ISOLATION OF PURE AND STABLE RENIN FROM HOG KIDNEY*

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SUMMARY: Pure renin from hog kidney was isolated as an electrophoretically homogeneous preparation. An affinity column suitable for this purpose was prepared by coupling pepstatin to aminohexyl agarose in an organic solvent mixture. Crude extract, treated with mixtures of protease inactivators to eliminate proteases, was purified on this column followed by gel filtration and ion-exchange chromatography. Hog renin, thus obtained after 180,000-fold purification at an overall yield of 25%, is stable at pH6.35 and -20°.

In view of the importance of renin (EC. 3.4.4.15) in blood pressure regulation numerous attempts have been made to isolate this enzyme from the kidney (for example 1-4). These studies have invariably encountered great difficulties presumably due to the extremely small amount of renin in kidney and due to rapid destruction of this enzyme as it is purified. Various affinity chromatographic techniques have been developed to cope with the difficulty (5-8). The strong affinity of renin to the acylated pentapeptide pepstatin (6, 9-11) has been utilized to prepare affinity columns for renin purification (5, 6). In view of the various shortcomings of these earlier affinity gels an improved pepstatin agarose gel has been prepared, and a complete purification procedure for hog renal renin has been developed. This method, consisting of the selective

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extraction of renin by a modification of the method of Rubin (12), use of protease inactivators to minimize proteolytic destruction, affinity chromatography and three steps of conventional column chromatography, enabled us to isolate electrophoretically homogeneous and stable renin from hog kidney after a 180,000-fold purification.

EXPERIMENTALS

Preparation of the affinity column -- Pepstatin (0.4 mmoles) was esterified with N-hydroxysuccinimide (0.8 mmoles) in the presence of dicyclohexyl carbodiimide (0.8 mmoles) in purified dimethylformamide (32 ml) at 4° for 16 hours. Aminohexyl agarose, (13) (18 g suction-dry weight) suspended in dry, peroxide-free dioxane (32 ml) after repeated filtration and suspension in the same solvent, was added to the dimethyl formamide solution of the activated pepstatin and the coupling of the peptide to the gel was allowed to proceed for 22 hours at room temperature with a gentle magnetic stirring under exclusion of atmospheric moisture. The gel was filtered and washed with 1 liter of dimethyl formamide dioxane mixture (1:2 v/v) to remove trapped pepstatin followed by a wash with 1 liter of 1 M NaCl and stored in the same salt solution in the cold. One ml of the wet gel was found to contain 1.8 μmole of covalently bound pepstatin.

Purification of renin from hog kidneys -- Hog renin was extracted by the modification of the method of Rubin (12). Frozen hog kidneys (6 kg) partially thawed, freed from fat and medulla, minced through an electric meat grinder, frozen with liquid nitrogen, freeze-dried, pulverized in a blender and extracted with ether, was suspended in 16 liters of the cold 30% (v/v) ethyleneglycol monomethylether-water mixture containing the following protease inactivators: sodium tetrathionate (5 mM) diisopropyl phosphorofluoridate (0.05 mM), phenylmethanesulfonyl fluoride (2 mM) and EDTA (5 mM). After gentle stirring for 1 hour the extract was collected by centrifugation; renin activity was adsorbed to DEAE cellulose (1 kg, Whatman DE-52) with gentle stirring for 40 min at pH between 6.5 and 7; cellulose was collected by filtration and washed in 8 liters of dilute acetic acid (pH 5.3) for 30 min. Renin was eluted twice in two 8 liter portions of 0.1 M Na-acetate buffer, pH 4.8, containing 0.2 M NaCl and collected by precipitation with 72% saturation of ammonium sulfate. Precipitates were collected by centrifugation, dissolved in 480 ml of 0.1 M Tris-HCl buffer, pH 7.5, dialyzed overnight against 12 liters of 0.01 M Tris-HCl buffer, pH 7.5. All buffers used up to this step after the first extraction contained the mixture of the protease inactivators at one-twentieth the concentrations used in the initial extraction.

The solution was diluted with an equal volume of 0.01 M Na-pyrophosphate buffer, pH 6.5, containing 0.1 M NaCl; pH was adjusted to pH 7.0 and then applied to the pepstatin-agarose column (0.5 x 14.5 cm). The column was eluted by stepwise changes in pH and ionic concentration as described in the legend of Fig. 1. The renin containing fractions were then subjected to gel filtration chromatography on a column of Sephadex G-75 as shown in Fig. 2. Only the middle peak fractions exhibited significant renin activity. These fractions were purified on a DEAE-cellulose column (Fig. 3) at pH 6.35 with a concentration

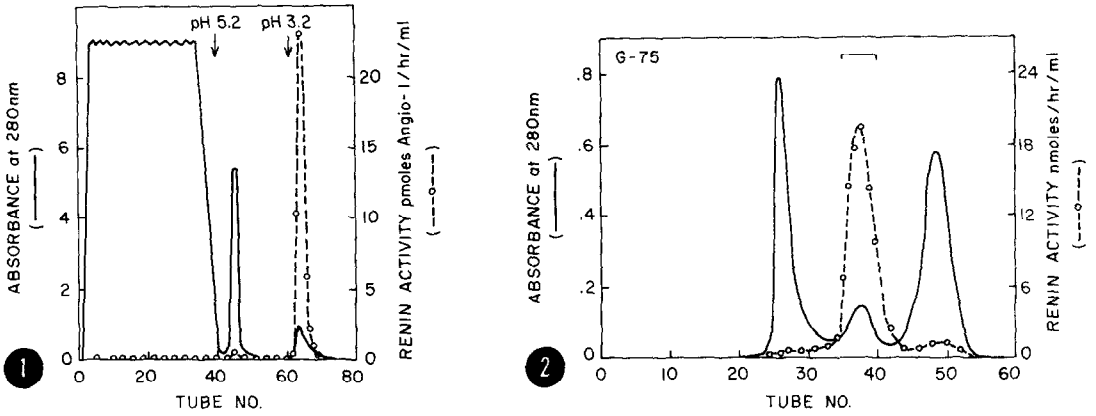

Fig. 1. Affinity chromatography of the ammonium sulfate fraction of renin on pepstatin-aminohexylagarose gel. The column (1.5 x 14.5 cm) had been equilibrated with 0.1 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl. After the application of the sample the column was thoroughly washed with the same buffer to exhaust proteins in eluate; then washed with 0.02 M Na-acetate buffer, pH 5.2, containing 1.0 M-NaCl to elute non-renin proteins bound to the column. Renin containing fractions were eluted with 0.1 M acetic acid of pH 3.2. Renin activity was determined by the radioimmunoassay of angiotensin I generated at pH 5.4 by the method of Haber et. al. (16) using New England Nuclear kit and crude renin substrate from the hog (Miles Laboratories).

Fig. 2 Gel-filtration chromatography on a Sephadex G-75 column. The active fractions from the affinity chromatography (Fig. 1) were concentrated by pressure filtration and applied to a column (2.5 x 110 cm) of Sephadex G-75 equilibrated with 0.01 M Na-pyrophosphate buffer, pH 6.5, containing 0.1 M NaCl. The column was eluted with the same buffer. Renin activity was determined by the method of Reinharz and Roth (17).

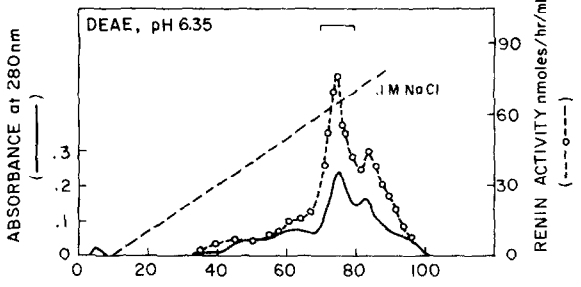

Fig. 3 Chromatography on a DEAE-cellulose column. The renin containing middle peak fractions from the gel filtration chromatography (Fig. 2) were concentrated and equilibrated with 0.02 M Na-phosphate buffer pH 6.35 by the pressure filtration, then applied to a DEAE-cellulose column (0.9 x 20 cm, Whatman DE-52) equilibrated with the same buffer. A linear concentration gradient was generated by mixing 100 ml of the same buffer in the mixing flask with 100 ml of the same buffer containing 0.1 M NaCl. The renin activity was determined as in Fig. 2.

Table 1. Purification of Renin from the Hog Kidney

Purification Step	Total Protein mg	Specific Activity* μg Ang. I/μg Protein/hr.	Purification	Yield %
Kidney Powder**	425,000	0.003	1.0	100
Crude Extract	90,000	0.013	4.3	93
DEAE-batch	25,000	0.038	13	75
Affinity Column	50	11	3,700	45
Sephadex G-75	5.0	100	33,000	39
DEAE-Cellulose	0.87	480	160,000	33
CM-Cellulose	0.60	530	180,000	25

* Rennin activity was determined by the radioimmunoassay (16) of angiotensin I generated at pH 5.4 (See the legend to Fig. 1).

** Protein concentration of all fractions except for the crude extract was determined spectrophotometrically at 280 nm using pure submaxillary gland renin A (15) as reference standard. Frozen hog kidney (6 kg) was used as starting material. Finely dispersed suspension was used for the activity assay and protein concentration determination by biuret method. The suspension was soluble in the alkaline biuret medium.

gradient of NaCl (See the legend for details). The active fractions indicated by the brackets in Fig. 3 were further purified on a carboxymethyl cellulose column at pH 5.05 with a NaCl concentration gradient. As shown in Fig. 4 a major renin peak was resolved from several minor peaks which also possessed renin activity.

RESULTS AND DISCUSSION

As summarized in Table 1, an 180,000-fold purification was achieved to obtain the pure renin from the hog kidney at a remarkable overall yield of 25%. The approximately 300-fold purification achieved by the affinity chromatography on the pepstatin-agarose column greatly facilitated the present purification work. The pepstatin-agarose column could be used repeatedly after washing with 6.5 M urea.

The major renin peak fraction obtained by chromatography on CM-cellulose (Fig. 4) gave a single band on electrophoresis in polyacrylamide gel (Fig. 5). The purified hog renal renin is stable. When stored frozen at -20° in 0.02 M Na-

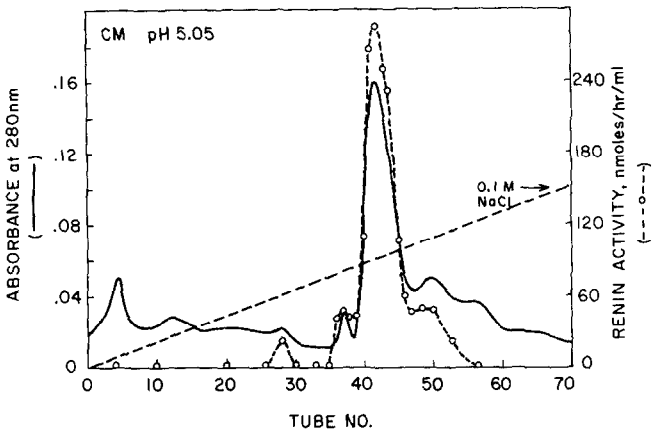

Fig. 4 Chromatography on CM-cellulose. The major renin peak fractions shown by the bracket in Fig. 3 were pooled, concentrated to 4 ml and equilibrated with 0.02 M Na-acetate buffer, pH 5.05, then applied to a CM-cellulose column (0.9 x 19 cm, Whatman CM-52) previously equilibrated with the same buffer. It was eluted by a linear concentration gradient generated by mixing 125 ml of 0.02 M Na-acetate, pH 5.05, in the mixing flask with 125 ml of the same buffer containing 0.15 M NaCl. Renin activity was determined as in Fig. 2.

phosphate buffer, pH 6.35 containing 0.1 M NaCl, no detectable loss of activity occurred over a period of 7 weeks. No appreciable loss of activity was noted during the chromatographic purification as indicated by the very good recovery of the renin activity for each of the chromatographic steps. This stability no doubt contributed to the very high overall yield. Thus the notorious instability of renal renin documented in early literature (1, 2) does not seem to be the intrinsic property of renin, but seems to be due to contaminating proteases. In order to prevent the proteolytic destruction of renin, measures were taken to chemically eliminate contaminating proteases by using a mixture of several protease inactivators of different specificity in the extraction medium and in subsequent adsorbtion and dialysis steps. Since exposure of renin to acid pH is known to produce multiple peaks of renin derivatives (14) presumably due to limited proteolysis by cathepsins, the acid treatment was avoided in the extraction procedure. Renin prepared by the present method seems to

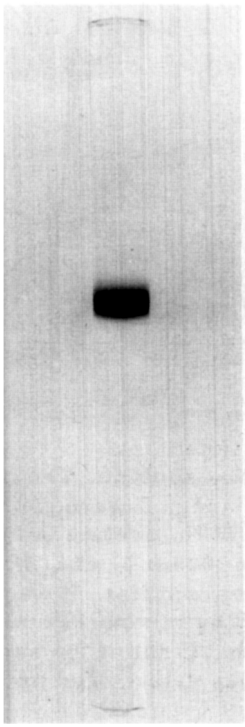

Fig. 5 Polyacrylamide gel electrophoresis of hog renal renin from the major peak fractions of Fig. 4. Electrophoresis was performed on 7.5% cross-linked gel at pH 9.5 with 3mA of current per tube for 45 min.

consist of one major component and several minor components. In this preliminary study these minor components have not been purified to the highest purity. The specific activity of 530 μg angiotensin I produced/μg of enzyme/hour and a preliminary molecular weight of 40,000 (to be published) allow us to estimate a turnover number of 4.7 sec^{-1}.

Although mouse submaxillary gland renin has been purified to a stable and pure state in our laboratory (15), renal renin has never been available at a comparable purity and stability. The present studies have opened the new possibility to provide well defined renin for its characterization and to isolate the pure renin from other species of animals.

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