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# CHROMATOGRAPHIC AND KINETIC PROPERTIES OF ACID- AND PEPSIN-ACTIVATED INACTIVE RENIN FROM HUMAN AMNIOTIC FLUID*

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**Abstract**—The chromatographic and kinetic properties of acid- and pepsin-activated inactive renin from human amniotic fluid were determined. Acid-activated inactive renin, like inactive renin, was found to be bound to an Affi-Gel Blue affinity column. Pepsin-activated inactive renin, on the other hand, did not bind to such a column. The $K_m$ values of endogenous active renin, acid-activated inactive renin, and pepsin-activated inactive renin with bovine substrate were 0.11, 0.12 and 0.05 $\mu$M respectively. With hog substrate, the $K_m$ values were 0.16, 0.19 and 0.10 $\mu$M respectively. These results suggest that there is a difference between the active form of renin obtained from acid activation of inactive renin and the active renin obtained after pepsin treatment.

Human amniotic fluid contains an inactive form of renin which can be activated by treatment either with acid [1] or with proteolytic enzymes such as pepsin, cathepsin D and trypsin [2, 3]. Although Morris [3] indicated that the degree of activation with acid treatment was very similar to that observed with pepsin treatment, we observed [4] during the course of our purification of inactive renin that the extent of activation with acid treatment was always significantly less than that observed with pepsin activation. Our observations suggested that the active form of renin obtained from acid activation of inactive renin was different from the active form obtained from pepsin activation. In an effort to determine whether or not this is indeed the case, we have, in the present study, examined both the chromatographic and kinetic properties of acid-activated and pepsin-activated inactive renin.

## MATERIALS AND METHODS

*Materials.* Human amniotic fluid was obtained at 22 weeks of gestation. Hog plasma renin substrate was obtained from Miles Laboratories, Inc., Elkhart, IN. [$^{125}$I]angiotensin I was purchased from New England Nuclear, Boston, MA. Angiotensin I for the radioimmunoassay was obtained from the National Institute for Biological Standards, Holy Hill, London, England. Affi-Gel Blue was purchased from Bio-Rad Laboratories, Richmond, CA. Pepsin was obtained from the Sigma Chemical Co., St. Louis, MO.

*Inactive renin preparation.* Amniotic fluid obtained from three patients at 22 weeks of gestation was centrifuged at 7000 rev/min for 15 min. The supernatant fractions were decanted, pooled (total volume equal to 410 ml) and then treated with ammonium sulfate until a concentration of 3 M was achieved. The mixture was centrifuged for 15 min at 10,000 rev/min. The precipitate was dissolved in 10 mM sodium phosphate buffer (pH 7.0), 0.15 M NaCl, and 5 mM sodium azide (hereafter referred to as PNA buffer), and this solution was dialyzed against this same buffer system overnight.

The above sample (50 ml) was placed on an Affi-Gel Blue column (2.5 × 60 cm) which had been equilibrated previously with PNA buffer. The column was washed with PNA buffer and the elution of protein from the column was followed by measuring the absorbance of the eluate at 280 nm. After 500 ml of eluent had been collected, elution was begun with 1.4 M NaCl in PNA buffer. The collected fractions were assayed for renin activity both before and after pepsin treatment, using a method described by us previously [4]. The fractions obtained from the 1.4 M NaCl elution were pooled, concentrated using an Amicon PM-10 filter, and dialyzed overnight against PNA buffer. This concentrated material served as the starting material for the comparative studies described below.

The amount of inactive renin in this starting preparation was calculated by subtracting the renin activity present in the sample before pepsin treatment from the renin activity present after pepsin activation. Using this method, the concentrate was found to have 85 per cent of its renin activity in the form of inactive renin.

*Pepsin activation of inactive renin.* Pepsin activation of the concentrated Affi-Gel Blue fraction was carried out by incubating 0.8 ml of the material to be activated with 0.2 ml of pepsin (240 $\mu$g/ml) in 0.48 M sodium acetate buffer, pH 4.8. After 30 min at 37°, the incubation mixture was mixed with 1 ml of 0.5 M Tris–HCl buffer, pH 7.4. This solution was subsequently dialyzed overnight against PNA buffer.

*Acid activation of inactive renin.* Acid activation of the inactive renin samples was carried out by dialysis against 50 mM glycine HCl buffer, pH 3.3, for 24 hr. After this time the material was dialyzed against PNA buffer overnight.

* The Human Investigations Committee of the University of Kansas Medical Center has approved the experiments performed in this study.

*Affi-Gel Blue chromatography of acid- and pepsin-activated inactive renin.* A portion of inactive renin obtained after chromatography on Affi-Gel Blue was activated with pepsin using the method described above. The activated material was applied to an Affi-Gel Blue column (0.7 × 29 cm). The column was washed with PNA buffer until ten 3-ml fractions had been collected. At this time, elution was begun with 1.4 M NaCl in PNA buffer. The fractions which were collected were assayed for renin activity as described below.

The same chromatographic procedures were used for samples of inactive renin which were activated as follows: (1) acid activation only, (2) acid activation followed by pepsin treatment, and (3) pepsin activation followed by acid treatment.

*Kinetic studies.* The material used for the kinetic studies was prepared in the following manner. Fifteen ml of the concentrated Affi-Gel Blue preparation of inactive renin was applied to a pepstatin affinity column (1.0 × 22 cm) which had been prepared using the method of Kasakova and Orekhovich [5]. The column was washed with PNA buffer until 59 ml of eluent had been collected. At this time, elution was carried out with 4 M urea in PNA buffer. The fractions which were collected during the urea elution were combined, dialyzed against PNA buffer, and then concentrated using an Amicon PM-10 filter. This material was labeled endogenous renin.

The fractions which were collected in the PNA wash of the pepstatin column were also combined and concentrated. This material, which consisted of inactive renin, was divided into two portions. One portion of the inactive renin was acid activated, while the second portion was pepsin activated. The resulting two preparations were termed acid-activated inactive renin and pepsin-activated inactive renin respectively.

Analysis of the kinetic properties of these three preparations of active renin was carried out by determining the reaction velocities of each preparation with various concentrations of substrate. Two substrates were employed in this study, bovine plasma renin substrate and hog plasma renin substrate. For each substrate, the concentration was expressed in term of the number of nmoles of angiotensin I equivalents/ml. This value was determined by incubating a known volume of substrate with an excess of renin. For bovine plasma substrate, this value was 1.28 nmoles of angiotensin I equivalents/ml; the stock solution of hog plasma substrate contained 4 nmoles/ml. The concentration of bovine substrate was varied between 0.51 and 0.043 $\mu$M, while the concentration of hog plasma substrate was varied between 0.4 and 0.04 $\mu$M. The enzymatic reaction was carried out in a manner analogous to that described below.

Reaction velocities for each substrate concentration were expressed as the number of $\mu$moles of angiotensin I generated/ml/hr. The results were used in a Lineweaver–Burk plot of $1/V$ vs $1/S$; each point represents an average of three determinations. The lines were calculated by linear regression analysis, and the $K_m$ value of each preparation was obtained from the negative reciprocal of the $x$-axis intercept.

*Assay of renin activity.* The fractions obtained from the Affi-Gel Blue column were assayed for renin activity in the following manner. Fifty $\mu$l of each fraction was incubated at 37° with 50 $\mu$l of bovine serum substrate in 150 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA and 20 mM dithiothreitol. Bovine serum substrate was prepared according to the methods of Lever *et al.* [6] and was found to contain 1.28 nmoles of angiotensin I equivalents/ml. After 30 min the enzymatic reaction was stopped by placing the incubation mixtures on ice and diluting them with 0.8 ml of 0.1 mM Tris–acetate buffer, pH 7.4. The angiotensin I produced was assayed by radioimmunoassay, using a procedure described previously by us [7] except that the supernatant fractions were counted using a Packard gamma counter instead of by liquid scintillation. Each fraction was assayed in duplicate and the enzymatic activity was expressed as $\mu$g angiotensin I/ml/hr.

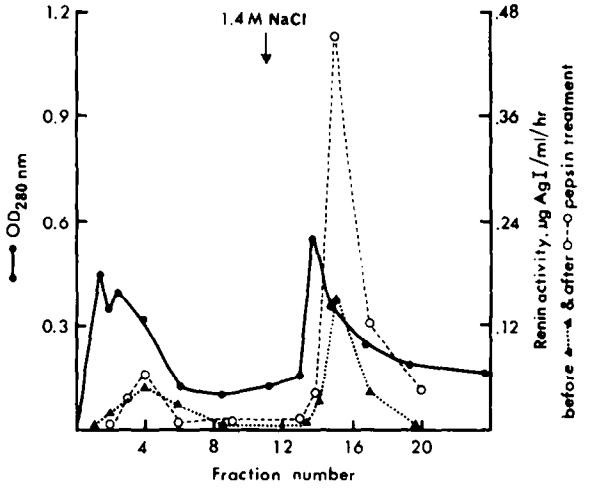


Fig. 1. Affi-Gel Blue column chromatography of the 3 M ammonium sulfate fraction of human amniotic fluid. Elution was with 10 mM sodium phosphate buffer (pH 7.0), 0.15 M NaCl and 5 mM $NaN_3$. At fraction number 11 elution was begun with 1.4 M NaCl in PNA buffer.

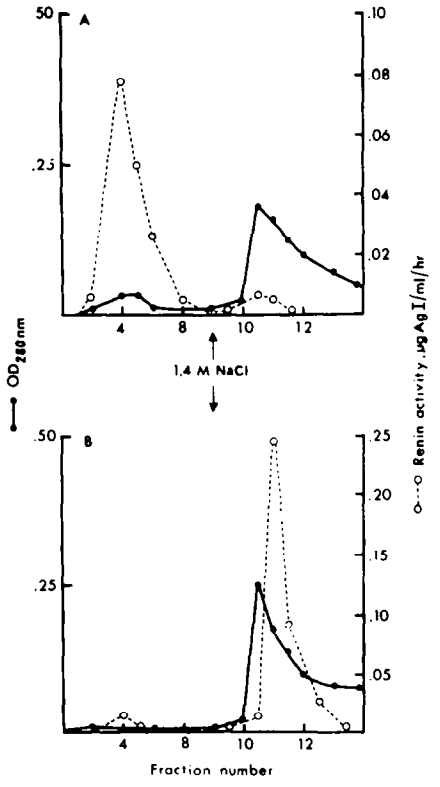


Fig. 2. Affi-Gel Blue column chromatography. Panel A: pepsin-activated inactive renin; and panel B: acid-activated inactive renin.

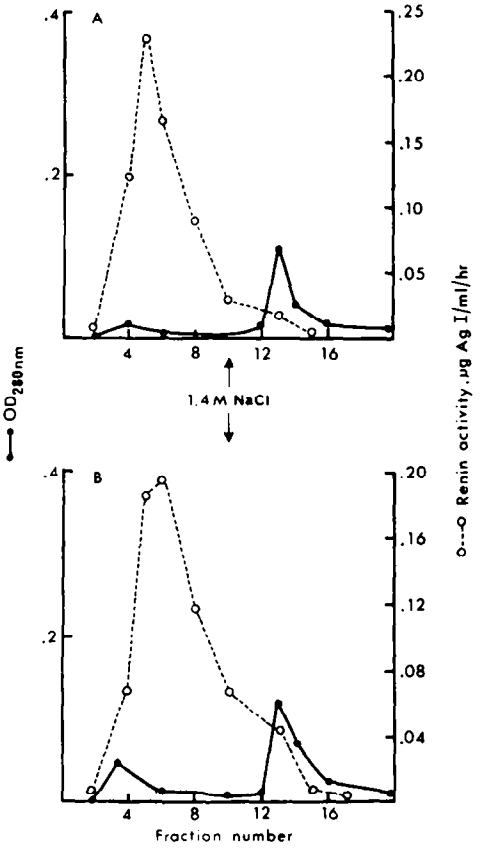


Fig. 3. Affi-Gel Blue column chromatography. Panel A: acid-activated inactive renin after treatment with pepsin; and panel B: pepsin-activated inactive renin after acid treatment.

## RESULTS

The behavior of inactive renin on an Affi-Gel Blue column is illustrated in Fig. 1. Most of the inactive renin, as well as the active renin already present in amniotic fluid, was retained by the column. Both could be eluted from the column with 1.4 M NaCl. When this retained material was activated with pepsin and then reapplied to the Affi-Gel Blue column, 93 per cent of the enzymatic activity now appeared in the initial wash fractions (Fig. 2A). If, on the other hand, the inactive renin was activated with acid treatment instead of pepsin, 84 per cent of the activated material was still retained on the Affi-Gel Blue column (Fig. 2B). Like inactive renin, this acid-activated inactive renin was eluted from the column with 1.4 M NaCl.

When we treated the acid-activated inactive renin with pepsin, we found that the resulting material was now no longer retained by the Affi-Gel Blue column (Fig. 3A). A similar effect is seen in Fig. 3B where the pepsin-activated inactive renin was treated with acid.

A comparison of the kinetic properties of endogenous renin, pepsin-activated inactive renin, and acid-activated inactive renin with bovine plasma renin substrate is shown in the Lineweaver–Burk plot of Fig. 4. With this substrate, the $K_m$ values of the three enzyme preparations were found to be 0.11 $\mu$M for the endogenous renin, 0.12 $\mu$M for the acid-activated inactive renin, and 0.05 $\mu$M for the pepsin-activated inactive renin. Similar results were obtained when the substrate was from hog plasma. With this substrate the $K_m$ value for the endogenous renin was 0.16 $\mu$M, while the $K_m$ values for the acid-activated and pepsin-activated inactive renin were 0.19 and 0.10 $\mu$M respectively.

## DISCUSSION

In the present study, we have examined the chromatographic and kinetic properties of acid- and pepsin-

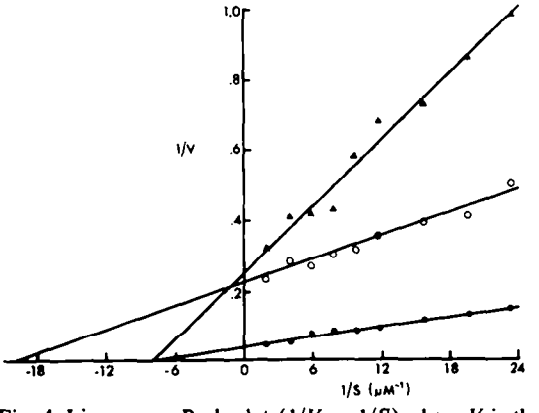


Fig. 4. Lineweaver–Burk plot ($1/V$ vs $1/S$) where $V$ is the reaction velocity ($\mu$moles angiotensin I/ml/hr) determined at varying concentrations of bovine plasma renin substrate. Key: (▲) acid-activated inactive renin; (○) pepsin-activated inactive renin; and (●) endogenous renin.

activated inactive renin in an attempt to determine whether or not the active renin obtained with acid activation differs from that obtained with pepsin treatment. We observed in our early studies [4] with partially purified inactive renin that the molecular weight of the inactive material, as well as that for the acid- and pepsin-activated forms, was around 39,000. Similar results have recently been reported by Shulkes *et al.* [8]. We also observed in our previous studies that both the acid- and pepsin-activated forms were bound to a pepstatin affinity column, while the inactive form was not. In spite of these similarities, however, one significant difference was observed. That difference was that the degree of activation seen with pepsin was on the average 1.8 times that observed with acid treatment [4]. This latter observation thus suggested that, in spite of the similarities in molecular weight and behavior on a pepstatin affinity column, the acid-activated inactive renin was somehow different from the pepsin-activated inactive renin.

In the present study, we have compared the behavior of acid-activated inactive renin and pepsin-activated inactive renin on an Affi-Gel Blue affinity column. The preparation of inactive renin which was used in this study was partially purified by ammonium sulfate precipitation and Affi-Gel Blue chromatography. These two steps, as has been shown previously [4], result in a 16-fold purification. Our results have demonstrated that acid-activated inactive renin is chromatographically different from pepsin-activated inactive renin since the former was bound by the Affi-Gel Blue column while the latter was not. Thus, acid-activated inactive renin behaves in a manner similar to the active and inactive forms of renin which are found in amniotic fluid, since all three forms are bound to the Affi-Gel Blue column.

Affi-Gel Blue consists of a sulfonated polyaromatic blue dye coupled to an agarose matrix. The nature of the binding between the dye and the above forms of renin is unknown. Although several investigators [9, 10] have observed that this gel has a high affinity for enzymes which possess nucleotide binding sites, it seems unlikely that this would account for the affinity of the above forms of renin for Affi-Gel Blue since nucleotides such as ATP, cAMP and NAD, which are able to remove nucleotide-requiring enzymes from the gel [9], are unable to elute inactive renin from the column (R. L. Johnson, N. W. Fleming and A. M. Poisner, unpublished results). It may be that in this instance Affi-Gel Blue is acting as a cation exchange resin since the blue dye which is attached to the column contains several sulfonic acid moieties.

Regardless of the nature of the binding between inactive renin and Affi-Gel Blue, acid treatment must not fundamentally alter the binding site involved since the acid-activated material still binds to the column. Pepsin treatment of inactive renin and of acid-activated inactive renin, however, must destroy the binding site involved since in both cases the resulting material no longer binds to Affi-Gel Blue.

Our results would also suggest that there are kinetic differences between acid-activated inactive renin and pepsin-activated inactive renin. The $K_m$ value of the acid-activated inactive renin was approximately two times that obtained for the pepsin-activated inactive renin regardless of whether the substrate used was from bovine plasma or hog plasma. Whether or not this difference is due to a difference in the affinity of the activated enzymes for the substrate or to a difference in the rate constant of the breakdown of the enzyme–substrate complex going to product cannot be determined from these experiments.

Interestingly, the $K_m$ value of the acid-activated material was similar to that for the active renin which was already present in the amniotic fluid and which could be separated from the inactive renin present by pepstatin affinity chromatography. This observation coupled with the chromatographic similarites between these two active enzymes would suggest that acid activation of inactive renin yields an active renin which is similar to the endogenous active renin found in amniotic fluid. In this regard, Shulkes *et al.* [8] have reported similar findings, even though their experimental conditions are somewhat different.

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