Classification and Antihypertensive Activity of Angiotensin I-Converting Enzyme Inhibitory Peptides Derived from Food Proteins

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ABSTRACT: Angiotensin I-converting enzyme (ACE)-inhibitory peptides from the thermolysin digest of chicken muscle and the peptic digest of ovalbumin were isolated. However, some of them failed to show antihypertensive activity in spontaneously hypertensive rats (SHR). To clarify this discrepancy, ACE-inhibitory peptides from various sources were preincubated with ACE before measurement of ACE-inhibitory activity and classified into 3 groups: (1) inhibitor type, IC_{50} values of peptides that are not affected after preincubation with ACE; (2) substrate type, peptides that are hydrolyzed by ACE to give peptides with weaker activity; and (3) prodrug-type inhibitor, these peptides are converted to true inhibitors by ACE or gastrointestinal proteases. Peptides belonging to the 1st and the 3rd groups exert antihypertensive activities even after oral administration in SHR.

Key Words: angiotensin I-converting enzyme inhibitor, spontaneously hypertensive rat, ovalbumin, chicken muscle, bonito

Introduction

THE ANGIOTENSIN I-CONVERTING ENZYME (ACE) CATALYZES the formation of angiotensin II, a strong vasopressor, from angiotensin I, together with inactivation of bradykinin possessing hypotensive activity (Ondetti and others 1977). Such dual effects of ACE inhibitors are contributory to their potent antihypertensive activity in spontaneously hypertensive rat (SHR) or hypertensives (Case and others 1978). Commercially available antihypertensive drugs such as captopril and enarapril are very potent ACE inhibitors. Recently, ACE-inhibitory peptides derived from casein (Maruyama and others 1985, 1987a, 1987b, 1989), fish muscle (Kohama and others 1988; Suetuna and others 1991), and other proteins (Oshima and others 1979; Maruyama and others 1989) were isolated. It was revealed by the present authors that thermolysin digest of dried bonito (katsuo-bushi), a Japanese traditional seasoning made of bonito muscle, had potent ACE-inhibitory activity, and 8 ACE-inhibitory peptides were isolated from it (Yokoyama and others 1992). The digest exerted antihypertensive activity after oral administration in SHR (Fujita and others 1995, 1999) and borderline hypertensive subjects (Fujita and others 1997). The digest "Katsuo-bushi oligopeptide" was approved as Foods for Specified Health Use in Japan. Then, we also found that the thermolysin digest of chicken muscle and the peptic digest of ovalbumin possessed potent ACE-inhibitory activities, and we isolated ACE-inhibitory peptides from them. In spite of potent ACE-inhibitory activities, some of them failed to show antihypertensive activity after oral administration in SHR. In this paper, possible causes responsible for such conflicting activities were investigated.

Results and Discussion

Purification of ACE-inhibitory peptides from enzymatic digests of food proteins

Thermolysin digest of chicken muscle. The thermolysin digest of chicken muscle showed almost the same potent ACE-inhibitory activity (IC_{50} = 45.0 μg/mL, Table 1) as that of the dried bonito (IC_{50} = 29 μg/mL), tempting us to purify the ACE-inhibitory peptides from it. The chicken muscle digest was fractionated on an octadecyl silica (ODS) column (Fig. 1A). Inhibitory fractions (A, B, C, D, E) were eluted at 19% to 30% acetonitrile (Fig. 1A); subsequently, the active peaks were further purified on phenyl and cyanopropyl columns. Peak C was additionally purified on an ODS column at pH 7.0 in the presence of 10 mM phosphate buffer. After all chromatography steps, 7 ACE-inhibitory peptides (LKA, LKP, LAP, IKW, FQKPKR, FKGRYYP, and IVGRPRHQG) were isolated. Among them, IVGRPRHQG was also isolated from the thermolysin digest of dried bonito in our previous study (Table 2). The other 6 peptides (LKA, LKP, LAP, IKW, FQKPKR, and FKGRYYP) were new ACE-inhibitory peptides. IKW showed the most potent ACE-inhibitory activity (IC_{50} = 0.21 μM, Table 2) among them. The LKP sequence constitutes a part of the LKPNM sequence; incidentally, the latter is an ACE-inhibitory peptide found in the thermolysin digest of dried bonito. These sequences are found in primary structure of chicken fructose-bisphosphate aldolase (230–234).

Table 1—ACE inhibition by enzymatic digests of food proteins

Substrates	Protease	IC_{50} (μg/mL)
Chicken muscle	Thermolysin	45.0
Ovalbumin	Thermolysin	83.0
	Pepsin	45.3
	Trypsin	>1000
	Chymotrypsin	>1000

Peptic digest of ovalbumin. We also tried to obtain ACE-inhibitory peptides from enzymatic digest of ovalbumin, a typical egg protein. Ovalbumin was hydrolyzed by pepsin, trypsin, chymotrypsin, and thermolysin. Among the digests obtained, the peptic digest exerted the most potent inhibitory activity for ACE (IC_{50} = 45.3 μg/mL, Table 1). The peptic digest of ovalbumin was fractionated on an ODS column, and inhibitory fractions (A, B, C, D) were eluted at 25% to 36% acetonitrile (Fig. 1B). The active peaks were further purified on phenyl and cyanopropyl columns, while Peaks A and C were additionally purified on an ODS column at pH 7.0 in the presence of 10 mM phosphate buffer. Six ACE-inhibitory peptides were isolated, and their structures are shown in Table 2. Of these peptides, ERKIKVYL showed the most potent ACE-inhibitory activity (IC_{50} = 1.2 μM, Table 2).

Antihypertensive activity in SHR

We investigated whether these peptides showed antihypertensive activities after intravenous administration at a dose of 10 mg/kg in SHR. As an example in Fig. 2, IKP showed antihypertensive activity after the administration. IY, LW, IKW, LAP, LKP, LKPNM, and IWHHT (IC_{50} = 2.1, 6.8, 0.21, 3.5, 0.32, 2.4, and 5.8 μM, respectively) also showed similar antihypertensive activities (Table 3). On the other hand, FFGRCVSP, ERKIKVYL, FKGRYYP, and IVGRPRHQG failed to exert antihypertensive activities under the same conditions.

We also investigated whether these peptides showed antihypertensive activities after oral administration at a dose of 60 mg/kg in SHR. FFGRCVSP, ERKIKVYL, and FKGRYYP failed to exert antihypertensive activities after oral administration. On the other hand, IY, LW, IKW, LKP, LKPNM, IWHHT, IVGRPR, and IVGRPRHQG showed antihypertensive activities (Table 4). It should be noted that IVGRPRHQG, which was inactive after intravenous administration, lowered blood pressure after oral administration. Among these peptides, dipeptides, such as IY, exhibited maximal blood pressure reduction 2 h after the administration; tripeptides, such as IKW, 4 h after administration; pentapeptides, such as LKPNM, 6 h after administration; and nonapeptide IVGRPRHQG 8 h after oral administration, respectively (Fig. 3).

Classification of ACE-inhibitory peptides by preincubation method

As briefed above, some peptides isolated from different sources failed to show antihypertensive activities in SHR although they exhibited seemingly ACE-inhibitory activities. There are many ACE substrates in the enzymatic digest of food proteins because the substrate specificity of ACE is very broad. The substrates for ACE also show seemingly ACE-inhibitory activities tentatively in the assay used for the screening. Accordingly, the ACE substrates can only be detected in in vitro assay. When they are orally given, they are inactive. In order to discriminate the substrates from true inhibitors, peptides were preincubated with ACE before measurement of ACE-inhibitory activity.

Table 2—Purification of active peaks from enzymatic digests of chicken and OVA

	CH_3CN conc. elution (%)					
ODS[1]	Phenyl[1]	CN[1]	ODS[2]	Structure	IC_{50} (μM)	Origin
Thermolysin digest of chicken muscle						
A:19	15	7		LKA	8.5	Creatine Kinase
B:20	21	7		LKP	0.32	Aldolase
C:21	21	7	14	LAP	3.5	—
	25	15		FQKPKR	14	(Myosin)*
D:26	24	18		IVGRPRHQG	2.4	Actin
	28	28		FKGRYYP	0.55	Creatine kinase
E:30	34	30		IKW	0.21	—
Peptic digest of ovalbumin						
A:25	34	29	27	FGRCVSP	6.2	Ovalbumin
B:30	37	37		ERKIKVYL	1.2	Ovalbumin
C:31	38	39	36	FFGRCVSP	0.4	Ovalbumin
	44	43		LW	6.8	Ovalbumin
D:36	51	45		FCF	11	Ovalbumin
	52	46		NIFYCP	15	Ovalbumin

[1]Containing 0.1% trifluoroacetic acid.
[2]Containing 10 mM phosphate buffer (pH7.0)
*A sequence homologous to FQKPKR, FQKPJQ was found in the primary structure of rat myosin.

Table 3—Inhibitory activities for ACE and antihypertensive activities of peptides following intravenous administration in SHR

Structure	IC_{50} (μM)	Antihypertensive activity (ΔmmHg, i.v.*)
Thermolysin digest of dried bonito		
IY	2.1	−45
IKP	1.6	−70
LKPNM	2.4	−80
IWHHT	5.8	−60
IVGRPRHQG	2.4	0
Thermolysin digest of chicken		
IKW	0.21	−50
LAP	3.5	−40
LKP	0.32	−75
FKGRYYP	0.55	0
Peptic digest of ovalbumin		
LW	6.8	−45
ERKIKVYL	1.2	0
FFGRCVSP	0.4	0

*Decrease of systolic blood pressure after intravenous administration (10 mg/kg)

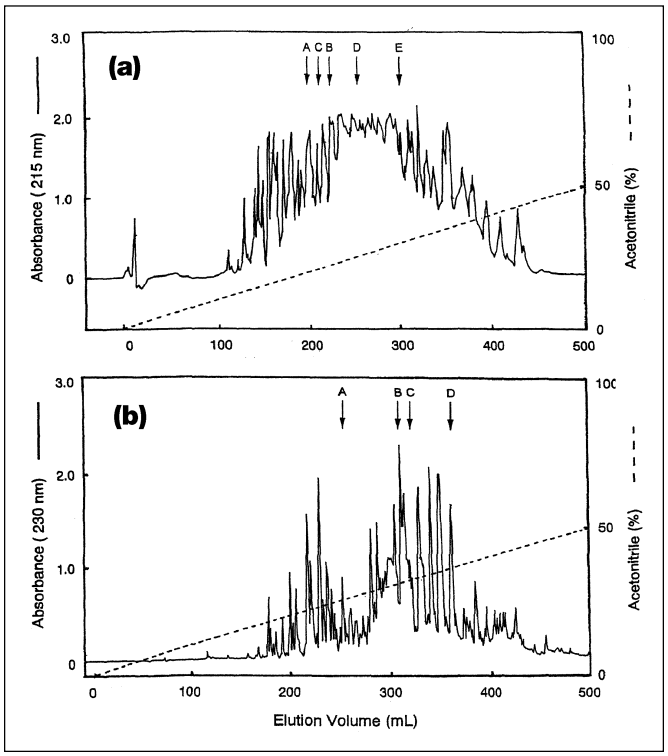

Fig. 1— HPLC of enzymatic digest of food proteins on ODS column. Reverse phase HPLC of the thermolysin digest of chicken muscle (A) and the peptic digest of ovalbumin (B). These digests were fractionated on an ODS column (Cosmosil 5C18-AR, 20 × 250 mm, Nacalai Tesque). Active fractions are indicated by arrows.

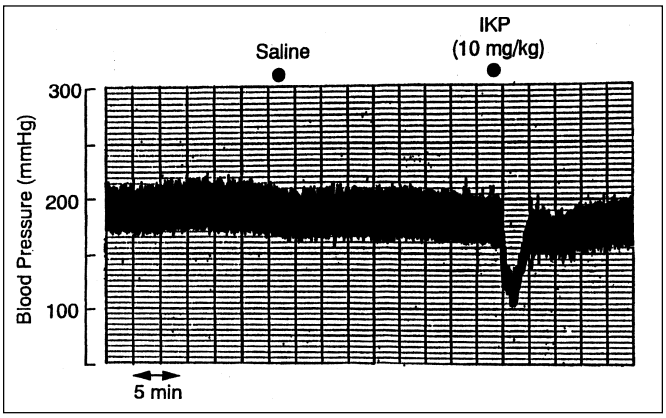

Fig. 2—Antihypertensive activities of IKP after intravenous administration in SHR. IKP (10 mg/kg) was injected into femoral vein. Carotid blood pressure was determined with a pressure transducer.

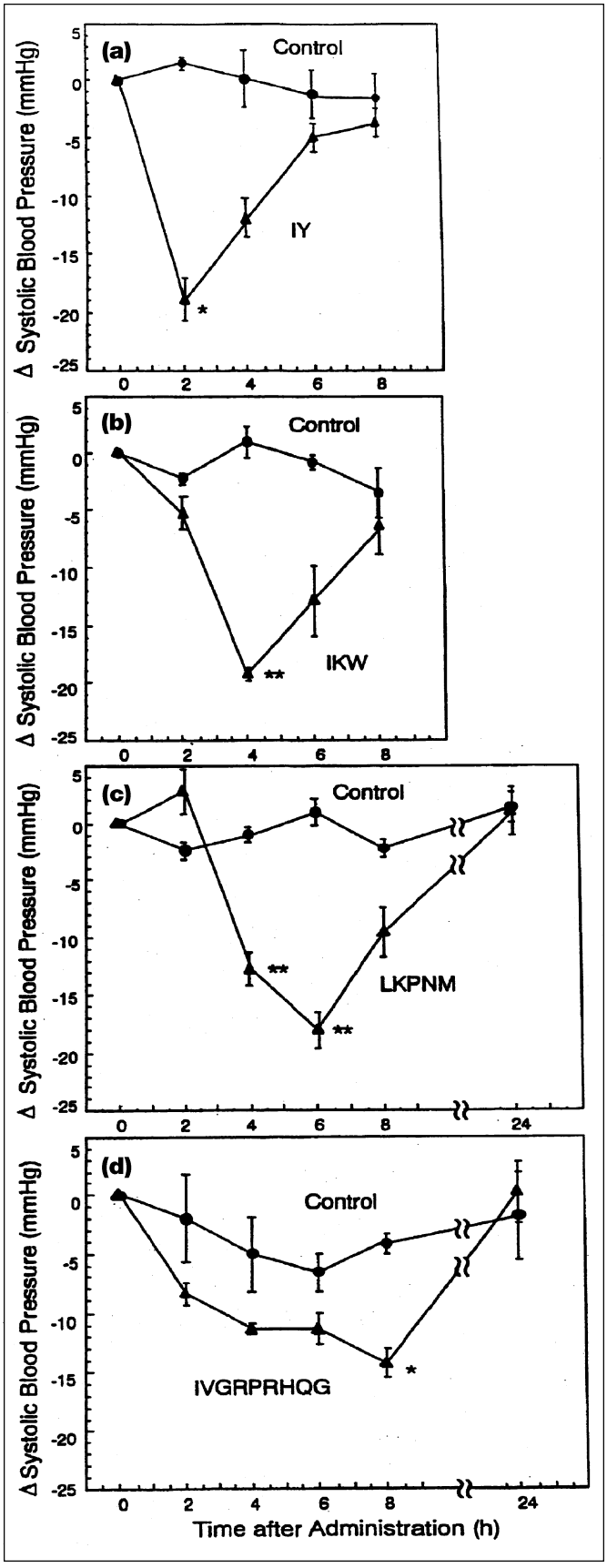

Fig. 3—Antihypertensive activities of ACE-inhibitory peptides after oral administration in SHR. Peptides were administered at a dose of 60 mg/kg as a solution in saline. Control (●), IY (▲), IKW (▲), LKPNM (▲) and IVGRPRHQG (▲). Changes of systolic blood pressure from zero time were expressed with mean ± S.E. *, indicate significant differences against control (*p < 0.05, **p < 0.01; *t*-test, n = 5).**

IC_{50} values of the true inhibitors are not altered by preincubation with ACE, while those substrates for ACE are altered by preincubation with ACE. FKGRYYP, ERKIKVYL, and FFGRCVSP are considered to be the substrates because their IC_{50} values increased after preincubation with ACE itself. To take FKGRYYP (IC_{50} = 0.55 μM) as an example, this heptapeptide was hydrolyzed by ACE into FKG, RY, and YP (Fig. 4), and the IC_{50} value increased to 34 μM. Similarly, FFGRCVSP (IC_{50} = 0.4 μM) was hydrolyzed into FF, GR, CV, and SP, giving an apparent IC_{50} value of 4.6 μM. Finally, ERKIKVYL (IC_{50} = 1.2 μM) was hydrolyzed into ER, KI, KV, and YL, giving an apparent IC_{50} value of 6.0 μM. These hydrolyzed products did not exert any antihypertensive activities as described above. It is elucidated that the potent ACE-inhibitory activities of these substrates for ACE may be eroded by rapid hydrolysis by ACE in vivo. It should be noted that FFGRCVSP and ERKIKVYL did not show antihypertensive activity in spite of the fact that their apparent IC_{50} values after the preincubation (4.6 μM and 6.0 μM, respectively) are still fairly small.

Interestingly, the IC_{50} values of LKPNM and IWHHT were decreased by the preincubation with ACE; IC_{50} value of LKPNM became 1/3 after the preincubation with ACE ,whereas a part of LKPNM (IC_{50} = 2.4 μM) was hydrolyzed by ACE to produce LKP (IC_{50} = 0.32 μM) and NM (Fig. 5). This result indicates that LKPNM is slowly activated and hydrolyzed by ACE into LKP, which in turn inhibits ACE itself. Given the fact that LKPNM is not susceptible to hydrolysis with pepsin or pancreatin (data not shown), it is anticipated that LKP is involved in the onset of antihypertensive activity of LKPNM. LKPNM showed maximal decrease of blood pressure 6 h after oral administration in SHR, while LKP developed its maximal effect 4 h after the administration (Fig. 3). This 2-h delay may be explained by the time lag required for enzymatic conversion of LKPNM by ACE into the true inhibitor LKP in vivo, suggesting that LKPNM can be regarded as a prodrug-type ACE inhibitory peptide. ACE was found also in jejunum (Bay 1993). Whether LKPNM is converted into LKP before or after the intestinal absorption remains to be elucidated.

IWHHT (IC_{50} = 5.8 μM) was also hydrolyzed into IWH (IC_{50} = 3.5 μM) and HT by ACE (data not shown). In similar manners, IWHHT might be hydrolyzed into IW and HHT by chymotrypsin,

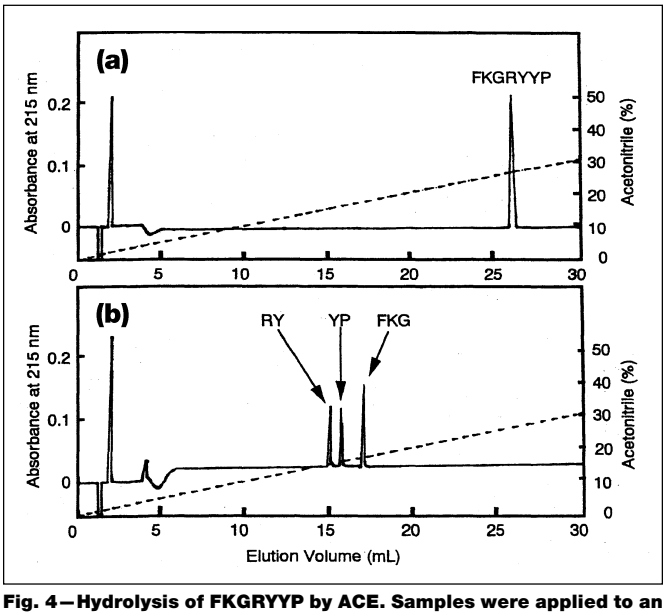

Fig. 4—Hydrolysis of FKGRYYP by ACE. Samples were applied to an ODS column (Cosmosil 5C_{18}-AR, 4.6 mm × 150 mm, Nacalai Tesque). Chromatograms before preincubation (A) and after preincubation (B). Peptide (10 mg/mL) was incubated with 28.8 mU ACE (37 °C, 3 h).

Table 4—IC_{50} for ACE and antihypertensive activities of peptides following intravenous and oral administration in SHR

Peptides	Origin	IC_{50} (μM)		Stability for ACE by HPLC (%)	Antihypertensive activity		
		−Preinc.	+Preinc.		i.v.[1] (ΔmmHg)	p.o.[2] (max,	ΔmmHg)
Substrate type							
FKGRYYP	Chicken	0.55	34	0	0	0	
FFGRCVSP	OVA	0.4	4.6	0	0	0	
ERKIKVYL	OVA	1.2	6.0	0	0	0	
Inhibitor type							
IY	Bonito	2.1	1.9	100	−45	−19	2h
LW	OVA	6.8	6.6	100	−45	−22	2h
IW	Bonito	5.1	5.1	100	−55	−22	2h
IKW	Chicken	0.21	0.18	>95	−50	−17	4h
IKP	Bonito	1.6	1.8	100	−70	−20	6h
LKP	Bonito, Chicken	0.32	0.32	100	−75	−18	4h
IWH	Bonito	3.5	3.5	100	−70	−30	4h
IVGRPR	Bonito	300	300	100	−25	−17	6h
Prodrug type							
LKPNM*	Bonito	2.4	0.76	80	−80	−23	6h
IWHHT*,**	Bonito	5.8	3.5	80	−60	−26	6h
IVGRPRHQG***	Bonito	2.4	23	0	0	−14	8h

[1] Decrease of systolic blood pressure after intravenous administration (10 mg/kg).
[2] Decrease of systolic blood pressure after oral administration (60 mg/kg).
*Activated by ACE.
**Activated by chymotrypsin.
***Activated by trypsin.

however, IW also possesses inhibitory activity for ACE (IC_{50} = 5.1 μM). It would appear likely that antihypertensive activity of IWHHT might be mediated by IWH or IW. IWHHT showed maximal decrease of blood pressure 6 h after oral administration in SHR, while IWH and IW showed their maximal effect 4 h and 2 h (30 mmHg, $p < 0.01$; 22 mmHg, $p < 0.01$) after administration, respectively. Viewed together, it is conceivable that IWHHT may also classified as a prodrug-type ACE inhibitor.

Interestingly, IVGRPRHQG showed antihypertensive activity after oral administration in SHR, though its IC_{50} value increased from 2.4 μM to 23 μM by the preincubation with ACE. Although IVGRPRHQG was hydrolyzed by ACE into IVG, RP, RH, and QG (Fig. 6), IVGRPRHQG was alternatively hydrolyzed by trypsin into HQG and IVGRPR, which exerted antihypertensive activity after oral administration in SHR (Table 4). IVGRPR (IC_{50} = 300 μM) was the true inhibitor for ACE because it was not hydrolyzed by ACE (Fig. 6). Therefore, IVGRPRHQG is a prodrug-type ACE inhibitor specifically activated by trypsin. After intravenous administration in SHR, IVGRPR lowered blood pressure, while the

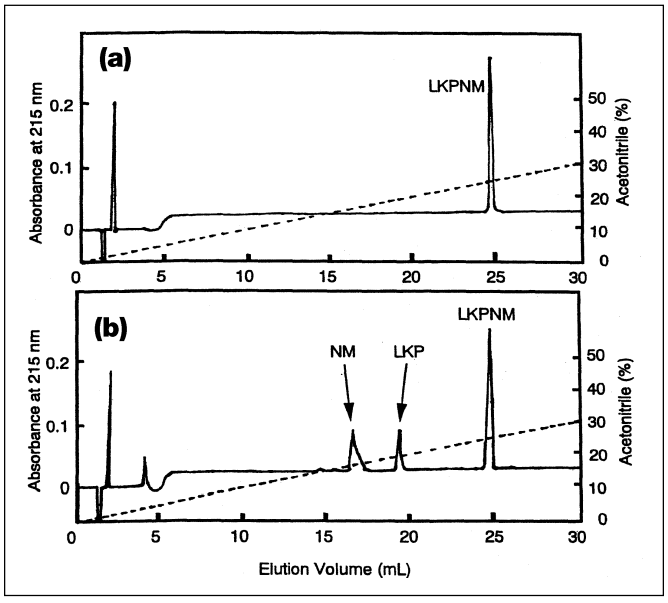

Fig. 5—Hydrolysis of LKPNM by ACE. Samples were applied to an ODS column (Cosmosil 5C_{18}-AR, 4.6 mm × 150 mm, Nacalai Tesque). Chromatograms before preincubation (A) and after preincubation (B). Peptide (10 mg/mL) was incubated with 28.8 mU ACE (37 °C, 3 h).

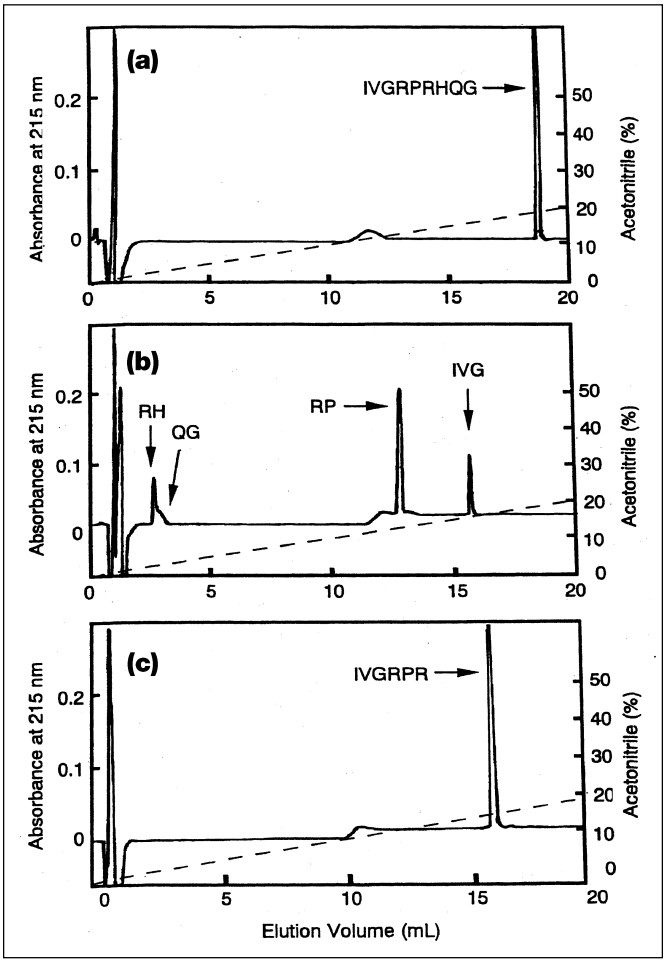

Fig. 6—Hydrolysis of IVGRPRHQG and IVGRPR by ACE. Samples were applied to an ODS column (Cosmosil 5C_{18}-AR, 4.6 mm × 150 mm, Nacalai Tesque). Chromatograms before preincubation (A) and after preincubation (B) and after preincubation of IVGRPR (C). Peptides (10 mg/mL) were incubated with 28.8 mU ACE (37 °C, 3 h).

parent peptide, IVGRPRHQG, failed to show antihypertensive activity (Table 4). These results suggest that conversion of IVGRPRHQG into IVGRPR by trypsin is essential for onset of its antihypertensive activity after oral administration. It was rather unexpected that IVGRPR would show an antihypertensive effect in spite of its weak ACE-inhibitory activity. IVGRPRHQG produced a maximal decrease of blood pressure 8 h after oral administration in SHR, while IVGRPR showed its maximal effect 6 h after the administration.

All of these findings are summarized in Table 4. Isolated peptides exhibiting seeming inhibition of the enzyme were classified into 3 groups. Both inhibitor-type and prodrug-type peptides are characterized with long-lasting antihypertensive activities after oral administration in SHR. It should be noted that all of the ACE-inhibitory peptides, isolated from the thermolysin digest of dried bonito, are either of the inhibitor (5 peptides) or the prodrug type (3 peptides). This may be the reason why the thermolysin digest of dried bonito (Katsuo-bushi oligopeptide) effectively shows antihypertensive activity after long-term administration in SHR (Fujita and others 1995) and humans (Fujita and others 1997).

Conclusions

IN SUMMARY, THIS REPORT OUTLINES NECESSARY CONDITIONS permitting the onset of antihypertensive activity of **seeming** ACE-inhibitory peptides after oral administration. Nevertheless, behavior of peptides toward various peptidases and susceptibility to intestinal absorption may deserve attention in determining oral effectiveness of ACE-inhibitory peptides derived from food proteins.

Materials and Methods

Materials

Pepsin (porcine stomach mucosa, 3700 unit/mg protein), trypsin (bovine pancreas, Type I, 10,000 units/mg protein), chymotrypsin (bovine pancreas, Type II, 50 units/mg protein), and ovalbumin were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) Thermolysin (Bacillus Thermoproteolyticus, 8000 units/mg protein) and hippuryl-histidyl-leucine (HHL) were purchased from the Peptide Institute Inc. (Japan). *t*-Butyloxycarbonyl amino acids were purchased from Watanabe Chemical Inc., Ltd. (Japan). Partially purified ACE, a dipeptidyl-carboxy peptidase (code No. A6778, 2 units/mg protein) that hydrolyzes peptide bond between the second and the third residue from the carboxyl terminus, was also from Sigma Chemical Co. This enzyme did not show any other protease activity (trypsin, chymotrypsin, and neutral endopeptidase).

Digestion of food proteins

Five grams of chicken muscle were suspended in 45 mL of distilled water and homogenized with a Polytron (Kinematica GmbH PT 10/35, Switzerland) for l min. The homogenate was boiled for l0 min then digested with 50 mg thermolysin at 37 °C for 3 h (pH 7.5). Ovalbumin (100 mg/mL) was digested with various proteases (pepsin, trypsin, chymotrypsin, and thermolysin; 880 μg/mL) at 37 °C for 3 h. Before pepsin digestion, ovalbumin was adjusted to pH 2.0 with hydrochloric acid, and the pH was maintained every 30 min during hydrolysis. Before other proteases digestion, ovalbumin was adjusted to pH 7.5 with sodium hydroxide, and the pH was maintained every 30 min with sodium hydroxide during hydrolysis. The digests were boiled for 10 min to inactivate the protease. The pepsin digest was adjusted at pH 7.0 with sodium hydroxide before boiling.

Purification of peptides from the digests of food proteins

The digests were applied to an octadecyl silica (ODS) column (Cosmosil 5C_{18}-AR, 20 mm × 250 mm, Nacalai Tesque Inc., Japan). The column was developed at a flow rate of l0 mL/min by a linear gradient of acetonitrile (0% to 50% / 50 min) containing 0.l% trifluoroacetic acid. Individual fractions were dried with a centrifugal concentrator, and their inhibitory activities were measured. The active fractions were purified on a phenyl silica (Ph) column (Cosmosil 5Ph, 4.6 mm × 250 mm, Nacalai Tesque Inc.), which was developed at a flow rate of l mL/min by a linear gradient of acetonitrile (0% to 40% / 40 min) containing 0.l % trifluoroacetic acid. The active peaks from the phenyl column were purified on a cyanopropyl silica (CN) column (Cosmosil 5CN-R. 4.6 × 250 mm, Nacalai Tesque Inc.), which was developed as described above. When the obtained peptide was not sufficiently purified, despite using 3 HPLC steps, the peptide was finally chromatographed on an ODS column (Cosmosil 5C_{18}-AR, 4.6 mm × 150 mm, Nacalai Tesque Inc.) and developed at a flow rate of l mL/min by a linear gradient of acetonitrile (0% to 40%/40 min) containing 10 mM phosphate buffer (pH 7.0).

Amino-acid sequence analysis

The amino-acid sequence of the purified peptide was analyzed by a protein sequencer (477A, Applied Biosystems Inc., Foster, Calif, U.S.A.)

Synthesis of peptides

In this study, all results were obtained using synthetic peptides. Peptides were synthesized by a solid phase method with a peptide synthesizer (Biosearch SAM TWO, San Rafael, Calif., U.S.A.) *t*-Butyloxycarbonyl amino acids were successively coupled in the presence of N,N'-diisopropylcarbodiimide. Peptides were deprotected by the anisole/hydrogen fluoride method and purified on HPLC.

ACE-inhibitory activity

ACE-inhibitory activities were measured using HHL as a substrate and extracts of rabbit lung acetone powder by the method of Cushman and Cheung (1971) with a minor modification by Yamamoto and others (1980). Each assay mixture contained the following components at the indicated final concentration: borate buffer (pH 8.3), l00 mM; sodium chloride, 400 mM; HHL, 5 mM; and enzyme 3 mU per 250 μL of assay volume. This assay mixture was incubated at 37 °C for 30 min. The IC_{50} value is the concentration of peptide that resulted in 50% ACE inhibition in the reaction mixture.

Stability of peptides for ACE

Individual peptides (10 mg/mL) were incubated with 28.8 mU of ACE at 37 °C for 3 h, and the reaction was stopped by boiling for 10 min; thereafter, IC_{50} values were compared before and after preincubation. Stability of the inhibitory peptides for ACE was also tested by HPLC equipped with an ODS column.

Antihypertensive activity after intravenous or oral administration in SHR

Antihypertensive activities of peptides were determined as

follows: After intravenous administration of peptides, SHR were anesthetized with α-D-chrolarose (40 mg/kg, i.v.) Peptides dissolved in saline were injected into the femoral vein using a catheter (10 mg/kg), followed by determination of carotid-artery blood pressure with a pressure transducer (Ohmeda Pte Ltd, Singapore). On the other hand, following oral administration of peptides dissolved in saline via a gastric metal zonde (60 mg/kg), antihypertensive activities were also measured by the tail cuff method using UR-5000 (Ueda Seisakusho, Japan).

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