A dipeptide YY derived from royal jelly proteins inhibits renin activity

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Abstract. Renin is the rate limiting enzyme in the renin-angiotensin (RA) system that regulates blood pressure and electrolyte balance. In this study, we investigated the renin inhibitory effect of a royal jelly (RJ)-derived peptide. A dipeptide YY was isolated from the digested fraction of RJ proteins by proteases and was found to inhibit human renin activity. The inhibition constant (K_i) of YY was estimated to be 10 μM when the K_m was 0.16 μM using sheep angiotensinogen as the substrate. The peptide was observed to lower blood pressure in spontaneously hypertensive rats.

Introduction

The renin-angiotensin (RA) system is known to regulate blood pressure and the electrolyte balance (1). Renin (EC 3.4.23.15) secreted from the kidney reacts with angiotensinogen to release the decapeptide, angiotensin I (2), which is converted to the octapeptide, angiotensin II, by angiotensin converting enzyme (ACE, EC 3.4.15.1) (3). The octapeptide then acts via type-1 angiotensin receptors to increase arterial tone, adrenal aldosterone secretion, renal sodium reabsorption, sympathetic neurotransmission and cellular growth (4). The RA system has recently been observed to stimulate angiogenesis (5), and angiotensin II has also been reported to contribute to key events of the inflammatory process (6). On the other hand, royal jelly (RJ) which is renowned worldwide as a popular traditional health food (7), acts as an anti-inflammatory drug on streptozotocin-induced diabetic rats during acute and chronic phases of inflammation (8).

RJ has been used for many years in medical and cosmetic products as a health food and a dietary supplement (7). Some of the peptides from RJ have recently shown antihypertensive effects in spontaneously hypertensive rats (SHR) by inhibiting ACE (9-12). The inhibitors of ACE have been studied previously (13,14), but their potential interaction with the kallikrein system (15,16) and their inhibition of other enzymes unrelated to the RA system cloud interpretation of their physiologic responses (17). Many peptides derived from the prosegment sections have been demonstrated to have an inhibitory effect on renin activity (18). Competitive inhibitors of renin, based on the sequence of the natural substrate angiotensinogen, have been synthesized (19), but they have been of insufficient potency and solubility for *in vivo* studies (20). As renin catalyzes the rate limiting step of the RA system, renin inhibition possibly has a higher priority than ACE inhibition (21). Therefore, potent and selective inhibitors of renin can be employed as a new supplement with less side effects than ACE inhibitors (22). In this study, a dipeptide YY was isolated from proteolytically digestive RJ proteins to evaluate its inhibitory effects on renin activity *in vitro* using highly sensitive human renin substrate, sheep angiotensinogen (23), and to investigate its possible blood pressure-lowering capacity.

Materials and methods

Preparation of the RJ protein fraction and its digestion by protease. Fresh RJ was collected from *Apis mellifera* L. fed primarily on nectar and pollen from *Brassicaceae Brassica campestris* L. in the Yangtze valley, P.R. China. Fresh RJ (1,000 g) with a moisture content of ~67% was first extracted with 2,000 ml of ethanol by stirring for 2 h at room temperature and then filtered to remove the soluble fraction. An additional 2,000 ml of ethanol was then added to the insoluble fraction and stirred for a further 2 h at room temperature. The insoluble precipitate designated as the RJ protein fraction was collected by filtration and evaporated to dryness. The RJ protein fraction (60 g) was suspended in 570 ml of water, adjusted to pH 7.0 by NaOH and incubated with 1 mg/ml of protease N (Amano Enzyme Inc., Nagoya, Japan) for 7 h at 50˚C. After boiling for 10 min to stop the enzymatic reaction, the solution was lyophilized for the isolation of active peptides.

Isolation of dipeptide YY by high performance liquid chromatography (HPLC). Protease digests of the RJ protein fraction were dissolved in 30% acetonitrile to a concentration

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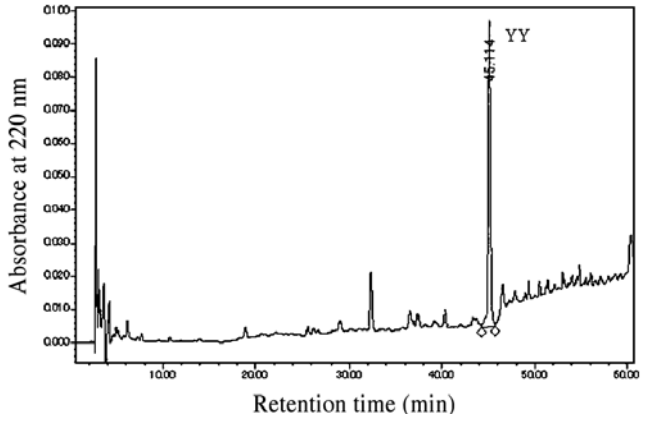

Figure 1. Final purification step of dipeptide YY from the protease digests of RJ proteins by HPLC. The dipeptide YY was eluted at ~45 min of the retention time.

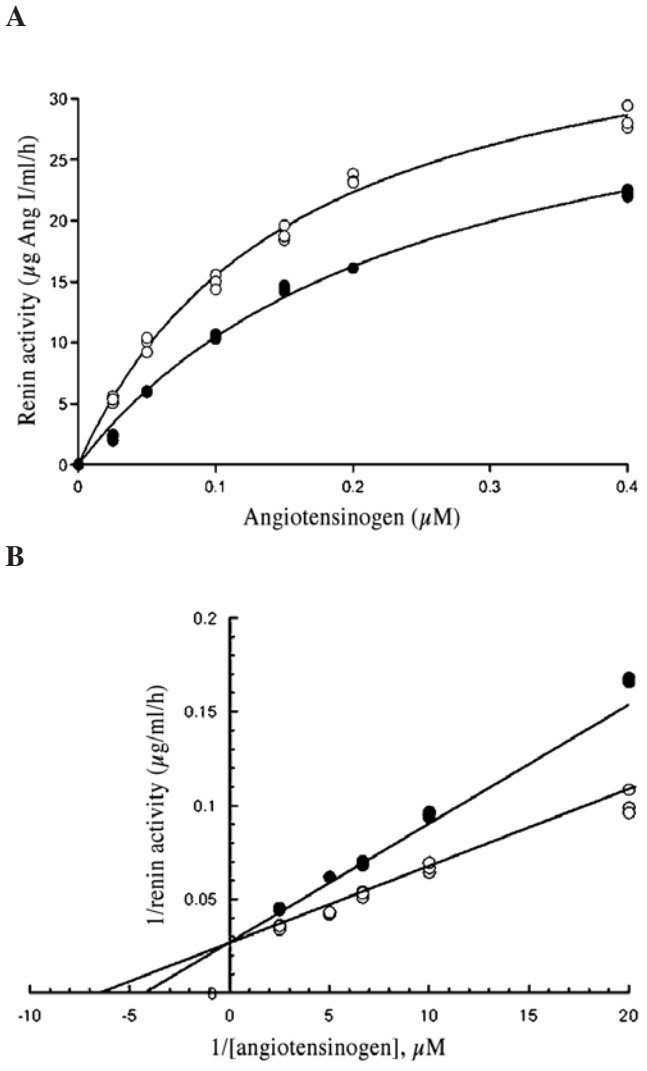

Figure 2. (A) Inhibition of renin activity by the dipeptide YY. The renin activity was measured with (●) and without (○) peptide YY at various concentrations of angiotensinogen (μM). (B) Lineweaver-Burk plots of the renin activities obtained in A with (●) and without (○) the dipeptide YY under the standard assay conditions.

of 30 mg/ml, and 100 μl was applied to an HPLC system (Model 600S and 486, Waters) with a gel filtration column (Superdex Peptide 10/300; 10x300 mm; Pharmacia Biotech) equilibrated with solvent A containing 30% acetonitrile and 0.1% trifluoroacetic acid (TFA). The retained peptides were eluted with solvent A at 0.3 ml/min. A renin inhibitor was eluted from the column at the retention time of 51-70 min. The inhibitor fraction was evaporated to dryness, dissolved in a small amount of 2% acetonitrile, and then applied to an HPLC system with a Capcell Pak AG-120 (Shiseido) C18 column (4.6x250 mm) equilibrated with solvent B containing 2% acetonitrile and 0.1% TFA. The retained peptides were eluted with a linear gradient of 2-30% acetonitrile in solvent B at 1 ml/min for 60 min. The renin inhibitor was eluted from the column at the retention time of 15-30 min. The inhibitor fraction was evaporated to dryness, dissolved in a small amount of 1% acetonitrile, and applied again to an HPLC system with a Capcell Pak AG-120 equilibrated with solvent C containing 1% acetonitrile and 0.1% TFA. The retained peptides were eluted with the linear gradient of acetonitrile (1%, 0-10 min; 1-8%, 10-40 min; 8-30%, 40-60 min) in solvent C at 1 ml/min, and the observed major peaks were individually collected by detection at a wavelength of 220 nm (Fig. 1). As a result of renin assay, we obtained a single peak at the retention time of 45 min with renin inhibitor and identified it as a dipeptide YY by mass spectrometry (API 365, Applied Biosystems) which showed the spectra consistent with those observed following analysis of the comparable commercially available synthetic YY (Bachem AG).

Expression and purification of sheep angiotensinogen. Chinese hamster ovary (CHO) cells containing sheep angiotensinogen cDNA were obtained as described previously (23,24). The expression vector was transfected into CHO cells by a phosphate-mediated method (23). CHO cells transfected with wild-type sheep angiotensinogen cDNA were routinely cultured in DMEM supplemented with 5% FBS, 0.1 mM non-essential amino acids, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 2.3 mM MTX at 37˚C in a CO_2 incubator. Sheep angiotensinogen secreted in the medium was collected and purified by CM-Toyopearl column chromatography (25,26).

Expression and purification of recombinant human renin. The purified recombinant renin was obtained from the human prorenin cDNA harboring in the CHO cells and was followed by purification after trypsin treatment under the same conditions as described previously (24).

Renin assay. Human renin was incubated with 0.4, 0.2, 0.15, 0.1, 0.05 and 0.025 μM of sheep angiotensinogen for 30 min at 37˚C in 1.0 mM phosphate buffer (pH 7.0) containing 5 mM EDTA.2Na and 10 mM diisopropylfluorophosphate with and without 6 μM of the dipeptide YY isolated in this study. Resultantly, angiotensin I generated by the renin reaction was measured by angiotensin I-ELISA as described previously (27). The renin activity was represented as the angiotensin I generated amounts for 1 h in the 1 ml of original renin source.

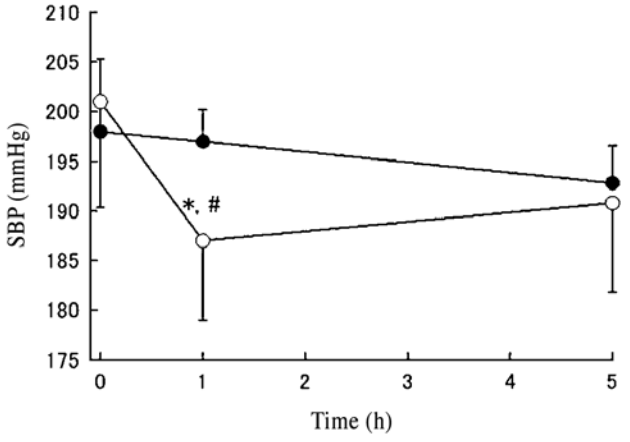

Figure 3. Effect of the dipeptide YY on lowering blood pressure. Single oral administration of YY (10 mg/kg) (○) or vehicle (●) was carried out in 11-week rats, and systolic blood pressure (SBP) was measured 0, 1 and 5 h after the administration. Statistical differences of SBP compared before administration ($^{*}p<0.05$) and with the vehicle control ($^{\#}p<0.05$) were evaluated by Dunnett's test. Values represent the mean ± SD (n=6).

Animals and measurements. Male spontaneously hypertensive rats (SHR) (9 weeks old) were obtained from Hoshino Experimental Animal Breeding Farm, Inc. (Saitama, Japan). All rats were housed at 23±1˚C with 55±10% humidity and fed a standard laboratory rodent diet (CRF-1; Oriental Yeast Co., Ltd., Japan). Single oral administration of the dipeptide YY was carried out in 11-week rats with systolic blood pressure (SBP) >180 mmHg, in which the dosage of 10 mg/5 ml/kg in 5% gum arabic solution was injected by incubation with a nutritional catheter. Control rats were administered the same volume of gum arabic solution. SBP was measured with a tail-cuff method 0, 1 and 5 h after the administration as described previously (28).

Results

Inhibition constant (K_i) of dipeptide YY. The K_i of dipeptide YY was estimated to be 10 μM by Lineweaver-Burk plots when the K_m of the substrate of renin was determined to be 0.16 μM, as shown in Fig. 2B. These plots also indicated the competitive inhibition of renin activity by this peptide.

Single oral administration of dipeptide YY in SHR. One hour after the single oral administration of the dipeptide YY (10 mg/kg), the systolic blood pressure was significantly lowered in the SHR ($p<0.05$) and returned to the control level after 5 h, as shown in Fig. 3. The diastric pressure was only slightly changed after 1 h even after the dose.

Discussion

Hypertension is one of the major risk factors of cardiovascular diseases and is associated with stroke and/or heart attack, which affects 20-30% of the total world population (29). In most studies, it has been demonstrated that the time course of the blood pressure lowering activity parallels the inhibition of plasma renin activity and the concomitant decrease in plasma levels of angiotensin I and II (30). In this study, we found that a dipeptide YY inhibited the human renin activity at the physiological pH. As shown in Fig. 3, the

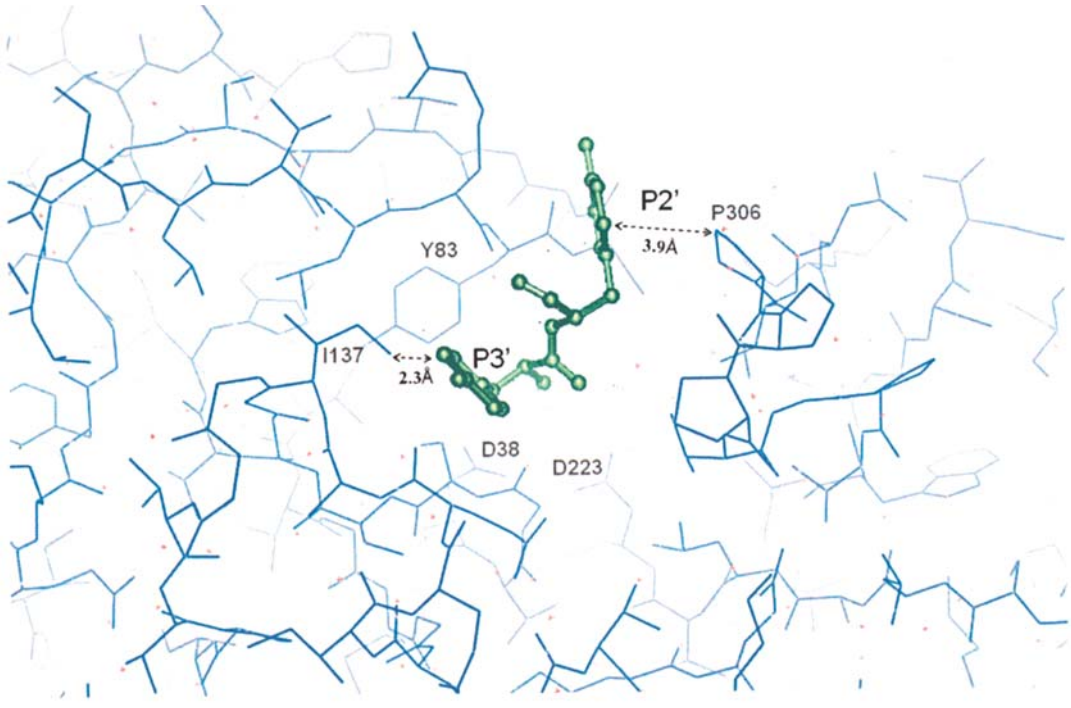

Figure 4. Possible stereostructure of the complex of human renin with rat renin inhibitor CH66. The putative complex structure was designed by highlighting human renin and the YY region of CH66 after the complex structure (PDB code: 1SMR) of mouse renin with CH66 was superimposed with the complex structure (PDB code: 1RNE) of human renin with C60. The compound C60 is a renin inhibitor, transition state analog, and CH66 is a renin inhibitor derived from sequence P5' to P4' in rat angiotensinogen. The YY sequence corresponds to the P2' and P3' positions in the rat angiotensinogen. The structures of human renin and the YY sequence are indicated by a wire frame and a ball and stick model, respectively.

dipeptide YY lowered the systolic blood pressure in SHR after its oral administration. The dipeptide YY was previously observed to inhibit ACE (10). The phenomenon associated with the blood pressure lowering effect must be the dual inhibitory function of the dipeptide YY against renin and ACE. As the peptide was prepared from the protein fraction by protease, the peptide could be generated in the gastrointestine after an oral dose of the RJ, possibly as described by Matsui *et al* (31).

In this study, we determined the K_i of the dipeptide YY to human renin to be 10 μM when the K_m was 0.16 μM at a pH 7.0 (Fig. 2). As shown by the Lineweaver-Burk plots, the peptide was found to inhibit the renin activity competitively (Fig. 2). The value of K_i was more than 60 times higher than that of K_m and was much higher than that of other potent renin inhibitors (32,33). The K_m was in the similar range as reported previously (24). Therefore, the dipeptide may be useful as a mild effective supplement.

The stereostructure of a complex of human renin and dipeptide YY could not be directly demonstrated in this study in order to indicate the counterpart of the peptide in the human renin molecule. The atomic coordinates of a complex of mouse renin with a renin inhibitor, CH66 (PIV-HPFHL-OH-LYYS) derived from the sequence of P5' to P4' in the rat angiotensinogen, is available in the Protein Databank (PDB code, 1SMR). That of human renin with a renin inhibitor, transition state analog C60, is also available in the databank (PDB code, 1RNE). In this study, a human renin structure superimposed with mouse renin confirmed that both renin structures are similar in these main chains. In Fig. 4, the possible positioning of the YY dipeptide (corresponding to P2' and P3' in the substrate angiotensinogen) is indicated in the human renin. These spaces of the renin molecule are blocked by the YY dipeptide so that angiotensinogen cannot come into the active cleft in the renin molecule. The space of YY is >5 Å from the active sites of renin, Y83, D38 and D223, (34,35). On the other hand, the distances from P3' to I37 and P2' to P306 are 2.31 and 3.86 Å, respectively (Fig. 4). These data suggest that these residues associate with hydrophobic bonds to form the complex of renin and dipeptide YY.

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