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EXPERIMENTAL ARTICLES

Isolation, Purification, and Resolution of the Extracellular Proteinase Complex of *Aspergillus ochraceus* 513 with Fibrinolytic and Anticoagulant Activities

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Abstract—The extracellular proteinase complex of the microscopic fungus *Aspergillus ochraceus* 513 was isolated, purified, and resolved by affinity chromatography on bacillichin-silochrom and subsequent column chromatography on DEAE-Toyopearl 650M. The extracellular enzyme of the protein C activator type had a molecular mass of 36.5 kDa and activity close to that of the *Agkistrodon* snake venom protein C activator. The fibrinolytic and anticoagulant activities of the enzyme were investigated.

Key words: extracellular proteinases, protein C activator, affinity chromatography, column chromatography.

Recent years have seen increasing medical interest in microbial thrombolytic enzymes. They act as a plasmin, which directly dissolves human blood clots [1–7], or as tissue activators of the blood plasminogen [8–10].

With the discovery of the role of protein C in human hemostasis, this anticoagulant system that prevents from blood hyperclotting has become the subject of particular research interest [11]. Until recently, protein C activators have been isolated from the *Agkistrodon* snake venom [12]. The addition of these activators to the blood serum prolongs the partial thromboplastin time due to inactivation of the clotting factors V and VIII necessary for the formation of thrombin. Experiments on animals showed that the protein C activator from the *Agkistrodon* snake venom possesses anticoagulant activity [13]. Microbial protein C activators may appear more promising for the use in clinical practice due to their low cost.

The aim of the present work was to isolate, purify, and separate the extracellular proteinase complex of the microscopic fungus *Aspergillus ochraceus* 513, whose anticoagulant activity is of the protein C activator type.

MATERIALS AND METHODS

The microscopic fungus *Aspergillus ochraceus 513* used in the work was selected in the course of our previous investigations as possessing high anticoagulant activity. The plasmin-like and the plasminogen activator–like activities of this strain are low [14]. To obtain proteolytic enzymes, the fungus was grown at 28°C for 7 days in a submerged mode in 750-ml flasks with 100 ml of a nutrient medium on a rotary shaker (200 rpm). The nutrient medium contained (%) glucose, 3.5; starch, 1.0; soybean meal, 2.0; peptone, 0.5; meat extract, 0.5; NaCl, 0.2; KH_2PO_4, 0.05; and $MgSO_4 \cdot 7H_2O$, 0.05. Each flask was inoculated with 5 ml of the 2-day-old culture grown in a medium containing 250 ml of wort, 20 g/l glucose, and 1 g/l peptone per liter. The mycelium was separated from the culture liquid by filtration and centrifugation.

The protein concentration was determined either by the method of Lowry as described by Landau and Egorov [10] or spectrophotometrically at 280 nm [19].

Proteolytic activity was assayed by a modified Anson's method using casein as the substrate (λ = 280 nm; incubation time, 1 min; enzyme preparation volume, 1 ml) [10].

Anticoagulant activity was determined as an increase in the partial thromboplastin time (PTT) using our modification of the method described by Strukova *et al.* [15]. The reaction mixture containing 0.1 ml of the human blood serum, 0.1 ml of a thromboplastin suspension, and 0.1 ml of kaolin was incubated in a water bath at 37°C for 2 min and then rapidly mixed with 0.1 ml of 0.277% $CaCl_2$. The time of the blood serum clotting was determined using a stopwatch. Instead of the tested preparation, the control mixture contained an equivalent amount of physiological saline solution (0.86% NaCl). The relative PTT was calculated using the formula $[(A–B)/B] \times 100\%$, where A and B are the times (in s) of the blood serum clotting in the presence

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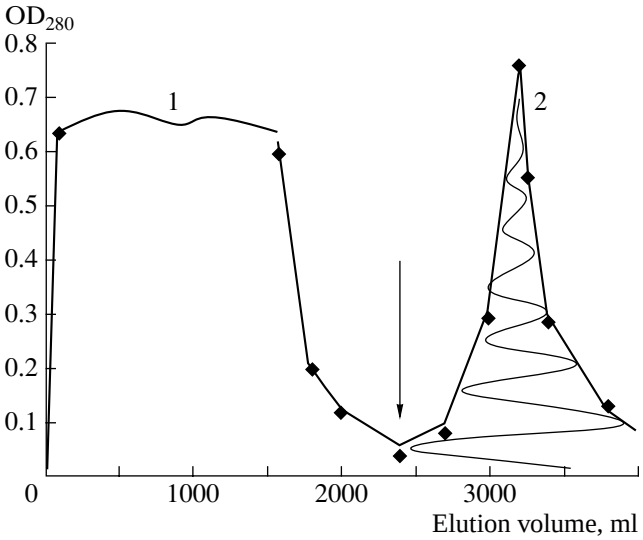

Fig. 1. Chromatography of the proteinase complex of *A. ochraceus* 513 on bacillichin-silochrom: (1) nonadsorbed material and (2) material eluted with a 25% solution of isopropanol in 0.05 M Tris–HCl buffer (pH 7.5) containing 2 mM Ca acetate and 1 M NaCl (the arrow indicates the time of the beginning of this elution). Peak 2 possesses proteolytic activity.

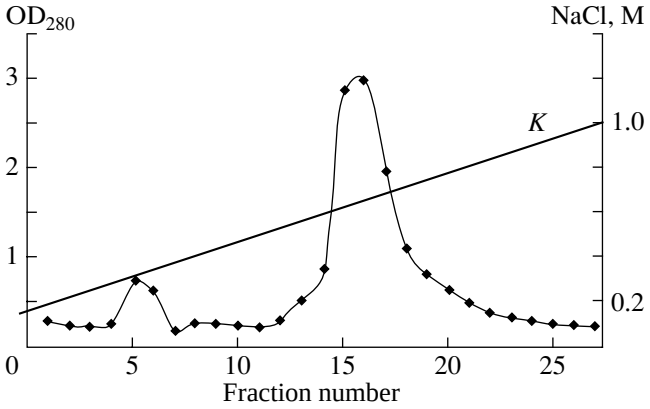

Fig. 2. Ion-exchange chromatography of the proteinase complex of *A. ochraceus* 513 on DEAE-Toyopearl 650 M.

and absence of the microbial proteinase, respectively. All measurements were carried out in triplicate.

Pigments from the culture liquid were removed by treating it with FAF anionite (Olaine, Latvia) in Cl^- form (bead size, 160–300 μm; swelling coefficient, 4.0; capacity, 36 mg-equiv/g) [16].

Affinity chromatography was carried out using bacillichin-silochrom, an affinity sorbent with a ligand content of 12 μg/g [17].

To obtain proteinase, 1360 ml of the neutralized culture liquid (pH 7.0) was passed at room temperature through a column packed with FAF anionite in Cl^- form. The material passed through this column was applied to a (4 × 25 cm) column with the affinity sorbent bacillichin-silochrom equilibrated with 0.05 M Tris–HCl buffer (pH 7.5) containing 2 mM Ca acetate (buffer A). The column was washed with the same buffer. Active enzymes were eluted with a 25% solution of isopropanol in buffer A containing 1 M NaCl. The eluate was dialyzed against 2 mM Ca acetate at 6°C for one day and lyophilized.

Proteinases were further separated by ion-exchange chromatography on DEAE-Toyopearl 650 M. The lyophilized preparation (100 mg) dissolved in 1 ml of 0.1 M ammonium acetate buffer (pH 8.0) was applied to a (2.5 × 30 cm) column with DEAE-Toyopearl 650 M equilibrated with 0.1 M ammonium acetate buffer (pH 8.0). The column was washed with this buffer until the eluate absorbance at 280 nm fell to almost zero. Enzymes were then eluted with a linear gradient of 0.15–1 M NaCl in the same buffer.

Disc-electrophoresis in PAAG was carried out using Tris–glycine buffer (pH 8.3) as described by Davis [20]. The molecular mass of enzymes was determined by denaturing electrophoresis in 10% PAAG in the presence of SDS [18]. Prior to treatment with SDS, the enzymes were denatured with 90% phenol, precipitated with cold acetone, and washed with acetone and ether. Phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) (Serva, Germany) were used as molecular weight markers.

RESULTS AND DISCUSSION

The microbial proteinase complex was isolated, purified, and then fractionated by controlling its specific enzymatic activity of the protein C activator type in terms of the so-called PTT test.

The culture liquid of hydrolytic microorganisms usually contains cellulases and dextranases, which may degrade the polysaccharide sorbents used for their purification. In view of this, in the present work we used an affinity sorbent with the inorganic matrix aminosilochrom, which is resistant to the action of hydrolytic enzymes. This sorbent is specific to serine, thiol, carboxylic, and metalloproteinases. This allowed the extracellular proteolytic complex of strain 513 to be isolated from the culture liquid in one chromatographic step.

To remove pigments from the culture liquid, it was passed through FAF anionite in Cl^- form. The depigmented culture liquid was subjected to affinity chromatography on bacillichin-silochrom (Fig. 1). As a result, the specific proteolytic activity of the preparation determined with casein increased by a factor of 12 with an yield of 14%. At the same time, the specific anticoagulant activity increased by a factor of 34 with a yield of 39%.

After subsequent dialysis against 2 mM Ca acetate at 6°C for one day, the activity of the proteinase com-

Isolation of proteinase from the culture liquid of *A. ochraceus* 513

Purification step		Culture liquid	Depigmentation on FAF anionite	Affinity chromatography on bacillichin-silochrom	Dialysis
Anticoagulant activity (PTT test)	Activity yield, %	100	70	39	35
	Purification factor	1	1.25	34	36
	Total activity, units	89760	62640	35020	31769
	Specific activity, units/ml	12	15	412	427
	Activity, %	65.7	43	350	205
Caseinolytic activity	Activity yield, %	100	63	14	13.8
	Purification factor	1	1.1	12	14
	Total activity, units	130900	82267	18275	18154
	pecific activity, units/ml	17.5	19.7	215	244
	Activity in μg tyrosine/(ml min)	96	57	183	117
Total protein, mg		7480	4176	85	74.4
Protein concentration, mg/ml		5.5	2.9	0.85	0.48
Volume, ml		1360	1440	100	155

plex not only failed to decrease but even slightly increased. As a result, the specific caseinolytic activity of the complex after affinity chromatography and subsequent dialysis increased by a factor of 14 with a yield of 13.8%, whereas its specific anticoagulant activity increased by a factor of 36 with a yield of 35% (see table). After dialysis against 2 mM $Ca(CH_3COO)_2$, this proteinase preparation was lyophilized and stored at –20°C until required.

The lyophilized proteinase represented a light cream-colored powder, easily soluble in water and physiological saline. The proteinase solution was slightly yellow colored and completely transparent. The protein content of this proteinase was 40%.

The denaturing electrophoresis of the proteinase complex of *A. ochraceus* 513 purified by affinity chromatography showed the presence of three protein bands with molecular masses of ~36.5, 29, and 14.4 kDa.

Further purification by ion-exchange chromatography on DEAE-Toyopearl 650 M in a linear gradient of 0.15–1 M NaCl allowed the proteinase to be purified of the ballast material and to be separated into two proteins with different enzymatic activities (Fig. 2). The measurement of proteolytic activity showed that both preparation 1 (chromatographic fractions 4 through 7) and preparation 2 (fractions 14 through 21) lacked caseinolytic activity, whereas the activity of the protein C activator type was detected only in the preparation 2.

The analysis of this preparation by denaturing electrophoresis showed the presence of only one protein band with a molecular mass of about 36500 Da. Thus, we succeeded in isolating the extracellular proteinase of the microscopic fungus *A. ochraceus* 513 with the activity of the protein C activator type.

A comparison of this enzyme with the protein C activator from the *Agkistrodon* snake venom showed that they are almost equally efficient in prolongating the thrombin formation time [12, 13, 15]. Further studies are necessary to understand the mechanism of the action of the *A. ochraceus* 513 proteinase on the protein C system and to elaborate a diagnostic kit for assaying the activity of protein C.

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