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KININ AND ANGIOTENSIN METABOLISM BY PURIFIED RENAL POST-PROLINE CLEAVING ENZYME

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Abstract—Post-proline cleaving enzyme (PPCE; EC 3.4.21.26) is a proline specific endopeptidase capable of hydrolyzing biologically active peptides. The present studies examined the hydrolysis of kinin- and angiotensin-related peptides by cytosolic PPCE purified from porcine kidney. PPCE hydrolysis of the synthetic substrate Z-Gly-Pro-MCA (30.7 ± 0.3 μmol · min^{-1} · mg^{-1}) was competitively inhibited by saralasin, bradykinin, des(Arg^9)bradykinin, [Leu^8],des(Arg^9)bradykinin and angiotensin II (IC_{50} = 0.5 to 7.0 μM). Qualitative TLC studies demonstrated that each peptide was degraded by hydrolysis on the carboxyl side of proline residues (positions 7 or 8). Quantitative HPLC studies established that peptide degradation was optimal at pH 8.2 to 8.7 and was inhibited by the specific PPCE inhibitor Z-Pro-prolinal (IC_{50} = 0.8 ± 0.1 nM). Conversely, degradation was unaffected by inhibitors of aminopeptidases (amastatin), neutral endopeptidase (phosphoramidon), carboxypeptidase N (MERGETPA) or angiotensin I converting enzyme (captopril). Apparent K_m values, obtained from Lineweaver–Burk analysis, were comparable for all kinin and angiotensin peptides (K_m = 5.5 to 12.8 μM), whereas V_{max} values ranged from 1.7 μmol · min^{-1} · mg^{-1} for angiotensin II to 0.44 μmol · min^{-1} · mg^{-1} for saralasin. These data are consistent with a role for PPCE in the degradation of kinins and angiotensin *in vivo*.

Kinins and angiotensins are potent vasoactive peptides implicated in the pathogenesis of hypertension and other phenomena involving vascular smooth muscle. In addition to the kidney, kinins and angiotensins are reported to be synthesized in both the vascular wall [1, 2] and the CNS [3, 4]. Although angiotensin I converting enzyme (EC 3.4.15.1) and carboxypeptidase N (EC 3.4.17.3) are established kinin and angiotensin metabolizing enzymes [5, 6], less is known about the possible contribution of other peptidases to kinin and angiotensin metabolism.

These vasoactive peptides contain a high proportion of proline residues which make them resistant to hydrolysis by most other peptidases. Post-proline cleaving enzyme (PPCE; EC 3.4.21.26), first discovered by Walter and co-workers [7], has been characterized as an endopeptidase which specifically hydrolyzes peptides on the carboxyl side of internal proline residues [8]. PPCE has been identified in a variety of sites including the kidney [9, 10], brain [11, 12] and vasculature [13]. Since a previous study had found that bradykinin and angiotensin II inhibited renal PPCE [14], we examined the ability of this purified enzyme to metabolize kinins and angiotensins.

MATERIALS AND METHODS

Materials. The PPCE substrate *N*-benzyloxycarbonyl-Gly-Pro-methylcoumarinyl-7-amide (Z-Gly-Pro-MCA) was obtained from Bachem (Torrance, CA). Kallidin, bradykinin, des(Arg^9)bradykinin, [Leu^8],des(Arg^9)bradykinin, Phe-Arg, saralasin, phenanthroline, amastatin, phosphoramidon [*N*-(α-rhamnopyranosyl-oxyhydroxyphosphinyl-L-leucyl-L-tryptophan)], dithiothreitol (DTT), crystalline bovine serum albumin (BSA) and 7-amino-4-methylcoumarin (MCA) were obtained from the Sigma Chemical Co. (St. Louis, MO). The angiotensin I converting enzyme inhibitor captopril was obtained from Squibb (Princeton, NJ). The plasma carboxypeptidase N inhibitor MERGETPA (D-L-mercaptomethyl-3-guanidino-ethylthiopropanoic acid [15] was from Calbiochem-Behring (San Diego, CA). Amino acid standards, *o*-phthalaldehyde crystals and *o*-phthalaldehyde reagent solution (OPA) were obtained from the Pierce Chemical Co. (Rockford, IL). The MN 300 Uniplates used for TLC were from Analtech, Inc. (Newark, DE).

Z-Gly-Pro-MCA hydrolysis. Porcine kidney PPCE was purified as previously described and routinely assayed using Z-Gly-Pro-MCA as substrate [14]. The reaction (400 μl) consisted of PPCE in 20 mM Tris/HCl buffer (pH 8.3) containing 1 mM EDTA, 1 mM sodium azide, 10 mM DTT, crystalline bovine serum albumin (10 μg/ml) and 0.15 mM Z-Gly-Pro-MCA. The fluorescence of the MCA product was followed continuously at 37° using a Perkin Elmer spectrofluorometer with excitation and emission set at 383 and 455 nm respectively. During the initial reaction (20%), increases in fluorescence were directly proportional to both time of incubation and amount of enzyme added. Fluorescence was converted into μmol of product produced using a standard curve of MCA. For competitive substrate experiments, a specified concentration of peptide (e.g. bradykinin) was added simultaneously with various concentrations of Z-Gly-Pro-MCA substrate, and reaction

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rates were plotted as $1/V$ vs $1/S$ [16]. Enzyme specific activities are expressed as units/mg protein where one unit equals the hydrolysis of one μmol Z-Gly-Pro-MCA substrate per min. Protein was determined with the protein assay kit of Bio-Rad (Richmond, CA) [17].

Peptide metabolism—TLC. Qualitative assays of peptide metabolism were carried out by TLC on MN 300 cellulose plates [18]. The reaction (60 μl) consisted of PPCE, the above buffer and peptide substrate (1 mM). At sequential time intervals, 5-μl aliquots were spotted on the plate and immediately dried. Plates were developed in butanol–acetic acid–water (4:1:5), and the products were visualized by staining with 0.4% (w/v) ninhydrin in acetone.

Peptide metabolism–HPLC. Quantitative analysis of peptide metabolism was carried out by HPLC as previously described [18, 19]. The standard reaction (300–1000 μl) consisted of 100 mM sodium phosphate buffer (pH 8.3) containing, as above, 1 mM EDTA, 1 mM sodium azide, 10 mM DTT, crystalline bovine serum albumin (10 μg/ml) and peptide substrate (250 μM). At sequential time intervals, 80 to 200-μl aliquots of the reaction mixture were immersed in a boiling water bath (6 min) to terminate the reaction, cooled on ice and centrifuged in a Brinkmann table top centrifuge (5 min); the supernatant fractions were collected for analysis. Control experiments established that recoveries of metabolites [i.e. des(Phe^8,Arg^9)bradykinin, Phe-Arg, amino acids] were greater than 92%. Reaction rates, calculated during the first 15% of the reaction, were linear with respect to both amount of enzyme used and the time of incubation. Specific activities are expressed as units/mg protein where one unit equals the hydrolysis of one μmol of peptide per min.

For pH experiments, a 100 mM sodium acetate buffer was used over the pH range 4.0 to 6.0, and the above 100 mM phosphate buffer was used from pH 6.5 to 8.5. pH studies were also conducted with the Britton and Robinson type [20] universal buffer (pH 5.5 to 10.5). For inhibition studies, inhibitors were preincubated with enzyme and buffer for 20 min at 37°. For K_m determinations, measurements of the initial velocity of hydrolysis were determined over a range of substrate concentrations (4–40 μM). Data were plotted according to the Lineweaver–Burk method ($1/V$ vs $1/S$) and fit to the best straight line [16].

A high performance liquid chromatograph (Waters Associates, Milford, MA) consisting of two model 6000A pumps, a model 730 Data Module, a model 721 System Controller, a model 710B WISP Autosampler, and a model 420 Fluorescence Detector were employed for the HPLC analysis. Standards and unknowns (40–120 μl) were automatically derivatized with OPA solution (20 μl) 3 min prior to chromatography (Pre-column Derivatization Program, Waters 710B WISP Autosampler) and subsequently separated on a reverse phase column. Separation of amino acid metabolites was performed on a Waters, 10 μ/C_{18}-Radial-PAK (8 mm × 10 cm) column at a constant flow rate of 5 ml/min utilizing a linear gradient from 100% Buffer A to 40% Buffer A/60% Buffer B (12 min). Buffer A was 10 mM sodium phosphate (pH 7.0), and Buffer B was a 50/50 (v/v) mixture of Buffer A and acetonitrile. Prior to use, both buffers were degassed by filtration through a Millipore AP Pre-Filter. Separation of Phe-Arg, des(Phe^8,Arg^9)bradykinin and des(Phe^9, Arg^{10})kallidin was performed on a Waters 4 μ/C_{18} NOVA-PAK (8 mm × 10 cm) column at a constant flow rate of 4 ml/min utilizing a linear gradient from 100% Buffer A to 100% Buffer B (15 min). After each separation, the column was washed with 100% Buffer B (4 min) and re-equilibrated in 100% Buffer A (4 min). Integration of sample peak areas and quantitation of metabolites against the last-run standards (run every sixth injection) were automatically calculated by the data module.

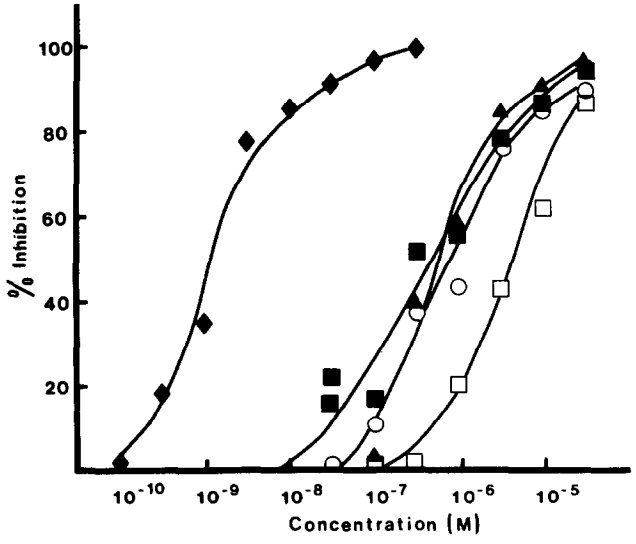

Fig. 1. Inhibition (%) of renal post-proline cleaving enzyme hydrolysis of Z-Gly-Pro-MCA by Z-Pro-prolinal (◆), saralsin (■), bradykinin (▲), des(Arg9)bradykinin (○) and angiotensin II (□). Reactions were carried out in 20 mM Tris/HCl buffer (pH 8.3) containing 1 mM EDTA, 1 mM sodium azide, 10 mM DTT and BSA (10 μg/ml) with 0.15 mM Z-Gly-Pro-MCA substrate. Inhibitors (Z-Pro-prolinal or peptides) were added simultaneously with substrate. Values shown are the averages of two determinations.

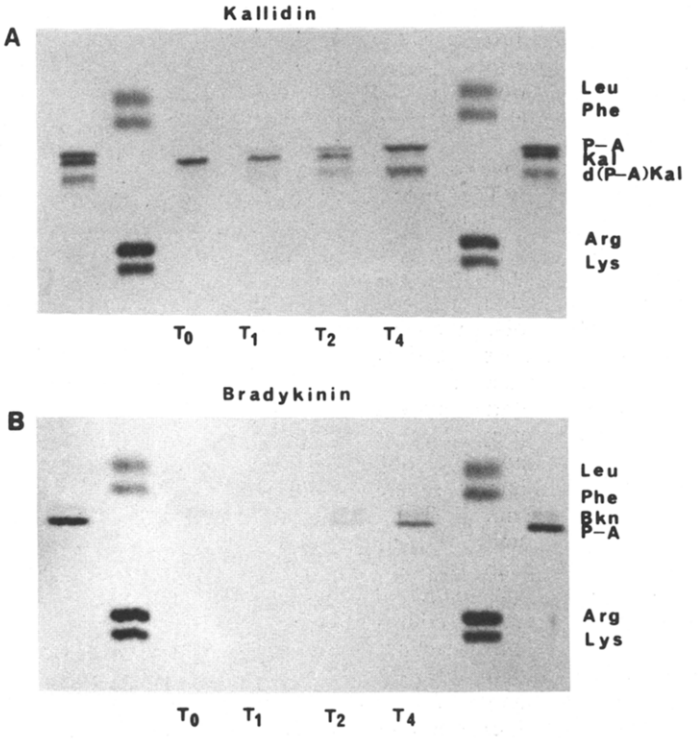

Fig. 2. Incubation (0–4 hr) of purified renal post-proline cleaving enzyme with (A) kallidin and (B) bradykinin. Reaction aliquots (5 μl) and standards were spotted on MN 300 cellulose plates and immediately air dried. Plates were developed in butanol–acetic acid–water (4:1:5), and the products were visualized by staining with 0.4% (w/v) ninhydrin in acetone. Identified standards include kallidin [Kal], bradykinin [Bkn], Phe-Arg [P-A] and des(Phe^9,Arg^{10})kallidin [d(P-A)Kal].

The OPA solution was made fresh daily by mixing 3.8 ml *o*-phthalaldehyde reagent solution, 0.1 ml methanol, 0.1 ml β-mercaptoethanol and 16.8 ml *o*-phthalaldehyde crystals.

RESULTS

Z-Gly-Pro-MCA hydrolysis. As previously found [14], the pH optimum of Z-Gly-Pro-MCA hydrolysis was 8.3, and the apparent K_m and V_{max} values obtained from Lineweaver–Burk analysis were 52.5 ± 7.3 μM and 30.7 ± 0.3 μmol · min^{-1} · mg^{-1} (N = 3) respectively. The IC_{50} of the specific PPCE inhibitor Z-Pro-prolinal was 0.8 nM (Fig. 1). Although less potent, saralasin, bradykinin, des(Arg^9) bradykinin and angiotensin II were effective competitive inhibitors with IC_{50} values of 0.5, 0.65, 0.8 and 7.0 μM respectively. Conversely, a peptide containing no proline residues (Met^5-enkephalin; 10^{-4}M) did not inhibit PPCE significantly (≤ 5%).

Peptide metabolism—TLC. In preliminary experiments, purified PPCE was incubated with a number of kinin and angiotensin analogs, and the metabolites were separated and identified by TLC. As seen in Fig. 2A, kallidin was progressively converted to metabolites which co-migrated with Phe-Arg and des(Phe^9,Arg^{10})kallidin. Similarly, incubation of bradykinin resulted in a metabolite co-migrating with Phe-Arg (Fig. 2B). The absence of any other visible product was expected since control experiments had established that a des(Phe^8,Arg^9)bradykinin standard stained only very faintly with ninhydrin. These results demonstrate that PPCE was hydrolyzing the Pro-Phe bond of both kallidin and bradykinin. Consistent with these results, Phe was released from the B_1-agonist des(Arg^9)bradykinin (Fig. 3A; left side) and Leu was released from the B_1-antagonist [Leu^8],des(Arg^9)bradykinin (Fig. 3A; right side).

Incubation of PPCE with angiotensin II resulted in the production of Phe and a second metabolite [presumably des(Phe^8)angiotensin II] (Fig. 3B; left side). Similarly, Ala was produced from the angiotensin antagonist (Sar^1,Ala^8)angiotensin II (saralasin) (Fig. 3B; right side). In contrast to the above, no peptide or amino acid metabolites were detected when incubations were carried out in the absence of peptide substrates. Collectively, these data demonstrate that all six peptides were being inactivated by hydrolysis on the carboxyl side of internal proline residues (Fig. 4).

Peptide metabolism—HPLC. As expected from the qualitative TLC results, kallidin was hydrolyzed to metabolites that co-migrated with Phe-Arg and des(Phe^9,Arg^{10}) kallidin. Bradykinin was hydrolyzed to metabolites co-migrating with Phe-Arg and des (Phe^8,Arg^9)bradykinin. Similarly, Phe, Leu, Phe and Ala were produced from des (Arg^9)bradykinin, [Leu^8],des(Arg^9)bradykinin, angiotensin II and saralasin respectively. Control experiments demonstrated that the rate of production of each product

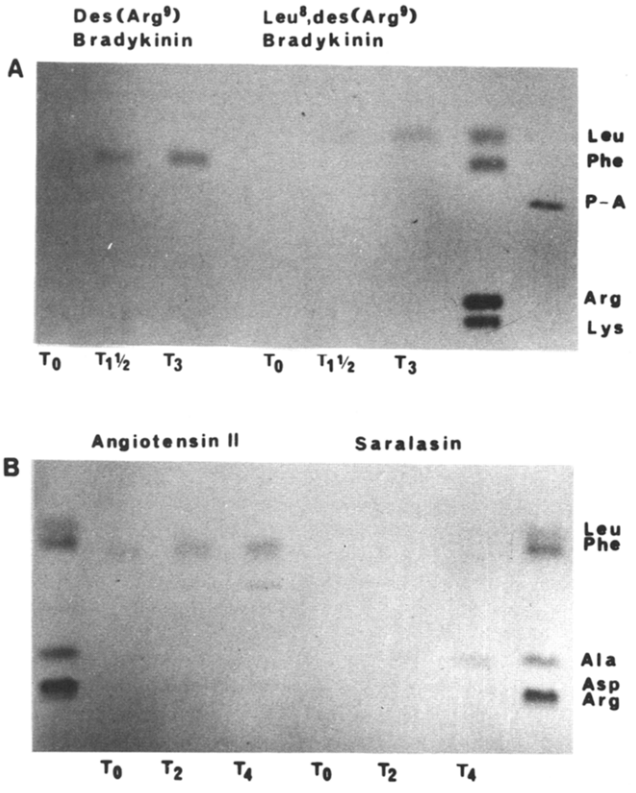

Fig. 3. Incubation (0–3 hr) of purified renal post-proline cleaving enzyme with (A: left side) des(Arg^9)bradykinin; (A: right side) [Leu^8]des(Arg^9)bradykinin; (B: left side) angiotensin II and (B: right side) (Sar^1,Ala^8)angiotensin II. Chromatographic separation was carried out as described in Fig. 2. Identified standards include Phe-Arg [P-A].

was directly proportional to time of incubation (0–75 min) and amount of PPCE protein added (25–175 ng). Where two products were measured [e.g. Phe-Arg and des(Phe^8,Arg^9)bradykinin], both were produced in equimolar amounts.

Depending on the buffer used, the pH optimum for bradykinin degradation was 8.2 to 8.7 (Fig. 5). Comparable pH optima were found for the metabolism of all other peptides (not shown). Thus, all subsequent studies were carried out in 100 mM sodium phosphate buffer (pH 8.2).

Z-Pro-prolinal [21] was a potent inhibitor of PPCE-mediated peptide degradation with a mean IC_{50} of 0.8 ± 0.1 nM (0.5 to 1.1) for all six peptides (Fig. 6; three peptides shown). However, degradation was not affected by *o*-phenanthroline (1 mM) or inhibitors of aminopeptidases (amastatin), neutral endopeptidase (phosphoramidon), carboxypeptidase N (MERGETPA) or angiotensin I converting enzyme (captopril) (i.e. less than 5 and 8% inhibition, respectively, at 10 and 100 μM final concentrations).

The rates of kinin and angiotensin degradation were determined over a range of peptide concentrations. As shown in Fig. 7 (individual experiments) and Table 1, the maximal velocities of hydrolysis were comparable for bradykinin ($V_{max} = 1.37 \pm 0.05$

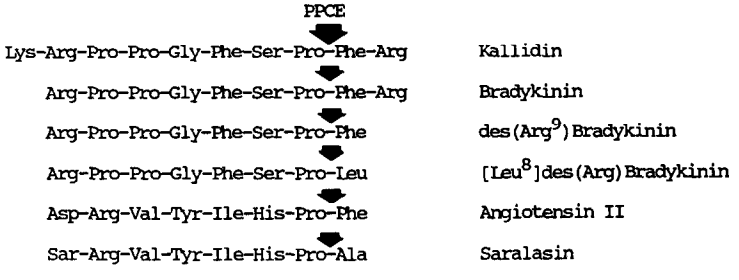

Fig. 4. Sites of hydrolysis of kinin and angiotensin peptides by purified renal post-proline cleaving enzyme as established by thin-layer chromatography (Figs. 2 and 3) and confirmed by high pressure liquid chromatography.

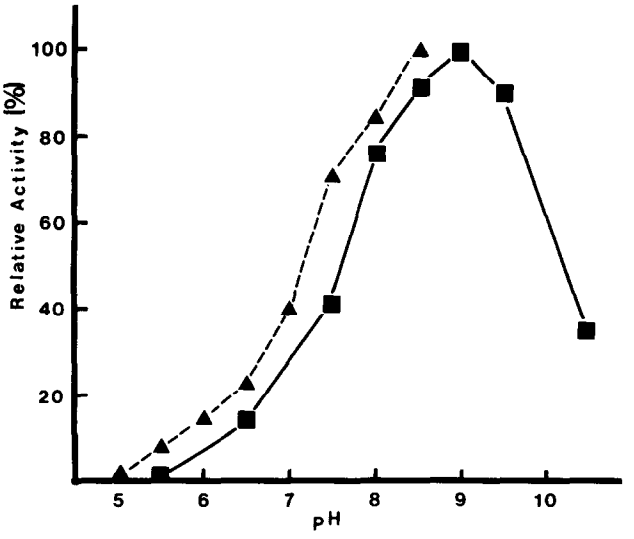

Fig. 5. Effect of pH on bradykinin degradation by purified renal post-proline cleaving enzyme. Activity is expressed as percent maximal activity in either 100 mM sodium acetate/sodium phosphate (▲) or Britton and Robinson type universal buffer (■). Buffers contained 1 mM EDTA, 1 mM sodium azide, 10 mM DTT and BSA (10 μg/ml).

μmol · min^{-1} · mg^{-1}), des(Arg^9)bradykinin (1.52 ± 0.18), [Leu^8],des(Arg^9)bradykinin (1.15 ± 0.16) and angiotensin II (1.70 ± 0.09), whereas saralasin was hydrolyzed more slowly (0.44 ± 0.07). Further, the apparent K_m values of each peptide for PPCE were similar (K_m = 5.5 to 12.8 μM).

DISCUSSION

Circulating angiotensin I is converted to angiotensin II by plasma and vascular angiotensin I converting enzyme [5, 6]. Although converting enzyme also degrades kinins, the principal enzyme in plasma responsible for kinin hydrolysis is reported to be carboxypeptidase N [22] which inactivates the classic (B_2) kinins to form des(Arg)kinins (B_1-agonists) [23]. However, little is known about the enzyme(s) responsible for degrading circulating angiotensin II or des(Arg)kinins. Similarly, few studies have examined the metabolism of kinins and angiotensins in the vascular wall [1, 2] and CNS [3, 4] or in tissues such as the placenta [24, 25] where access to plasma and vascular angiotensin I converting enzyme and carboxypeptidase N may be restricted.

Although originally identified and characterized in the kidney [7], PPCE is widely distributed throughout the body including the vasculature [13], CNS [11, 12], placenta [26] and plasma [27]. Since bradykinin and angiotensin II were found previously to inhibit purifed PPCE [14], we examined the metabolism of kinin and angiotensin by this purified renal enzyme.

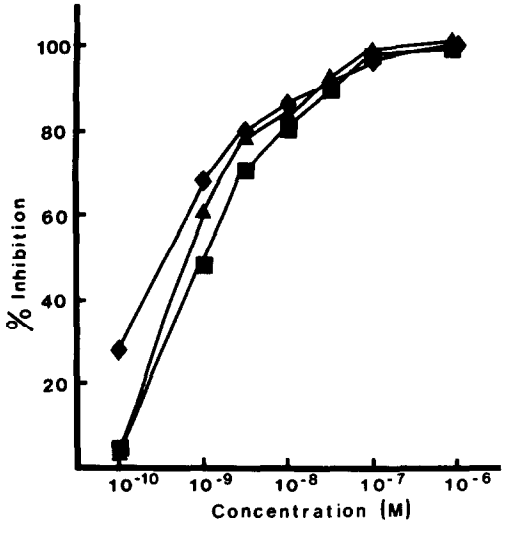

Fig. 6. Inhibition (%) of renal post-proline cleaving enzyme degradation of bradykinin (◆), des(Arg^9)bradykinin (■) and angiotensin II (▲) by Z-Pro-prolinal. Incubations were carried out in 100 mM sodium phosphate buffer (pH 8.3) containing 1 mM EDTA, 1 mM sodium azide, 10 mM DTT and BSA (10 μg/ml). The inhibitor (Z-Pro-prolinal) was preincubated for 20 min (37°) with enzyme and buffer before addition of peptide substrate (250 μM).

Table 1. Kinetics of kinin and angiotensin metabolism by purified renal post-proline cleaving enzyme

Peptide	K_m (μM)	V_{max} (μmol·min^{-1}·mg^{-1})
Bradykinin	7.5 ± 1.7 (3)	1.37 ± 0.05 (3)
des(Arg^9)Bradykinin	10.1 ± 1.0 (4)	1.52 ± 0.18 (4)
[Leu^8],des(Arg^9) Bradykinin	12.8 ± 1.8 (4)	1.15 ± 0.16 (4)
Angiotensin II	5.7 ± 0.9 (3)	1.70 ± 0.09 (3)
(Sar^1,Ala^8),Angiotensin II (saralasin)	5.5 ± 1.0 (3)	0.44 ± 0.07 (3)

Incubations were carried out over a range of substrate concentrations (4–40 μM) in 100 mM sodium phosphate buffer (pH 8.3) containing 1 mM EDTA, 1 mM sodium azide, 10 mM DTT and BSA (10 μg/ml). Values were calculated by Lineweaver–Burk analysis [16] as depicted in Fig. 7 and are given as the means ± SEM. The number of experiments are given in parentheses.

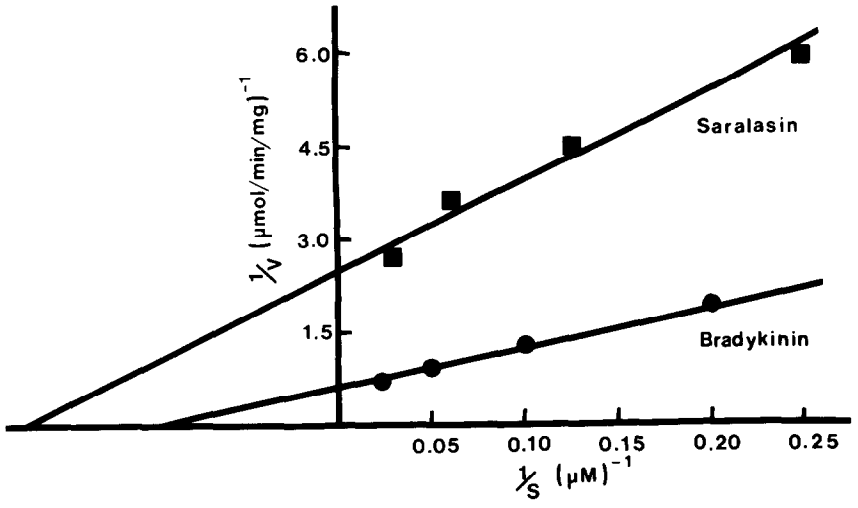

Fig. 7. Lineweaver–Burk plot of the rates of degradation of saralasin (■) and bradykinin (●) by purified renal post-proline cleaving enzyme. Incubations were carried out in 100 mM sodium phosphate buffer (pH 8.3) containing 1 mM EDTA, 1 mM sodium azide, 10 mM DTT and BSA (10 μg/ml).

All kinin and angiotensin peptides examined were competitive inhibitors of PPCE with IC_{50} values of 0.5 to 7.0 μM. Consistent with competitive inhibition and the reported substrate specificity of PPCE [8], each peptide was hydrolyzed on the carboxyl side of proline residues. Based on the known biologic actions of these kinin and angiotensin sequences, such hydrolysis *in vivo* would result in termination of both agonist and antagonist activities.

Peptide degradation by PPCE was optimal at a neutral pH. Degradation was unaffected by aminopeptidase, neutral endopeptidase, carboxypeptidase or converting enzyme inhibitors, but it was inhibited by the specific PPCE inhibitor Z-Pro-prolinal [21]. Further, the potency of Z-Pro-prolinal as an inhibitor of peptide degradation (IC_{50} = 0.8 ± 0.1 nM) was comparable to that previously found for inhibition of Z-Gly-Pro-MCA hydrolysis [14].

Maximal velocities of degradation were similar for both kinin agonists [bradykinin and des(Arg^9)bradykinin; V_{max} = 1.37 ± 0.05 and 1.52 ± 0.18 μmol · min^{-1} · mg^{-1} respectively] and the B_1 kinin antagonist [Leu^8],des(Arg^9)bradykinin (1.15 ± 0.16). Conversely, the rate of degradation of angiotensin II (1.70 ± 0.09) was significantly more rapid than that of the angiotensin antagonist saralasin (0.44 ± 0.07).

The apparent K_m of each peptide for PPCE ranged from 5.5 to 12.8 μM. Thus, although tissue and species differences may be involved, these peptides may have somewhat higher affinities for renal PPCE than those reported for rabbit brain endo-oligopeptidase B (K_m = 35 and 63 μM, bradykinin and angiotensin II, respectively), an enzyme apparently identical to PPCE [12, 28]. The K_m of bradykinin for renal PPCE (7.5 μM) is comparable to that reported for angiotensin I converting enzyme (0.9 to 4.4 μM [29, 30] and is considerably lower than that reported for porcine and human neutral endopeptidase (92 to 120 μM) [31, 32]. Further, in contrast to reports that des(Arg^9)bradykinin has a low affinity for converting enzyme (K_m = 130–240 μM) [29, 30], the K_m of des(Arg^9)bradykinin for PPCE (10.1 μM) is comparable to that of bradykinin.

Unlike the cell surface localization of numerous peptidases [33], PPCE is found largely in cytosol [7, 8]. Thus, the importance of PPCE to *in vivo* metabolism may be confined to the degradation of kinins and angiotensins that are taken up and/or synthesized within the cell [1–4]. Nevertheless, a role in extracellular metabolism should not be ruled out since PPCE is present in the circulation [27], and some PPCE (or like) activity has been reported to be associated with membrane-enriched fractions [10, 34, 35].

In summary, the results of the present study demonstrate that purified renal PPCE degrades kinin and angiotensin agonists and antagonists, and that it has the kinetic properties that would enable it to play a role in the degradation of such peptides *in vivo*. Nevertheless, studies of the effects of Z-Pro-prolinal *in vivo* [21] will be required to determine the physiological significance of such metabolism.

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