

## Antihypertensive Activities of Royal Jelly Protein Hydrolysate and Its Fractions in Spontaneously Hypertensive Rats

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Angiotensin I-converting enzyme (ACE) inhibitory and hypotensive effects of 7 peptide fractions (Fr) of royal jelly protein hydrolysate (RJPH) were studied in comparison with those of RJPH alone. Fr 4 and Fr 5 were the highest in ACE inhibitory activity and yield, respectively. Molecular weights (MWs) of RJPH and Fr 1-Fr 7 were distributed from 100 to 5,000 and those of Fr 1-Fr 7 increased in order from Fr 1 to Fr 7. RJPH, Fr 3 and Fr 4 at doses of 10, 30 and 100 mg/kg i.v. and Fr 5 and Fr 6 at doses of 30 and 100 mg/kg i.v. caused transiently significant hypotensive effects in spontaneously hypertensive rats (SHR). Fr 3, Fr 4, Fr 5 and Fr 6 at a dose of 1,000 mg/kg also caused significant hypotensive effects 3h, 4-5h, 7-8h and 8h after oral administration in SHR, respectively. RJPH caused a long-lasting hypotensive effect in proportion to the magnitude of the MWs of RJPH fractions. The hypotensive pattern of RJPH was similar to the combined pattern of Fr 3-Fr 6. From these results, it can be concluded that the long-lasting hypotensive effect of oral administration of RJPH is dependent on the MWs of its ACE inhibitory peptides and the time required to digest them.

**Key words:** royal jelly, peptide, ACE inhibitory activity, hypotensive effect, spontaneously hypertensive rat

It is well known that hypertension is a major risk factor for cerebral stroke, heart failure, acute myocardial infarction and diabetic nephropathy. On the other hand, food products containing sardine dipeptide, lacto-tripeptides, or dried bonito oligopeptides, all of which exhibit angiotensin I-converting enzyme (ACE) inhibitory activity, have a hypotensive effect comparable to that of antihypertensive medications [1-3]. Therefore, the potential use of these products to maintain low blood pressure has been attracting attention in middle-aged and older people.

However, the results of one of the main studies supporting this use of peptide-containing foods have not been reproducible. In the study in question, the sardine peptide Val-Tyr and the lacto-tripeptide Ile-Pro-Pro were shown to have hypotensive effects in spontaneously hypertensive rats (SHR), and the lack of reproducible results may be related to differences in the methods used to measure blood pressure between this and subsequent studies [4]. Currently, therefore, there is insufficient information on the hypotensive effect of protein hydrolysate in *in vivo* studies, although many studies have shown that some peptides had potent ACE inhibitory activity.

Royal jelly of the honeybee *Apis mellifera L.* is a yogurt-like bee milk secreted by worker bees as food

for their queen, and is also used worldwide as a popular health food [5]. Royal jelly contains various proteins, including essential amino acids, sugar, fatty acids and vitamins [6]. Scientific reports have shown that royal jelly exhibits bioactivities such as antitumor activity, a preventive effect against osteoporosis, and amelioration of menopausal disorder [7–9]. The hypotensive effect of royal jelly has recently been studied in detail; that is, 11 ACE inhibitory peptides have been found in a pepsin/trypsin/chymotrypsin hydrolysate of royal jelly protein (RJP), and the hydrolysate decreased blood pressure in SHR [10]. Recently, a microbial protease-treated royal jelly protein hydrolysate containing ACE inhibitory peptides was shown to decrease blood pressure in SHR [11] and in humans [12] with high-normal blood pressure or mild hypertension in a 3-month ingestion test.

We have also studied a trypsin-treated royal jelly protein hydrolysate (RJPH) desalted by a column wash, which contains many kinds of ACE inhibitory peptides [13]. In this study, we compared the hypotensive effects of 7 peptide fractions separated from RJPH with that of RJPH alone in SHR in order to investigate the correlation between the hypotensive pattern of RJPH and the molecular weight of its peptides.

## Materials and Methods

**Animals and chemicals.** Male SHR aged 8–10 weeks were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All animals were maintained in an air-conditioned room with controlled temperature ( $23 \pm 2^\circ\text{C}$ ) and humidity (30–80%). They were housed individually in metal cages and kept under a light-dark cycle (lights on from 6:30 to 18:30), and they were fed with normal rat chow (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and given water *ad libitum*. The rats were 10–13 weeks old (body weight 267–324 g). All procedures involving animals were performed in accordance with the “Standards Relating to the Care and Management, *etc.* of Experimental Animals” issued by the Prime Minister’s Office, Japan. RJP was obtained from Yamada Apiculture Center, Inc. (Okayama, Japan) and Ile-Tyr was from Kokusan Chemical Co., Ltd. (Tokyo, Japan).

**Preparation of RJPH.** RJPH was prepared as previously reported [13]. Ethanol-denatured RJP was

dissolved in 10 volumes of water, and the solution was adjusted to pH 8.0 with 20% sodium hydroxide (NaOH) solution at  $37^\circ\text{C}$  and added to 0.25 wt% trypsin (Novozymes A/S, Bagsvaerd, Denmark), and reacted for 24 h. This reaction solution was heated at  $100^\circ\text{C}$  for 30 min to inactivate the enzyme, and then adjusted to pH 4.5 with 50% hydrochloric acid (HCl). The filtrate was subjected to a synthetic adsorbent Diaion HP20 (Mitsubishi Chemical Co., Tokyo, Japan) column and the peptides adsorbed were eluted with 80–100% ethanol. The elution was concentrated by an evaporator at  $40^\circ\text{C}$ , and then spray-dried to powder.

**Preparation of HP20-column fractions of RJPH.** The peptides from RJP were sequentially eluted with about 3,000 ml of 20, 40, 60, 80 and 100% methanol, monitoring the absorbance at 275 nm. The Frs were prepared as follows: aqueous washing, Fr 1 + Fr 2; 20% methanol elution, Fr 3; 40% methanol elution, Fr 4; 60% methanol elution, Fr 5; 80% methanol elution, Fr 6; and 100% methanol elution, Fr 7. Each Fr was concentrated using an evaporator at  $40^\circ\text{C}$ , followed by freeze drying, and the yields of the Frs were calculated.

**Measurement of ACE inhibitory activity.** ACE inhibitory activity was assayed according to the method of Lieberman, with some modifications [14]. In short, hippuric acid released from hippuryl-L-histidyl-L-leucine by ACE was extracted with ethyl acetate, and the concentration was measured by HPLC. The ACE inhibitory activity of each substance (%) was measured at the concentration of 0.5 mg/ml.

**Molecular weight (MW) distribution.** High performance gel filtration chromatography was performed with a Shimadzu LC-10A system (Shimadzu Co., Kyoto, Japan) under the following conditions: column, TSK 2000SW<sub>XL</sub> (7.8 mm I.D.  $\times$  30 cm, Tohso Co., Tokyo, Japan); mobile phase, 0.1% TFA-containing 45% acetonitrile; flow rate, 1 ml/min; column oven temperature,  $25^\circ\text{C}$ ; detection, UV 220 nm. The MW distribution was determined from the calibration curve plotted from the correlation between peptide and small MW-protein markers and the elution time.

**Measurement of blood pressure.** Blood pressure was measured by a direct measuring method through the left carotid artery under thiobutabarbital sodium (Inactin<sup>®</sup>; Wako, Ltd., Osaka, Japan) anes-

thetia (120 mg/kg, i.p.) using an amplifier (AP-641G; Nihon Kohden Co., Tokyo, Japan) and a pressure transducer (TY-400T; Nihon Kohden Co., Tokyo, Japan). For intravenous injection, 5 rats were used in each group, and test substances were injected into the femoral vein at a rate of 0.05 ml/min. Blood pressure and heart rate were measured for 30 min after injection. The ED<sub>20</sub> value was defined as the dose decreasing the systolic blood pressure by 20 mmHg. For oral administration, 7 rats were used in each group. The substances were administered at a volume of 10 ml/kg. Blood pressure and heart rate were measured for 24 h after administration.

**Statistical analysis.** Values are the means  $\pm$  standard error of the means (S.E.M.). Paired-*t* test and one-way analysis of variance followed by Dunnett's multiple comparison test were used to analyze the hypotensive effects by intravenous and oral administration, respectively. Values of  $p < 0.05$  were considered to indicate statistical significance.

## Results

**Preparation of HP20 fractions.** The chromatogram of HP20-column fractionation of RJPH using 0, 20, 40, 60, 80 and 100% methanol is shown in Fig. 1. The yields, ACE inhibitory activity and contribution rate (yield  $\times$  ACE inhibitory activity of each Fr/ACE inhibitory activity of RJPH) of Fr 1–Fr 7 are shown in Table 1. The yield of Fr 5 was the highest, followed by Fr 4. The yield of Fr 7 was very

low. The ACE inhibitory activity of Fr 4 was the highest, followed by Fr 5 and Fr 3. The ACE inhibitory activity of Fr 1 + Fr 2 was very low. There was no significant correlation between the MWs of RJPH in HP20 fractions and their ACE inhibitory activities. The inhibitory activity of RJPH before HP20 fractionation was  $49.6 \pm 4.3\%$ . The contribution ratio of Fr 5 was the highest, followed by Fr 4, Fr 3 and Fr 6. The contribution ratios of Fr 1 + Fr 2 and Fr 7 were very low. The MWs of RJPH and Fr 1–Fr 7 were almost all distributed from 100 to 5,000, and the MWs of the fractions increased in order from Fr 1 to Fr 7 (Fig. 2).

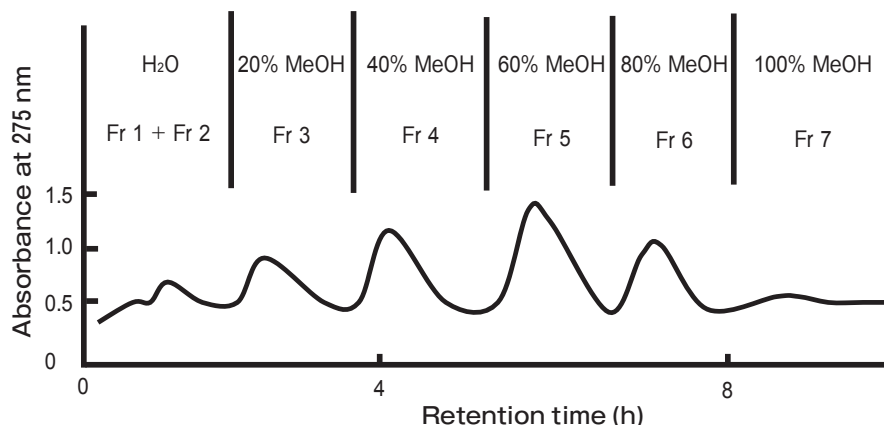
**Table 1** Yields, ACE inhibitory activity and contribution ratio of fractions separated from royal jelly protein hydrolysate

Fraction	Yield (%)	ACE inhibitory activity (%)	Contribution ratio (%)
RJPH	–	$49.6 \pm 4.3$	–
Fr 1 + Fr 2	$16.5 \pm 12.1$	$10.0 \pm 5.2$	3.3
Fr 3	$14.8 \pm 2.0$	$59.1 \pm 4.0$	17.6
Fr 4	$21.6 \pm 2.4$	$65.0 \pm 3.7$	28.2
Fr 5	$27.0 \pm 1.9$	$59.8 \pm 1.9$	32.5
Fr 6	$15.1 \pm 2.7$	$44.8 \pm 0.4$	13.6
Fr 7	$0.5 \pm 0.1$	$38.8 \pm 13.6$	0.4

Values are the means  $\pm$  S.E.M (n=3). The ACE inhibitory activity of each substance was measured at the concentration of 0.5 mg/ml.

Fr, fraction; RJPH, royal jelly protein hydrolysate.

Contribution ratio: yield  $\times$  ACE inhibitory activity of each Fr/ACE inhibitory activity of RJPH.



**Fig. 1** Chromatogram of royal jelly protein hydrolysate fractionated by methanol-stepwise elution using an HP20 column. Fr, fraction; MeOH, methanol.

**Hypotensive effects by intravenous administration.** When Fr 3–Fr 6 were administered intravenously, systolic blood pressure decreased in a transient and dose-dependent manner, reaching the lowest level 1 min after administration and recovering to the previous level within 5 min. Significant decreases of Fr 3 and Fr 4 were observed at doses of 10, 30 and 100 mg/kg. Fr 5 and Fr 6 showed significant effects at doses of 30 and 100 mg/kg (Table 2). However, the hypotensive effects of Fr 3 and Fr 4 were slightly higher than those of Fr 5 and Fr 6, almost the same ED<sub>20</sub> values were observed in Fr 3–Fr 6. RJPH also significantly and transiently decreased blood pressure at doses of 10, 30 and 100 mg/kg. The effect was slightly lower than those of Fr 3–Fr 6, but almost the same ED<sub>20</sub> values were observed in RJPH and Fr 3–Fr 6. There was no correlation between the MWs of Fr 3–Fr 6 and their hypotensive effects. Transient hypotensive activities were also observed by Ile-Tyr at a dose of 10 mg/kg, but this effect was not significant. Heart rates were not remarkably affected by the intravenous administrations of RJPH, Fr 3–Fr 6 and Ile-Tyr (Table 2).

**Hypotensive effects by oral administration.** The time course of systolic blood pressure by oral administrations of RJP, Ile-Tyr, RJPH and Fr 3–Fr 6 is shown in Fig. 3. Blood pressure slowly decreased with time by about 20 mmHg from the previous level after 8 h in the control group. Almost identical changes were observed in the RJP at a dose of 1,000 mg/kg. Ile-Tyr at a dose of 300 mg/kg had no significant effect, but Ile-Tyr at a dose of 1,000 mg/kg significantly decreased blood pressure at 3 h after administration. RJPH and Fr 3–Fr 6 showed hypotensive effects in a dose-dependent manner at doses of 300 and 1,000 mg/kg. At a dose of 300 mg/kg, RJPH, Fr 3, Fr 4 and Fr 6 had no significant effect, but Fr 5 showed a significant effect after 8 h. On the other hand, RJPH at a dose of 1,000 mg/kg decreased blood pressure gradually with time and a significant decrease was observed 7 and 8 h after administration. Fr 3 at a dose of 1,000 mg/kg decreased blood pressure at 2–4 h after administration, reaching the maximum hypotensive effect at 3 h; this effect was statistically significant. Fr 4 at a dose of 1,000 mg/kg also decreased blood pressure at 3–6 h, reaching the lowest level at 5 h. A significant effect was observed after 4 and 5 h. Fr 5 at a dose of 1,000 mg/kg caused a gradual

decrease in blood pressure after administration, with the lowest blood pressure occurring after 8 h; the results after 7 and 8 h were statistically significant. Fr 6 at a dose of 1,000 mg/kg decreased blood pressure at 4–8 h after administration, showing a significant and maximum decrease after 8 h. RJPH caused a long-lasting hypotensive effect in proportion to the magnitude of the MWs of RJPH fractions. Heart rates were not markedly affected by the oral administrations of RJPH, Fr 3–Fr 6 and Ile-Tyr (Fig. 4).

## Discussion

In the present study, it was found that the yields of Fr 3 (20% methanol elution), Fr 4 (40% methanol elution), Fr 5 (60% methanol elution) and Fr 6 (80% methanol elution) were high, and accounted for about 90% of RJPH. On the other hand, the ACE inhibitory activity of these fractions was also high. Contribution ratios, which indicate the rate of ACE inhibitory activity in each Fr to that of RJPH, were also high in Fr 4 and Fr 5, accounting for about 60% of RJPH. From these findings, it was thought that active components of RJPH were dissolved in 20–80% methanol. As described above, the MWs of RJPH and Fr 1–Fr 7 were distributed from 100 to 5,000, and the MWs were successively higher from Fr 1 to Fr 7. In other words, each Fr separated from RJPH was eluted with an increase in the methanol concentration. It is well known that Fr 3 contains many low MW peptides, such as dipeptide and tripeptide, Fr 4 contains larger peptides and Fr 5 and Fr 6 contain peptides consisting of 6 or more amino acids.

It was also found that intravenous injection of RJPH and Fr 3–Fr 6 caused a dose-dependent and transient hypotensive effect, and the hypotensive effects of these Frs were similar. Ile-Tyr used as a reference drug also showed a transient hypotensive effect, and similar short transient hypotensive effects have been reported after intravenous injection of Ile-Lys-Pro (10 mg/kg [15]), Ile-Tyr (10 mg/kg [15]), and Leu-Arg-Pro (30 mg/kg [16]). The findings suggest that these peptides were incorporated into the blood, and were rapidly (within 5 min) metabolized and degraded by peptidase in the blood.

Oral administration of Fr 3, Fr 4, Fr 5 and Fr 6 at a dose of 1,000 mg/kg also resulted in lower blood pressure, and a significant hypotensive effect was

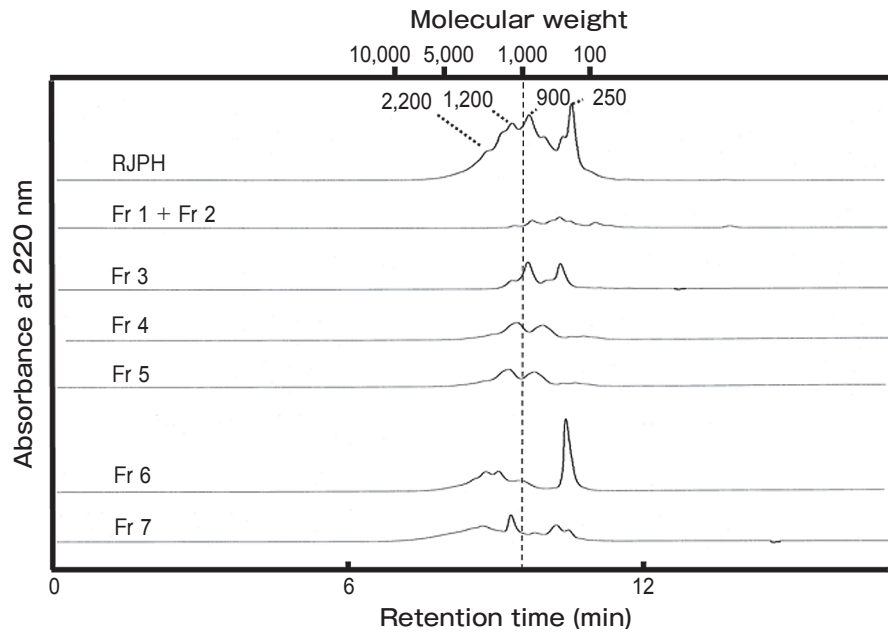
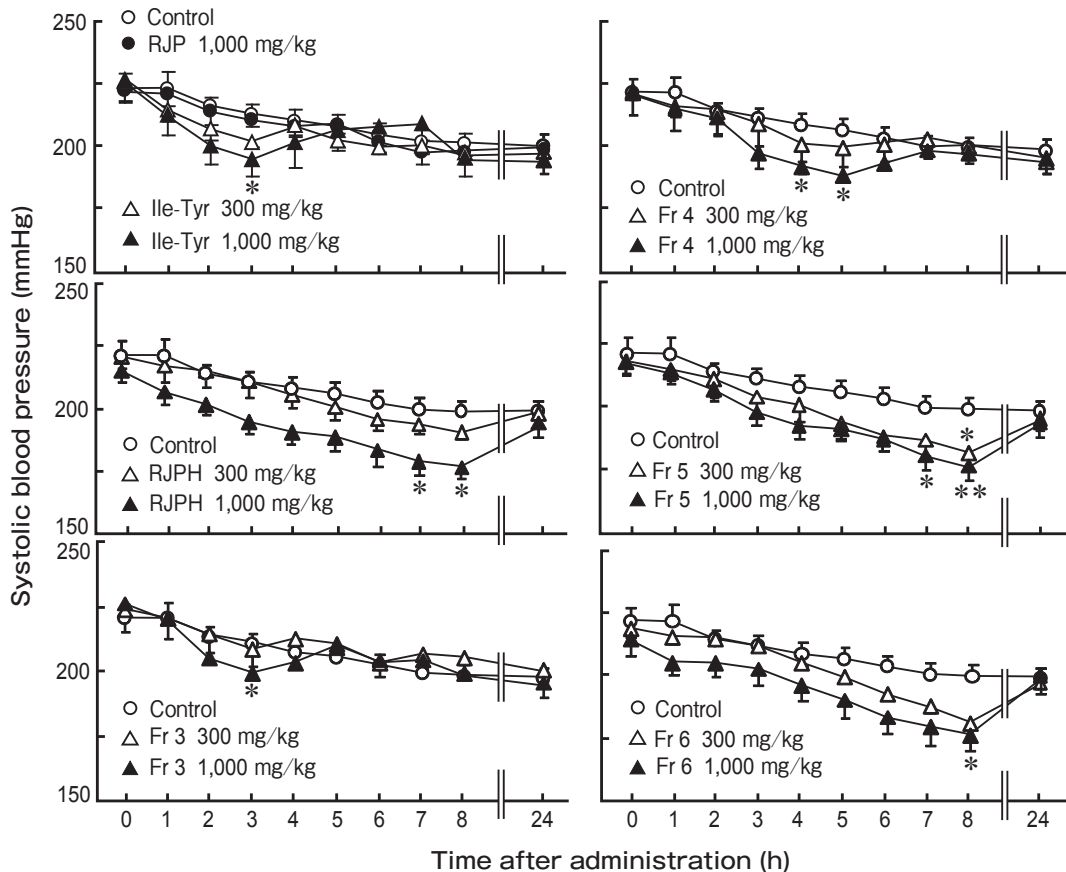


Fig. 2 High performance gel filtration chromatography of royal jelly protein hydrolysate and its fractions Fr, fraction; RJPB, royal jelly protein hydrolysate.

Table 2 Effects of intravenous injections of royal jelly protein hydrolysate and its fractions on blood pressure and heart rate in anesthetized SHR

Test sample	Dose (mg/kg, i.v.)	Systolic blood pressure (% of previous value)		ED <sub>20</sub> (mg/kg)	Heart rate (% of previous value)	
		After 1 min	After 5 min		After 1 min	After 5 min
Control	—	100.2 ± 1.0	99.3 ± 0.1	—	98.2 ± 0.4	98.6 ± 0.6
RJPB	10	94.0 ± 1.4*	103.4 ± 3.0	18.9	100.4 ± 0.1	98.2 ± 1.6
	30	83.9 ± 2.7**	111.8 ± 3.2		100.8 ± 0.2	101.2 ± 1.5
	100	81.4 ± 4.7*	114.1 ± 2.8		100.9 ± 0.1	102.7 ± 0.9
Fr 3	10	92.6 ± 2.3*	99.9 ± 1.1	18.7	100.8 ± 0.7	99.5 ± 0.3
	30	79.7 ± 5.8*	105.7 ± 3.7		97.9 ± 2.3	98.0 ± 0.5
	100	67.8 ± 1.5**	102.1 ± 2.1		100.8 ± 0.7	102.7 ± 2.1
Fr 4	10	91.2 ± 2.7*	99.3 ± 2.1	19.3	101.1 ± 0.3	99.8 ± 0.7
	30	81.3 ± 5.2*	105.0 ± 0.9		101.4 ± 0.5	101.9 ± 0.9
	100	75.5 ± 2.3**	104.6 ± 2.3		101.9 ± 0.6	101.8 ± 1.5
Fr 5	10	93.6 ± 2.1	101.2 ± 0.7	20.2	100.8 ± 0.4	99.2 ± 0.5
	30	85.1 ± 4.0*	101.7 ± 2.4		101.6 ± 0.4	99.9 ± 1.4
	100	75.6 ± 3.9**	102.3 ± 1.2		99.2 ± 1.8	100.0 ± 1.8
Fr 6	10	93.1 ± 3.2	107.7 ± 3.7	19.6	97.8 ± 2.9	100.2 ± 1.9
	30	83.6 ± 2.0**	105.8 ± 2.3		100.4 ± 2.8	96.9 ± 1.2
	100	75.5 ± 0.9**	112.1 ± 1.2		101.4 ± 0.9	100.4 ± 0.6
Ile-Tyr	10	87.4 ± 6.8	104.2 ± 1.0	—	99.7 ± 0.2	99.6 ± 1.1

Values are the means ± S.E.M. of 5 rats. The control group was injected with physiological saline. SHR were anesthetized before measurement of blood pressure and heart rate. RJPB, royal jelly protein hydrolysate; ED<sub>20</sub> (mg/kg), the dose to decrease systolic blood pressure by 20 mmHg. \**p* < 0.05 (v.s. previous level, paired *t*-test), \*\**p* < 0.01 (v.s. previous level, paired *t*-test).



**Fig. 3** Effects of oral administration of royal jelly protein, Ile-Tyr, royal jelly protein hydrolysate and Fr 3 and Fr 6 on blood pressure in anesthetized SHR. Values are the means  $\pm$  S.E.M. of 7 rats. SHR were anesthetized before measurement of blood pressure. RJP, royal jelly protein; RJPH, royal jelly protein hydrolysate; Fr, fraction. \* $p < 0.05$  (v.s. control, Dunnett's multiple comparison test), \*\* $p < 0.01$  (v.s. control, Dunnett's multiple comparison test).

observed after 3h in Fr 3, 4–5h in Fr 4, 7–8h in Fr 5 and 8h in Fr 6. These findings indicate that the time of decrease in blood pressure was delayed with an increase in the MWs of these peptides. It is well recognized that some short-acting peptides, such as dipeptide and tripeptide, are absorbed directly, and many long peptides are first digested and subsequently absorbed as shorter fragment peptides or amino acids [17], which is the reason why long peptides such as Fr 5 and Fr 6 showed a delayed hypotensive effect. RJPH also caused a potent hypotensive effect 1–8h after administration, and a significant difference was observed at 7 and 8h. The hypotensive effect of RJPH was attributed to trypsin treatment of RJP, because RJP alone did not show such an effect. By comparing the hypotensive effect of RJPH and Fr 3

and Fr 6, it was found that the hypotensive pattern of RJPH closely resembled to the combined pattern of Fr 3–Fr 6.

Crude royal jelly contains protein of about 13%, which is about 1/6 the content of RJP. However, as shown in Fig. 3, RJP showed no hypotensive effect even at a dose of 1,000mg/kg. RJPH is a purified substance produced by trypsin hydrolyzation and desalting of RJP, and this compound caused a hypotensive effect at a dose of 1,000mg/kg. It seems likely, therefore, that crude royal jelly showed no hypotensive effect even at a dose of 6,000mg/kg.

On the other hand, Ile-Tyr, which was used as a reference, caused a slight hypotensive effect 3h after oral administration at a dose of 300mg/kg, but it was not significant. Fujita *et al.* [15] reported much more

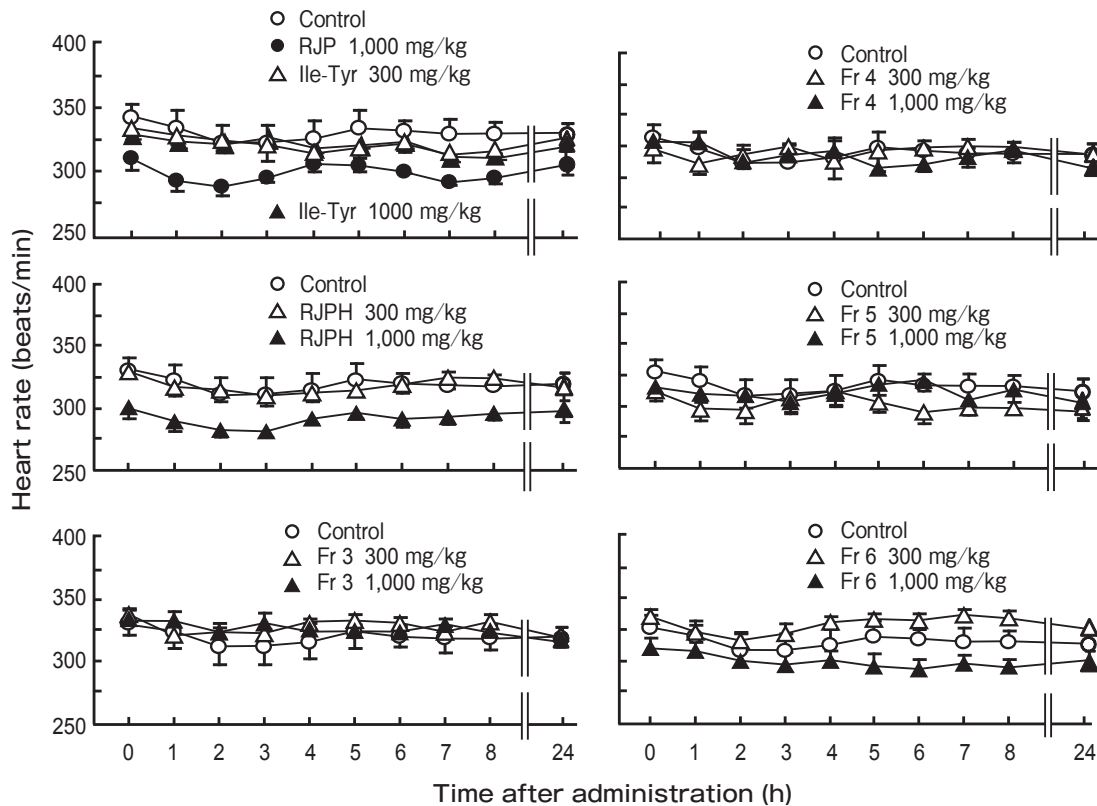


Fig. 4 Effects of oral administration of royal jelly protein, Ile-Tyr, royal jelly protein hydrolysate and Fr 3 and Fr 6 on heart rate in anesthetized SHR. Values are the means  $\pm$  S.E.M. of 7 rats. SHR were anesthetized before measurement of heart rate. RJP, royal jelly protein; RJPH, royal jelly protein hydrolysate; Fr, fraction.

potent effects of Ile-Tyr; in their study, Ile-Tyr induced significant hypotension even at a dose of 60 mg/kg. At present, we are uncertain as to why Ile-Tyr caused such a potent hypotensive effect in their study, but the discrepancy may be related to differences in the methods used to measure blood pressure.

In the present work, RJPH caused a long-lasting hypotensive effect in proportion to the magnitude of MWs of RJPH fractions. From these findings, it can be concluded that the long-lasting hypotensive effect of RJPH is related to the MW of its ACE inhibitory peptides and the time required to digest them, and that Fr 4 and Fr 5 make the main contributions to the persistent hypotensive effect of orally administered RJPH.

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