CRYSTALLIZATION OF CATHEPSIN D

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Received July 29, 1976

SUMMARY

Cathepsin D from chicken liver purified to apparent homogeneity by the method of affinity chromatography on pepstatin-Sepharose, was crystallized, upon gradual precipitation with ethanol, from 1.5% protein solution in slightly acid media corresponding to the isoelectric point of the enzyme.

A number of intracellular proteinases of animal origin, or cathepsins, have been obtained in the highly purified state, but so far none of them have been crystallized.

Cathepsin D (EC 3.4.4.23) was isolated from a variety of sources by several groups of authors (1-4). Since the number of isosymes in different preparations essentially varies, it still remains to be solved whether the multiple forms observed actually exist in vivo or whether they are the products of partial autolysis appearing in the course of the isolation procedure.

Having synthesized the biospecific sorbent on the basis of pepstatin, a competitive inhibitor of acid proteinases, we managed to obtain a homogeneous preparation of cathepsin D from chicken liver, which was subsequently crystallized.

MATERIALS AND METHODS

Pepstatin, produced by Banyu Co (Japan) was kindly given by Prof. Umezawa. AN-Sepharose was produced by Pharmacia (Sweden). The synthesis of the carrier (5,6):8g of AN-Sepharose 4B was washed off with 0.5 M NaCl solution (500 ml) and with water (500 ml). Pepstatin (30 mg) was dissolved in 3 ml of dimethylformamide with subsequent addition of hydroxysuccinimide. Dicyclohexylcarbodiimide (20 mg) was dissolved in 1 ml of formamide. The solutions were cooled up to $4^{\circ}C$, poured together and allowed to stand for 1 hr in the cold room. Added to the reaction mixture were AN-Sepharose and 8 ml of solution consist-

ing of equal volumes of 0.1 M phosphate buffer, pH 6.1, and of dimethylformamide. The mixture was stirred at room t^0 for 18 hrs, the liquid was separated and the remaining gel was washed twice with small volumes of dimethylformamide. The unfixed pepstatin was determined in the aliquots by inactivation of crystalline pepsin. The gel was washed with a mixture consisting of equal volumes of dioxan and dimethylformamide and with large amounts of water. The gel was applied to a column of 20 X 100 mm and washed with water and citrate buffer, pH 3.9. Under these conditions about 20 mg of pepstatin was found to be fixed to the carrier. The activity of cathepsin D was determined by splitting of hemoglobin. One activity unit was defined as the amount of enzyme increasing the extinction of trichloroacetic filtrates at 280 nm by 1.0 per hr, with the sample containing: 2 ml of 1% hemoglobin solution (the preparation of Reanal) in 0.1 M citrate buffer, pH 3.0; 0.1 ml of the enzyme in solution; 2 ml of 5% trichloroacetic acid.

RESULTS AND DISCUSSION

Chicken livers were extracted with 0.05 M citrate buffer, pH 2.7, and fractionated with ammonium sulfate. The fraction precipitating at 40-70% saturation was desalted in Sephadex G-25 columns and adjusted to pH 4.0 with 1 M CH_3COOH; the precipitate was removed, the remaining transparent solution was passed through a pepstatin-Sepharose column, and complete adsorbtion of cathepsin D occured. The column was then washed with 0.1 M acetate buffer, pH 4.0, containing 0.5 M NaCl (solution A) in order to remove unspecifically adsorbed proteins, and with a small

Table 1. Purification of cathepsin D on pepstatin-Sepharose

	Total fraction (40-70% saturation)	Preparation obtained from pepstatin-Sepharose
Protein (mg)	16 000	30
Specific activity (units)	0,8	300
Yield from total activity (%)	100	70

volume of water. As demonstrated earlier (7), the cathepsin D-pepstatin complex completely dissociates in slightly alkaline media. The enzyme was eluted from the column with 0.1 M Na bicarbonate solution containing 0.5 M NaCl (solution B). The yield of the enzyme by protein and by specific activity is presented in Table 1.

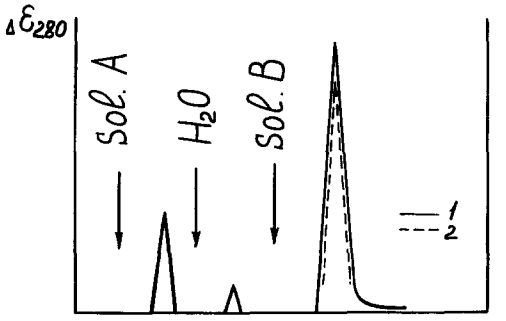

Fig. 1. Isolation of cathepsin D on pepstatin-Sepharose
1 - optical density at 280 nm
2 - proteolytic activity
For conditions and other details see the text

The preparations obtained had one band upon electrophoresis in polyacrylamide gel (Fig.2). Their activity was 50-70% that of crystalline hog pepsin, as measured by splitting of hemoglobin. In some cases, however, the slight traces of pigment were seen, imparting a yellowish colour to the solution; to eliminate these, the affinity sorption was repeated twice in the course of the crystallization procedure. The solution of cathepsin D obtained from the pepstatin-Sepharose complex was dialyzed for 3-4 hrs on a magnetic stirrer, its pH was adjusted to 4.0 by gradual adding 0.5 M CH_3COOH, and the resulting solution was applied to the pepstatin-Sepharose column, previously washed with 4 M urea and 0.1 M citrate buffer, pH 3.9. After the second elution, the enzyme was desalted by dialysis and concentrated by polyethyleneglycol (mol. wt. 20000, produced by Merck) to the volume of 1.5-2 ml, the concentration of the enzyme in solution reaching 1.5-1.7%. The solutions thus prepared were used to crystallize the enzyme.

As will be seen from the electrophoretic patterns presented (Fig.2), the preparations contained no isosymes of cathepsin D. It seems hardly possible that the isosymes, having similar physico-chemical properties,

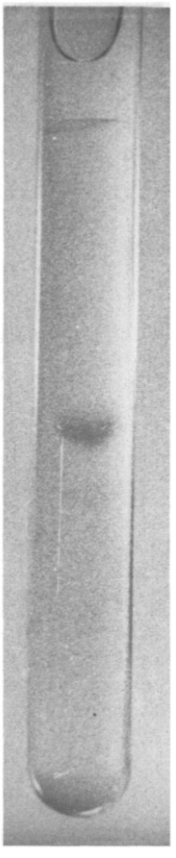

Fig. 2. Electrophoresis of purified cathepsin D in polyacrylamide gel. Electrophoresis was conducted in Tris-glycine buffer, pH 8.9. Potential gradient was 5.5 mA per sample, time was 120 min

could be separated and discarded at the ammonium sulfate fractionation step. Besides, the fractions discarded were all characterized by a very low activity of cathepsin D. The data were interpreted by us as indicating that multiple forms of cathepsin D found in chicken liver by other authors (2) are not pre-formed in vivo but arise from partial autolysis of the enzyme in the course of the preparation procedure.

Before crystallization, to the protein solution was added ethanol (up to 30% by volume). The slight dimness seen in the solution was removed by centrifugation. Crystallization was carried out in capillaries 40 mm long and 2 mm in diameter. The capillaries with the enzyme solution were covered from one end with dialysis membranes, fixed by elastic rings, and from the other end with the paraffin film, "Parafilm M". Glass vessels were filled with solution containing 25 ml of one of the following 0.05 M

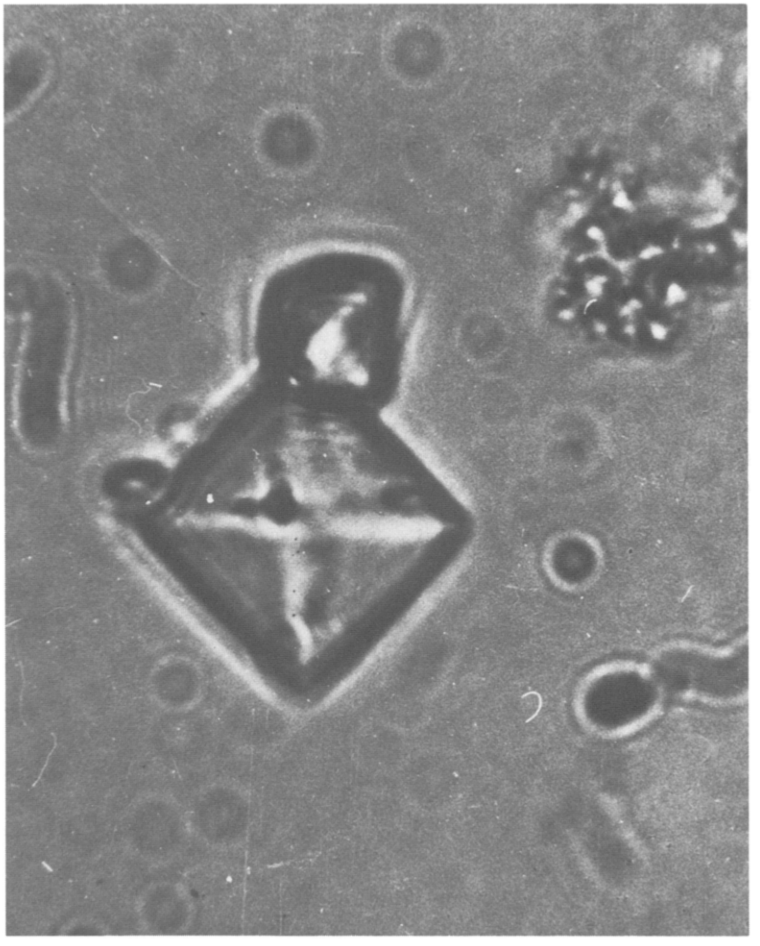

Fig. 3. Crystals of cathepsin D magnified 8000-fold.

buffers: citrate (pH 4.0), acetate (pH 4.5, 5.0, 5.5) or phosphate (pH 6.0, 6.5, 7.0) and 25 ml of ethanol (the final concentration 50%). The capillaries were placed into the vessels which were stoppered and allowed to stand in the cold room for several days. In the capillary placed into the vessel with the acetate buffer, pH 5.0, where the enzyme was under conditions closest to its isoelectric point, small rhomb-like crystals grew (Fig. 3). In other capillaries the enzyme precipitated as amorphous grains.

The procedure for obtaining larger crystals of cathepsin D to be used in X-ray structure studies is currently being developed.

ACKNOWLEDGEMENTS

We would like to thank Dr. K.F. Firfarova and Dr. M.B. Garber for helpful discussions.

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