INHIBITION OF CATHEPSIN D BY DIAZOACETYLNORLEUCINE METHYL ESTER

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1. Introduction

Diazo compounds have been employed for a long time in the modification and inhibition of enzymes. Doscher and Wilcox [1] used diazoacetamide to esterify the carboxyl groups of chymotrypsinogen. For a similar modification of ribonuclease, Riehm and Sheraga [2] employed diazoacetylglycinamide. Delpierre and Fruton [3] inactivated pepsin by diphenyldiazomethane and postulated that this reaction specifically blocks a carboxyl group in the active site of the enzyme. Rajagopalan et al. [4] studied the reaction of pepsin with diazoacetylnorleucine methyl ester and arrived at similar conclusions. Bayliss et al. [5], using radioactive diazoacetylphenylalanine methyl ester, were able to provide evidence for esterification of an aspartic acid residue in the active site of pepsin and to determine the amino acid sequence of a heptapeptide derived from the active site of this enzyme. Stepanov and Orekhovich [6] reported the inhibition of pepsin and the partial inhibition of cathepsin D by *N*-diazoacetyl-*N*-2,4-dinitrophenylethylene diamine.

Since certain similarities in the enzymatic characteristics of pepsin and cathepsin D exist [7, 8], we have investigated the effect of a specific inhibitor of pepsin (diazoacetylnorleucine methyl ester) on the activity of cathepsin D.

2. Materials and methods

Cathepsin D (EC 3.4.4.23) was isolated from bovine spleen in this laboratory. The homogeneity of the preparation was tested by disc electrophoresis at pH 8.3 and 4.5, sedimentation analysis in the ultracentrifuge, and by the results of *N*-terminal end-group analysis which showed glycine to be the only *N*-terminal amino acid [7, 9].

Diazoacetylnorleucine methyl ester was synthesized according to Rajagopalan et al. [4] in this Institute.

2.1. *Inhibition of the enzyme*

The enzyme solution (0.005 mM), was adjusted to pH 5 by the addition of 4 M acetate buffer and was mixed with a solution of copper acetate. The mixture was allowed to stand for 15 min at room temperature and a methanolic solution of diazoacetylnorleucine methyl ester was added. In control experiments, the corresponding volume of methanol was added. Samples of the mixture were withdrawn at different intervals for determination of proteolytic activity.

2.2. *Activity toward hemoglobin*

0.5 ml of a 1% solution of hemoglobin, denatured by urea at pH 3.8, was incubated with 10 µl of cathepsin D (approximately 5×10^{-5} µmole assuming a M.W. of 58,000). After 15 min of incubation at 38°C, digestion was terminated by addition of 1 ml of 5% trichloracetic acid. The mixture was centrifuged and absorbance of the supernatant measured at 280 nm.

2.3. *Activity towards the synthetic pentapeptide Ac-Gly-Phe-Leu-Gly-Phe*

The pentapeptide was synthesized by Dr. Bláha in this institute. The peptide is cleaved only between the 2nd and 3rd amino acid residue. 0.1 ml of enzyme solution (0.005 µmole in 1 ml) was added to 0.5 ml of the substrate solution (1 mg/ml at pH 5.0) and the

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mixture was incubated for 60 min at 38°C. Digestion was terminated by addition of 20 µl of 4 N NaOH and the quantity of liberated tripeptide, Leu.Gly.Phe, was determined by ninhydrin.

2.4. *Ninhydrin colorimetry*

The reaction mixture was heated with 0.5 ml of ninhydrin reagent in a boiling water bath for 20 min. The mixture was cooled and diluted with 2.5 ml of 60% ethanol. The absorbance of the solution at 570 nm was measured using a blank in which enzyme had been added after ninhydrin.

3. Results and discussion

Cathepsin D has a number of characteristics similar to those of pepsin e.g. the optimum pH for cleavage of protein substrates by cathepsin D lies in the acidic pH-region; the B-chain of oxidized insulin is cleaved at very similar sites by both enzymes, the most susceptible peptide bond being that at the carboxyl side of phenylalanine. On the other hand, cathepsin D requires a larger peptide substrate than pepsin. The smallest peptide substrate of cathepsin D known is a pentapeptide [10] while pepsin cleaves simple di- and tripeptides. These differences can possibly be explained by differences in the organization of the binding sites; this, however, does not eliminate the possibility that the mechanism of the catalytic reaction may be the same for both enzymes.

Cathepsin D cannot be classified either as an SH-enzyme or as an enzyme of the serine type. We could not inhibit cathepsin D by *p*-bromphenacyl bromide which reversibly blocks the carboxyl group at the active site of pepsin [11]. On the other hand, cathepsin D is blocked irreversibly by diazoacetylnorleucine methyl ester in the presence of Cu^{2+} (fig. 1). The concentration of copper acetate (a 100 to 500-fold molar excess) in the reaction mixture does not markedly effect the proteolytic activity of cathepsin D. The same is true for diazoacetylnorleucine methyl ester in the same molar excess. If, however, the enzyme is allowed to react with diazoacetylnorleucine methyl ester after preincubation with the copper salt, it loses its activity. Since diazoacetylnorleucine methyl ester is unstable in aqueous media and since we used considerably diluted solutions of the enzyme, we preferred higher concentrations of reagents and shorter incubation periods. We do not yet know how Cu^{2+} participates in

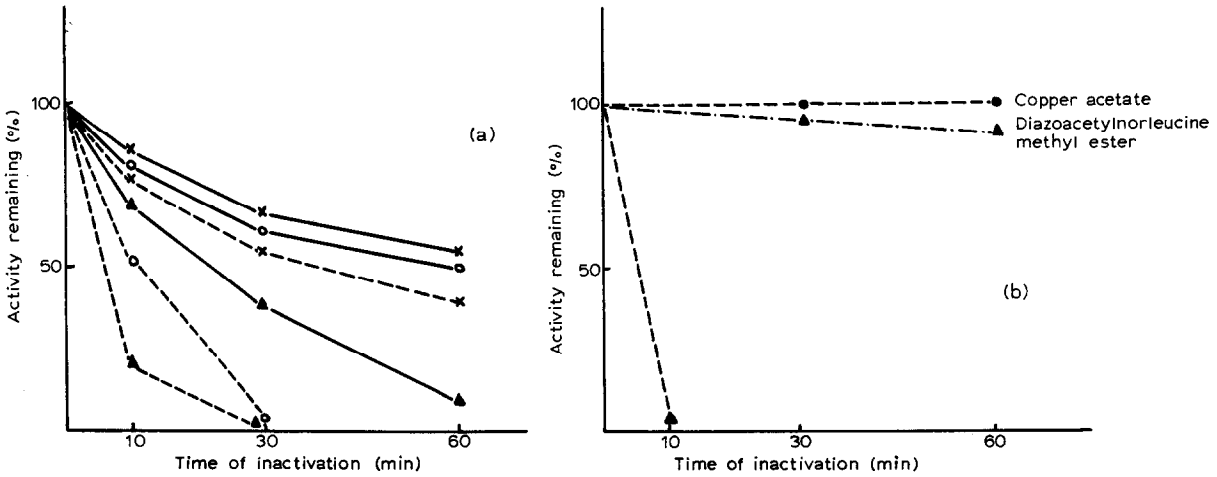

Fig. 1. Inactivation of cathepsin D by diazoacetylnorleucine methyl ester
a) Proteolytic activity toward hemoglobin
b) Proteolytic activity toward pentapeptide Ac-Gly-Phe-Leu-Gly-Phe
—— 100-fold molar excess for copper acetate.
- - - 500-fold molar excess of copper acetate.
x 100-fold molar excess of diazoacetylnorleucine methyl ester.
o 250-fold molar excess of diazoacetylnorleucine methyl ester.
▲ 500-fold molar excess of diazoacetylnorleucine methyl ester.

the reaction. An allosteric effect on the enzyme molecule may be involved but it is possible that Cu^{2+} exerts a catalytic effect on the reaction of the enzyme with the diazo compound. It has been reported that 1 mole of pepsin can bind 9.9 moles of Cu^{2+} at pH 5.6, but we do not know how this binding affects the three-dimensional arrangement of the molecule [12].

The fact that cathepsin D, similarly to pepsin, is inhibited by diazoacetylnorleucine methyl ester provides additional evidence that the mechanisms of action of these two enzymes are similar. Since the carboxyl group of the aspartate residue in the active site of pepsin has, by use of a similar reagent, been unambiguously demonstrated to play a key role, by analogy, a similar mechanism of inhibition of cathepsin D is assumed. The final proof of this assumption requires the isolation of a conveniently labeled peptide derived from the active site and determination of its amino acid sequence. This problem will be further studied.

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