Antihypertensive Effect of Peptides from Royal Jelly in Spontaneously Hypertensive Rats

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We have shown that Protease N treated Royal Jelly (ProRJ) and peptides from ProRJ (Ile-Tyr (IY), Val-Tyr (VY), Ile-Val-Tyr (IVY)) inhibited angiotensin I-converting enzyme (ACE) activity and they have an antihypertensive effect in repeated oral administration for 28 d on spontaneously hypertensive rats (SHR). We investigated the contributive ratio of these peptides in ProRJ for antihypertensive effect in single oral administration on SHR. In single oral administration of each peptide and peptides mixture (MIX; IY, VY and IVY) at doses of 0.5, 1 and 10 mg/kg, systolic blood pressure (SBP) of SHR was reduced dose-dependently. This antihypertensive effect was held for 8 h. These results suggest that peptides contributed to the antihypertensive effect of ProRJ. And the contributive ratio of MIX in ProRJ for antihypertensive effect was computed to be about 38%. Therefore it is considered that intake of peptides, as a functional food would be beneficial for improving blood pressure in people with hypertension.

Key words Royal Jelly; antihypertensive effect; angiotensin I-converting enzyme activity; spontaneously hypertensive rat

Royal Jelly (RJ) from the honey bee, *Apis mellifera*, is a popular traditional health food all over the world. Components of RJ are proteins, sugar, fatty acids including 10-hydroxy-2-decenoic acid, and so on.[1] RJ has several pharmacological functions such as hypotensive activity, insulin-like action,[2] antitumor activity,[3] anti-allergic activity,[4] vasodilative activity[5] and so on. We have shown that Protease N treated RJ (ProRJ) and peptides (IY, VY, IVY) from ProRJ inhibited angiotensin I-converting enzyme (ACE) activity[6] and they have an antihypertensive effect in repeated oral administration for 28 d on spontaneously hypertensive rats (SHR).[7] Their ACE inhibitory activity (IC_{50}) of IY, VY and IVY are 0.008, 0.020 and 0.018 (mg/ml) respectively.[5]

Generally, the Renin-angiotensin (RA) system is well known to regulate blood pressure (BP) in the circulatory system and localized organs. ACE is important in the RA system, which regulates blood pressure. Captopril and Enalapril are known as antihypertensive drugs with ACE inhibitory activity. Therefore, it plays important role to investigate the regulation of the RA system by retarding the catalytic action of ACE. Recently, many ACE inhibitory peptides were found from food such as sour milk,[8] sardine,[9] wakame[10] and dried bonito[11] *etc*.

From this point of view, we quantified ACE inhibitory peptides such as IY, VY and IVY in ProRJ, in order to investigate contributive ratio of peptides in ProRJ for antihypertensive effect. In addition, we investigated the contributive ratio of these peptides in ProRJ for antihypertensive effect in SHR.

MATERIALS AND METHODS

Materials RJ hydrolysate by Protease N was prepared as previously described.[12] Protease N "Amano" (from *Bacillus subtillis*) was purchased from Amano Enzyme Inc., (Aichi, Japan). VY, IY and IVY were purchased from Sigma Chemical Co., (St. Louis, MO, U.S.A.), BACHEM AG, (Bubendorf, Switzerland), Peptide Institute Inc., (Osaka, Japan) respectively. MIX was prepared according to the ratio of the amount of each peptide (IY, VY, IVY) that exists in ProRJ.

Quantification of Peptides in ProRJ In order to investigate the contributive ratio of peptides in ProRJ for antihypertensive effect, the contents of peptides in the ProRJ were measured. The quantification of peptides in ProRJ was carried out by high performance liquid chromatography (HPLC) system, which equipped with a model 600 controller, 600 pump and 996-photodiode-array detector (Waters, Osaka). Sample was dissolved in water containing 5% acetonitrile (CH_3CN) and 20 μl of it was injected in the system. Chromatography was performed at 35 °C using a CAPCELL PAK C18 column (250×4.6 mm i.d., 5.0 μm particle size; Shiseido, Tokyo). The elution was made in a stepwise gradient mode using deionized water containing 0.1% trifluoro acetic acid (TFA) (solvent A) and 50% CH_3CN containing 0.1% TFA (solvent B) (gradient program; time, solvent B concentration; 0 to 10 min, 6% hold, 10 to 100 min, 6 to 24%) at a flow rate of 1.0 ml/min.

Animals and Measurement of Blood Pressure SHR (10-week-old, male, SHR/Hos, SPF, 200—255 g B.W.) were purchased from Hoshino Laboratory Animals (Saitama, Japan). SHR were housed individually in steel cages in a room kept at 25 °C with a 12 h light and dark cycle (lights on 8:00—20:00), and fed a laboratory diet (Charles River Japan, Kanagawa). Water was freely available. SBP and heart rate (HR) were measured by tail-cuff method with a Softron BP system (Softron BP-98A, Tokyo, Japan) after warming SHR in a chamber maintained at 37 °C for 10 min.

Single Oral Administration of ProRJ and MIX in SHR SHR with tail SBP over 180 mmHg were used. ProRJ was dissolved in deionized water at each concentration of 10 and 100 mg/10 ml. MIX was also dissolved in deionized water at each concentration of 1 and 10 mg/10 ml. These solutions were administered orally only once. Control rats were given the same volume of deionized water. SBP and HR were measured at 0, 1, 2, 4, 6, and 8 h after administration.

Single Oral Administration of Peptides (IY, VY, IVY) in SHR SHR with tail SBP over 180 mmHg were used. MIX and peptides were dissolved in deionized water to each

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concentration of 1 and 10 mg/10 ml. These solutions were administered orally only once. Control rats were given the same volume of deionized water. SBP and HR were measured at 0, 1, 2, 4, 6, and 8 h after administration.

Data Analysis The results were expressed as mean and standard deviations (S.D.) ($n=6$). The significance of the differences in SBP at each time after administration was analyzed by Student's paired *t*-test. Statistical analysis was performed using StatLight#04 (Yukums Co. Ltd., Japan).

RESULTS

Quantification of Peptides The retention time and the UV spectrum of each peptide were confirmed to be completely identical with those of synthetic peptides. Retention time of IY, VY and IVY were 25.1, 41.8 and 80.9 min, respectively (Fig. 1). Calibration curves were prepared by HPLC using the synthetic peptides to determine the content of each peptide in ProRJ. Contents of IY, VY and IVY in ProRJ were 0.07, 0.11 and 0.20%, respectively.

Measurement of Blood Pressure and Judgment of Antihypertensive Activity SBP of all groups decreased most at 1 h after administration and the antihypertensive effect was held for 8 h. Therefore, it was judged that the group to which SBP decreased significantly 1 h after administration as compared with an initial value showed antihypertensive activity.

Single Oral Administration of ProRJ in SHR We investigated the existence of antihypertensive activity and dose dependence of ProRJ by measuring SBP of SHR at each time interval after administration.

The prepared ProRJ sample was subjected to the single oral administration experiment in 12-week SHR. Single oral administration of ProRJ was done at doses of 10, 50 and 100 mg/kg. Consequently, SBP was reduced at doses of 100 mg/kg at 1 h after administration of ProRJ and the effect was held for 8 h (Fig. 2). And single oral administration of MIX was done at doses of 0.25, 0.5, 1 and 10 mg/kg. The result was shown that although SBP was reduced significantly at doses of 1 and 10 mg/kg at 1 h after administration of MIX, it was not reduced at doses of 0.25 and 0.5 mg/kg. And the effect was held for 8 h (Fig. 3). During this experiment, HR was not changed.

Single Oral Administration of Peptides in SHR The prepared peptide samples were subjected to the single oral

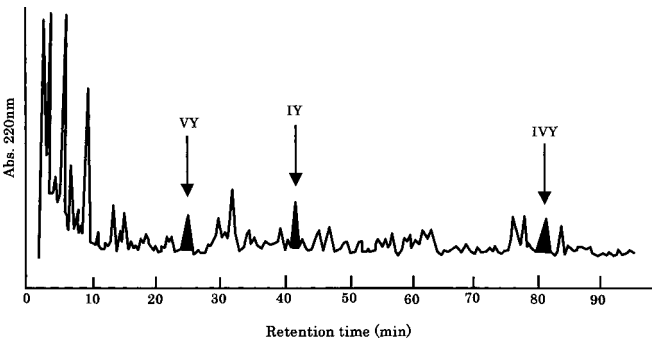

Fig. 1. HPLC Chromatogram of ProRJ Analysis at 220 nm

VY: Val-Tyr (25.1 min), IY: Ile-Tyr (41.8 min), IVY: Ile-Val-Tyr (80.9 min) (R.T.). The elution was done in the stepwise gradient mode of 50% CH_3CN containing 0.1% TFA (6% hold, 0→10 min, 6→24%, 10→100 min) at a flow rate of 1.0 ml/min.

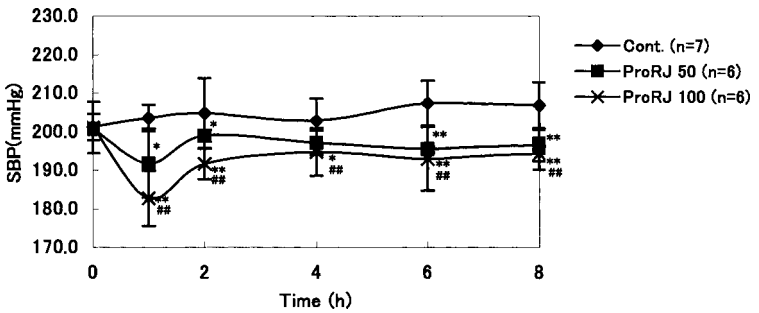

Fig. 2. Effect of Single Oral Administration of Protease Treated Royal Jelly on Systolic Blood Pressure in SHR

SBP: systolic blood pressure, ProRJ 100: protease treated Royal jelly 100 mg/kg administration, ProRJ 50: protease treated Royal jelly 50 mg/kg administration. Data were analyzed by Student's paired *t*-test. Significant differences from the control were evaluated by Student's paired *t*-test: $*p<0.05$; $**p<0.01$. Significant differences from the administration 0 d were evaluated by Student's paired *t*-test: $^{\#\#}p<0.01$. Each point with a vertical bar represents the mean±S.D.

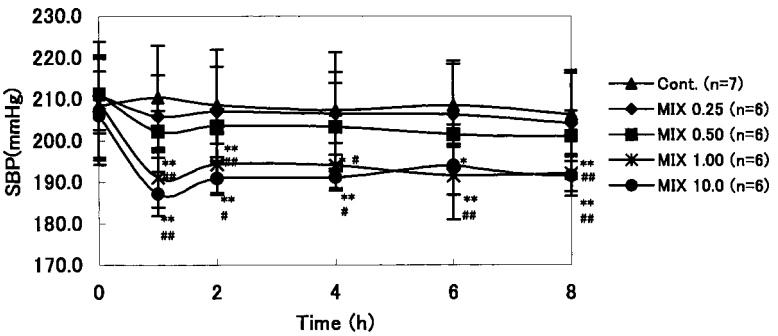

Fig. 3. Effect of Single Oral Administration of MIX on Systolic Blood Pressure in SHR

SBP: systolic blood pressure, MIX 0.25, 0.50, 1.00, 10.0: MIX 0.25, 0.50, 1.00, 10.0 mg/kg administration. Significant differences from the control were evaluated by Student's paired *t*-test: $*p<0.05$; $**p<0.01$. Significant differences from the administration 0 d were evaluated by Student's paired *t*-test: $^{\#}p<0.05$; $^{\#\#}p<0.01$. Each point with a vertical bar represents the mean±S.D.

Table 1. Changes of SBP at 1 h after Administration

Administration conc. (mg/kg)	Change of SBP at 1 h after administration (mmHg) ($n=6$)				
	ProRJ	MIX	IY	IVY	VY
100	−18.4±9.8**	—	—	—	—
50.0	−8.9±8.8	—	—	—	—
10.0	+0.8±13.4	−18.9±9.4**	−16.8±2.5**	−16.1±7.6**	−14.8±4.0**
1.0	—	−18.0±7.0**	−15.1±8.4**	−14.3±5.6**	−11.3±7.2**
0.5	—	−9.0±2.6	−11.1±4.7**	−7.7±5.4	−6.2±6.0
0.25	—	−5.1±3.2	—	—	—

Significant differences from the administration 0 d were evaluated by Student's paired *t*-test: ** $p<0.01$.

administration study in 12-week SHR. Single oral administration of peptides were done at doses of 0.5, 1 and 10 mg/kg. In the results showed that although SBP of VY and IVY administration groups were reduced at doses of 1 and 10 mg/kg in SHR, it was not reduced at doses of 0.25 and 0.5 mg/kg. However, only in the IY administration group, SBP was reduced significantly at doses of 0.5 mg/kg. SBP was reduced at 1 h after administration of peptides, and the effect was held for 8 h. During this experiment, HR was not changed.

Calculation of the Contributive Ratio for ProRJ of Peptides The contributive ratio is one of the important standards which evaluates activity in an application of the Food for specified health uses. The contributive ratio shows the ratio, which peptides have contributed to antihypertensive activity of ProRJ. The contributive ratio of peptides in ProRJ for antihypertensidve effect was calculated to the below equation according to the results of antihypertensive effect. The below equation was referred to the equation of contributive ratio reported by Seki *et al.*[9]

contributive ratio of MIX (w/w %)=[amount (mg) of administration of MIX in effective ProRJ (mg)]/[amount (mg) of administration of effective MIX (mg)]×100

[amount (mg) of administration of MIX in effective ProRJ (mg)]; In single oral administration of ProRJ, SBP was reduced at the dose of 100 mg/kg. The average of body weight of SHR was 300 g. Consequently effective ProRJ is 30 (mg). And total contents of IY, VY and IVY in ProRJ were 0.38%. Therefore amount of administration of MIX is 0.114 mg. In the same way, [amount (mg) of administration of MIX in effective ProRJ (mg)] is 0.3 (mg).

DISCUSSION

Recently, several ACE inhibitory peptides have been evaluated from food hydrolysates. We have also reported purification and identification of ACE inhibitory peptides from ProRJ. Their ACE inhibitory activity (IC_{50}) of IY, VY and IVY are 0.008, 0.020 and 0.018 (mg/ml) respectively.[5] They have an antihypertensive effect in repeated oral administration for 28 d on SHR.[6] In this study, we investigated the contributive ratio of these peptides in ProRJ for antihypertensive effect and MIX in single oral administration using SHR.

There were several reports of the single oral administration of VY,[9,10] IY[10,11] and IVY[13] in SHR. But IY and VY were not focused on the RJ. This study appeared firstly that IY and VY in RJ played antihypertensive action. In single oral administration of ProRJ and peptides, their antihypertensive effects appeared at 1 h after administration. We confirmed that ProRJ had resistance to digestion against gastrointestinal proteases[12] and ACE inhibitory peptides had resistance to digestion against gastrointestinal proteases. It was known that dipeptides and tripeptides are absorbed in their intact form from the intestinal tract.[14—16] And we confirmed that these peptides were absorbed in their intact form and exist in aorta, lung and plasma after single oral administration.[17] From this point of view, it was suggested that ACE inhibitory peptides, which we isolated from ProRJ could be promptly absorbed in their intact form from the intestinal tract. And it was thought that ACE inhibitory activity was acting at 1 h after administration. SBP was reduced dose-dependently at 1 h after administration as opposed to reduce not having been seen, as for the control group in single oral administration of MIX at doses of 0.25, 0.5 and 1.0 mg/kg (Table 1). However, in administration of MIX at dose of 1.0 and 10 mg/kg, SBP reduction was −18.9 and −18.0 mmHg, respectively. Their antihypertensive effect was equivalent to the action when administration of ProRJ was at the dose of 100 mg/kg. According to these results, the contributive ratio of MIX in ProRJ for antihypertensive effect was 38%. In this experiment of peptides, statistically significant SBP reductions of −11.1 mmHg for IY, −14.3 mmHg for IVY and −11.3 mmHg for VY were observed at lower doses (0.5, 1.0, 1.0 mg/kg, respectively) (Table 1). In the same way, the contributive ratio of VY, IY and IVY in ProRJ for antihypertensive effects were 22, 20 and 7.0%, respectively. Since MIX showed a high contributive ratio, it was assumed that IY, VY and IVY are the main ingredients, which show antihypertensive effect in ProRJ.

These results suggest that antihypertensive effect of ProRJ is related to peptides. Therefore it is considered that intake of peptides, as a functional food would be beneficial for improving blood pressure in people with hypertension.

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