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Secreted Protease of the Entomopathogenic Fungus *Cordyceps militaris*. II. Enzyme Properties and Adsorption on the Insect Cuticle

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Abstract—Electrophoretically pure preparation of a secreted subtilisin-like protease was isolated from the culture liquid of an entomopathogenic fungus *C. militaris* strain KP-97-2. The enzyme had an optimum activity at 45°C and pH 7–9. Data were collected on the substrate specificity of the enzyme and its stability at different values of pH and temperature. Adsorption and desorption of the extracellular protease on the cuticle of *Nauphoeta cinerea* was studied in function of pH.

Keywords: entomopathogenic fungi, proteolytic enzymes, *Cordyceps militaris*.
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Wide use of entomopathogenic fungi of the genus *Cordyceps* both in medicine and in agriculture provokes interest in the compounds they secrete, particularly the proteolytic enzymes. Extracellular proteases presumably play the key role in the permeation of insect pathogens through the cuticle of the host insects; the strains with the introduction of additional genes encoding secreted proteolytic enzymes had a considerably higher capacity for attacking the insects [1]. Study of the enzymatic properties and adsorption ability of the extracellular proteases of insect pathogens is of theoretical as well as practical importance; by taking into account the conditions of the optimum activity of the secreted proteolytic enzymes, it would allow for the development of more efficient biopesticides.

Few extracellular proteases of insect pathogens have been characterized as yet; little is known about their role in pathogenesis, mechanisms of action, and regulation at the level of synthesis. Detailed study of the enzymatic properties of extracellular proteases was performed only for the entomopathogenic fungi most widely used as biopesticides, such as *Metarhizium anisopliae* and *Beauveria bassiana* [2–4]. On the other hand, study of the enzymatic properties and adsorption ability of the proteases secreted by other fungi would allow for a wider range of applied entomopathogens and the development of more active and efficient biopesticides.

In the present work, the enzymatic properties of an extracellular subtilisin-like protease of *C. militaris* and its adsorption on the cuticle of insects at different pH values was studied.

MATERIALS AND METHODS

Isolation of the electrophoretically homogeneous preparation of the secreted protease. Purification of the secreted protease of *C. militaris* was performed, as is reported in the accompanying paper "Secreted Protease of an Entomopathogenic Fungus *Cordyceps militaris*. I. Selection of Medium Components and Development of Purification Procedure". The extracellular subtilisin-like protease was isolated from the culture liquid of an entomopathogenic fungus *C. militaris* strain KP-97-2 grown on Czapek medium modified with 10 g/l gelatin. Protease activity was measured by spectrophotometry using a *p*-nitroanilide substrate Glp-Ala-Ala-Leu-pNa. Affinity chromatography on bacitracin–silochrome followed by gel filtration on a Superdex G-75 column resulted in a homogeneous preparation of the enzyme, which was confirmed by denaturing electrophoresis.

The enzyme pH optimum was determined as follows: 160 μl of 0.1 M universal buffer (pH range of 2.8–11.4) and 10 μl of the substrate were added to 35 μl of the enzyme solution. The reaction mixture was incubated at 37°C. Optical density was measured at 405 nm using a plate reader.

The enzyme pH stability was determined by incubation of 15 μl enzyme and 45μl 0.1 M buffer (universal, for pH values between 2.9 and 8.7 and glycine–NaOH buffer, for pH 8.6–11.3) at 37°C for 40 min. Then, 140 μl 0.5 M phosphate buffer (pH 7.0) and 10 μl Glp-AAL-pNa were added to each sample. The reaction

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Substrate specificity of the protease secreted by *C. militaris*

Substrate	Activity, nmol/min	K_m
Clp-AAL-pNa	336	0.96
Suc-AAPF-pNa	1746	0.4
Suc-AALF-pNa	1147	0.33
L-pNa, Suc-F-pNa, N-ac-LL-pNa, F-pNa, Glp-FA-pNa	0	–

mixture was incubated at 37°C. Optical density was measured at 405 nm using a plate reader.

The enzyme temperature optimum was determined by incubation of the samples containing 500 μl buffer (pH 7.0), 50 μl enzyme, and 10 μl substrate for 30 min at 20, 30, 35, 40, 45, 50, and 60°C. Activity was determined by hydrolysis of a *p*-nitroanilide substrate Glp-AAL-pNa.

The enzyme temperature stability was determined by incubation of the samples containing 500 μl buffer (pH 7.0) and 50 μl enzyme for 30 min at 30, 40, 50, 60, 70, and 80°C followed by cooling down to room temperature. Then, 10 μl Glp-AAL-pNa was added and the activity was measured on a plate reader.

The Michaelis constant (K_m) for the reaction of hydrolysis of synthetic substrates Glp-AAL-pNa, Suc-AAPF-pNa, and Suc-AAPL-pNa was determined from the analysis of kinetic curves. The samples to obtain kinetic curves of hydrolysis of the synthetic substrates were prepared as follows: various aliquots of the substrate solution were added to 20 μl enzyme and the volume was adjusted to 200 μl with phosphate buffer, pH 7.0. Optical density of the solutions was measured at 405 nm using a plate reader. For each substrate, the reaction rate was plotted against the substrate concentration and the Michaelis constant was calculated.

Protease adsorption on the cuticle of *Nauphoeta cinerea* depending on pH. Adsorption of the protease on the cuticle was studied as follows: 50 μl 0.1 M buffer (citrate–phosphate, for pH values 3–7, and phosphate, for pH 6–8) and 7 mg cuticle were added to 15 μl enzyme. The samples with the enzyme substituted by 15 μl phosphate buffer, pH 7.0 and the samples lacking a cuticle were used as controls. The samples were incubated for 1 h on a shaker and then centrifuged at 11600 *g* for 5 min. Twenty microliter of the supernatant was withdrawn from each sample and added to 100 μl 0.5 M phosphate buffer, pH 7.5, and 7 μl Glp-AAL-pNa. Optical density of the solutions was measured at 405 nm using a plate reader. For each pH value, the percent ratio of the enzyme adsorbed on the cuticle was calculated. Then, 100 μl 0.1 M phosphate buffer was added to the cuticle-containing sediment and the enzyme was washed off during 1 h. The samples were centrifuged and 7 μl Glp-AAL-pNa was added to the supernatant. Optical density of the solutions was measured at 405 nm using a plate reader.

RESULTS AND DISCUSSION

Physicochemical properties of the enzyme and kinetics of the catalyzed reaction were studied on an electrophoretically homogeneous preparation of the protease. Physicochemical properties of the protease under study are presented on Figure 1.

The data on the effect of the incubation temperature on the enzyme activity show that the enzyme optimum temperature was in the range from 35 to 55°C with a maximum at 45°C, which is in agreement with the results obtained for most of the entomopathogenic fungi. For example, the protease secreted by *B. bassiana* was characterized by an optimum temperature of 37–42°C and the chymoelastase Pr1 of *M. anisopliae* was most active at 50°C [5]. At temperatures above 55°C, the activity of the enzyme under study decreased sharply.

Study of the enzyme temperature stability demonstrated that the protease was stable up to 50°C, while an increase in the temperature to 60°C during 30-min incubation resulted in almost complete loss of the enzymatic activity. Such dependency is typical of the extracellular proteases of entomopathogenic fungi. According to the literature data, chymoelastase secreted by *M. anisopliae* lost 5% of its activity at 50°C, while heating to 60°C resulted in 80-% loss of the activity. Incubation of the protease isolated from *B. bassiana* for 15 min at 60°C also resulted in a considerable decrease in the enzyme activity [5].

It should be noted that duration of incubation also influenced the temperature profile of the enzyme activity. For example, after 1-h incubation at 40°C, a sharp decrease in the activity of a chymotrypsin-like protease from *C. militaris* was observed [6].

Study of the pH optimum of the enzyme was performed in the pH range from 2.6 to 11.4. As follows from our data, the enzyme was active in a wide range of pH values with the maximum activity at alkaline pH 7.0–9.0, which is typical of the serine proteases and, in general, agrees with the data obtained for other entomopathogenic fungi. For example, a chymotrypsin-like serine protease isolated from *C. militaris* was active in the pH range of 4.0–10.0 with the maximum at pH 7.4 [6]. The observed pH optimum for the protease of *B. bassiana* was within the pH range of 7.5–9.5 [7] which is also in good agreement with our data. Although the pH optimum of the trypsin-like protease of *C. militaris* determined by Japanese researchers was in the range of pH 8.5–12.0 [8], the observed high activity of the enzyme at pH 11–12 possibly resulted from spontaneous hydrolysis of the substrate at highly alkaline conditions, which was not taken into account by the authors.

The results of the substrate specificity studies are presented in the table. The purified enzyme did not hydrolyze *p*-nitroanilides of one or two amino acids L-pNa, Suc-F-pNa, N-ac-LL-pNa, F-pNa, or Glp-FA-pNa, and the maximum activity was observed

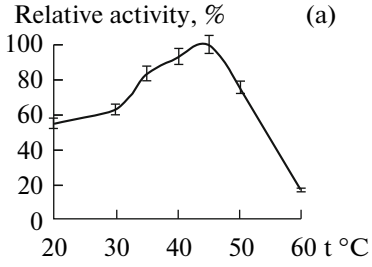
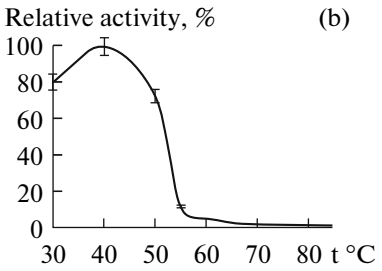
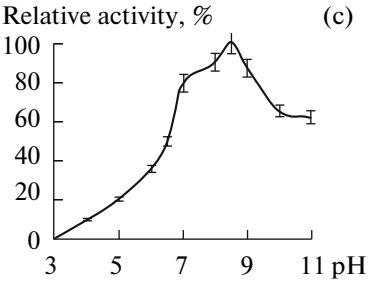
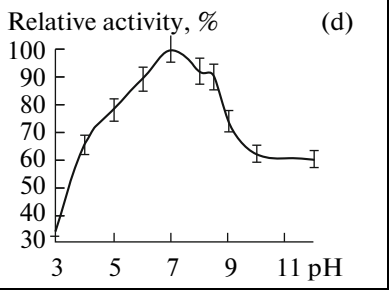
t °C optimum	45°C	
t °C stability	до 60°C	
pH optimum	7–9	
pH stability	6.3–8.7	

Fig. 1. Physicochemical properties of the protease secreted by *C. militaris*. Effect of temperature (a) and pH (c) on the enzyme activity. Stability of the enzyme depending on the temperature (b) and pH (d).

upon cleavage of Suc-AAPF-pNa. Apparently, high endopeptidase activity toward Suc-AAPF-pNa is due to the enzyme similarity to chymoelastases isolated from other entomopathogenic fungi, *M. anisopliae* and *B. bassiana* [7]. Chymoelastase is known to be responsible for the penetration of *M. anisopliae* and, probably, other entomopathogenic fungi through the insect cuticle [5].

The Michaelis constant values were determined for the substrates susceptible to the enzyme under study (see table). The data show that Suc-AALF-pNa, with its lowest K_m value, is characterized by the highest affinity to the enzyme. One substrate, different by a single amino acid, Suc-AAPF-pNa, exhibited lower affinity. More efficient binding of the substrate with leucine at P_2' site may be due to the steric hindrances created by the proline residue.

The synthetic substrate characterized by the lowest affinity to the enzyme was Glp-AAL-pNa, which was probably due to its three-peptide nature. The substrates containing four amino acid residues probably bind the enzyme more efficiently than those with three residues, which is manifested in their higher K_m values. Probably, the substrate-binding region of the enzyme is elongated and the interactions between distant amino acid residues of the substrate with the enzyme affect the binding characteristics. Thus, a subtilisin-like protease secreted by *M. anisopliae* is also known to be more efficient in cleavage of the longer-chain synthetic peptides [5].

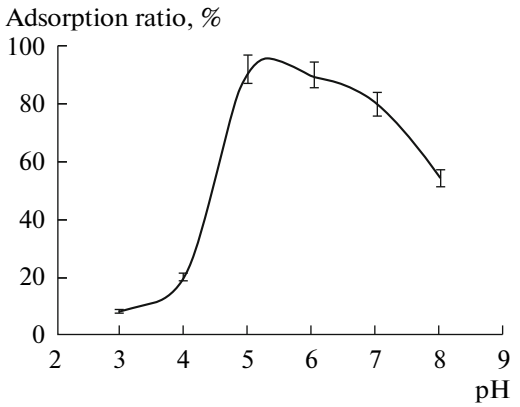


Fig. 2. Adsorption of the protease on the cuticle of *Nauphoeta cinerea*.

Protease adsorption on the cuticle of an insect is important for the infection process and is an indirect indication of the pathogenicity of a strain. Therefore, study of adsorption ability of proteases is of both theoretical and applied interest. Adsorption of the proteolytic enzymes responsible for the penetration of entomopathogenic fungi through the cuticle of the host insect and capable of hydrolysis of the cuticle proteins is the primary stage of the action of the fungal hydrolytic enzymes on the insect covers. The results of the experiments on adsorption of the enzyme under study on the cuticle of *Nauphoeta cinerea* depending on pH are presented on Figure 2.

Maximum adsorption of the protease on the cuticle was observed at pH values between 5 and 7. No adsorption took place under strongly acidic conditions. Under alkaline conditions, the adsorption ability decreased gradually. Adsorption of the proteases of *M. anisopliae, B. bassiana*, and *V. lecanii* on the cuticle of *Melanoplus sanguinipes* reached its maximum at pH values between 4 and 7. Under acidic conditions (pH 2.9 and below), proteases of entomopathogenic fungi did not adsorb on the insect cuticle. At pH 7 and 8, a slight decrease in adsorption ability was observed [9]. The protease under study was removed from the cuticle by 0.1 M phosphate buffer, pH 7.0. Desorption ratio was 20 to 50%.

The results of the experiments on the physicochemical, enzymatic, and adsorption properties of the protease under study are in agreement with the data obtained from studying the proteolytic enzymes isolated from other entomopathogenic fungi, particularly of chymoelastase Pr1 from *M. anisopliae*. Similar properties of these proteases are probably explained by the similar functions these enzymes perform in nature, and indirectly suggests the role of the subtilisin-like protease of *C. militaris* in pathogenesis in insects. Further studies are required to determine the functional role of the protease in the process of infection by *C. militaris*, and widen our perception of the role of hydrolytic enzymes in pathogenesis mechanisms of entomopathogenic fungi.

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