THE ESTERASE AND PEPTIDASE ACTIVITIES OF AN ELASTOLYTIC ENZYME OF Actinomyces rimosus

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We have previously reported the isolation of the homogeneous elastolytic enzyme of Actinomyces rimosus and a comparison of its hydrolytic specificity with that of pancreatic elastase [1, 2]. It is known that the latter exhibits a high specificity for amino-acid sequences containing aliphatic amino acids, particularly alanine, both in natural and in synthetic substrates. On this basis, a number of authors [3, 4] have proposed as highly specific substrates for pancreatic elastase various esters and amides of N-acetylmono-, di-, tri-, and -tetraalanines (with $-OCH_3$, $-OC_6H_5$, $-OC_6H_4-NO_2$, etc., groups).

The task of the present work was to give a comparative estimate of the elastase activities of the homogeneous elastolytic enzyme of Actinomyces rimosus and pancreatic elastase with respect to synthetic substrates consisting of methyl esters of N-acetylmono-, -di-, and -trialanines. The substrates that we synthesized are soluble in water and are hydrolytically stable at pH 7-10. The interaction of the enzyme of Actinomyces rimosus with the substrates was performed under conditions similar to those for pancreatic elastase. It must be observed that, in contrast to the latter enzyme, the one isolated from Actinomyces rimosus showed no esterase or peptidease activity in relation to the methyl esters of N-acetylmono- and -dialanines even at an enzyme–substrate ratio of 2:1. Only in the case of the methyl ester of N-acetyltrialanine as substrate did the enzyme begin to exhibit a high esterase activity.

Below we give the results of a comparison of the values of K_m of the esterase activity of the enzyme from Actinomyces rimosus, which we determined by a colorimetric method at pH 8 [7, 8], and K_m from pancreatic elastase [5] and the combined proteolytic enzymes from Streptomyces griseus which are known as "pronase" [6], determined by a potentiometric method [5]:

Enzyme	K, mM	V, mM/sec
Actinomyces rimosus	1,8	$0{,}78 \cdot 10^{-3}$
Pancreatic elastase	0,43	—
Pronase	1,5	—

The values of K_m for all three elastases are of the same order, which shows their related specificity in relation to the methyl ester of N-acetyltrialanine.

In order to determine whether the hydrolysis of the ester bond is the only reaction or if cleavage of the peptide bond also takes place, we performed a parallel determination of the amine nitrogen by the method of Ryle and Porter [9]. As the results showed, at pH 8 the amount of amine nitrogen did not increase in the first ten minutes, which shows the absence of cleavage of the peptide bonds in the substrate, and the same thing is observed in the case of pancreatic elastase [5]. Subsequently, an increase in the amount of amine nitrogen does take place as the result of the hydrolysis of the peptide bond. This two-stage nature of the process is apparently due to the fact that in the case of this substrate the esterase activity is expressed more strongly than the peptidase activity. It is also likely that the peptidase activity begins to be shown only when sufficiently large amounts of N-acetyltrialanine have been formed and is not shown in relation to its methyl ester. The kinetics of the liberation of amine nitrogen are linear up to 50% conversion of the substrate which, in this case, must be considered to be N-acetyltrianiline. The dependence of the rate of hydrolysis of the peptide bonds on the concentration of the substrate obeys the Michaelis–Menten equation: K_m and V are 14.6 mM and $0.95 \cdot 10^{-5}$ M/sec·mg, respectively. Thus, the elastase of Actinomyces rimosus is largely similar to pancreatic elastase. However, the fact that it is inactive with respect to such substrates as the methyl esters of N-acetylmono- and -dialanines indicates that its active center has fundamental differences. This is possibly connected with the fact that, as has been shown previously [1], the elastase of Actinomyces rimosus is metalloenzyme.

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EXPERIMENTAL

The substrates were synthesized from L-alanine. The purity of the compounds was checked by thin-layer chromatography in a fixed layer of silica gel (250 mesh, plates 7.5 × 2.5 cm) in the following systems: 1) water–acetic acid–secondary butanol (30 : 10 : 100) and 2) secondary butanol–3% ammonia (100 : 44). The elastase of Actinomyces rimosus was isolated as described previously [1]; its activity was 180 units/mg, evaluated from the hydrolysis of elastin obtained from bovine nuchal ligament and stained with the dye Remazol Brillant Blau – RBB (Hoechst, GFR), which is called RBB-elastin.

The esterase activity was determined by Hestrin's colorimetric method [7, 8] on an SF-16 spectrophotometer with thermostated cells. The hydrolysis of the substrate by the enzyme was performed at 20°C in 0.025 M tris-HCl (pH 8) containing $1 \cdot 10^{-4}$ M potassium ions; the concentration of the substrate was 0.5-1.33 mg/ml and that of the enzyme 10 μg/ml. The amount of substrate hydrolyzed was determined from a calibration curve plotted for various concentrations of the substrate. The peptidase activity was monitored from the increase in the amount of amine nitrogen during the hydrolysis of the methyl esters of N-acetyldi- and -trialanines [9].

The enzymatic hydrolysis was stopped by the addition of 0.5 ml of 0.5 N HCl, 0.5 ml of 0.15 M tris-HCl, 1 ml of ninhydrin reagent, and 3 ml of 60% ethanol. The measurements were performed at 670 nm. The concentration of substrate was 0.33-1.33 mg/ml and of enzyme $1.5 \cdot 10^{-2}$ mg/ml. The values of K_m and V both for the esterase and for the peptidease activities were determined by the method of Lineweaver and Burk [10]. The mean square deviations of K_m and V were 0.5%.

Synthesis of the Substrates. The hydrochloride of alanine methyl ester (I) was obtained by the standard method in methanol solution from alanine and thionyl chloride; yield 82%, mp 108-111°C.

Acetylalanine methyl ester (II) was obtained by the standard method from (I), treated with IRA-401 in the OH^- form, and acetic anhydride in absolute pyridine; yield 63%, mp 89-92°C, R_f 0.65 (system 1) and 0.8 (system 2).

The methyl ester of benzyloxycarbonylalanylalanine (III) was obtained by the mixed-anhydride method starting from 3 g of benzyloxycarbonylalanine dissolved in chloroform, 1.41 ml of isobutyl chloroformate, and 1.85 ml of triethylamine; yield 84%, mp 105-106°C (from a mixture of ethyl acetate and hexane), R_f 0.9 (system 1) and 0.8 (system 2).

The hydrochloride of dialanine methyl ester was obtained from (III) by hydrogenation over catalytic palladium in methanol solution; yield 96%. mp 108-109°C (from methanol). R_f 0.56 (system 1) and 0.55 (systen 2).

The methyl ester of benzyloxycarbonylalanylalanylalanine was obtained from (III) in a similar manner to the synthesis of (III) with a yield of 68%, mp 175-176°C (from ethanol), R_f 0.8 (system 1) and 0.83 (system 2)

The hydrochloride of trialanine methyl ester (IV) was obtained in a similar manner to the synthesis of the hydrochloride of dialanine methyl ester, yield 76%, mp 149-151°C (from methanol), R_f 0.58 (system 1) and 0.65 (system 2).

The methyl ester of acetyltrialanine was obtained from (IV) in the same way as (II) with a yield of 71%, mp 248-250°C, R_f 0.65 (system 1) and 0.75 (system 2).

SUMMARY

1. It has been shown that the values of K_m for the esterase activity of elastase of Actinomyces rimosus and for pancreatic elastase are of the same order of magnitude.

2. The elastase of Actinomyces rimosus, unlike the pancreatic enzyme, is capable of hydrolyzing only the methyl ester of N-acetyltrialanine, and not the mono and di derivatives.

3. It has been found that in the initial stage of the hydrolysis reaction it is mainly the ester bonds that are cleaved.

LITERATURE CITED

1. Yu. A. Rassulin, L. R. Radzhabov, E. D. Kaverzneva, V. A. Shibnev, and G. S. Erkomaishvili, Izv. Akad. Nauk SSSR, Ser. Khim., No. 3, 687 (1974).
2. L. R. Radzhabov, Yu. A. Rassulin, L. I. Mar'yash, and V. A. Shibnev, Bioorg. Khim., 1, 554 (1975).
3. D. M. Shotton, Methods in Enzymology, 19, 113 (1970).
4. A. Gertler and T. Hofman, Can. J. Biochem., 51, 121 (1973).
5. A. Gertler and T. Hofman, Can. J. Biochem., 48, 384 (1970).
6. A. Gertler and M. Trop, Europ. J. Biochem., 19, 90 (1973).
7. J. Bieth and J. Meyer, Anal. Biochem., 51, 121 (1973).
8. S. Hestrin, J. Biol. Chem., 180, 249 (1949).
9. A. P. Ryle and R. R. Porter, Biochem. J., 73, 75 (1959).
10. H. Lineweaver and D. Burk, J. Am. Chem. Soc., 56, 658 (1934).