ON THE ISOZYMES OF CATHEPSIN B_1

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1. Introduction

Like with many other enzymes, also in the case of cathepsins – the intracellular proteinases – the existence of isozymes has been observed. Press et al. [1] reported at least 10 different forms of cathepsin D observed on ion-exchange chromatography; they all showed the same enzymatic specificity when assayed with the B-chain of oxidized insulin as substrate. Using isoelectric focusing, Barrett [2] has been able to obtain three isozymes of cathepsin D with isoelectric points of 5.7, 6.0, and 6.5. Otto [3] has described the isolation of two cathepsins, B_1 and B_2, from bovine spleen. The two enzymes differ in molecular weight and in specificity when assayed with low molecular weight substrates. Cathepsin B_1 occurs in several forms, as follows from our own preceding experiments [4, 5] and also from the data of Franklin and Metrione [6]. The latter authors have obtained 4 to 5 forms of cathepsin B_1 by ion-exchange chromatography. All these forms showed the same molecular weight, the same N-terminal amino acid, and were susceptible to the same activators and the same inhibitors. The authors, however, did not investigate the enzymatic specificity of the individual forms.

In this paper we describe the modification of our earlier isolation procedure, the preparation of four forms of cathepsin B_1, their final purification by affinity chromatography on a gel with covalently bound mercury, the determination of their N-terminal amino acids, and the comparison of the specificities of these isozymes assayed with the B-chain of oxidized insulin as substrate.

Abbreviations:
BAPN : N^{α}-benzoyl-L-arginine-p-nitroanilide;
EDTA : ethylene diaminetetracetic acid;
Tris : tris(hydroxymethyl)aminomethane;
Dansyl: 1-dimethylaminonapthalene-5-sulfonyl.

2. Material and methods

A homogenate of fresh bovine spleen served as starting material for the preparation of cathepsin B_1. The extraction and precipitation with ammonium sulfate has been carried out according to Otto [3].

2.1. *Gel filtration on Sephadex G-150*

The precipitate obtained at 46–56% saturation with ammonium sulfate was dialyzed against water, concentrated by ultrafiltration, and separated by gel filtration on a column of Sephadex G-150 (Pharmacia Uppsala) in 0.01 M phosphate buffer at pH 6.9. The elution volumes of human serum albumin, egg albumin, and bovine ribonuclease were determined on the same column under identical conditions of the experiment.

2.2. *Chromatography on DEAE-Sephadex A-50*

The effluent from the above described column, corresponding with respect to its elution volume to a product of molecular weight of 24,000–25,000 and showing enzymatic activity when assayed with BAPN as substrate, was fractionated further by chromatography on a column of DEAE-Sephadex A-50 (Pharmacia, Uppsala), equilibrated in 0.01 M phosphate at pH 6.9. The proteins were eluted from the column by a stepwise gradient of NaCl (0.1 M and 0.2 M) in the same buffer.

2.3. *Affinity chromatography*

To Sepharose 4B (Pharmacia, Uppsala) activated with cyanogen bromide [7] 1-aminophenylmercuric acetate (a product of Aldrich) was bound according to Sluyterman and Wijdenes [8]. The affinity chromatography of cathepsin B_1 was carried out under conditions analogous to those used by these authors for papain.

2.4. *Assay of proteolytic activity*

To 50 μl of cathepsin B_1 solution, 1.5 ml of 0.1 M phosphate buffer at pH 6.0, 1 mM in EDTA and 25 mM in cysteine was added and the sample was activated 5 min at 40°. A solution (50 μl) of BAPN in dimethylformamide (40 mg/ml) was added to the activated sample. After 10–15 min of incubation at 40° the digestion was discontinued by the addition of 100 μl of glacial acetic acid. The *p*-nitroaniline liberated was determined photometrically at 405 nm. One unit we consider the quantity of cathepsin B_1 which under the described conditions will raise the absorbance by 0.1 in 1 min.

2.5. *Digestion of B-chain of oxidized insulin*

The B-chain (300 μg) of oxidized insulin was digested by 2 cathepsin B_1 units in 500 μl of 0.05 M pyridine acetate buffer at pH 6.0, 1 mM in EDTA and 5 mM in dithiothreitol, 17 hr at 39°. The digest was taken to dryness. The digest was taken to dryness. The specificity of the cleavage was examined by the method of peptide maps. The digest was separated on Whatman No. 3MM paper. High-voltage electrophoresis at pH 1.9 in the mixture acetic acid–formic acid–water (15:5:80, v/v) was used for the first direction and chromatography in the system butanol:acetic acid:pyridine:water (15:3:10:12, by vol) for the second direction. The technique of small 20 × 20 cm maps, prepared under analogous conditions, was used for routine analyses. The peptides were detected on the paper by 0.2% solution of ninhydrin in acetone.

2.6. *N-terminal end-group analysis*

The N-terminal amino acids were determined by a modification [9] of the Dansyl technique. The Dansyl-amino acids were identified by chromatography on 5 × 5 cm polyamide layer sheets, using the modification described by Hartley [10].

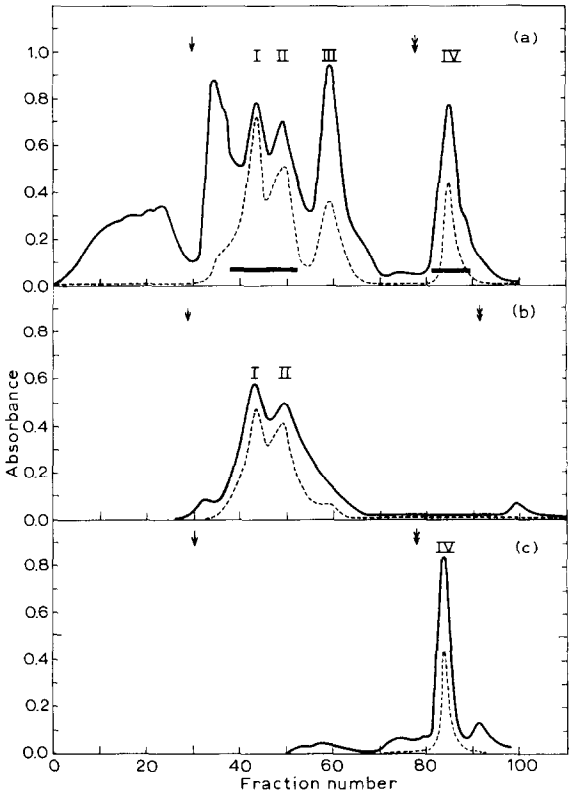

Fig. 1. a) Chromatography on DEAE-Sephadex A-50. 1200 ml of the Sephadex G-150 fraction active toward BAPN was charged on a DEAE-Sephadex A-50 column (4 × 17 cm); the colum was eluted by 0.01 M phosphate buffer at pH 6.9, 0.1 M NaCl in the same buffer (↓) and 0.2 M NaCl in the same buffer (↓), fractions 26 ml/30 min. (——) Absorbance at 280 nm; (– – – –) activity toward BAPN-(absorbance at 405 nm). b) Rechromatography on DEAE Sephadex A-50 of combined peaks no. I and II from the column presented in a. See a for experimental conditions. c) Rechromatography on DEAE-Sephadex A-50 of peak IV from the column presented in a. See a for experimental conditions.

2.7. *Disc electrophoresis*

Disc electrophoresis in 15% polyacrylamide gel was carried out in Tris-glycine buffer at pH 8.3 according to Davis [11].

3. Results and discussion

The starting material used for the preparation of cathepsin B_1 in our earlier studies [4] was the acetone

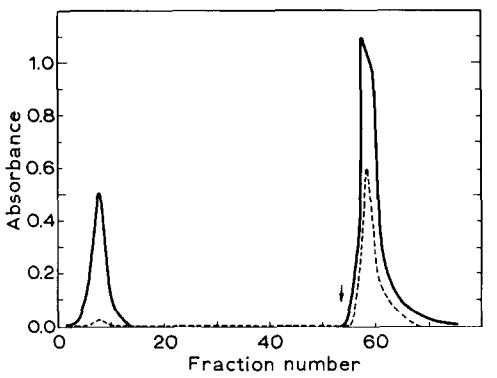

Fig. 2. Affinity chromatography of cathepsin B_1 on Sepharose mercurial column. Purification of 100 units of cathepsin B_1-IV on a Sepharose 4B mercurial column (1.6 × 12 cm), fractions 2 ml/min. ↓ Addition of $HgCl_2$ to the standard buffer.

powder of bovine spleen; this material was fractionated by precipitation with ammonium sulfate, then subjected to chromatography on CM-Sephadex, DEAE-Sephadex, and gel filtration on Sephadex G-100. In this study we have modified this procedure: we have started with a homogenate of fresh bovine spleen and used fractionation on Sephadex G-150 as another purification step following the precipitation with ammonium sulfate. This operation has enabled us a very good resolution of cathepsin C, D, and B_1 [12]; cathepsin B_1 is equilibrated by this operation in a solution suitable for the subsequent chromatographic fractionation on DEAE-Sephadex. When we fractionated cathepsin B_1 from bovine spleen on DEAE-Sephadex in our earlier isolation experiments, we always observed several protein peaks showing enzymatic activity when assayed with BAPN as substrate. Of these peaks we employed only the most acidic form of cathepsin B_1 since it showed the relatively highest homogeneity.

Cathepsin B_1, which hydrolyzes BAPN, emerges from the DEAE-Sephadex A-50 column under the conditions of ion-exchange chromatography described in the experimental part at least in four fractions (fig. 1a). The first three forms are eluted by 0.1 M NaCl, the fourth form is displaced by 0.2 M NaCl.

The occurrence of enzymes in several forms leads us always to the question as to whether we are not dealing with artifacts formed during the isolation process. In an effort to verify that the individual active forms are not artifacts and that their elution volume on chromatography is reproducible, we rechromatographed combined forms I and II (fig. 1b) and form IV (fig. 1c) under identical conditions. A comparison of fig. 1a, b, and c shows the complete reproducibility of the positions of peaks active toward BAPN as substrate. We wish to stress that we

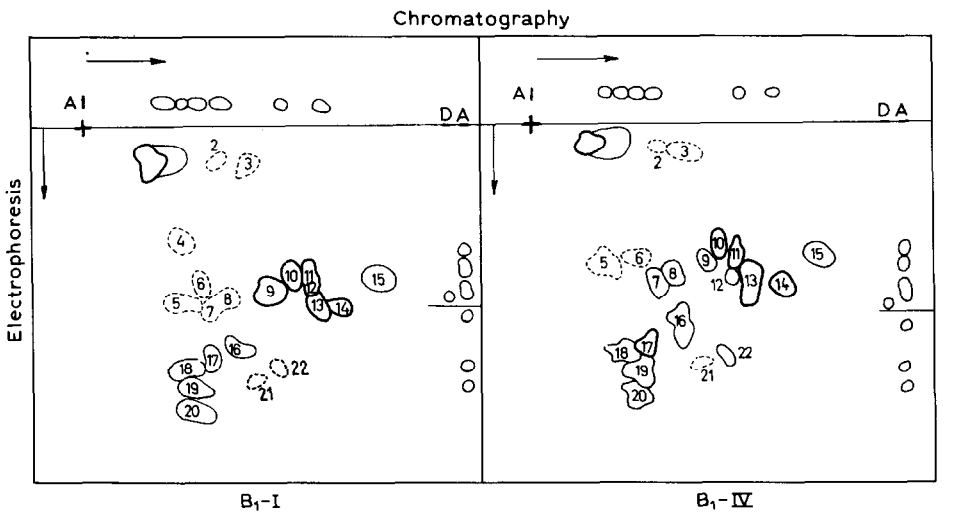

Fig. 3. Peptide maps of B-chain of oxidized insulin digested by cathepsin B_1-I and B_1-IV, see text for further details; + = application of the digest, A = reference mixture of amino acids, D = di(2,4-dinitrophenyl-)ethylenediamine as marker.

have observed analogous enzyme components during our repeated isolation experiments with cathepsin B_1 in the past few years, even in cases where entirely different conditions of isolation were used (such as, e.g. when the acetone powder of spleen was used or when the fractionation with ammonium sulfate was replaced by fractionation with acetone). It is also important that similar results were obtained also in other laboratories [6].

The examination of forms I through IV by disc electrophoresis showed their inhomogeneity. We used therefore affinity chromatography on a column of Sepharose 4B with covalently bound mercury and were able to achieve a two- to three-fold purification of all fractions of cathepsin B_1 which is an SH-enzyme. Cathepsins B_1 are bound by the SH-group of their active site to the Hg-modified carrier whereas proteins lacking free SH-groups are not retarded in the column and are eluted by the starting buffer. Active cathepsin B_1 is displaced from the column by 0.5 mM $HgCl_2$ (fig. 2). This operation will not only separate exogenous proteins contaminating cathepsin B_1 but at the same time also that part of cathepsin B_1 which no longer can be activated. Another advantage of this operation is that the unstable SH-enzyme is converted into its Hg-form which, in analogy to papain, is more stable than the SH-form. From the practical viewpoint we find that it is better to use affinity chromatography at the final stage of the purification process or at least after other high molecular weight proteins with a free SH-group (such as, e.g. cathepsin C), which considerably decrease the capacity of the carrier for cathepsin B_1, have been removed.

We have determined the N-terminal end groups of all the forms isolated by the sensitive Dansyl-technique. Leucine has been shown unambiguously to be the N-terminal amino acid of all four forms. At this instance we wish to correct our earlier information [4] on cathepsin B_1-IV reporting isoleucine and valine as its N-terminal amino acids. The N-terminal amino acids were originally examined by the phenylthiohydantoin technique and the results were misinterpreted because of incomplete separation of PTH-leucine and PTH-isoleucine. Valine was obviously derived from a contaminating, enzymatically inactive protein.

To illustrate the specificity of the forms, fig. 3 shows schematically peptide maps of the B-chain of oxidized insulin digested by cathepsins B-1 and B-IV, i.e. by the forms which differ most on chromatography on DEAE-Sephadex. There is no essential difference in the maps of digests prepared with cathepsins B_1-I and B_1-IV and with the remaining cathepsins, B_1-II and B_1-III. This leads us to postulate that the specificity of all four forms toward this substrate is essentially the same.

The results presented above, especially the finding that the individual forms of cathepsin B_1, isolated from the same tissue by different procedures, show the same N-terminal amino acid, the same specificity when examined with the B-chain of oxidized insulin, and the same molecular weight yet differ in elution volumes on ion-exchange chromatography, permit us to define [13] these forms of the enzyme as the isoenzymes of cathepsin B_1.

It remains to be shown whether the same isozymes can be obtained also from other tissues or from tissues of organisms at pathological states.

Acknowledgement

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