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EXPERIMENTAL ARTICLES

Preparations of Extracellular Proteinases from *Aspergillus ochraceus* 513 and *Aspergillus alliaceus* 7 dN1

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Abstract—Preparations of extracellular proteolytic enzymes with high anticoagulant activity resembling protein C activators were isolated from the culture liquids of *Aspergillus ochraceus* 513 and *Aspergillus alliaceus* 7 dN1 by precipitation with ammonium sulfate and subsequent purification from ammonium ions by gel filtration on a column with Sephadex G-25. The pH and temperature activity optima and stability of the proteolytic enzymes from *A. ochraceus* 513 and *A. alliaceus* 7 dN1 were determined.

Key words: extracellular proteinases, pH and temperature activity optima, pH and temperature stability.

Proteinases exhibiting high fibrinolytic and anticoagulant activities are prominent among microbial extracellular proteolytic enzymes due to their probable high therapeutic effect on thromboembolism [1]. The functional role of protein C in human hemostasis has recently been established; the activation of this anticoagulant system seems extremely promising for the treatment, prophylaxis, and diagnostics of thrombogenesis [2]. By now, activators of protein C have been isolated from the *Agkistrodon* snake venoms [3]. However, microbial proteinases may be less expensive and even more efficient in the activation of the protein C system.

The aim of the present work was to study some characteristics of fungal extracellular proteinases.

MATERIALS AND METHODS

Strains *Aspergillus ochraceus* 513 and *Aspergillus alliaceus* 7 dN1 were from the Collection of the Microbiology Department, Biological Faculty, Moscow State University. These cultures were chosen because of their high anticoagulant activity resembling protein C activator activity. No plasmin-like activity or plasminogen activation were revealed in these strains [4]. Fungi were grown in 750-ml flasks with 100 ml of the medium containing (%) glucose, 3.5; starch, 1.0; soybean flour, 2.0; peptone, 0.5; meat extract, 0.5; NaCl, 0.2; KH_2PO_4 – 0.05, $MgSO_4 \cdot 7H_2O$, 0.05. Flasks were inoculated with 5% of a 2-day culture grown in the medium containing (g/l) glucose, 20; peptone, 1.0 and wort, 250 ml. Cultivation was performed on a rotary shaker (200 rpm) at 28°C for 7 days. To separate mycelium, culture liquid was filtrated or centrifuged.

Protein content was assayed by the Lowry method [5] or spectrophotometrically at 280 nm [6].

Caseinolytic activity was analyzed by a modified Anson method; the amount of tyrosine formed per minute in the presence of 1 ml of the enzyme solution was measured spectrophotometrically at 280 nm [5].

Anticoagulant activity was estimated from the activated partial thromboplastin time (APTT) of human blood plasma. The value of APTT was determined by the method recommended by the Research Institute of Hematology and Blood Transfusion (Vyatka) [7] in our modification. The relative value of APTT was calculated by the formula: $((A–B)/B) \times 100\%$, where A and B are the times (s) of plasma coagulation in the presence of fungal proteinases and in the control, respectively. Analyses were carried out in triplicate.

The pH optima for the enzyme activities was determined using 0.2 M phosphate buffer (pH 6.0–7.0) and 0.05 M Tris–HCl buffer (pH 8.0–9.0). To study the pH stability of the enzymes, caseinolytic and anticoagulant activities were assayed after the incubation of the preparations in the same buffers over a pH range from 6.0 to 9.0 for 1, 3, and 5 h.

The temperature optima for caseinolytic and anticoagulant activities were studied over a temperature range from 4 to 85° C (4, 20, 30, 37, 50, and 85°C).

To determine thermostability of proteolytic enzymes, the preparations were preincubated at 4, 20, 30, 37, 50, and 85°C for 1, 3, and 5 h.

To estimate the molecular weights of the enzymes, disk-electrophoresis was performed in 10% PAAG in the presence of sodium dodecyl sulfate (DS-Na) [8]. The following markers were applied: albumin (67000 Da), ovalbumin (43000 Da), chymotrypsinogen (25000 Da), and α-lactalbumin (14000 Da) (Serva, Germany).

RESULTS AND DISCUSSION

Proteinases of *A. ochraceus* 513 and *A. alliaceus* 7 dN1 were precipitated from the culture liquid filtrates with ammonium sulfate at 80% of saturation and harvested by centrifugation. Sediments colored dark-brown (from *A. ochraceus* 513) or light-brown (from *A. alliaceus* 7 dN1) were dissolved in distilled water (insoluble precipitates were removed) and purified from ammonium ions by gel filtration on a column with Sephadex G-25 (rough, Pharmacia, Sweden).

Complexes of proteolytic enzymes from the fungal strains studied exhibited pronounced caseinolytic and

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anticoagulant activities within a pH range from 6.0 to 9.0. Proteinases from *A. ochraceus* 513 showed optimum of both activities at pH 8.0, whereas the enzyme preparation from *A. alliaceus* 7 dN1 had the optima of anticoagulant and caseinolytic activities at pH 7.0 and 8.0, respectively.

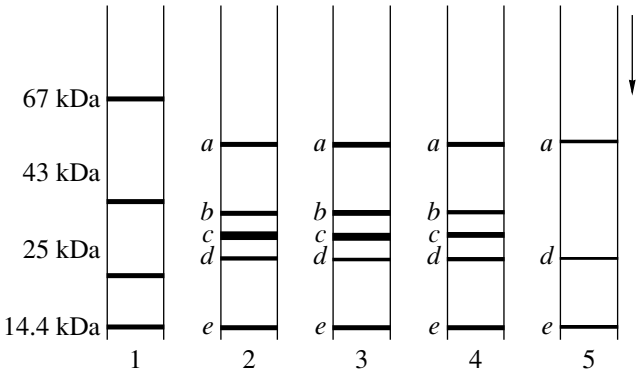
The study of the pH stability of the fungal proteinases revealed that they lost activities with an increase in the acidity of the medium and were more stable at alkaline pH. The caseinolytic activity of proteinases from *A. alliaceus* 7 dN1 remained unaltered after a 3-h incubation of the preparation at pH 8.0 and decreased slightly (by 4%) at pH 9.0. The incubation of the preparation from *A. ochraceus* 513 at pH 8.0 for 1 h produced no effect on the enzyme activities, whereas a 3-h incubation resulted in a decrease in caseinolytic and anticoagulant activities by 6 and 12%, respectively. The preincubation of the preparations at pH values ranging from 6.0 to 8.0 for a longer period (5 h) caused sometimes a more than twofold decrease in both enzyme activities.

Both caseinolytic and anticoagulant activities of the proteinases from *A. ochraceus* 513 and *A. alliaceus* 7 dN1 were maximum at 50°C and remained unaltered after the incubation of the preparations at 5 to 50°C for 1 h or at 6 to 20°C for 3 h. A pronounced inactivation of the enzymes was observed after a 5-h incubation of the preparations at 50°C.

The proteolytic complex from *A. alliaceus* 7 dN1 was more tolerant to a 3-h incubation at 50°C than proteinases from *A. ochraceus* 513; the caseinolytic activities of these preparations decreased by 28 and 61%, respectively.

Proteinases from both fungal strains were rapidly inactivated at 85°C; after a 1-h incubation, their caseinolytic and anticoagulant activities decreased by 35 and 32.5%, respectively, and were almost completely lost after 2- or 3-h incubations at this temperature.

The examination of the proteinase complex from *A. ochraceus* 513 by electrophoresis in PAAG revealed the presence of five protein bands with molecular weights of 55000, 41500, 36500, 30000, and 14400 Da, which are denoted in the figure as (*a*), (*b*), (*c*), (*d*), and (*e*), respectively (figure). To study the correlation between proteolytic activities and the protein profile of the enzyme complex, the preparation was incubated for 5 days at –20, 4°C, or at room temperature and then subjected to electrophoresis. The incubation of the preparation at –20°C produced no effect on the enzyme activities, whereas at 4°C, caseinolytic and anticoagulant activities decreased by 75 and 67%, respectively. After a 5-day incubation of the preparation at room temperature, caseinolytic activity decreased by 95%, whereas anticoagulant activity determined in the APTT test completely disappeared. In this case, electrophoretic examination of the preparation did not reveal proteins with molecular weights of 41500 and 36500 Da, which are designated in the figure as (*b*) and (*c*), respectively. It is conceivable that one of these proteins or both are associated with the anticoagulant activity of the proteinases from *A. ochraceus* 513, resembling activators of protein C.



Electrophoresis in PAAG in the presence of sodium dodecyl sulfate of the enzyme preparations from *A. ochraceus* 513. (1) Markers: albumin (67000 Da); ovalbumin (43000 Da); chymotrypsinogen (25000 Da), and α-lactalbumin (14400 Da). (2) Initial enzyme preparation; (3), (4), and (5) enzyme preparations after a 5-day incubation at –20°C, 4°C, and room temperature (about 20°C), respectively.

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