A Sensitive Radiochemical Assay for Angiotensin-Converting Enzyme (Kinase II)

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(Received 7 April 1975)

$[[^{125}\text{I}]\text{Tyr}^8]\text{Bradykinin}$ is degraded by angiotensin-converting enzyme to $[[^{125}\text{I}]\text{Tyr}\text{-Arg}]$. The reaction product can be separated completely and recovered nearly quantitatively from unchanged substrate by cation-exchange chromatography. Thus it is possible to use $[[^{125}\text{I}]\text{Tyr}^8]\text{bradykinin}$ at high specific radioactivity (about 400 Ci/mmol) to measure the small quantities of angiotensin-converting enzyme encountered in small-scale cultures of pulmonary endothelial cells.

The enzyme that converts angiotensin I into angiotensin II is also capable of inactivating bradykinin (Dorer *et al.*, 1972, 1974a; Nakajima *et al.*, 1973). The enzyme acts as a dipeptide carboxypeptidase: angiotensin I is converted, by removal of its C-terminal dipeptide, into its potent lower homologue angiotensin II. Bradykinin is inactivated by removal of its C-terminal dipeptide. Although the enzyme is named angiotensin-converting enzyme, Dorer *et al.* (1974a) have shown that bradykinin is the preferred substrate when substrate concentrations are in the range of first-order kinetics.

In the present study, we have examined the reactivity of pig lung angiotensin-converting enzyme with a radioactive analogue of bradykinin, $[[^{125}\text{I}]\text{Tyr}^8]\text{-bradykinin}$ (H-Arg-Pro-Pro-Gly-Phe-Ser-Pro- $[[^{125}\text{I}]\text{Tyr}\text{-Arg}\text{-OH}]$), of high specific radioactivity. The study was begun to develop a highly sensitive assay using a substrate available from commercial sources. Emphasis was placed on sensitivity to assist current efforts to identify angiotensin-converting enzyme in isolated cell lines and in cell culture (e.g. Ryan & Smith, 1973; Lewis *et al.*, 1973).

The $[[^{125}\text{I}]\text{Tyr}^8]\text{bradykinin}$ (about 400 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. The substrate was seldom pure as received, but its impurities (primarily free iodide) could be removed by chromatography on Bio-Gel P-2 (Ryan *et al.*, 1971). The purity of the substrate was examined by paper electrophoresis at pH 5 (Whatman 3MM; 0.1 M-acetic acid adjusted to pH 5 with pyridine; 22 V/cm for 1 h) and by t.l.c. [silica gel developed with butan-1-ol-water-pyridine (8:1:1, by vol.) and cellulose MN/300 developed with butan-

1-ol-acetic acid-water (78:5:17, by vol.)]. These are systems A, B and C respectively of Table 1. The silica-gel thin-layer plates were purchased from Eastman Kodak Co., Rochester, N.Y., U.S.A., and the cellulose plates from Brinkman Instruments, Westbury, N.Y., U.S.A. Tyrosyl-arginine was purchased from Cyclo Chemical Co., Los Angeles, Calif., U.S.A., and was iodinated by the method of Hunter & Greenwood (1962). Pig lung angiotensin-converting enzyme was purified as described by Dorer *et al.* (1972) and was equivalent to their fraction G (approx. 35% pure). The enzyme was dissolved in 10 mM-sodium Hepes [2-(N-2-hydroxyethyl)piperazin-N'-yl]-ethanesulphonic acid] buffer, pH 7.0. The stock solution had a protein concentration of 0.6 mg/ml (measured by an automated modification of the method of Lowry *et al.*, 1951) and a specific activity of 6.33 $\mu\text{mol/min}$ per mg of protein with hippuryl-Gly-Gly as substrate (F. E. Dorer, J. R. Kahn, K. E. Lentz, M. Levine & L. T. Skeggs, unpublished work). A sample of the stock solution of enzyme was diluted (250- to 10000-fold) for each day's experiments. The enzyme was diluted in 50 mM-Hepes buffer, pH 8.0, containing 0.1 M-NaCl and 0.1% lysozyme (egg-white lysozyme, crystallized three times; Sigma Chemical Co., St. Louis, Mo., U.S.A.).

Except where noted, the reaction mixtures were prepared as follows: 25 μl of the diluted enzyme was incubated with 75 μl of the sodium Hepes buffer for 10 min at 37°C, and then 100 μl of $[[^{125}\text{I}]\text{Tyr}^8]\text{bradykinin}$ (70–100 pM in the Hepes buffer) was added to start the reaction. The substrate solution was heated to 37°C before addition to the enzyme. Reactions were

Table 1. *Electrophoretic and chromatographic mobilities of [125 I]Tyr 8 bradykinin and related compounds*

System A: paper electrophoresis, pH 5, 22 V/cm for 60 min. System B: thin-layer silica gel developed with butan-1-ol-water-pyridine (8:1:1, by vol.). System C: thin-layer cellulose developed with butan-1-ol-acetic acid-water (78:5:17, by vol.). For further details, see the text.

	System		
	A R_{Arg}	B R_F	C R_F
[125 I]Tyr 8 Bradykinin	0.56	0.0	—
[Tyr 8]Bradykinin	0.66	0.0	0.11
[I]Tyr-Arg	0.58	0.15	0.16
Tyr-Arg	0.68	—	0.18
Monoiodotyrosine	0.09	0.42	0.57
Di-iodotyrosine	0.09	0.44	0.72

terminated by the addition of 10 μ l of 3M-HCl. Samples of each reaction mixture were diluted with water (0.5ml) and then applied to a small column (0.4cm $\times$ 6cm) of Bio-Rad AG 50W-X2 (Bio-Rad Laboratories, Rockville Centre, N.Y., U.S.A.) prepared as described by Boucher *et al.* (1964). The column was washed with 4.0ml of water and then the reaction product, [125 I]Tyr-Arg, was eluted with 6.0ml of 0.05M-NH $_3$ solution. Unchanged substrate was strongly bound by the resin and was not eluted with concentrations of NH $_3$ solution up to 2.0M. 125 I was measured in a gamma scintillation well counter (Iso-Lect; Dade Reagents, Miami, Fla., U.S.A.). Buffered substrate, without enzyme, did not deteriorate during the course of the incubation, and blank values did not exceed 6% and were subtracted from the experimental values.

Samples of the reaction mixtures were also chromatographed on the Bio-Gel P-2 column. A representative result is shown in Fig. 1. Recovery of radioactivity after chromatography was approx. 83%. The reaction product was characterized by using the electrophoresis and t.l.c. systems described above and was compared with authentic tyrosine, Tyr-Arg, [I]Tyr-Arg, [125 I]Tyr 8 bradykinin, [Tyr 8]bradykinin, monoiodotyrosine and di-iodotyrosine. All amino acids and amino acid residues were in the L form. In each of three lots examined, the [125 I]-tyrosine residue of [125 I]Tyr 8 bradykinin occurred in the monoiodo form (more than 85%). The electrophoretograms and chromatograms were developed with ninhydrin (0.2% in acetone).

The reaction product was indistinguishable from [I]Tyr-Arg. Although other possible lower homologues of [125 I]Tyr 8 bradykinin were not available for comparison, our results ruled out the presence of the C-terminal tripeptide, Pro-[I]Tyr-Arg, and larger

Fig. 1. (a) Molecular-sieve chromatography of [125 I]Tyr 8 bradykinin and (b) of the reaction product formed on incubation of [125 I]Tyr 8 bradykinin with angiotensin-converting enzyme

(a) The substrate was chromatographed on a column (1.1cm $\times$ 100cm) of Bio-Gel P-2; 2ml fractions were collected. The recovery of radioactivity from the column was approx. 85%. (b) Essentially all of the radioactivity eluted from the column occurred in the form of the dipeptide, [125 I]Tyr-Arg. The recovery of radioactivity from the column was approx. 83%. Monoiodotyrosine and di-iodotyrosine are eluted from the column after fraction 80.

C-terminal homologues. Leucine aminopeptidase (Mitz & Schlueter, 1958) released [125 I]tyrosine, and [125 I]tyrosine was the only phenylthiohydantoin

residue recovered when the reaction product was submitted to a one-step Edman degradation reaction (Edman, 1970).

The assay of angiotensin-converting enzyme was based on the finding that the reaction product is readily eluted from the cation-exchange resin whereas the substrate is not eluted. This finding provides the possibility of measuring very low reaction rates in terms of percentage utilization of substrate. Thus the major practical limit to the sensitivity of the assay is that of having sufficient reaction product for accurate radioactivity counting.

The reaction rate was linear with increasing times of incubation, with increasing concentrations of substrate (0.1–1 nM) and with increasing amounts of enzyme (1.5–12 ng of enzyme, 0.1–0.9 fmol/min when the initial substrate concentration was 0.27 nM). Over the range of substrate concentrations studied, the reaction obeyed first-order kinetics.

One of the major problems of using a substance such as bradykinin or its [125 I]Tyr⁸ analogue as a substrate for a peptidase enzyme is that contaminating peptidase enzyme can obscure the result. In this study we used an angiotensin-converting enzyme preparation having no such contamination. However, biological fluids and tissue homogenates are known to contain enzymes capable of degrading bradykinin (e.g. see Ryan *et al.*, 1970). To decrease interference by such enzymes, we modified [125 I]Tyr⁸bradykinin by treating it with maleic anhydride (Butler & Hartley, 1972). The maleyl derivative should be resistant to degradation by enzymes capable of removing the *N*-terminal arginine of the substrate. Maleylation had little effect on the enzyme-substrate reaction. Formation of [125 I]Tyr-Arg from the maleyl derivative proceeded at the same rate as from [125 I]Tyr⁸bradykinin itself.

In an attempt to simplify the assay, we immobilized [125 I]Tyr⁸bradykinin, via its α -amino group, on glass beads [*N*-hydroxysuccinimide-glass beads (1 μ m) provided through the courtesy of Roger N. Piasio, Corning Glass Works, Medfield, Mass., U.S.A.]. The purpose of this part of our study was to provide substrate in solid phase such that the reaction product could be separated from the unchanged substrate by centrifugation and decantation. In addition, by binding the *N*-terminus, the substrate would be protected from enzymes capable of removing the *N*-terminal arginine. However, angiotensin-converting enzyme was unreactive with the immobilized substrate. This finding may be consistent with the earlier observation that higher homologues of bradykinin, extended at the *N*-terminal end (e.g. Lys-bradykinin and Met-Lys-bradykinin), are less reactive with the enzyme than is bradykinin itself (Dorer *et al.*, 1974b). Larger homologues such as Gly-Arg-Met-Lys-bradykinin and Polistes kinin, and 18-residue polypeptide having bradykinin as its *C*-terminal

moiety, are even less reactive. Although angiotensin-converting enzyme acts as a dipeptide carboxypeptidase, it apparently interacts with the *N*-terminal region of bradykinin. Alternatively, steric hindrance may have prevented the enzyme from approaching the substrate bound to glass.

As indicated in the introduction, an aim of this study was to develop a highly sensitive assay such that angiotensin-converting enzyme could be assayed in isolated cell lines. Endothelial cells of the main-stem pulmonary artery of a rabbit were isolated on strips of cellulose acetate paper and were maintained in culture for 18 days (Ryan & Smith, 1973; Lewis *et al.*, 1973). The cells were washed free of medium (which contained foetal calf serum, a possible source of converting enzyme) and were then incubated in Hanks basic salt solution, pH 7.4, at 37°C for 60 min with [125 I]Tyr⁸bradykinin (0.125 pmol) in a final volume of 0.5 ml. Samples were assayed after 15, 30 and 60 min of incubation. Hydrolysis of the substrate proceeded linearly at a rate of 0.84 fmol/min, and the sole reaction product was [125 I]Tyr-Arg (cf. Ryan & Smith, 1973). Incubation mixtures that omitted cells did not degrade the substrate. In these experiments the reaction mixtures contained approx. 1×10^4 cells. Thus it appears possible to use the assay to measure angiotensin-converting enzyme on a per-cell basis.

The assay is highly sensitive and reasonably precise. On the basis of results obtained so far, the assay appears to be specific. The assay allows the use of [125 I]Tyr⁸bradykinin in concentrations at or below the concentrations of bradykinin thought to occur *in vivo* (Mashford & Roberts, 1972). We have not used the assay for the measurement of converting enzyme in biological fluids or tissue homogenates. Possibly, modifications of the present assay will be required to eliminate the influence of other peptidase enzymes.

This work was supported in part by grants from the U.S. Public Health Service (HL 15691 and Contract N01 HR3 3015), the John A. Hartford Foundation, the Council for Tobacco Research—U.S.A., New York, U.S.A., the Veterans Administration (Project no. 7963-01) and by an Established Investigatorship award to U.S.R. from the American Heart Association.

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