Separation of Dog Brain Renin-Like Activity from Acid Protease Activity

MOHAMED Y. OSMAN, PH.D., ROBERT R. SMEBY, PH.D., AND SUBHA SEN, PH.D., D.Sc.

SUMMARY A renin-like enzyme and acid protease (cathepsin) from whole and saline-perfused dog brains were separated by CM-cellulose chromatography with a linear NaCl gradient. Plasma renin and cathepsin were also separated using the same system. During the separation steps (in all the above cases) the specific activity of the brain renin-like enzyme was increased, while the specific activity of the brain cathepsin was decreased. Approximately a 70-fold increase in the specific activity of brain renin-like enzyme and a sixfold decrease in brain cathepsin specific activity was obtained from saline-perfused brain. The separation made it possible to study the pH optimum of the brain renin-like enzyme and acid protease. The brain renin-like enzyme showed optimal activity in the range of pH 6–7. Immunologically, the renin-like enzyme was distinctly different from dog kidney renin. (Hypertension 1: 53-60, 1979)

KEY WORDS • brain renin • cathepsin • plasma renin • acid protease • brain

PRESENT evidence indicates that angiotensin II has a variety of effects on central nervous system structures and may be involved with the long-term regulation of systemic blood pressure. The suggestion has been made that these central nervous system effects of angiotensin II may be far more important than originally envisioned by Bickerton and Buckley several years ago, particularly with respect to its possible role in the pathogenesis of arterial hypertension. If endogenous angiotensin II has an action in the central nervous system, the natural question is how does it reach the sites at which it is active? While angiotensin generally does not cross the blood-brain barrier, there is some proof that the hormone can cross at specific, limited sites. The suggestion has also been made that the brain possesses an endogenous renin-angiotensin system that may act independently of its counterpart in the kidney. While the data presently available are not sufficient to establish the validity of this suggestion, both renin substrate and converting enzyme have been detected in brain of mammals. On the other hand, the existence of renin in the brain has been widely debated and this controversy has been recently reviewed by Reid. Proteolytic activity capable of producing angiotensin I from renin substrate has been observed in crude extracts of dog, rat and human brain. Because a wide variety of neutral and acid proteases have also been observed in brain extracts, and renin-like activity has not been separated from cathepsin, Day and Reid have concluded that the activity observed in brain extracts could merely be the lysosomal acid protease, cathepsin D. Hackenthal et al. have clearly demonstrated that acid protease closely related to cathepsin D from rat brain will produce angiotensin I from sheep and tetradecapeptide renin substrate. These observations require that the assignment of a physiological role for renin in the brain awaits the demonstration of the existence of renin that is distinct from nonspecific brain proteases. The present report demonstrates that brain renin-like activity can be chromatographically separated from both brain acid protease activity and from plasma renin. Preliminary results on the existence of brain renin-like activity have been reported by other investigators in abstracts.
Methods

In this report, renin-like activity is defined as the enzymatic production of angiotensin I from renin substrate at pH 6.5 and failure to hydrolyze hemoglobin at pH 3.5. Acid protease activity (cathepsin) is defined as the enzymatic hydrolysis of denatured hemoglobin at pH 3.5. Since we have not conducted studies to determine the peptide bonds split by the acid protease(s) of dog brain, we have not properly distinguished between cathepsin D and cathepsin E or other possible cathepsins.\(^\text{20}\)

Separation of Renin-like Activity from Dog Brain

In order to compare our experiments with those reported previously,\(^\text{11}\) initial processes of extraction, such as homogenization, centrifugation, dialysis, precipitation with ammonium sulfate and lyophilization, were performed as described by Day and Reid,\(^\text{11}\) using whole dog brains (five brains with an average weight of 90 g) from animals killed with an overdose of pentobarbital.

Further purification was achieved using CM-cellulose as described previously.\(^\text{21}\) Briefly, the CM-cellulose was prepared by washing with 0.001 M phosphate buffer, pH 5.5, and adding 10 mg of the crude protein per g of CM-cellulose. After stirring for 30 minutes, the CM-cellulose was removed by centrifugation, and washed with 10 ml of phosphate buffer (0.01 M, pH 5.5) per g of CM-cellulose. The wash was removed by filtration and discarded. The protein was eluted from the CM-cellulose by stirring with 5% NaCl in 0.01 M phosphate buffer at pH 5.5 for 30 minutes (approximately 10 ml of eluting solvent/g of CM-cellulose), the mixture was filtered and the CM-cellulose rewashed with the same volume of the buffer containing 5% NaCl. The eluate and wash solutions were combined (total volume 4 liters) and concentrated to a small volume by evaporation at reduced pressure and dialyzed overnight against phosphate buffer (0.01 M; pH 5.5). After dialysis, the solution was further concentrated under reduced pressure and dialyzed overnight against the same buffer (final volume 25 ml). This solution was applied to CM-cellulose column (2.2 X 60 cm) which had been well washed with 0.02 M phosphate buffer (pH 5.2). The protein was eluted using an approximate linear sodium chloride concentration gradient generated by placing 250 ml of sodium phosphate buffer (0.02 M, pH 5.2) in the mixing flask (500-ml round-bottom flask, with magnetic stirrer) with 250 ml of the same buffer containing 0.15 M NaCl replacing the buffer flow to the column. Fractions of 6 ml were collected and each assayed for cathepsin activity, renin activity and protein content.

To eliminate blood contaminants from contributing to the brain tissue enzyme activity, the procedures described above were repeated with whole brains perfused in situ with saline to remove as much blood as possible. Three dogs were anesthetized with pentobarbital sodium (30 mg/kg, I.V.), heparinized and, after opening the chest, a large bore tube was quickly inserted into the beating left ventricle. The ascending aorta was cross-clamped just distal to the origin of the subclavian artery and the right atrium was slit open. Saline was pumped at a rate of 800 ml/min with a roller pump. Perfusion continued until the fluid emanating from the open right atrium was clear of any noticeable blood (15 to 20 minutes). The brains (average weight 90 g) were then rapidly removed and frozen at −20°C until extracted. In addition, 500 ml plasma from normal dogs, collected with disodium ethylenediaminetetraacetic acid (EDTA) as an anticoagulant, were fractionated as described above.

To indicate the effect of protease inhibitors on the separation of brain renin-like activity from cathepsin D, an amount of 500 mg protein obtained (after (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} purification step) from non-perfused dog brain was dissolved in 25 ml of phosphate buffer (pH 5.2, 0.02 M) containing the following protease inhibitors: EDTA, 5 mM; N-ethyl maleimide (EMI), 5 mM; and phenyl methyl sulphonyl fluoride (PMSF), 2 mM. The solution was then fractionated with CM-cellulose and chromatographed on CM-cellulose as described above. All buffers used up to this step contained the mixture of protease inactivators at one tenth the concentrations mentioned above.

Enzyme Assay Systems

Acid protease (cathepsin) activity was measured by the method of Anson.\(^\text{22}\) In this method, samples were incubated in 1 ml of 0.1 M sodium acetate buffer (pH 3.5) containing 5–10 mg acid denatured hemoglobin, freshly prepared daily. Incubation was done for 60 minutes at 37°C. The protein was precipitated with 0.5 ml of 10% trichloroacetic acid (TCA), and the mixture centrifuged for 20 minutes at 1000 × g. The tyrosine content of the supernatant was measured by the Lowry method.\(^\text{23}\) Protease activity is expressed as \(\mu\)mol TCA soluble tyrosine produced/ml/hr, or per mg protein.

Cathepsin pH optimum activity was performed in 0.1 M NaAc buffer (pH 3.5) adjusted to appropriate pH with 1 M HCl below pH 3.5, and with 0.5 M NaAc above pH 3.5.

Renin-like activity in initial experiments was determined by measuring the amount of angiotensin I produced by incubating the samples with rat renin substrate at pH 6.5 under the conditions described by Boucher et al.\(^\text{24}\) In this assay, samples (0.1 ml) were incubated in the presence of 3 ml of substrate solution (150 mg of rat substrate dissolved in 2.9 ml distilled water, 0.04 ml Na\textsubscript{2}CO\textsubscript{3} [1%] and 0.04 ml EDTA [15%], then adjusted to pH 6.5 with 1 M NaOH), and 1 ml resin (Dowex 50 W-X2 [NH\textsubscript{4}]\textsuperscript{+}) suspension. The incubation was conducted at 37°C for 2 hours and the angiotensin I produced was measured by either bioassay in the rat\(^\text{28}\) or by radioimmunoassay\(^\text{24}\) after elution from the resin.\(^\text{24}\) Renin-like activity is expressed as pmoles angiotensin I produced per hour incubation per ml sample or per mg sample protein.
Renin-like pH optimum activity was performed as described below. The assay system consisted of 100 μl brain fraction, 100 μl substrate solution, containing 20 mg/ml of dog substrate protein prepared as described, and 100 μl of buffer. The buffers used were 0.05 M citrate-0.05 M phosphate for the pH range 2.5 to 7.0 and 0.05 M Tris-0.05 M phosphate for pH range 6.0 to 8.5. All buffers contained 0.58 mM PMSF, 5 mM EDTA and 5 mM EMI. To conserve enzyme protein the samples were incubated for 16 hours at 37° C. The reaction was stopped by heating the samples in a boiling water bath for 10 minutes. The denatured protein was removed by centrifugation and the angiotensin I in the supernatant was determined by radioimmunoassay using commercial reagents.*

Immunological Study using Kidney Renin Antibody

To 20 μl of brain renin-like solution (obtained from P1, fig. 2) were added 10 μl of diluted rabbit serum (1:500 dilution of #1769 from Dr. Inagami), and the samples were incubated at 4° C for 16 to 18 hours. Then 100 μl dog renin substrate solution (see above) and 170 μl of buffer (0.1 M phosphate, pH 6.5) were added. The samples were incubated at 37° C for 1 hour. The reaction was stopped by heating the samples in a boiling water bath for 10 minutes. A shorter incubation time was chosen because of a larger amount of enzyme. The denatured proteins were removed by centrifugation and the angiotensin I in the supernatant was determined by radioimmunoassay using commercial reagents.*

Results

The amount of protein and enzyme activity in fractions obtained during fractionation of non-perfused whole brain tissue are shown in table 1. During fractionation the specific activity of renin-like enzyme increased approximately 50-fold, while that of cathepsin decreased approximately threefold.

The chromatographic separation of 750 mg of protein from non-perfused whole dog brain is shown in figure 1. Three major protein peaks were observed which were collected into three pools and identified as P1, P2 and P3. It was found that P1 (Fr 30-37) contains both renin-like and cathepsin activities, while P3 (Fr 38-45) had only cathepsin activity and P2 (Fr 45-55) had only renin-like activity (table 1).

The chromatographic separation of 450 mg of protein from saline-perfused dog brain, which should not be contaminated with blood, is shown in figure 2. Again, three major protein peaks were observed and were collected into three pools. However, after removal of blood from the brain, only renin-like activity was found in P1 (Fr 30-37), and only cathepsin activity was obtained in P3 (Fr 38-45), while P2 (Fr 50-60) showed only slight cathepsin activity and no renin-like activity. The data obtained on fractionation of the perfused brain are given in table 2, which indicates that renin specific activity increased 57-fold while cathepsin specific activity decreased 6 times.

Examination of figures 1 and 2 shows that two enzyme activities disappeared following brain perfusion, the cathepsin activity of P1, and the renin-like activity of P3 (fig. 1). The absence of these two peaks following perfusion was concluded to be due to the removal of blood in the brain by the saline perfusion. The validity of this conclusion was tested by collecting plasma from normal dogs and fractionating it as described for the brain tissue extracts. Figure 3 shows the CM-cellulose chromatographic separation of 1500 mg of protein from plasma. Again, fractions were collected, assayed and pooled as before. Fractions 30-37 (P1) contained cathepsin activity and Fr 50-60 (P3) had renin-like activity, respectively, which correspond to the activity peaks that disappeared when blood was removed from the brain by saline perfusion (fig. 2). There are slight changes in the tube numbers corresponding to the peak positions, since we used three different columns and there were differences in column height and protein concentration from one ex-

*The commercial reagents used were initially from E. R. Squibb & Co., but when these reagents were recently modified, more satisfactory results were obtained using those from New England Nuclear Co.
**Figure 1.** Separation of non-perfused dog brain protein on a CM-cellulose column (2.2 × 60 cm). Solid circle = protein concentration; solid square = cathepsin activity; solid triangle = renin-like activity.

**Table 2.** Purification of Saline-Perfused Dog Brain Renin-Like and Cathepsin Activity

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Cathepsin</th>
<th>Renin-like activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Specific activity (pmole/mg protein/hr)</td>
<td>Yield (%)</td>
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<tr>
<td>Homogenization</td>
<td>4000</td>
<td>0.95</td>
<td>1.13</td>
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<td>(NH₄)₂SO₄ precipitate</td>
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<td>0.34</td>
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<td>CM-cellulose batch</td>
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<td>CM-column</td>
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<td></td>
</tr>
<tr>
<td>P₁</td>
<td>6</td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>P₂</td>
<td>30</td>
<td>0.15</td>
<td>0.12</td>
</tr>
<tr>
<td>P₃</td>
<td>3</td>
<td>0.02</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**Table 3.** Purification of Dog Plasma Renin and Cathepsin

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Cathepsin</th>
<th>Renin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Specific activity (nmole/mg protein/hr)</td>
<td>Yield (%)</td>
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<tr>
<td>Plasma</td>
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<td>(NH₄)₂SO₄ precipitate</td>
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<td>CM-cellulose batch</td>
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<td>16.09</td>
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<td>CM-column</td>
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<td>P₁</td>
<td>700</td>
<td>6.16</td>
<td>6.79</td>
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<tr>
<td>P₂</td>
<td>500</td>
<td>0.55</td>
<td>1.43</td>
</tr>
<tr>
<td>P₃</td>
<td>10</td>
<td>—</td>
<td>0</td>
</tr>
</tbody>
</table>
periment to another. The data obtained on fractionation are shown in table 3, which indicates that plasma renin specific activity increased 46 times, while the plasma cathepsin activity decreased to its half activity. The chromatographic separation of 250 mg protein obtained from non-perfused dog brains, dialyzed and eluted with buffer containing protease inhibitors, gave only renin-like activity in the area of P1 (fig. 1), while the plasma cathepsin activity in these fractions was inhibited. Also only one active cathepsin peak was observed in the area of P1 compared with two obtained in the absence of inhibitors (figs. 1 and 2).

The hydrolysis of acid denatured hemoglobin by partially purified cathepsin obtained from P1 (fig. 1) resulted in a higher pH optimum at 3.5 and a distinct second pH optimum in the range of 4.0–5.0 (fig. 4).

The examination of partially purified brain renin-like activity (obtained from P1, fig. 1), as the pH is

FIGURE 2. Separation of saline-perfused dog brain protein on a CM-cellulose column (2.2 × 60 cm). Solid circle = protein concentration; solid square = cathepsin activity; solid triangle = renin-like activity.

FIGURE 3. Separation of dog plasma protein after passage through CM-cellulose column (2.2 × 70 cm). Solid circle = protein concentration; solid square = cathepsin activity; solid triangle = renin-like activity.
Figure 4. Brain renin-like (solid triangle) and cathepsin (solid square) activity at different pH. Each point is an average of three determinations.

Discussion

A cerebral counterpart to the components of the renin-angiotensin system of kidney and peripheral blood were initially reported by Fischer-Ferraro et al. and Ganten et al. in 1971. These observations, later confirmed by other investigators, led them to believe that there may be local formation of angiotensin I and II in the brain of mammals by an “iso-renin” system. The data gave credence to the possibility that extra-renal sources of renin may have a role in the regulation of arterial blood pressure and vascular reactivity. Since the brain possesses powerful mechanisms engaged in the regulation of cardiovascular activity, a cerebral renin angiotensin system could have either physiological or pathological consequences.

While crude extracts of brain tissue are capable of forming angiotensin, Reid has recently questioned the physiological significance of this observation. This questioning is largely based on his inability to separate acid proteinase activity, which he identified as cathepsin D without proper substrate specificity studies, from renin-like activity of brain. This questioning is perhaps strengthened by the recent work of Hackenthal et al., which demonstrated that both bovine spleen cathepsin D and brain acid protease, closely related to cathepsin D, have the ability to form angiotensin I from tetradecapeptide and sheep renin substrates.

Does an enzyme with renin-like activity occur among the non-lysosomal, neutral proteinases found in brain? This question, not answered by the demonstration that cathepsin D can form angiotensin I, must be answered to establish a physiological role for the brain renin angiotensin system. The present experiment provides the first evidence that extracts from whole dog’s brain have renin-like activity that is not due to acid protease activity. This conclusion was obtained by 1) separation of renin-like activity from acid protease activity; 2) demonstration that renin-like activity and cathepsin activity of crude brain extracts was in part due to blood contamination; 3) separation of brain renin-like activity from plasma renin; and 4) demonstration of the formation of angiotensin I by a brain enzyme having a pH optimum between pH 6 and pH 7.

During all the separation steps of brain renin-like activity from cathepsin, in all cases (non-perfused brain with and without inhibitors, saline-perfused brain and plasma) the specific activity of renin-like activity increased, while the specific activity of cathepsin decreased. This is in contrast to the fractionation results obtained by Day and Reid in which the specific activity of these two enzyme activities increased together.
The hydrolysis of hemoglobin with cathepsin fractions showed two distinct pH optima; one at pH 3.5, which resembled that obtained by Day and Reid11 for cathepsin D, and a second between pH 4 and 5. In this connection, Schwabe and Kalnitsky,77 Woessner and Shamberger,28 Barrett,28 and Cunningham and Tang28 found that on the less acidic side, the pH optimum curves with hemoglobin show a distinct shoulder or even a second optimum, in the range of pH 4–5. Cunningham and Tang28 have shown that the position of the pH optimum is influenced by an effect of ionic strength on hemoglobin, and when an oligopeptide (peptide D) was used as substrate instead of hemoglobin, the salt effect diminished. Using purified cathepsin D from procine spleen, they indicated that the two-optima phenomenon is a property of a single enzyme species.30

The pH optimum curve (fig. 4) obtained with brain renin-like activity (non-perfused dog brain) showed a broad shoulder or approximately optimum pH in the range of 4–5; moreover a second pH optimum in the range of 6–7 was obtained. The first optimum (pH 4–5) resembled that obtained by Day and Reid11 for brain renin, but they mentioned that there is no activity for the brain renin above pH 6.0. Contrary to this, an optimum was obtained in the range of pH 6–7, indicating that there is a reaction in the physiological pH range. The activity at pH 2.5 is due to the plasma cathepsin as has been proved by plasma fractionation (fig. 3). At this pH (2.5) cathepsin was able to hydrolyze dog plasma substrate. Since this enzyme apparently hydrolyzes hemoglobin poorly and has a pH optimum below pH 3, it may be cathepsin E,30 but further work must be done to establish this identification. We used non-perfused brain in studying the pH optimum of cathepsin and renin in order to compare our results with those previously published.8, 9, 11, 18

Since we wanted to observe all protease activity under the same conditions used previously by Day and Reid,11 the present experiments do not tell us whether renin might exist in the cell in a high molecular weight range of 6–7 was obtained. The first optimum (pH 3.5) resembled that obtained by Day and Reid11 for brain renin, but they mentioned that there is no activity for the brain renin above pH 6.0. Contrary to this, an optimum was obtained in the range of pH 6–7, indicating that there is a reaction in the physiological pH range. The activity at pH 2.5 is due to the plasma cathepsin as has been proved by plasma fractionation (fig. 3). At this pH (2.5) cathepsin was able to hydrolyze dog plasma substrate. Since this enzyme apparently hydrolyzes hemoglobin poorly and has a pH optimum below pH 3, it may be cathepsin E,30 but further work must be done to establish this identification. We used non-perfused brain in studying the pH optimum of cathepsin and renin in order to compare our results with those previously published.8, 9, 11, 18

Since we wanted to observe all protease activity under the same conditions used previously by Day and Reid,11 the present experiments do not tell us whether renin might exist in the cell in a high molecular weight form as has been reported for the kidney.31 There could have been destruction of renin by acid protease during extraction so the amount of renin-like activity reported cannot be used for quantitative comparisons of renin-like activity.

While brain renin has been reported to have a pH optimum of 5.0,8, 4 the renin-like activity in our experiments after separation from acid proteases possessed optimal activity at pH range of 6 to 7. Although further investigation is required to determine the nature and role of the angiotensin-forming enzyme in the brain, our experiments indicate that there is in the dog brain an enzyme that generates angiotensin distinct from the cathepsins and immunologically distinct from dog kidney renin. Since the cerebral enzyme that produces angiotensin is not inhibited by antibody as readily as the kidney enzyme, either a different protein has been produced by the brain or the protein from kidney has been modified in the brain (such as glycosylation).

Addendum

After submission of this manuscript, the demonstration that rat brain contains an enzyme which generates angiotensin I from rat renin substrate with a pH optimum at 6.0 to 7.5 and is neutralized by hog renin antibody has been reported by Hirose and co-workers (Hirose S, Yokosawa H, Inagami T: Immunochimical identification of renin in rat brain and distinction from acid proteases. Nature 274: 392, 1978).

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