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Extracellular Alkaline Proteinase of *Colletotrichum gloeosporioides*

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Abstract—The main proteinase of the filamentous fungus *Colletotrichum gloeosporioides* causing anthracnoses and serious problems for production and storage of agricultural products has molecular mass of 57 kD and was purified more than 200-fold to homogeneity with the yield of 5%. Maximal activity of the proteinase is at pH 9.0-10.0, and the enzyme is stable at pH 6.0-11.5 (residual activity not less than 70%). The studied enzyme completely kept its activity to 55°C, with a temperature optimum of 45°C. The purified *C. gloeosporioides* proteinase is stable at alkaline pH values, but rapidly loses its activity at pH values lower than 5.0. Addition of bovine serum albumin stabilizes the enzyme under acidic conditions. Data on inhibitor analysis and substrate specificity of the enzyme allow its classification as a serine proteinase of subtilisin family. It is demonstrated that the extracellular proteinase of *C. gloeosporioides* specifically effects plant cell wall proteins. It is proposed that the studied proteinase – via hydrolysis of cell wall – provides for penetration of the fungus into the tissues of the host plant.

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Key words: fungi, *Colletotrichum gloeosporioides*, plants, serine proteinase, pathogens

Anthracnoses caused by imperfect fungi from the genus *Colletotrichum* represent a serious problem for the production and storage of agricultural products. These fungi are pathogens for seedlings and fruit trees, they cause maculation and fall of leaves, fading of offsets, and decay of fruits during storage. They are causal agents for coffee plants, citriculture, grapes, etc. A pathogenic fungus, *Colletotrichum gloeosporioides* is usually regarded as a weak pathogen, but it has a broad spectrum of hosts (more than 68 species) and survives well in soil on decaying plant material for several years. Conidia and sclerotia of the fungus can infect tissue of the host plant, which is in direct contact with infected soil, or reach tissue of the host plant with water drops and irrigation water, as well as can be spread with wind and insects. *C. gloeosporioides* is especially harmful for countries specialized on monoculture, when this monoculture is sensitive to anthracnose. Losses of yam yield in West-African countries, the main world producers of this culture, can reach from 80 to 90% [1]. The harm is aggravated by the ineffectiveness of benzimidazole fungicides due to the appearance of fungicide-resistant strains of *C. gloeosporioides* [2].

This fungus is used as mycoherbicide for inhibition of furrow weeds. Thus, Collego preparation manufactured in USA, a hygroscopic powder containing fungal conidia, is used to control Virginia jointvetch (*Aeschynomene virginica*) in rice and soybean seedlings, and BioMal preparation manufactured in USA and Canada and containing of dry spores of the fungus *C. gloeosporioides f. sp. malvae* mixed with re-hydrating agents is used against dwarf mallow in wheat, flax, and lentil plantings.

Infection with *C. gloeosporioides* presents a complex multifactorial process. The fungus uses a broad arsenal of chemical substances to prevent resistance of plants – from excreted enzymes to pathological toxins (phytotox-

Abbreviations: Ac) acetyl; BTEE) N-benzoyl-L-tyrosine ethyl ester; Bz) benzoyl; Glp) pyroglutamyl; IAA) iodoacetamide; PMSF) phenylmethylsulfonyl fluoride; pNa) *p*-nitroanilide; Suc) succinyl; TLCK) N_{α}-tosyl-L-lysine chloromethyl ketone hydrochloride; TPCK) N-*p*-tosyl-L-phenylalanine chloromethyl ketone; Z) benzoyl oxycarbonyl.

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ic secondary metabolites). As in the case of anthracnoses caused by *C. coccodes* [3], *C. lindemithianum* [4], and *Glomerella cingulata* [5], one of stages determining invasion of pathogenic *C. gloeosporioides* can be associated with action of hydrolases secreted by the fungus providing its penetration into the plant tissue as well as "fitting" to its own transport systems necessary for nutrition of plant cells. Proteolytic enzymes amplifying the possibilities of the fungus to use various organic nitrogen sources take an important place among hydrolases. Extracellular proteinase is a significant pathogenetic and virulent factor of *C. coccodes*, and its removal by mutagenesis converts a virulent pathogen into a nonpathogenic endophyte [3].

There is no data on extracellular proteinases of *C. gloeosporioides* and regulation of their activity during culture growth. The question has not been regarded of how endogenous plant protease inhibitors, which play an important role in plant defense against phytopathogen attack, influence the activity of these proteinases.

In the present work, results are given on purification and characterization of one major proteinase secreted by *C. gloeosporioides* during its growth.

MATERIALS AND METHODS

The fungus *C. gloeosporioides* was cultivated in 250-ml Erlenmeyer flasks containing 100 ml of modified Czapek's liquid medium (g/liter): sucrose, 30; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4$, 0.01; and 0.1 M phosphate buffer, pH 7.3, in which 1% casein solution sterilized for 30 min under 0.5 atm was added instead of mineral nitrogen source. A suspension obtained by washout from seven-day culture with 10 ml of sterile distilled water was used as an inoculum. Spores for inoculation of liquid media were produced on solid Czapek's medium. After inoculation, the Erlenmeyer flasks were cultivated on a shaker at 23°C and 100 rpm for 15-20 days to obtain maximum values of extracellular proteolytic enzymatic activities. The grown mycelium was removed by filtration on Buchner funnel, and the obtained culture liquid was used for determination of proteolytic activity.

Activity of excreted proteolytic enzymes was determined by the method of Erlanger et al. [6] by measuring of hydrolysis extent of synthetic substrates N_α-Bz-DL-Arg-pNa (BApNa), Glp-Ala-Ala-Leu-pNa (Glp-AALpNa), and others (the substrate concentration was 5 mM), as well as by trinitrophenylation [7] of hydrolysis products for substrates such as the protein gelatin (1% solution) or Z-Gly-Lys and Z-Gly-Pro—the substrates for carboxypeptidases. In the first case, the amount of the enzyme that increases the optical absorption of the solution at 410 nm by 0.01 due to the hydrolysis of a substrate under the indicated conditions (1 h, 37°C, 0.1 M phosphate buffer, pH 8.0) was taken as one unit of enzymatic activity. In the second case, one unit of enzymatic activity corresponded to the enzyme amount releasing 1 nmol of amino groups per 1 h with glycine used as a standard.

The procedure for purification of *C. gloeosporioides* extracellular protease consisted of several stages. First, the proteins were precipitated from the culture medium with ammonium sulfate (80% saturation), and the pellet was dialyzed against 0.01 M phosphate buffer, pH 7.1, for 16 h at 4°C. Then the dissolved protein was separated from the pellet by centrifugation at 8000*g* for 30 min and subjected to "batch" chromatography on DEAE-Sephadex A-50. Non-sorbed protein was removed with 0.01 M phosphate buffer, pH 7.1, and the sorbed protein was eluted with 0.6 M NaCl solution. The proteins were desalted and concentrated on an Amicon cell (The Netherlands) with PM-10 membrane at 4°C. Ion-exchange chromatography of the obtained protein mixture was carried out on a Mono Q column (Pharmacia, Sweden) installed into an FPLC system and equilibrated with 0.01 M phosphate buffer, pH 7.5, at the flow rate of 1 ml/min using buffers A (0.01 M phosphate buffer, pH 7.5) and B (0.01 M phosphate buffer, pH 7.5, containing 1 M NaCl) to form the following gradient of NaCl: 0-12% for 5 min, 12-25% for 15 min; 25-35% for 25 min, and 35-50% for 30 min of buffer B.

Molecular mass of the enzyme was determined on a Superose-12 HR 10/30 (Pharmacia) column installed into an FPLC system and equilibrated with 0.01 M phosphate buffer, pH 6.8, containing 0.5 M NaCl. BSA dimer (136 kD), BSA (68 kD), ovalbumin (45 kD), soybean trypsin inhibitor (21.5 kD), RNase (14 kD), and cytochrome *c* (12.4 kD) were used as protein standards.

A universal buffer, 200 mM, pH 2-11, was used to determine the pH-dependence of the activity of highly purified proteinase in the hydrolysis reaction with synthetic substrates. Aliquots of the enzyme preparation were incubated in 50 mM universal buffer at various pH values for 40 min to determine pH stability of the enzyme. Then 10 volumes of 100 mM phosphate buffer, pH 8.0, and substrate were added to each sample. Enzymatic activity was determined as described above.

Thermostability of the enzyme was determined by measurement of its activity after 5-min incubation at 20-80°C. Electrophoresis under non-denaturing conditions was carried out in 7.5% polyacrylamide gel at pH 8.9 [8], 5 mA per tube for 1 h. The gels were stained with 0.02% Coomassie blue G-250.

Electrophoresis under denaturing conditions was carried out in 8% polyacrylamide gel with SDS, 25 mA per gel for 1.5 h [9]. The gel was then stained with 0.02% Coomassie blue R-250 solution. A marker protein set (Helikon, Russia) consisting of cellulase (94.6 kD), BSA (66.2 kD), ovalbumin (45 kD), carboanhydrase (31 kD), trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD) was used for the enzyme molecular mass determination.

Gelatin (0.026% solution) was co-polymerized with polyacrylamide gel for post-electrophoretic within-the-gel determination of enzymatic activity. The protein was

applied onto the gel without heating, and electrophoresis was carried out at 4°C and constant current of 25 mA. After the electrophoresis, the gel was washed twice with 2.5% Triton X-100 solution, then twice with 20 mM Tris-HCl, pH 8.0, and incubated for 2 h in the same buffer at 37°C. After the incubation, the gel was stained with 0.1% Coomassie blue R-250 solution in methanol–acidic acid– water (4 : 1 : 5) and washed out with the same solution without the dye. Proteinase was revealed as a light band on the dark background.

The effects of inhibitors specifically binding with proteases of various classes on *C. gloeosporioides* extracellular protease was studied as follows: 10 μl of inhibitor solution of a particular concentration and 90 μl of 0.1 M phosphate buffer, pH 7.3, were added to 2 μl of enzyme solution, and the mixture was incubated for 20 min at room temperature. Then the substrate solution was added, and residual activity was determined as specified above. Inhibitors of cysteine proteinases (iodoacetamide (IAA) and E-64), metal-dependent proteinases (EDTA), and serine proteinases (phenylmethylsulfonyl fluoride (PMSF), N-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and N_{α}-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK)) were used in the study.

Albumin and globulin fractions from the flour of buckwheat and wheat seeds were prepared via extraction with 0.1 M phosphate buffer, pH 7.0, containing 2 M NaCl, followed by exhaustive dialysis from distilled water for precipitation of globulins. Plant cell walls were kindly provided by Dr. N. R. Meychik (Department of Plant Physiology, Moscow State University). Cell wall proteins were extracted with 0.1 M phosphate buffer, pH 7.0, containing 0.5 M NaCl.

All experiments were performed in triplicate.

RESULTS AND DISCUSSION

As seen from Table 1, the *C. gloeosporioides* extracellular proteinase was purified 203-fold as a result of a series of stages with the yield of 5%. Note a substantial elevation (approximately by 38%) of total enzymatic activity after chromatography on DEAE-Sephadex that is probably due to elimination of an inhibitor of the determined proteolytic activity. Similar increase in activity in the course of purification was earlier observed upon isolation of buckwheat seed metal-dependent proteinase [10], whose inhibitor was then isolated and characterized [11]. Repeated FPLC proved to be the most effective for the enzyme purification. A typical FPLC chromatogram of the *C. gloeosporioides* proteinase purified on the Mono Q column is presented in Fig. 1. Fractions 23-26 corresponding to the studied enzyme were pooled and analyzed. The enzyme was separated from the main bulk of inactive protein by using a NaCl stepped gradient. Notice that similar patterns of separation of active fractions were observed when either synthetic tripeptide Glp-Ala-Ala-Leu-pNa or gelatin was used as substrate. This is the evidence that the studied extracellular proteinase, the activity of which was determined mainly via hydrolysis of the synthetic substrate Glp-Ala-Ala-Leu-pNa, is able to hydrolyze protein substrates as well.

The purity of the isolated enzyme was evaluated by electrophoresis in polyacrylamide gel under non-denaturing and denaturing conditions, as well as post-electrophoretic determination of its activity by hydrolysis of gelatin copolymerized with polyacrylamide gel. In all cases, the purified fraction was revealed as a single band, the mobility of which on polyacrylamide gel electrophoresis under denaturing conditions coincided with the band possessing activity of the enzyme under study determined directly in the gelatin-containing gel after the electrophoresis (Fig. 2). SDS-PAGE with 2-mercaptoethanol and gel filtration on Sepharose-12 demonstrated that the isolated enzyme has molecular mass of 54 kD. This molecular mass differs from those of most fungal proteinases of this type [12]. Similar increase in molecular mass (78 kD) was detected in the enzyme secreted by the pathogen *C. coccodes* causing anthracnose in tomato [3].

Table 1. Sequential stages of *C. gloeosporioides* proteinase purification

Purification stage	Protein amount, mg	Specific activity, U/mg protein	Total activity, U	Purification extent	Yield, %
Initial preparation after precipitation with ammonium sulfate	194	33.7	6540	1	100
Batch-chromatography on DEAE-Sephadex A-50	61.56	147	9045	4.36	138
Concentration on Amicon cell	19.6	253.3	4964	7.5	75.9
Ion-exchange chromatography on Mono Q column	0.048	6843	328.5	203	5

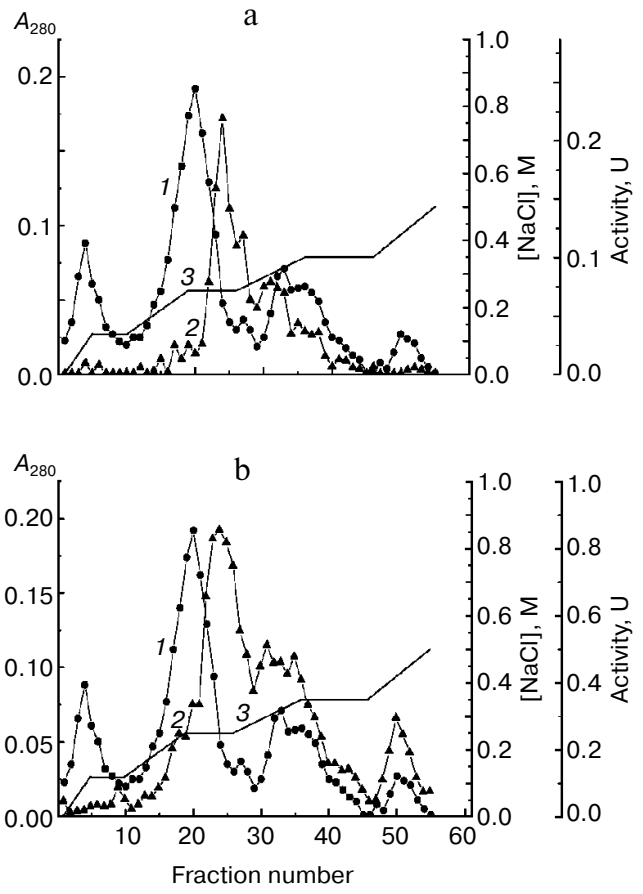

Fig. 1. FPLC of *C. gloeosporioides* extracellular proteinase on a Mono Q column (pH 7.5). Glp-Ala-Ala-Leu-pNa (a) and gelatin (b) were used as substrates for the enzymatic activity determination. *1*) A_{280}; *2*) activity; *3*) NaCl concentration.

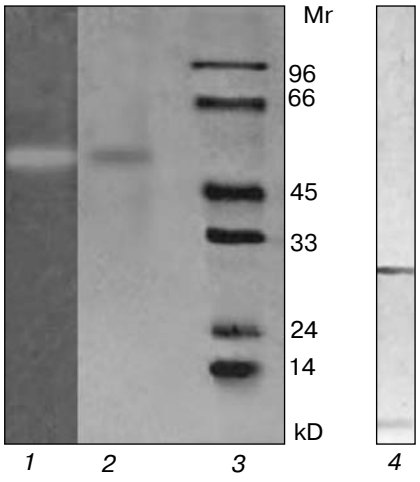

Fig. 2. Electrophoregram of purified *C. gloeosporioides* extracellular proteinase: *1*) zymogram in polyacrylamide gel containing 0.026% gelatin; *2*) polyacrylamide gel with SDS; *3*) protein markers; *4*) polyacrylamide gel without SDS.

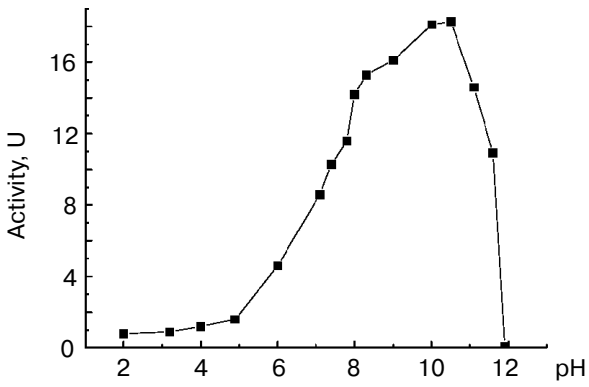

Fig. 3. Effect of pH on the activity of purified *C. gloeosporioides* proteinase determined from hydrolysis of Glp-Ala-Ala-Leu-pNa.

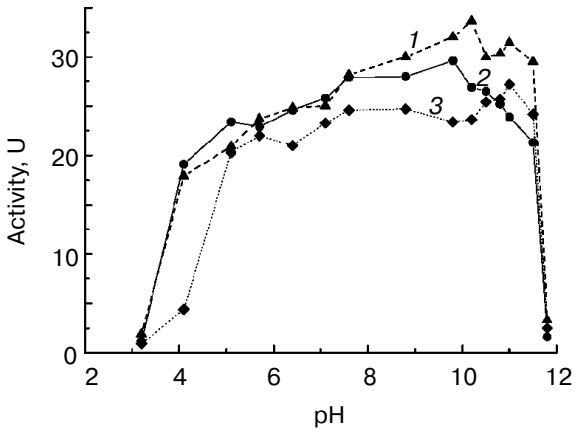

Fig. 4. The pH stability of purified *C. gloeosporioides* proteinase, the activity being determined from hydrolysis of Glp-Ala-Ala-Leu-pNa at 37°C: *1-3*) incubation for 1, 4, and 24 h, respectively.

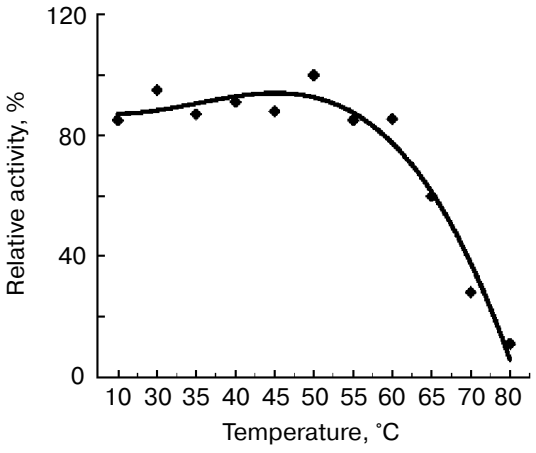

Fig. 5. Temperature stability of the *C. gloeosporioides* extracellular proteinase, activity being determined from hydrolysis of Glp-Ala-Ala-Leu-pNa.

The pH dependence of activity of the purified enzyme was determined using Glp-Ala-Ala-Leu-pNa as a substrate (Fig. 3). Extracellular proteinase hydrolyzed the substrate at pH 6.0-11.5 with an obvious maximum of the activity in the pH range 9.0-10.0. At pH 7.0 and 11.0, the enzymatic activity comprised ~50% of the activity at the pH optimum. Maximum activity of the enzyme with gelatin was detected at pH 9.0. One can point out that the alkaline and rather broad pH optimum for activity is typical for a number of excreted proteinases of some *Aspergillus* [12, 13] and *Fusarium* [14] species.

The enzyme was stable at pH 6.0-11.5 (residual activity comprised 70% of the initial activity) (Fig. 4). Even at more acidic pH value (4.5) the enzyme retained ~40% of its activity at 22 and 37°C for 1-4 h. Drastic decrease in enzymatic activity was observed at pH 11.8.

The *C. gloeosporioides* extracellular proteinase retained virtually all of its activity up to 55°C (Fig. 5). The enzymatic activity decreased to ~25% after 5 min incubation at 70°C. The temperature optimum of the activity was 45°C (Fig. 6). A similar type of dependence of the activity on temperature was detected for serine proteinase of subtilisin type from *T. harzianum* Rifai [15], although its stability and optimum of its activity were somewhat lower than those in the studied enzyme.

The purified *C. gloeosporioides* proteinase was stable at alkaline pH values but rapidly became inactive at pH < 5.0. The enzyme lost 50% of its initial activity after 1 h incubation at pH 3.8 and 37% of its initial activity at pH 4.5. Addition of calcium (10-100 mM) did not stabilize the extracellular proteinase at acidic pH values. However, addition of BSA had a stabilizing effect. The enzyme lost only 5-7% of its initial activity after 1 h incubation in the presence of BSA (40 μg/ml), and retained all its activity at BSA concentration of 160 μg/ml. BSA (and possibly other proteins) may facilitate the preservation of proteinase activity by preventing conformational changes. The partial loss of the enzymatic activity during its purification may be associated with separation of protein admixtures stabilizing the secreted proteinase in the culture mixture. Neither calcium nor BSA protected the enzyme from inactivation at temperature above 65°C.

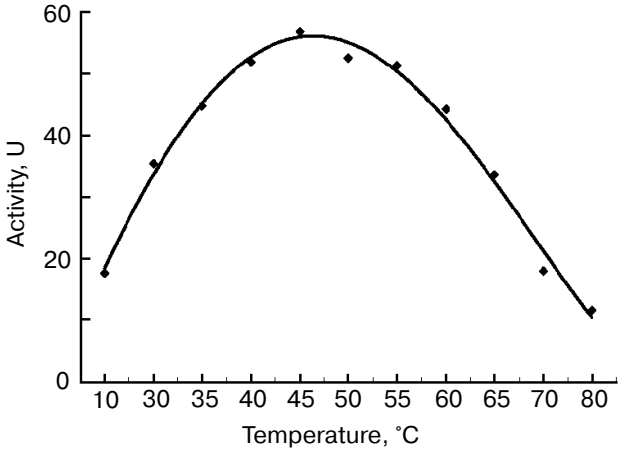

Fig. 6. Effect of temperature on the activity of the purified *C. gloeosporioides* proteinase determined from hydrolysis of Glp-Ala-Ala-Leu-pNa.

Table 2. Effects of inhibitors on the activity of the *C. gloeosporioides* extracellular proteinase

Inhibitor	Concentration, mM	Inhibition, %
PMSF	10^{-4} 10^{-5}	100 95
TPCK	10^{-3}	0
TLCK	10^{-3} $5 \cdot 10^{-4}$	15 1
E-64	10^{-5}	19
Iodoacetamide	10^{-3} $5 \cdot 10^{-4}$	24 6
Dithiothreitol	10^{-3}	5
EDTA	10^{-2}	9

Inhibitor analysis carried out for determination of functional groups in the active center revealed that the *C. gloeosporioides* extracellular proteinase using Glp-Ala-Ala-Leu-pNa as a substrate could be inhibited only by PMSF (Table 2). Only an insignificant inhibition was observed in the presence of IAA and E-64. These results indicate that the studied enzyme belongs to the serine proteinase class. The observed effect of cysteine proteinase inhibitors can be associated with the presence of SH-group(s) in the studied proteinase (outside of its active center), and modification of such group(s) influences to some extent the enzymatic activity (steric or due to conformational changes), as well as with the presence of some admixture of cysteine proteinase in the preparation. However, the second supposition becomes unlike when taking into account the lack of any activating effect of dithiothreitol, an activator of cysteine proteinases, on the enzyme.

The study of the *C. gloeosporioides* proteinase action on a number of synthetic substrates demonstrated that the enzyme hydrolyzes well the substrates for subtilisin- and chymotrypsin-like proteinases and possesses low esterase activity (Table 3). The enzyme preferably cleaved longer substrates (3-4-membered) and caused virtually no hydrolysis of shorter ones (Ac-Leu-pNa, Glp-Phe-pNa, etc.). This enzyme feature, more typical for subtilisin-like proteinases, in combination with the lack of inhibitory effect of TPCK, a specific inhibitor for chymotrypsin-like proteinases, on its activity (Table 2), suggests that the iso-

Table 3. Substrate specificity of *C. gloeosporioides* extracellular proteinase

Substrate	Concentration, mM	Activity, U/h
Bz-Arg-pNa	20	0
Ac-Leu-pNa	20	0
Glp-Phe-pNa	20	0
Z-Ala-Phe-Arg-pNa	20	5.7
ABZ-Ala-Ala-Leu-pNa	20	24.8
Glp-Ala-Ala-Leu-pNa	20	328.5
Suc-Ala-Ala-Pro-Phe-pNa	20	205.7
BTEE	20	7.2

Table 4. Protein hydrolysis by the purified *C. gloeosporioides* proteinase

Proteins	Activity, U	Specific activity, U/mg
Wheat globulins	2.3	92
Buckwheat globulins	2.5	100
Wheat albumins	6.66	266
Buckwheat albumins	5.16	206
Cell wall proteins		
mung bean	18.4	736
wheat	8.2	328
Gelatin	7.5	300
Casein	8.33	333

lated and purified extracellular proteinase belongs to the subtilisin family.

This conclusion was supported partially by the analysis of the effect of protein proteinase inhibitors of plant and animal origin on the purified preparation of the *C. gloeosporioides* excreted proteinase. Activity of the studied proteinase could be inhibited by subtilisin inhibitors revealed in a fraction isolated from wheat seeds by affinity chromatography on trypsin-Sepharose and purified by ion-exchange chromatography on Mono Q column, whereas the soybean trypsin inhibitor, which could inhibit chymotrypsin by 64% [14], had virtually no effect on the studied proteinase. A collagenase inhibitor from king crab (which was purified by affinity chromatography on collagenase immobilized on aminosilochrom and able to hydrolyze subtilisin substrates) inhibited the fungal excreted proteinase by approximately 50%.

The purified enzyme actively hydrolyzed various proteins, among which proteins of mung bean and wheat cell walls should be noted (Table 4). The hydrolysis of cell wall proteins enables the fungus to solve a double task: to provide for its own nutrition with nitrogen compounds and to facilitate penetration of the fungal hypha into cells of the host plant.

Thus, an excreted proteinase was purified to homogeneity and characterized from culture medium of the anthracnose-causative agent *C. gloeosporioides*. The data indicate that it is a serine proteinase of the subtilisin family. The effective hydrolysis of cell wall proteins by the studied enzyme indicates its possible involvement in the penetration of the fungus into plant cells. Further study for determination of its role in pathogenesis and the search for effective inhibitors, which are able to elevate plant resistance against infection by *C. gloeosporioides*, will be carried out.

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