ISSN 0026-2617, Microbiology, 2011, Vol. 80, No. 6, pp. 884–888. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © T.A. Semenova, M.A. Belozerskii, G.A. Belyakova, B.A. Borisov, S.A. Semenova, Ya.E. Dunaevskii, 2011 published in Mikology and Phytopathology.

ARTICLES FROM THE RUSSIAN JOURNAL
MIKOLOGIYA I FITOPATOLOGIYA
(MYCOLOGY AND PHYTOPATHOLOGY)

Secreted Protease of an Entomopathogenic Fungus *Cordyceps militaris*. I. Selection of Medium Components and Development of Purification Procedure

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Abstract—Extracellular serine proteases from the culture liquids of two strains of an entomopathogenic fungus *Cordyceps militaris* were isolated and characterized. Their activity was demonstrated to depend on the composition of the culture medium. Electrophoretically homogeneous preparations of the secreted enzymes were obtained by affinity chromatography on bacitracin–silochrome and gel filtration on Superdex G-75. Molecular weight of the proteins was 24 kDa.

Keywords: entomopathogenic fungi, proteolytic enzymes, *Cordyceps militaris*.

DOI: 10.1134/S0026261711060178

Entomopathogenic fungi of the genus *Cordyceps* play an important role in ecological associations and are widely-used in agricultural control of insect pests. The entomopathogenic fungus *C. militaris* (Fr.) Link, which is common in the northern temperate zone [1] is used to protect forests from the pine sawfly (*Dendrolimus pini*); it secretes compounds which induce apoptosis in certain tumor cells and thus exhibit antitumor activity [3]. Application of *C. militaris* both in agriculture and pharmacology dictates the interest to the compounds it secretes.

Osmotrophic nutrition typical of fungi determines the high secretion rate in *C. militaris*. Intensive production of hydrolytic enzymes, mainly proteases, is due to their involvement in the insect pathogenesis. Extracellular proteases are known to be the key enzymes in the process of fungus invasion through the insect cuticle, destroying the chitin-binding proteins and thus making chitin available to chitinases.

Optimization of the parameters of cultivation to provide for the optimal conditions and the highest yield of the fungal secreted proteases in vitro, along with the development of an efficient procedure for their purification, are of theoretical importance in view of investigation of the fungal metabolic pathways, as well as of practical significance in relation to the wide application of proteases in light industry.

In the present work, the effect of the medium composition on secretion of the extracellular proteases by two strains of *C. militaris* was investigated and a procedure for chromatographic isolation of the protease under study was developed.

MATERIALS AND METHODS

Cultivation conditions. The strains *C. militaris* SM-2-05 and KP-97-2 were stored at 4°C. The fungi culture was maintained on Czapek medium supplemented with mannose and yeast extract and thus containing the following (g/l distilled water): KH_2PO_4, 1; $NaNO_3$, 2; KCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; mannose, 30; yeast extract, 10; and agar, 22. The flasks were sterilized at 1 atm and inoculated with the mycelia.

Culturing medium composition. Enzymatic activity was determined in 1–12-day culture liquids of *C. militaris* grown at 23°C on a rotary shaker (130 rpm). The cultures were grown in 750- and 250-ml Erlenmeyer flasks containing 200 and 60 ml of the modified Czapek medium of the following composition (g/l distilled water): KH_2PO_4, 1; KNO_3, 3; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; tryptone, 10; and gelatin, 10.

To determine the chitinolytic activity, the cuticle of *Nauphoeta cinerea* (10 g/l) was added to the medium. Preliminarily, *N. cinerea* exoskeleton was detached and soaked in potassium tetraborate solution for 2 h at 21°C, then rinsed with distilled water and dried at 87°C [4].

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Biomass accumulation and enzyme activity curves. Enzyme activity of the culture liquid depending on the growth stage of the fungus was analyzed in each strain in at least three repeats. Equal amounts of sporulating mycelia were inoculated. Grown mycelium was separated by 25-min centrifugation at 14000 *g* filtration through paper filters. Then 8-% sodium azide solution in water was added to the culture liquid to the final concentration of 0.02% and it was stored at 4°C or frozen at –20°C for long-term storage. Filtered mycelium was dried at 85°C to constant weight.

Proteolytic activity was assessed in the culture liquid of the fungus shake culture with *p*-nitroanilide substrates [5]: Glp-Ala-Ala-Leu-pNa (Glp-AAL-pNa), Suc-Ala-Ala-Pro-Phe-pNa (Suc-AAPF-pNa), and Suc-Ala-Ala-Pro-Leu-pNa (Suc-AAPL-pNa). To measure the activity, 10–100 μl of the culture liquid or enzyme preparation was added to 700 μl phosphate buffer (pH 7.0, 0.1 M) and 10 μl of the substrate solution in dimethylformamide (10 mg/ml) and incubated at 37°C. The solution absorbance was measured at time zero and time *t* at 410 nm in a 1-cm cuvette. In some cases, the activity was measured in a plate reader at 405 nm upon addition of 10–35 μl enzyme to 150–180 μl buffer and 7 μl substrate. The enzyme activity unit was defined as the amount of the enzyme hydrolyzing 1 μmol substrate during 1 min under the experimental conditions.

Chitinolytic activity was determined in 500 μl culture liquid upon addition of 0.2 mg chitin azure and 200 μl 0.1 McIlvain (citrate–phosphate) buffer, pH 5.2, and incubation at room temperature for 45 min at constant shaking. The reaction was stopped by the addition of 200 μl 0.1 M HCl to each sample. The samples were then centrifuged for 5 min at 8000 *g*. The absorbance was measured at 560 nm [6].

Affinity chromatography on bacitracin–silochrome. The column with bacitracin–silochrome (4 × 7.5 cm) was washed with Tris–HCl buffer, pH 7.5, containing 20% isopropanol and 1 M NaCl and with distilled water, then 45 ml of the culture liquid was applied. The column was washed with 0.1 M phosphate buffer, pH 7, to remove unbound protein and the protease was eluted with the same phosphate buffer containing 1 M NaCl. In the fractions obtained, protein content was determined by measuring the optical density at 280 nm and proteolytic activity. Active fractions were concentrated and desalted by ultrafiltration.

FPLC gel chromatography on a Superdex G-75 column. For further purification, 200 μl of the enzyme solution was applied to the column (1 × 30 cm) equilibrated with 0.01 M phosphate buffer (pH 7.0). In a number of experiments, 0.5 M NaCl was added to the buffer since it affected the enzyme affinity to the adsorbent.

Denaturing PAGE. The samples were treated with 7% TCA to precipitate the proteins, cooled for 20 min, and centrifuged at 2500 *g* for 15 min. The supernatant was removed and the protein pellet was resuspended in 1 ml acetone and again centrifuged at 2500 *g* for 5 min. Then, the supernatant was removed again. Excessive TCA was removed by treating twice with acetone. The protein was dried on air. Then, 15–20 μl sample buffer (0.0625 M Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.4 ml 1% bromophenol blue) was added and the samples were boiled on a water bath for 5 min. Electrophoresis was performed in vertical gels, 80 × 70 × 0.75 mm, in a Mini-protean III electrophoresis cell (BioRad, United States) at 10 mA. To determine the enzyme molecular mass, 16-% separating and 8-% stacking gels were used. The electrode buffer contained 0.062 Tris, pH 8.3, 0.33 M glycine, and 0.1% SDS. Electrode and sample buffers were prepared according to the BioRad manual.

Phosphorylase B (97000 Da), bovine serum albumin (66200 Da), ovalbumin (45000 Da), carbonic anhydrase (31000 Da), soy trypsin inhibitor (21500 Da), and lysozyme (14 400 Da) were used as standard protein markers.

The gels were fixed and stained in 0.15% Coomassie brilliant blue R-250 solution in ethanol–acetic acid–water, 3 : 1 : 6. Then, the gels were washed with ethanol–acetic acid–water, 1 : 2 : 16 and distilled water.

PAGE activity staining. In order to measure the enzyme activity upon electrophoresis, the protein was applied to the gel without heating and electrophoresis was performed at 4°C and constant current of 25 mA. Then, the gel was washed in 2.4% triton solution during 30 min and in phosphate buffer at 37°C, for 20 min. A membrane soaked in the substrate solution (130 μl Glp-AAL-pNa in 10 ml 0.1 M phosphate buffer, pH 7.0) was applied to the dried gel and put into a thermostat for 3 h. Then, the membrane was placed sequentially into solution A (0.01 g $NaNO_2$ in 10 ml 0.1 M HCl) for 5 min, solution B (0.05 g ammonium amidosulfate in 10 ml 0.1 M HCl) for 5 min, and solution C (0.005 g N-(1-naphtyl)ethylenediamine hydrochloride in 10 ml 50% ethanol) until pink color appeared. Pink bands against the white background indicated the enzyme activity.

RESULTS AND DISCUSSION

Effect of the medium composition on the extracellular proteolytic activity. Results of the preliminary analysis of the effect of the medium composition on the extracellular proteolytic activity of *Cordyceps* sp. are presented in Table 1. The data show that the basal level of proteolytic activity was registered even upon growth on minimal medium while the addition of potassium nitrate resulted in a slight increase in the extracellular activity. Introduction of protein to the medium intensified protease secretion considerably, resulting in a 5–10-fold increase in proteolytic activity. An additional increase was registered in *Cordyceps* culture growth on the medium containing cuticle, a substrate specific for entomopathogenic fungi. Introduction of carbohy-

drates led to a sharp decrease in the proteolytic activity, which was probably due to the accessibility of more easily available sources of nutrients and energy. Higher activity was retained upon growth on glycerol, which is rather poorly consumed by fungi, than on other more easily available compounds such as sucrose and glucose.

Intense synthesis of hydrolytic enzymes is known to be induced by emergence of mono- and oligomers formed in the process of lysis of the fungal cell wall polysaccharides [7]. Since the most active protease synthesis was observed during transition of the culture to the stationary growth phase, independently of the medium composition, the culture liquid of 5–7-day cultures with the maximum protease activity was used for isolation and study of the proteases (see figure). Upon addition of the insect (*N. cinerea*) cuticle, the peak of chitinolytic activity was observed much later than the peak of proteolytic activity. The results are in agreement with the suggestion that chitinase synthesis is induced by the fragments of the proteins lysed by proteases [8], and the early secretion of proteases is necessary for the destruction of the proteins protecting chitin of the insect covers from chitinases.

In all modifications of the medium, proteolytic activity was inhibited almost completely by phenylmethylsulfonyl fluoride (PMSF, 0.8 mM) indicating the serine protease nature of the enzyme.

Purification of the proteases secreted by *C. militaris*. In order to isolate and study extracellular proteases, Czapek medium modified with tryptone and gelatin was chosen for the cultivation of *C. militaris*. The medium, which is widely used to study the enzymes of entomopathogenic fungi [9], contained no insoluble components in contrast to the media with cuticle and made it possible to obtain high levels of proteolytic activity in the culture liquid. A two-step technique was developed to purify the most abundant protease of the culture liquids of both *C. militaris* strains (Table 2). The first step was affinity chromatography on bacitracin–silochrome, one of the most efficient known methods to isolate and purify proteolytic enzymes. Proteases of numerous fungi including representatives of the genera *Trichoderma, Acremonium, Aspergillus, Coprinus*, and *Saccharomyces* were purified by this technique [10].

Although the enzyme bound tightly to the bacitracin–silochrome adsorbent equilibrated with distilled water, no binding was observed in the column equilibrated with 0.05 M phosphate buffer (pH 7.4).

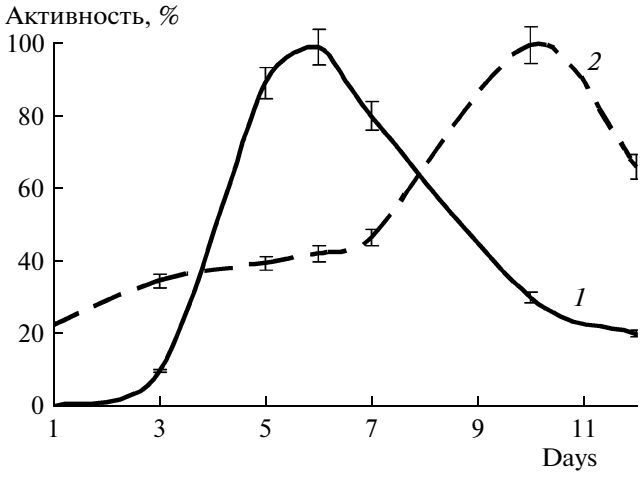
Quantitative characteristics of purification of the proteases of both strains are presented in Table 2. Affinity chromatography made it possible to use considerable amounts of the original material (culture liquid) facilitating further purification by concentrating the active fraction via ultrafiltration and gel filtration on Superdex G-75.

Table 1. Effect of the medium composition on the extracellular activity

Supplements to the minimum medium			Activity, nmol/min
KNO_3			129
Cuticle			1050
Tryptone			280
»	Gelatin		450
»	Casein		320
»	Sucrose		105
»	Glycerol		173
»	»	Glucose	14
»	Cuticle		750
»	»	Sucrose	65
»	»	Glycerol	81

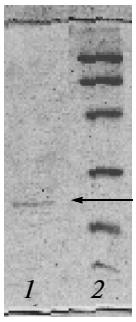
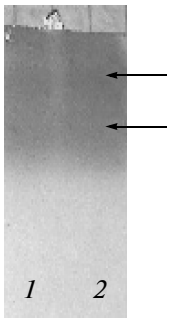
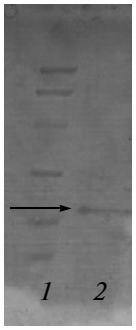
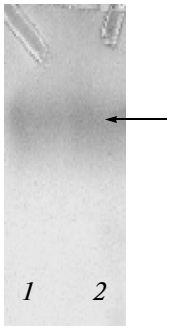
Note: The minimum medium composition (g/l) was: KH_2PO_4, 0.2; $MgCl_2 \cdot 7H_2O$, 0.1; NaCl, 0.1, and $FeSO_4 \cdot 7H_2O$, 0.002. Activity value exhibited upon growth on the minimum medium was 85 nmol/min. The supplements were added at 1% concentration.

Electrophoresis of the active protein fractions obtained by gel chromatography provided evidence of electrophoretic homogeneity of the preparations. As follows from the electrophoresis pictures, the protein band under study corresponds to the molecular mass of 24 kDa. Yield values calculated for the proteases of two strains differed twofold. This is probably the result of strain SM-2-05 secreting two proteases capable of Glp-AAL-pNa cleavage into the culture liquid, while strain KP-97-2 produces a single enzyme active towards the substrate, as was demonstrated in the experiment on post-electrophoresis activity determination. Samples of 5- and 12-day culture liquid did



Proteolytic (*1*) and chitinolytic (*2*) activities in the process of *C. militaris* growth on the cuticle-containing medium.

Table 2. Purification of the proteases secreted by *C. militaris*

Strain		Purification step		Picture of SDS-PAGE	Postelectrophoretic staining
		Affinity chromatography	Gel filtration		
SM-02-05	Purity degree	24	99		
	Yield, %	13	8		
KP-97-02	Purity degree	59	93		
	Yield, %	35	14		

Note: Electrophoresis pictures of the protease preparations and standard markers in SDS-PAGE (strain SM-02-05, *1* and *2*, respectively, and KP-97-02, *2* and *1*, respectively). Protein markers in PAGE are (top-to-bottom): phosphorylase B (97000 Da), bovine serum albumin (66200 Da), ovalbumin (45000 Da), carbonic anhydrase (31000 Da), soy trypsin inhibitor (21500 Da), and lysozyme (14400 Da). Postelectrophoretic staining of proteases. Electrophoresis pictures of the preparations isolated from 5-day and 12-day culture liquid preparations (*1* and *2*, respectively). Arrows indicate the activity manifested as pink bands against the white background.

not differ by the set of enzymes active toward the Glp-AAL-pNa substrate (Table 2).

By now, two proteases of an entomopathogenic fungus *C. militaris* have been isolated and characterized: a chymotrypsin-like [11] and a trypsin-like [12]. A subtilisin-like protease was also detected in the culture liquid of *C. militaris* by Japanese researchers, although homogeneous preparations of this protease could not be isolated and characterized even after a four-step purification procedure [12]. The development of an efficient purification procedure for the protease under study will make it possible to investigate its characteristics and its possible functional role in pathogenesis of insects. Studies on the enzyme activity and functions of proteases secreted by *C. militaris* will allow for the deeper understanding of the mechanisms of pathogenesis and provide for better control over the infection process.

ACKNOWLEDGMENTS

The work was supported by grants of the Russian Foundation for Basic Research, projects no. 08-04-00545 and 10-04-00739, and of the International Science and Technology Center, project no. 3455.

REFERENCES

1. Lesso, T., *Griby: Opredelitel'* (Fungi: Identification Guide), Moscow: AST, 2003.
2. Alicja, S., Towards an Integrated Management of *Dendrolimus pini* L., in *Proc.: Population Dynamics, Impacts, and Integrated Management of Forest Defoliation Insects, USDA Forest Service General Technical Report NE-247*, 1998, pp. 129–142.
3. Park, C., Hong, S.H., Lee, J.Y., Kim, G.Y., Choi, B.T., Lee, Y.T., Park, D.I., Park, Y.M., Jeong, Y.K., and Choi, Y.H., Growth Inhibition of U937 Leukemia Cells by Aqueous Extract of *Cordyceps militaris* through Induction of Apoptosis, *Onkology Rep.*, 2005, vol. 13, pp. 1211–1216.
4. Bidochka, M.J. and Khachatourians, G.G., Protein Hydrolysis in Grasshopper Cuticles by Entomopathogenic Fungal Extracellular Proteases, *J. Invertebrate Pathol.*, 1993, vol. 63, pp. 7–13.
5. Erlanger, B.F., Kokowsky, N., and Cohen, W., The Preparation and Properties of Two New Chromogenic

Substrates of Trypsin, *Arch. Biochem. Biophys.*, 1961, vol. 95, pp. 271–278.

6. Weisskopf, L., Tomasi, N., Santelia, D., Martinoia, E., Langlade, N.B., Tabacchi, R., and Abou-Mansour, E., Isoflavonoid Exudation from White Lupin Roots Is Influenced by Phosphate Supply, Root Type and Cluster-Root Stage, *New Phytol.*, 2006, vol. 171, pp. 657–668.
7. Braga, G.U.L., Destefano, R.H.R., and Messias, C.L., Protease Production during Growth and Autolysis of Submerged *Metarhizium anisopliae* Cultures, *Revista Microbiol.*, 1999, vol. 30, pp. 107–113.
8. Borisov, B.A., Serebrov, V.V., Novikova, I.I, and Boikova, I.V., Entomopathogenic Ascomycetes and Deuteromycetes, in *Patogeny nasekomykh: strukturnye i funktsional'nye aspekty* (Insect Pathogens: Structural and Functional Aspects), Moscow: Kruglyi god, 2001.
9. Urtz, B.E. and Rice, W.C., Purification and Characterization of a Novel Extracellular Protease from *Beauveria bassiana, Mycol. Res.*, 2000, vol. 104, pp. 180–186.
10. Rudenskaya, G.N., Affinity Chromatography of Proteinases, *Bioorg. Khim.*, 1994, vol. 20, pp. 213–229.
11. Kim, J.S., Sapkota, K., Park, S.E., Choi, B.S., Kim, S., Hiep, N.T., Kim, C.S., Choi, H.S., Kim, M.K., Chun, H.S., Park, Y., and Kim, S.G., A Fibrinolytic Enzyme from the Medicinal Mushroom *Cordyceps militaris, J. Microbiol.*, 2006, vol. 44, pp. 622–631.
12. Hattori, M., Isomura, S., Yokoyama, E., Ujita, M., and Hara, A., Extracellular Trypsin-Like Proteases Produced by *Cordyceps militaris, J. Biosci. Bioengin*, 2005, vol. 100, pp. 631–636.