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EXPERIMENTAL ARTICLES

Extracellular Proteinases of Filamentous Fungi as Potential Markers of Phytopathogenesis

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Abstract—he presence of proteins in the culture liquid of filamentous fungi under study was found to induce the secretion of proteinases. The inhibitory analysis of the major extracellular proteinases of the saprotrophic fungus *Trichoderma harzianum* and the phytopathogenic fungus *Alternaria alternata* showed that they both belong to the group of serine proteinases. The substrate specificity of these proteinases and their sensitivity to inhibitors suggest that the enzyme of *T. harzianum* is a subtilisin-like proteinase and the enzyme of *A. alternata* is a trypsin-like proteinase. This difference between the proteinases may reflect the physiological difference between their producers (saprotroph and phytopathogen).

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The interest of researchers in secretory fungal proteases is due to several reasons. First, they play an important part in the metabolism of producing fungi. The specific osmotrophic type of nutrition of fungi makes the secretion of their proteolytic enzymes vitally important [1, 2]. Indeed, the secretion of hydrolytic enzymes, including proteases, by fungi considerably improves their nutrition, since these extracellular enzymes make macromolecular compounds occurring in the medium suitable for nutrition. Second, extracellular proteolytic enzymes are likely to be necessary for the penetration of fungi into host tissues and, hence, may play an important role in various forms of pathogenesis [3–5]. It should be noted, however, that this suggestion has not yet received strong experimental confirmation, as the derivation of a mutant deficient in a particular secretory protease leads to increased synthesis of an alternative proteinase [6]. Third, fungal proteases are widely used in the food industry, skinnery, and pharmaceutics, as well as in the manufacture of detergents [7].

The aim of this work was to comparatively study the secretory proteases of saprotrophic and phytopathogenic filamentous fungi (*T. harzianum* and *A. alternata*, respectively) in order to elucidate the relationship between the extracellular proteases of the fungi and their phytopathogenicity.

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MATERIALS AND METHODS

The filamentous fungi used in this study were obtained from the collection at the Department of Mycology and Algology, Faculty of Biology, Moscow State University. The fungus *Alternaria alternata* (Fr.) Keissl was isolated from buckwheat seeds and the fungus *Trichoderma harzianum* Rifai (courtesy of A.N. Likhachev), from soil samples.

The fungi *A. alternata, Botrytis cinerea* Pers., *Penicillium chrysogenum* Thom, *P. terlikowskii* K.M. Zalessky, *Urocladium botrytis*, and *T. harzianum* were cultivated in 250-ml Erlenmeyer flasks with 100 ml of a modified version of liquid Czapek medium containing (in g/l) sucrose, 30; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5, KCl, 0.5; $FeSO_4$, 0.01; and casein, 10 in 0.1 M phosphate buffer (pH 7.3).

The solution of casein was sterilized separately at 0.5 atm for 30 min.

Spores for inoculation were washed off from 7-day-old plate cultures with 10 ml of sterile distilled water. *T. harzianum* cultures for inoculation were grown on malt extract agar (200 ml of malt extract, 20 g of agar, and 800 ml of water). All other fungi for inoculation were grown on Czapek agar. After inoculation, the Erlenmeyer flasks were incubated at 24°C on a shaker (100 rpm) for 8–10 days, when extracellular proteolytic activity in the culture liquid was at a maximum. Prior to measuring proteolytic activity, the mycelium was removed from the culture liquid by filtration through a

Table 1. Major proteinases secreted by various micromycetes under standard cultivation conditions

Micromycete	Activity, U/g dry mycelium	
	Trypsin-like activity with BAPNA	Subtilisin-like activity with Glp-AAL*p*Na
Alternaria alternata	678	276
Botrytis cinerea	3873	1291
Ulocladium botrytis	1209	492
Trihoderma harzianum	0	1843
Penicillium terlikowskii	0	2738
Penicillium chrysogenum	0	913

Table 2. The effect of the concentration of casein in the cultivation medium on the activity of proteinases (in U/g dry mycelium) secreted by *T. harzianum* (measured with Glp-AAL*p*Na) and *A. alternata* (measured with BAPNA)

Fungus	Casein, %				
	0	0.1	0.5	1.0	2.0
Trichoderma harzianum	0	1212	1218	1843	1633
Alternaria alternata	0	442	455	468	213

Buchner funnel. To calculate specific proteolytic activity, the mycelium was dried to a constant weight.

Proteolytic activity was measured with synthetic substrates (5 mM): Nα-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), Glp-Ala-Ala-Leu-*p*Na (BA*p*Na), and others [8], as well as with 1% casein and carboxypeptidase substrates (Z-Gly-Lys and Z-Gly-Pro) [9]. In the former case, one unit of enzymatic activity was defined as the amount of enzyme in the culture liquid that increased the optical density of the reaction mixture measured at 410 nm by 0.01 optical unit per 1 h of incubation in 0.1 M phosphate buffer (pH 8.0) at 37°C. In the latter case, one unit of enzymatic activity corresponded to the amount of enzyme liberating, under similar conditions, 1 nmole of amino groups (with glycine as the standard) per 1 h.

The extracellular proteases of *T. harzianum* and *A. alternata* were purified by procedures including protein precipitation with ammonium sulfate at 80% saturation or with acetone (20 vol %), affine chromatography on bacitracine-silochrome, ion-exchange chromatography on Mono Q (Pharmacia, Sweden) in the case of the *T. harzianum* protease, and gel filtration on Sephadex G-50 and Superose 6 in the case of the *A. alternata* protease [10, 11].

The molecular mass of the purified enzymes was determined by gel filtration on the Superdex 75 HR 10/30 column (Pharmacia) in 0.01 M phosphate buffer (pH 6.8) containing 0.5 M NaCl. The marker proteins were bovine serum albumin (BSA) dimer (136 kDa), BSA (68 kDa), ovalbumin (45 kDa), soybean trypsin inhibitor (22 kDa), RNase (14 kDa), and cytochrome *c* (12.4 kDa).

The optimum pH for the activity of the purified enzymes with the synthetic substrates was determined using a 200 mM universal buffer at pH values ranging from 2 to 11.

The optimum pH for the stability of the enzymes was determined as follows: aliquots of the enzymes were incubated in 50 mM universal buffer at different pH values for 40 min and then proteolytic activity was measured in tenfold volumes of 100 mM phosphate buffer (pH 8.0) as described above.

The optimum temperature for the enzymes was determined by measuring their activity after 5 min of incubation at temperatures ranging from of 20 to 80°C.

All measurements were performed in triplicate.

RESULTS AND DISCUSSION

The study of the proteases secreted by various filamentous fungi showed that saprotrophic fungi (*T. harzianum, P. terlikowskii*, and *P. chrysogenum*) mostly secrete subtilisin-like proteinases active with the substrate Glp-AAL*p*Na, whereas phytopathogenic fungi (*A. alternata, B. cinerea*, and *U. botrytis*) secrete not only subtilisin-like proteinases but also a trypsin-like proteinase active with BAPNA (Table 1). No variations in the cultivation conditions (pH, medium composition, degree of aeration) could induce extracellular trypsin-like proteinases in saprotrophs [12], indicating that the secretion of trypsin-like proteinases is a hallmark of phytopathogenic fungi.

The study of the synthesis of the subtilisin-like proteinase of the saprotrophic fungus *T. harzianum* and the trypsin-like proteinase of the phytopathogenic fungus *A. alternata* showed that the presence of a protein substrate (in our case, casein) at an optimum concentration in the cultivation medium is a necessary condition for the synthesis of secretory proteases in these fungi. When the cultivation medium contained no casein (but had a mineral source of nitrogen instead), the extracellular proteolytic activity of the culture liquid of the fungi was zero (Table 2).

A comparative inhibitor analysis of the purified proteinases of *T. harzianum* and *A. alternata* [10, 11] showed that phenylmethylsulfonyl fluoride (PMSF, an inhibitor of serine proteases) completely inhibited the activity of both enzymes measured with casein or specific chromogenic substrates (Glp-AAL*p*Na and BAPNA, respectively) (Table 3). Further studies showed that Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) inhibited the activity of the extracellular enzyme of *A. alternata* by 60–100%, depending on the enzyme substrate used (synthetic one or casein). At the same time, TLCK virtually did not inhibit the extracellular enzyme of *T. harzianum.* By contrast, Nα-*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK) inhib-

Table 3. The inhibitor analysis of the extracellular proteinases of (I) *T. harzianum* and (II) *A. alternata*

Inhibitor	Concentration, mM	Residual activity (%) with the substrates			
		Glp-AAL*p*Na (I)	BA*p*Na (II)	Casein	
				I	II
None		100	100	100	100
EDTA	10	90	97	105	98
o-Phenanthroline	6	99	98	109	100
PMSF	0.1	7	58	15	45
″	1.0	0	3	0	2
TLCK	0.14	100	0	100	43
″	1	100	0	91	5
TLPK	0.5	83	ND*	93	ND
″	1	58	99	83	100
p-CMB	1	100	95	100	76
Dithiothreitol	1	97	98	99	100
Pepstatin	0.01	100	100	100	100

* ND stands for "not determined."

ited the activity of the enzyme of *T. harzianum* but did not affect the enzyme of *A. alternata.* The inhibitors of cysteine proteases (*p*-chloromercury benzoate, *p*-CMB), aspartate proteases (pepstatin), and metalloproteases (EDTA) inhibited neither of the proteases under study.

The structural differences in the active centers of the two proteases revealed by the inhibitor analysis were likely responsible for the differences in the substrate specificity of these enzymes (Table 4). Indeed, the protease of *T. harzianum* was very specific to the substrates of subtilisin-like proteinases, which contain leucine or phenylalanine residues at the P_1 position, whereas the protease of *A. alternata* was specific to the substrates of trypsin-like proteinases, which contain arginine residues at the P_1 position. Both enzymes cleaved the specific substrates of other proteinase types very slowly, if at all. The preferable substrates contained more than one amino acid residue in their molecules.

The proteases of the saprotrophic and phytopathogenic fungi also differed in their physicochemical properties (Table 5). However, only the differences in the structure of the active centers and in the substrate specificity of these proteases are fundamental and may reflect differences in the type of nutrition of these two groups of fungi [12].

It should be noted that subtilisin-like proteinases were detected in the culture liquid of both saprotrophic and phytopathogenic fungi on the fourth–fifth day of cultivation, while the trypsin-like proteinase of phytopathogens was detected only on the seventh day [12, 13]. This observation can be explained as follows: subtilisin-like proteinase is less specific to the type of cleaved bonds and can attack a range of peptide bonds, thus making specific regions of proteins' substrates available to the attack of trypsin-like proteinase, which appears in the medium later than the former proteinase. It is also possible that the late appearance of trypsin-

Table 4. The substrate specificity of the extracellular proteinases of (I) *T. harzianum* and (II) *A. alternata*

Substrate*	Initial hydrolysis rate, μmol/(min ml)	
P_1 P'_1	I	II
Z-Gly-Ala-Phe-*p*Na	4.5	0
Z-Gly-Gly-Leu-*p*Na	1.5	0
Glp-Ala-Ala-Leu-Phe-*p*Na	3.0	0
Glp-Ala-Ala-Leu-*p*Na	10.6	0
Ac-Tyr-*p*Na	0	0
Suc-Phe-*p*Na	0	0
Leu-*p*Na	0	0
Z-Gly-Lys	0	0
Z-Gly-Pro	0	0
Z-D-Pro-Phe-Arg-*p*Na	0	2.4
Z-D-Val-Leu-Lys-*p*Na	0	1.9
Nα-Bz-DL-Arg-*p*Na	0	0.3
Nα-Bz-DL-Lys-*p*Na	0	0.04
Nα–Bz-DL-Arg-βNa	0	0.04
Nα-Bz-L-Arg-OEt	0	1.5

* Abbreviations: Glp, pyroglutamyl; Z, N-carbobenzoxy; Bz, benzoyl; Ac, acetyl; Suc, succinyl; *p*Na, *p*-nitroanilide; βNa, β-naphthylamide; Oet, ethyl ether.

Table 5. The main properties of the extracellular proteinases of *T. harzianum* and *A. alternata*

Fungus	Enzyme	Active center	M_r, kDa	pH		Optimal *T*°C
				Optimum	Stability	
A. alternata	Trypsin-like	Serine type	33	8.0	6–10	48
T. harzianum	Subtilisin-like	Serine type	73	8.5	6–11	40

like proteinase is due to its specific role in phytopathogenesis, which shows up after the cell wall of the host plant has been disrupted by other hydrolytic enzymes, including subtilisin-like proteinase. It should be noted that earlier studies revealed a similar successive action of cuticle-degrading enzymes in the entomopathogenic fungus *Metarhizium anisopliae* [14].

Thus, the presence of proteins in the cultivation medium of the filamentous fungi induces the synthesis and secretion of rather stable specific serine proteinases. *T. harzianum* and other saprotrophic fungi secrete only subtilisin-like proteinases, whereas *A. alternata* and other phytopathogens secrete trypsin-like proteinases as well. These data, together with those obtained for *Fusarium oxysporum* [15] and other fungi [2, 13], indicate that the production of trypsin-like proteinases is a specific feature of phytopathogens, whereas the production of subtilisin-like proteinases is a specific feature of saprotrophic and entomopathogenic fungi.

The presence of appreciable extracellular trypsin-like proteolytic activity in filamentous fungi may serve as a marker of their phytopathogenicity.

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