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ISORENIN, PSEUDORENIN, CATHEPSIN D AND RENIN

A COMPARATIVE ENZYMATIC STUDY OF ANGIOTENSIN-FORMING ENZYMES

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Summary

1. Renin was purified 30 000-fold from rat kidneys by chromatography on DEAE-cellulose and SP-Sephadex, and by affinity chromatography on pepstatinyl-Sepharose.

2. The enzymatic properties of isorenin from rat brain, pseudorenin from hog spleen, cathepsin D from bovine spleen, and renin from rat kidneys were compared: Isorenin, pseudorenin and cathepsin D generate angiotensin from tetradecapeptide renin substrate with pH optima around 4.9, renin at 6.0. With sheep angiotensinogen as substrate, isorenin, pseudorenin and cathepsin D have similar pH profiles (pH optima at 3.9 and 5.5), in contrast to renin (pH optimum at 6.8).

3. The angiotensin-formation from tetradecapeptide by isorenin, pseudorenin and cathepsin D was inhibited by albumin, α- and β-globulins. These 3 enzymes have acid protease activity at pH 3.2 with hemoglobin as the substrate. Renin is not inhibited by proteins and has no acid protease activity.

4. Renin generates angiotensin I from various angiotensinogens at least 100 000 times faster than isorenin, pseudorenin or cathepsin D, and 3000 000 times faster than isorenin when compared at pH 7.2 with rat angiotensinogen as substrate.

5. The 3 'non-renin' enzymes exhibit a high sensitivity to inhibition by pepstatin ($K_i < 5 \cdot 10^{-10}$ M), in contrast to renin ($K_i \approx 6 \cdot 10^{-7}$ M), at pH 5.5.

6. It is concluded from the data that isorenin from rat brain and pseudorenin from hog spleen are closely related to, or identical with cathepsin D.

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Introduction

In the preceding publication the purification of rat brain isorenin by affinity chromatography on pepstatinyl-Sepharose has been described [1]. The enzymatic properties of the purified enzyme indicated that it may be a cathepsin D. Therefore, in the present study, the enzymatic properties of rat brain isorenin, rat kidney renin and cathepsin D from bovine spleen were compared. The enzyme pseudorenin, which has been detected by Skeggs and his colleagues [2] in plasma and kidneys of man, in kidneys and spleen of pigs and in most tissues of the rat, was also included in the study, since its reported properties exhibit some similarity to those of rat brain isorenin.

Methods

Enzyme preparations studied

Isorenin from rat brain was purified to homogeneity by a three-step procedure including affinity chromatography on pepstatinyl-Sepharose, as described previously [1].

Pseudorenin, purified from hog spleen, was kindly given to us by Dr. Lentz and Dr. Skeggs (Cleveland). This preparation contained 1.22 units/mg protein. According to Skeggs et al. [2] 1 unit is defined as the amount of enzyme, which generates 1 μmol of angiotensin I from tetradecapeptide renin substrate in 60 min in citrate buffer (pH 4.3) containing 0.15 M NaCl. This preparation does probably not represent a pure enzyme, since in polyacrylamide gel electrophoresis 2 protein bands could be separated.

Cathepsin D (EC 3.4.23.5) from bovine spleen was purchased from Sigma, München. According to the manufacturer, the preparation had a specific activity of 10 units per mg of protein. 1 unit is defined as the amount of enzyme producing an increase in $E_{280}^{1\,cm}$ of 1.0 per min per ml at pH 3.0 at 37°C, measured as trichloroacetic acid-soluble products formed from hemoglobin as the substrate. This preparation also displayed 3 protein bands in polyacrylamide gel electrophoresis.

Renin (EC 3.4.99.19) was purified from rat kidneys as described subsequently.

Purification of renin

Kidneys (20 g wet weight), obtained from male Sprague Dawley rats (180—250 g) were cut into quarters, lyophilized, ground in a mortar and extracted twice with 100 ml of ether. The dried residue was suspended in 90 ml of a homogenization medium, which was similar to that described by Murakami and Inagami [3] (30%, v/v) ethylenglycol/monomethylether in distilled water, sodium tetrathionate 5 mM, phenylmethansulfonylfluoride 2 mM, EDTA 5 mM, and *o*-phenanthroline 3 mM (pH 7.2)). The suspension was homogenized with an ultraturrax (Jahnke and Kunkel, Germany), stirred at 0°C for 1 h and centrifuged at 33 000 × *g* for 15 min. The supernatant was kept and the sediment re-extracted with 50 ml of the homogenization medium. To the combined supernatants 20 g of moist DEAE-cellulose (Whatman DE 52) were added and the slurry was stirred for 15 min. The DEAE-cellulose cake was

washed on a Buchner funnel with homogenization medium diluted 10-fold with 5 mM tris(hydroxymethyl)aminoethane/NaOH (TES/NaOH, pH 6.5). The renin-containing fraction was eluted with the same medium, but containing 0.25 M NaCl. The eluate was dialyzed against 20 mM Tris · HCl (pH 7.3) and applied to a column (2.5 × 80 cm) of DEAE-cellulose (Whatman DE 52), equilibrated with the same buffer. The column was eluted with a linear gradient of NaCl (0—0.5 M) in 20 mM Tris · HCl (pH 7.3) (total volume 1000 ml, 6 ml fractions, flow rate 0.6 ml/min). Renin-containing fractions, eluting between 0.12 and 0.15 M NaCl, were pooled, dialyzed against 5 mM sodium phosphate buffer (pH 5.5), and applied to a column (1.5 × 60 cm) of sulfopropyl-Sephadex (SP-Sephadex C-50, Pharmacia, Sweden). The column was eluted with a linear gradient of NaCl (0—0.3 M, total volume 450 ml) in 5 mM sodium phosphate (pH 5.5), in fractions of 5.8 ml at a flow rate of 0.52 ml/min. Renin eluted from the column at about 0.1 M NaCl. Active fractions were pooled, dialyzed against 10 mM ammonium bicarbonate (pH 7.8) and lyophilized. For further purification by affinity chromatography, pepstatinyl-Sepharose was prepared as has been described previously [1]. The enzyme preparation was dissolved in 3 ml of 0.1 M sodium phosphate buffer (pH 5.8), containing 0.2 M NaCl, and applied to a column containing 4 ml of the affinity gel. The column was washed with 25 ml of the same buffer and renin was eluted with 3 ml of 2 M urea in 0.1 M sodium phosphate (pH 7.6). The preparation was dialyzed against 5 mM sodium phosphate (pH 6.0), concentrated by partial lyophilisation and kept frozen until used. Unless otherwise indicated, dilutions of this renin preparation were made with buffers containing 1.0 mg/ml human serum albumin as a stabilizer.

Enzyme assays

For comparison of the 4 enzymes the following assay systems were used:

In assay system A the generation of angiotensin I from tetradecapeptide renin substrate was measured at pH 5.0. This is similar to the pseudorenin assay described by Skeggs et al. [2], except that Skeggs et al. measured the rate of formation of the tetrapeptide fragment rather than the decapeptide angiotensin I, and used a pH of 4.3.

In assay system B, the rate of angiotensin formation from sheep angiotensinogen was measured at pH 5.5, the pH optimum for the rat brain isorenin [1]. This assay is also suitable for the estimation of renin.

Assay system C is the acid protease assay, commonly used for the estimation of cathepsin D [4], in which denatured human hemoglobin serves as substrate at pH 3.2. All three assay systems have been described in detail [1].

For the quantification of renin during purification from rat kidney, samples were incubated for 15 min with rat angiotensinogen in 0.1 M TES/NaOH (pH 7.0) containing 5 mM EDTA and 1.6 mM dimercaptopropanol. Following incubation, an aliquot was diluted with 0.1 M Tris/acetate buffer (pH 7.4), kept in a boiling water wath for 10 min, and centrifuged. Angiotensin I in the supernatant was estimated in a radioimmunoassay, which has been described elsewhere [5].

Angiotensinogen preparations

Sheep angiotensinogen was prepared from plasma of sheep, which had been

nephrectomized 48 h previously, following the procedure described by Boucher [6]. The preparation had a specific concentration of 170 ng angiotensin I per mg of protein. Rat angiotensinogen was prepared from plasma of rats, nephrectomized 24 h previously, by ammonium sulfate fractionation. The preparation had a specific concentration of approx. 140—180 ng angiotensin I-equivalents/mg protein. Hog renin substrate, obtained from Miles Laboratories had a specific concentration of 350 ng angiotensin I/mg protein.

Protein determination

In samples with a high protein content, the method of Lowry et al. [7] was used. In all other samples protein was measured with the sensitive method of Bramhall et al. [8], which permits the estimation of 0.4 μg of protein. In this method, protein is precipitated and washed with trichloroacetic acid on filter paper, stained with Coomassie Blue R 250, eluted with methanol and measured at 610 nm against paper blancs. A standard curve is prepared from dried human serum albumin.

Results

Purification of renin

Renin was purified 33 000-fold from rat kidney with an overall yield of 20%, as summarized in Table I. The DEAE-cellulose chromatography was similar to that described elsewhere [5], the SP-Sephadex step is illustrated in Fig. 1. The most efficient purification step was the affinity chromatography on pepstatinyl-Sepharose. This affinity material has previously been used in the purification of renin from hog kidneys by Murakami and Inagami [3,9]. However, when attempting to elute the enzyme at pH 3.2, as described by these authors, the rat kidney enzyme was found to be very unstable, and the pH of 7.6 was selected for the elution. Although the observed decrease in affinity of pepstatin to the enzyme with increasing pH (see later) permitted the elution with buffer at pH 7.6 alone, large volumes were necessary to elute significant proportions of the enzyme. Therefore, a phosphate buffer (pH 7.6) containing 2 M urea was used, by which the enzyme was eluted in a volume of 3 ml with a recovery of

TABLE I

PURIFICATION OF RENIN FROM RAT KIDNEY

Renin activity is expressed in nmol of angiotension formed/15 min from rat angiotensinogen at pH 7.0. See Methods for details.

Step	Protein (mg)	Total renin activity (nmol angiotensin/15 min)	Specific activity (nmol angiotensin/mg)	Recovery (%)
Homogenate	3350	3367	1.0	100
Crude extract	2497	3351	1.34	99
DEAE-batch eluate	705	3050	4.33	90.5
DEAE-column eluate	42.7	2164	50.7	64.3
SP-Sephadex	6.7	1599	239	47.5
Pepstatin-affinity chromatography	0.020	670	33 500	20.0

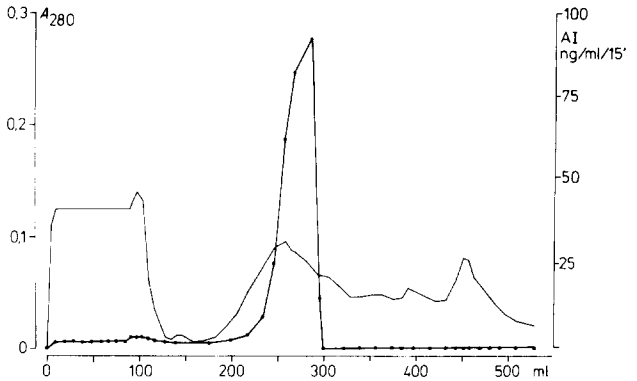

Fig. 1. Purification of renin from rat kidney. The active fractions from DEAE-cellulose chromatography were applied to a column (1.5 × 60 cm) of sulfopropyl-Sephadex, and fractionated with a linear salt gradient (0—0.3 M NaCl in 5 mM sodium phosphate (pH 5.5), total volume 450 ml, flow rate 0.25 ml/min, fractions of 5.8 ml). Elution was monitored by measuring the absorbance at 280 nm (———) and estimating the renin concentrations (●———●). The active fractions under the peak were further purified by pepstatin-affinity chromatography (see Methods). AI, angiotensin I.

40%. A similar system (6 M urea, pH 6.5) has been described by Corvol et al. [10] for the elution of hog renin from pepstatin-Sepharose columns. The final enzyme preparation does probably not represent a pure enzyme. A detailed analysis of this preparation with regard to homogeneity and molecular weight was not possible, because of the limited amount of enzyme protein available. However, no interfering enzymatic activities, such as angiotensinases, converting enzyme or protease activity could be detected in the preparation.

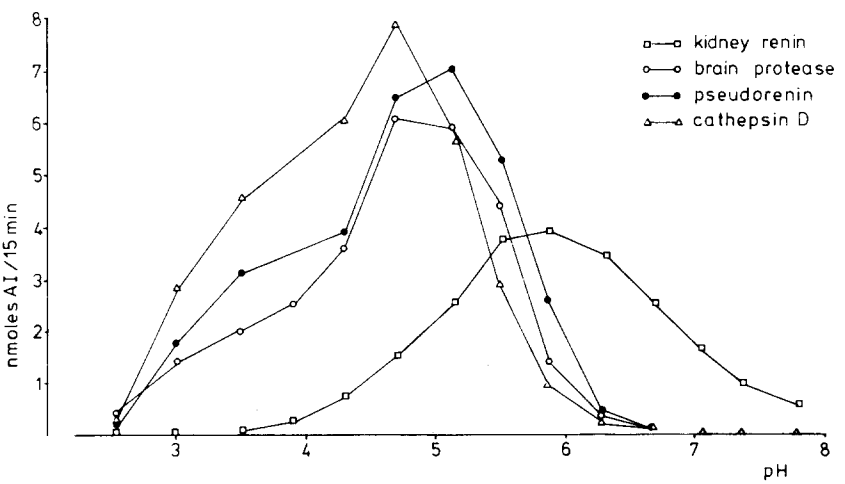

Fig. 2. pH-dependence of angiotensin-formation from tetradecapeptide renin substrate. Incubation of angiotensin-forming enzymes at varying pH values with tetradecapeptide as the substrate. The pH gradient was obtained by titrating a mixture of equal volumes of formic, acetic, and phosphoric acid (0.5 M each) with 5 M NaOH to the desired pH. The actual pH of incubates was ascertained after incubation. AI, angiotensin I.

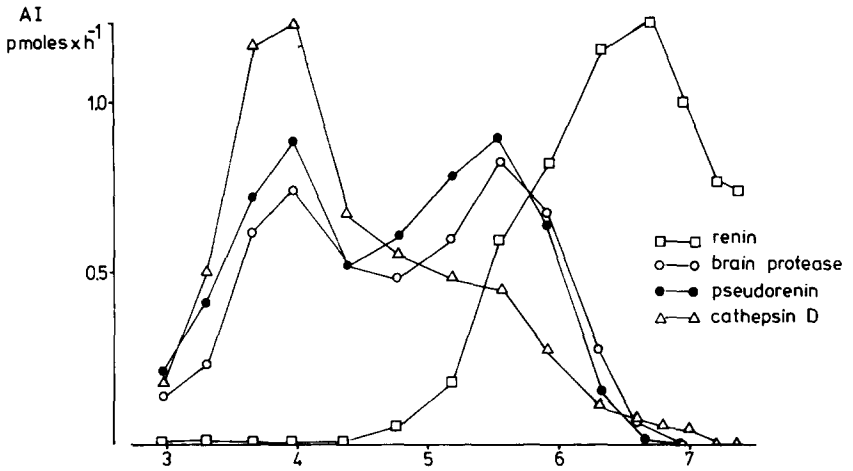

Fig. 3. pH dependance of angiotensin (AI) formation from sheep angiotensinogen. The pH-gradient was obtained as described in the legend to Fig. 2. Except for the buffer system, standard assay conditions as described in Methods were used.

pH profile of angiotensin formation

A comparison of angiotensin formation by the 4 enzymes at varying pH values is shown in Figs. 2 and 3. It is obvious that not only renin, pseudorenin and rat brain isorenin, but also cathepsin D can generate angiotensin I, both from tetradecapeptide and sheep angiotensinogen. It is also apparent from Fig. 2, that the pH-profile of rat kidney renin with tetradecapeptide as the substrate (pH-optimum at pH 6.0) is quite different from that of the three other enzymes, which have their pH optima at pH 4.7—5.2. With sheep angiotensinogen as the substrate, a more complex picture is obtained (Fig. 3). The enzymes pseudorenin and rat brain isorenin exhibit 2 pH-optima at pH 3.9 and 5.5, whereas cathepsin D has its pH optimum at pH 3.9 and only a shoulder at pH 5.5. Again rat kidney renin is clearly different with a single pH optimum at pH 6.8.

Product identification

The identity of the product of the enzymatic activity of the 3 'non-renin' enzymes with angiotensin I was ascertained by the following experiments:

Limiting amounts of tetradecapeptide were incubated both at pH 3.9 and pH 5.5 with the 3 enzymes and the time course of formation of immunoreactive angiotensin was followed. After 40 min of incubation any remaining tetradecapeptide was converted to angiotensin I by the addition of a large amount of hog renin. The result of the experiment performed at pH 5.5 is shown in Fig. 4. During 40 min of incubation most of the tetradecapeptide is converted to angiotensin I. By the addition of hog renin, the total amount approaches the theoretical value to be expected from complete conversion of tetradecapeptide to angiotensin I. If any of the three enzymes in this experiment would generate a peptide different from angiotensin I, this peptide must have complete cross-reactivity with the angiotensin I antibody in order to produce the result shown

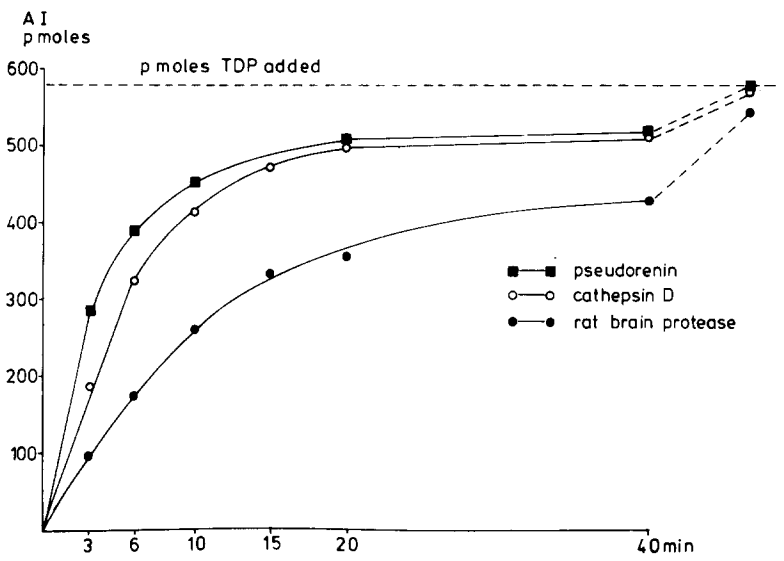

Fig. 4. Identification of the product formed from tetradecapeptide. Tetradecapeptide (TDP) (567 pmol) was incubated at pH 5.5 with pseudorenin, cathepsin D and rat brain isorenin (brain protease). At the intervalls indicated aliquots were withdrawn and analyzed for angiotensin I (AI) content by radioimmunoassay. At 40 min, 20 munits of hog renin (NBC) were added, and 5 min later, the angiotensin I content of the incubate was analyzed again. The data are calculated for the initial incubation volume.

in Fig. 4. This is a very unlikely possibility since the antibody used has a high degree of specificity [1]. Essentially the same result was obtained, when the experiment was performed at pH 3.9 (not shown). Further confirmation of the identity of the product with angiotensin was obtained by estimation of the reaction product with the rat blood pressure assay. The differences between the values obtained by radioimmunoassay and bioassay were less than 10%, i.e. within the limits of the experimental error. Also with sheep angiotensinogen as a substrate at pH 5.5, the amount of angiotensin I estimated by radioimmunoassay was the same as that by bioassay. No angiotensin II or crossreacting peptides could be detected in the incubated samples by a sensitive radioimmunoassay for angiotensin II [11]. It can be concluded from these results that the reaction product formed by cathepsin D, pseudorenin, and rat brain isorenin, is angiotensin I.

Substrate specificity

An interesting observation made during the purification of rat brain isorenin [1] was the very low rate of angiotensin I formation from angiotensinogen as compared to tetradecapeptide. If the possible function of any of the enzymes as an angiotensin-forming enzyme in vivo is considered, the reaction rate with angiotensinogen would be the more important parameter, since the tetradecapeptide is not naturally available to the enzymes in vivo. Therefore a quantitative comparison of angiotensin formation from different substrates was made. From the data of Table II it is evident that the main property common to rat brain isorenin, cathepsin D and pseudorenin is the very low rate of angiotensin formation from different angiotensinogens. Even at pH 5.5 the reaction

TABLE II

SUBSTRATE SPECIFICITY OF ANGIOTENSIN-FORMING ENZYMES

Angiotensin formation is expressed in nmol/h per mg enzyme preparation. Assay conditions are described in Methods.

Preparation	Tetradeca-peptide renin substrate pH 5.5	Origin of angiotensinogen and pH of incubation				
		Sheep pH 5.5	Sheep pH 7.0	Hog pH 5.5	Rat pH 5.5	Rat pH 7.2
Isorenin rat brain	$4.1 \cdot 10^7$	1390	4	1200	93	10
Cathepsin D bovine spleen	$8.4 \cdot 10^6$	161	19	108	31	3
Pseudorenin hog spleen	$1.0 \cdot 10^7$	184	0.2	112	44	1
Renin rat kidney	$4.2 \cdot 10^7$	$8.5 \cdot 10^7$	$1.5 \cdot 10^7$	$1.4 \cdot 10^6$	$1.9 \cdot 10^7$	$3.3 \cdot 10^7$

rates with homologous angiotensinogens are at least 100 000-fold less than those with tetradecapeptide at the same pH. This property of the three 'non-renin' enzymes is in striking contrast to that of kidney renin which has a high rate of angiotensin-formation with all angiotensinogens and with tetradecapeptide.

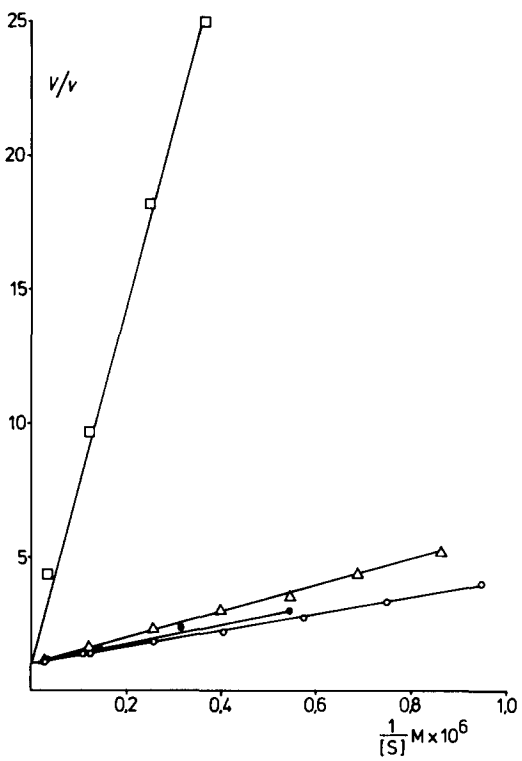

Fig. 5. Estimation of apparent Michaelis constants. The enzymes rat renin (□———□), pseudorenin (△———△), cathepsin D (●———●) and rat brain isorenin (○———○) were incubated with varying concentrations of tetradecapeptide at pH 5.0. Angiotensin I formation was estimated as described in Methods. Data are plotted in a double reciprocal plot. For better comparison the rate of angiotensin formation is given as relative rate ($V = 1$). The absolute values for V were: brain protease 0.256 nmol/15 min cathepsin D 0.625 nmol/15 min, pseudorenin 0.317 nmol/15 min and rat renin 1.67 nmol/15 min. Substrate concentrations are expressed in μM.

Acid protease activity

By using denatured human hemoglobin as a substrate at pH 3.2, rat brain isorenin, cathepsin D and pseudorenin were found to have acid protease activity of 14, 7 and 2.5 units/mg, respectively. No acid protease activity could be measured in the kidney renin preparation.

Substrate affinity

The apparent affinity of tetradecapeptide to the four angiotensin-forming enzymes was derived from double reciprocal plots shown in Fig. 5. For comparability, the rates of angiotensin formation have been normalized by expressing the reaction rate as fractions of V. From this plot apparent K_m values of 3.0, 3.5, 4.7, and 33 μM have been obtained for rat brain isorenin, cathepsin D, pseudorenin and rat kidney renin, respectively. For comparison, an apparent K_m of 1.85 μM has been reported for the reaction of human pseudorenin with tetradecapeptide at pH 4.5 by Skeggs et al. [2].

Inhibition of angiotensin formation by proteins

Cathepsin D can cleave a variety of different peptide bonds in many proteins with a preference for bonds, which constitute a hydrophobic region in the peptide chain (for review see ref. 4). In view of the similarity of pseudorenin and brain isorenin with cathepsin D, especially with regard to their acid protease activity, it may be predicted that proteins, such as albumin or globulins, would act as competitive inhibitors of the hydrolysis of the Leu-Leu bond in the tetradecapeptide by these enzymes. This is indeed the case, as shown in Fig. 6. The inhibition obtained with hog α-globulin and bovine β-globulin (both from Serva, Heidelberg) was even more pronounced than that by albumin. The rate of angiotensin-formation from tetradecapeptide at pH 5.0 by rat brain isorenin, cathepsin D and pseudorenin was inhibited by α-globulin (8 mg/ml) to 85, 69 and 81%, respectively, and by β-globulin (8 mg/ml) to 82, 66 and 79%, respectively. In contrast, no inhibition of renin by any of the proteins was observed. This again documents the extreme specificity of this enzyme.

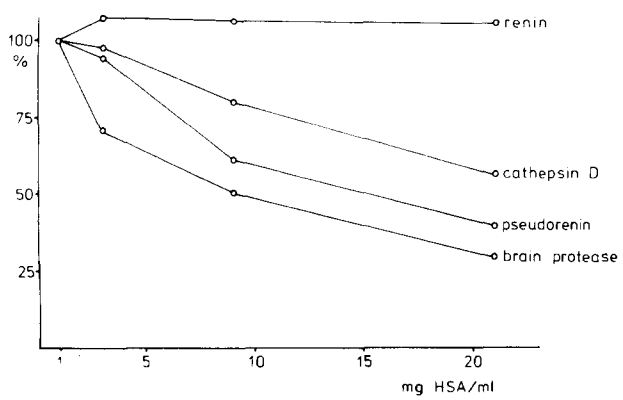

Fig. 6. Inhibition of angiotensin formation from tetradecapeptide by albumin. To standard assay systems at pH 5.0 with tetradecapeptide as the substrate, varying concentrations of human serum albumin (HSA) (Behringwerke, Marburg) were added. The rate of angiotensin formation is expressed in percent of the values obtained in the standard assay system, which contains 1 mg/ml human serum albumin.

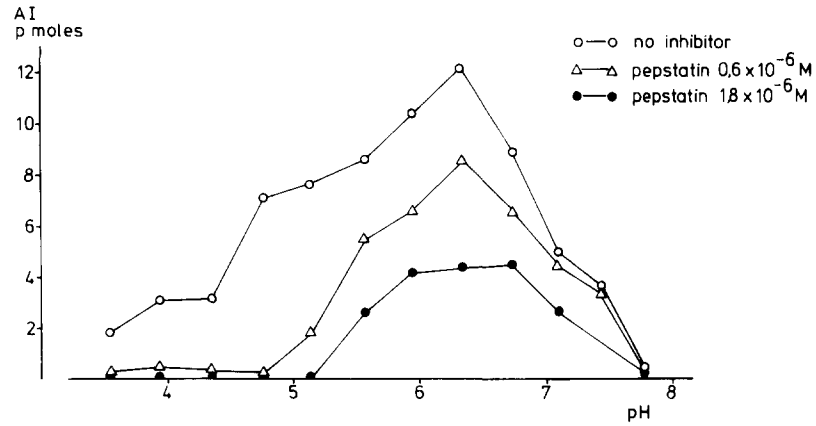

Fig. 7. pH-dependence of inhibition of pepstatin of rat kidney renin. The pH gradient was obtained as described in the legend to Fig. 3. Tetradecapeptide was used as the substrate. Pepstatin was added at the concentrations given in the figure. AI, angiotensin I.

Inhibition by pepstatin

It has been described previously [1] that the inhibitory effect of pepstatin on rat brain isorenin (brain protease) decreases with increasing pH. This phenomenon is also observed with rat renin, pseudorenin and cathepsin D. Two examples are shown in Figs. 7 and 8. Interestingly, much higher concentrations of pepstatin are required to inhibit renin (Fig. 7) than for a comparable inhibition of pseudorenin (Fig. 8), cathepsin D (not shown), or rat brain isorenin (shown previously [1]). The relative insensitivity of renin to inhibition by pepstatin as compared to the 3 'non-renin' enzymes brain isorenin, pseudorenin and cathepsin D is even more evident, when the inhibition by pepstatin is examined with varying amounts of enzyme. As shown in Fig. 9A, B and C, the apparent concentrations of pepstatin required for a given degree of inhibition decrease with decreasing enzyme concentration. This phenomenon is probably due to a high affinity of the inhibitor to the enzyme, i.e. the concentrations

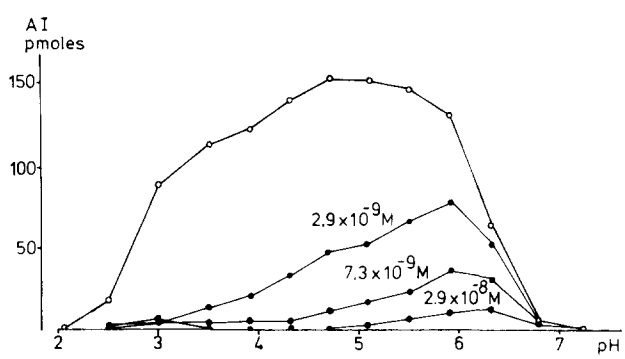

Fig. 8. pH-dependence of inhibition by pepstatin of hog spleen pseudorenin. The pH gradient was obtained as described in the legend to Fig. 3. Tetradecapeptide was used as the substrate. Pepstatin was added at the concentrations given in the figure. AI-angiotensin I.

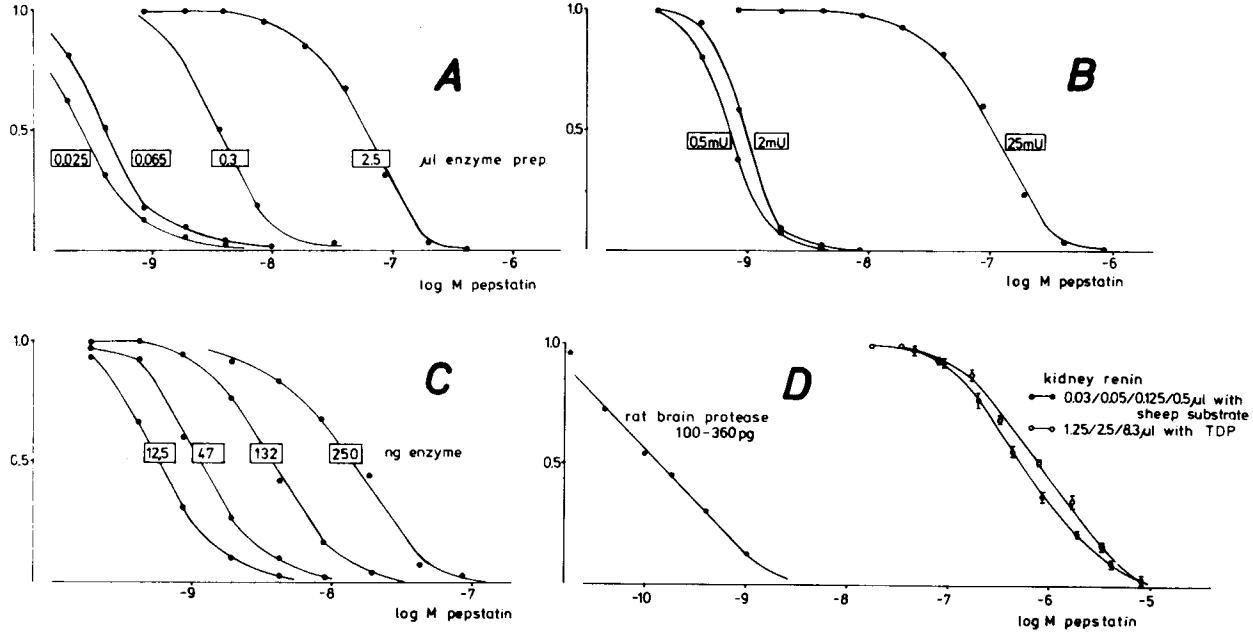

Fig. 9. Influence of enzyme dilution on the inhibitory effect of pepstatin on angiotensin formation. In standard assay system with tetradecapeptide as the substrate, dose vs. inhibition curves with pepstatin were established for different enzyme concentrations. The data were calculated as fractional activity of the uninhibited reaction. Pepstatin additions given as the negative logarithm of the total concentration. A: Hog spleen pseudorenin. 1 µl of enzyme preparation contained 1.6 µg of protein. B: Bovine spleen cathepsin D: Specific activity 10 units/mg protein. C: Rat brain isorenin. D: Rat kidney renin. 1 µl of enzyme preparation contained 0.04 µg of enzyme protein. With sheep angiotensinogen (•) renin was varied from 0.03 to 0.5 µl, with tetradecapeptide (TDP) (○) from 1.25 to 8.3 µl per incubate. Values are the means ±S.D. For comparison, the data obtained with the lowest concentrations of rat brain isorenin (100 pg) are included.

given in these figures do not represent the concentrations of free inhibitor in solution, because most of the pepstatin is bound to the enzyme. With less enzyme present, the same absolute amount of pepstatin can inhibit a larger percentage of enzyme. This "mutual depletion system", as described by Webb [12] does not permit the estimation K_i. However, from the dose vs. inhibition curves of the lowest enzyme concentrations, it can be derived that the K_i of pepstatin for rat brain isorenin, pseudorenin and cathepsin D is below $5 \cdot 10^{-10}$ M. This conclusion is justified not only for a non-competitive type of inhibition, but also for competitive inhibition, since substrate concentrations were well above K_m. A direct evaluation of the type of inhibition and the estimation of K_i of pepstatin is very difficult to accomplish, because this would require enzyme concentrations well below the K_i of pepstatin, in order to establish concentrations of free inhibitor appropriate for the kinetic analysis. The same problem was encountered by others when the inhibition of pepsin by pepstatin was studied [13]. In striking contrast to brain isorenin, pseudorenin and cathepsin D, the inhibition of renin by pepstatin is independent of the amount of enzyme (Fig. 9D) regardless of the substrate used. From Fig. 9D a K_i of pepstatin for renin can be derived of approx. $6 \cdot 10^{-7}$ M. For comparison, the lowest enzyme dilution of rat brain isorenin studied, is included in this figure. It is apparent that the sensitivity of this enzyme to pepstatin is at least 1000-fold higher than that of renin.

Discussion

The enzyme brain isorenin has been considered as an enzyme which is synthetized in the brain and which participates in the central regulation of blood pressure by local i.e. intracerebral formation of angiotensin I [14,15]. The enzymatic activity studied in crude preparations from dog and rat brain exhibited significant biochemical differences from renin with respect to pH-optima and utilization of angiotensinogens from different species [16—18]. Nevertheless, these enzymes have been considered as closely related to renin [14].

In the present study a purified preparation of rat brain isorenin exhibited properties which are quite dissimilar from those of renin, but are very similar to those of bovine cathepsin D. The similarity to cathepsin D is evident from the substrate specificity, the inhibition of angiotensin formation from tetradecapeptide by proteins, the acid protease activity, the high sensitivity to inhibition by pepstatin, and the pH-dependence of angiotensin formation. The bimodal pH characteristics of the rat brain enzyme and pseudorenin are in accordance with the reported properties of several species of cathepsin D, which show a distinct shoulder or a second pH-optimum near pH 5.0 with hemoglobin as a substrate [4,13,19,20]. In view of these similarities there remains little doubt that rat brain isorenin is identical with cathepsin D and that it is not an isoenzyme of renin, as implicated by the designation "isorenin". The same suggestion has been made by Day and Reid [21] concerning the relationship of dog brain isorenin to cathepsin D. The conclusion that the rat brain enzyme is not an isoenzyme of renin, appears to be at variance with the observation that antibodies elecited against hog kidney renin inhibit the angiotensin formation

by dog brain isorenin [18]. It has been suggested by Tang [22] that the active sites of acid proteases, such as pepsin, cathepsin D or renin are strongly homologous in their amino acid composition and structure. A certain degree of cross-reactivity of the antibody may therefore exist. Another, more likely explanation, however, for the observed inhibition by the antiserum, is a lack of specificity. It is a common observation that semipurified preparations of renin, such as has been used as antigen for the immunization [18], do contain acid protease activity as contaminant (refs. 3 and 23 and Hackenthal, E., Hackenthal, R., unpublished). As a consequence, the antiserum raised against such a preparation would contain antibodies against both renin and cathepsin D. The inhibition of dog brain 'isorenin' would thus represent inhibition of cathepsin D by specific cathepsin D antibodies. The proposed identity of rat brain isorenin with cathepsin D raises the question, whether this observation can be extended to other extrarenal 'isorenins'. There is no doubt that cathepsin D can generate angiotensin both from tetradecapeptide and angiotensinogens, a property which has hitherho been considered a specific function of renin. It is therefore to be expected, that several extrarenal 'isorenins' will eventually be identified as cathepsin D, in particular, if their previous identifications as 'isorenin' were based on assay conditions, which are favorable for the assay of cathepsin D, i.e. low pH, synthetic substrates or heterologous angiotensinogens. The identification of rat brain isorenin as a cathepsin D does not exclude the possibility that one of the biological functions of this enzyme in the brain may be the local formation of angiotensin I. However, there are several arguments in favor of the opposite view: Cathepsin D is an ubiquitous intracellular lysosomal enzyme, that is considered responsible for intracellular protein degradation [4, 24,25]. Its general proteolytic activity, and as a consequence, the competitive inhibition of angiotensin formation by proteins, would make this enzyme an unsuitable candidate for the selective and controlled formation of this peptide with a high biological activity. Also, enzymatic activity of the enzyme is very low at pH values above pH 6.0 with homologous angiotensinogen, an observation also made by Day and Reid [21]. The 'unsuitability' of rat brain cathepsin D as a specific angiotensin-forming enzyme as compared to renin is further illustrated by the observation that renin can generate angiotensin I from rat angiotensinogen at pH 5.5 at least 200 000 times faster, and, at pH 7.2, 3000 000 times faster per weight unit, than does the rat brain protease under the same conditions (Table III).

Two reports in the literature appear to be at variance with results of the present study. The high sensitivity to inhibition by pepstatin of human renin (K_i = $1.3 \cdot 10^{-10}$ M) observed by McKnown et al. [23] is in contrast to the low sensitivity of rat renin ($K_i = 6 \cdot 10^{-7}$ M), found in the present study, The same authors observed a strong inhibitory effect of the competitive type of various proteins on human renin [26], whereas in the present study no such inhibition of rat renin was seen. These apparent discrepancies are easily explained by the fact, that, as stated by these authors, their preparation of human renin was contaminated with pseudorenin. This enzyme contributed 80% to the total enzymatic activity measured with the synthetic polymeric substrate assay at pH 5.5 [26]. Consequently, it was the inhibition of pseudorenin by pepstatin and proteins and not that of renin, which has been analyzed in these studies. Since

pseudorenin has been identified as cathepsin D in the present study, the results reported by Gregerman and colleagues [23,26] are pertinent to cathepsin D and are thus in accordance with the results obtained in our study. Furthermore, other investigators also observed a low sensitivity of renin to inhibition by pepstatin (K_i approx. $5 \cdot 10^{-7}$ M for hog renin [27,28]). In agreement with our data, a significantly higher affinity ($K_D = 5 \cdot 10^{-10}$ M) for human liver cathepsin D at pH values below 5.0 has been reported [25]. Interestingly, a decline of affinity with increasing pH values has also been found with hog renin [27].

Another conclusion to be drawn from the results of this study is that pseudorenin from hog spleen is identical with or closely related to cathepsin D. This conclusion is based on the same arguments, as those listed for the identity of rat brain isorenin with cathepsin D. There was no significant difference in the pH dependence of angiotensin formation, in substrate specificity, inhibition by protein, sensitivity to inhibition by pepstatin, and acid protease activity.

Although only pseudorenin from hog spleen has been studied here, it appears reasonable to extend this conclusion to the pseudorenin found in human kidney and plasma, and various organs of the rat, since the properties of the enzyme described by Skeggs et al. [2] are essentially the same as those found with the hog spleen enzyme in this study. In view of these findings it is to be expected that at least in some tissues the three enzymatic activities described under the designations pseudorenin, isorenin and cathepsin D by different investigators will turn out to be one enzyme.

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