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ESTEROLYTIC, ELASTASE-LIKE ACTIVITY OF PURIFIED ALKALINE PROTEINASE FROM *ASPERGILLUS SOJAE*

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SUMMARY

1. The ester bond of acetyl-L-alanyl-L-alanyl-L-alanine methyl ester is hydrolysed by the alkaline proteinase from *Aspergillus sojae* in the pH range of 7–10. The K_m and k_{cat} values at 30°, pH 8.5, are 21.6 mM and 2800 sec^{-1}.

2. The alkaline proteinase possesses weak esterolytic activity on α-*N*-benzoyl-L-arginine ethyl ester and *N*-acetyl-L-tyrosine ethyl ester. This activity is not inhibited by natural and synthetic inhibitors of trypsin and chymotrypsin.

3. Alkaline proteinase does not hydrolyse elastin since its slightly acidic nature prevents its adsorbtion on insoluble elastin.

Alkaline proteinase from *Aspergillus sojae* is a DFP-sensitive enzyme with a molecular weight similar to that of pancreatic DFP-sensitive serine proteinases[1,2]. However, unlike trypsin (EC 3.4.4.4) or chymotrypsin (EC 3.4.4.5) but similarly to elastase (EC 3.4.4.7), it is unstable below pH of 3–4 and is not inactivated by tosyl-L-lysine and tosyl-L-phenylalanine chloromethylketones[1,3]. The recent introduction of a highly specific esteratic substrate of elastase[4], acetyl-L-alanyl-L-alanyl-L-alanine methyl ester ($AcAla_3OMe$), enabled us to compare the esterolytic activity of the alkaline proteinase to that of elastase. The esterolytic activities on α-*N*-benzoyl-L-arginine ethyl ester (BzArgOEt) and on *N*-acetyl-L-tyrosine ethyl ester (AcTyrOEt) were also tested.

The esterolytic activity on $AcAla_3OMe$, BzArgOEt and AcTyrOEt was estimated titrimetrically. The reaction was carried out in KCl–Tris–$CaCl_2$ buffer[5] (pH 8.5) at 30° in a pH-stat Radiometer pH meter 26 with Titrator 11. The volume of the reaction mixture was 1 ml. Since the *A. soja* alkaline proteinase is strongly adsorbed on the electrodes, they were rinsed after each assay with dilute detergent for 1 min and only then washed with water. K_m values were calculated from Lineweaver–Burk plots obtained with 4–40 mM substrate concentrations. The k_{cat} values were calculated from the v_{max} values and the concentration of the enzyme. The *A. sojae* alkaline proteinase concentrations were determined spectrophotometrically at 280 nm

Abbreviations: $AcAla_3OMe$, acetyl-L-alanyl-L-alanyl-L-alanine methyl ester; BzArgOEt, α-*N*-benzoyl-L-arginine ethyl ester; AcTyrOEt, *N*-acetyl-L-tyrosine ethyl ester.

TABLE I

KINETIC DATA CALCULATED FOR *A. sojae* ALKALINE PROTEINASE SUBSTRATES AS COMPARED TO THOSE FOR ELASTASE, TRYPSIN AND CHYMOTRYPSIN

Enzyme used	*Substrate*	K_m *(mM)*	k_{cat} *(sec^{-1})*	*Reference*
A. sojae alkaline proteinase	$AcAla_3OMe$	21.6	2800	This work
	BzArgOEt	8.3	26	This work
	AcTyrOEt	10.9	30	This work
Porcine elastase*	$AcAla_3OMe$	0.43	73	GERTLER AND HOFMANN[4]
Bovine trypsin*	BzArgOEt	0.05	22	CUNNINGHAM[6]
Bovine α-chymotrypsin*	AcTyrOEt	0.70	193	CUNNINGHAM[6]

* The data for these enzymes were calculated for pH 8.0 and 25°.

assuring a specific extinction $E^{1\%}_{1\,cm}$ of 8.98 and a molecular weight of 22 600 (see ref. 2).

The kinetic data compared to those of elastase, trypsin and chymotrypsin are presented in Table I. Although the K_m value of the alkaline proteinase on $AcAla_3OMe$ is significantly higher than that of elastase, indicating weaker affinity, the k_{cat} is extremely high. The alkaline proteinase also possesses some esterolytic activity on BzArgOEt and AcTyrOEt. In order to find out whether these two activities result from contaminating trypsin- and chymotrypsin-like enzymes or are intact properties of the enzyme itself, the alkaline proteinase was preincubated with synthetic or natural trypsin and chymotrypsin inhibitors, and the esterolytic activity on all three substrates was examined. The preincubation of enzyme *plus* inhibitor was carried out in 25 mM sodium phosphate buffer (pH 7.6) at 25°. The enzyme concentration was $2.5 \cdot 10^{-6}$ M (64 μg/ml); the tosyl-L-lysine and tosyl-L-phenylalanine chloromethylketones were added in excess in a 100:1 molar ratio, and the crystalline soybean trypsin inhibitor of KUNITZ[7] or pure soybean trypsin and α-chymotrypsin inhibitor AA[8] were added in 10:1 weight ratio. Since no significant decrease in activity on any of the esteratic substrates could be observed even after 30 h of incubation and since the enzyme is homogeneous in ultracentrifugal and electro-

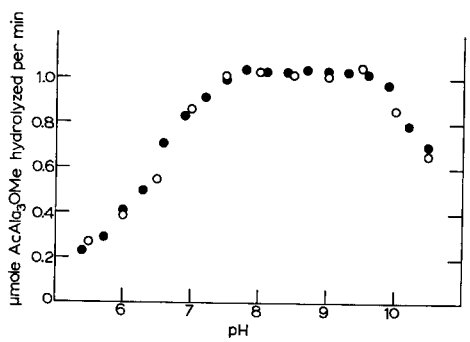

Fig. 1. pH dependence of hydrolysis of $AcAla_3OMe$ (20 mM) by *A. sojae* alkaline proteinase (0.25 μg/ml) at 30°. ○ and ●, two separate experiments.

phoretic analyses[1,2], it may be concluded that the esterolytic activity on BzArgOEt and AcTyrOEt is its intact property.

The pH dependence of the reaction was studied in the pH range 5.5–10.5 with 20 mM $AcAla_3OMe$ as a substrate (Fig. 1). As can be seen there is a broad activity optimum between pH 7.5 and 10.0 much resembling porcine elastase[4]. In order to ascertain that only the ester bond of $AcAla_3OMe$ and not any of the peptide bonds are split under assay conditions, hydrolysis of the substrate was allowed to proceed to completion at pH 7.5 and 10.0. Since the total alkali uptake corresponded to 1 mole/mole substrate in both pH values, it was concluded that only the ester bond is hydrolyzed under the assay conditions. Cleavage of any of the peptide bonds would have produced up to one additional ion at pH 10.0. No free alanine could be detected in amino acid analysis of $AcAla_3OMe$ after enzymatic hydrolysis.

In order to find out whether only the synthetic or also the natural substrate of elastase, elastin, is hydrolysed by the alkaline proteinase, its elastolytic activity was assayed by the Congo-Red elastin method[9]. It was found that the alkaline proteinase was completely inactive on this insoluble substrate. The lack of activity may however be explained by the fact that the alkaline proteinase, unlike elastase and other basic proteins, is not adsorbed on elastin at the pH range 5.5–9.0 in low ionic strength environment[10]. The adsorbtion of elastase and other basic proteins was found to be a non-specific electrostatic process that occurs between negative-charged groups of elastin and positive-charged groups of these proteins[10]. Since *A. sojae* alkaline proteinase is a slightly acidic protein with p*I* of 5.1 (see ref. 2), it is not adsorbed on elastin, and although its specificity resembles that of elastase, it cannot hydrolyse the insoluble elastin.

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