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PURIFICATION AND PARTIAL CHARACTERIZATION OF RAT BRAIN ACID PROTEINASE (ISORENIN)

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Summary

1. Isorenin was purified 2000-fold from rat brain by a simple 3-step procedure involving affinity chromatography on pepstatinyl-Sepharose. The preparation appears as a homogeneous protein in analytical polyacrylamide gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis indicated an apparent molecular weight of 45 000. Isoelectric focusing separated isoenzymes with isoelectric points at pH 5.45, 5.87, 6.16 and 7.05.

2. The enzyme generates angiotensin I from tetradecapeptide (pH optimum 4.7) and from sheep angiotensinogen (pH optima 3.9 and 5.5). The rate of angiotensin I formation from tetradecapeptide was 30 000 times higher than that from sheep angiotensinogen. The enzyme has acid protease activity at pH 3.2 with hemoglobin as the substrate and pepstatin is a potent inhibitor of the enzyme with a K_i of less than 10^{-9} M.

3. The properties of the enzyme strongly suggest that it is identical with cathepsin D.

Introduction

The enzyme renin (EC 3.4.99.19) is synthesized and stored in the juxtaglomerular cells of the kidney [1—4]. Renin exerts its function by a highly specific endoprotease activity, splitting a leucyl-leucyl bond in its natural protein substrate, angiotensinogen, to yield the decapeptide angiotensin I, which is subsequently converted to the main effector of the system, the octapeptide angiotensin II, by a specific converting enzyme.

During the past 15 years, several enzymatic activities in extrarenal tissues have been described, which share with renin the capacity to generate angioten-

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sin I from appropriate substrates under suitable conditions (for review see refs. 5 and 6). This capacity to generate angiotensin has been considered as the specific physiological function of such enzymes. Based on this assumption, it has recently been postulated that the locally formed 'renin-like' enzymes or 'isorenins' participate in local regulatory systems, independent from the plasma renin-angiotensen system [5,6].

However, with the exception of the mouse submaxillary gland isorenin, none of these enzymes has been purified and characterized with respect to their relationship to renin.

In this respect, the brain isorenin system is of particular interest, since it has repeatedly been implicated in the central regulation of systemic blood pressure and possibly other physiological functions [5—8]. Recently, its putative function as an angiotensin-forming enzyme has been questioned and a possible relationship to cathepsin D suggested [9]. In this paper, the purification of the enzyme from rat brain by affinity chromatography on pepstatinyl-Sepharose and some of its properties are described. A short communication of some of the data in this paper has been published [10].

Methods

Preparation of pepstatinyl-Sepharose

The preparation of pepstatinyl-Sepharose for affinity chromatography was based on the procedure described by Murakami and Inagami [11] with the introduction of several minor modifications. Pepstatin A, 73 μmol (Protein Research Foundation, Osaka, Japan) was dissolved in 4 ml redistilled dimethylformamide and added to 150 μmol *N*-hydroxysuccinimide in 2 ml of the same solvent. To this mixture, 150 μmol dicyclohexylcarbodiimide in 2 ml dimethylformamide were added and left for 18 h at 4°C in a desiccator.

The active hydroxysuccinimide ester was added to 9 g of wet aminohexyl-agarose (AH-Sepharose, Pharmacia, Sweden), swollen previously in 0.5 M NaCl, washed with dioxane, and suspended in a total volume of 15 ml of dioxane. The mixture was stirred at room temperature for 20 h. The gel-preparation was washed exhaustively with dimethylformamide/dioxane (1 : 2, v/v) and subsequently with 0.5 M NaCl. Since this gel still contained unreacted aminohexyl groups which interfered with subsequent experiments, these groups were reacted with 5 mM CH_3COONa (final concentration) by the addition of 500 mg ethyl dimethylaminopropyl carbodiimide (Sigma) at pH 5.6 for 20 h. The gel was washed with 0.5 M NaCl and stored as a suspension in 1.0 M NaCl at 4°C until used.

Purification of rat brain isorenin

Whole brain tissue, from male Wistar rats (250—300 g) (Ivanovas, Germany) was obtained after decapitation and kept frozen at −25°C until used. In a typical purification experiment (Table I) the tissue was thawed and homogenized by a knife-blade homogenizer (Ultraturrax, Jahnke and Kunkel, Germany) two times for 15 s with 4 vols. of 50 mM sodium phosphate buffer (pH 7.0), containing 5 mM EDTA and 0.5 mM diisopropylfluorophosphate. The homogenate was subjected to ultrasonic treatment (MSE, 450 W), for 15 s, allowed to cool

for 1 min, then a further 15 s. The homogenate was then centrifuged as 30 000 $\times g$ for 15 min. The supernatant was decanted and the pellet reextracted twice with 3 and 2 vols. of the homogenization buffer. To the combined supernatants, acetone (precooled to −20°C) was slowly added with continuous stirring to give an acetone concentration of 20% (v/v). The precipitate formed was removed by centrifugation and discarded; to the supernatant cold acetone was slowly added with stirring, until the acetone concentration reached 70% (v/v). This precipitate was also collected by centrifugation, washed with cold acetone and dried in vacuo. The dried material was suspended in distilled water (3 ml/g tissue), homogenized, and the pH adjusted to pH 5.5 with 0.1 M H_3PO_4. This homogenate was centrifuged at 30 000 $\times g$ for 15 min, the supernatant was then decanted, and the pellet reextracted with 0.1 M phosphate buffer (pH 5.5, 2 ml/g tissue). To the combined supernatants, NaCl was added to a final concentration of 0.2 M. This clear yellow solution was fractionated by affinity chromatography on pepstatinyl-Sepharose. A small column was packed with 4 ml of the pepstatinyl-Sepharose and equilibrated with 0.1 M sodium phosphate buffer (pH 6.0) containing 0.2 M NaCl. Following application of the sample, the column was washed with 25 ml of the starting buffer and eluted with a linear gradient of 0—3 M urea in 50 mM sodium phosphate buffer (pH 7.6) (flow rate 0.6 ml/min, 8-ml fractions). The eluted fractions were dialyzed individually against 10 mM phosphate buffer (pH 5.6). The fractions were analyzed for enzymatic activity and for protein distribution by polyacrylamide gel electrophoresis. Usually, three consecutive fractions, showing as single protein band and containing the highest enzymatic activity, were pooled, lyophilized and dissolved in one tenth of the initial volume. These were then dispensed in small volumes and stored frozen at −70°C until used. For most of the subsequent analytical work, (except protein estimations, polyacrylamide gel and SDS gel electrophoresis) the enzyme preparation was diluted with buffer containing 1 mg/ml human serum albumin, as stabilizer.

With this procedure, 3 separate purification experiments have been performed, starting with 22, 20 and 150 g of whole rat brain and no significant differences were observed between the batches.

Estimation of angiotensin I formation from tetradecapeptide

In this assay, the tetradecapeptide renin substrate, which represents the amino-terminal part of the natural protein substrate angiotensinogen [12], was used as a substrate for the enzyme. The formation of the decapeptide angiotensin I from this substrate was measured by radioimmunoassay. Since the substrate is not removed prior to the radioimmunoassay, this assay requires an antiserum with a high specificity for angiotensin I. The antibody used in this study had a cross-reactivity with the tetradecapeptide of less than 0.1%, as compared to angiotensin I (Hackenthal, E. unpublished). The incubation mixture contained 50 μl of enzyme in incubation buffer and 5 nmol of tetradecapeptide renin substrate (Bachem, Switzerland) in a total volume of 250 μl (0.1 M sodium citrate buffer (pH 5.0), 0.1 M NaCl, 5 mM EDTA, 1 mg/ml human serum albumin (Behring, Marburg, Germany). An aliquot of 50 μl of the incubation mixture served as a control. Following incubation for 15 min at 37°C, another 50 μl were withdrawn, added to 450 μl of 0.1 M Tris/acetate (pH 7.4)

containing 0.02% neomycin sulfate and 1 mg/ml human serum albumin. This aliquot was then heated to 100°C for 10 min and centrifuged at 10 000 × *g* for 5 min. The angiotensin I content in the supernatant was estimated by radioimmunoassay, as described elsewhere [13].

Estimation of angiotensen I formation from sheep angiotensinogen

The incubation mixture contained 25 μl of the enzyme solution, appropriately diluted with the incubation buffer, 0.71 mg of a sheep angiotensinogen preparation obtained from 48 h nephrectomized sheep by $(NH_4)_2SO_4$ fractionation (specific concentration 95 ng angiotensin I/mg protein), 2 mM phenylmethansulfonylfluoride, all in a total incubation volume of 50 μl of incubation buffer (0.2 M sodium phosphate buffer (pH 5.5) containing 5 mM EDTA and 1 mg/ml human serum albumin). Following incubation for 1 h at 37°C, 200 μl Tris/acetate buffer (pH 7.4) containing 1 mg/ml human serum albumin was added and the reaction stopped by immersion in a boiling water bath for 10 min. The tubes were cooled, centrifuged at 10 000 × *g* for 5 min and the angiotensin I content estimated by radioimmunoassay.

Estimation of acid protease activity

Acid protease activity was measured at pH 3.2 with denatured human hemoglobin as the substrate. The method described by Barrett [14] was used with the following modifications: The sample of 50 μl was incubated with 100 μl of 1.0 M sodium formate buffer (pH 3.2) and 50 μl of denatured hemoglobin (40 mg/ml distilled water). The incubation was stopped after 10 min by the addition of 150 μl of 6% trichloroacetic acid, the protein precipitate was removed by centrifugation and the concentration of acid soluble peptides in the supernatant estimated by the method of Lowry et al. [15].

In this procedure 10 munits of a commercial preparation of cathepsin D (bovine spleen, Sigma Chem.) produced an $E^{1\,cm}_{750\,nm}$ of 0.070. This preparation was used as a reference for the expression of data in units. For comparison, 1 unit as defined by Barrett (measuring the increase in absorbance at 280 nm) is equivalent to 48 units in our system. Denatured hemoglobin was prepared by titration of a solution of human hemoglobin (Serva, Germany) 60 mg/ml in distilled water, to pH 1.8 with dilute H_3PO_4. The solution was kept at this pH for 1 h at room temperature and centrifuged at 10 000 × *g* for 15 min. The supernatant was dialyzed against distilled water.

Protein determination

Protein was estimated by the method of Lowry et al. [15], except for the estimation of very low protein contents, where the more sensitive method by Bramhall et al. [16] was used.

Column isoelectric focusing

The LKB-system 8160-20 was used, according to the instructions of the manufacturer. After focusing in a pH gradient from 3.5 to 8 (41 h at 2°C, 300—500 V, cathode at upper end of column), the column was eluted at a flow rate of 0.7 ml/min. Fractions of 1.3—1.5 ml were collected and the pH measured at 2°C. The enzymatic activity in the eluted fractions was estimated with tetradecapeptide as substrate (see above).

Gel electrophoresis and molecular weight estimation

Polyacrylamide gel electrophoresis was performed as described by Davis [17] with modifications described by Reisfeld and Small [18] at pH 9.3. The molecular weight was estimated according to the method of Weber and Osborn [19]. Following a 2 h incubation in 1% sodium dodecylsulfate and 1% 2-mercaptoethanol at 37°C, the samples were subjected to electrophoresis in 10% polyacrylamide gels, which were then stained with Coomassie Blue R 250. Bovine serum albumin (M_r = 67 000), ovalbumin (M_r = 45 000), chymotrypsinogen (M_r = 25 000), and myoglobin (M_r = 17 000) were used to prepare a 'standard' curve. All biochemicals used were of reagent grade and were obtained from Serva Heidelberg.

Results

Purification of rat brain isorenin

The result of an affinity chromatography experiment with pepstatinyl-Sepharose is shown in Fig. 1. The enzyme is almost completely retained, while the bulk of protein from a crude preparation is not retarded on the column. The enzyme was then eluted with a urea gradient (0—3 M, in 50 mM sodium phosphate (pH 7.6)). The elution of the enzyme was completed at a urea concentration in the eluate of about 1.2 M. Interestingly, the three enzymatic activities (angiotensin formation from tetradecapeptide and from sheep substrate and acidic protease activity) elute in a parallel fashion. This indicates the possibility that the three enzymatic activities belong to one enzyme. A purification schedule of brain isorenin is summarized in Table I. It can be seen that the overall purification is about 2000-fold. Furthermore, it is evident that the

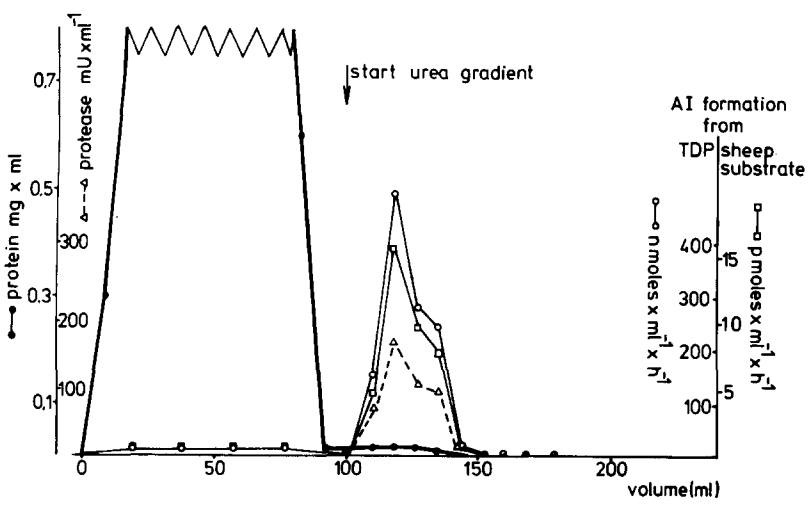

Fig. 1. Affinity chromatography of rat brain isorenin on pepstatinyl-Sepharose. The pH 5.5 extract from the acetone precipitation was adjusted to 0.2 M NaCl and chromatographed on a column with 4 ml of pepstatinyl-Sepharose. Fractions of 8 ml were analyzed for protein (●———●), angiotensin I-formation from tetradecapeptide (TDP) (○———○), and from sheep angiotensinogen (□———□) and acid protease activity (△———△). Elution of the enzyme from the column was started as indicated by the arrow with a gradient from 0—3 M urea at pH 7.6. See Methods for further details.

TABLE I

PURIFICATION OF RAT BRAIN ISORENIN

See Methods for details. A: angiotensin I - formation from tetradecapeptide-substrate, pH 5.0; B: angiotensin I - formation from sheep angiotensinogen, pH 5.5; C: acid protease activity with denaturated hemoglobin as substrate, pH 3.2; AI; angiotensin; TDP: tetradecapeptide.

Step	Total protein (mg)	Total activity			Specific activity			Recovery (%)		
		A (TDP assay) (nmol AI · h^{-1})	B (Sheep substrate) (pmol AI · h^{-1})	C (Acid protease) (munits)	A (nmol/mg)	B (pmol/mg)	C (munits/mg)	A	B	C
Homogenate	1910	34 130	1316	13 040	17.9	0.69	6.8	100	100	100
Crude extract	1228	26 490	947	10 080	21.6	0.77	8.2	77	72	77
Acetone precipitate	382	16 370	658	5 576	42.8	1.72	14.6	48	50	43
Extraction pH 5.5	129	13 550	395	3 782	105.	3.1	29.3	40	30	29
Pepstatin affinity chromatography	0.23	9 484	303	3 187	41 230	1 317	13 858	28	23	24
Purification factor					2 302	1 909	2 038			

three enzymatic parameters measured are purified in parallel. This again suggests that these three enzymatic activities are properties of the same enzyme. Another interesting aspect is the observation that the ratio of angiotensin I formation from the tetradecapeptide substrate to that from sheep angiotensinogen is about 30 000 to 1 in all fractions.

The final preparation was analyzed by analytical polyacrylamide gel electrophoresis and a single band was observed, indicating a homogenous preparation. The identification of this stained band with the enzyme was accomplished by slicing a gel which had been developed under exactly the same conditions. The location of the enzymatic activity, which had been eluted from another gel (sliced into 3-mm pieces), coincided with that of the stained protein. SDS gel electrophoresis confirmed the homogeneity of the enzyme preparation and, by comparison with proteins of known molecular weight, indicated a molecular weight of 45 000 ± 2500 for the rat brain enzyme (Fig. 2).

Isoelectric focusing

The pattern shown in Fig. 3 was obtained by preparative column isoelectric focusing of the purified enzyme preparation. The preparation is heterogeneous with respect to isoelectric points. The main fractions (isoelectric points 5.45, 5.87, 6.16 and 7.05), probably represent isoenzymes of the same enzyme. In order to support this conclusion, the peak fraction of the isoelectric focusing experiment were pooled as indicated by bars 1—6 in Fig. 3, dialyzed free of the ampholyte, concentrated by partial lyophilization and analyzed again. All 6 fractions from the isoelectric focusing experiment have acidic protease activity and generate angiotensin I from both the tetradecapeptide and angioten-

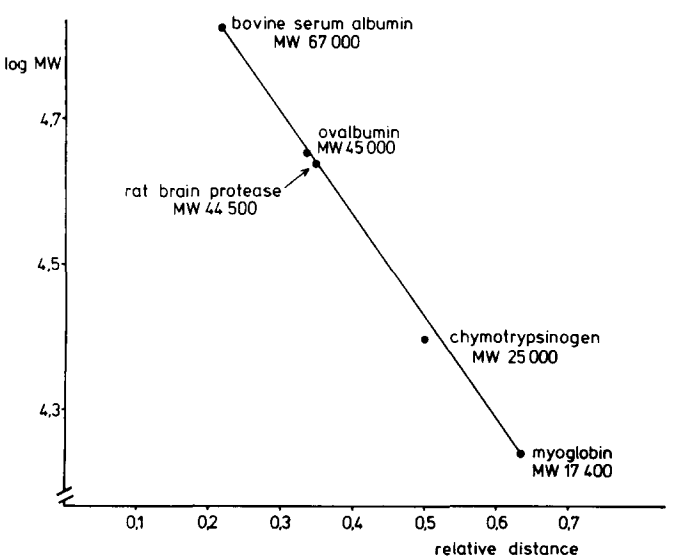

Fig. 2. Estimation of apparent molecular weight by SDS polyacrylamide gel electrophoresis. A sample of rat brain isorenin (3 μg) and reference proteins of known molecular weight were incubated with 1% sodium dodecyl sulfate and 1% mercaptoethanol for 2 h at 37°C and subjected to SDS gel electrophoresis. The relative mobilities of the proteins are plotted against log molecular weight.

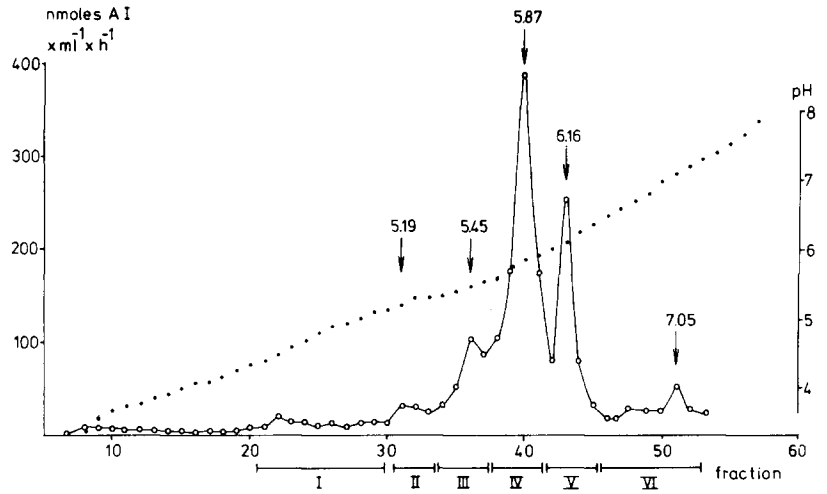

Fig. 3. Column isoelectric focusing of purified rat brain isorenin. The LKB system 8160-20 was used with a pH gradient of 3.5—8 in a sucrose density gradient (41 h, final voltage 500 V, cathode at upper end, 2°C). Fractions of approximately 1.4 ml were eluted at a flow rate of 0.7 ml/min. The pH was measured at 2°C. Aliquots of the fractions were diluted 200-fold and the rate of angiotensin (AI) formation from tetradecapeptide was measured, as described in Methods.

sinogen in about the same proportions. This result confirms the isoenzyme relationship of these fractions. Essentially the same pattern of isoelectric focusing was obtained with a crude extract. This indicated that heterogeneity of isoelectric points in the pure preparation is not an artifact produced during purification.

Product identification

The enzymatic activity of brain isorenin is measured in this study as the rate of angiotensin I formation from tetradecapeptide or protein substrates, assuming that the enzymatic product measured in the radioimmunoassay is indeed angiotensin I. Since the crossreactions of the antibody used with peptides related to angiotensin are very low (e.g. 0.1% with angiotensin II, 0.06% with tetradecapeptide, 0.05% with the (2—8)-heptapeptide (Hackenthal, E. unpublished)), this appears to be a reasonable assumption, as long as renin is measured. However, since the properties of the brain isorenin, as described here, indicate that it is different from renin in many aspects, additional confirmation of the identity of the product with angiotensin I was desirable. For a direct chemical identification sufficient amounts of product have not been obtained. However, immunological data support the identification as angiotensin I: the estimation of the reaction product with two different angiotensin I-antisera gave identical results. Serial dilutions of the reaction product are superimposable with the angiotensin I standard curve of the radioimmunoassay. No immunoreactivity was seen in a radioimmunoassay for angiotensin II.

The experiment described in Fig. 4 also indicates the identity of the reaction product with angiotensin I, since the theoretical amount of angiotensin I was recovered from incubations of a limited amount of tetradecapeptide with an excess of the enzyme both at pH 3.9 and 5.5. Furthermore, the amount of

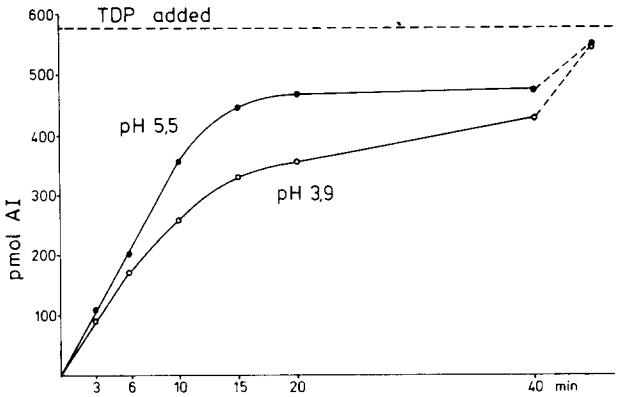

Fig. 4. Product identification. Rat brain isorenin was incubated with 572 pmol of tetradecapeptide (TDP) both at pH 3.9 and 5.5. Aliquots were withdrawn at intervals and analyzed for angiotensin I content by radioimmunoassay. At 40 min hog renin (20 munits) was added, and 5 min later the angiotensin I (AI) content of the incubate was analyzed again. The data are calculated for the initial incubation volume.

angiotensin I found by radioimmunoassay was confirmed by a rat blood pressure bioassay [20]. This agreement between radioimmunoassay and bioassay was also observed, when sheep angiotensinogen was used as the substrate at pH 5.5.

Reaction velocity and pH

As shown in Fig. 5, the pH optimum for the reaction with the tetradecapeptide is at pH 4.7 with a single sharp peak. In contrast, the reaction with sheep angiotensinogen exhibits two maxima at pH 3.9 and pH 5.5. This double peak was found with different batches of the purified enzyme.

Inhibition by pepstatin

The pentapeptide pepstatin, which is a potent inhibitor of cathepsin D [21]

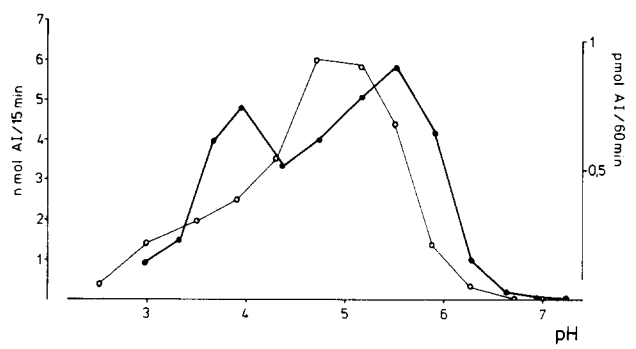

Fig. 5. pH dependence of angiotensin (AI) formation. Rat brain isorenin was incubated with tetradecapeptide (○) or sheep angiotensinogen (●) at varying pH values. The pH gradient was obtained by titrating a mixture of equal volumes of formic, acetic, and phosphoric acids (0.5 M each) with 5 M NaOH to the desired pH. The actual pH of each incubate was confirmed after incubation. The rate of angiotensin formation from tetradecapeptide is given in nmol/15 min (left ordinate) and from sheep angiotensinogen in pmol/60 min (right ordinate). See Methods for further details.

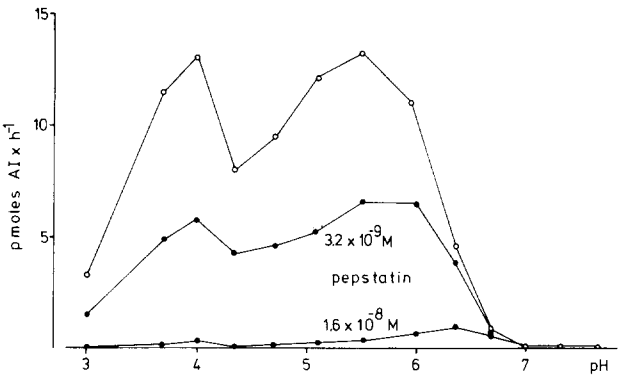

Fig. 6. pH dependence of pepstatin inhibition. Rat brain isorenin was incubated with tetradecapeptide at varying pH values in the absence or presence of pepstatin ($3.2 \cdot 10^{-9}$ M and $1.6 \cdot 10^{-8}$ M). The pH gradient was obtained as described in the legend to Fig. 5. AI, angiotenin.

and pepsin [22], is also an inhibitor of the rat brain enzyme. The pH-dependence of inhibition is shown in Fig. 6. With sheep angiotensinogen as substrate, the enzyme is inhibited to about 50% by pepstatin at $3.2 \cdot 10^{-9}$ M. Above pH 5.5 the inhibitory effect of pepstatin becomes less. This apparent decrease in affinity of the inhibitor to the enzyme with increasing pH, which has also been observed with hog renin [23] and cathepsin D [24], was used to advantage in the purification of the enzyme, by eluting the enzyme from the affinity column at pH 7.6 (see Fig. 1).

The degree of inhibition produced by a given amount of pepstatin proved to be a function of the amount of enzyme protein present, as illustrated in Fig. 7. The most likely explanation for this phenomenon is a very high affinity of the inhibitor to the enzyme and the curves of Fig. 7 actually represent 'titration' curves of different amounts of the enzyme with pepstatin. This situation has been described by Webb [25] as a mutal depletion system, and permits the estimation of the molarity of an enzyme in solution [25]. By plotting the

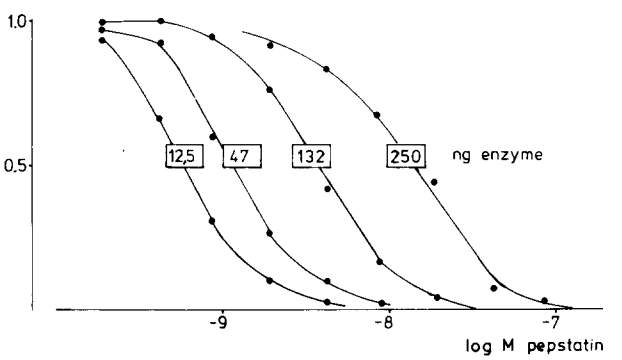

Fig. 7. Effect of enzyme dilution on the inhibition by pepstatin. The standard assay system with tetradecapeptide as substrate was used as described in Methods. The rate of angiotensin formation by the different enzyme dilutions is expressed as the fractional rate with respect to the uninhibited reaction (ordinate). The amount of enzyme in each experiment is indicated in the figure. Total pepstatin concentrations in the incubates are plotted as negative logarithms.

amount of pepstatin required for 50% inhibition against enzyme concentration, it was calculated that 14 nmol of pepstatin are bound to 1 mg of enzyme preparation. This is close to the calculated value of 22.2 nmol per mg protein assuming a molecular weight of 45 000, a pure enzyme preparation and a stochiometric 1 : 1 reaction. A similar approach has been used by McKown and colleagues [26] for the 'titration' of an impure human renin preparation. Also, Knight and Barrett [24] have shown that pepstatin is a good titrant for the determination of the molarity of solutions of cathepsin D. From the present data, the type of inhibition of rat brain isorenin cannot be identified. It may be assumed that the inhibition is reversible, since reversible inhibition has been demonstrated for other acid proteases such as pepsin [22], renin [23] and cathepsin D [24]. The inhibitory constant K_i of pepstatin for the rat brain isorenin at pH 5.5 is probably lower than $5 \cdot 10^{-10}$ M, which is the lowest concentration used in the experiment described in Fig. 7.

Discussion

The term "isorenin" has been introduced by Werle and coworkers [27] for a renin-like enzyme found in the submaxillary glands of white mice, to suggest an isoenzyme relationship of this enzyme with the classical enzyme renin for the kidney. This close relationship could be confirmed by the recent pioneering work of Cohen, Inagami, Taylor, Murakami and Michelakis [28,29], who purified the enzyme to homogeneity and found it indistinguishable in its biochemical properties from renin. In addition to the submaxillary gland of mice, "renin-like", "renin-related" or angiotensin-forming enzymes have been detected in most tissues of many mammalian species examined (for review see refs. 5 and 30).

Unfortunately, the term "isorenin" has been extended to all of these enzymes of extrarenal occurrence [5,6], although none of these enzymes has been characterized sufficiently to permit such a classification. Actually, the few available biochemical data of some of the enzymes indicate significant differences from renin.

This is also true for the 'isorenin' found in the brain of dogs and rats [7,31–34]. For example, the rat brain enzyme had its maximal activity with protein substrates at pH 5.5 [34], whereas rat kidney renin has its optimum at pH 6.7 (Hackenthal, E., Hackenthal, R., unpublished). The enzyme in rat brain extracts was found to react with heterologous angiotensinogens (dog and sheep) at a much higher rate than with homologous angiotensinogen, whereas plasma and kidney renin exhibited the opposite behaviour [34]. Actually, these reported properties have led us to use the assay system with sheep angiotensinogen at pH 5.5 as a guide in the purification of the enzyme. The purification, which was undertaken to clarify the relationship of this enzyme to renin, consisted of a simple 3 step procedure, including fractionated acetone precipitation of the crude extract, extraction of the acetone precipitate at low pH, and affinity chromatography on pepstatinyl-Sepharose columns. This latter step has recently been used by Murakami and Inagami [11] in the complete purification of renin from hog kidney. Other purification steps such as anion exchange chromatography and gel filtration have been used in preliminary experiments,

but have been omitted from the final procedure, because they did not improve the recovery or facilitate the purification of the enzyme.

The homogeneity of the purified enzyme is indicated by the presence of a single protein band in polyacrylamide gel electrophoresis and also in SDS gel electrophoresis. By the latter procedure an apparent molecular weight of 45 000 was obtained, and by isoelectric focusing at least 4 isoenzymes were separated.

Three enzymatic parameters of the enzyme have been measured: angiotensin I formation from tetradecapeptide renin substrate and from sheep angiotensinogen (ratio 30 000 : 1), and acid protease activity at pH 3.2 with hemoglobin as substrate. These three enzymatic activities have been purified in parallel from the crude homogenate to the homogenous enzymes preparation, and appear also in the same proportions in the fractions separated by isoelectric focusing. It can be concluded from these data that the 3 enzymatic activities belong to one enzyme, and that no significant amounts of any enzyme exhibiting only one or two of these enzymatic activities could have been lost during the purification. This latter conclusion also implies that no significant amounts of renin (which has no acid protease activity) could have been present in the starting material, i.e. the brain homogenate.

A final classification of the enzyme on the basis of the present data is not yet possible. An apparent molecular weight of about 40 000—45 000 and the existence of isoenzymes has been reported for cathepsin D from various sources [21] as well as for kidney renin. However, the proteolytic activity at pH 3.2 with hemoglobin as substrate and the high affinity of the inhibitor pepstatin ($K_i < 10^{-9}$ M) at pH 5.5 are typical properties of cathepsin D [21,35], whereas kidney renin has no proteolytic activity at pH 3.2 and the affinity of pepstatin is approximately three orders of magnitude lower (about 10^{-6} M) [23]. Thus, the present study strongly supports the suggestion made by Day and Reid [9] that the brain enzyme described under the designation "isorenin" is in fact cathepsin D. For further confirmation a direct comparison of the enzyme with cathepsin D appears necessary. Such experiments will be described in a separate publication. From the present study it can be concluded that the renin-related enzyme isolated from rat brain is not an isoenzyme of renin, therefore, the designations "brain renin", "intrinsic renin" or "isorenin" should no longer be used.

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