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THE LIMITED PROTEOLYSIS OF RABBIT MUSCLE ALDOLASE BY CATHEPSIN B1

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Summary: Rabbit muscle aldolase is inactivated by cathepsin B1 to approximately 10 percent of the original activity for fructose-1,6-bisphosphate cleavage without change in the fructose-1-phosphate cleavage activity. Activity loss is related to release of one mole of the dipeptide, alanyl-tyrosine, per mole of the enzyme. The additional three moles of the peptide are released without further loss of the residual activity.

Tissue proteinases have been considered to play an important role in the degradation of intracellular proteins. Cathepsin B1 which is characterized to hydrolyze α-N-benzoyl-L-argininamide[1] and also α-N-benzoyl-DL-arginine-p-nitroanilide was reported to inactivate some enzymes involved in the glycolysis pathway such as glucokinase, pyruvatekinase and fructose-1,6-bisphosphate aldolase (1) and also to modify fructose-1,6-bisphosphatase (2).

Rabbit muscle aldolase may provide a significant information for the studies on the degradation of proteins by cathepsin B1 at molecular level, because the primary structure of subunits of the enzyme has been elucidated (3).

In the present report we reveal that the inactivation of aldolase by the cathepsin was attributed to the hydrolysis of a specific peptide linkage near the COOH-terminal region of the polypeptide chain, suggesting a possibility that cathepsin B1 is concerned with the initial stage in aldolase degradation and with the regulation of aldolase activity in tissues.

MATERIALS AND METHODS

Rabbit muscle aldolase was prepared according to Taylor (4) as modified by Lai (5) and was recrystallized three times. Cathepsin B1 was prepared from rabbit liver by the method of Otto (1) and further purification was performed as follows. After Sephadex G-75 gel filtration, cathepsin B1 fraction was chromatographed on a DEAE-Sephadex A-50 column with a linear gradient elution of NaCl concentration (0-0.4 M) in 10 mM phosphate buffer, pH 6.5, containing

[1] The following abbreviations are employed: BAA, α-N-benzoyl-L-argininamide; BAPA, α-N-benzoyl-DL-arginine-p-nitroanilide.

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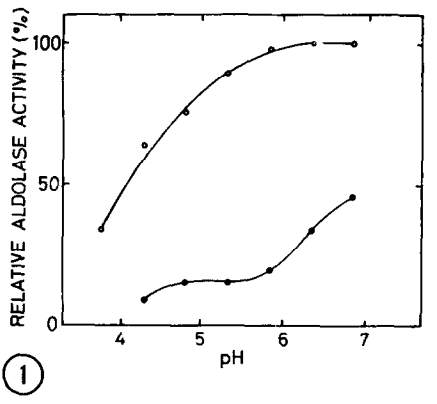
1 mM each of EDTA and β-mercaptoethanol. Cathepsin B1 was eluted in the fraction of 0.08-0.12 M NaCl concentration and the preparation was BAPA-hydrolyzing activity of 1.1 μmoles per hour per mg of protein under the assay condition of Otto (1). Aldolase activity was determined according to the method of Racker (6) using 1 mM Fru-P_2 or 10 mM Fru-1-P as substrates. The peptide was preparatively obtained as follows. Aldolase (80 mg) was incubated with cathepsin B1 (2 mg) in 0.1 M citrate buffer, pH 5.3, containing 1 mM each of EDTA and β-mercaptoethanol in a total volume of 8 ml at 27° for 30 minutes and was applied directly on a Sephadex G-25 column (3 x 75 cm) equilibrated with 0.05 M ammonium bicarbonate, pH 8.0 and eluted with the same solution. After protein fraction was eluted, the peptide fraction obtained was lyophilyzed and subjected to preparative paper electrophoresis at 25 v/cm for 3 hours in the buffer at pH 3.6 (pyridine:acetic acid:water=1:10:89). Amino acid analysis was carried out according to Spackman et al. (7). Sequence of the peptide was determined by the method of Edman (8) with a slight modification as described by Lai (5). For the identification of phenylthiohydantoin derivatives of amino acids, thin layer chromatography with silica gel plates (Merk 60 F-254) was performed as described by Jeppsson and Sjöquist (9). Protein concentration was determined by measuring the absorbance at 280 nm using an extinction coefficient of 0.91 for aldolase (10) and by the method of Lowry et al. (11) for cathepsin B1 using bovine serum albumin as a standard. Peptide was determined by the Fluorescamine method (12) using a dipeptide, L-alanyl-L-tyrosine, as a standard.

RESULTS AND DISCUSSION

Fig. 1 shows the effect of pH on the inactivation of aldolase with cathepsin B1. The lower the pH of the reaction mixture was, the more enzymatic inactivation was observed. However, remarkable decrease in aldolase activity without the addition of the cathepsin was observed below pH 5.0. By comparing the control activity with the residual activity after the cathepsin treatment, apparent pH optimun for the enzymatic inactivation of aldolase was determined to be 5.3. This value is intermadiate between 6.2 and 4.5 reported as optimum pH of cathepsin B1 activity with BAA or BAPA and with hemoglobin as substrates, respectively (1).

In order to confirm that the inactivation is due to the hydrolysis of the peptide chain, the isolation and identification of peptide released were carried out from a trichloroacetic acid soluble fraction of the reaction mixture in which aldolase was treated with the cathepsin at pH 5.3 for 30 minutes. On paper electrophoresis at pH 3.5 the fraction was found to contain only a single ninhydrin positive peptide. From the result of amino acid analysis it contained an equivalent amount of alanine and tyrosine, and the amount of the peptide released at 30 minutes of incubation was estimated to be



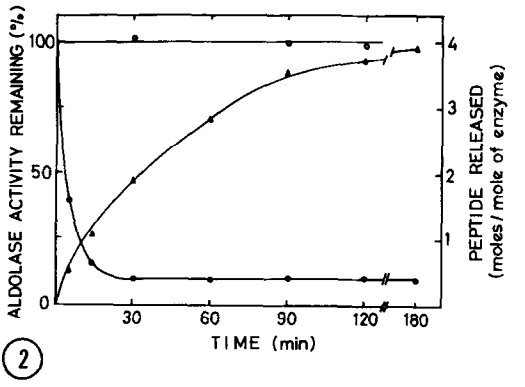


Fig. 1. Effect of pH on aldolase inactivation with cathepsin B1. Aldolase (0.6 mg) was incubated with (—●—) and without (—○—) cathepsin B1 (5 μg) in 0.1 M sodium citrate buffer containing 1 mM each of EDTA and β-mercaptoethanol at various pHs at 27° for 30 minutes. Aliquots were assayed for Fru-P_2 cleavage activity. The pH of each reaction mixture was measured immadiately after an aliquot was removed.

Fig. 2. Time dependence of inactivation of aldolase with cathepsin B1. The reaction mixture (2.0 ml) containing aldolase (17.3 mg), cathepsin B1 (0.45 mg), 1 mM EDTA and 1 mM β-mercaptoethanol in 0.1 M sodium citrate buffer, pH 5.3, was incubated at 27°. Aliquots were analyzed for Fru-P_2 cleavage activity (—●—) and for Fru-1-P cleavage activity (—○—). For the determination of peptide released (—▲—) aliquots (0.1 ml) were precipitated by the addition of 10% trichloroacetic acid (0.1 ml) and centrifuged at 3,000 rpm for 10 minutes. The supernatant (0.05 ml) were used for the Fluorescamine method (12).

1.8 moles per mole of aldolase. By Edman degradation the structure of the peptide was determined to be alanyl-tyrosine. The peptide was also identified with authentic sample of L-alanyl-L-tyrosine by thin layer chromatography.

No change in patterns of sodium dodecylsulfate disc gel electrophoresis was observed between the original and the cathepsin-treated enzymes. This finding shows little change in the primary structure of aldolase subunits and the peptide release from the COOH-terminal in consideration of established amino acid sequence of aldolase molecule (3). The specificity of cathepsin B1 for protein substrate is difficult to be established. However, it is noteworthy that of two histidyl-alanine linkages (residues 235 to 236 and 359 to

360, respectively) in a polypeptide chain of aldolase subunits, the second peptide linkage near the COOH-terminal is susceptible to be hydrolyzed by cathepsin B1 under the condition employed.

The time-dependent inactivation of aldolase with cathepsin B1 is shown in Fig. 2. The Fru-P_2 cleavage activity rapidly decreased within 15 minutes to about 10 percent of the original activity, and no further inactivation was observed. On the other hand, the activity with Fru-1-P was not altered during the treatment with the cathepsin. The amount of the peptide released increased progressively with time after the residual activity with Fru-P_2 was reduced to a constant value as shown in Fig. 2, and finally reached approximately four moles equivalent per mole of aldolase.

A tyrosine residue at the COOH-terminal of aldolase subunit has been reported to play a role in maintaining a conformation of catalytic site (13). Removal of tyrosine residues by carboxypeptidase digestion results in a decrease of the Fru-P_2 cleavage activity to about 5 to 7 percent of the original with little or no change in the Fru-1-P cleavage activity. Tyrosine released is limited to three moles per mole of aldolase, followed by release of two moles of alanine and activity loss is proportional to tyrosine released (14, 15). In the case of lobster aldolase (16), tyrosine is the only amino acid released and loss of activity with Fru-P_2 is complete when the first tyrosine is removed. However, catalytic properties of the modified aldolase by the cathepsin are similar to those of rabbit aldolase digested with carboxypeptidase. The results obtained in this paper suggest that a decrease in the Fru-P_2 cleavage activity is related to release of the first dipeptide per four subunits of aldolase and the proteolytic modification finally extends over the other three subunits without further change in the catalytic property. The differences of susceptibility with cathepsin B1 between the Fru-P_2 and Fru-1-P cleavage activities, and the retaining activity with Fru-P_2 also suggest that the limited proteolysis in the COOH-terminal region together with a minor change in aldolase molecule may control the primary function of aldolase in glycolysis.

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