Angiotensin I-Converting Enzyme Inhibitor Derived from an Enzymatic Hydrolysate of Casein. II. Isolation and Bradykinin-potentiating Activity on the Uterus and the Ileum of Rats

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Inhibitors of angiotensin I-converting enzyme were isolated from an enzymatic hydrolysate of bovine casein. The amino acid sequences of these inhibitors were Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys (CEI_{12}), Phe-Phe-Val-Ala-Pro (CEI_5), and Ala-Val-Pro-Tyr-Pro-Gln-Arg ($CEI_{\beta 7}$). CEI_5 is a penta-peptide derived from the hydrolysate of CEI_{12} with proline-specific endopeptidase, and $CEI_{\beta 7}$ is a hepta-peptide derived from β -casein. These inhibitors potentiated bradykinin in the contraction of the uterus and the ileum of rats. The ileum was more sensitive to these inhibitors than the uterus. The bradykinin-potentiating activity of these inhibitors on the ileum lasted for more than 90 min even after washing the organ.

Angiotensin I-converting enzyme [kininase II, peptidyl dipeptide hydrolase, EC 3.4.15.1] (ACE) catalyzes both the production of the vasoconstrictor angiotensin II and the inactivation of the vasodilator bradykinin.

ACE inhibitors were first discovered in snake venom.^{1,2)} All of the ACE inhibitors from snake venom have both ACE inhibitory and bradykinin-potentiating activities. Ondetti *et al.* developed captopril (D-3-mercapto-2-methylpropanoyl-L-proline), an orally effective inhibitor, based upon a hypothetical model of the binding site on the enzyme,³⁾ and many workers also developed many such inhibitors.^{4,5)}

We found a peptide inhibitor of ACE (CEI_{12} , Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys) from the tryptic hydrolysate of bovine casein,⁶⁾ and now we have isolated other ACE inhibitors from the hydrolysate that are more potent than CEI_{12} .

This report is on the isolation of these inhibitors and their effects as bradykinin-potentiators on the uterus and the ileum of rats.

MATERIALS AND METHODS

Materials. Bradykinin, bradykinin-potentiator B, bradykinin-potentiator C²⁾ and hippuryl-L-histidyl-L-leucine were purchased from the Protein Research Foundation, Osaka, Japan; bradykinin-potentiating nonapeptide SQ20881¹⁾ was from Peninsula Laboratories Co. Rabbit lung acetone powder was obtained from Sigma Chemicals Co., Proline-specific endopeptidase from *Flavobacterium meningosepticum* was from Seikagaku Kogyo Co., Ltd., Japan; trypsin was from P-L Biochemicals, Inc. and carboxypeptidase Y was from Oriental Yeast Co., Ltd., Japan. Casein was the product of Wako Pure Chemical Co., Japan.

Assay of ACE inhibitor. The activity of the ACE inhibitor was assayed by a modification of the method of Cushman and Cheung as described in our previous report.⁶⁾ Five millimolar hippuryl-L-histidyl-L-leucine and ACE inhibitor were dissolved in 100 mM sodium borate buffer, pH 8.3, containing 300 mM NaCl, and incubated with 8 m units ACE at 37°C for 30 min. The concentration of ACE inhibitors needed to inhibit 50% of ACE activity was defined as the ID_{50} value.

The ACE used in our experiments were crude, extracted from rabbit lung acetone powder, or partially purified with calcium phosphate gel and Sepharose 6B from crude ACE.

Isolation of $CEI_{\beta 7}$. One gram of casein from bovine milk

was hydrolyzed with 2.5 mg of trypsin from bovine pancreas by the method described in our previous report.⁶⁾ After 18 hr of digestion at 37°C the reaction was terminated by the addition of conc. HCl to 0.5 M concentration. After the precipitate was removed by filtration, the supernatant was adjusted to pH 7.0 with NaOH. The hydrolysate was put on a Sephadex LH-20 column (3 × 112 cm) and eluted with distilled water. The most active fraction was put in 2 batches on a SP-Sephadex C-25 column (2 × 47 cm) and eluted with a linear gradient using 200 ml each of distilled water and 0.5 M ammonium formate (pH 7.0). Two active peaks were observed. The 1st peak was CEI₁₂, and the 2nd peak was further chromatographed with a Sephadex LH-20 column (2 × 62 cm) and eluted with distilled water.

Isolation of CEI₅. Four milligrams of CEI₁₂ purified by the method described in our previous paper was dissolved in 1 ml of phosphate buffer (0.04 M, pH 7.0) and 2 units proline-specific endopeptidase was added. After 3 hr of incubation at 37°C, the enzyme was inactivated by boiling for 10 min. The denatured enzyme was removed by ultrafiltration with CF25 (Amicon Co.). The hydrolyzed sample was put on a column (Radial-PAK C₁₈, Waters Inc.) for high pressure liquid chromatography (HPLC). It was eluted at a flow rate of 1 ml/min with phosphate buffer (10 mM KH₂PO₄, 50 mM Na₂SO₄, pH 3.0)/CH₃CN (60:40, v/v) monitoring at 210 nm. An active peak was collected and salts were removed by a Sep-PAK C₁₈ cartridge (Waters Inc.).

Determination of amino acid sequences of ACE inhibitors. The amino acid composition of each ACE inhibitor was analyzed with a Hitachi 835 high-speed amino acid analyzer after hydrolysis in 6 N HCl at 110°C for 24 hr. Sequential analysis of CEI₅ was done by a modification of the method of Edman.⁷⁾ The N- and C-terminal amino acid sequences of CEI_{β7} were determined by the direct method of Edman and by treatment with carboxypeptidase Y, respectively.

Assay of the bradykinin-potentiating activity of ACE inhibitors on the uterus of rat. The bradykinin-potentiating activity of ACE inhibitors was measured on the isolated rat uterus. The uterus of a virgin rat (7 to 11 weeks old, Wistar) was mounted under 1 g load, in a 5-ml muscle bath at 27°C containing aerated de Jalon solution (NaCl 152 mM, KCl 5.6 mM, CaCl₂ 0.4 mM, NaHCO₃ 6.0 mM, glucose 2.8 mM). After mounting, the uterus was equilibrated for 30 min before the beginning of the experiments. ACE inhibitor was added before addition of standard bradykinin solution, and bradykinin induced contraction was measured with an isotonic transducer (Natsume Co., Ltd. KN-259).

Assay of the bradykinin-potentiating activity of ACE inhibitors on the ileum of rat. The ileum from a rat (7 to 11

weeks old, Wistar) was mounted under 2 g load in a 5-ml muscle bath at 30°C containing aerated Tyrode solution (NaCl 8.0 g/liter, KCl 0.2 g/liter, CaCl₂ 0.2 g/liter, MgCl₂ 0.1 g/liter, NaHCO₃ 1.0 g/liter, NaH₂PO₄ 0.05 g/liter, glucose 1.0 g/liter). The effects of the inhibitors were assayed in the same way as the uterus. 'Sensitizing activity' of the inhibitors was assayed according to a modification of the method of Kato and Suzuki²⁾ after washing the inhibitors out from the organ bath.

RESULTS

Isolation of CEI_{β7}

Isolation of CEI_{β7} from the tryptic hydrolysate of casein was attempted by the procedure described in MATERIALS AND METHODS. Figure 1 shows the separation of CEI₁₂ and CEI_{β7} by SP-Sephadex C-25 from the active peak of Sephadex LH-20 chromatogram. Fractions of CEI_{β7} were further fractionated with Sephadex LH-20 chromatography. The amino acid composition of CEI_{β7} hydrolyzed with 6 N HCl was: Ala (1.00), Val (0.95), Tyr (1.01), Arg (0.95), Pro (1.81), Glu (1.14). The amino acid sequence determined by the direct method of Edman and by digestion with carboxypeptidase Y is listed in Table I. This heptapeptide corresponds to the sequence 177~183 of β-casein.

Isolation of CEI₅

Isolation of CEI₅ was done as described in MATERIALS AND METHODS. Figure 2 shows a HPLC chromatogram of a solution of CEI₁₂ hydrolyzed with proline-specific endopeptidase. Potent inhibition appearing in the 3rd large peak (indicated as CEI₅) was collected. The amino acid composition of CEI₅ was: Ala (1.00), Val (1.10), Phe (1.84), Pro (0.90). The amino acid sequence determined by the direct method of Edman is also listed in Table I. This peptide was the 5 amino acid residues at the N-terminal of CEI₁₂.

The ID₅₀ values of CEI_{β7} and CEI₅ in our assay condition using crude ACE as enzyme were 15 μM and 6.0 μM, respectively. The ID₅₀ value of ACE inhibitors determined using partially purified ACE (0.8 unit/mg protein) was about 9/10 of the ID₅₀ value determined using crude ACE.

FIG. 1. Purification of $\text{CEI}_{\beta 7}$ on a SP-Sephadex C-25 Column.

After gel filtration on Sephadex LH-20, the sample was chromatographed on a SP-Sephadex C-25 column (2 × 47 cm) with linear gradient elution from 0 to 0.5 M ammonium formate buffer, pH 7.0. Eluate was collected as 4 ml fractions at a flow rate of 24 ml/hr. The effluent was assayed for inhibitory activity (—○—), and absorbance at 280 nm was monitored with an ISCO UA5 absorbance monitor (—).

FIG. 2. Purification of CEI_5 on a HPLC Column.

Twenty μl of the enzymatic hydrolysate of CEI_{12} by proline-specific endopeptidase was injected (see MATERIALS AND METHODS). The most active peak was collected (indicated as CEI_5). Conditions for chromatograms: Column, Radial-PAK C_{18} (10 μ); mobile phase, phosphate buffer (10 mM KH_2PO_4 , 50 mM Na_2SO_4 , pH 3.0)/ CH_3CN (60:40, v/v); flow rate, 1 ml/min; detector, UV 210 nm, 2.56; sample, 20 μl .

Bradykinin-potentiating activity of ACE inhibitors on the rat uterus

ACE inhibitors were tested by the contraction of the rat uterus for their bradykinin potentiation. Figure 3 shows SQ 20881, bradykinin-potentiator B, and CEI_{12} increase the effect of bradykinin at the indicated concentration (*i.e.*, $0.13 \times \text{ID}_{50}$ value of each ACE

FIG. 3. Bradykinin-potentiating Activity of ACE Inhibitors on the Rat Uterus.

Bradykinin activity was measured by the contraction of the rat uterus in aerated de Jalon solution at 27°C. For assay of bradykinin-potentiating activity, each ACE inhibitor (at the concentration of $0.13 \times \text{ID}_{50}$ value) was added before addition of 4.0×10^{-10} M bradykinin. BK, bradykinin 4.0×10^{-10} M; BK × 2, bradykinin 8.0×10^{-10} M; SQ, SQ 20881 6.5×10^{-8} M; BP-B, bradykinin-potentiator B 4.3×10^{-7} M; CEI_{12} , CEI_{12} 1.0×10^{-5} M; $\text{CEI}_{\beta 7}$, $\text{CEI}_{\beta 7}$ 2.0×10^{-6} M; $\text{CEI}_{\beta 7} \times 3$, $\text{CEI}_{\beta 7}$ 6.0×10^{-6} M; CEI_5 , CEI_5 7.8×10^{-7} M; $\text{CEI}_5 \times 6$, CEI_5 4.7×10^{-6} M.

inhibitors). $\text{CEI}_{\beta 7}$ and CEI_5 , however, had little bradykinin-potentiating activity at the concentration of the same proportion of the

TABLE I. AMINO ACID SEQUENCE AND ID₅₀ VALUE OF ACE INHIBITORS

	Amino acid sequence	ID ₅₀ μ M	Ref.
CEI ₁₂	Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys	77	6
CEI _{β7}	Ala-Val-Pro-Tyr-Pro-Gln-Arg	15	This paper
CEI ₅	Phe-Phe-Val-Ala-Pro	6.0	This paper
SQ 20881	Pyr-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro	0.50	1
BP-B	Pyr-Gly-Leu-Pro-Arg-Pro-Lys-Ile-Pro-Pro	3.3	2
BP-C	Pyr-Gly-Leu-Pro-Pro-Gly-Pro-Pro-Ile-Pro-Pro	17	2

BP-B, bradykinin-potentiator B; BP-C, bradykinin-potentiator C; Pyr, pyroglutamic acid.

FIG. 4. Bradykinin-potentiating Activity of ACE Inhibitors on the Ileum of Rat.

Bradykinin-potentiating activity was assayed by the contraction of the ileum of rat in aerated Tyrode solution at 30°C.

(a): To 5 ml volume of muscle bath 0.10~0.40 μ M bradykinin was added.

(b~d): ACE inhibitors (at the concentration of $0.13 \times \text{ID}_{50}$) were added just before addition of 0.10 μ M bradykinin.

b), 10 μ M CEI₁₂; c), 2.0 μ M CEI _{β 7}; d), 0.78 μ M CEI₅.

ID₅₀ value (*i.e.* $0.13 \times \text{ID}_{50}$ value of each peptide), but CEI _{β 7} and CEI₅ increased the effect of bradykinin when a higher concentration was added to the muscle bath.

Thus, CEI₅ and CEI _{β 7} are weak as bradykinin-potentiator in comparison with its inhibitory activity on ACE.

Bradykinin-potentiating activity of ACE inhibitors on the ileum of rat.

All ACE inhibitors derived from casein potentiated the effect of bradykinin on the contraction of the isolated ileum of rats at the indicated concentration (*i.e.* $0.13 \times \text{ID}_{50}$ value of each ACE inhibitor) (Fig. 4). Thus, ACE inhibitors were more effective on the ileum than on the uterus. The effects of these in-

hibitors lasted for more than 90 min even after the inhibitor was washed out from the organ bath. Figure 5 (sensitizing activity of CEI₁₂ on the ileum of rat) shows the effect of 20 μ M CEI₁₂ lasted for 90 min after CEI₁₂ was washed out from the bath.

DISCUSSION

Many of the natural inhibitors derived from snake venom have Pro-Pro or Ala-Pro sequences at their C-terminal. CEI₅ also has an Ala-Pro sequence at its C-terminal. Therefore, the inhibitory activity of CEI₅ is more potent than CEI₁₂, and the ID₅₀ value of CEI₅ is almost equal to the bradykinin-potentiator derived from the venom of *Agkistrodon halys*

FIG. 5. Sensitizing Activity of CEI_{12} on the Ileum of Rat.

Sensitizing activity was assayed by the contraction of the ileum of rat in aerated Tyrode solution at 30°C .

(1): To 5 ml volume of muscle bath $0.1\ \mu\text{M}$ bradykinin was added, and contraction was measured.

(2): Then the ileum was washed with Tyrode solution. $20\ \mu\text{M}$ CEI_{12} and $0.1\ \mu\text{M}$ bradykinin were added, and contraction was measured.

(3~5): And then, the ileum was washed 5 times with Tyrode solution. 10, 60, and 90 min later after washing, $0.1\ \mu\text{M}$ bradykinin was added, and contraction was measured.

blomhoffii (The ID_{50} value of these inhibitors were listed in Table I). On the other hand, CEI_{12} and $\text{CEI}_{\beta 7}$ do not have a common structure at their C-terminals. However, it is unclear which amino acid residues are important for their inhibitory activity.

ACE is a zinc metalloprotein, and similar in many ways to carboxypeptidase A of bovine pancreas, but our inhibitors (at the concentration of $400\ \mu\text{M}$) did not inhibit carboxypeptidase A.

Bradykinin-potentiating activity was measured using the guinea pig ileum by many investigators.^{2,8)} In the rat uterus, bradykinin potentiation by ACE inhibitor was less investigated, because it requires a higher concentration of ACE inhibitor than the guinea pig ileum.²⁾ Our ACE inhibitors potentiated the activity of bradykinin on the uterus of rat. But the concentration of $\text{CEI}_{\beta 7}$ and CEI_5 required for the potentiation on the uterus were higher than required on the ileum.

Cushman *et al.* measured the concentration of ACE in tissues of the rat. They reported that high specific activities of ACE were found in the lung and ileum of rats, and the uterus contained some peptidases other than ACE.⁹⁾ Our data may reflect the concentration of ACE in the tissues of rats.

Kato and Suzuki observed that the response of the isolated ileum of guinea pig to bradykinin was enhanced by bradykinin-potentiator B even after this peptide was washed out.²⁾ This residual effect was termed 'sensitization'. All our ACE inhibitors had this sensitizing activity on the ileum of rats. SQ20881 and CEI_5 also showed this effect on the uterus of rats, however, CEI_{12} and $\text{CEI}_{\beta 7}$ showed little of this effect, for unknown reasons.

Recently, it has been reported that there are biologically active peptides in enzymatic hydrolysates of casein, such as β -casomorphin¹⁰⁾ and exorphin.¹¹⁾ Our peptides derived from casein also inhibit ACE, and it is interesting that biologically active peptides are made from food by enzymes in the digestive tract.

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