

## Comparative Study of Proteases of Pathogenic and Saprophytic Filamentous Fungi

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### Abstract

The presence of protein in the culture medium induced secretion of proteases in the studied filamentous fungi species. Comparative analysis of extracellular proteases expressed *in vivo* by saprotrophic (*Trichoderma harzianum*, *Penicillium terlikowskii*) and pathogenic (*Alternaria alternata*, *Botrytis cinerea*, *Ulocladium botrytis*) filamentous fungi species has been carried out. All isolated enzymes were classified as serine proteases on the basis of inhibitor analysis data. According to substrate specificity and the effect of some inhibitors it is proposed that enzymes from *T. harzianum* and *P. terlikowskii* are subtilisin-like proteases and enzymes from *A. alternata*, *B. cinerea*, *U. botrytis* are trypsin-like proteases. This fact is, apparently, one of the main characteristic properties of saprophytic and phytopathogenic fungi species. Participation of extracellular fungal proteases in pathogenesis is discussed.

**Keywords:** *Alternaria alternata*; *Botrytis cinerea*; filamentous fungi; protease; *Penicillium terlikowskii*; proteolytic activity; secretion; *Trichoderma harzianum*; *Ulocladium botrytis*

### INTRODUCTION

Secretion of hydrolytic enzymes enhances the possibilities of the fungus for self provision with food products (PETRINI *et al.* 1992; ST. LEGER *et al.* 1997). Proteases, together with other hydrolytic enzymes, secreted to the environment, are able to make macromolecular substrates ready for use. Besides, extracellular hydrolases are necessary for penetration into the host tissue. And, finally, the interest to these enzymes is due to participation of proteases in various forms of pathogenesis (ST. LEGER 1995; RHODES 1995; LARCHER *et al.* 1996). The present paper deals with the study of secreted proteases of the saprophytic (*T. harzianum*, *P. terlikowskii*) and pathogenic (*A. alternata*, *B. cinerea*, *U. botrytis*) filamentous fungi to understand the peculiarities of phytopathogens and with some data confirming the

possible participation of extracellular proteases of the fungi in pathogenesis.

### MATERIALS AND METHODS

**Cultivation of the fungi.** The filamentous fungi *T. harzianum*, *P. terlikowskii*, *A. alternata*, *B. cinerea*, *U. botrytis* were cultivated in Erlenmeyer flasks, containing 250 ml of liquid Chapek medium, to which sterilized (30 min, 0.5 psi) 1% casein solution was added instead of mineral source of nitrogen (NaNO<sub>3</sub>).

**Determination of proteolytic activity.** Secreted proteolytic activity was measured, using 5 mM synthetic substrates Bz-Arg-pNa (BAPA), Suc-Phe-pNa (SPPA), Z-Ala-Ala-Leu-pNa (Z-AALPA), Leu-pNa (LPA) (ERLANGER *et al.* 1961) and by trinitrophenylation (HABEEB 1966) with 1% casein as protein substrate.

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**Isolation of proteases.** The main extracellular proteinases of *A. alternata*, *B. cinerea*, *U. botrytis*, *T. harzianum*, *P. terlikowskii* were purified by means of affinity chromatography on bacitracin-silochrome, gel-filtration on Superose-12 and ion-exchange chromatography on Mono Q under FPLC conditions.

## RESULTS AND DISCUSSION

Earlier, studying the defense mechanisms of plant, we have demonstrated the *in vitro* effect of protease inhibitors from the seeds of buckwheat on secreted proteases of phytopathogenic fungi (DUNAEVSKY *et al.* 1998). As prolongation of these investigations, in the present work we demonstrated that isolated protease inhibitors (0.5–3.0 mg/ml) were able to inhibit hypha growth and spore germination of the studied fungi. In this case spore germination of phytopathogenic species was suppressed 2–3-fold more effective as compared to saprotrophs. Since extracellular proteases of filamentous fungi are the most probable target for the studied inhibitors we purified and performed a comparative study of these enzymes.

It was demonstrated that only substitution of inorganic nitrogen ( $\text{NaNO}_3$ ) in the Chapek medium for protein provoked secretion of a number of proteolytic activities into the medium, the level of which depended on concentration of the substrate in the medium. The secreted proteolytic activity was heterogeneous, being represented by a number of endo- and exopeptidases. However, in all studied cases activity of only one proteolytic enzyme significantly dominated among activities of all secreted proteases. In the case with *A. alternata*, *B. cinerea*, *U. botrytis* it was a proteinase, active towards BAPA, which is the substrate for trypsin-like serine and some cysteine proteases. In the case with *T. harzianum* and *P. terlikowskii* it was a proteinase, active towards Z-AALPA, which is the

substrate for subtilisin-like proteases. These enzymes were obtained highly purified (320–1300-fold), with the yield of 5–8%. The results of comparative analysis of physico-chemical characteristics of the isolated enzymes are summarized in Table 1.

Comparative analysis of substrate specificity of the studied proteases revealed that these enzymes possessed narrow specificity but towards different groups of substrates. The proteinases from *T. harzianum* and *P. terlikowskii* hydrolyzed well only the substrates of subtilisin-like proteases with preference to leucine residue at position  $P_1$ . On the other side, the enzymes from *A. alternata*, *B. cinerea*, *U. botrytis* were highly active towards substrates of trypsin-like proteases with preference to an arginine residue at position  $P_1$ . Substrates of proteases of other types were hydrolyzed by the studied enzymes either weakly or were not hydrolyzed at all. All studied proteinases evidently preferred the substrates, containing more than one amino acid residue.

Totality of the data presented above makes it possible to conclude that the presence of protein in the cultivation media of the studied filamentous fungi leads to synthesis and secretion by the fungi of relatively stable, narrow specific serine proteases in dominating amounts. In one case these are trypsin-like (for *A. alternata*, *B. cinerea*, *U. botrytis*) and in the other – subtilisin-like enzymes (for *T. harzianum*, *P. terlikowskii*). These results together with the data obtained for *Fusarium oxysporum* (DUNAEVSKY *et al.* 1995) correlate well with results of ST. LEGER *et al.* (1997), demonstrating that production of trypsin-like proteases is, apparently, characteristic of plant pathogens, whereas a higher level of subtilisin-like activity is more often found for saprotrophs and entomopathogenic fungi. Apparently, the presence of a dominating extracellular trypsin-like activity may serve as molecular marker of phytopathogenicity for filamentous fungi.

Table 1. Main properties of the secreted proteolytic enzymes of the studied filamentous fungi

Fungus	Enzyme	Active center	$M_r$ (kDa)	pH		Temperature (°C)	
				optimum	stability	optimum	stability
<i>A. alternata</i>	trypsin-like	serine	33	8.0	6–10	48	to 30
<i>B. cinerea</i>	trypsin-like	serine	17	7.0	3–7	30	to 30
<i>U. botrytis</i>	trypsin-like	serine	25	9.5	9–11	50	to 40
<i>T. harzianum</i>	subtilisin-like	serine	73	8.5	6–11	40	45–50
<i>P. terlikowskii</i>	subtilisin-like	serine	15	7.0	3–11	37	to 40

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