

RAPID ISOLATION OF CATHEPSIN D BY AFFINITY CHROMATOGRAPHY ON THE IMMOBILIZED SYNTHETIC INHIBITOR

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1. Introduction

In previous work we have shown, that a rapid isolation of lysosomal proteinase cathepsin D (EC 3.4.4.23) is a prerequisite for obtaining this enzyme in undegraded form [1]. An immobilized substrate, such as hemoglobin is a suitable affinity ligand for this purpose, although immobilized inhibitors are more commonly used for the isolation of enzymes by affinity chromatography. Renin [2,3] and cathepsin D [4,5] were purified on the affinity column packed with immobilized pentapeptide pepstatin, a strong inhibitor of acid proteinases.

Synthetic enantiomeric substrate analogues are known to be competitive inhibitors of various proteinases [6–8] and they can serve as convenient ligands in affinity chromatography. Bound to Sepharose, they were used for the affinity chromatography of pepsin and acid proteinase from *Aspergillus awamori* [7]. We have found that synthetic octapeptide Gly-Glu-Gly-Phe-Leu-Gly-D-Phe-Leu is a competitive inhibitor of cathepsin D [9]. This report describes its use as an affinity ligand for the simple and rapid isolation of cathepsin D from tissue extracts in a single or double step procedure.

2. Materials and methods

Octapeptide inhibitor was synthesized by Merrifield solid phase synthesis [10]. It was easily soluble in water in concentrations up to 20 mg/ml at

an alkaline pH. Once dissolved the solution could be acidified to pH 5.0 without precipitation of the peptide. The purity of the inhibitor was verified by paper chromatography, end group determination and amino acid analysis. The sample contained at least 95% of the octapeptide. CH-Sepharose 4B was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The coupling agent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was obtained from Calbiochem, Los Angeles, Calif. All other chemicals were reagent grade. Redistilled water was used in all experiments.

Tissue extracts were prepared from fresh bovine thymus or spleen by homogenization with 3 parts of ice-cold water. Three parts of the supernatant, obtained after centrifugation at $17\,000 \times g$ for 40 min, were brought to 1 M NaCl in 0.125 M sodium acetate at pH 4.0 by the addition of one part of 4 M NaCl in 0.5 M sodium acetate buffer, pH 4.0. The precipitate formed at pH 4.0 was removed by centrifugation and the clear supernatant was applied to a Sepharose-inhibitor column. Proteolytic activity was assayed by a modified Anson's method, using hemoglobin as the substrate [1]. All experiments were performed at 4°C.

3. Results and discussion

For the preparation of the immobilized inhibitor, 1.5 g of dry CH-Sepharose 4B were swollen in 0.5 M NaCl, washed with 500 ml of the same solution and with 500 ml of water. Thirty milligrams of octapeptide inhibitor were dissolved in 6 ml of water,

the pH adjusted to 5.0 and added to the CH-Sepharose. To this slurry, 1 ml of EDC solution (290 mg/ml water) was added dropwise while stirring, the pH was kept at 5.0, using a pH-stat (Radiometer, Copenhagen). After 1 h the pH was stabilized and the suspension was left overnight at room temperature. The following day, 25 μ l of ethanol diamine was added in order to block the remaining free carboxylic groups. After 1 h the immobilized octapeptide was washed alternatively with 0.1 M Na acetate, pH 4.0 in 0.5 M NaCl and 0.1 M phosphate, pH 8.5 in 0.5 M NaCl. The gel was packed to 2.5 cm in a column of 1.7 diameter.

Acid proteinase was bound to immobilized octapeptide inhibitor at pH 4.0 in 0.125 M Na-acetate and 1.0 M NaCl. Acidification of the crude tissue extract below pH 3.5 resulted in a substantial loss of cathepsin D and was avoided. The proteolytically active fraction washed from the column with 0.15 M phosphate buffer, pH 8.0 in 1 M NaCl. All elution buffers were prepared in 1 M NaCl, in order to prevent non-specific protein-protein interactions. Figure 1 shows the elution pattern of the crude thymus extract of cathepsin D. The insignifi-

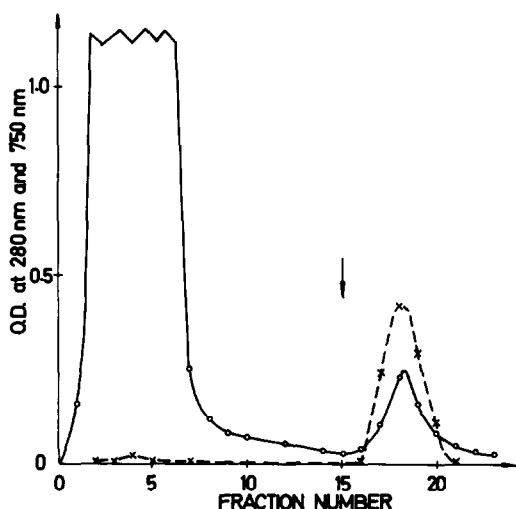


Fig.1. Elution pattern of 10 ml of crude thymus extract on 6 ml of inhibitor-CH-Sepharose, column size 1.7 \times 2.5 cm, equilibrated with 0.125 M Na-acetate in 1 M NaCl, pH 4.0. The arrow indicates the change of elution buffer to 0.15 M phosphate in 1 M NaCl, pH 8.0. Proteolytic activity (measured at 750 nm) is indicated by dotted line.

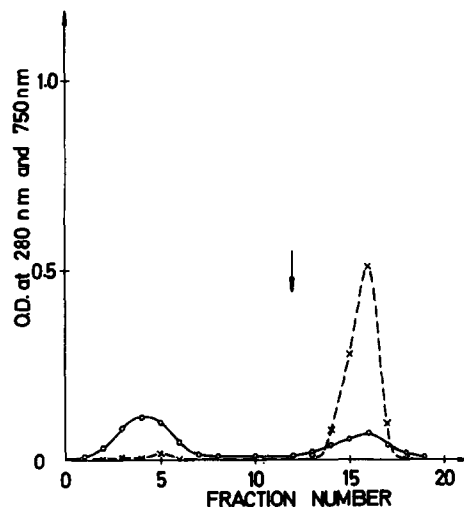


Fig.2. The second peak of fig.1 was rechromatographed on the same column under the same experimental conditions.

cant amount of cathepsin D activity in the first peak indicates that cathepsin D was selectively retarded. In order to purify cathepsin D further, fractions of the second peak were collected in two or three runs, dialyzed against the starting buffer, pH 4.0, applied to the same column previously equilibrated with starting buffer and eluted in the same manner (fig.2). Further purification was achieved by this procedure. Purification steps were monitored by disc electrophoresis and it was found that the cathepsin D was of the same purity as the one previously isolated by immobilized pepstatin [5]. In the first step a 30-fold purification was obtained and in the second a further 3-fold purification was obtained as calculated from the protein peak areas of figs.1 and 2. Similar results were obtained with the spleen extract.

CH-Sepharose 4B contains a six-carbon long spacer with a free carboxyl group for the coupling of a ligand with the free amino group. The octapeptide inhibitor was coupled by its amino terminal side, which enables positioning of its active part at a distance from the solid matrix. Although the inhibition constant of the inhibitor ($K_i = 5.2 \times 10^{-4}$ M) was slightly higher than, for example, the Michaelis constant for hemoglobin substrate ($K_M = 2.7 \times 10^{-5}$ M) [9] the affinity column effectively worked.

In a separate experiment it was found that the non-specific hydrophobic adsorption to the six-carbon spacer is of minor importance. To demonstrate this, 1 g of CH-Sepharose 4B was prepared as described and ethanolamine coupled to it by the carbodiimide procedure. A column was prepared, washed with the same buffers and the same tissue extract applied. It was found that less than 0.3% of the applied material was retarded and it had no cathepsin D activity.

The column with the immobilized octapeptide inhibitor binds cathepsin strongly up to pH 4.5, its binding capacity decreases with an increasing pH and becomes negligible above pH 5.0. The same column was used in our laboratory for several months without an appreciable loss of capacity. After prolonged use however, lumping of the gel occurred, probably due to an excessive irreversible adsorption of some unknown material which diminished the rate of flow. By occasional washing the column with a 10% solution of isopropanol in the pH 8.0 buffer, most of this material could be removed and the original flow rate restored.

These results indicate that immobilized enanthio-meric octapeptide inhibitor can be successfully used for the rapid isolation of cathepsin D from various tissue extracts.

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