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Isolation and Antihypertensive Effect of Angiotensin I-Converting Enzyme (ACE) Inhibitory Peptides from Spinach Rubisco

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Four new inhibitory peptides for angiotensin I-converting enzyme (ACE), that is, MRWRD, MRW, LRIPVA, and IAYKPAG, were isolated from the pepsin−pancreatin digest of spinach Rubisco with the use of HPLC. IC_{50} values of individual peptides were 2.1, 0.6, 0.38, and 4.2 μM, respectively. MRW and MRWRD had an antihypertensive effect after oral administration to spontaneously hypertensive rats. Maximal reduction occurred 2 h after oral administration of MRW, whereas MRWRD showed maximal decrease 4 h after oral administration at doses of 20 and 30 mg/kg, respectively. IAYKPAG also exerted antihypertensive activity after oral administration at the dose of 100 mg/kg, giving a maximum decrease 4 h after oral administration. IAYKP, IAY, and KP, the fragment peptides of IAYKPAG, also exerted antihypertensive activity. LRPVIA did not show any antihypertensive effect at a dose of 100 mg/kg despite its potent ACE-inhibitory activity.

KEYWORDS: Rubisco; spinach; ACE-inhibitory peptides; antihypertensive effect; spontaneously hypertensive rats (SHR)

INTRODUCTION

Angiotensin I-converting enzyme (ACE, peptidyldipeptide hydrolase, EC 3.4.15.1) plays a key physiological role in the regulation of blood pressure by virtue of two different reactions it catalyzes: conversion of the inactive decapeptide angiotensin I into a powerful vasoconstrictor and salt-retaining octapeptide, angiotensin II; and inactivation of the vasodilator nonapeptide bradykinin, which is conducive to lowering blood pressure (*1*). Eventually it was demonstrated that ACE inhibitors exhibit antihypertensive activity in spontaneously hypertensive rats (SHR) and hypertensive patients (*2*). Recently, many studies have focused on various ACE-inhibitory peptides derived from casein (*3*−*8*), fish muscle (*9*−*12*), silk fibroin (*13*), egg yolks (*14*), gelatin (*15*), plasma (*16*, *17*), and plant proteins (*18*−*25*).

Plants are renewable sources of food protein. Seed proteins are mainly used for the production of protein-rich foods. However, the green parts of plants, due to the mass production, are potentially sources of valuable protein. One of the greatest constituents of biomass proteins is ribulose bisphosphate carboxylase/oxygenase (Rubisco), which catalyzes the primary step in photosynthetic CO_2 fixation. Rubisco is the most abundant protein on the earth and is an important source of peptides because of its amino acid composition. Although many biologically active peptides have been isolated from different proteins, Rubisco was never extensively searched from this point of view. The aim of our study was to check whether Rubisco contains biologically active peptides. Previously, two opioid peptides, rubiscolin 5 and rubiscolin 6, have been isolated from spinach Rubisco (*26*). In this study, spinach Rubisco was tested for ACE-inhibitory peptides having antihypertensive effect. To avoid disturbance by other plant constituents, studies were performed with the use of commercially available ribulose bisphosphate carboxylase isolated from spinach leaf.

MATERIALS AND METHODS

Angiotensin I-converting enzyme (EC 3.4.15.1) was obtained from Sigma. Spinach Rubisco was obtained from Sigma. Pancreatin from porcine pancreas and pepsin from porcine stomac mucosa were obtained from Sigma. Hippuryl-histidyl-leucine (HHL) was obtained from the Peptide Institute, and synthetic peptides were obtained from American Peptide Co.

Enzymatic Hydrolysis of Spinach Rubisco. Spinach Rubisco (10 mg/mL) was adjusted to pH 2.0 with 1.0 N HCl and digested by pepsin (E/S = 1/100) for 5 h at 37 °C. After the pH had been adjusted to 7.5 with 1.0 N NaOH, the reaction was stopped by boiling for 10 min. Pancreatin digest was prepared identically like pepsin digest. The only difference was pH 7.5. When additional digestion with pancreatin was made following pepsin digestion, the pH was adjusted to 7.5, pancreatin (E/S = 1/100) was added, and further digestion was carried out for 5 h at 37 °C. The reaction mixture was boiled to stop reaction, and then centrifuged.

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Purification of Peptides. The digest was separated by reverse-phase high-performance liquid chromatography (RP-HPLC) on an octadecyl silica (ODS) column (Cosmosil 5C18-AR-II, 20 × 250 mm, Nacalai Tesque). The column was eluted with a linear gradient of acetonitrile (1%/min), containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 10 mL/min. The elution was monitored at 230 nm, and each peak was collected as a separate fraction. All fractions were dried with a centrifugal concentrator, and their ACE-inhibitory activity was measured. Active fractions were further purified on phenethyl silica (5PE-MS, 4.6 × 250 mm, Nacalai Tesque), which was developed using the same gradient at a flow rate of 1 mL/min. Active fractions from the phenethyl column were further purified on a cyanopropyl silica (CN) column (Cosmosil 5 CN-R, 4.6 × 250 mm, Nacalai Tesque) and a nitrophenethyl column (5NPE, 4.6 × 150 mm, Nacalai Tesque) with the same gradient at a flow rate of 1 mL/min.

Amino Acid Sequence Analysis and Peptide Synthesis. The amino acid sequence of the purified peptide was determined automatically by Edman's degradation procedure with the use of a 492 protein sequencer (Applied Biosystems), whereby PS3 (Rainin) peptide synthesizer was used to synthesize the peptide. Peptides were synthesized using the solid phase method. Fmoc-amino acids were successively coupled in the presence of HBTU. Peptides, after deprotection, were purified using an HPLC ODS column (Cosmosil 5C18-AR-II, 20 × 250 mm, Nacalai Tesque) with identical conditions as for purification of peptides from digest. The identity of peptides was confirmed by sequence analysis. Purity of peptides was evaluated with the use of HPLC equipped with photodiode array detector. Analysis was performed on an analytical ODS column (Cosmosil 5C18-AR-II, 4.6 × 150 mm, Nacalai Tesque) in a linear gradient of acetonitrile (0−40%, 1%/min), containing 0.1% TFA at a flow rate of 1 mL/min.

Measurement of ACE-Inhibitory Activity. ACE-inhibitory activity was determined using the method reported by Cushman and Cheung (*27*) with minor modification by Yamamoto (*28*) and expressed in terms of IC_{50}.

Characterization of ACE-Inhibitory Peptides. The character of ACE-inhibitory peptides was differentiated through evaluation of IC_{50} for ACE inhibition before and after preincubation of individual peptides [0.1 mM in borate buffer (pH 8.3)] with 32 mU of ACE at 37 °C for 3 h. Additionally, reaction mixtures were analyzed using HPLC following preincubation with ACE. Samples were injected on an ODS column (Cosmosil 5C18-AR-II, 4.6 × 150 mm, Nacalai Tesque). The column was eluted with a linear gradient of acetonitrile (0−40%; 1%/min), containing 0.1% TFA at a flow rate of 1 mL/min

Determination of Antihypertensive Effect of Peptides Following Oral Administration. Male SHR (16−24 weeks old) were employed. Peptides dissolved in saline were administered orally to SHR via a gastric metal zonde in a volume of 1.0 mL. Following oral administration of the peptide, the blood pressure was measured by the tail cuff method using the MK-2000 blood pressure meter (Muromachi Kikai) to ascertain antihypertensive activity, while only saline was administered, as a control.

Data Analysis. All results are expressed as means ± SEM. Statistical comparisons of the results between the two groups were made with Student's *t* test.

RESULTS AND DISCUSSION

ACE-Inhibitory Activity of Spinach Rubisco Digests. The spinach Rubisco was digested by pepsin, pancreatin, and pepsin−pancreatin, respectively. Pepsin digest (IC_{50} = 64 μg/mL) and pepsin−pancreatin digest (IC_{50} = 72 μg/mL) showed potent ACE-inhibitory activities, whereas the pancreatin digest showed a weaker activity (IC_{50} = 170 μg/mL), suggesting the importance of pepsin action.

Isolation of ACE-Inhibitory Peptides from the Pepsin—Pancreatin Digest of Spinach Rubisco. The pepsin−pancreatin digest was selected for the isolation of active peptides as this method of hydrolysis is a model for in vivo digestion. The pepsin−pancreatin digest of spinach Rubisco was separated on an ODS column. Many active fractions were isolated from pepsin−pancreatin digest as shown in **Figure 1**. Of the fractions with highest relative ACE-inhibitory activity, fractions I, II, and III were eluted at 22−31% acetonitrile. Subsequently, the active peaks were further purified on phenethyl, cyanopropyl, and nitrophenethyl columns, respectively. After the completion of all chromatography steps, the following ACE-inhibitory peptides were isolated: MRWRD, IAYKPAG, MRW, and LRIPVA (**Figure 2**). Among them, LRIPVA (IC_{50} = 0.38 μM) and MRW (IC_{50} = 0.6 μM) showed the most potent ACE-inhibitory activities (**Table 1**). MRW constitutes a part of MRWRD, which is also isolated from the pepsin−pancreatin digest of spinach Rubisco. Sequences of all peptides can be found in the primary structure of small and large subunits of spinach Rubisco (**Table 1**).

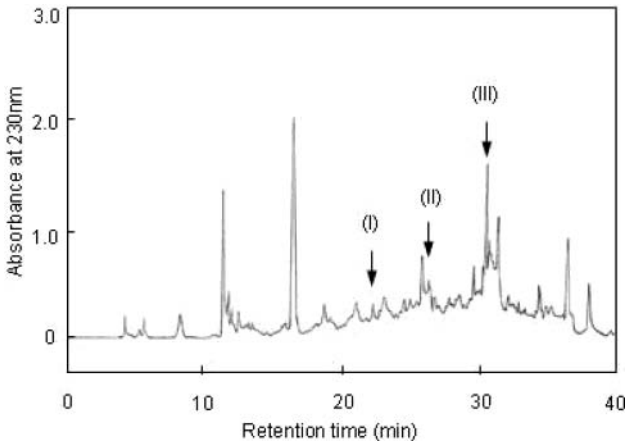

Figure 1. HPLC separation of Rubisco pepsin–pancreatin digest on an ODS column. Active fractions I, II, and III are shown with arrows.

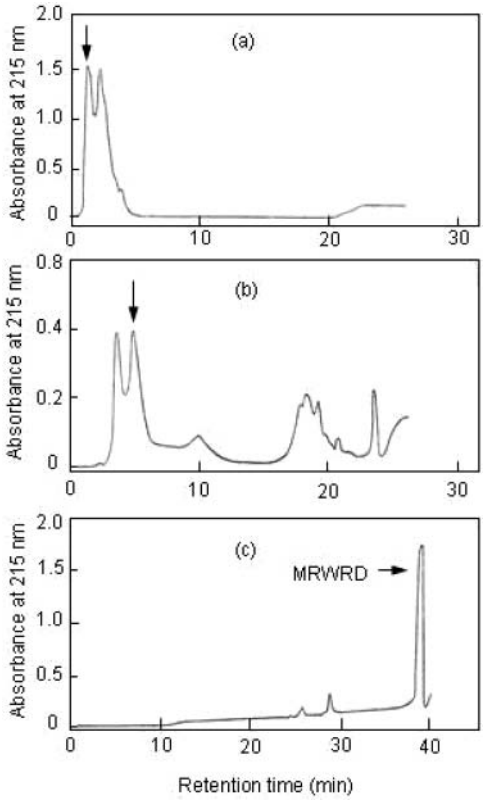

Figure 2. Purification of MRWRD from fraction II on phenethyl (a), cyanopropyl (b), and nitrophenyl (c) columns, respectively. Arrows show active fraction.

Table 1. IC_{50} for ACE Inhibitory Peptides Derived from Spinach Rubisco

CH_3CN concn elution (%)					IC_{50}	
ODS	phenyl	CN	N-PhA	structure	(μM)	origin
I:22	23	4	22	IAYKPAG	4.2	small subunit (116–122)
II:26	1	5	39	MRWRD	2.1	large subunit (212–216)
III:31	2	9	32	MRW	0.6	large subunit (212–214)
III:31	2	13	30	LRIPVA	0.38	large subunit (138–143)

Classification of ACE-Inhibitory Peptides by Preincubation Method. ACE-inhibitory peptides can be classified into the following groups according to their interaction with ACE: (i) true inhibitors, which are resistant to cleavage by ACE; and (ii) substrates for ACE, which are hydrolyzed by ACE. According to ACE-inhibitory properties of peptides released during the hydrolysis of substrates by ACE, they can be divided into "real substrates", which are hydrolyzed by ACE with the release of inactive fragments or fragments having a markedly lower activity, and "pro-drugs", which are converted by ACE with the release of highly active fragments (*11*, *29*). Previous studies have proved that the real substrates, which show apparent ACE-inhibitory activities in assay used for screening, are inactive after being administered orally because they are hydrolyzed by ACE to inactive fragments (*11*, *29*). To discriminate the substrates from true inhibitors, peptides were preincubated with ACE prior to the measurement of ACE-inhibitory activity. IC_{50} values of the true inhibitors are not affected by preincubation with ACE, whereas substrates for ACE are altered by preincubation with ACE.

Among isolated peptides MRW is a true inhibitor because its IC_{50} value was unaltered by preincubation ($IC_{50} = 0.6$ μM). In contrast, the IC_{50} value of MRWRD was decreased from 2.2 to 0.84 μM by preincubation with ACE. HPLC analysis showed that MRWRD was almost totally hydrolyzed by ACE, producing 87.6% of MRW ($IC_{50} = 0.6$ μM) and 12.4% of RW ($IC_{50} = 22$ μM) (**Figure 3**). This result indicates that MRWRD is a pro-drug type of ACE-inhibitory peptide and is slowly activated and hydrolyzed by ACE into MRW, which in turn inhibits ACE itself.

IC_{50} values of both LRIPVA and IAYKPAG were increased after preincubation, suggesting that these are substrates for ACE. The IC_{50} value of LRIPVA increased by >120 times following preincubation with ACE itself. LRIPVA ($IC_{50} = 0.5$ μM) was hydrolyzed into LR ($IC_{50} \sim 2.2$ mM), IP ($IC_{50} \sim 1.8$ mM), and VA ($IC_{50} \sim 0.9$ mM) (**Figure 4**), giving an apparent IC_{50} of 62 μM following preincubation. The very low ACE-inhibitory activities of peptides released during incubation with ACE prove that LRIPVA is a real substrate.

An interesting case is IAYKPAG, which had its IC_{50} value increased by only a factor of 2 (from 4.6 to 10 μM) by preincubation with ACE (**Table 2**), suggesting that this peptide is also a substrate for ACE. However, through the digestion of IAYKPAG by ACE, two ACE inhibitors, IAY and KP (**Figure 5**), were released, with IC_{50} values of 12.5 and 30.4 μM, respectively. IAYKP, an obligatory intermediate product during ACE preincubation, has a higher ACE-inhibitory activity ($IC_{50} = 2.2$ μM), which means that preincubation with ACE activates IAYKPAG by releasing IAYKP with higher ACE-inhibitory properties, which subsequently releases the two ACE inhibitors IAY and KP.

Antihypertensive Activities of Peptides after Oral Administration in SHR. To investigate whether isolated peptides can exert antihypertensive activity following oral administration

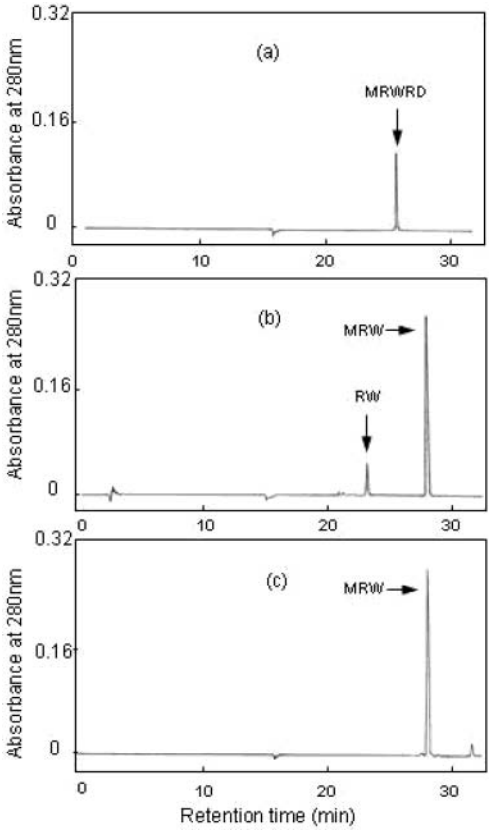

Figure 3. Hydrolysis of MRWRD by ACE. Peptide (0.1 mM) was incubated with 32 mU of ACE (37 °C, 3 h): (a) before preincuation; (b) after preincubation; (c) MRW standard. The sample was applied to an ODS column.

to SHR, the effect of the most potent ACE-inhibitory peptides, MRW and MRWRD, was tested. In the case of MRW, maximum reduction of systolic blood pressure was observed only 2 h after oral administration, with the minimum effective dose being 20 mg/kg as shown in **Figure 6**. MRWRD exerted antihypertensive activity following oral administration with the minimum effective dose being 30 mg/kg. The maximum decrease in systolic blood pressure due to MRWRD occurred 4 h after oral administration (**Figure 6**). Considering these results together, it is evident that MRWRD exerts long-lasting antihypertensive activity (4 h) compared with MRW (2 h). It is conceivable that the long-lasting antihypertensive effect of MRWRD is contributed to the time required for enzymatic conversion of pro-drug into true inhibitor in vivo.

The antihypertensive effect of IAYKPAG, which had its IC_{50} value increased by a factor of 2 following preincubation with ACE, was tested in SHR. The effects of IAYKP and of IAY and KP, which are the intermediate and final products during the preincubation of IAYKPAG, respectively, were also tested. As shown in **Figure 7a**, IAYKPAG exerted antihypertensive activity after oral administration with its minimum effective dose being 100 mg/kg. Maximum decrease in systolic blood pressure by IAYKPAG occurred 4 h after oral administration (−15.0 mmHg), with a decrease that was 1.5 times greater than at 2 h after oral administration (−9.8 mmHg). The antihypertensive effect of IAYKPAG after oral administration in SHR is probably a result of the antihypertensive activity of IAYKP, IAY, and KP, which are released during IAYKPAG digestion in vitro and have the capability to lower blood pressure when administered at dosages equivalent to an effective dose (100 mg/kg) of IAYKPAG. The maximum decrease in systolic blood pressure

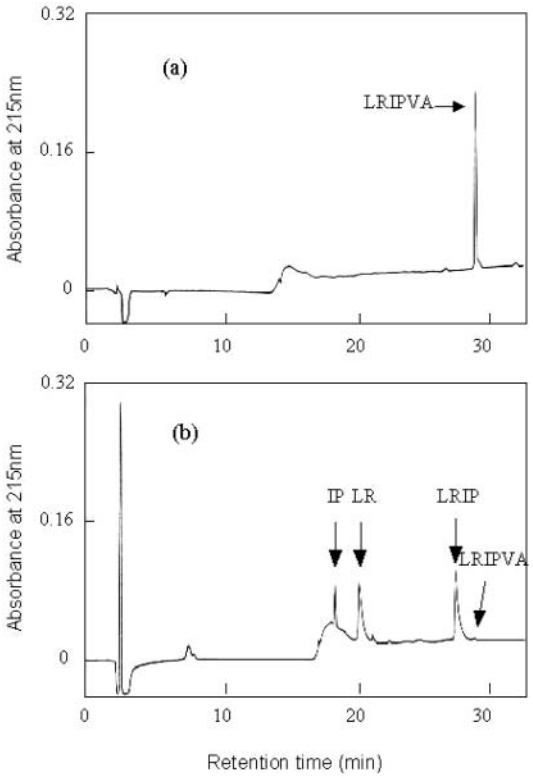

Figure 4. Hydrolysis of LRIPVA by ACE. Peptide (0.1 mM) was incubated with 32 mU of ACE (37 °C, 3 h): (a) before preincuation; (b) after preincubation. The sample was applied to an ODS column.

Table 2. IC_{50} for ACE Inhibition and Antihypertensive Activities of Peptides Following Oral Administration in SHR

	IC_{50} (μM)		antihypertensive activity after oral administration	
peptide	− preinc	+ preinc	max ΔmmHg	time of max effect (h)
IAYKPAG	4.6	10.0	−15[a]	4
LRIPVA	0.5	62.0	0	–
MRW	0.6	0.6	−20[b]	2
MRWRD	2.2	0.84	−13.5[b]	4

[a] Decrease of systolic blood pressure after oral administration (100 mg/kg). [b] Decrease of systolic blood pressure after oral administration (30 mg/kg).

by 80 mg/kg IAYKP (equivalent to a dose of 100 mg/kg IAYKPAG) occurred 4 h after oral administration, which is the same as for 100 mg/kg IAYKPAG. The mixture of IAY and KP exerted antihypertensive activity at doses of 50 mg/kg IAY and 30 mg/kg KP (equivalent to the dose of 100 mg/kg IAYKPAG) even though IAY and KP failed to show effective antihypertensive activity when administered separately at the same dosages. Maximum decrease in systolic blood pressure due to the mixture of IAY and KP occurred 2 h after oral administration (**Figure 7b−d**). Results show that some peptides, such as IAYKPAG, have an ACE-inhibitory activity that decreases following preincubation with ACE, which indicates that they are substrates for this enzyme, and may have an antihypertensive effect in vivo. This probably occurs when inhibitors of ACE are released during preincubation. ACE inhibitory activity determined in vitro for peptides being hydrolyzed by ACE is the result of activity of a mixture of the original peptide itself and activities of fragments released by ACE. Composition of the hydrolysate and the resulting inhibitory activity depends on the incubation period. IAYKPAG (IC_{50} = 4.6 μM) is first hydrolyzed into IAYKP, which has a

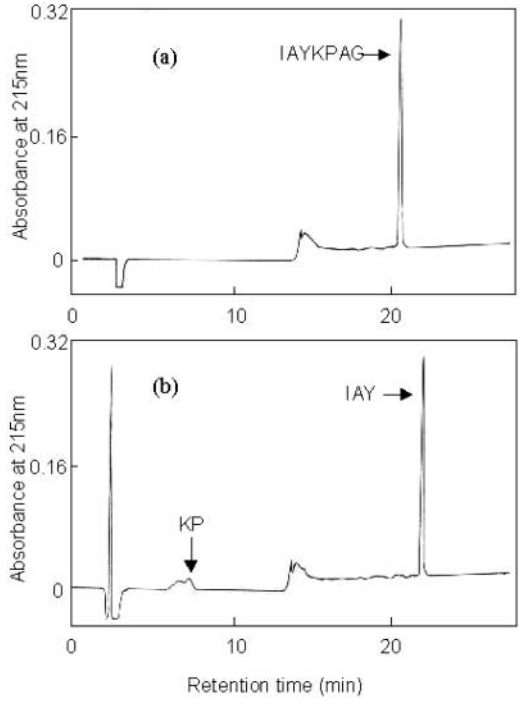

Figure 5. Hydrolysis of IAYKPAG by ACE. Peptide (0.1 mM) was incubated with 32 mU of ACE (37 °C, 3 h): (a) before preincuation; (b) after preincubation. The sample was applied to an ODS column.

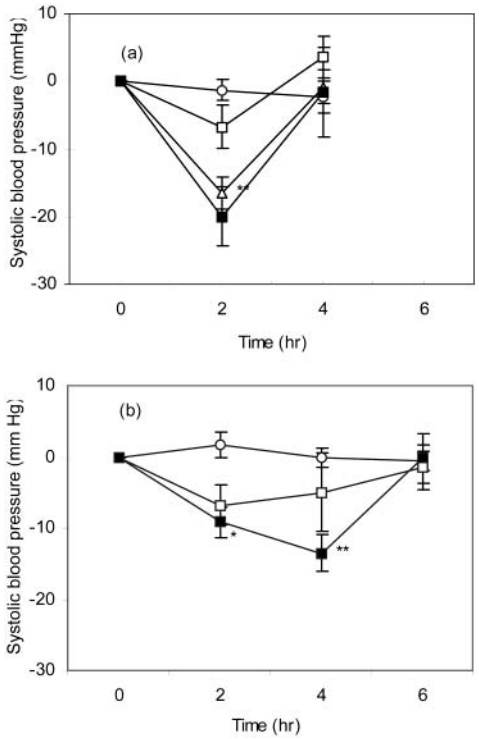

Figure 6. Antihypertensive activity of MRW (a) and MRWRD (b) after oral administration in SHR. Peptides were administered as a solution in saline at doses of (○) 0 mg/kg (control), (□) 10 mg/kg, (△) 20 mg/kg, and (■) 30 mg/kg. Changes of systolic blood pressure from time zero were expressed with mean ± SEM. *, ** indicate significant differences against control (*, $p < 0.05$; **, $p < 0.01$).

higher ACE inhibitory activity (IC_{50} = 2.2 μM), and finally converted to IAY and KP with lower activities (IC_{50} values of 12.5 and 30.4 μM, respectively). The same effect could be observed for antihypertensive activity of peptides in vivo. This

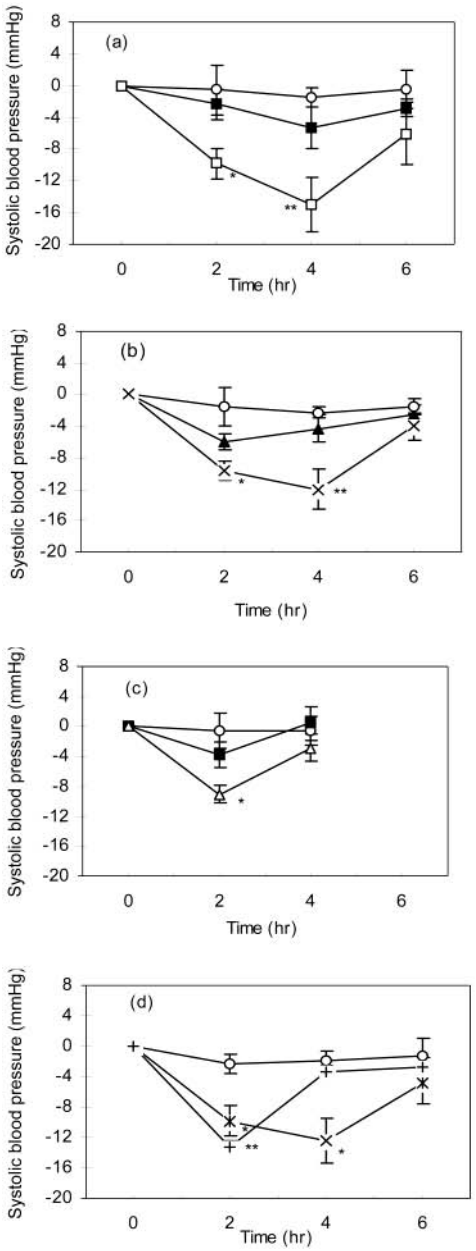

Figure 7. Antihypertensive activities of IAYKPAG, IAYKP, IAY, and KP after oral administration to SHR: (a) IAYKPAG at doses of (■) 30 mg/kg and (□) 100 mg/kg; (b) IAYKP at dose of (×) 80 mg/kg and IAY at a dose of (▲) 50 mg/kg; (c) KP at doses of (■) 30 mg/kg and (△) 60 mg/kg; (d) IAYKP at a dose of (×) 80 mg/kg and IAY + KP at a dose of (+) (50 + 30) mg/kg; (○) 0 mg/kg (control). Changes of systolic blood pressure from time zero were expressed as mean ± SEM. *, ** indicate significant differences against control (*, $p < 0.05$; **, $p < 0.01$).

is probably why IAYKPAG can exert an antihypertensive effect in vivo, although the effective dosage is relatively high at 100 mg/kg. However, it should be noted that the situation in vivo is much more complicated than that in vitro because, apart from ACE, other peptidases also may participate in the degradation of peptides and release more or less active fragments.

LRIPVA, which is converted during preincubation with ACE into peptides with very low ACE-inhibitory activities, similarly to other substrates for ACE (*11*, *29*), failed to show an antihypertensive effect in SHR even at a dosage of 100 mg/kg.

In summary, the obtained results prove that enzymatic hydrolysis of spinach Rubisco releases ACE-inhibitory peptides having antihypertensive activity following oral administration to SHR. Sequences of Rubiscos from different plant species show very high homology; therefore, Rubisco, the most abundant protein on earth, may be important in preventing hypertension. Our studies proved antihypertensive properties of peptides released by pepsin−pancreatin digestion of spinach Rubisco. However, it is interesting whether undigested Rubisco has the same effect.

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