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Note

Purification of human renin by renin inhibitor (ES-305) affinity chromatography

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Renin (EC 3.4.23.15) is an aspartic protease which catalyses hydrolytic release of decapeptide angiotensin I from the amino-terminal end of angiotensinogen. Angiotensin-converting enzyme (EC 3.4.15.1), in turn, converts angiotensin I (AI) to the octapeptide angiotensin II (AII), which is the potent pressor and the stimulator of aldosterone secretion [1]. The purification of renal renin is difficult because of its low concentration in the kidney and the instability of the enzyme during purification. Several biospecific affinity chromatographic techniques have been successfully employed in the purification of the enzyme using an aspartic protease inhibitor, pepstatin [2,3], and a peptide inhibitor of human renin, H.77 [4], as affinity ligands. Recently, we have developed several small peptide derivatives as highly potent inhibitors of human renin [5–7]. One of these inhibitors, a dipeptide derivative, bis[(1-naphthyl)methyl]acetylhistidylstatine 2(*S*)-methylbutylamide (ES-305), competitively inhibited human renin with a K_i value of 1.7 n*M* and had little effect on other aspartic proteases such as cathepsin-D and pepsin at a concentration of 10 μM [7]. In this work, we prepared an affinity column using ES-305 as an affinity ligand and purified stable human renin from kidney.

EXPERIMENTAL

Preparation of the affinity column

ES-305 (300 mg) was dissolved in 15 ml of dry dioxane and then diluted with 0.1 *M* carbonate buffer (pH 10) to a final concentration of 50%. Epoxy-activated Sepharose 6B (Pharmacia, Uppsala, Sweden) was suspended in 50% dioxane in

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0.1 *M* carbonate buffer (pH 10) after being swollen and repeatedly washed with the same solvent. The solution of ES-305 was then mixed with Sepharose gel (30 ml of sedimented gel) and incubated for 16 h at 40 °C with continuous shaking. The gel was filtered and washed with 500 ml of 50% dioxane in 0.1 *M* carbonate buffer (pH 10). The remaining reactive groups were blocked with 1 *M* ethanolamine (pH 8.5) for 4 h at room temperature, followed by washing alternatively with 0.1 *M* acetate buffer (pH 4.0) and 0.1 *M* borate–carbonate buffer (pH 8.0), each containing 0.5 *M* sodium chloride. The amount of ES-305 coupled to the gel was calculated to be approximately 1.2 μmol per ml of gel. The gel was stored in 20 m*M* sodium phosphate buffer (pH 6.5) at 4 °C in the presence of 0.02% sodium azide.

Purification of human renin from kidney

Human cadaver kidneys were obtained at autopsy and stored at −20 °C until used. The frozen kidneys were partially thawed and their cortices were dissected. All procedures were carried out at 4 °C unless stated otherwise. The renal cortices (2.4 kg) were homogenized in four volumes (w/v) of 20 m*M* potassium phosphate buffer (pH 7.8) containing protease inhibitors of 5 m*M* ethylenediaminetetraacetic acid (EDTA), 2 m*M* phenylmethylsulphonyl fluoride (PMSF), 5 m*M* sodium tetrathionate and 10 m*M* benzamidine hydrochloride. The homogenate was stirred for 1 h and centrifuged at 700 *g* for 20 min. The supernatant was adjusted to pH 5.0 with acetic acid, stirred for 30 min and then centrifuged at 15 000 *g* for 20 min. The supernatant (pH 5) was acidified to pH 3.3 with 2 *M* sulphuric acid and stirred for an additional 3 min. After the pH had been adjusted to 7.0 with 4 *M* sodium hydroxide solution, the insoluble material was removed by centrifugation at 15 000 *g* for 30 min. Subsequently the proteins that were precipitated between 35 and 65% saturation with ammonium sulphate were collected by centrifugation and dissolved in and dialysed against 20 m*M* sodium phosphate buffer (pH 7.4) containing the protease inhibitors described above.

The crude renin extract was mixed with 1 l of sedimented DEAE-cellulose gel (DE-52; Whatman, Maidstone, U.K.) which had been equilibrated with the dialysis buffer. After gentle stirring for 2 h at 4 °C, the gel was washed with the dialysis buffer on a glass filter and then packed into two columns (40×5 cm I.D.). The columns were washed again with 500 ml of the same buffer and developed with a linear gradient of sodium chloride (0–0.3 *M*) in the same buffer. The active fractions were collected and concentrated by ultrafiltration with a PM 10 membrane on an Amicon ultrafiltration apparatus.

The concentrate was dialysed against 20 m*M* Tris–HCl (pH 7.6) containing 1 m*M* calcium chloride, 1 m*M* magnesium chloride, 1 m*M* manganese(II) chloride and 0.2 m*M* sodium chloride and then applied to a column (30×2.6 cm I.D.) of concanavalin A–Sepharose (Pharmacia). Renin adsorbed to the column was eluted with 0.2 *M* glucose and 0.2 *M* mannose solution in the starting buffer. The fractions containing renin were pooled, concentrated by ultrafiltration and dialysed against 20 m*M* sodium phosphate buffer (pH 6.5) containing 0.2 *M* sodium chloride and the protease inhibitors.

The dialysed sample was applied to the ES-305 affinity column (25×1.2 cm

I.D.) equilibrated with 20 m*M* sodium phosphate buffer (pH 6.5) containing 0.2 *M* sodium chloride and the protease inhibitors. The column was eluted stepwise with the equilibration buffer, followed by 0.1 *M* sodium acetate buffer (pH 5.5) containing 1 *M* sodium chloride and 0.1 *M* acetic acid (pH 3.2). Renin was tightly bound to the column and eluted with 0.1 *M* acetic acid (pH 3.2). Immediately after fractionation, the pH of each fraction was neutralized to 7.0 with 1 *M* Tris base. The renin-containing fractions were pooled and concentrated by ultrafiltration.

The final step in purification was carried out in a Pharmacia FPLC system on a Superose-6 column (Pharmacia). The column was equilibrated with 20 m*M* sodium phosphate buffer (pH 6.8) containing 0.15 *M* sodium chloride. A 400-μl volume of the sample was injected and developed with the same buffer at a flow-rate of 0.5 ml/min. The renin activity peak was eluted at 29.0 min. Several injections were made and the peak eluted at 29.0 min was collected, concentrated and stored at $-80\,^{\circ}$C. A portion of the renin preparation was stored at 4 °C and pH 6.8 in the presence of 0.02% sodium azide.

Assays

Renin activity was determined by radioimmunoassay of generated angiotensin I during incubation with nephrectomized sheep plasma. The reaction was carried out in 0.1 *M* phosphate buffer (pH 7.3) for 10–30 min at 37 °C in the presence of 10 m*M* EDTA and 3.4 m*M* 8-hydroxyquinoline [8].

Protein concentration was determined by the method of Lowry et al. [9] with bovine serum albumin as the standard.

Non-specific acid protease activity was measured by the method of Barrett [10] using bovine haemoglobin as the substrate and bovine spleen cathepsin-D (13 U/mg) (Sigma, St. Louis, MO, U.S.A.) as the reference.

RESULTS AND DISCUSSION

The purification steps and results are summarized in Table I. The pH 5 supernatant of homogenate of human kidney cortices contained a specific renin activity of 0.44 μg AI per h per mg of protein. The following five purification steps resulted in a 9800-fold increase in the specific activity of renin with an overall recovery of 13.2%. The purified enzyme had a specific activity of 4318 μg AI per h per mg of protein.

During purification on an ES-305 affinity column (Fig. 1), a majority of proteins were eluted during washing with 20 m*M* phosphate buffer (pH 6.5) containing 0.2 *M* sodium chloride. Renin activity was tightly bound to the column and was eluted with 0.1 *M* acetate buffer (pH 3.2). The specific activity of renin increased from 38.9 to 3156 μg AI per h per mg of protein, achieving an 81-fold purification in this step. Renin activity could also be eluted with high ionic and alkaline buffers, including 0.5 *M* Tris–HCl buffer (pH 7.6), other than at pH 3.2, but the renin activity was incompletely resolved with these buffers (data not shown). The column could be used for at least eight cycles without loss of the binding capacity of renin. When the crude extract of renin from ammonium sul-

TABLE I

PURIFICATION OF RENIN FROM HUMAN KIDNEY

Purification step	Total activity (μg AI per h)	Total protein (mg)	Specific activity (μg AI per h per mg)	Purification (*n*-fold)	Recovery (%)
pH 5 supernatant	7130	16205	0.44	1	100
Ammonium sulphate fractionation	5222	2712	1.93	4.4	73.2
DEAE-cellulose	3682	729.6	5.05	11.5	51.6
Concanavalin A-Sepharose	2275	58.5	38.9	88.4	31.9
ES-305-Sepharose	1136	0.36	3156	7173	15.9
FPLC (Superose-6)	747	0.173	4318	9814	13.2

phate fractionation was applied directly to the column and eluted under the same conditions, the renin activity increased from 1.39 to 494 μg AI per h per mg of protein, resulting in a 256-fold increase in the specific activity. However, a considerable amount of contaminating proteins bound non-specifically to the column and emerged with renin activity at pH 3.2.

Renal renin has been successfully purified from human [11,12] and hog kidneys [13,14] using an affinity chromatographic procedure with an aminohexyl pepstatin as a ligand. However, renin is unstable in crude preparation and, in particular, after the aminohexyl pepstatin affinity chromatography, possibly owing to the presence of contaminating acid proteases such as cathepsin-D-like activity [11,12]. This problem was overcome by the use of a haemoglobin–Sepharose column to remove destructive proteases in an early step of the purification [11]. In this study, the renin preparation (200–300 μg/ml) after the affinity chroma-

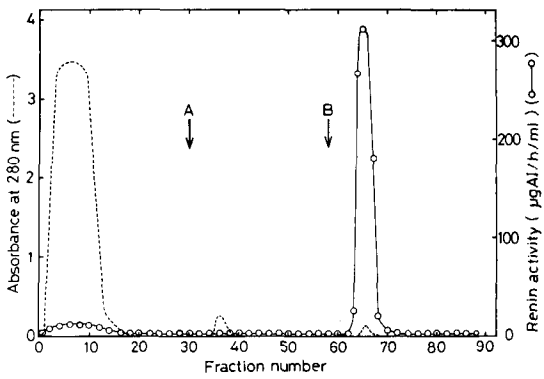

Fig. 1. Biospecific affinity chromatography of human renin on an ES-305-Sepharose column. The renin preparation from concanavalin A-Sepharose was applied to the ES-305-Sepharose column. The column (25 × 1.2 cm I.D.) was washed with 20 m*M* sodium phosphate buffer (pH 6.5) containing 0.2 *M* sodium chloride. The elution buffers were changed at the arrows A and B: 0.1 *M* acetate buffer (pH 5.5) containing (A) 1 *M* sodium chloride and (B) 0.1 *M* acetic acid (pH 3.2).

tography was found to be stable for several weeks at either 4 or −80 °C in 20 m*M* phosphate buffer (pH 6.8) in the presence of 0.02% sodium azide.

The dipeptide derivative ES-305 is a specific inhibitor of human renin, representing a K_i value of 1.7 n*M* for human renin [5]. It inhibits only 4–7% of bovine cathepsin-D and porcine pepsin at the concentration of 10 μM [5]. Non-specific acid protease activity was measured in the samples before and after the ES-305 affinity chromatography using bovine haemoglobin as substrate. No acid protease activity was detected in the renin preparation after the chromatography, while the sample before the chromatography (from concanavalin A–Sepharose) contained acid protease activity of 1.54 U/mg of protein when bovine spleen cathepsin-D was used as the reference. McIntyre et al. [4] and more recently Shinagawa et al. [15] have purified human renin from the kidney employing an affinity chromatographic technique with a specific peptide inhibitor of human renin (H.77) as an affinity ligand. Their renin preparation was free from non-specific acid proteases and stable after purification. The absence of non-specific acid protease activity accounts for the stability of our renin preparation.

In conclusion, affinity chromatography using a specific human renin inhibitor, ES-305, as a ligand is useful for the purification of stable human renin.

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