Purification of human renin by affinity chromatography using a new peptide inhibitor of renin, H.77 (d-His-Pro-Phe-His-Leu<sup>8</sup>-Leu-Val-Tyr)

Gordon D. McIntyre,* Brenda Leckie,* Allan Hallett† and Michael Szkelke†
*MRC Blood Pressure Unit, Western Infirmary, Glasgow G11 6NT, Scotland, U.K., and †Department of Chemical Pathology, Royal Postgraduate Medical School, Hammersmith Hospital, London W12 OHS, U.K.

(Received 21 January 1983/ Accepted 11 March 1983)

A new affinity column for renin was prepared by coupling the isosteric peptide inhibitor of renin, H.77 (d-His-Pro-Phe-His-Leu<sup>8</sup>Leu-Val-Tyr, where R is a reduced isosteric bond, -CH<sub>2</sub>-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 $\alpha_2$-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 (Pear et al., 1966). The application of affinity-chromatographic techniques to this problem, and, in particular, the use of the serylated 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<sup>8</sup>Leu-Val-Tyr. H.77 is an analogue of the His<sup>6</sup>-Tyr<sup>13</sup> 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 bond (R: -CH<sub>2</sub>-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., 1982a), 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, $\beta$-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., 1982b).

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 (10 g) was swollen and washed at room temperature with 2
litres of 1 mM-HCl on a sintered-glass funnel. H.77 (100 mg) was dissolved in 30 ml of 0.1 M-NaHCO₃ (pH 8.0)/0.5 M-NaCl (coupling buffer) and mixed with the washed gel. The gel suspension was gently shaken for 16 h at 4°C, after which excess ligand was washed from the gel with coupling buffer (500 ml). The remaining reactive groups were blocked by treatment of the gel in 100 ml of 1 M-ethanolamine, pH 9.0, for 1 h at 20°C. The H.77–Sepharose was washed with five cycles of coupling buffer (200 ml) followed by 0.1 M-sodium acetate (pH 4.0)/0.5 M-NaCl, and finally equilibrated with 50 mM-Tris/HCl, pH 7.4.

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

**Purification of human renin**

All buffers contained 20 mM-benzamidine, 5 mM-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 1 μg-unit (see under ‘Assays’ below) of renin/mg wet weight of renal cortex were used in the present study.

Renal cortex (30 g) was subjected to two cycles of freezing and thawing and was then homogenized in 40 ml of 50 mM-Tris/HCl (pH 7.4)/1 mM-phenylmethanesulphonyl fluoride/0.5 mM-N-ethylmaleimide with a Polytron PT 20s homogenizer (5 min; 25 000 rev./min). The homogenate was centrifuged for 30 min at 100 000g and 4°C. The supernatant solution was passed through a glass microfibre filter (Whatman GF/C) and immediately applied to a column (1.6 cm × 5 cm) of H.77–Sepharose equilibrated with extraction buffer. The column was washed with 100 ml of the same buffer, with 100 ml of 0.1 M-Tris/HCl (pH 7.4)/1 M-NaCl and finally with 100 ml of 0.1 M-sodium acetate (pH 6.0)/1 M-NaCl. Renin was eluted with a pH gradient generated by allowing 75 ml of 0.1 M-acetic acid to mix with 75 ml of 0.1 M-sodium acetate (pH 6.0). The flow rate was 0.2 ml/min and 5 ml fractions were collected.

The renin-containing fractions were pooled and adjusted to pH 6.8 by adding 2 M-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 100 μg/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 182.8 μg/h in the above assay system.

Acid-proteinase activity was assayed by measuring the release of 3H-labelled peptides from [3H]acetylhaemoglobin. The labelled substrate (35 × 10⁶ d.p.m./mg) was prepared as described by Hille et al. (1970). Assays were carried out by incubating 10 μl of enzyme solution at 37°C with 50 μl of 1 M-sodium formate, pH 3.3, and 40 μl of a substrate solution prepared by dissolving 87 mg of [3H]acetylhaemoglobin and 1 g of unlabelled haemoglobin in water to a final volume of 20 ml. The reaction was terminated by adding 0.5 ml of 3% (w/v) trichloroacetic acid, and precipitated protein was removed by centrifugation at 1000g and 4°C for 30 min. A 0.2 ml sample of the supernatant solution was then removed for liquid-scintillation counting in 8 ml of Packard scintillator 299. One unit of enzyme activity was defined as the activity exhibited by 1 μg 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 pH 7.4 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 pH 6.0 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; 89 μg 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).
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 [3H]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. 1. Affinity chromatography of renin on H.77-Sepharose](image)

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). $\Delta$, [Protein]; $\bullet$, acid-proteinase activity; $O$, [renin]; $\square$, pH.

### Table 1. Purification of human renal renin

<table>
<thead>
<tr>
<th></th>
<th>Renin activity (units)</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>102</td>
<td>645</td>
<td>0.158</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Renin eluted from H.77-Sepharose</td>
<td>77.5</td>
<td>0.089</td>
<td>871</td>
<td>5512</td>
<td>76</td>
</tr>
</tbody>
</table>

Discontinuous electrophoresis was carried out in a 1mm-thick polyacrylamide slab gel (stacking gel, 3% polyacrylamide; separating gel, 10% polyacrylamide). Track 2, 4.4 μg of crude kidney-cortex extract; track 3, 100ng of purified renin; tracks 1 and 4, standard $M_r$ marker proteins: 50ng each of bovine serum albumin (68000), pyruvate kinase (57000), aldolase (40000), carbonic anhydrase (30000), $\beta$-lactoglobulin (17500) and lysozyme (14300). (Submit $M_r$ values taken from Righetti & Caravaggio, 1976).
presence of fragments of renin with apparent $M_r$ values of 25,000 and 20,000 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.

References

Nomenclature Committee of the International Society of Hypertension (1979) Hypertension 1, 654