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Short Communication

Purification of two proteinases from *Aspergillus terreus* by affinity chromatography

Miglena E. Stefanova*

Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, 142292 Pushchino, Moscow Region (Russian Federation)

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ABSTRACT

A simple and effective method for purification of proteinases from *Aspergillus terreus* is described. A combination of three chromatographic techniques is proposed, starting with affinity chromatography on bacitracin–silochrome. This first stage is crucial for the enzyme separation and leads to almost full removal of contaminating proteins. It is followed by gel permeation and ion-exchange chromatography. Using this method, two extracellular proteinases (I and II) were purified from the culture filtrate of *A. terreus*. They had molecular masses of approximately 37 000 and 21 000 as determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The inhibitory analysis revealed that proteinase I is a serine thiol-dependent enzyme and proteinase II is a metalloproteinase. The latter was activated by the addition of magnesium and cobalt ions.

INTRODUCTION

Proteolytic enzymes have been attractive for many years because of their key role in cell metabolism as well as their practical application. Their capacity for selective protein modification (*e.g.* of enzymes and hormones) by means of limited cleavage suggests that some proteinases have regulatory functions in cellular processes [1]. Various methods of purification of proteinases from different sources have been proposed in the literature, including ammonium sulphate, ethanol and acetone precipitation, a variety of conventional chromatographic techniques (*e.g.* ion exchange, gel filtration) and preparative electrophoretic techniques [2,3]. Most of them are time and labour consuming because they include too many steps. The application of affinity sorbents, especially at earlier stages of separation, considerably simplifies the procedure and increases its effectiveness. In the present paper we report the development of a simple three-step scheme for purification of two extracellular proteinases from *Aspergillus terreus* using affinity chromatography and describe some properties of these enzymes.

EXPERIMENTAL

Chemicals

Materials were obtained from the following sources: bacitracin–silochrome was a generous

* Address for correspondence: Department of Enzyme Biosynthesis, Institute of Microbiology, Bulgarian Academy of Sciences, Acad. G. Bontchev str., Bl. 26, 1113 Sofia, Bulgaria.

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gift from Dr. G. Rudenskaya (Faculty of Chemistry, Lomonosov Moscow State University, Moscow, Russian Federation); Sephadex G-25, ribonuclease A and chymotrypsinogen A were from Pharmacia (Sweden); ovalbumin, Coomassie brilliant blue (CBB) R-250, phenylmethylsulphonyl fluoride (PMSF), 1,4-dithiothreitol (DTT), sodium dodecyl sulphate (SDS) and CM-cellulose, sodium salt, with degree of polymerization (DP) 500, were purchased from Serva (Germany); DEAE–Spheron 1000 and 4-hydroxymercuribenzoic acid, sodium salt (PHMB), were from Chemapol (Czechoslovakia). All other chemicals were of reagent grade.

Organism and culture conditions

The strain *Aspergillus terreus* VKM F-2200 D was grown in a medium with the following composition (in %, w/v): peptone (0.05), KH_2PO_4 (0.14), $CaCl_2$ (0.03), $MgSO_4 \cdot 7H_2O$ (0.03), urea (0.01), CM-cellulose, sodium-salt (1), $FeSO_4$ (0.0005), $MnSO_4$ (0.00016), $ZnSO_4$ (0.00014), $CuSO_4$ (0.00014). Fermentation was carried out at 28°C for 48 h with an agitation speed of 240 rpm.

Enzyme purification

Buffers used were as follows: (A) 0.1 *M* sodium acetate pH 5.0; (A_1) 0.05 *M* sodium acetate pH 5.0; (B) 0.025 *M* Tris–HCl pH 9.3. All purification steps were carried out at 4°C. Cells were removed by centrifugation (1800 *g*, 30 min). The pH of the cell-free medium was adjusted to 5.0, and 2600 ml were applied to a chromatographic column (10 × 1.4 cm) containing bacitracin–silochrome equilibrated with buffer A. The elution was performed with 1 *M* sodium chloride in 25% isopropanol. Fractions containing proteinase activity were pooled (total volume 95 ml). A 190-ml volume of eluate from two chromatographic runs was concentrated by ultrafiltration (Amicon UM 2 membrane). The concentrate thus obtained was dialysed on a Sephadex G-25 column (25 × 5 cm) equilibrated with buffer B, at a flow-rate of 120 ml/h. Fractions of 5.8 ml were collected. The protein fraction was collected and loaded onto a column of DEAE-Spheron equilibrated with the same buffer. The elution was performed using a linear gradient of 0–0.3 *M* sodium chloride in buffer B. The active fractions were pooled and stored at −10°C for several months without any loss of activity.

Enzyme and protein assays

Protein was determined by the method of Bradford [4] with bovine serum albumin as a standard. Protein concentrations in the column eluates were monitored by measuring the absorbance at 280 nm.

Proteinase activity was measured by the release of tyrosine from 1% casein. A 1-ml aliquot of enzyme was incubated with 1 ml of casein (prepared according to the procedure of Hammarsten [16] 1% in 0.1 *M* Tris–HCl pH 7.0) at 40°C for 20 min. The enzyme reaction was stopped with 1 ml of 10% trichloroacetic acid. Samples were filtered and the absorbance in filtrates was measured at 280 nm against an appropriate blank. One unit of enzyme activity was defined as the amount of enzyme required to catalyse the liberation of 1 μM tyrosine for 1 min at 40°C and pH 7.0.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-disc PAGE was performed according to Laemmli [5] after boiling the samples with β-mercaptoethanol and SDS using 15% acrylamide (separating gel) and 6% stacking gel. A Pharmacia electrophoresis system (EPS 500/400, GE-2/4) was used. The electrophoresis was done at 150 V, 2 mA per gel rod. Gels were stained for proteins with CBB R-250. Ovalbumin (M_r 43 000), chymotrypsinogen A (25 000) and ribonuclease A (13 700) were used as molecular mass marker proteins.

RESULTS AND DISCUSSION

Separation and purification of proteinase components

The results of a typical purification procedure are presented in Table I. The crucial purification stage was the affinity chromatography on bacitracin–silochrome. The pH of the supernatant fluid had to be adjusted to 5.0 beforehand so

TABLE I

PURIFICATION OF *ASPERGILLUS TERREUS* PROTEINASES

Step	Total volume (ml)	Total activity ($U \times 10^{-3}$)	Specific activity ($U \times 10^{-3}$/mg)	Yield (%)	Purification (*n*-fold)
Culture supernatant	5200	41 340	72	100	1
Bacitracin–silochrome	190	32 656	1814	79	25
Sephadex G-25	142	20 708	5597	50	78
DEAE–Spheron					
Proteinase I	25	8138	5425	20	75
Proteinase II	45	2344	1116	6	15

that the proteinases could completely adsorb on the affinity sorbent. At the original pH (6.5–7.0) the enzymes were easily washed out of the column with acetate buffer. Except for proteinases, peptides and some endoglucanase activity (measured by the decrease in the viscosity of CM-cellulose) were retained on bacitracin–silochrome at pH 5.0 too. The latter was removed by extensive washing of the column with diluted acetate buffer (0.05 *M*) before performing the elution. Our attempts to desorb the proteinases with 1 *M* sodium chloride only failed (data not shown). This suggested that lowering the dielectric constant of the medium could perhaps increase the electrostatic interaction of the proteins with the eluent and thus facilitate the elution. Indeed proteinases were eluted with isopropanol solution of high ionic strength (Fig. 1). The enzyme preparation thus obtained was almost completely free from contaminating proteins, as indicated by the considerable increase (25-fold) in the specific proteinase activity (Table I).

Bacitracin-based affinity sorbents (Sepharose and silochrome) have been described previously as suitable matrices for purification of proteinases of different type and origin [6]. They provide both a high purification factor and a high yield of activity [7,8]. In our case bacitracin–Sepharose was unsuitable because of the presence of cellulases secreted by the fungus. First, they would adsorb on Sepharose, thus complicating the purification procedure and, second, could degrade it. But when the affinity ligand was coupled to silochrome (macroporous silica compound) it proved to be very effective in purification of *A. terreus* proteinases, providing a 79% recovery. This high effectiveness is due to the specific interaction between the peptide antibiotic bacitracin and the substrate-binding site of proteinases [9].

Further proteinases were concentrated by ultrafiltration and desalted on a Sephadex G-25 column. This was accompanied by the removal of the peptides from the enzyme preparation. The protein fraction (void volume of the column)

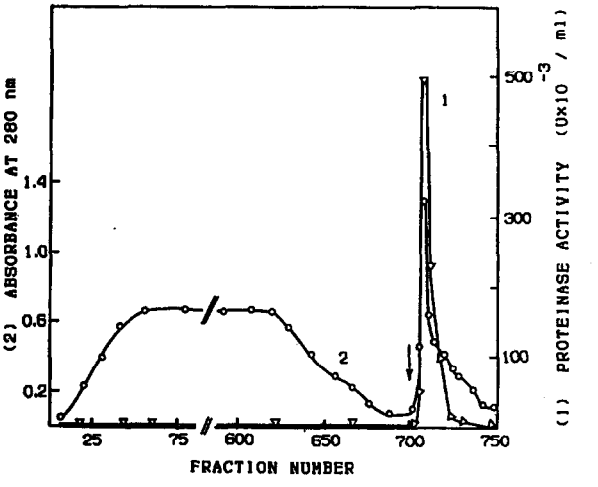

Fig. 1. Chromatography on the affinity sorbent of *A. terreus* proteinases. The column (10 × 1.4 cm) was prepacked with bacitracin–silochrome and equilibrated with buffer A. The supernatant fluid at pH 5.0 was applied to the column at a flow-rate of 40 ml/h. After washing the column with 200 ml of buffer A_1 the enzymes were eluted with 1 *M* sodium chloride in 25% isopropanol at the same flow-rate. Fractions of 4 ml were collected. The arrow indicates the beginning of the elution.

was fully adsorbed on an anion exchanger, DEAE–Spheron, at pH 9.3 and eluted with a linear sodium chloride gradient as two proteinase peaks (I and II) at 0.11 *M* and 0.22 *M* sodium chloride concentration, respectively (Fig. 2).

On SDS-PAGE proteinase II migrated as a homogeneous band (Fig. 3) with a molecular mass of 21 000, while proteinase I was purified to near homogeneity. It contained a major protein with molecular mass of 37 000 and a minor one with lower molecular mass.

Thus, two proteinases, I and II, were separated and purified from the culture filtrate of *A. terreus* with purification factors of 75 and 15 and recoveries of 20% and 6%, respectively. A considerable loss of activity of proteinase II after ion-exchange chromatography was observed. Most probably this is caused by chelating of the co-factor (see below) by the resin (Spheron is based on polymethacrylic acid) under the experimental conditions.

In most cases procedures for obtaining homogeneous and highly purified proteins include at least 4–5 different steps. In this paper we propose a simple and effective purification scheme of three stages which produces two purified proteinases by means of affinity ligand. Usually, starting the separation with a biospecific

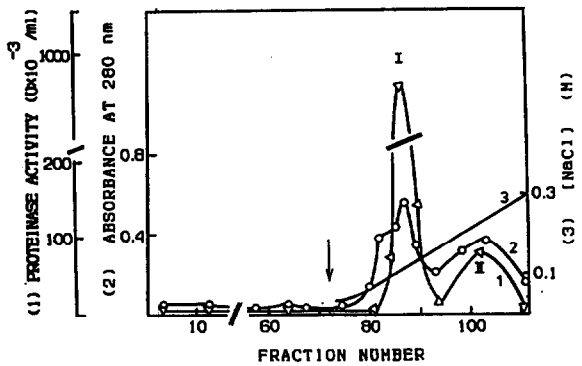

Fig. 2. Ion-exchange chromatography of proteinases from *A. terreus*. The protein fraction obtained after gel-permeation chromatography was loaded onto a column (10 × 1.4 cm) containing DEAE–Spheron previously equilibrated with buffer B. The column was washed with three bed volumes of the same buffer. Chromatography conditions were as follows: flow-rate, 25 ml/h; fraction volume, 2.5 ml; eluent, linear gradient 0–0.3 *M* sodium chloride in the equilibrating buffer. The arrow indicates the beginning of the elution.

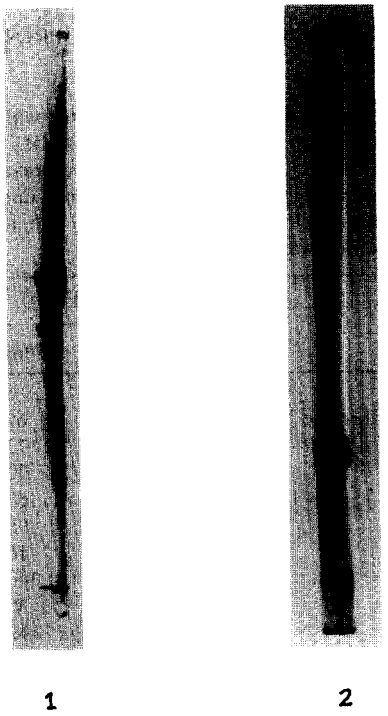

Fig. 3. SDS-PAGE pattern of the purified proteinases from *A. terreus* after chromatography on DEAE–Spheron. Experimental conditions are described in the text. 1 = Proteinase I; 2 = proteinase II.

adsorbent considerably simplifies the procedure while at the same time keeping yield high. Mosolova *et al.* [8] acquired homogeneous Glu, Asp-specific proteinase from *Actinomyces* sp. in three stages using chromatography on bacitracin–silochrome. Homogeneous carboxylic proteinase from *Aspergillus niger* was obtained in three steps by chromatography on pepstatin–Sepharose [10].

Effect of some reagents on proteinase activity

Proteinase I was fully inhibited by PMSF (Table II), which indicates the presence of serine residues in the active centre. Like most serine proteinases, it was insensitive to the chelating agents EDTA and 1,10-phenanthroline. The cysteine-binding inhibitor PHMB reduced the enzyme activity to zero, while in the presence of the reducing agent DTT a 30% increase in activity was observed. These findings indicated that the cysteine residue is essential for enzyme

TABLE II

EFFECT OF SOME REAGENTS ON PROTEINASE ACTIVITY

Aliquots of 1 ml of the enzyme were incubated with 50 μl of each reagent solution (final concentration 10 m*M*) for 1 h at room temperature. After that the enzyme reaction was carried out according to the standard assay procedure and the residual enzyme activity was determined. The activity of untreated enzyme was taken as 100%.

Reagent	Residual activity (%)	
	Proteinase I	Proteinase II
EDTA	95	6
1,10-Phenanthroline	98	66
PMSF	0	73
PHMB	0	32
DTT	130	60
Ca^{2+}	72	98
Cd^{2+}	12	16
Co^{2+}	44	140
Cu^{2+}	37	35
Mg^{2+}	81	142
Mn^{2+}	62	92
Zn^{2+}	0	27

activity and that proteinase I may be related to the subfamily of thiol-dependent serine proteinases described by Stepanov *et al.* [11]. The only enzyme of this group from *Aspergillus* fungi reported until now seems to be the intracellular proteinase from *A. niger* [12]. Cd^{2+} and Zn^{2+} almost completely inhibited enzyme activity, while the other metal ions tested were less effective —28–63% inhibition.

The inhibitory analysis of proteinase II showed more complicated results (Table II). The residual activity after EDTA treatment was 6%, suggesting that proteinase II is a metalloproteinase. At the same time 1,10-phenanthroline caused only 44% loss of activity.

Some authors have reported inhibitory effect of reducing agents (DTT, cysteine, β-mercaptoethanol) on neutral metalloproteinases because of their ability to chelate metal ions [13]. The metalloproteinase II from *A. terreus* was inhibited by DTT (Table II).

Proteinase II was activated by Mg^{2+} and Co^{2+} and strongly inhibited by Cd^{2+}, Cu^{2+} and Zn^{2+}. The enzyme activity was not influenced by Ca^{2+}. The same effect of metal ions tested was observed with the "acid" metalloproteinase from *A. oryzae* [14]. The metalloproteinase from *Penicillium roqueforti* was activated twice in the presence of 10 m*M* Co^{2+} [15]. This and some other properties of the *A. terreus* enzyme —low M_r (about 20 000) and pH optimum about 6–7 (data not shown)— are characteristic of the "acid" fungal metalloproteinases from *Aspergillus* and *Penicillium* [14,15].

Bearing in mind the effect of chelating agents and metal ions mentioned above, it is quite possible that the present (and the others described as "acid" in the literature) metalloproteinase may contain Mg^{2+} or Co^{2+} as a cofactor since both these ions increase enzyme activity to an equal extent and, in contrast to EDTA, are very weakly (if at all) chelated by 1,10-phenanthroline.

To summarize, in present communication we report on the purification of two novel proteinases, first identified in a strain of *Aspergillus terreus*. Further biochemical and enzymological characterization of these enzymes will be the subject of a subsequent paper.

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