PEPSTATIN, AN INHIBITOR FOR RENIN PURIFICATION BY AFFINITY CHROMATOGRAPHY

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1. Introduction

Pepstatin A is a new inhibitor which has been isolated from a culture media of various species of *Streptomyces* [1]. The structure of this pentapeptide is isovaleryl—L-valyl—L-valyl—4-amino-3-hydroxy-6-methylheptanoyl-L-alanyl—4-amino-3-hydroxy-6-methylheptanoic acid [1]. It is a powerful inhibitor of several acid proteases [2, 3] and of renin [4], a renal enzyme which hydrolyses angiotensinogen to liberate angiotensin I. Gross et al. [4], Miller et al. [5] and Aoyagi et al. [6] have demonstrated an *in vivo* and *in vitro* inhibition of renin by Pepstatin. Miller et al. mentioned that this inhibition was of the competitive type.

The purpose of this paper was to further characterize the inhibitory activity of Pepstatin *in vitro* on hog renin and to demonstrate that Pepstatin was a suitable ligand for renin purification by affinity chromatography. We found that renin could be purified with quantitative recovery on a Pepstatin—hexamethylene-diamino-Sepharose column.

2. Materials and methods

Hog renal renin was purified according to Peart et al. [7], step IV, and contained 4.4 Goldblatt units (G.U.) per mg of protein. This renin was tested against hog renin supplied by Nutritional Biochemical (N.B.C.) with a specific activity of 17 G.U. per mg of protein. Renin substrate, free of renin and angiotensinases was prepared from binephrectomized rat plasma according to Haas and Goldblatt [8]. This plasma renin substrate (PRS) contained 40 pm of angiotensinogen per mg of protein. Pepstatin A was a generous gift from Dr. H. Umezawa. Sepharose 4B was purchased from Pharmacia.

2.1. *Renin assays*

2.1.1. Radioimmunoassay

Incubations were performed in siliconized pyrex glass tubes containing 145 pmmol of PRS in 500 µl of phosphate buffer, 0.2 M, pH 6.5 (buffer A). Ten µl of renin were added and the incubation performed 15 min at 37°C. The reaction was stopped by immersion in a boiling water bath for 10 min. The amount of angiotensin I generated was measured in the supernatant by a radioimmunoassay (RIA) [9]. In all experiments the amount of renin did not hydrolyze more than 5% of the available substrate, in order to maintain a zero order kinetic reaction.

2.1.2. Bioassay

Renin pressor activity was assayed in the rat, according to Peart [10]. Hog renin N.B.C. (0.05 G.U./assay) was used as a standard in a four point assay.

2.2. *Characteristics of renin inhibition by Pepstatin*

10^{-3} G.U. were incubated with increasing amounts of PRS (6 to 60 pm) with or without 10^{-7} M Pepstatin in buffer A. Results were plotted according to the method of Lineweaver—Burk.

2.3. *Preparation of a Pepstatin—Sepharose derivative*

Pepstatin was coupled to hexamethylene-diamino-Sepharose according to a modified technique of

Cuatrecasas and Parikh [11]: 50 mg of Pepstatin dissolved in dimethylformamide (DMF) were allowed to react with 2 equivalents of *N, N'*-dicyclohexylcarbodiimide and with 2 equivalents of *N*-hydrosuccinimide. The *N*-hydroxysuccinyl–Pepstatin was then coupled to 16 ml of hexamethylene-diamino-Sepharose prepared as described by Cuatrecasas [12]. The gel was extensively washed with large volumes of dioxane: DMF (v:v), then with water. The deaerated gel was packed in a small column and washed before any experiment with the same buffer as those used for elution. All experiments were performed at 4°C.

No method was available to directly measure the amount of covalently bound Pepstatin. For this reason, the efficacy of such a column was tested by its ability to retain about 90% of commercial purified pepsin (Sigma, 2X crystallized) whereas 10% of unactive protein(s) were directly eluted (C. Devaux, P. Corvol et al., unpublished data).

2.4. *Miscellaneous*

Polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Rodbard and Chrambach [13]. System B was used (operative pH: 10.2, 0°C). Gels were stained by Coomassie Blue [13] or sliced transversally and the renin content was tested in each slice. Protein concentration was measured by the method of Lowry et al. [14].

3. Results and discussion

3.1. *Renin inhibition by Pepstatin: kinetic characteristics*

In a preliminary experiment, we found that the concentration of Pepstatin giving a 50% inhibition of 10^{-3} G.U. of renin was 0.3×10^{-7} M. As shown in fig. 1, renin is inhibited competitively by Pepstatin. The concentration of Pepstatin required for 50% inhibition of renin is in the range of that found by other authors [4–6] using less sensitive methods. The present assay besides being highly reproducible, has the advantage of using a renin substrate free of renin and of angiotensinases and can be utilized for large-scale studies.

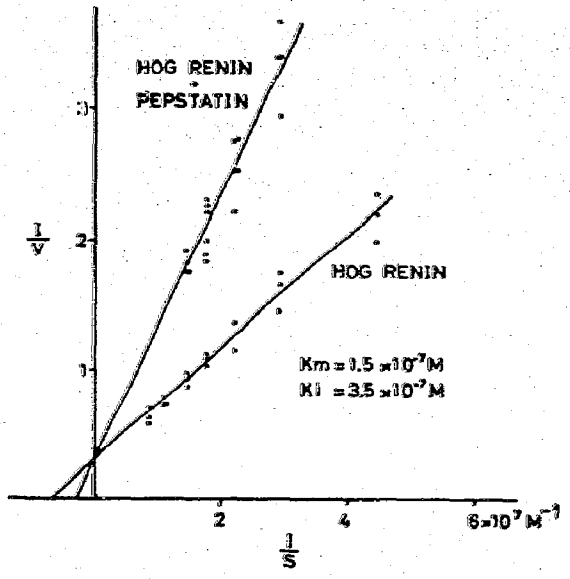

Fig. 1. Lineweaver–Burk representation of hog renin (10^{-3} G.U.) inhibition by Pepstatin (10^{-7} M). Velocity is expressed in pm of angiotensin I per min.

3.2. *Renin purification by affinity chromatography*

31.5 mg of proteins (140 G.U. of renin) dialyzed overnight against buffer A were applied to Pepstatin–hexamethylene-diamino-Sepharose column (fig. 2c). A first protein peak (I) was eluted with buffer A; another peak (II) was obtained with KCl–HCl buffer 0.2 M, pH 2. Renin was finally eluted with 6 M area in buffer A (peak III). Less than 1% of renin was detected in the first two peaks. A specific activity of 130 G.U./mg of protein was found by RIA in peak III, corresponding to a thirty times purification with a 70% recovery.

Attempts to elute renin with 0.1 N HCl, N HCl and 3 M KSCN were unsuccessful. The use of 6 M urea did not decrease renin activity when the solution was immediately dialyzed against buffer A.

In control experiments, 15 G.U. of renin were applied to a column of unsubstituted Sepharose (fig. 2a) and of hexamethylene-diamino-Sepharose (fig. 2b). All the renin activity was recovered in the first peak in both experiments. Some proteins were retained on hexamethylene-diamino-Sepharose and eluted with KCl–HCl buffer.

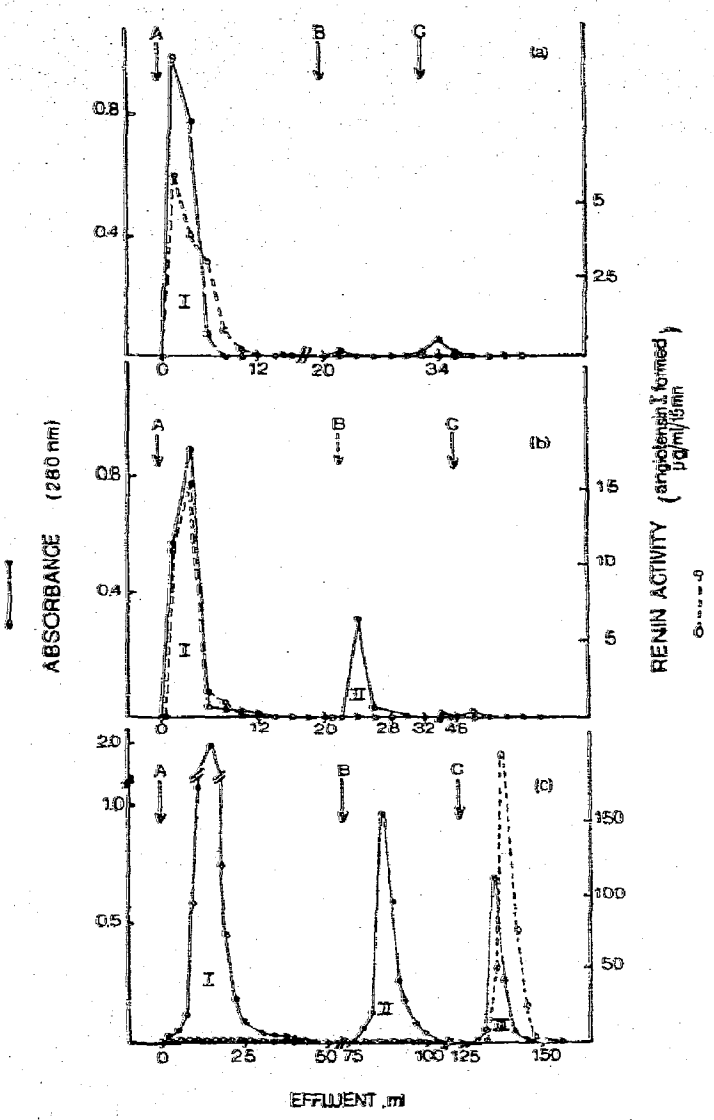

Fig. 2. Affinity chromatography of renin. 15 G.U. were applied on a 2 ml column of (a) unsubstituted Sepharose, (b) hexamethylene-diamino-Sepharose. 140 G.U. were applied on a 16 ml column of Pepstatin-hexamethylene-diamino-Sepharose (c). Arrows indicate the different buffers used for elution; A: buffer A, B: KCl–HCl 0.2 M, pH 2.0; C: 6 M urea in buffer A.

3.3. *Renin characterization*

Renin was characterized from peak III by its ability to increase rat blood pressure by bioassay and to generate angiotensin I as measured by RIA, when incubated with PRS. PAGE of peak III showed two major

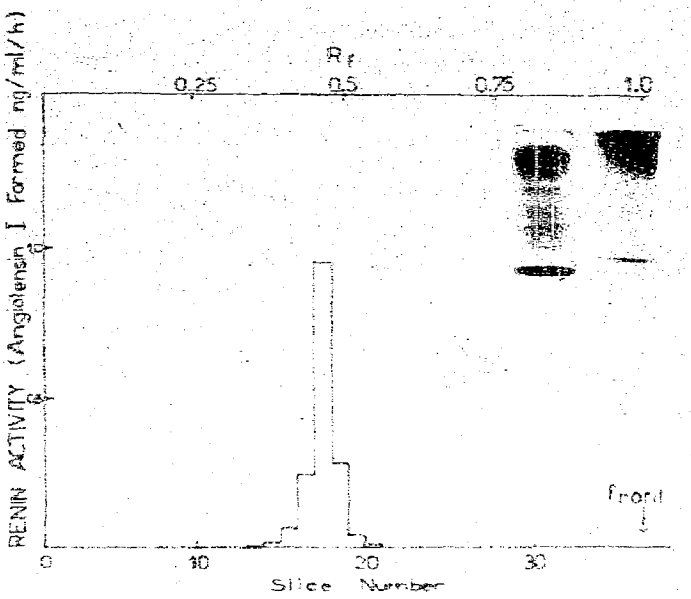

Fig. 3. Polyacrylamide gel electrophoresis of renin from peak III. 5×10^{-3} G.U. were submitted to electrophoresis at pH 10.2 and after electrophoresis each slice was incubated with PRS in 1 ml of buffer A. Angiotensin I formed was measured by RIA. Insert: PAGE of renin solution applied to the affinity column (left) and of peak III (right).

bands. The faster had the same R_f (0.49) as renin detected in the gel (fig. 3). This R_f was identical to the R_f of the starting material (0.48).

Renin purified by affinity chromatography was not homogeneous on PAGE and still contained at least one major contaminant. This might be due to the simultaneous adsorption and elution of renal acid protease(s) inhibited by Pepstatin, such as cathepsins. However, no pepsin nor cathepsin-like activity was detectable in peak III, using casein [1] or hemoglobin [15] as a substrate. Other mechanisms could explain the retention of proteins on Pepstatin–Sepharose such as strong hydrophobic binding. Hydrophobic binding on Sepharose substituted with *N*-alkylamines has been recently reported [16]. This phenomenon is in agreement with the retention of peak II proteins, which occurs on hexamethylene-diamino-Sepharose whether Pepstatin is coupled or not (fig. 2).

Pure renal renin has never been obtained by conventional methods. Affinity chromatography is a very powerful tool, allowing in a single step a thirty times purification. This could be used in conjunction with

already described methods, in order to obtain a complete purification of renin.

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