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PROPERTIES OF THE ACTIVATION BY PEPSIN OF INACTIVE RENIN IN HUMAN AMNIOTIC FLUID

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Summary

1. The renin present in human amniotic fluid was found to have an apparent M_r of 58 000 by gel filtration and is thus bigger than renin in untreated kidney extracts and plasma ($M_r \approx 40\,000$).

2. Treatment with pepsin (40 μg/ml, pH 4.8, 2 h, 22°C) caused a 6-fold increase in activity of this renin species, although M_r was not very different (57 000).

3. Unlike renal renin, renin in human amniotic fluid was not a glycoprotein and behaved similarly on concanavalin A-Sepharose before and after activation by pepsin.

4. Ion-exchange chromatography demonstrated a small change in the ionization properties of human amniotic fluid renin after activation by pepsin.

5. Pepsin-mediated activation resulted in a five-fold increase in V, but only a small decrease in the K_m of renin to 39% of normal, so that the increase in activity observed was not due to an increase in the affinity of the enzyme for its substrate. The kinetic data were consistent with the theory of noncompetitive inhibition.

6. The activation of human amniotic fluid renin by pepsin may be caused by a change in the tertiary structure of the molecule subsequent to a proteolytic action that does not remove detectable polypeptide components.

Introduction

'Inactive' renin was discovered by Lumbers in human amniotic fluid and plasma and could be activated by acidification to pH 3.3 [1]. In 1971 Morris

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and Lumbers showed that activation could also be evoked by carboxyl or serine proteases, viz by adding pepsin at pH 4.5 or trypsin at pH 7.5, and that it was the action of an endogenous protease at pH 3.3, not H^+ per se, that was responsible for 'acid' activation [2,3]. The endogenous activator in amniotic fluid was, like pepsin, still active after treatment to pH 1.5, a pH at which renin is irreversibly inactivated. 'Inactive' renin has since been found in kidney extracts and plasma of a number of species and this literature has been reviewed recently [4]. Activation by exogenous pepsin and trypsin was later confirmed by Day and Luetscher in studies with Wilm's tumor plasma [5] and activation by exogenous trypsin added to normal human plasma has also been observed [5]. The endogenous activator at pH 3.3 in human plasma appears to be pepsin (Day, R.P. and Morris, B.J., unpublished results). The pepsin-like intracellular protease, cathepsin D, can also activate 'inactive' renin [7], thus making tenable an intracellular mechanism [3,8,9]. More recently, treatment of human amniotic fluid [10] or plasma [11—13] to temperatures approaching their freezing points has been reported to activate 'inactive' renin and there is evidence that a protease is reponsible for this activation by cold also, although the enzyme is probably a serine protease [14,15].

The molecular mechanisms occurring during proteolytic activation of 'inactive' renin are not known. Therefore, the aim of the present study was to determine the properties of renin in human amniotic fluid both before and after pepsin-mediated activation.

Materials and Methods

Materials

Human amniotic fluid free from contamination by blood was collected following amniotomy and stored at −20°C before use. It contained a low concentration of angiotensinogen (70 pmol/ml) and low angiotensinase activity. Pepsin (EC 3.4.4.1) from hog stomach mucosa was purchased from Sigma Chemical Co., St. Louis, MO (Lot No 26 C-8100) as a twice-crystallized and lyophilized powder and was stored dessicated at −20°C before use.

Renin assay

Renin was measured by the rate at which it produced angiotensin I upon incubation of 0.025—0.10 ml sample (diluted where appropriate) at 37°C for a period in the range of 1—8 h (depending on the concentration of renin in the sample) with 0.5 ml nephrectomized dog plasma containing 50 mM sodium phosphate buffer (pH 7.4)/15 mM EDTA/1.6 mM 2,3-dimercaptopropanol/3.4 mM 8-quinolinol [16]/>1000 pmol angiotensinogen/ml/0.2 pmol angiotensin I · h^{-1}/ml renin and undetectable angiotensinase activity. Reaction mixtures were then diluted with 0.5 ml cold distilled water, placed in boiling water for 5 min, centrifuged at 1000 × *g* for 10 min, and the angiotensin I content of 0.025 and 0.10 ml supernatant was quantified by an established radioimmunoassay technique [17]. The reaction was linear with regard to time and volume of sample and renin was expressed as pmol angiotensin I · h^{-1}/ml sample.

Detection of protein

Distribution of protein eluting from columns was determined by measuring

the absorbance of each fraction at 280 nm using a Gilford Spectrophotometer, Model 2400-S.

Treatment with pepsin

Amniotic fluid was thawed and then dialysed in 8/32 cellophane casings for 3 h at 22°C against 20 vols pH 4.6 buffer (26.7 mM citric acid, 46.6 mM Na_2HPO_4, 5 mM EDTA, 81.7 mM NaCl). This adjusted the pH to 4.8, which is too high for 'acid' activation to occur [1], since endogenous pepsin is still combined with its inhibitor, and yet is within the pH range of activity of pepsin, whose activity falls to zero above pH 6 [18]. Samples of pH 4.8 amniotic fluid were incubated at 22°C alone and with 40 μg/ml pepsin for 0—5 h. The reaction of separate portions was terminated by the addition of 2 vols. 200 mM sodium phosphate buffer (pH 7.4) and the samples stored at —20°C overnight before renin assay.

Determination of Michaelis constant

Samples of 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ml plasma from nephrectomized dogs were made up to a total volume of 0.5 ml with 50 mM sodium phosphate buffer (pH 7.4) and then mixed with 0.1 ml sample containing either activated or nonactivated renin (viz. human amniotic fluid incubated either with or without pepsin, 40 μg/ml for 2 h at 22°C and subsequently diluted with 2 vols. 200 mM sodium phosphate buffer (pH 7.4) to stop the reaction of pepsin). The mixtures were incubated at 37°C for 1 h. The reaction of renin was then stopped by standing the reaction tubes in boiling water for 5 min, and after sedimentation of insoluble precipitate by centrifugation at $1000 \times g$ for 10 min angiotensin I was quantified by radioimmunoassay. The Michaelis constant (K_m) was determined using a Hewlett-Packard computer program based on the method of Wilkinson [19].

Gel filtration

A sample of 1 ml human amniotic fluid was applied to a column of Sephadex G-100 (Pharmacia) (1.6 × 80 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.4 at 4°C) and 2.3-ml fractions were collected using a Gilson Micro Fractionator; flow rate, 0.1 ml/min. A portion (approx. 1.2 ml) of each fraction was dialysed against two changes of 300 ml 50 mM citrate/phosphate buffer (pH 4.6) at 22°C for 3 h, to adjust the pH to 4.8. The samples were decanted and 0.4 ml was pipetted into two sets of Wasserman tubes. Pepsin (10 μl 1.6 mg/ml solution in pH 4.6 buffer) was added to one set (final concentration of pepsin = 40 μg/ml) and the mixtures were incubated at 22°C for 2 h. The pH was then adjusted to stop the reaction of pepsin, by adding 0.8 ml 200 mM sodium phosphate buffer (pH 7.4) and the samples were stored at —20°C overnight before renin assay.

In a subsequent experiment, 1 ml amniotic fluid was dialysed to pH 4.8 and then incubated at 22°C with pepsin (40 μg/ml, final concentration) for 5 h. The reaction was stopped by adding 2 ml 200 mM sodium phosphate buffer (pH 7.4). Sample was applied to the Sephadex G-100 column (3.2-ml fractions) and the renin was assayed.

The Sephadex G-100 column was calibrated by determining the elution

volume (V_e) of aldolase (M_r 158 000), ovalbumin (45 000), chymotrypsinogen (25 000) and ribonuclease (13 700). Void volume (V_o) was measured using Blue Dextran. Apparent molecular weights were determined from a plot of $V_e - V_o$ against the M_r, on a log scale, of the protein standards.

Ion-exchange chromatography

A 5-ml aliquot of human amniotic fluid was dialysed overnight in column buffer at 4°C and then applied to a column of DEAE-cellulose (DE52, Whatman) equilibrated with 50 mM Tris-HCl buffer (pH 8.4 at 4°C)/30 mM NaCl. A gradient of NaCl (30—150 mM) was formed using 100 ml column buffer (30 mM NaCl) and 100 ml column buffer/150 mM NaCl (flow rate 0.4 ml/min, 6.5-ml fractions). An aliquot (approx. 1.2 ml) of each was adjusted to pH 4.8 by dialysis for 3 h against 1 l pH 4.6 buffer at 22°C. 0.4-ml aliquots were then incubated at 22°C for 2 h in the presence and absence of pepsin (40 μg/ml, final concentration). The reaction was stopped by adjusting the pH with 2 vols. 200 mM sodium phosphate buffer (pH 7.4). After storage overnight, renin was assayed.

5 ml amniotic fluid was also pretreated with 40 μg/ml pepsin for 2 h at 22°C and pH 4.8, followed by overnight dialysis in column buffer (pH 8.4 at 4°C) before chromatography as above (5.7-ml fractions).

The NaCl gradient was analysed by measuring the concentration of Na^+ in the fractions with a Flame Photometer (model 143, Instumentation Laboratory, Inc., Lexington, Mass., U.S.A.).

Lectin affinity chromatography

A 5-ml aliquot of amniotic fluid was applied to a 25-ml column of concanavalin A-Sepharose (Sigma) kept at 4°C and equilibrated in 10 mM NaCl/10 mM sodium acetate/1 mM $MnCl_2$/1 mM $CaCl_2$/0.01% merthiolate and titrated to pH 6.0 with acetic acid. Gradient elution was performed at 4°C using 100 ml column buffer alone and 100 ml 0.1 M glucose in column buffer (flow rate, 0.3 ml/min, 6.8-ml fractions). A portion (approx. 1.2 ml) of each was adjusted to pH 4.8 by dialysis for 3 h against 1 l pH 4.6 buffer at 22°C. Samples of 0.4 ml were then incubated at 22°C for 2 h in the presence or absence of pepsin (40 μg/ml, final concentraton). The reaction was stopped by adjusting the pH with 2 vols. 200 mM sodium phosphate buffer (pH 7.4). After storage overnight at —20°C, renin was measured in the fractions.

In a further experiment, 5 ml amniotic fluid was dialysed to adjust the pH to 4.8 and then incubated with 40 μg/ml pepsin for 2 h at 22°C. After dialysis overnight in column buffer (pH 6.0) at 4°C, the sample was applied to the column and elution performed as above. The renin content of each fraction was then measured.

The behavior of semipurified human kidney renin on concanavalin A-Sepharose was also examined. The purification used steps 1—3 of the procedure of Haas et al. [20]. Elution was performed by forming a continuous ascending gradient of 0.2 M α-methyl-D-mannoside and 0.2 M 1-*O*-methyl-α-D-glucopyranoside.

The osmotic pressure of the fractions was measured with a Wide Range Advanced Osmometer, Model 3W, to indicate the carbohydrate gradient.

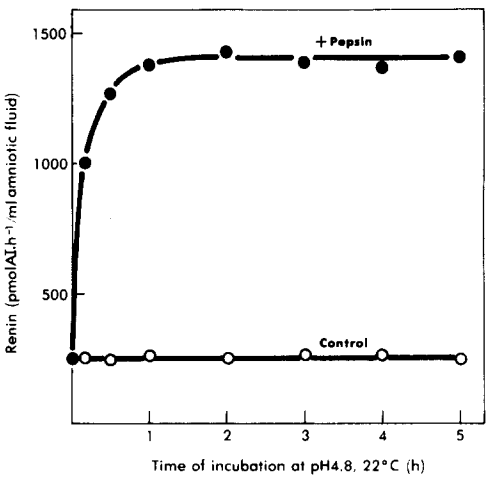
Results

Treatment with pepsin

Incubation of human amniotic fluid with 40 μg/ml pepsin at pH 4.8 at 22°C for up to 5 h, resulted in the generation of renin, with a maximum formation of 5.6 times the initial level being attained within 2 h (Fig. 1). Control samples incubated at pH 4.8 without pepsin had no increase in renin. Consumption of angiotensinogen by renin in 0.025 ml of sample (diluted 1 in 3) was <3% of that present. Formation of angiotensin I during incubation at pH 4.8 was small: <3% of the amount formed during renin assay at pH 7.4, and was taken into account in Fig. 1 so that the values represent only angiotensin I formed during renin assay. Pepsin can form angiotensin I from angiotensinogen [21,22]. However, this did not occur during assay of renin because at the pH of assay, 7.4, pepsin has negligible activity against proteins [18]. Furthermore, the similarity in zero time points in Fig. 1 in the presence and absence of pepsin indicates that no detectable formation of angiotensin I by pepsin occurred.

Determination of Michaelis constant

Activated and non-activated renin generated angiotensin I at different rates when incubated with dog angiotensinogen. Fig. 2 is a Lineweaver-Burk plot of 1/initial velocity vs. 1/concentration of angiotensinogen. After analysing the data using a computer program the K_m was calculated as 771 pmol/ml before activation and 302 pmol/ml after activation by pepsin, V was 6 pmol angiotensin I · h^{-1}/ml before activation and 29 pmol angiotensin I · h^{-1}/ml after activation by pepsin.



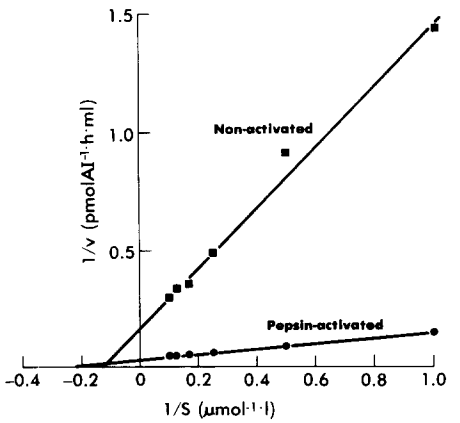


Fig. 1. Generation of renin during incubation of human amniotic fluid without (○) and with pepsin, 40 μg/ml (●) at pH 4.8 and 22°C. Renin was measured at pH 7.4 by its rate of hydrolysis of dog angiotensinogen (pmol angiotensin I · h^{-1} /ml of amniotic fluid). AI, angiotensin I.

Fig. 2. Lineweaver-Burk plot of initial velocity of reaction (v) of pepsin-activated human amniotic fluid renin and non-activated renin with different concentrations of dog angiotensinogen (S). AI, angiotensin I.

Gel filtration

Renin in human amniotic fluid emerged from Sephadex G-100 in a region corresponding to a M_r of 58 000 (Fig. 3). When column fractions were treated with pepsin there was a 6.5-fold increase in the amount of renin in the elution peak. Control measurements of angiotensin I formed during incubation of eluates with pepsin at pH 4.8 were too low to be of significance in the renin assay. The additional renin activity observed corresponded in position to a M_r of 59 000. When amniotic fluid previously treated with pepsin was applied to the column, renin emerged in a similar region, corresponding to a M_r of 57 000 (Fig. 3). In this case the amount of renin in the peak was similar to that for pepsin-treatment after chromatography. The extensive hydrolysis of proteins in this pepsin-treated sample is seen by the appearance of a large region of absorbance in the 28 000 M_r region of the column eluate with a large reduction in the size of protein peaks in the 76 000 M_r region and void volume. Some of the absorbance in the lower panel of Fig. 3 may also be due to pepsin (M_r 34 600 [23]).

Ion-exchange chromatography

In control fractions, a broad peak of renin was found at 60—67 mM NaCl

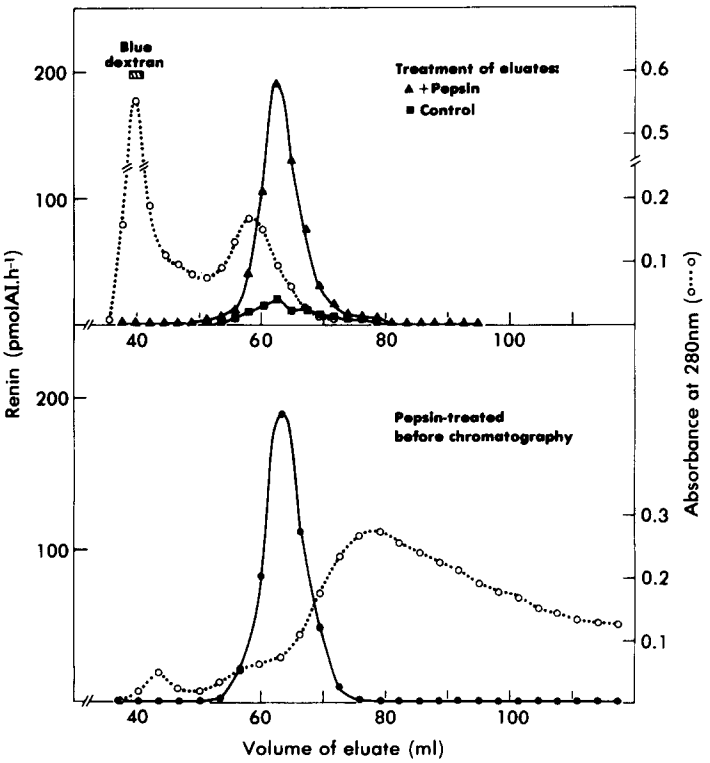


Fig. 3. Elution of renin in human amniotic fluid applied to a column of Sephadex G-100. Upper panel: 1 ml of amniotic fluid was applied and fractions were then incubated without or with pepsin (40 μg/ml) for 2 h at 22°C and pH 4.8. Lower panel: 1 ml of amniotic fluid previously treated with pepsin was applied and renin measured in the fractions. Protein is indicated by absorbance at 280 nm. Void volume is indicated by elution of Blue Dextran. AI, angiotensin I.

(Fig. 4). The concentration of angiotensin I in fractions treated to pH 4.8 was also measured and was less than 0.3 pmol/ml, indicating undetectable generation of angiotensin I by renin acting on endogenous angiotensinogen, which is present in amniotic fluid in low concentrations, during the pH 4.8, 22°C incubation. Angiotensin I was also measured in fractions treated with pepsin and was less that 0.3 pmol/ml in fractions eluting before 100 ml of eluate. However, succeeding fractions contained measurable angiotensin I, but this was always less than 1.2 pmol/ml, and indicates formation of angiotensin I by pepsin acting on endogenous angiotensinogen during the pH 4.8, 22°C incubation. This amount of angiotensin I was too low to affect the measurement of angiotensin I made after incubation of sample with a high concentration of exogenous angiotensinogen at pH 7.4, 37° C, during the renin assay. The amount of renin in column fractions treated with pepsin was 5 times greater than control fractions (Fig. 4). However, the position of the major peak of renin was similar: 60—67 mM NaCl. In the next experiment, where amniotic fluid was run after it had been treated with pepsin, the major peak of renin again appeared near 67

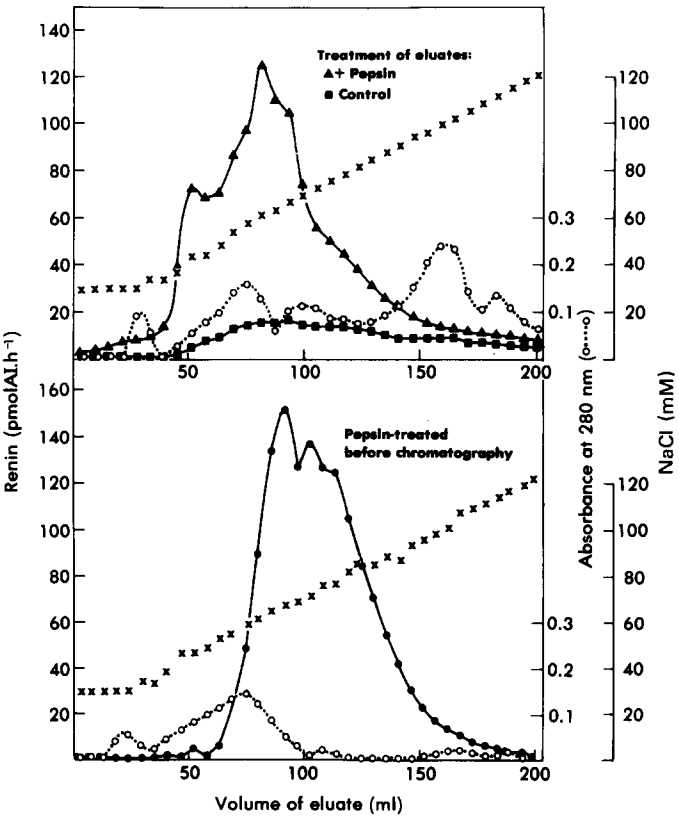


Fig. 4. Elution of renin in human amniotic fluid applied to a column of DE52 cellulose. Upper panel: 5 ml of amniotic fluid was applied and fractions were subsequently incubated without or with pepsin (40 μg/ml) for 2 h at 22° C and pH 4.8. Lower panel: 5 ml of amniotic fluid previously treated with pepsin was applied and renin measured in the fractions. Protein is indicated by absorbance at 280 nm. The NaCl gradient is shown by the crosses. AI, angiotensin I.

mM NaCl, but more renin activity eluted at NaCl > 70 mM and the amount of renin was 5.5 times higher than control fractions (Fig. 4).

Treatment with pepsin before chromatography also markedly reduced the size of the later peaks of protein. Low M_r fragments do not appear, as these would have been removed during analysis of sample prior to chromatography.

Lectin affinity chromatography

Renin in human amniotic fluid emerged in the void volume, i.e., did not bind to concanavalin A-Sepharose (Fig. 5). In contrast, renin partially purified from human kidneys did bind and eluted as several peaks using a gradient of 0.2 M α-methyl-D-mannoside mixed with 0.2 M 1-*O*-methyl-α-D-glucopyranoside (Fig. 6). Treatment of amniotic fluid with pepsin before chromatography resulted in a 5-fold increase in renin, but did not alter the elution behavior from the affinity column (Fig. 5). Treatment of fractions with pepsin after chromatography resulted in a 6.1-fold increase in renin in the same position of elution as controls. Recovery of renin in these experiments was 97—102% of that expected, thus eliminating the possibility that some renin may not have been eluted from the column by the carbohydrate used.

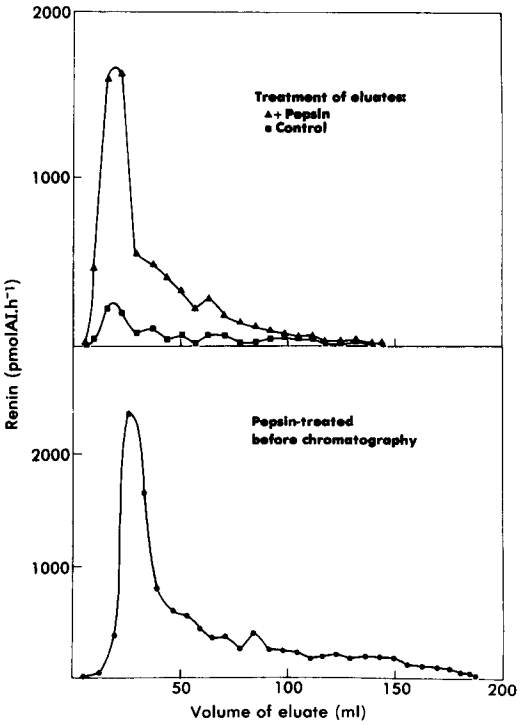


Fig. 5. Elution of renin in human amniotic fluid applied to a column of concanavalin A-Sepharose. Upper panel: 5 ml of amniotic fluid was applied and fractions were then incubated with or without pepsin (40 μg/ml) for 2 h at 22°C and pH 4.8. Lower panel: 0.5 ml of amniotic fluid previously treated with pepsin was applied and renin measured in the fractions. AI, angiotensin I.

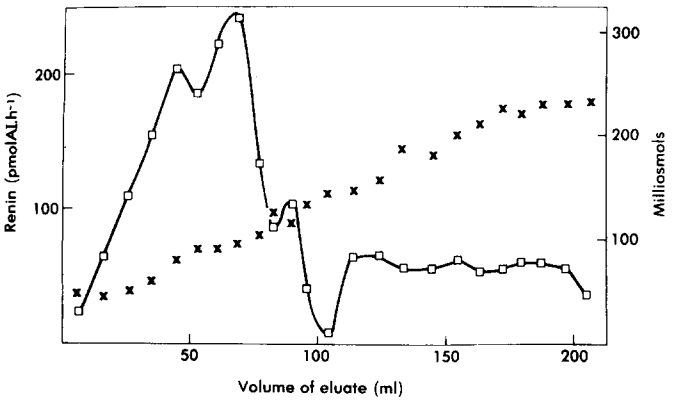


Fig. 6. Elution of semi-purified human kidney renin from a column of concanavalin A-Sepharose with a continuous linear gradient of 0.2 M 1-*O*-methyl-α-D-glucopyranoside and 0.2 M α-methyl-D-mannoside. The latter is indicated by measuring the osmolarity of the fractions (X). AI, angiotensin I.

Discussion

Treatment of renin-constaining samples with pepsin (or acid or cold) results in the appearance of a new activity for hydrolysing angiotensin I from angiotensinogen. Although other proteases, namely pepsin [21,22] and cathepsin D [24,25], can also hydrolyse angiotensin I from angiotensinogen, there is good evidence to suggest that the new 'renin-like activity' is due to the action of a molecule related to renin: (1) the pH-activity profile and pH optima of this activity is similar to that of renin, with considerable activity at pH 7.4 (refs. 10, 13, 26 and Morris, B.J. and Lumbers, E.R., unpublished results), but differs markedly from other carboxyl proteases, which have lower pH optima and negligible activity against angiotensinogen at pH 7.4 [24], (2) the activity is neutralized by antibodies to renal renin [3,27,28], (3) the activity has been shown to be determined by the same factors that affect renal renin [12], and (4) the K_m of the activity is of the same order of magnitude as that of ordinary renal or plasma renin, whereas cathepsin D, for example, generates angiotensin I from angiotensinogen at a rate of 1/100 000th that of renin (at pH 5.5) and 1/3000 000th at pH 7.2 [24]. Renin has no measurable protease activity apart from its hydrolysis of angiotensin I from its substrate [24].

Although the 'renin-like activity' activated by treatment of human amniotic fluid with pepsin is related to renin in untreated kidney extracts, the present study has demonstrated that the enzyme possessing the activity differs at the molecular level in two ways: (1) it has an apparent M_r of 58 000 compared with a M_r of approximately 40 000 for kidney renin [5,29] and (2) it is not a glycoprotein, since it does not bind to concanavalin A-Sepharose, whereas kidney renin does [30,31].

The present results also show that pepsin activates this human amniotic fluid renin by a mechanism that does not bring about a noticeable change in the M_r of the molecule, while causing marked degradation of other proteins present. Furthermore, the activation by pepsin does not alter the K_m appreciably. The decrease observed (from 771 to 309 pmol/ml, i.e. to 39% of normal) would be

too small to account for the large increase in activity seen, since the concentration of angiotensinogen in the renin assay mixtures gave close to zero-order conditions with respect to substrata. A large increase in V with little change in K_m makes unlikely a role for competitive inhibition, but is consistent with the theory of noncompetitive inhibiton. Ion-exchange chromatography showed that there was no major change in the ionization properties of human amniotic fluid renin after its activation by pepsin. Minor alterations were apparent, however, which suggest a slight increase in negative charges on human amniotic fluid renin when it is incubated with pepsin.

A larger form of human amniotic fluid renin (M_r 63 000) has also been reported by Day and Luetscher in gel filtration studies of plasma and kidneys from patients with Wilm's tumor and with other renal dysfunctions [5,27,33]. This large renin in plasma could be activated by treatment to pH 3.3 or by pepsin, with acid activation resulting in a decrease in K_m to 33% of normal (calculated from ref. 5), which is similar to the small change found in the present study (39%), and with no detectable change in M_r. Treatment to pH 3.3 appears to have a similar effect to treatment with pepsin, most likely because the low pH serves merely to activate an endogenous protease which in turn activates the renin [2,3]. A large form of renin (M_r 60 000) has also been found in normal human kidneys [28]. This could be activated six-fold by acidification, but only in less pure preparations, and antibodies to the large form inhibited both the large form and the 40 000-dalton form.

In contrast to the above findings, there are reports of large forms of renin of M_r 58 000 in pig kidneys [34] and of M_r 54 000 in rabbit kidneys [35], which are converted by acid treatment to forms of M_r 40 000 and 37 000, respectively, due to dissociation of a polypeptide fragment, with an increase in renin activity. In one study K_m was determined and found to be similar for each form thus excluding a role of competitive inhibition [34]. Other studies with hog kidneys, in which protease inhibitors were used during extraction, have revealed large forms of renin of M_r 140 000 and 61 000, having specific activities of <1% and 20%, respectively, of that of the 40 000-dalton form [36]. In subsequent studies these investigators found that all of the renin in rat kidney was exclusively in a 63 000-dalton form [37]; omission of *N*-ethylmaleimide during extraction resulted in complete conversion to a 41 000-dalton form, but with only a relatively small increase in the renin activity of the sample. A role for a sulfhydryl protease in the conversion was therefore proposed [37].

The differences in the results obtained by different investigators may be related to differences in chemical composition, pH, temperature and combination of proteases in the tissue sample examined. Reactions in which carboxyl protease activity predominates may produce an activation of large forms of renin with little change in size, whereas a predominance of sulfhydryl protease activity may diminish the size of the molecule with little change in its activity.

The lack of carbohydrate groups in large forms of renin, the larger size, and the capacity to be activated by proteases are consistent with these forms being a biosynthetic precursor of renin. After synthesis of protein precursors in the rough endoplasmic reticulum, they might move to the smooth endoplasmic reticulum where carbohydrate residues are added [38] and proteases could then act to process the molecule into the form in which it is secreted [39—41].

Serum albumin for example is synthesized by way of 'proalbumin' which is converted into serum albumin by the proteolytic removal of a pentapeptide just before secretion [40,41].

The present study has shown that pepsin activates human amniotic fluid renin in a manner that does not alter appreciably the size or substrate affinity of the active site of the enzyme. Pepsin may activate human amniotic fluid renin by hydrolysing peptide bonds distal to the active site, but without causing the separation of large polypeptide fragments. If peptide(s) are removed at all, then these would probably consist of fewer than 10 amino acids. A structural change in the large form of renin could lead to an increase in its activity and this possibility is supported by the slight increase in charge after activation by pepsin.

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