ISOLATION AND SPECIFICITY OF CATHEPSIN B

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1. Introduction

Cathepsin B belongs to a group of proteolytic enzymes whose active sites involve an SH-group. The enzyme was purified and described first by Fruton who observed that cathepsin B cleaves benzoyl-arginine amide, a trypsin substrate, in the presence of cysteine [1]. A detailed procedure for the purification of the enzyme was reported by Greenbaum and Fruton [2]. These authors also found other activators and inhibitors of this enzyme. They were able to activate cathepsin B by dimercaptopropanol, mercaptoethanol and glutathione while inhibition could be achieved by iodacetamide, $HgCl_2$ and partly also by *p*-chloromercuribenzoate. By contrast, cathepsin B is not inhibited either by diisopropyl-fluorophosphate or by soy-bean trypsin inhibitor. Similarly to trypsin, cathepsin B cleaves benzoyl-arginine amide and benzoyl-lysine amide. It does not hydrolyze, however, the simpler substrates of trypsin, such as L-arginine amide or L-arginine methyl ester. Cathepsin B activates trypsinogen to trypsin and catalyzes transamidation reactions [3].

This part of our more extensive study on cathepsins, predominantly on their specificity, was aimed at the isolation of homogeneous cathepsin B and at the determination of the specificity of cleavage of a polypeptide substrate, the B-chain of oxidized insulin, by this enzyme.

2. Material and methods

As starting material for the preparation of cathepsin B served the beef spleen acetone powder [4] which was extracted with 0.01 M phosphate buffer at pH 6.

2.1. *Precipitation with ammonium sulfate*

The phosphate extract was centrifuged and the supernatant was saturated to 10% of saturation with ammonium sulfate. The precipitate was removed and the supernatant was adjusted to 75% saturation with ammonium sulfate. The precipitate formed was dissolved in a minimum volume of water and the bulk of ammonium sulfate was removed by dialysis against water.

2.2. *Chromatography on* CM-*sephadex* C-25

The material was equilibrated with 0.01 M phosphate buffer at pH 6.1 by passage over a column of Bio-Gel P-10 and then chromatographed on a CM-sephadex column equilibrated with the same buffer. Under these conditions all proteolytically active components emerged in the first peak and the largest portion of contaminating proteins remained adsorbed on the resin.

2.3. *Chromatography on* DEAE-*sephadex* A-50

The active fraction was chromatographed on a DEAE-sephadex A-50 column equilibrated with 0.01 M phosphate at pH 6.4. The fraction which cleaved benzoyl-arginine-*p*-nitranilide was present both in the first peak and also in the material eluted by 0.1-0.2 M NaCl. The first fraction contained in addition to cathepsin B the bulk of cathepsin D present in the original mixture. Therefore only the material emerging as the second peak was treated further.

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2.4. *Gel filtration*

High molecular weight proteins and a part of cathepsin D were removed by gel filtration through a Sephadex G-100 column equilibrated with 0.01 M phosphate buffer at pH 7.5.

2.5. *Rechromatography on* DEAE-*sephadex* A-50

The column was equilibrated with 0.01 M phosphate at pH 7.5. The rest of cathepsin D was eluted by 0.1 M NaCl under these conditions while cathepsin B emerged in 0.2 M NaCl. This material, rich in cathepsin B, was subjected to one additional rechromatography run under the same conditions. Cathepsin B was eluted again by 0.2 M NaCl and emerged as a sharp peak containing only one proteolytically active fraction.

2.6. *Assay of proteolytic activity*

(1) The 1% solution (1 ml) of hemoglobin which had been urea-denaturated in the McIlvain buffer at pH 3.8 and the enzyme solution (0.2 ml) were incubated for 1 hr at 37°. After the addition of 3 ml of 5% trichloroacetic acid to the incubation mixture, the precipitate formed was filtered off and the absorbance of the filtrate at 280 μm was measured.

(2) A mixture containing 0.4 ml of the benzoyl-arginine-*p*-nitranilide solution (1 mg/1 ml H_2O), 0.4 ml of 0.2 M phosphate buffer containing 0.04 M cysteine at pH 5.2 and 0.4 ml of the enzyme solution was incubated for 2 hr at 37°. The amount of *p*-nitranilide liberated was determined spectrophotometrically at 410 mμ.

Disc-electrophoresis in polyacrylamide gel was carried out according to Davis [5].

2.7. *Molecular weight determination*

The molecular weight was calculated from the elution volume in which the enzyme emerged from a Bio-Gel P-60 column equilibrated with 0.01 M acetate buffer at pH 4.5, either in the presence of 0.04 M cysteine or in the absence of cysteine. As standard samples ribonuclease, ovalbumin, and serum albumin were used.

The N-terminal end group was determined by the Edman degradation technique [6].

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

The B-chain (40 mg) of oxidized insulin was dissolved in 0.05 M trimethylamine acetate buffer at pH 5.2 containing 0.04 M cysteine. The digestion with cathepsin B was allowed to proceed for 8 hr at 37°. The digest was lyophilized and cysteine from the buffer was oxidized by performic acid to cysteic acid. The material was then applied to a Dowex 50-X2 column equilibrated at 25° with pyridine formate buffer at pH 3.0. The peptides were eluted by buffers of increasing molarity and pH. Aliquots of the eluates were examined by paper chromatography in the system pyridine-*n*-butanol-acetic acid-water (10:15:3:12). The mixture of peptides was fractionated by paper electrophoresis or by high voltage electrophoresis at 4,000 V. Qualitative and quantitative amino acid analyses of peptides were performed on an amino acid analyzer according to Spackmann, Moore and Stein.

3. Results and discussion

It has been shown by Bouma and Gruber [7] that spleen represents a very rich source of cathepsin B. The ability of this enzyme to cleave the trypsin substrate, benzoyl-arginine-*p*-nitranilide, in the presence of cysteine enables us to isolate selectively this enzyme from the mixture of other proteolytic enzymes which show different specificities. When the purest cathepsin B preparation, which we obtained by systematic fractionation based on this specific test, was subjected to disc-electrophoresis in polyacrylamide gel at pH 8.4, the presence of the main zone and of one additional minor zone was observed. Additional rechromatography did not increase the purity of the preparation which, on the contrary, was sometimes even more heterogeneous, most likely due to partial autolysis during the experiment. The purest preparation of cathepsin B showed the presence of two N-terminal amino acids, i.e. of isoleucine and valine at an approximately equimolar ratio. This finding seems to suggest the existence of two polypeptide chains linked together by disulfide bonds. When subjected to gel filtration on Bio-Gel P-60 in the absence of cysteine, cathepsin B gives two active components of molecular weight 24,000 and 40,000. In the presence of 0.04 M cysteine the equilibrium is shifted significantly in favor of the component of lower molecular weight. The two components do not differ in the value of pH of optimum cleavage of the specific substrate nor in their behaviour during the interaction with inhibitors.

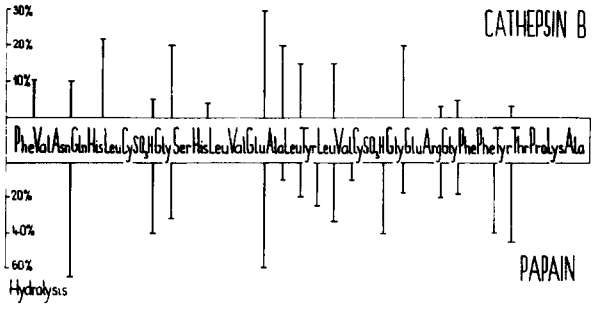

Fig. 1. Degree of hydrolysis of the B-chain of oxidized insulin with cathepsin B and papain.

Hence, the components probably represent two different quaternary structures of the same active enzyme whose existence is based on the reactivity of SH-groups.

Greenbaum and Fruton [2] found that the optimum pH for the cleavage of benzoyl-arginine amide by cathepsin B lies between pH 4.2 and 5.8. The pH optimum of cleavage of a similar substrate, benzoyl-arginine-*p*-nitranilide, by our enzyme preparation, was between pH 5 and 6. It has been shown that cathepsin B cleaves still other synthetic substrates such as e.g. leucyl-leucyl-*p*-nitranilide. This rather broad specificity of cathepsin B resembles that of papain. Another feature in common is the presence of an SH-group at the active site.

In an effort to elucidate the function of cathepsin B in more detail we assayed its specificity toward the B-chain of oxidized insulin as substrate and compared the specificity with that of papain toward the same substrate as reported by Johansen et al. [8]. As can be seen in fig. 1, the specificity of cathepsin B is rather broad and resembles that of papain. The main sites of cleavage are at the carboxyl side of glycine, alanine, leucine and glutamic acid. A fact deserving interest is that cathepsin B cleaves readily a simple substrate, i.e. benzoyl-arginine-*p*-nitranilide or amide while the bond Arg-Gly in the B-chain of oxidized insulin is cleaved only very little. The same holds true for papain.

The difference in the mode of cleavage of a simple synthetic substrate and of a polypeptide substrate can be confronted with the data reported by Gerwin, Stein and Moore [9] and by Schechter and Berger [10]. According to these and other authors [11] the cleavage is affected not merely by the nature of the two residues which form the given bond but also by other amino acid residues distant from the cleaved bond. Thus, e.g. the streptococcal proteinase cleaves preferentially the bond X-Y if X is preceded by a hydrophobic amino acid residue [9]. A similar observation was made with papain. Schechter and Berger were able to show that the peptide X.Phe.Y.Z is a good substrate for papain. The phenylalanyl residue does not participate in the cleaved bond but it is in the close environment of the amino acid residue at whose carboxyl side the papain cleavage takes place. The substrate X.Phe.Z can be bound by the enzyme but it is not cleaved and acts as a competitive inhibitor.

The fact that cathepsins like other proteinases can "perceive" in the substrate amino acids which are located at a considerable distance from the cleaved bond has been observed by us [11] with cathepsin D. When we substituted individual amino acids of the substrate by the corresponding D-amino acids we were able to show that a D-amino acid which is located four residues apart from the cleaved peptide bond (with respect to the carboxyl-terminal end of the peptide) can inhibit by 95% the cleavage of the peptide. It would thus appear that the binding site as regarded with respect to the carboxyl end of the given peptide involves at least four amino acid residues.

The situation may become even more complicated with polypeptide substrates, such as the B-chain of insulin, where the arising peptides can act as inhibitors by blocking the binding site of the enzyme.

The results of our studies on the specificity of cathepsin B cleavage do not permit as yet its regularities to be defined in such detail as with papain. A fact which in our opinion deserves consideration from a general biological point of view is the existence of such considerable similarities in the enzymatic properties of proteinases isolated from streptococci, from a plant, and from animal sources. A more detailed study on the specificity of cathepsin B may in future provide evidence in favor of the hypothesis postulating the existence of similarities in the structure of binding sites and active sites of proteinases isolated from species which are very distant from a genetic and evolutionary point of view.

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