metabolite, Val-Tyr, by aminopeptidases in rat and human plasma [23]. Thus, Ile-Val-Tyr was thought to be one of the main contributors in PCT-RJ hydrolysate toward the in vivo ACE inhibition.

We demonstrated the in vivo depressor effect of PCT-RJ hydrolysate in 10-week SHR (Figure 5). After oral administration of PCT-RJ sample (1 g/kg dose), a marked SBP reduction of 22.7 mmHg at 2 hr ($P<0.05$ vs. 0 hr) was found, and the effect continued for 6 hr. Favorable intact intestinal or portal absorption of di- and tri-peptides through the integral membrane peptide transporter [24] rather than amino acids has been made clear by many investigators [25–27]. We have also proved that Val-Tyr was absorbed intact into normotensive human blood [15]. Thus, the DTP fraction or identified ACE inhibitory peptides in the PCT-RJ sample might be the main candidate for this depressor action. However, further considerations on the direct involvement of the identified ACE inhibitory peptides (Table 2) in lowering BP would be needed, since some peptides are susceptible to hydrolytic degradations by intracellular peptidases or to translational modification such as sulfation [28] in the liver. The depressor ability of 22.7 mmHg was higher than that of the same dose of sardine muscle hydrolysate (13.4 mmHg) [29], though both preparations showed the

long term depressor effect for 6 hr-run experiment. The administration of sake lee hydrolysate also resulted in a significant and long term SBP reduction for 6 hr [30]. However, according to the fact that the $t_{1/2}$ of peptide, e.g., Ile-Val-Tyr was 1.5 hr in rat plasma [23], circulatory ACE inhibition induced by these natural peptide inhibitors was presumed to be short-term effect. Okunishi et al [31] have demonstrated that the prolonged depressor effect induced by the single intake of spirapril as a long term oral therapeutic drug was correlated to the suppression of ACE activities in blood vessels. This indicated that some of natural inhibitory peptides, in particular PCT-RJ peptides, could accumulate at the vessel, and exert a regulation of secretion of pressor active substances such as nitric oxide, endothelins, or prostaglandins [32]. Further studies on the influence of isolated natural peptides on various organs in SHR are now under investigation. Dose-dependency of PCT-RJ hydrolysate on antihypertensive effect is also investigated to estimate its in vivo depressor power.

Consequently, RJ protein was a latent natural resource with in vivo antihypertensive effect, and produced many ACE inhibitory peptides during the digestion after the intake so as to potentiate depressor effect.

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