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Purification of human renin by affinity chromatography using a new peptide inhibitor of renin, H.77 (D-His-Pro-Phe-His-Leu^{R}Leu-Val-Tyr)

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A new affinity column for renin was prepared by coupling the isosteric peptide inhibitor of renin, H.77 (D-His-Pro-Phe-His-Leu^{R}Leu-Val-Tyr, where R is a reduced isosteric bond, $-CH_2-NH-$), to activated 6-aminohexanoic acid–Sepharose 4B. Chromatography of a crude extract of human kidney cortex on this material resulted in a 5500-fold purification of renin in 76% yield. The purified enzyme (specific activity 871 units/mg) was free of non-specific acid-proteinase activity and was stable at pH 6.8 and −20°C over a period of several weeks.

Renin (EC 3.4.99.19) is an aspartic proteinase of $M_r \sim 40000$ that is synthesized mainly in the juxtaglomerular cells of the renal cortex. It is released into the bloodstream, where it cleaves the α_2-globulin angiotensinogen in a highly specific manner to produce the inactive decapeptide, angiotensin I. Subsequently, angiotensin I is cleaved by a peptidyl dipeptide hydrolase to give the active octapeptide, angiotensin II, which has important roles in sodium and fluid homoeostasis and in the regulation of blood pressure (Leckie *et al.*, 1979).

In spite of its importance, renin has been purified to homogeneity only recently. Early attempts at renin purification were hindered by the low concentration of renin in the kidney (Haas *et al.*, 1954) and by the instability of the enzyme during purification (Peart *et al.*, 1966). The application of affinity-chromatographic techniques to this problem, and, in particular, the use of the acylated pentapeptide pepstatin as an affinity ligand, has resulted in the isolation of renin from the renal cortex of a number of species, including man (Inagami *et al.*, 1980).

Abbreviations used: H.77, D-His-Pro-Phe-His-Leu^{R}Leu-Val-Tyr [H.77 is an analogue of the His^{6}–Tyr^{13} octapeptide sequence of equine angiotensinogen in which the *N*-terminal histidine residue has been replaced with D-histidine and the normal cleavage site has been modified by the introduction of a reduced isosteric bound (R; $-CH_2-NH-$) in place of the peptide bond]; SDS, sodium dodecyl sulphate. The names 'angiotensin I' and 'angiotensin II' were accepted by the Nomenclature Committee of the International Society of Hypertension (1979). The IUPAC-recommended equivalents are 'proangiotensin' and 'angiotensin' respectively.

However, the purification of renin remains a lengthy procedure, involving several steps of conventional chromatography as well as affinity chromatography (Slater, 1981).

In the present paper we report the use of a new potent peptide inhibitor of renin, H.77 (Szelke *et al.*, 1982*a*), as an affinity ligand to achieve the rapid isolation of a highly purified and stable renin preparation from human renal cortex.

Materials and methods

Chemicals and other materials were obtained either from the sources described in the appropriate references or from BDH Chemicals, Poole, Dorset, U.K., except for the following: bovine serum albumin, aldolase, pyruvate kinase and carbonic anhydrase were from Boehringer; haemoglobin, β-lactoglobulin, lysozyme and activated 6-aminohexanoic acid–Sepharose 4B (catalogue no. A9019) from Sigma; human spleen cathepsin D was kindly donated by Dr. A. J. Barrett, Strangeways Research Laboratory, Cambridge, U.K.

H.77 was synthesized and purified as previously described (Szelke *et al.*, 1982*b*).

Preparation of H.77–Sepharose

H.77 was coupled to Sepharose through the *N*-terminal amino group by reaction with the *N*-hydroxysuccinimide ester of 6-aminohexanoic acid–Sepharose 4B (activated 6-aminohexanoic acid–Sepharose 4B). Activated Sepharose (10g) was swollen and washed at room temperature with 2

litres of 1mM-HCl on a sintered-glass funnel. H.77 (100mg) was dissolved in 30ml of 0.1M-$NaHCO_3$ (pH8.0)/0.5M-NaCl (coupling buffer) and mixed with the washed gel. The gel suspension was gently shaken for 16h at 4°C, after which excess ligand was washed from the gel with coupling buffer (500ml). The remaining reactive groups were blocked by treatment of the gel in 100ml of 1M-ethanolamine, pH9.0, for 1h at 20°C. The H.77–Sepharose was washed with five cycles of coupling buffer (200ml) followed by 0.1M-sodium acetate (pH4.0)/0.5M-NaCl, and finally equilibrated with 50mM-Tris/HCl, pH7.4.

The amount of peptide coupled to the gel was estimated by amino acid analysis after hydrolysis of 0.5ml of H.77–Sepharose in 2ml of 6M-HCl for 20h at 110°C; 0.9μmol of H.77 were coupled per ml of gel.

Purification of human renin

All buffers contained 20mM-benzamidine, 5mM-EDTA and 0.01% Tween 20.

Human kidneys were obtained at autopsy from individuals with no history of renal disease. Preliminary experiments were carried out to determine the renin content of individual kidneys, and only those kidneys containing more than 1m-unit (see under 'Assays' below) of renin/mg wet weight of renal cortex were used in the present study.

Renal cortex (30g) was subjected to two cycles of freezing and thawing and was then homogenized in 40ml of 50mM-Tris/HCl (pH7.4)/1mM-phenylmethanesulphonyl fluoride/0.5mM-*N*-ethylmaleimide with a Polytron PT 20s homogenizer (5min; 25000rev./min). The homogenate was centrifuged for 30min at 100000*g* and 4°C. The supernatant solution was passed through a glass-microfibre filter (Whatman GF/C) and immediately applied to a column (1.6cm × 5cm) of H.77–Sepharose equilibrated with extraction buffer. The column was washed with 100ml of the same buffer, with 100ml of 0.1M-Tris/HCl (pH7.4)/1M-NaCl and finally with 100ml of 0.1M-sodium acetate (pH6.0)/1M-NaCl. Renin was eluted with a pH gradient generated by allowing 75ml of 0.1M-acetic acid to mix with 75ml of 0.1M-sodium acetate (pH6.0). The flow rate was 0.2ml/min and 5ml fractions were collected.

The renin-containing fractions were pooled and adjusted to pH6.8 by adding 2M-Tris. The enzyme was then concentrated by ultrafiltration through an Ultra Thimble type UH 100/10 (Schleicher and Schüll) and stored at −20°C in 50% (v/v) glycerol at a concentration of 100μg/ml.

Assays

Renin concentration was measured by using the antibody-trapping method of Millar *et al.* (1980) to determine the rate of angiotensin generation during incubation of samples with excess ox renin substrate. Assay results were expressed in international units of renin by comparison with the International Reference Standard Preparation of Human Renin 68/356 (Bangham *et al.*, 1975). One unit of renin generated angiotensin I at a rate of 182.8μg/h in the above assay system.

Acid-proteinase activity was assayed by measuring the release of ^{3}H-labelled peptides from [^{3}H]acetylhaemoglobin. The labelled substrate (35×10^6d.p.m./mg) was prepared as described by Hille *et al.* (1970). Assays were carried out by incubating 10μl of enzyme solution at 37°C with 50μl of 1M-sodium formate, pH3.3, and 40μl of a substrate solution prepared by dissolving 87mg of [^{3}H]acetylhaemoglobin and 1g of unlabelled haemoglobin in water to a final volume of 20ml. The reaction was terminated by adding 0.5ml of 3% (w/v) trichloroacetic acid, and precipitated protein was removed by centrifugation at 1000*g* and 4°C for 30min. A 0.2ml sample of the supernatant solution was then removed for liquid-scintillation counting in 8ml of Packard scintillator 299. One unit of enzyme activity was defined as the activity exhibited by 1μg of pure human spleen cathepsin D in the above assay system.

Protein concentration was measured using the dye-binding method of Bradford (1976) as modified by Macart & Gerbaut (1982). Bovine serum albumin was used as a standard.

Gel electrophoresis

Electrophoresis in the presence of 0.1% SDS was carried out by using the method of Laemmli (1970). Protein bands were stained as described by Wray *et al.* (1981).

Results and discussion

Crude extract of human renal cortex containing 102 units of renin activity was applied to a column of H.77–Sepharose. At pH7.4 the renin activity was bound tightly by the immobilized H.77 (Fig. 1). In contrast, non-specific acid-proteinase activity did not bind and emerged from the column with the bulk protein fraction. After washing the column to remove weakly bound proteins, renin activity was eluted with a pH gradient from pH6.0 to 3.2. This procedure resulted in an increase in the specific activity of renin from 0.158 units/mg of protein in the crude material to 871 units/mg; 89μg of purified enzyme was obtained, representing a yield of 76% (Table 1). In two subsequent purifications, renin preparations with specific activities of 785 units/mg and 815 units/mg were obtained in similar yields (70% and 81% respectively).

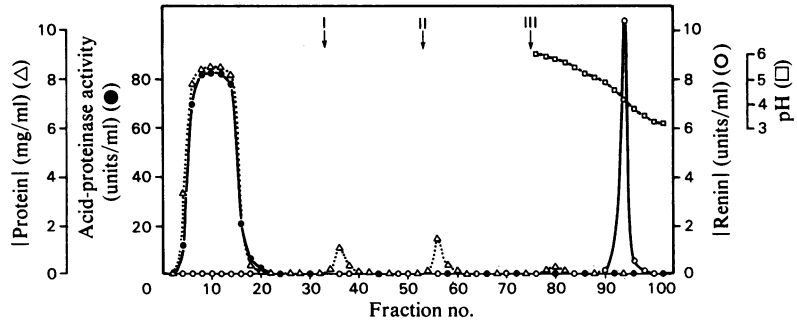

Fig. 1. *Affinity chromatography of renin on H.77–Sepharose*
Crude extract of human kidney cortex was applied to a column of H.77–Sepharose equilibrated with extraction buffer. The column was washed with 0.1 M-Tris/HCl (pH 7.4)/1 M-NaCl (arrow I) and then with 0.1 M-sodium acetate (pH 6.0)/1 M-NaCl (arrow II). Renin was eluted with a pH gradient from pH 6.0 to pH 3.2 in 0.1 M-acetate buffer (arrow III). △, [Protein]; ●, acid-proteinase activity; ○, [renin]; □, pH.

Table 1. *Purification of human renal renin*

	Renin activity (units)	Protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude homogenate	102	645	0.158	1	100
Renin eluted from H.77–Sepharose	77.5	0.089	871	5512	76

The instability of renin encountered in previous studies has been attributed to the presence of contaminating proteinases and, in particular, to cathepsin D-like activity (Inagami *et al.*, 1980). The purified renin obtained in the present study exhibited no non-specific proteolytic activity towards [^{3}H]acetylhaemoglobin and was found to be stable over a period of several weeks at pH 6.8 and −20°C.

The specific activity of human renin has been reported to be 400 units/mg by Slater *et al.* (1978). These authors used the fluorescamine assay of Bohlen *et al.* (1973) to determine the protein content of the pure enzyme. Yokosawa *et al.* (1980), who used the method of Lowry *et al.* (1951) to determine protein concentration, obtained a specific activity of 950 units/mg for pure human renin. The purified renin obtained in the present study was found to have a similar specific activity (871 units/mg), the dye-binding method of Bradford (1976) being used to measure protein concentration. However, examination of the purified enzyme preparation by electrophoresis in the presence of 1% SDS (Fig. 2) showed that it was not homogeneous. It consisted of two proteins with apparent M_r values of 40000 and 17000. The larger protein probably corresponds to the 40000-M_r renin purified previously (Slater *et al.*, 1978; Yokosawa *et al.*, 1980). The nature of the 17000-M_r protein is not clear at present, although it may represent a fragment of renin formed by limited proteolysis. Galen *et al.* (1979) have reported the

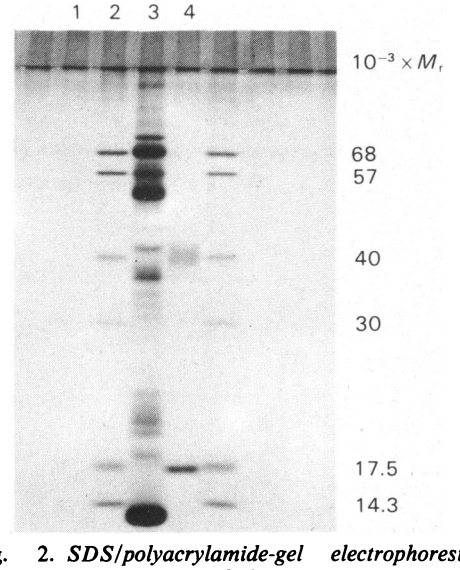

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of purified renin*
Discontinuous electrophoresis was carried out in a 1 mm-thick polyacrylamide slab gel (stacking gel, 3% polyacrylamide; separating gel, 10% polyacrylamide). Track 2, 4 μg of crude kidney-cortex extract; track 3, 100 ng of purified renin; tracks 1 and 4, standard M_r marker proteins: 50 ng each of bovine serum albumin (68000), pyruvate kinase (57000), aldolase (40000), carbonic anhydrase (30000), β-lactoglobulin (17500) and lysozyme (14300). (Submit M_r values were taken from Righetti & Caravaggio, 1976).

presence of fragments of renin with apparent M_r values of 25000 and 20000 in a renin preparation (specific activity 860 units/mg) isolated from a juxtaglomerular cell tumour of human renal cortex.

Affinity chromatography of renin using the isosteric peptide inhibitor of renin, H.77, as an affinity ligand has proved to be a simple and rapid method for the purification of human renal renin.

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