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Full Papers

Cleavage of des-Arg^9-bradykinin by angiotensin I-converting enzyme from pig kidney cortex[1, 2]

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Summary. Fast and very slow hydrolyses of des-Arg^9-bradykinin and angiotensin II by angiotensin I-converting enzyme were detected by high performance liquid chromatography. The Michaelis constants of the enzyme, K_m values, for des-Arg^9-bradykinin and bradykinin were found to be 0.24 mM and 4.4 μM, and the maximum velocities, V_{max} values (μmol·min^{-1}·mg protein^{-1}) for these compounds to be 3.24 and 0.34, respectively.
The enzyme also hydrolyzed Z-Gly-Pro-Gly-Gly-Pro-Ala to a tripeptide that was identified as dansyl-Gly-Pro-Ala by TLC on polyamide. These observations show that the enzyme hydrolyzes the peptides at the bond before the prolyl residue in the penultimate position.
Key words. Pig kidney cortex; des-Arg-bradykinin; angiotensin I-converting enzyme.

Introduction. The angiotensin I-converting enzyme (ACE; kininase II) is known to cleave a wide variety of peptides including angiotensin I and kinins[20], releasing a dipeptide from the C-terminus. This enzyme, therefore, is called peptidyl dipeptide hydrolase or dipeptidyl carboxypeptidase [EC 3.4.15.11][3]. The number of possible substrates of ACE is limited, because ACE cannot hydrolyze peptide bonds involving the imino group of proline[3, 18, 21]. Thus, angiotensin II is thought not to be hydrolyzed after its release from angiotensin I by ACE. A HPLC method was developed in this laboratory for measuring the concentrations of metabolites of bradykinin and angiotensin I and II and characterizing them. After separation of peptides by single step, reversed-phase HPLC, the guanidino groups of arginyl residues in the peptides were detected fluorometrically by reaction with alkaline ninhydrin[9]. As reported previously, human plasma cleaved bradykinin to produce other peptides besides des-Arg^9-BK and des-[Phe^8-Arg^9]-BK, products of kininases I and II, respectively. Human plasma also hydrolyzed des-Arg^9-BK to form an unidentified peptide that differed from the heptapeptide des-[Phe^8-Arg^9]-BK. The formation of the unknown peptide from bradykinin seemed to be correlated with the disappearance of des-Arg^9-BK by human plasma. Since degradation of des-Arg^9-BK by human plasma was strongly inhibited by EDTA, an exopeptidase, probably a carboxypeptidase, was thought to be responsible for the hydrolysis[9].
We purified ACE to homogeneity from pig kidney cortex, and found that it converted angiotensin I to II, inactivated bradykinin and hydrolyzed several smaller synthetic peptide substrates[15, 17]. Thus, it was a typical peptidyl dipeptide hydrolase. The purified preparation of ACE was essentially free from aminopeptidase, carboxypeptidase and other dipeptidases[16]. This enzyme preparation hydrolyzed des-Arg^9-BK at a rate comparable to that of bradykinin. This paper reports the hydrolyses by ACE from pig kidney cortex of the antepenultimate peptide bonds of Z-Gly-Pro-Gly-Gly-Pro-Ala to

Gly-Pro-Ala, and probably also of des-Arg^9-BK and angiotensin II which have a prolyl residue in the penultimate position, to the tripeptides Ser-Pro-Phe and His-Pro-Phe, respectively.

Materials and methods. Materials. Bradykinin, Z-Gly-Pro-Gly-Gly-Pro-Ala, L-Ala-L-Ala; L-Pro-L-Phe, L-Pro-L-Ala, Gly-Gly and Gly-L-Pro-L-Ala were obtained from Sigma Chemical Co., St. Louis, Mo., USA. Angiotensins I and II, des-Arg^9-BK and des-[Phe^8-Arg^9]-BK were purchased from the Protein Research Foundation, Minoh-shi, Osaka 562, Japan. Captopril was obtained from Sankyo Co., Shinagawa-ku, Tokyo 140, Japan. Dansyl chloride was obtained from Tokyo Kasei Co., Chuo-ku, Tokyo 103, Japan. Polyamide sheets were from E. Merck AG, Darmstadt, West Germany. ACE from pig kidney cortex was purified as described previously[17]. The purified ACE appeared to be homogeneous on disc and sodium dodecyl sulfate polyacrylamide gel electrophoresis, and had a specific activity of 27.3 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ with Bz-Gly-Gly-Gly as substrate. The preparation seemed to be free from peptidases including amino- and carboxy-peptidases and dipeptidase[16].

Hydrolysis of peptides by ACE. The reaction mixture contained 10 nmol peptide and 3.0 µg of ACE in 0.1 ml of 10 mM Tris-HCl (pH 7.4) containing 0.1 M NaCl. After incubation at 37 °C, 25 µl of 30% trichloroacetic acid was added and an aliquot (25 µl) was subjected to HPLC under essentially the same conditions as described previously[9]. Kinetic analyses in 2 min with 0.3 mg of ACE were made at substrate concentrations of 0.5 to 2.0 µM bradykinin and 25 to 200 µM des-Arg^9-BK, and K_m and V_{max} values were determined from double reciprocal plots[11].

Identification of peptides. Z-Gly-Pro-Gly-Gly-Pro-Ala (100 nmol) was treated overnight with ACE (28 µg) at 37 °C in 0.1 ml of 0.1 M HEPES-NaOH (pH 8.0) containing 0.2 M NaCl and 1% dimethyl sulfoxide. Dansyl chloride (10 µmol/0.1 ml acetone) was added to the digest which was then shaken for 2 h at room temperature. The mixture was dried in vacuo, and dansylamine was removed by three consecutive extractions with ether. The residue was acidified with 20 µl of 1 M HCl and extracted twice with ethyl acetate. TLC on polyamide sheets was done with several solvent systems, the best separation being obtained with benzene: acetic acid (9:1).

Results and discussion. Bradykinin, des-Arg^9-BK and angiotensin II were incubated with ACE from pig kidney cortex, and then unhydrolyzed peptides in the reaction mixture were measured by HPLC as described previously[9]. Bradykinin was rapidly hydrolyzed to des-[Phe^8-Arg^9]-BK (fig. 1) and probably the pentapeptide Arg-Pro-Pro-Gly-Phe. Des-Arg^9-BK was also rapidly and extensively hydrolyzed by the enzyme.

The product of des-Arg^9-BK and des-[Phe^8-Arg^8]-BK were eluted as two incompletely separated peaks under the conditions described previously[9]. ACE hydrolyzed angiotensin II slightly on prolonged incubation. This slow hydrolysis of angiotensin II by ACE was demonstrated by the appearance of small new peaks in the elution profile in addition to the large peak of remaining angiotensin II: Since the authentic hexapeptide des-

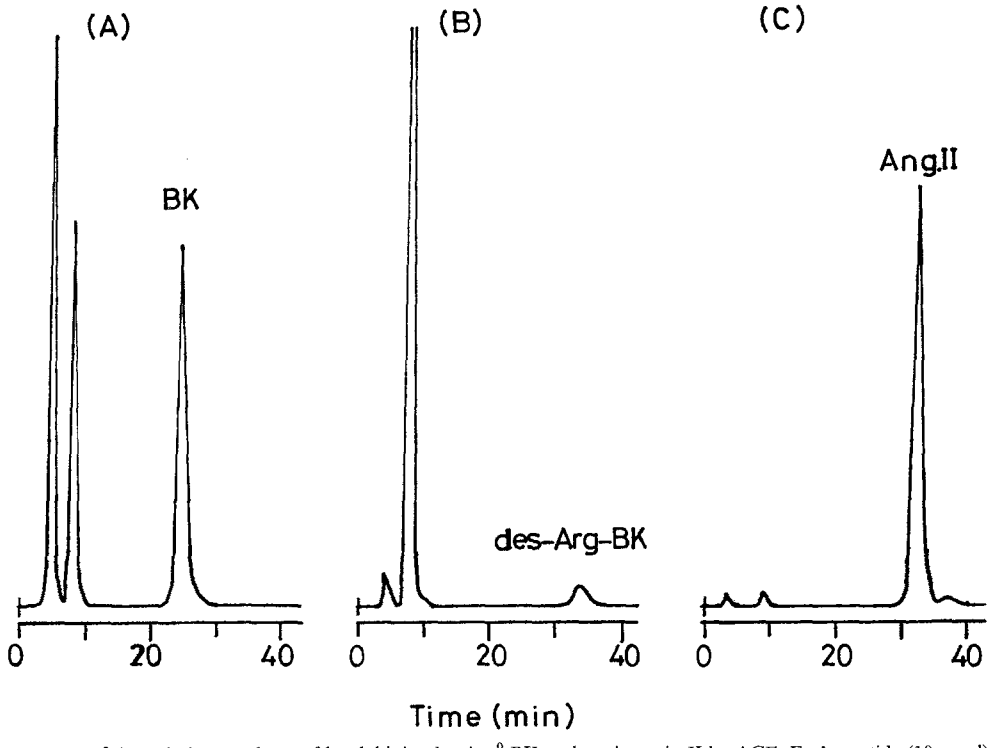

Figure 1. Chromatogram of degradation products of bradykinin, des-Arg^9-BK and angiotensin II by ACE. Each peptide (10 nmol) was hydrolyzed for 20 min at 37 °C by 3.0 µg ACE in 0.1 ml of 10 mM Tris-HCl (pH 7.4) with 0.1 M NaCl. The digestion products were analyzed by HPLC. (A) bradykinin; (B) des-Arg^9-BK; (C) angiotensin II.

[Pro^7-Phe^8]-angiotensin II was not available, these small peaks could not be identified by HPLC.

The rates of hydrolysis of 10 nmol of bradykinin, des-Arg^9-BK and angiotensin II were determined from the amounts of these peptides remaining after the reaction (Table). Des-Arg^9-BK was hydrolyzed more rapidly than the other two peptides tested by ACE, while angiotensin II was hydrolyzed slowest. The apparent rate of hydrolysis of bradykinin by ACE was about one-tenth of that of Bz-Gly-Gly-Gly under our assay conditions. In general, ACE hydrolyzed shorter peptide substrates more rapidly than angiotensin I[4,19]. Moreover, the enzyme had a much higher V_{max} for an N-blocked tripeptide than a further C-terminal tetrapeptide of angiotensin I[13]. Since the rate was determined with 0.1 mM bradykinin, i.e. far above the physiological concentration, relatively slow hydrolysis of bradykinin by ACE could be expected.

The hydrolyses of des-Arg^9-BK and angiotensin II by ACE were inhibited completely by 1 mM EDTA (data not shown). The hydrolysis of des-Arg^9-BK was also strongly inhibited by 1 mM Ala-Ala and 0.1 μM captopril. These inhibitions of ACE by EDTA, Ala-Ala[5,16] and captopril (SQ 14225)[14] can be used for identification of the enzyme. The ACE preparation used released Ala-Ala, but not free alanine, from N-blocked trialanine and tetraalanine[16]. These findings indicate that the ACE preparations was not contaminated by aminopeptidase [EC 3.4.11.1] or carboxypeptidase [EC 3.4.13.11]. Thus, des-Arg^9-BK was hydrolyzed specifically by ACE, and not by other contaminating proteases.

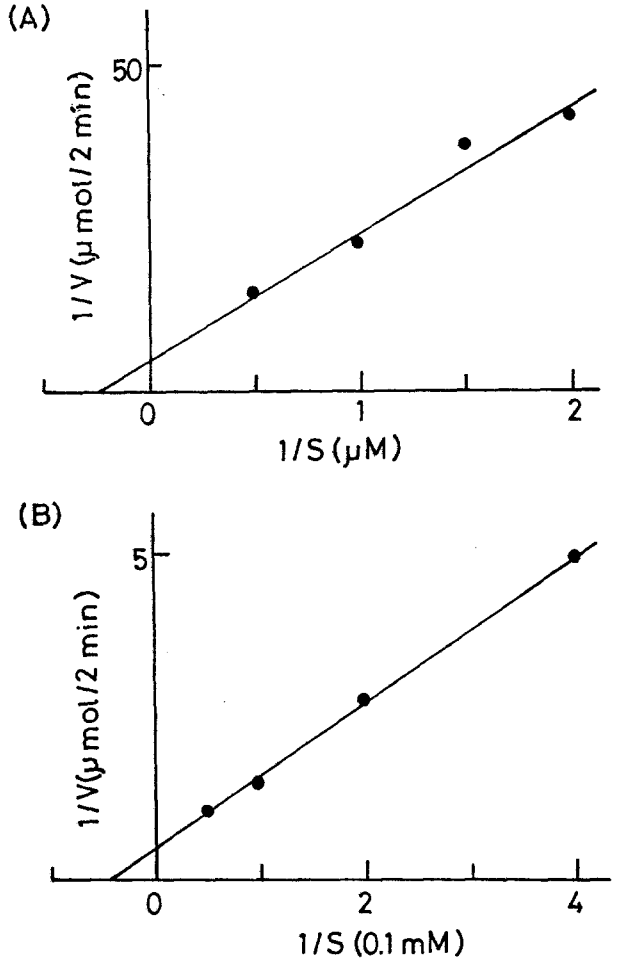

Figure 2. Lineweaver and Burk's plots of hydrolyses of bradykinin and des-Arg^9-BK by ACE. Bradykinin (0.5 to 2.0 μM) and des-Arg^9-BK (25–200 μM) were digested for 2 min at 37 °C with 0.3 mg ACE in 1.0 ml of 10 mM Tris-HCl (pH 7.4) with 0.1 M NaCl. (A) bradykinin; (B) des-Arg^9-BK.

The kinetic parameters of ACE for bradykinin and des-Arg^9-BK were determined by HPLC. The Michaelis constants, K_m values, of ACE for des-Arg^9-BK and bradykinin were calculated to be 240 μM and 4.4 μM, respectively. The maximum velocities, V_{max} values, of ACE for des-Arg^9-BK and bradykinin were 3.24 and 0.34 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$, respectively (fig. 2). The K_m value obtained for bradykinin was consistent with those reported[6,7]. The slight differences between reported values and our value could be due to species or organ differences. Our results showed that the K_m values of ACE for bradykinin and for des-Arg^9-BK were markedly different. The k_{cat} value of the enzyme was higher for des-Arg^9-BK than for bradykinin. Although the sequence of the C-terminal dipeptide of angiotensin II is the same as that of des-Arg^9-BK, only the latter peptide was cleaved by ACE at a measurable rate, and the hydrolysis of angiotensin II was too slow to allow determination of the kinetic parameters of the enzyme for this peptide.

Since ACE does not hydrolyze an imino-peptide bond in the penultimate position of several substrates, including angiotensin II[3,20], it was of interest to determine how it cleaves the peptide bond of des-Arg^9-BK with a prolyl residue in the penultimate position. The digest of des-Arg^9-BK contained at least three peptides (unhydrolyzed substrate and N- and C-terminal peptide fragments). Only Pro-Phe was available as an authentic peptide. Therefore, we used Z-Gly-Pro-Gly-Gly-Pro-Ala to deduce the cleavage site of des-Arg^9-BK and angiotensin II by ACE. This peptide has a prolyl residue in the penultimate position at the C-terminus and Pro-Ala, Gly-Gly and Gly-Pro-Ala are detected by TLC on polyamide after dansylation. The digest of Z-Gly-Pro-Gly-Gly-Pro-Ala by ACE contained only dansyl-Gly-Pro-Ala. The results indicated that when a prolyl residue was located in the penultimate position of the substrate, ACE hydrolyzed the antepenultimate peptide bond, beyond the resistant imino-peptide bond of the substrate. Recently, it was demonstrated that ACE does not have an absolute requirement for a free carboxyl group on the substrate, since it hydrolyzes several peptide derivatives with a C-terminal nitrobenzylamide residue[8].

Rates of hydrolysis of bradykinin, des-Arg^9-BK and angiotensin II by ACE. The peptides (10 nmol) were hydrolyzed for various periods at 37 °C by 3.0 μg ACE in 0.1 ml of 10 mM Tris-HCl (pH 7.4) and 0.1 M NaCl. Half-lives, $t_{1/2}$(min), were calculated by the following equation: $t_{1/2} = 0.693/k$

Substrate	$t_{1/2}$ (min)
Bradykinin	20.1
Des-Arg^9-BK	4.3
Angiotensin II	354

ACE thus preferentially hydrolyzes an antepenultimate peptide bond of a stubstrate having a resistant prolyl residue in its penultimate position. These findings suggest that ACE hydrolyzes the Phe[5]-Ser[6] and Ile[5]-His[6] bonds of des-Arg[9]-BK and angiotensin II, respectively. After completion of our work, the paper of Inoguchi and Nagamatsu[10] appeared reporting that ACE has tripeptidyl carboxypeptidase activity for several substrates including des-Arg[9]-BK with a prolyl residue in the penultimate position. Their results are consistent with our conclusion that ACE hydrolyzes the antepenultimate peptide bond of des-Arg[9]-BK. Since they could not detect any hydrolysis of angiotensin II or Bz-Gly-Gly-Pro-Phe by ACE, they concluded that ACE hydrolyzed hexa-peptides or larger peptides with seryl or alanyl and prolyl residues in the antepenultimate and penultimate positions, respectively[10]. However, ACE slowly hydrolyzed angiotensin II and Z-Gly-Pro-Gly-Gly-Pro-Ala in our conditions, indicating that peptides with a prolyl residue in the penultimate position but a residue other than a seryl or alanyl residue in the antepenultimate position are also hydrolyzed slightly by ACE. Since ACE shows strong stereo-specificity for the third amino acid residue from the C-terminus of the substrate peptide[16], it seems likely that this residue in a variety of peptide substrates determines the specificity of the peptides to ACE.

Recently, Marceau et al.[12] reported the degradation of des-Arg[9]-BK in plasma. They suggested that the peptide was hydrolyzed to des-[Phe[8]-Arg[9]]-BK by carboxypeptidase that was not inhibited by captopril, but our data and those of Inoguchi and Nagamatsu obtained by HPLC clearly showed that ACE was responsible for the hydrolysis of des-Arg[9]-BK. The above findings indicate that previous conclusions that ACE does not hydrolyze peptides with a prolyl residue in the penultimate position and that it does not hydrolyze any bonds in the substrate besides the Phe-His bond of angiotensin I[3] are incorrect. Thus, it is probable that angiotensin II is not a limit peptide for ACE and that in fact it is inactivated very slowly by the enzyme.

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2 Abbreviations used: Z, carbobenzoxy; Bz, benzoyl; ACE, angiotensin I-converting enzyme; BK, bradykinin; HPLC, high performance liquid chromatography.

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