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PURIFICATION OF A COMPLETELY INACTIVE RENIN FROM HOG KIDNEY AND IDENTIFICATION AS RENIN ZYMOGEN.

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Hog renal inactive renin was separated from active renin and completely purified to an electrophoretically homogeneous state by using a new procedure which consisted of affinity chromatography on pepstatin-Sepharose, octyl-Sepharose, Affil-Gel blue and Con A-Sepharose columns, ion exchange chromatography and gel filtration. By this method a 3,000,000-fold purification was obtained with a 6% recovery from a crude kidney extract. This pure preparation was totally inactive and underwent marked activation by trypsin. It is a glycoprotein as judged by affinity to concanavalin A and has an apparent molecular weight of 50,000 as determined by gel filtration on Sephadex G-100. Treatment of the inactive renin with guanidine, urea and Triton X-100 did not cause activation indicating that the inactive renin isolated in the present study is not a product of renin-inhibitor complex.

Renin (EC3.4.99.19) catalyzes the formation of angiotensin I from angiotensinogen. This is the first step in the cascade of reactions leading to the formation of the major pressor substances angiotensins II and III and to the release of aldosterone from the adrenal cortex. Thus, renin plays a key role in blood pressure regulation.

Since the discovery in human amniotic fluid of activatable renin (1), similar or different types of the activatable enzyme have been reported in plasma (2,3), kidney (4-10) and brain (11) of various species. It was not known whether the activatable renin was a regulatable enzyme with a potential for further enhancement of activity or a totally inactive enzyme. Affinity chromatographic separation of the activatable form from active renin in human plasma allowed us to demonstrate that it was a totally inactive substance (12). Question remained as to the inactive renin is the zymogen of renin

Abbreviations: ConA, concanavalin A; NEM, N-ethylmaleimide; DFP, diisopropylphosphorofluoridate; PMSF, phenylmethylsulfonyl fluoride; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; MES, 2-(N-morpholino) ethanesulfonic acid.

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or an enzyme-inhibitor complex. In spite of numerous studies, the nature of prorenin remained elusive, primarily because of the lack of pure material.

Definitive proof for the zymogen nature of inactive renin and the elucidation of its activation mechanism requires determination of its molecular properties with a completely purified preparation. By devising a new procedure involving affinity chromatographic steps, inactive renin was purified and properties compatible with its zymogen nature were demonstrated.

MATERIALS AND METHODS

Renin activity was determined by the radioimmunoassay of angiotensin I (13) generated in 20 min from 25 µl of 28 ng angiotensin I equivalent of the purified sheep angiotensinogen (14) incubated at 37°C in a mixture consisting of 160 µl of 0.2 M MES buffer, pH 6.1, 5µl of 0.15M PMSF, 10 µl of 0.2 M EDTA and 60 µl of the solution of renin. Inactive renin was determined similarly after activation by trypsin as described below.

Activation of inactive renin The inactive renin solution (25 µl) was incubated for 20 min at 22°C with 25 µl of a freshly mixed solution of 2.5 µg of TPCK-treated trypsin (Worthington) and 250 µg of crystalline bovine serum albumin (Sigma) in 0.1 M Tris HCl buffer, pH 7.5. The activation was terminated by the addition of 100 µg of soybean trypsin inhibitor (Sigma) in 10 µl of 0.1 M Tris- HCl, pH 7.5. For the determination of inactive renin in extracts containing large amounts of active renin, the latter was removed before assay by passing 2 ml of sample solutions through a column (2 ml) of pepstatin-Sepharose followed by washing with 0.02 M phosphate buffer, pH 6.1. The unabsorbed fractions and washing were combined, activated with trypsin and subjected to renin assay as above.

Purification procedures Hog kidney cortex (4.0 kg) was sliced and homogenized in a Waring blendor with 16 l of 0.01 M phosphate buffer, pH 6.5, containing 10 mM EDTA, 10 mM NEM, 1 mM DFP and 2 mM PMSF. After removal of insoluble materials by centrifugation at 5,000 x g for 30 min, the supernatant was adsorbed to DEAE-cellulose (1.5 kg, Whatman DE52) and eluted with 6 l of 0.02 M phosphate buffer, pH 6.5, containing 0.5 M NaCl, 0.1 mM DFP, 0.02 mM PMSF, 1 mM NEM and 1 mM EDTA. Unless stated otherwise the mixture of these protease inhibitors were added in buffers used throughout in the following purification procedures. The eluate was saturated with ammonium sulfate to 2.6 M. Precipitates collected by centrifugation were dissolved in 600 ml of 0.02 M phosphate buffer, pH 6.1 and dialyzed for 40 hrs against 8 changes of a total of 8 l of the same buffer. The pooled extract (4.5 l) from a total of 20 kg of fresh hog kidney cortex was applied to a pepstatin-Sepharose (15) column (2.5 x 20 cm) equilibrated with 0.02 M phosphate buffer, pH 6.1 and washed with 2 l of the phosphate buffer. The non-retained fractions and washing were pooled and directly applied to DEAE-Sephacel column (Pharmacia, 4.0 x 75 cm) previously equilibrated with 0.02 M phosphate buffer, pH 6.1, washed with the same buffer and eluted with a linear NaCl concentration gradient generated between 1.5 l each of the initial buffer and the same buffer containing 0.5 M NaCl. Fractions containing inactive renin were pooled and saturated with ammonium sulfate to 1.0 M. The clear supernatant obtained after centrifugation was applied to an octyl-Sepharose column (Pharmacia, 5 x 90 cm) previously equilibrated with 0.02 M phosphate buffer, pH 6.5 containing 1.0 M ammonium sulfate and washed with the same buffer (first arrow, Fig. 1). The column was eluted with 50% ethylene glycol in 0.01 M phosphate buffer, pH 6.5 (second arrow Fig. 1). Fractions containing the inactive renin (bracket, Fig 1)

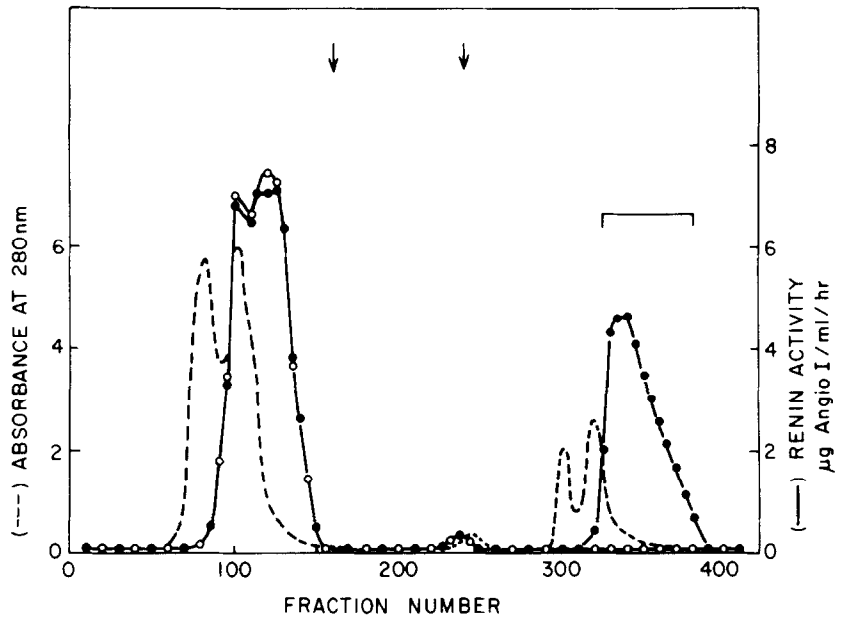

Fig. 1 Hydrophobic interaction chromatography on an octyl-Sepharose column. Renin activity was determined before (○) and after trypsin activation (●). The elution buffer was changed at the arrows.

were concentrated by pressure filtration through an Amicon PM-10 filter, equilibrated with 0.01 M pyrophosphate buffer, pH 6.5 containing 0.1 M NaCl, centrifuged to remove precipitates, and applied to a Sephadex G-100 column (Pharmacia, 5 x 90 cm) equilibrated with the pyrophosphate buffer. The inactive renin fractions were pooled, dialyzed against 0.02 M phosphate buffer, pH 6.5 and applied to an Affi-Gel blue column (Bio-Rad, 4 x 60 cm). The column was washed with 1.0 l of 0.02 M phosphate buffer, pH 6.5, then eluted with a linear NaCl concentration gradient generated between 1.5 l each of 0.02 M phosphate buffer, pH 6.5, and the same buffer containing 1.0 M NaCl (arrow, Fig 2). The fractions containing inactive renin were pooled, dialyzed against

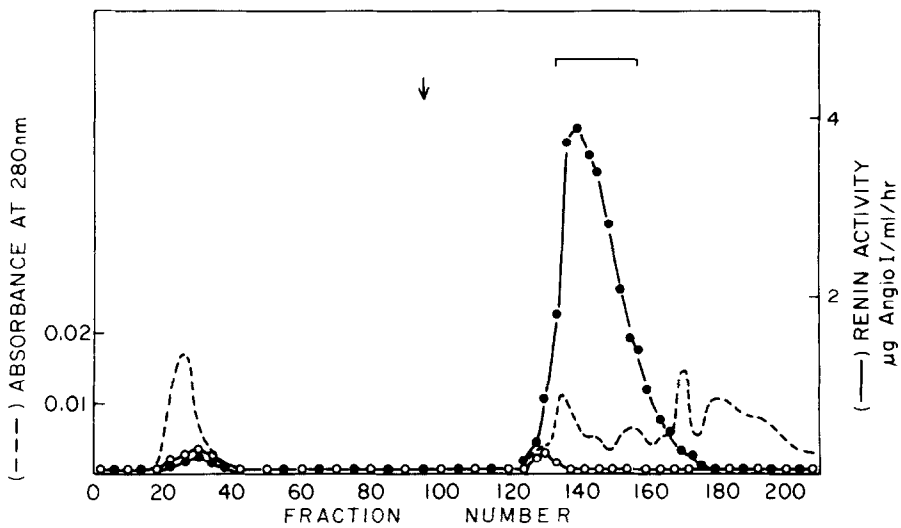

Fig. 2 Affinity chromatography on an Affi-Gel blue column.

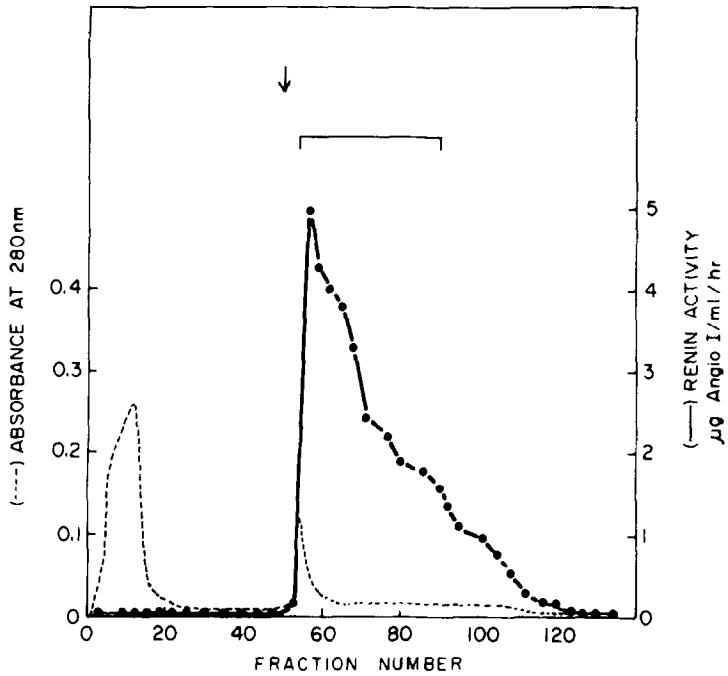

Fig. 3 Affinity chromatography on a ConA-Sepharose column.

0.02 M phosphate buffer, pH 6.5 and applied on Con A-Sepharose column (Pharmacia, 1.0 x 20 cm). The protease inhibitors were not used at this and subsequent stages. After a wash with 200 ml of 0.02 M phosphate buffer, pH 6.5, the column was eluted with 0.25 M α-methyl-D-mannoside in the phosphate buffer (arrow, Fig. 3). Fractions containing inactive renin were concentrated on Amicon PM-10 membrane and applied to a column (1.5 x 95 cm) of Sephadex G-100. Inactive renin fractions were pooled, dialyzed against 0.02 M phosphate buffer, pH 6.5, and applied to DEAE-Sephacel column (1.0 x 10 cm) equilibrated with the same buffer (Fig. 4). After a wash with the phosphate buffer, it was eluted with a linear gradient of NaCl in the same buffer (0 to 0.2 M). Fractions under the bracket (Fig. 4) were used as inactive renin preparations.

Analytical gel filtration A calibrated Sephadex G-100 column (2.5 x 90 cm) was employed to estimate the molecular weight of renin. Radiolabeled molecular weight standards were added to samples as internal reference as described previously (16).

Effect of rabbit antibodies to hog renin (17) on renin activity was determined after incubating 60 µl of the trypsin-activated inactive renin fraction with 10 µl of the diluted antibody (1:1,500) for 18 hrs at 4°C.

Protein concentration was determined by the method of Lowry et al (18) and of Waddel (19).

Polyacrylamide gel electrophoresis was performed at pH 8.4 using 10% gel according to the method of Davis (20).

RESULTS

The purification steps and the yield for a typical preparation of inactive renin are summarized in Table 1.

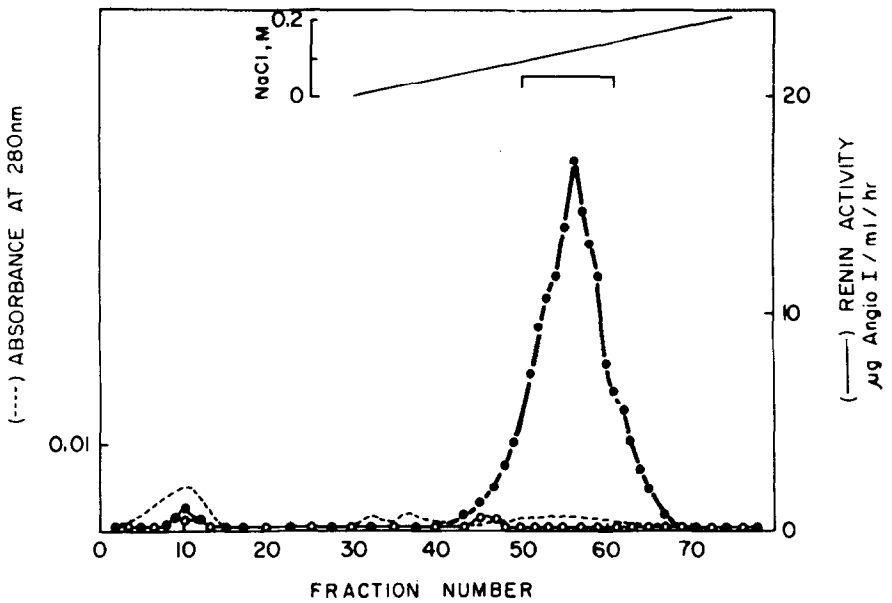

Fig. 4 Chromatography on a DEAE-Sephacel column. Renin activity was determined in the absence of (●), or in the presence of (○) anti-hog renal renin antibody after trypsin activation.

TABLE I

Purification of Inactive Renin From Hog Kidney

Step	Total Protein (mg)	Specific[b] Activity	Purification	Yield (%)
Crude Extract[a]	1,600,000	0.0015[c]	1	100
DEAE Batch	125,000	0.016[c]	10	82
Pepstatin-Sepharose	60,000	0.03	20	75
DEAE-Sephacel	18,800	0.08	53	63
Octyl-Sepharose	1,000	1.43	950	59
Sephadex G-100	208	5.29	3,530	46
Affi-Gel Blue	16.0	62.9	41,900	42
Con A-Sepharose	0.750	1,000	666,700	31
Sephadex G-100	0.170	2,400	1,600,000	17
DEAE-Sephacel	0.030	5,000	3,333,000	6.3

[a] Prepared from 20 kg of fresh hog kidney

[b] Activity of inactive renin as defined by, inactive renin = total renin activity after activation - renin activity before activation; expressed as μg angiotensin I/ mg of protein/hr

[c] Determined after removal of contaminating renin by using affinity chromatography; see details in "MATERIALS AND METHODS".

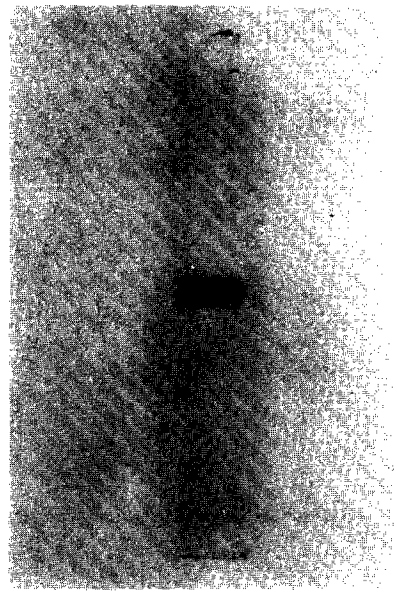

Fig. 5 Gel electrophoresis of inactive renin obtained from DEAE-Sephacel column (Fig. 4) on 10% polyacrylamide gel at pH 8.4.

Starting with 20 kg of fresh hog kidney, 30 μg of inactive renin was obtained with a 6% yield, which represents a 3,300,000-fold purification. Polyacrylamide gel electrophoresis of this preparation yielded a single protein band (Fig 5). The final product was stable for at least 10 days at pH 6.5 and -20°C in the presence of 0.05% bovine serum albumin.

A pepstatin-Sepharose was effective in removing 90% of active renin at the very beginning of the purification steps. Inactive renin was selectively bound to octyl-Sepharose (Fig 1) and Affi-Gel blue (Fig 2) while active renin was not. Thus, after the step of Affi-Gel blue the preparation was completely free from active renin. The inactive renin was firmly bound to concanavalin A-Sepharose and eluted by α-methylmannoside from the lectin-gel (Fig 3) indicating that it is a glycoprotein. Renin activity generated from inactive renin by trypsin was completely inhibited by antibodies to hog renal renin (Fig 4).

The inactive renin showed an apparent molecular weight of 50,000 as determined by gel filtration on a calibrated Sephadex G-100 column. Treatment of inactive renin in 0.5% bovine serum albumin with 3 M guanidine or 3 M urea for 30 min at pH 6.8 and 37°C or with 0.05% Triton X-100 for 30 min

at pH 6.8 and 4°C did not result in significant activation. The same treatment of active enzyme did not cause significant inactivation.

DISCUSSION

Affinity chromatographic procedures employed in the present studies permitted rapid isolation and demonstration of inactive renin in hog kidney extract. The pure inactive enzyme in the present study is distinct from previously isolated high molecular weight, partially active renin (4-10) in that it is totally inactive, that it undergoes marked activation by trypsin, and that it is not activated by procedures which have been shown to cause dissociation of renin-inhibitor complex (4-7).

The total lack of enzyme activity in this preparation, activation by proteolysis, a molecular weight (50,000) significantly greater than active renin (40,000) ref (21) and lack of activation by various dissociative processes known to activate a renin-inhibitor complex indicate that the inactive renin isolated from hog kidney has properties compatible with those of zymogen rather than an enzyme-inhibitor complex as was proposed earlier. The identification of inactive renin (prorenin) as a zymogen and the availability of pure preparation is expected to facilitate studies on its physiological role and biochemical mechanism of its activation.

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