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Separation of Renin-Like Activity from Acid Protease Activity in Different Parts of Rat Brain

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Abstract: A renin-like enzyme was separated from acid protease (cathepsin) from whole and different parts of rat brains on hemoglobin agarose resin. During the separation steps the specific activity of the brain renin-like enzyme was increased, while the specific activity of the brain cathepsin was decreased. More than 10-folds increase in the brain renin-like specific activity, and a 50% decrease in the brain cathepsin specific activity were obtained when compared to their activities obtained when separation on CM-cellulose column occurred. The brain renin-like enzyme showed optimal activity in the range of pH 6-7. The frontal cortex showed the highest renin activity, (5.4ng Angiotensin I/g tissue/hr).

INTRODUCTION:

The existence of renin-like activity in brain distinct from acid protease (cathepsin) is of great interest as there is evidence for the presence of angiotensinogen [(1)], angiotensin I and converting enzymes[(2)] in the brain. Also, the role of angiotensin II on the central nervous system is well recognized[(3-4)].

The effects of brain angiotensin II(Ang II) on blood pressure and fluid homeostasis are now well recognized. Although circulating Ang II can have effects on the circum-ventricular areas, because they are devoid of a blood-brain barrier, it has become apparent that local production of Ang II in the brain is also important. Immunoreactive Ang II has been detected in many areas of the brain that are inside the blood-brain barrier[(5)].

All components of the renin-angiotensin

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system (RAS) have been detected in the brain. Angiotensinogen (AGT) and angiotensin-converting enzyme (ACE) have been described to be present throughout the brain[6]. It was also reported that Ang II can be generated from over expression of angiotensinogen and renin in the brain[7].

A renin-like enzyme has been separated from acid protease on a CM-cellulose from saline perfused whole dog brain. The enzyme showed optimal activity in the pH range of 6-7 and during the purification of the enzyme, the specific activity was increased while the acid protease specific activity was decreased. Immunologically the dog renin-like enzyme was distinctly different from dog kidney renin[8].

This observation was extended by Husain et al[9] who observed such renin-like activity in tissue other than dog brain.

This paper demonstrates that rat brain renin-like activity can be separated from brain on hemoglobin-agarose affinity column chromatography.

The objective of this work was to develop a simple, rapid method to separate and assay renin-like enzyme activity from whole and different parts of rat brain, namely; medulla oblongata, pons, frontal cortex and cerebellum.

Material and methods

Chemicals

1- (CNBr-activated) Sepharose 4B (Pharmacia Fine Chemicals-Sweden).

The following chemicals were purchased from Sigma Chemical Co. St. Louis, MA

2- Recrystallized human hemoglobin.
3- Ethylenediamine tetra acetic acid (EDTA).
4- Phenylmethylsulfonyl fluoride (PMSF).
5- N-ethyl maleimide (EMI).

All other chemicals were purchased from BDH, England

Animals

Sixty male albino rats (weighing 150-200g) aged 2 months, were used in the

experiments. The rats were supplied by the Medical Research Institute animal house, Alexandria University (Egypt). Rats were housed in group cages (5 in each cage) and allowed free access to food and tap water. The rats were killed by decapitation after having been subjected to overnight fast with free access to water. Each brain weighed about 2.0gm. Twenty out of sixty brain (40g) were used for whole brain renin extraction. From the other forty brains, the parts under study were isolated, washed with ice-cold saline and weighed each separately as follows: cerebellum (20g), frontal cortex (12g), medulla oblongata (20g) and pons (18g).

Preparation of Hb-agarose affinity column:

The resin was prepared by the method of Smith and Turk [10] with some modification. CNBr-activated sepharose gel (7.5g) was swollen and washed for 15min on a glass filter with 0.001 M HCl (1.5 L). Recrystallized human hemoglobin (225mg) was dissolved in coupling buffer (0.1 M $NaHCO_3$ (pH 8.6) containing 0.5 M NaCl (150ml)) and mixed with the gel in an Erlenmeyer flask and the mixture was stirred for 2hrs at room temperature using a glass stirrer. Unbound materials were washed away with 2L of 1 M ethanolamine to react with any remaining active groups, followed by 3 washing cycles with coupling buffer (2L), acetate buffer (pH 3.5, 0.1M; 2L), coupling buffer (2L), borate buffer (pH 7.6 containing 1M Na Cl; 2L) and lastly coupling buffer (2L) , acetate buffer (pH 3.5, 0.05M, 2L).

Separation of renin from whole and different parts of rat Brain

The whole as well as different parts of rat brain were homogenized in 1L of saline containing the following protease inhibitors: 30 mM disodium EDTA, 2mM phenyl methyl sulphonyl fluoride and 2 mM N-ethyl maleimide at pH 7.0, then centrifuged for 30 min. at 1500xg. To the supernatant (800ml) ammonium sullphate was added until the

resulting mixture was 60% saturated at pH 6.4 using 0.02 M $NaHCO_3$ to adjust the pH. The precipitate was removed by centrifugation for 1h at 1500xg, redissolved in 100ml sodium acetate buffer (0.05M) pH 7.0 and dialyzed against 20 volumes of the same buffer for 24 hrs , followed by centrifugation. The supernatant was redialyzed against 20 volumes of 0.02 M Na citrate pH 7.0 for 48 hrs with change of the dialyzing medium at 24hrs at 4^0C .The insoluble residues were removed by centrifugation at 1500xg and the supernatant was lyophilized, dissolved in 25ml of distilled water, and dialyzed against 20 volumes of Na phosphate buffer (pH 5.2, 0.001 M) for 24 hrs. The dialysate was concentrated to 10 ml under reduced pressure and applied to CM cellulose column (1.8 × 50cm) which has been well washed with 0.02M phosphate buffer. The protein was eluted using 0.02 M phosphate buffer pH 5.2 containing 0.1 M NaCl and protease inhibitors (at 1/20 of the concentrations mentioned above)until the absorbance of the eluate reached 0.05. The pH of the eluate as well as the molarity of NaCl changed until it reached 4.2 and 0.2 M respectively by using linear concentration gradient[8]. Fractions of 6 ml were collected and the protein content was determined by measuring the absorbance at 280 nm. The active renin fractions were collected and dialyzed against 0.05 M sodium acetate buffer pH 3.5 and centrifuged at 2000xg then the precipitate was applied to the equilibrated Hb-agarose column (0.5 x 25cm) which has been well washed with the same buffer. The protein eluted with 0.05 M sodium acetate (pH 3.5) until the absorbance of the eluate reached 0.01, then the elution continued using 0.1 M tris buffer pH 8.6 containing 1M NaCl. Fractions were collected and assayed for both renin and cathepsin activities.

Enzyme assay system

Acid protease (cathepsin) activity was measured as described by Osman et al.[8]. In this method, samples were incubated for 60 min at 37ºC in 1 ml of 0.1 M sodium

acetate buffer (pH 3.5) containing 5 to 10mg acid-denatured hemoglobin, freshly prepared daily. The protein was precipitated with 0.5ml of 10% trichloroacetic acid, and the mixture was centrifuged for 20 min at 1000 x g. Protease activity is expressed as µmol/mg protein.

Renin-like activity was measured by incubating the various fractions with partially purified rat renin substrate prepared using plasma from 48-hour nephrectomized rats.[8]. The assay system consisted of 100µl of brain extract or fraction, 100µl of substrate solution containing 2 mg of protein, and 100 µl of 0.1 M phosphate buffer (pH 6.5). The samples were incubated at 37º for 2 hours and the reaction was stopped by heating 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 (New England Nuclear Co.).

Renin-like activity is expressed as pmoles angiotensin I produced/hour incubation/ml sample or mg sample protein.

Results

Table 1 and Figure 1 show the chromatographic separation on CM-cellulose column of 106 mg protein obtained from rat brains. Three protein peaks were obtained; the first peak (P_1) showed only acid protease activity while the second and the third peaks (P_2 and P_3) showed both renin and acid protease activities. When the fractions of the last two peaks collected, they were concentrated and rechromatographed on Hb-agarose affinity column, Two peaks were obtained using two different eluents (Fig. 2). The first protein peak which eluted at lower ionic strength and acidic pH 3.5 (namely F1) showed only a high renin specific activity, while the second protein peak which eluted at a high ionic strength 1 M NaCl and pH 8.6 (namely F2) showed a high acid protease specific activity with a

lower renin specific activity when compared with F1.

Table 2 indicates the amount of rennin in the different parts of rat brain (ng angiotensin 1/g tissue/hr) after incubation of the extracts with rat renin substrate at pH 6.5. The data indicated that the frontal cortex possessed the highest renin content while the cerebellum possessed the lowest(5.4 and4.11 ng Angiotensin I/g/hr) respectively.

It is noteworthy to mention that during all the purification steps the renin-like specific activity was increased while the acid protease specific activity was decreased (Table 1).

Table 1: Purification of rat whole brain renin-like and cathepsin activity.

Purification steps	Total protein mg	Cathepsin Sp. ac pmol/mg	Cathepsin yield %	Renin activity sp.ac pmol/mg	Renin activity yield %
Homogenization	1.700	0.42		0.30	
$(NH_4)_2 SO_4$ ppt	620	0.44	20	1.15	72
CM-column	106	0.37	5.8	2.25	46.7
Hb-agarose affinity column:					
F1	8			22.13	35
F2	6	0.20	3.2	3.00	7.6

Table 2: Assay of renin- like activity in different parts of rat brain.

Brain part	Renin(ng Angiotensin I/g tissue/hr)
	Average ±S.D
Frontal cortex	5.4 ± 0.48
Pons	5.1 ± 0.43
Medulla oblongata	4.6 ± 0.38
Cerebellum	4.11 ± 0.42

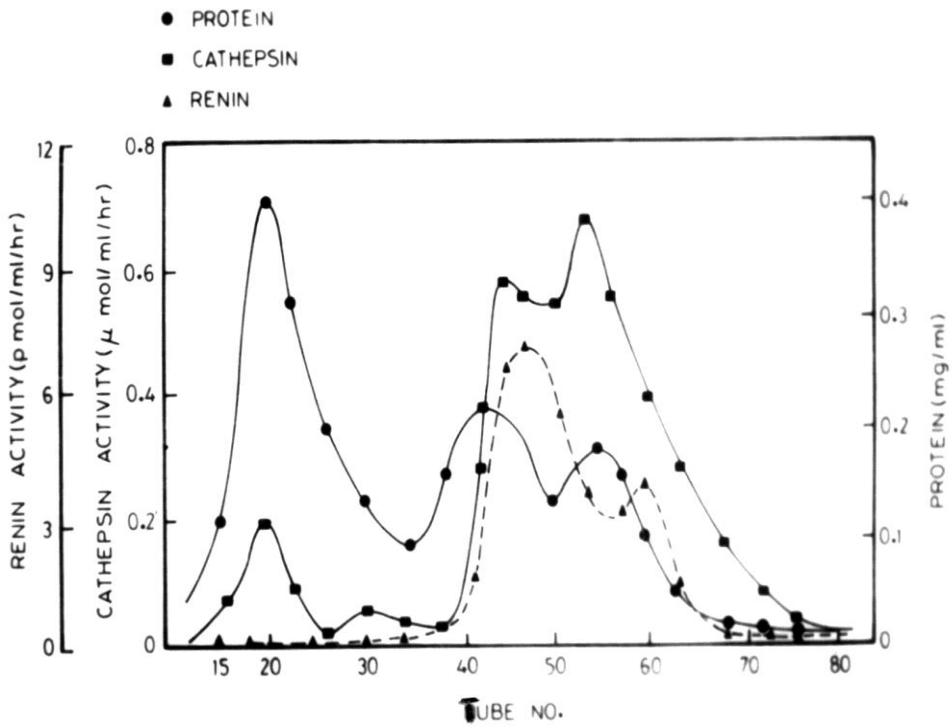

Figure 1: Separation of rat brain protein on a CM-cellulose column (1.8 × 50cm). • protein, ▪ cathepsin, ▲ renin.

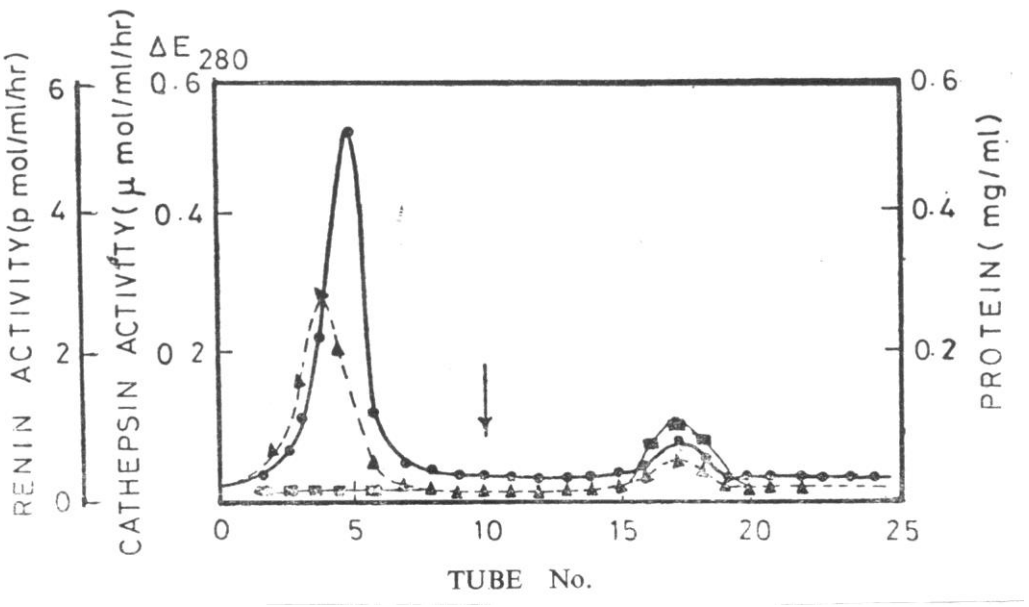

Figure 2: Hemoglobin-sepharose chromatography column (0.5 × 23cm) of the active renin fractions obtained from peaks 2 and 3 (fig. 1). The starting buffer 0.05 M NaAc, pH 3.5. At the point marked with an arrow, column bound 3.5. At the point marked with an arrow, column bound proteins were eluted with 0.1M tris buffer pH 8.5 containing 1M NaCl •protein, ▪ cathepsin, ▲renin

Discussion

The hydrolysis of renin substrate by F1 at pH 6-7 was due to the renin-like enzyme which was unaffected during the purification by the inhibitors used and not due to the acid protease cathepsin D whose activity was decreased during the extraction processes as it has been reported by some authors[11]. The hydrolysis of acid denatured hemoglobin occurred between pH 2.0 and 5.0 by F2 peak showed pH optimum at 3.0, then the activity increased from pH 4 to 5 with a possible optimum above this pH. The reaction could not be followed above pH 5 due to the instability of the acid denatured-Hb above this pH. It has been reported by

Cunningham and Tang[12] that purified cathepsin D had two optima and these two optima phenomena are properties of a single enzyme species. It is noteworthy to mention that at the end of the separation process, the yield of renin-like enzyme was 75% in F1 with 10 folds increase in its specific activity, while that of the acid protease activity in F1 disappeared due to its absorption on Hb-agarose and the unability of the acidic and low ionic strength of Na acetate buffer (pH 3.5; 0.05M) to elute it (fig.2). On the other hand, the yield of acid protease was 50% in F2 with a decrease to its half specific activity compared to the activities obtained after separation on CM-cellulose column.

On the same Hb-agarose affinity column and using 0.1 M tris buffer pH 8.6 containing 1M NaCl, Smith and Turk[10] were able to isolate purified cathepsin D from spleen and thymus. This indicates that the hydrolysis of the acid denatured-Hb by F_2 at pH 3.0 is due to the brain cathepsin D which chromatographed and eluted as previously described[10] .

The present experiment provides a good evidence that extracts from whole rat brain have renin-like activity that is not due to acid protease activity or blood plasma renin, and this activity could be separated on a simple hemoglobin agarose affinity column chromatography this conclusion was achieved by: 1) separation of renin-like activity (F1) from cathepsin activity (F2); 2) demonstration that brain renin-like enzyme has a pH optimum between pH 6 and pH 7; 3) during all the purification steps the specific activity of renin-like enzyme increased while the specific activity of cathepsin decreased.

The separation of renin from the different parts of rat brain in this study; revealed the presence of renin-like (isorenin) [13] enzyme in these parts with different concentrations varying from one part to another. The highest isorenin content was in case of frontal cortex extract

(5.4 ng Angiotensin I/g tissue/hr), while the lowest content was in case of cerebellum extract (4.11 ng Angiotensin I/g tissue/hr). In this connection, Julie et al[7] used immunohistochemistry to demonstrate the presence of rennin and angiotensin in close proximity in different areas in medulla oblongata. This provides the potential for local formation of angiotensin I.

The presence of different forms of renin in extrarenal tissues opens the possibility that the differential regulation of the enzyme occurs either at the level of the gene or by post-translational processing[9]. The importance of the systemic renin-angiotensin system (RAS) and the pivotal role of angiotensin (Ang) II in the pathogenesis of hypertension and other cardiovascular diseases are widely acknowledged. However, the traditional view that Ang II is the singular key product of the RAS has been questioned following the discovery of angiotensin-converting enzyme 2[14-17].

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