Vol. 96, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1980 Pages 907-914

PURIFICATION OF RAT RENAL RENIN FROM CRUDE KIDNEY EXTRACTS BY DIAMINOHEXAMETHYLENE-SEPHAROSE CHROMATOGRAPHY.

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Received July 31,1980

SUMMARY Renin from rat kidney extracts was adsorbed to diaminohexamethylene-sepharose columns at extremely low ionic strength and neutral pH. Renin was retarded while the column was developed in 1 mM sodiumpyrophosphate and extraneous proteins were removed. Elution of renin was performed using a linear gradient of sodiumpyrophosphate, 1 - 17 mM at pH 6.8. Renin was purified in a yield up to approx. 60 per cent of the applied activity and a purification factor between 5 - 122 depending on the specific activity of the applied sample. The specific activity after this single chromatography of crude rat kidney homogenate on diaminohexamethylene-sepharose showed a median of 11.3 Goldblatt units per mg protein in a range of 5.3 - 42.0 Goldblatt units per mg protein. The renin binding capacity of the column was 1 Goldblatt unit per ml wet gel. The purified renin was subjected to G-100 Sephadex chromatography demonstrating two molecular weight forms of 44 000 and 50 000 dalton. Polyacrylamide gel electrophoresis demonstrated three separate fractions of renin.

INTRODUCTION

Renin (EC 3.4.99.19) is the acidic protease which catalyzes the proteolytic cleavage of angiotensinogen in plasma to angiotensin I and an inactive protein.

The purification of renin from rat kidney has been the subject of several studies (1, 2, 3). Recently rat kidney renin has been completely purified in low yield by a multistep procedure including affinity chromatography using pepstatin coupled to a diaminohexamethylene-sepharose column (4). The present paper describes a one step method of partial purification of rat kidney renin in high yield.

Renin from crude kidney extracts adsorbed to diaminohexamethylene-sepharose at extremely low ionic strength, and was retarded during the development of the column in a low ionic strength buffer. Selective desorbtion of renin from the column was accomplished using a gentle slope gradient of increasing ionic strength at neutral pH. The forces involved in the binding of renin were only operational at extremely low ionic strength and neutral pH.

Diaminohexamethylene-sepharose:aminohexyl-sepharose.

0006-291X/80/180907-08$01.00/0

MATERIALS AND METHODS

Chemicals. AH-Sepharose 4B (diaminohexamethylene-sepharose) was from Pharmacia, Sweden (abbreviated aminohexyl-sepharose). N-cyclohexyl-N'-[2-(4-morpholinyl)-ethyl]-carbodiimide-methyl-p-toluolsulphonate (CMC) was from FLUKA, Switzerland. Standard hog renin was from Medical Research Council, Mill Hill, London, U.K. Angiotensin I (lot A 0413, $\varepsilon_{280}^{2} = 13.000$) was from Bechman, Geneva, Switzerland. Antiangiotensin I antibody and (^{125}I)-Angiotensin I was a gift from professor Knud Poulsen. NH_2-Leu-Leu-Val-Tyr-Ser was synthesized by one of us, I.R. Other chemicals used were of the highest grade available.

Kidney preparation. The crude kidney extract was prepared as described (5) by method 2, replacing the kidney homogenate:solvent ratio with 1:6 (w:v) and the dialysis buffer of step V with 1 mM $Na_4P_2O_7$/HCl, pH 6.8 and a conductivity of 200 - 300 μmho x cm^{-1}.

AH-Sepharose 4B was washed according to the manufacturer's instruction (Affinity chromatography. Principles and methods, Pharmacia, Sweden) at room temperature and swollen in 1 mM $Na_4P_2O_7$/HCl, pH 6.8, 200-300 μmho x cm^{-1} and equilibrated on the column at 4°C.

Coupling procedure of NH_2-Leu-Leu-Val-Tyr-Ser. 4 grams of AH-Sepharose 4B was washed as stated above and coupled to 150 mg NH_2-Leu-Leu-Val-Tyr-Ser according to the manufacturer's instruction. CMC was used as coupling reagent. The coupling suspension was stirred for 20 h at room temperature, washed and finally equilibrated in the application buffer at 4°C. The peptide was only partly soluble in dioxan, and in one experiment the vigorous stirring of the suspension prior to addition of the gel caused the complete substitution of the column. Amino acid analysis was performed on three of the five columns subjected to the coupling procedure. A blindcoupling was performed on one column omitting the peptide in the coupling procedure.

Separation procedure on aminohexyl-sepharose and on the NH_2-Leu-Leu-Val-Tyr-Ser--aminohexyl-sepharose. The following procedures were performed at 4°C. Typically 25 ml kidney extract was applied on the column, dimensions 2 cm^2 x 6.5 cm, during 24 h by recycling the effluent on the column, flow 10 - 17 ml per h. The column was developed for another 24 h in 1 mM $Na_4P_2O_7$/HCl, pH 6.8, conductivity 200 - 300 μmho x cm^{-1}. Finally renin was eluted using a continuous gradient of 1 - 17 mM $Na_4P_2O_7$/HCl, pH 6.8 from 200 - 3500 μmho x cm^{-1}, flow as indicated above, fractions approx. 3.4 ml. Between each run the columns were perfused with 3 volumes at 6 M Urea followed by equilibration with the application buffer until the pH 6.8 and the conductivity of 200 - 300 μmho x cm^{-1} was restored.

Renin activity was calculated in all samples by the rate of formation of angiotensin I in rat plasma as described in (6). The direct biological assay was performed in two experiments as described in (7) using female Wistar rats of 200 grams. Protein concentration was estimated as described in (8) using crystalline bovine serum albumin as standard. Molecular weight was estimated by G-100 Sephadex gel chromatography as described in (5) using the principles described in (9). The pooled purified renin from the aminohexyl-sepharose chromatography was applied directly on the G-100 Sephadex column. Polyacrylamide gel electrophoresis was performed as described in (10) omitting the stacking gel, in a 7 per cent total concentration of acrylamide (w:v), 2.6 per cent C (proportion of cross-linking agent) in the separation gel at pH 8.0, 2.5 mA for 2 h at room temperature. Purified pooled fractions from aminohexyl--sepharose chromatography were dialyzed against distilled water, lyophilized and dissolved in the electrophoresis buffer. 21 ug of protein was layered on the top of the gel and submitted to electrophoresis. The gels were scanned on a Zeiss gel scanner at 280 nm, frozen and sliced in 1 mm strips. Elution of each slice was performed at 4°C in saline for 12 h and the eluats were all tested for renin activity in the radioimmunoassay.

RESULTS

Renin was adsorbed to aminohexyl-sepharose only at extremely low ionic strength and neutral pH. The enzyme leaked from the column at ionic strength above 500 - 600 μmho x cm^{-1} and pH 6.8, but remained adsorbed during the development of the column with up to 0.5 l of the application buffer at 200 - 300 μmho x cm^{-1}. Thus differences of a few hundred μmho x cm^{-1} distinguishes the ionic strength where renin binds to the column from the renin leaking point. This emphasizes the importance of strict control of the ionic strength. Renin leaked from the aminohexyl-sepharose column using buffers of high ionic strength below pH 6.0 (unpublished results).

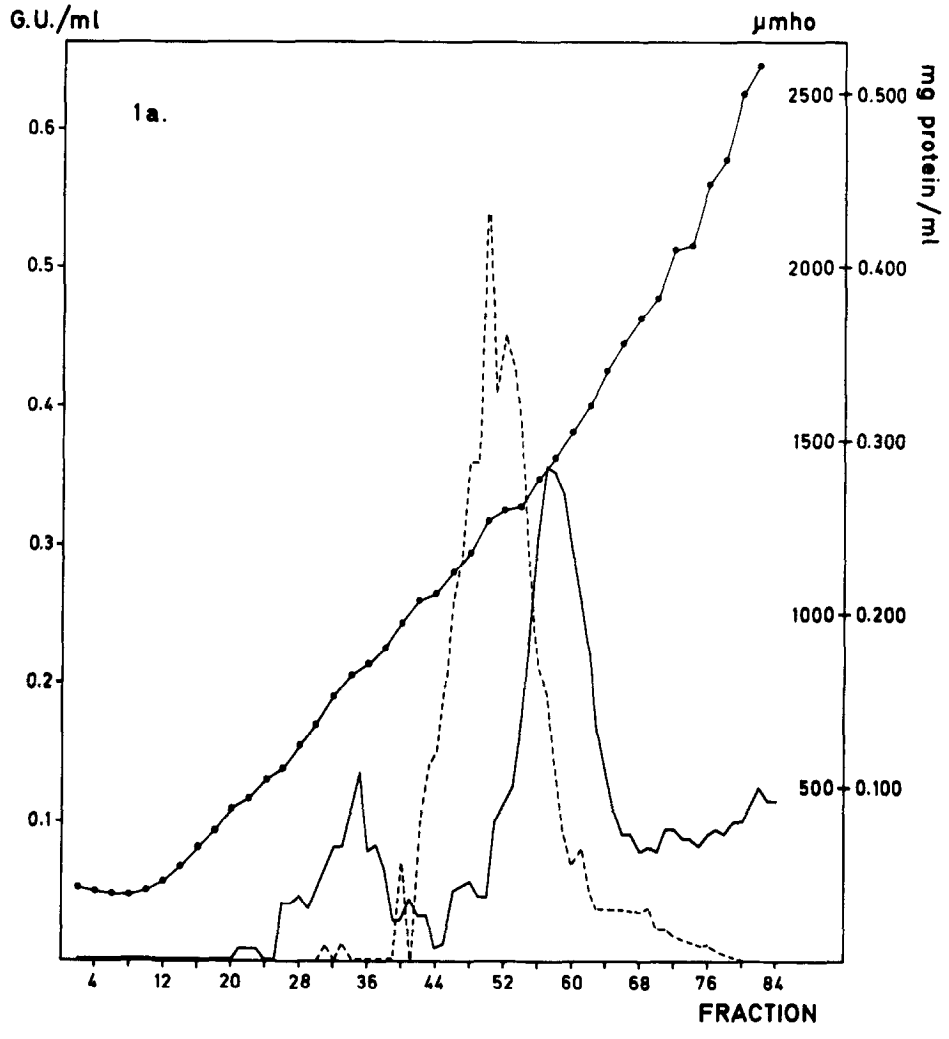
The gentle slope of the elution gradient was essential for the separation of renin from other proteins, which were eluted in the same range. An elution gradient of 3 - 9 μmho x cm^{-1} per ml was useful for separation of renin from contaminating proteins (figs 1a and 1b).

The aminohexyl-sepharose column demonstrated a purification of the peak fractions of 93 and 61 times the applied activity and a specific activity of 42.0 and 38.5 Goldblatt units per mg protein respectively (table 1).

The binding properties of the aminohexyl-sepharose gel towards renin was not improved by coupling with the substrate analog NH_2-Leu-Leu-Val-Tyr-Ser. The substitution degree determined for 3 of the 5 columns subjected to coupling with NH_2-Leu-Leu-Val-Tyr-Ser was 0.00, 0.08 and 12.61 umol peptide per ml wet gel. The columns showed a median purification of 30 times in a range of 5 - 122 times the applied activity, and the peak fractions exhibiting a median specific activity of 8.9 Goldblatt units per mg protein (p=0.05, n=11) (table 1).

The renin binding capacity of the columns was in all cases surmounted by the applied activity. The columns comprised 13 ml wet gel and showed a median recovery of 16.6 Goldblatt units, range 11.6 - 21.6 Goldblatt units (p=0.05, n=14). This indicates a renin binding capacity of approx. 1 Goldblatt unit per ml wet gel. The recovery and the yield of active purified renin increased to approx. 90 and 60 per cent respectively when the applied activity was below the renin binding capacity of the columns.

Renin activity of the purified fractions was demonstrated by formation of angiotensin I from homologous plasma substrate, and in two experiments by the direct biological assay which showed the characteristic blood pressure elevation pattern of standard renin. G-100 Sephadex chromatography of purified renin showed a major peak corresponding to a molecular weight of 44 000 and a shoulder on the ascending limb of the chromatogram at 50 000 dalton (fig 2) in accordance with previous findings (5). Polyacrylamide gel electrophoresis showed three distinct fractions of renin activity in the radioimmunoassay in accordance with previous findings of ours (3) and others (4).



DISCUSSION

The present results indicate that the weak interaction of renin and the aminohexyl-sepharose can be exploited in separation of renin from other proteins. The adsorbtion of renin from hog kidney to aminohexyl-sepharose was originally thought to be ruled out by experiments where a high ionic strength buffer and an extreme pH value (KCl-HCl, 0.2 M, pH 2.0) was applied (11). Later it was reported, however, that rabbit kidney renin in crude extracts was adsorbed to aminohexyl-sepharose columns (12). The present study confirms the latter results because rat kidney renin was shown to be adsorbed to aminohexyl-sepharose and purified in high yield. The method is easy, cheap and provides for a mild treatment of the enzyme. The purification of rat renin by other conventional chromatographic means cause an enhancement of the specific activity of renin of less

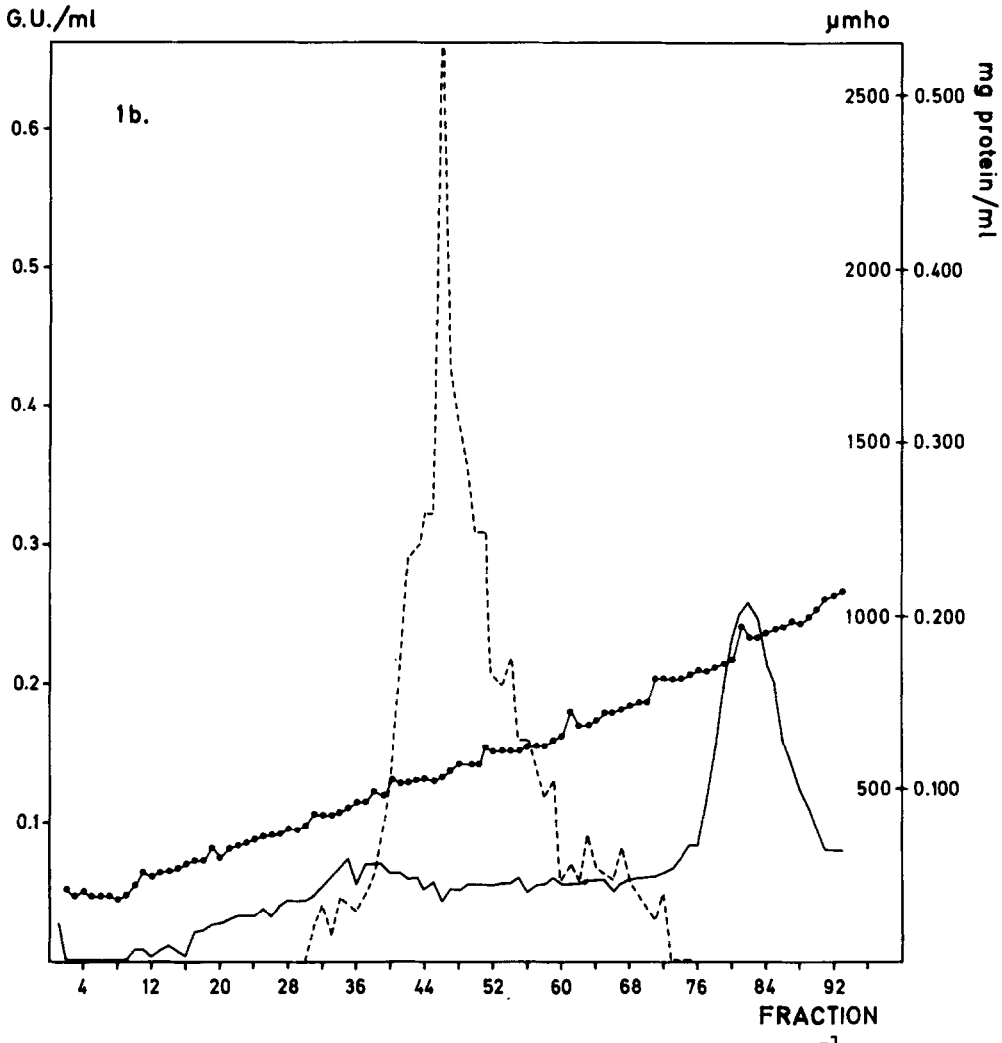


Fig 1. Two gradients with an extremely gentle slope of 9 µmho x cm^{-1} per ml (1a) and 3 µmho x cm^{-1} per ml (1b) useful for the elution of renin from two aminohexyl-sepharose columns subjected to blindcoupling (1a) and substitution with 0.08 umol peptide per ml gel (1b). The load was 25.0 Goldblatt units, 174 mg protein (1a) and 36.0 Goldblatt units, 102 mg protein (1b). Conductivity (●——●) umho x cm^{-1}. Renin activity (- - - -) Goldblatt units. Protein concentration (———), mg protein per ml.

than ten times in a single chromatographic step (1,2,3). Affinity chromatography on pepstatin-aminohexyl-sepharose columns (4) on the other hand shows a purification and yield of similar size as renin from crude extracts by chromatography on aminohexyl-sepharose columns. The renin binding capacity of the pepstatin--aminohexyl-sepharose columns are, however, several times higher than the columns used in the present study. We suggest that the present method using aminohexyl-sepharose columns in combination with affinity chromatography using pepstatin as a ligand will prove useful for the purification of renin in high yield.

Table 1. Purification of renin by chromatography on aminohexyl-sepharose and NH_2-Leu-Leu-Val-Tyr-Ser substituted aminohexyl-sepharose

Column (Run No.)		Application sample		Purified samples		
		Total activity G.U.	Spec. activity G.U./mg protein	Yield G.U.	Range of specific activity G.U./mg protein	Purification factor of peak fraction
Aminohexyl-sepharose	(1)	20.0	0.45	11.6	8.0 - 42.0	93
	(2)	36.0	0.63	15.5	7.3 - 38.5	61
Peptide coupled [a] aminohexyl-sepharose	(1)	24.0	0.12	6.9	4.4 - 7.6	63
	(2)	28.0	0.20	7.9	4.1 - 6.0	30
Peptide coupled [b] aminohexyl-sepharose	(1)	54.0	0.36	8.7	4.6 - 8.7	24
	(2)	45.0	0.32	12.5	4.7 - 8.9	28
	(3)	36.0	0.35	16.5	4.0 - 18.9	54
Peptide coupled [c] aminohexyl-sepharose	(1)	20.0	1.10	11.8	4.0 - 5.9	5
Peptide coupled [d] aminohexyl-sepharose	(1)	43.4	0.87	16.3	5.5 - 11.5	13
	(2)	33.0	0.55	7.5	4.0 - 19.0	35
Peptide coupled [d] aminohexyl-sepharose	(1)	34.0	0.15	13.7	4.2 - 9.6	64
	(2)	18.2	0.09	3.8	11.0	122
	(3)	30.2	0.18	0.7	5.3	29
Blind coupled aminohexyl-sepharose	(1)	25.0	0.36	10.4	4.1 - 21.4	59

Results are indicated for purified samples with a specific activity exceeding 4.0 Goldblatt units/mg protein. Substitution degree of 1 ml wet gel was for the columns indicated:
a) 0.00 µmol peptide, b) 0.08 µmol peptide, c) 12.61 µmol peptide, and d) were not subjected to amino-acid analysis.

The yield of the columns showed a rather uniform pattern except for two runs (table 1) which were below 25 and 3 per cent respectively (3.8 and 0.7 Goldblatt units). We have no explanation of this discrepancy, which might be due the instability of the enzyme or to association of renin with renin binding proteins (5). The finding of two molecular weight forms (44 000 and 50 000 dalton respectively) of the purified renin lends support to this possibility.

The forces involved in the adsorbtion of renin from crude kidney extracts to the aminohexyl-sepharose column, we suggest were of two types. The column initially would interact weakly by electrostatic forces with the enzyme at a conductivity below 300 μmho x cm^{-1} and neutral pH followed by weak hydrophobic interactions (13). Thus a mutual reinforcement of the weak electrostatic and hydrophobic forces during the extended application time might facilitate the binding of renin to the aminohexyl-sepharose. The enzyme did not exhibit a compound affinity (14) towards the NH_2-Leu-Leu-Val-Tyr-Ser-aminohexyl-sepharose. This indicates that the binding forces involved in the present study are not operational in affinity chromatographic systems using the same matrix but other buffers of high ionic strength, since no improvement was obtained in renin binding capacity nor purification using the substrate analog as a ligand. This indicates the presence of binding sites on renin operational at extremely low ionic strength, not confined to the active site of the enzyme.

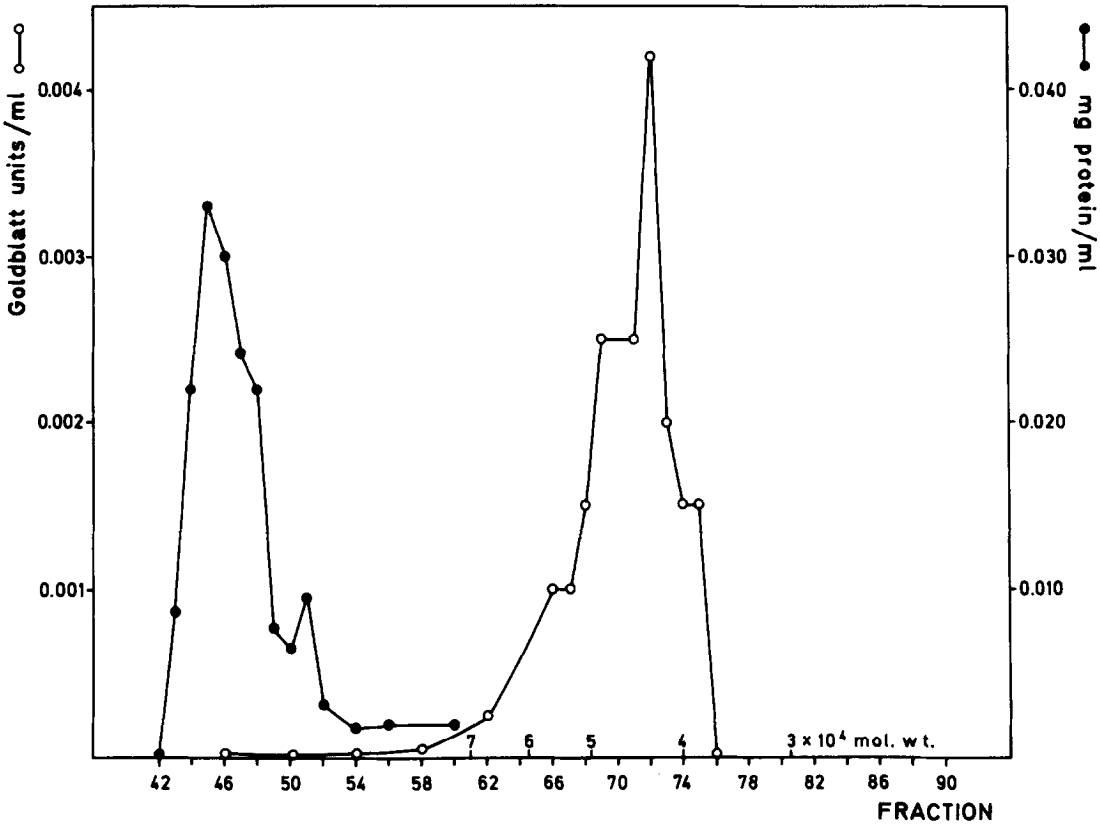


Fig 2. G-100 Sephadex chromatography of purified renin obtained upon aminohexyl-sepharose chromatography on the blindcoupled aminohexyl-sepharose column. The load was 9 ml of pooled purified renin with an activity of 11.6 Goldblatt units comprising approx. 0.5 mg protein.

ACKNOWLEDGEMENT We are indebted to the following: Dr. Poul K. Jensen, Dept. Biochemistry C, Panum Institute for valuable criticism and discussions; Mrs. Lone Hansen and Miss Ulla Thomsen for expert technical assistance. Professor K. Poulsen of the Institute for Biochemistry, Royal Dental College, Copenhagen for his gift of material for the radioimmunoassay. Clinical Chemistry Dept., Aalborg Sygehus Syd, Denmark for giving room to some of the experiments. This work was in part supported by the Danish Heart Foundation.

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