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ISOLATION OF ALKALINE PROTEINASES FROM *ASPERGILLUS ORYZAE* BY ONE-STEP AFFINITY CHROMATOGRAPHY ON OVOINHIBITOR–SEPHAROSE COLUMN

G. FEINSTEIN[a] AND A. GERTLER[b]

[a]*Department of Biochemistry, Tel Aviv University, Tel Aviv, and* [b]*Faculty of Agriculture, Hebrew University of Jerusalem, Rehovot (Israel)*

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SUMMARY

1. Alkaline proteinases from a crude preparation of *Aspergillus oryzae* were isolated by one-step affinity chromatography on a chicken ovoinhibitor–Sepharose column. The yield of the activity was 55% and the purified fraction had about 300-fold higher specific activity than the starting material.

2. The purified fraction was found to be homogeneous in size but consisted of at least four electrophoretically distinct bands. The amino acid composition was estimated and found to be very similar to other known alkaline proteinases from *Aspergillus* species, thus indicating a strong genetic relationship.

3. The esterolytic activity of the purified fraction was tested on specific esteratic substrates of pancreatic serine proteases. The purified fraction was active on all 3 substrates, thus indicating a rather broad specificity and the kinetic parameters were almost identical to those of pure alkaline proteinase from *Aspergillus sojae*. Like the latter, the purified fraction had an extremely high k_{cat} on acetyl tri-L-alanine methyl ester.

INTRODUCTION

Purification of extracellular alkaline proteinases from *Aspergillus oryzae*[1–3] and other *Aspergillus*[4–6] has been reported by several authors.

Inhibition of alkaline proteinases from *Aspergillus* by chicken ovoinhibitor was demonstrated by Matsushima[7], and recently we found that the alkaline proteinases from *Aspergillus sojae* and porcine pancreatic elastase are bound to the same or to two close inhibitory sites of ovoinhibitor[8]. It was also reported that ovoinhibitors from other avian species inhibit alkaline proteinase from *A. oryzae*[9].

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

Purification of trypsin and chymotrypsin by affinity chromatography on chicken and turkey ovomucoid–Sepharose resins was recently described by one of the authors[10,11] and the present report describes the use of a chicken ovoinhibitor–Sepharose column for isolation of alkaline proteinases from *A. oryzae*.

MATERIALS AND METHODS

Materials

α-*N*-Acetyl-L-tyrosine ethyl ester (AcTyrOEt) and α-*N*-benzoyl-L-arginine ethyl ester (BzArgOEt) were obtained from British Drug House (London, England) and *N*-acetyl-L-alanyl–L-alanyl–L-alanine methyl ester ($AcAla_3OMe$) from Miles-Yeda (Rehovot, Israel). Chicken ovoinhibitor was prepared from chicken ovomucoid by salt fractionation (Procedure C)[12], and ovoinhibitor–Sepharose was prepared by coupling the ovoinhibitor to Sepharose by the cyanogen bromide procedure[13], as described earlier[10]. Pure alkaline proteinase from *A. sojae*[5] was a gift of Dr K. Hayashi. A crude *A. oryzae* preparation Polidase-S was purchased from Schwarz/Mann Co. (Orangeburg, N.Y., U.S.A.).

Assay of enzymatic activities

The esterolytic activity on $AcAla_3OMe$, BzArgOEt and AcTyrOEt was estimated titrimetrically[14]. The reaction was carried out in KCl–Tris–$CaCl_2$ buffer (pH 8.5) at 30 °C in a pH-stat Radiometer pH meter 26 with Titrator 11. The volume of the reaction mixture was 1 ml. 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 values and the concentration of the enzyme. The *A. oryzae* alkaline proteinases concentrations were determined spectrophotometrically at 280 nm, assuming a specific extinction $E^{1\%}_{1\,cm}$ of 9.1 and a mol. wt of 23 800. The proteolytic activity was estimated by the casein-digestion method at pH 7.6 as described by Laskowski[15].

Purification procedure

All the following operations were carried out at 4 °C. 45 g of Polidase-S were dissolved in 900 ml of 0.1 M triethanolamine–HCl buffer (pH 8.0) and centrifuged at 9000 × *g* for 15 min. The clear supernatant was applied to an ovoinhibitor–Sepharose column (3 cm × 8 cm) previously equilibrated with the same buffer. After all the material was adsorbed, the column was washed with 700 ml of the same buffer and then the adsorbed proteinases were eluted with 0.2 M acetic acid. The flow rate was 50 ml/h and 10-ml fractions were collected. The protein content was estimated by determinating the absorbance at 280 nm. A protein solution giving an absorbance of 1.00 at 280 nm through a 1-cm path length cell was defined as possessing one absorbance unit ($A_{280\,nm}$) per ml. The combined eluate (see Tubes 165–183, Fig. 1) was dialyzed against water and lyophilized.

It should be noted that the same ovoinhibitor–Sepharose column could be regenerated and reused successively for at least 4–5 preparations.

Molecular weight estimation

The molecular weight determination was done by the equilibrium sedimen-

tation method. The enzyme was dissolved in 0.10 M sodium phosphate buffer (pH 7). The experiment was performed in a Beckman Model E analytical ultracentrifuge (20 000 rev./min, 20 °C). Three different protein concentrations were used and the molecular weight was determined from extrapolation to zero protein concentration.

Electrophoresis on cellulose acetate

The electrophoresis was performed in Beckman Micro-Zone Electrophoresis system Model R-100 in 0.08 M collidine acetate buffer (pH 6.9). Samples of 0.25–0.75 μl of 2–3% of protein solution were applied to the layer and electrophoresis was performed for 40 min at a potential of 400 V. The membranes were stained with Ponceu-S as described by the producers.

Amino acid analysis

The protein samples were hydrolyzed at 110 °C for 22 and 48 h in evacuated sealed tubes with glass-distilled constant-boiling HCl in the presence of known amounts of norleucine. After hydrolysis the acid was removed under reduced pressure and the residues were dissolved in sodium citrate buffer, pH 2.2. The amino acid analysis was performed by a single column accelerated method in BC-200 (BioCal) Amino Acid Analyzer. Tryptophan was estimated on a short column for basic amino acids after 22 h acid hydrolysis in the presence of 4% thiodiglycolic acid[16].

RESULTS AND DISCUSSION

A typical purification procedure is presented in Fig. 1 and Table I. As can be

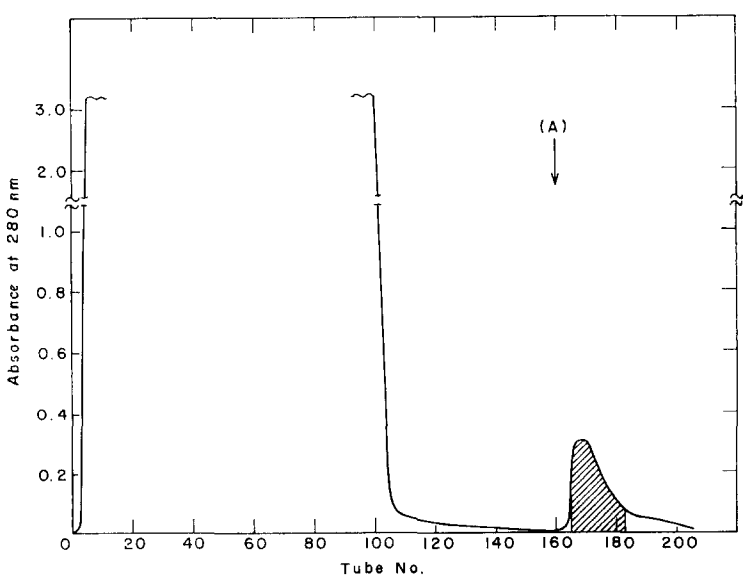

Fig. 1. Chromatography of crude Polidase-S solution on ovoinhibitor–Sepharose column (3 cm × 8 cm) equilibrated with 0.1 M triethanolamine–HCl buffer (pH 8.0). The elution was primarily performed with starting buffer and then with (A) 200 mM acetic acid. The flow rate was 50 ml/h and 10 ml fractions were collected. The shaded area indicates the pooled tubes designated as acetic acid eluate.

TABLE I

RECOVERY OF $AcAla_3OMe$ ESTERASE ACTIVITY DURING PURIFICATION OF *A. oryzae* ALKALINE PROTEINASES

Fraction	*ml*	$A_{280\ nm}$	*Total absorbance*	*$AcAla_3OMe$ activity**		*Yield of activities*
				per $A_{280\ nm}$	*Total*	
Polidase solution (5%)	900	20.50	18 450	11.0	203 000	100
Non-adsorbed fraction (Tubes 1–160)	1600	11.20	17 900	0.8	14 000	7
Acetic acid eluate (Tubes 165–183)	190	0.19	36	3120.0	112 000	55

* The activity was assayed in 12 mM substrate concentration which provides only about 40% of the *V*.

seen, the $AcAla_3OMe$ esterase activity was almost completely adsorbed on the column and the non-adsorbed fraction, which consisted of at least 97% of the applied material (as measured by absorption at 280 nm), contained only 7% of applied activity. On the other hand, the acetic acid eluate (see Fig. 1, Tubes 165–183) contained only 0.2% of applied material, but 55% of activity. The distribution of the caseinolytic activity was almost identical. It should be noted that although the recovery of the protein was almost 100%, only about 65% of activity was recovered. Since no active-site titrations of the purified enzyme(s) were performed, it is difficult to decide at present whether, during the purification procedure: (a) the enzymes were partially inactivated and the acidic eluate consist of a mixture of partially active molecules; (b) part of the enzymes was inactivate and eluted in the acidic eluate, which is therefore a mixture of fully active and inactive molecules; (c) part of the enzymes was inactivated and eluted in the non-adsorbed fraction or not eluted at all. However, since the specific $AcAla_3OMe$-se activity of the purifed proteinases is almost identical to that of alkaline proteinase from *A. sojae* (see Table II), it seems that the last possibility is the most probable one.

TABLE II

KINETIC DATA CALCULATED FOR *A. oryzae* ALKALINE PROTEINASES COMPARED TO THOSE FOR *A. sojae* ALKALINE PROTEINASE

Enzyme used	*Substrate*	K_m *(mM)*	k_{cat} (s^{-1})	k_{cat}/K_m $(s^{-1} \cdot M^{-1})$	*References*
A. oryzae purified alkaline proteinases	$AcAla_3OMe$	20.8	2850	137 000	This work
	BzArgOEt	7.1	41	5800	This work
	AcTyrOEt	12.8	43	3350	This work
A. sojae alkaline proteinase	$AcAla_3OMe$	21.6	2800	130 000	Gertler and Hayashi[14]
	BzArgOEt	8.3	26	3100	Gertler and Hayashi[14]
	AcTyrOEt	10.9	30	2750	Gertler and Hayashi[14]

The average yield of the purified proteinases from the different preparations varied from 30–40 mg obtained from 45 g of Polidase-S, and the specific extinction $E^{1\%}_{1\,cm}$ at 280 nm of the freeze-dried material was 9.1.

The esterolytic activity of the purified proteinases on various substrates, as compared to alkaline proteinase from *A. sojae* is summarized in Table II. As judged from the activity on a typical elastase, trypsin and chymotrypsin substrates, purified proteinases, similarly to the *A. sojae* alkaline proteinase, exhibit rather broad specificity. Moreover, the kinetic parameters of both enzymes are very similar, including the extremely high k_{cat} on $AcAla_3OMe$, thus indicating that those properties are probably shared by all four enzymes found in the purified fraction.

The proteolytic activity of the purified proteinases was tested on casein. The specific activity was found to be 10.5 Kunitz units[15] per mg and very similar (about 90%) to that of the *A. sojae* alkaline proteinase. The caseinolytic activity was not inhibited by EDTA (20 mM) thus indicating that the purified fraction does not contain neutral proteinases. Existence of neutral proteinases (which is characterized by its sensitivity to EDTA) in crude *A. oryzae* preparation, was reported by Misaki *et al.*[17].

The molecular weight of the purified fraction, estimated by extrapolation to zero concentration, was found to be 23 800. Partial specific volume, as calculated from the amino acid composition, was 0.72. It should be noted that the plot of ln con-

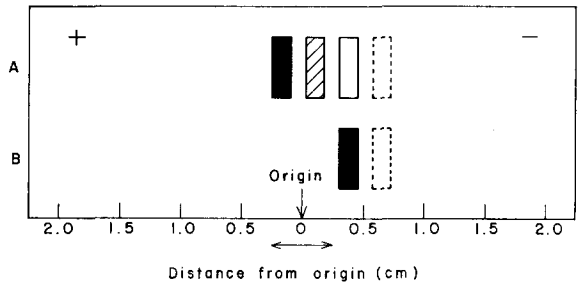

Fig. 2. Electrophoresis of alkaline proteinases from *A. oryzae* (acetic acid eluate) and alkaline proteinase from *A. sojae* on cellulose acetate membranes in 0.08 M collidine–acetate buffer (pH 6.9) for 25 min at 400 V. (A) Alkaline proteinases from *A. oryzae*. (B) Alkaline proteinase from *A. sojae*. Colour intensity: shaded areas, strong; hatched areas, mild; blank areas, weak; dotted areas, very weak.

centration *versus* X^2 resulted in all cases, in a straight line, thus indicating size homogeneity.

However, the electrophoresis on cellulose acetate membranes at pH 6.9 (see Fig. 2) revealed at least 4 distinct bands. The relative amounts of protein in each band, as judged by the color intensity, were not equal: the most basic band was the strongest one and the most acidic band was the weakest one. It should be noted that one of the bands corresponds to that of alkaline proteinase from *A. sojae*.

As mentioned earlier, two alkaline proteinases, namely Aspergillopeptidase B[2] and Aspergillopeptidase C[3] were purified from crude *A. oryzae* extract, and recently Bretschneider and Nordwig[18] have shown that *A. oryzae* culture media contain at least 4 proteinases capable to digest hemoglobin at pH 7.6, but distinct in their pI values (4.6, 5.9, 6.0 and 8.3, respectively) as measured by preparative isoelectric

TABLE III

AMINO ACID COMPOSITION OF PURIFIED PROTEINASE(S) FROM *A. oryzae* AS COMPARED TO OTHER PURE PROTEINASES FROM *A. species*

Amino acid	*Amino acid residues per 100 residues*				
	Purified proteinases (A. oryzae)[a]	*Aspergillo peptidase B (A. oryzae)*[b]	*Aspergillo peptidase C (A. oryzae)*[c]	*Alkaline proteinase (A. flavus)*[d]	*Alkaline proteinase (A. sojae)*[e]
Aspartic acid	12.2	12.2	11.4	12.0	12.3
Threonine	6.5	6.4	6.9	6.3	7.3
Serine	12.1	11.0	12.1	11.4	11.5
Glutamic acid	7.7	7.0	6.9	7.1	7.3
Proline	2.5	2.4	2.9	2.6	2.7
Glycine	11.3	11.0	11.1	11.4	11.0
Alamine	13.0	13.4	12.2	13.1	12.8
Half cystine	0.0	0.0	0.0	0.0	0.0
Valine	6.4	8.7	8.4	8.6	7.3
Methionine	0.7	0.0	0.5	0.6	0.9
Isoleucine	5.0	5.5	5.5	5.4	5.5
Leucine	5.2	5.2	5.3	5.1	5.9
Tyrosine	3.7	2.9	2.4	2.9	3.2
Phenylalanine	3.0	2.9	3.2	2.9	2.7
Lysine	5.6	6.7	6.3	6.3	5.5
Histidine	2.3	2.3	2.1	2.1	1.8
Arginine	1.7	1.2	1.6	1.1	1.4
Tryptophan	1.1	1.2	1.2	1.1	0.9

[a] This work.
[b] Subramamian and Kalnitsky[19].
[c] Nordwig and Jahn[3].
[d] Turkova *et al.*[4].
[e] Hayashi *et al.*[20].

focusing. Although these proteinases were not characterized according to their esterolytic activity on $AcAla_3OMe$ and their capability to be inhibited by chicken ovoinhibitor, it is quite possible that they are present in the acidic eluate from the ovoinhibitor–Sepharose column. This possibility is further substantiated by the finding that all the caseinolytic activity (at pH 7.6) found in the crude extract is completely inhibited by chicken ovoinhibitor.

Amino acid composition of the purified proteinases, as compared to pure alkaline proteinases from *A. oryzae, A. sojae* and *A. flavus* is presented in Table III. In order to make this table easier to compare, the published results were recalculated and expressed in residues per 100 residues.

As can be seen, the amino acid composition of the purified fraction, although not identical, is extremely similar to that of the pure enzymes, indicating a strong genetic relationship and thus giving a hint that all four proteinases present in the acidic eluate are probably homologous enzymes that developed through gene duplications, which were followed by only limited point mutations. Comparative studies on the proteinases from genus *Aspergillus*[21,22] recently published, show also a very close relation or even identity of these enzymes.

It should be noted that the data concerning the molecular weight, amino acid composition and kinetic properties represent average values of electrophoretically distinct components. However, in view of their size homogenity, their amino acid

composition similarity to other alkaline proteinases from *Aspergillus* species, their kinetic properties similarity to the alkaline proteinase from *A. sojae* and their inhibition by chicken ovinhibitor, it is quite likely that all four components share rather similar properties. The recent publication that alkaline proteinase from *A. sojae* exhibits rather wide specificity in hydrolysis of peptide bonds[23] further supports this view.

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REFERENCES

1 Bergkvist, R. (1963) *Acta Chem. Scand.* 17, 1521
2 Subramanian, A. R. and Kalnitzki, G. (1964) *Biochemistry* 3, 1861
3 Nordwig, A. and Jahn, W. F. (1968) *Eur. J. Biochem.* 3, 519
4 Turkova, J., Mikes, O., Gancev, K. and Boublik, B. (1969) *Biochim. Biophys. Acta* 178, 100
5 Hayashi, K., Fukushima, D. and Mogi, K. (1967) *Agr. Biol. Chem. Tokyo* 31, 1237
6 Danno, G. and Yoshimura, S. (1967) *Agr. Biol. Chem. Tokyo* 31, 1151
7 Matsushima, K. (1958) *Science* 127, 1178
8 Gertler, A. and Feinstein, G. (1971) *Eur. J. Biochem.* 20, 547
9 Liu, W. H., Means, G. E. and Feeney, R. E. (1971) *Biochim. Biophys. Acta* 229, 176
10 Feinstein, G. (1970) *FEBS Lett.* 7, 353
11 Feinstein, G. (1970) *Biochim. Biophys. Acta* 214, 224
12 Tomimatsu, Y., Clary, J. J. and Bartulovich, J. J. (1966) *Arch. Biochem. Biophys.* 115, 536
13 Axen, R., Porath, J. and Ernback, S. (1967) *Nature* 214, 1302
14 Gerler, A. and Hayashi, K. (1971) *Biochim. Biophys. Acta* 235, 378
15 Laskowski, M. (1955) *Methods Enzymol.* 2, 27
16 Matsubara, H. and Sasaki, R. M. (1969) *Biochem. Biophys. Res. Commun.* 35, 175
17 Misaki, T., Yarnada, M., Okazaki, T. and Sawada, J. (1970) *Agr. Biol. Chem. Tokyo* 34, 1383
18 Bretschneider, G. and Nordwig, A. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* 352, 1039
19 Subramanian, A. R. and Kalnitzki, G. (1963) *Biochemistry* 3, 1868
20 Hayashi, K., Fukushima, D. and Mogi, K. (1967) *Agr. Biol. Chem. Tokyo* 31, 1171
21 Bretschneider, G., Nordwig, A., Mikes, O. and Turkova, J. (1971) *Hoppe-Seyler's Z. Physiol. Chem.* 352, 1372
22 Turkova, J., Mikes, O., Hayashi, K., Danno, G. and Polgar, L. (1972) *Biochim. Biophys. Acta* 257, 257
23 Hayashi, K. and Terada, M. (1972) *Agric. Biol. Chem.* 36, 1755