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Induction of secretion of extracellular proteases from *Trichoderma viride*

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Abstract

Filamentous fungi possess an efficient hydrolytic system capable of utilize the wide spectrum of complex substrates. Cellulolytic enzyme systems produced by the different strains of *Trichoderma spp.* are the best examples of versatility and adaptability for the saprophytic strategy of surviving. Filamentous fungi are known to secrete serine-, aspartic- and metallo-proteases upon cultivation. In this study we aimed to answer the question whether the secretion of protease(s) in filamentous fungi is dependent on the type of protein used as substrate. We found that the secretion of protease(s) by *Trichoderma viride* is induced in the presence of yeast extract, or of purified proteins, such as bovine serum albumin, casein, or ovalbumin. We found furthermore that properties of proteases induced under cultivation conditions were different in various aspects (temperature- and pH-dependencies, substrate specificity, sensitivity to protease inhibitors, chromatographic properties). Protease activities were characterized by electrophoresis in polyacrylamide gel with co-polymerized gelatin. Multiple proteases bands with high-molecular weights (higher than 70 kDa) were detected by gelatin zymography. Patterns of protease bands were inducer-specific.

These results show that the induction of extracellular proteases in *Trichoderma viride* fungus is not purely nutritive process but it also includes an unknown molecular recognition process, which triggers a specific secretory response.

Keywords: secretion of proteases, *Trichoderma viride*

Introduction

Extracellular proteases (EPs) of filamentous fungi (FF) play essential role in post-secretion proteins processing (Eneyskaya et al. 1999), in aerial mycelium formation (Wosten et al. 1996), in nutrition (Archer and Peberdy 1997), in adaptation on environment (St Leger et al. 1997) and in the formation of fructification structures (Small and Bidochka 2005). EP secretion is regulated by environmental (Denison 2000, Tibbet et al. 1999) and nutrition (Paoletti et al. 1998) factors. EP production may be the response to induction by specific peptides (Paterson et al. 1994) but it may also play a role in the autolysis of dying or dead mycelia of FF (McIntyre et al. 2000). Genus *Trichoderma* lives in soil and plant root system. Some strains establish long-lasting colonization of root systems and penetrate into the epidermis (Yedidia et al. 1999) using hydrolytic enzymes as cellulases, chitinases, glucanases and proteases (De la Cruz et al. 1993). Some strains are opportunistic plant symbionts and mycoparasites of phytopatogenic fungi (Harman et al. 2004). Molecular mechanisms of mycoparasitism are not clear. It has been suggested that *Trichoderma* uses for this purpose a spectrum of responses, such as the ability to colonize appropriate environment and compete for substrates (Sivan and Chet 1989), produce antibiotics (Schirmböck et al. 1994), stimulate plant protection mechanism (Djonovic et al. 2006) and produce hydrolytic enzymes. *Trichoderma* is very effective against root-knot nematode *Meloidogyne*, economically the most harmful group of phytopatogens over the world (Suarez et al. 2004). Hydrolytic enzymes from FF are used in different industrial and agricultural areas (McIntyre et al. 2001). Proteases represent a group of commercially used hydrolases so new microbial sources for their biotechnological applications are searched. Genus *Trichoderma* is due to antagonistic potential a promising candidate.

There is information about isolation and characterization of EP from genus *Trichoderma*, however less is known about molecular mechanisms participating in the protease production. *Trichoderma* EP purification by affinity chromatography using bacitracin was reported first time (Stepanov et al. 1981). In 1993 first fungal gene coding alkalic protease, pbr1, was cloned from *T. atroviride* (Geremia et al. 1993) and homologue from *T. virens* (Pozo et al. 2004). Promoter of prb1 includes AreA and CreA sites for N- and C-regulation and 4 mycoparasitic response elements (Cortes et al. 1998). Prb1 is active if FF was grown in a medium with chitin or *R. solani* cell walls (Olmedo-Monfil et al. 2002). High-level expression of pbr1 was demonstrated in a dual culture experiment with *T. atroviride* and *R. solani* though there was no mutual contact of FF. It suggests that the host produces a

diffusible factor responsible for triggering PBR1 synthesis. The pbr1 expression is repressed by glucose and regulated by N-availability (Cortes et al. 1998). *T. virens* mutant study with deleted or overexpressed tvsp1 showed that no essential functions important for growth and development of FF are influenced and that it plays a role in biocontrol process (Pozo et al. 2004). *T. harzianum* strains secrete an entire complex of EP. Enzyme profiles of 3 trypsin-like (5-19 kDa) and 6 chymotrypsin-like proteases (12-43 kDa) were detected by fractionation of *T. harzianum* medium (Manczinger et al. 2002). Subtilisin-like serine protease (73 kDa) with temperature optimum 40°C (Dunaevsky et al. 2006) and chymotrypsin-like protease PRA1 (Suarez et al. 2004) were purified to homogeneity and characterized from medium of *T. harzianum*. A PRA1 analysis showed that it is 28 kDa monomer and contains 3 O-glycosylation sites. N-terminus amino acid sequence showed (60-85%) similarity with EP from other FF. Expression of pra1 is induced by fungal cell walls and subjected to N- and C-derepression. Serine proteases were found in *T. viride* (Uchikoba et al. 2001), *T. koningii* (Manonmani and Joseph 1993) and *T. longibrachiatum* (Kredics et al. 2004). Acid aspartic proteases were identified from *T. reesei* (Eneyskaya et al. 1999), *T. harzianum* (Delagdo-Jarana et al. 2002), *T. viride* (Simankova et al. 1998) and *T. asperellum* (Viterbo et al. 2004). EPs with various sensitivity to pepstatine A were characterized from *T. reesei* (2,40). Post-secretion limited proteolysis study showed that the pepsin-like protease (32 kDa) participates in activation of galactosidase and cellobiohydrolase; the degree of proteolysis depends on glycosylation level and pH. Glucose and cellobiose inhibit the activity of the aspartyl protease (Eneyskaya et al. 1999). Pitts et al. performed crystallization and X-ray analysis of trichodermapepsin from *T. reesei*. The gene encoding aspartyl protease papA was found in *T. harzianum* (Delgado-Jarana et al. 2002) and in *T. asperellum* (Viterbo et al. 2004). PapA promoter contains potential AreA, PacC and MYC sites responsible for regulation by N-source and pH. PapA expression is repressed by glucose, glycerol and $(NH_4)_2SO_4$ but organic N-source induces it. PAPA is synthesized in an inactive form which is processed to 36.7 kDa product. PAPA does not contain any potential post-translational modifications sites. PAPA role in mycoparasitism was confirmed by confrontation surface cultivation with *T. sperellum* and *R. solani* when 4-fold higher papA induction was observed in the presence of pathogen. EPs belonging between cysteine proteases and metalloproteases have not been described in *T. viride*.

The aim of this work is to characterize the ability of filamentous fungus of *Trichoderma viride* to secrete a specific extracellular protease in dependence on the protein(s) available in the environment. Our results show that the secretion of extracellular proteases in

T. viride fungus is not only general response as a consequence of nutrition requirements but it also includes an unknown molecular recognition process, which triggers a specific secretory response.

Experimental

Model microorganism

Trichoderma viride, strain CCM F-534 from the Czech Collection of Microorganisms, T.G. Masaryk University, Brno, Czech Republic, was used.

Cultivation of the fungus

Trichoderma viride was cultured under submerged conditions. Two hundreds mL of liquid Czapek-Dox medium supplemented with 0.1% (w/v) yeast autolysate (YA) and protein inducer (final concentration of 1%) in 500 mL flasks were inoculated with *T. viride* conidia (final concentration 1×10^6 conidia per mL), and the suspension was cultivated on a rotary shaker (240 rpm) for 7 days at 27°C. Purified proteins such as bovine serum albumin (BSA), ovalbumin (OVA) and casein (CAS) were used as protein inducers, which were sterilized together with Czapek-Dox medium.

Measurement of biomass concentration

During cultivation, 5 mL aliquots of submerged cultures were withdrawn (at times as indicated in the figure) and filtered through glass-fiber membranes (Whatman GF/A). Membranes with collected mycelia were washed two times with 4 mL deionized water and dried (at 70°C for 5 hours) to a constant weight. Biomass concentration is stated as the dry mycelial mass (in milligram per milliliter of submerged culture). Two experiments each with submerged culture of different origin were performed in duplicate. Data are expressed as average ± standard deviation.

Protease purification

For proteases purification, extracellular fluids of *T.viride* submerged cultures were used. The culture supernatant was collected by filtration on nylon net (pore diameter is 43 μm), concentrated by AMICON stirred ultrafiltration cell with PB membrane (50-kDa cut-off , Millipore), and extensively dialyzed against 20 mM Tris-HCl buffer (pH 7.5). The resulting solution containing approximately 50 mg of enzyme protein was applied to a DEAE

Sephadex A-50 column that was equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The column was washed with 180 mL of the equilibration buffer containing 80 mM of NaCl to remove unbound proteins and then the protein fraction bound to the matrix was eluted with two different linear NaCl gradients, one after the other. First, a 400 mL linear gradient of 0.08-0.6 M NaCl was used followed by a 200 mL linear gradient of 0.6-2.0 M NaCl both in Tris-HCl buffer (pH 7.5). Fractions containing the majority of protease activity were pooled, concentrated by membrane ultrafiltration and dialyzed against 20 mM potassium phosphate buffer, pH 7.4. The concentrated protein solution was loaded on a Superdex 200 column (1 x 30 cm) and eluted with the dialysis buffer. Those fractions containing protease activity were pooled, dialyzed and concentrated. All chromatographic runs were carried out on an AKTA FPLC chromatographic system from Amersham Pharmacia Biotech (Uppsala, Sweden) in the cold cabinet at 4°C.

Measurement of proteolytic activity

The protease activity was assessed in duplicate by measuring the release of trichloroacetic-acid soluble peptides from 0.25% (w/v) azocasein in 50 mM Tris-HCl buffer (pH 7.5) at 37ºC for 4 hours. The 0.5 mL-reaction was terminated by the addition of 0.4 mL of 10% trichloroacetic acid and then centrifuged at 10.000*g* for 10 min. After centrifugation, 850 μL of the supernatant was collected and mixed with of 320 μL of 1M NaOH. Proteolytic activity was determined by reading optical density of the resulting solution at 440 nm. Control assay without enzyme was used as a blank. One unit of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 440 nm equal to 1.0 in hour.

Effect of inhibitors on protease activity

The effect of standard protease inhibitors on protease activity was determined by the addition of the corresponding inhibitors (PMSF, Na_2EDTA, pepstatin, pCMB) to the reaction mixture at 0 time, and assayed under standard conditions.

Substrate specificity

Different synthetic p-nitroanilide derivates such as N-α-benzoyl-DL-arginine p-nitroanilide (BAPA), γ-L-glutamyl-p-nitroanilide (GlupNA), N-succinyl-L-alanyl-L-alanyl-L-alanine p-nitroanilide (STANA) were used as substrate to evaluate the substrate specificity of isolated proteases. The final assay mixture (0.25 mL) containing 2.5 mM of substrate, 0.1 M

of Tris-HCl buffer (pH 7.5), and enzyme proteins (10 μg of total proteins) was incubated for 12 hours at 37°C. The reaction was stopped by the addition of 0.75 mL of 10% (v/v) acetic acid, and the amount of released p-nitroaniline was measured spectroscopically at 405 nm. One unit of the enzyme activity was defined as the amount of enzyme required to liberate 1 μmol of p-nitroaniline per time unit under the conditions of the assay.

pH and temperature optima

The effect of pH on the proteolytic activity was investigated in 0.1 M acetate-Tris buffer (pH 4.0 to 5.5), 0.1 M Mes buffer (pH 6.0 to 7.5), and 0.1 M Tris-HCl buffer (pH 7.5 to 9.0) at 37°C. Other conditions of assay were same as described earlier. The effect of temperature on the activity of proteases was measured under standard conditions, except that reaction mixtures were maintained at various temperatures ranging from 5 to 50°C for 8 hours in 50 mM Tris-HCl buffer at pH 7.5.

Protein concentration

Protein concentration at different stages of purification was determined by absorbance at 280 nm as well as by method of Bradford (1976) using BSA as a standard.

Gelatin Zymography

Zymograms were prepared using gelatin (0.2%) co-polymerised in 7% acrylamide according to the method of Laemmli (1970). Samples, containing approximately 20 μg of protein, were mixed in Laemmli solubilizing solution without reducing agents and loaded into gel. Electrophoresis was performed at 28 mA and 4°C for 1 h and then, in order to remove SDS, the gels were soaked with gentle agitation for 2 x 20 min at room temperature in 25 mM Tris-HCl buffer, pH 7.5, containing 2.5% Triton X-100. The gels were shortly washed by distilled water and placed in the enzyme incubation buffer for at least 8 h at 37°C (25 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$ and 20 mM NaCl). The gels were finally stained for 30 min with 0.25% Coomassie Briliant Blue R-250 in methanol/H_2O/acetic acid solution (45:45:10; v/v) and then de-stained in methanol/H_2O/acetic acid solution (10:90:10; v/v) for 4 x 2 hours at room temperature on a rotary shaker to visualize the proteolytic activities as clear bands (areas of digestion) against the blue background of stained substrate.

Chemicals

The chemicals used were from the following sources: Tris(hydroxymethyl) aminomethane (Tris) from MP Biomedicals (Ohio, U.S.A.); azocaseine, N-α-benzoyl-DL-arginine p-nitroanilide (BAPA), γ-L-glutamyl-p-nitroanilide (GlupNA), bovine serum albumin (BSA), ovalbumin (OVA), and Coomassie Briliant Blue R-250 from SIGMA-Aldrich (Steinheim, Germany); casein (CAS) from Difco (Detroit, U.S.A.); N-succinyl-L-alanyl-L-alanyl-L-alanine p-nitroanilide (STANA) and phenylmethanesulphonyl fluoride (PMSF) from SERVA (Heidelberg, Germany). All other reagents were of analytical purity purchased from Lachema, Brno, Czech Republic.

Results and Disccusion

Production of extracellular proteases

In order to evaluate the relationship between properties of the protein inducer and protease secretion, purified proteins as inducers, for example bovine serum albumin (BSA), ovalbumin (OVA) and casein (CAS) were used. Presence of protein inducers in CzD medium during submerged cultivation did not dramatically affect growth or amount of *T.viride* biomass (Fig. 1A). It indicates that the nutritive value of protein cleavage is not essential for the growth of fungus in the presence of sucrose, nitrate and yeast autolysate (YA) that are components of CzD medium. However, addition of 1% protein inducer into growth medium of *T. viride* resulted in significantly increased secretion of proteolytic activity. In the presence of protein inducers, proteolytic activities in *T. viride* culture filtrates were measured after 40 hours followed by continuous increasing up to 7 days at least, although first proteolytic activity in the control experiment cultivated without addition of protein inducer began already after 5 days (Fig.1B). YA alone functions as source of vitamins for fungus and even if its presence stimulate the production protease(s), it does not have a crucial effect on protease secretion. Purified proteins induced secretion of protease(s) with different efficiency, BSA was shown as the most efficient protein inducer.

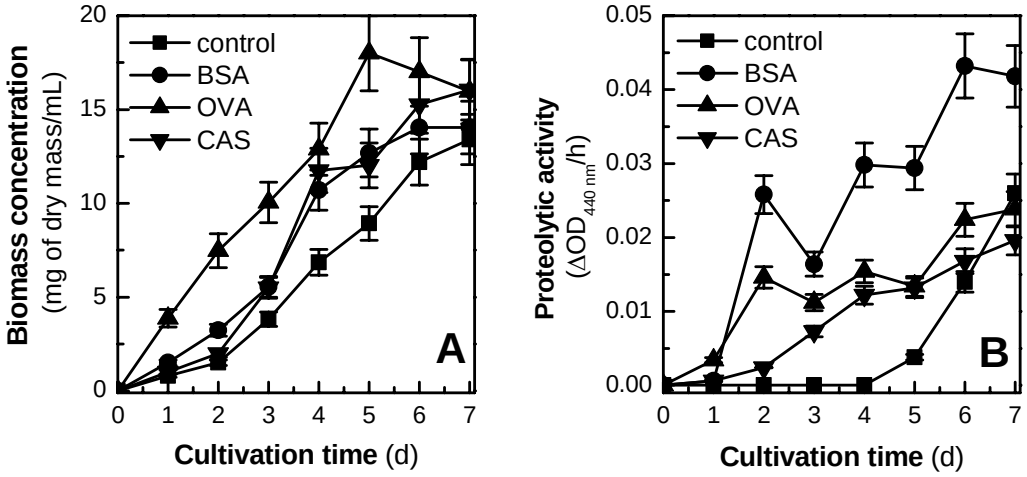

Fig. 1. Growth curves (A) and secretion of extracellular proteases (B) by *T. viride* during submerged cultivation of fungus in CzD medium containing purified protein inducers (final concentration of 1%): control experiment contains no inducer (control, ■), bovine serum albumin (BSA, ●), ovalbumin (OVA, ▲), and casein (CAS, ▼).

Purification of proteases

In order to estimate the differences in secreted proteases in dependence of protein inducers used, proteases from culture filtrates were purified by chromatography. In the first step ion-exchange chromatography was used followed by gel filtration. Differences in protease properties suggested their behavior during ion-exchange chromatography. Proteolytic activities eluted from DEAE Sephadex A-50 column at different retention volumes (Fig. 2). OVA and CAS induced proteolytic activities eluted almost in the dead volume of the column, whereas proteolytic activity in control experiment and BSA induced protease(s) came out from the column at 0.25 and 0.5 M of NaCl concentrations, respectively. This result suggests the molecular differences of secreted proteases.

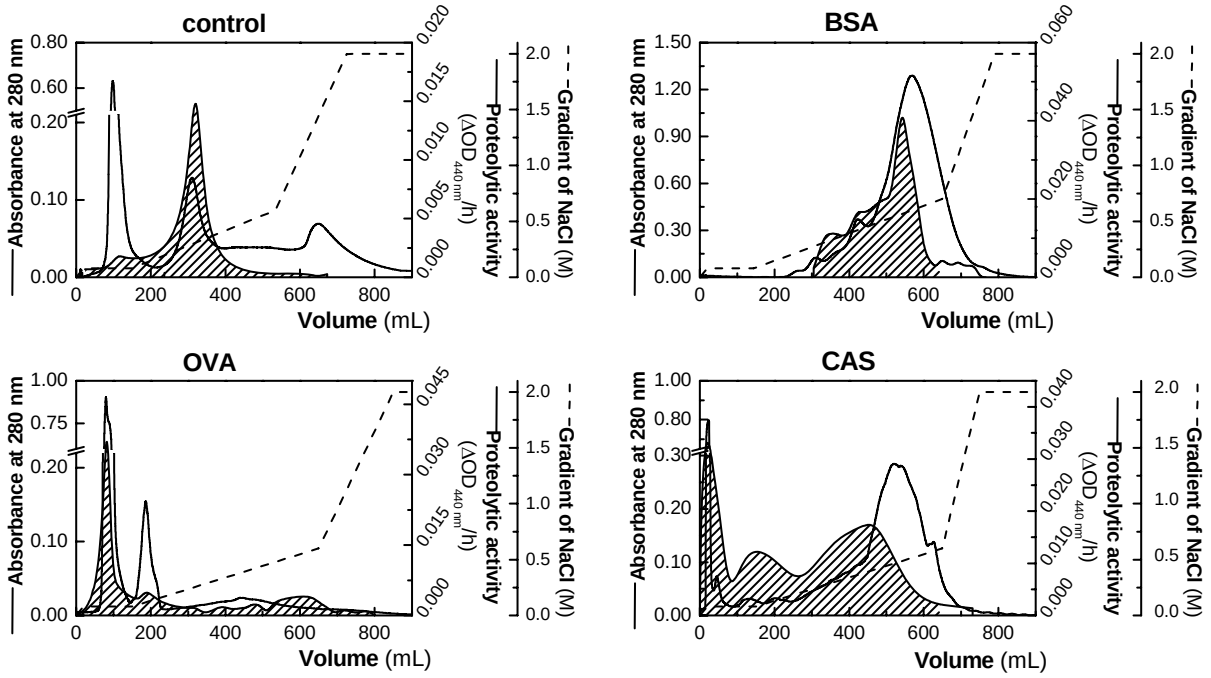

Fig. 2. Elution patterns of proteases secreted by *T. viride* on DEAE Sephadex A-50 column. Curve of absorbance at 280 nm is represented by the solid line. Proteolytic activity curve is represented by the filled area under the curve (hatch pattern). Curve of NaCl gradient is dashed line.

However, patterns of proteolytic activities during the gel filtration of proteases are almost identical to each other, and very close to the dead volume of Superdex 200 column, which indicates similarity in molecular weights of secreted proteases (Fig. 3).

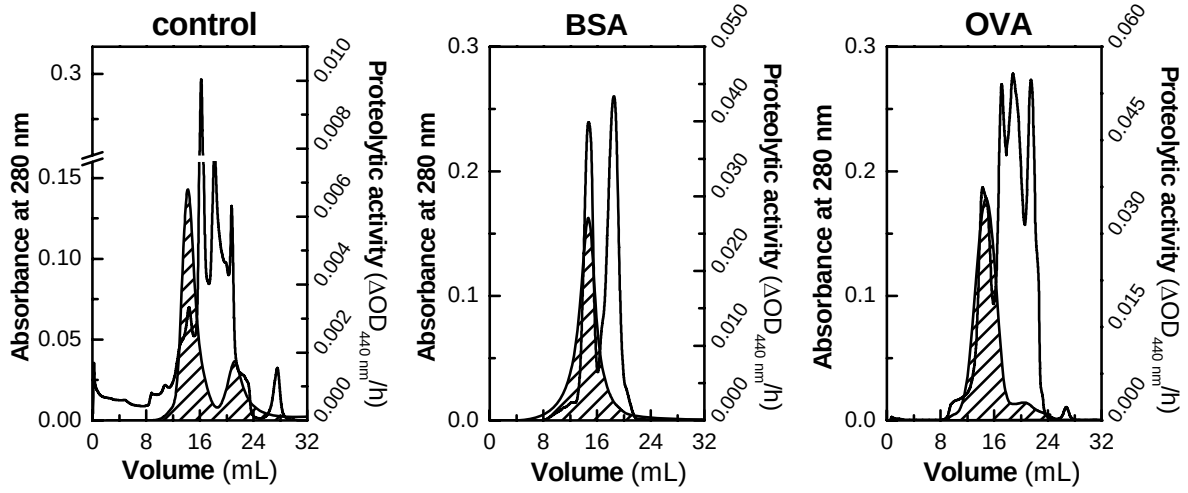

Fig. 3. Elution patterns of extracellular proteases produced by *T. viride* fungus on Superdex 200 column. Curve of absorbance at 280 nm is represented by solid line. Proteolytic activity curve is represented by the fill area under the curve (hatch pattern).

All induced proteases underwent autoproteolytic cleavage in each purification step and recovery of proteases after purification procedure ranged from 20 to 30%. Purity analysis of protease fractions by gel electrophoresis revealed that even after purification, proteases are not electrophoretically homogeneous and contain some low-molecular peptides, probably products of autoproteolysis (not shown). The addition of another purification step, to improve purity of fractions, usually resulted in a complete loss of proteolytic activity, probably due to massive autoproteolysis.

Biochemical characterization of proteases

Differences in profile and in catalytic properties of secreted protease(s) after the use of specific protein inducers were also confirmed by the measurement of substrate specificity, temperature dependence and sensitivity to protease inhibitors. Although from these results is not possible to identify type of proteases it indicates the existence of several proteases with different catalytic properties.

The substrate specificity of proteases was examined with three different chromogenic substrates (Fig. 4). Protease activities induced by different inducers were not strictly specific to any of tested substrates. CAS induced protease cleaved all substrates almost at the similar rate. GlupNA was an efficient substrate for protease induced by OVA, other substrates were far less effective (approximately 8-fold lower activity was observed with them). Protease induced by BSA cleaved particularly GlupNA, but almost no activity was observed with BAPA. In the control experiment, GlupNA and STANA were cleaved at the same rate.

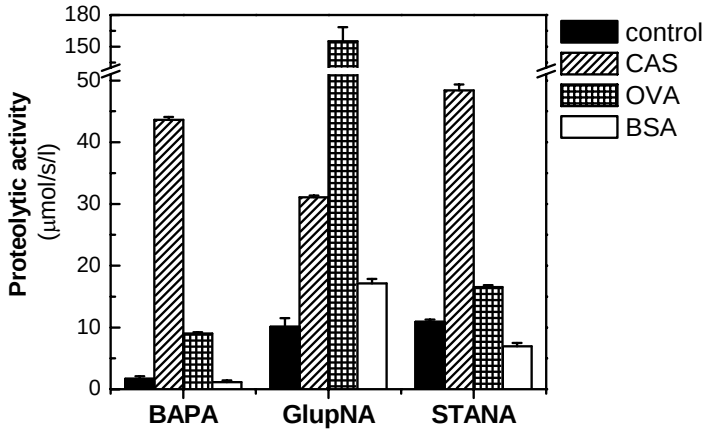

Fig. 4. Substrate specificity of proteases secreted by *T. viride*. p-nitroanilide substrates: N-α-benzoyl-DL-arginine-p-nitroanilide (BAPA), γ-L-glutamyl-p-nitroanilide (GlupNA), and N-succinyl-L-alanyl-L-alanyl-L-alanyl-p-nitroanilide (STANA) were used for characterization at 2.5 mM concentration.

Measurements of sensitivity to standard proteinase inhibitors: PMSF - inhibitor of serine proteases, pCMB - inhibitor of thiol proteases, EDTA - inhibitor of metalloproteases, and pepstatin A - inhibitor of aspartyl proteases, confirmed differences in catalytic properties of secreted proteases. Sensitivities of proteolytic activities induced by protein inducers were different (Fig. 5). Protease(s) in control experiment were sensitive to almost all inhibitors. CAS induced proteases were, similarly as in the control, sensitive to all inhibitors but at different level, with exception of pCMB that at the maximal concentration had only slight effect on proteolytic activity. OVA induced secretion of proteases with the least sensitive to all inhibitors. None of the standard protease inhibitors could completely inhibit the proteolytic activity induced by OVA.

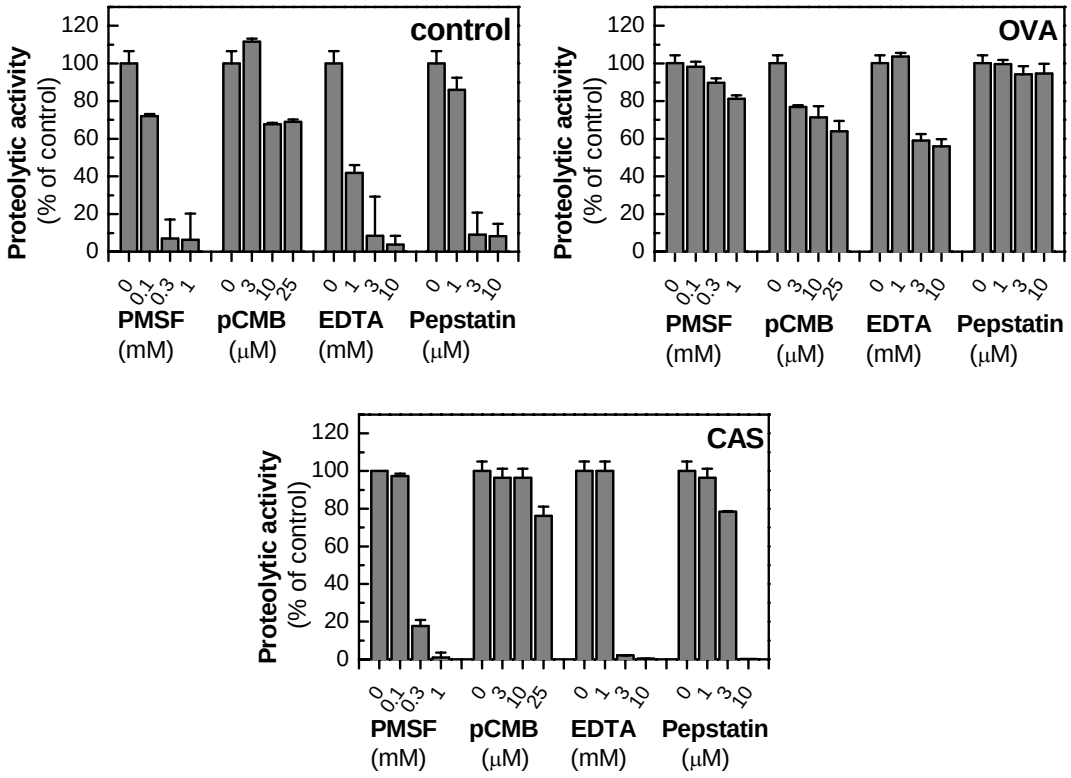

Fig. 5. Sensitivity of secreted proteolytic activities to protease inhibitors. Protease inhibitors were tested in concentration ranges as follows: PMSF from 0 to 1 mM, pCMB from 0 to 25 μM, Na_2EDTA from 0 to 10 mM, and pepstatin A from 0 to 10 μM.

Temperature- and pH-dependence measurements also yielded different patterns of activity stimulation by temperature and by pH (Fig. 6). Control and CAS optimal temperature was about 37°C, but ranges of optimal proteolytic activities in the dependence of temperature for both samples were different. CAS proteolytic activity abruptly decreased at temperatures

higher or lower than 37°C, whereas the temperature optimum of proteolytic activity in the control is much broader, from 20 to 45°C. BSA induced protease(s) with temperature optimum at lower temperature, about 20°C. Proteases secreted by *T. viride* in the presence of protein inducers are active at neutral or slightly alkaline pH with an optima of 7.0 to 8.0. The activity of the control was optimal at neutral pH, whereas BSA and CAS induced proteases showed maximal proteolytic activities at pH 7.5 and 8.0, respectively.

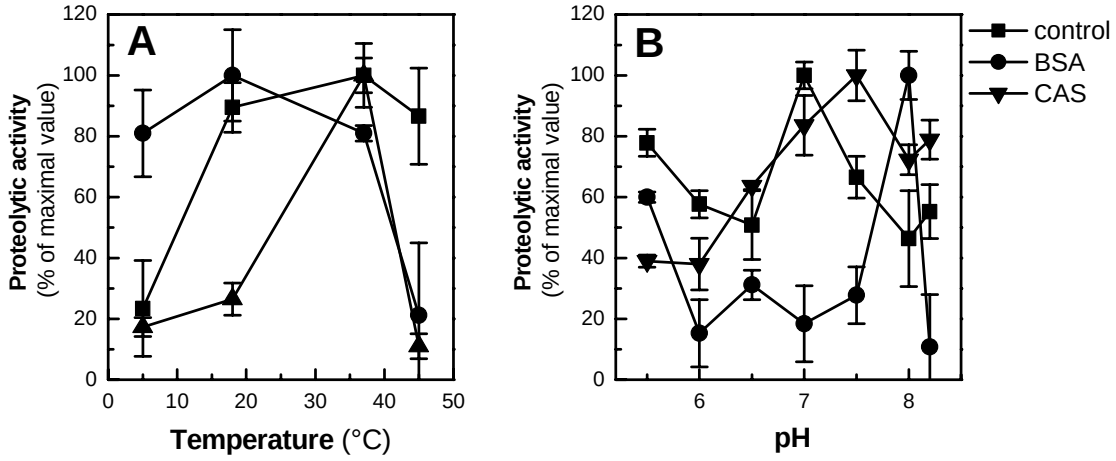

Fig. 6. The effect of temperature (A) and pH (B) on the activity of extracellular proteases of *T. viride* induced by various protein inducers: control experiment without protein inducer (■), BSA (●), and CAS (▼).

To characterize molecular properties of secreted proteases (molecular weights and profile of proteolytic activities), the gelatin zymography of proteases on 7% PAGE was carried out (Fig. 7). Profile of proteolytic activities showed, that *T.viride* fungus after contact with protein inducer(s) secretes several proteases with high-molecular weight in the range from 70 to 250 kDa. Low-molecular bands with proteolytic activity are probably cleavage products of high-molecular proteases. Important observation is that proteolytic activity profiles induced by each protein inducer are different to each other, and this supports the idea about specific production of proteases in dependence on type of protein inducer.

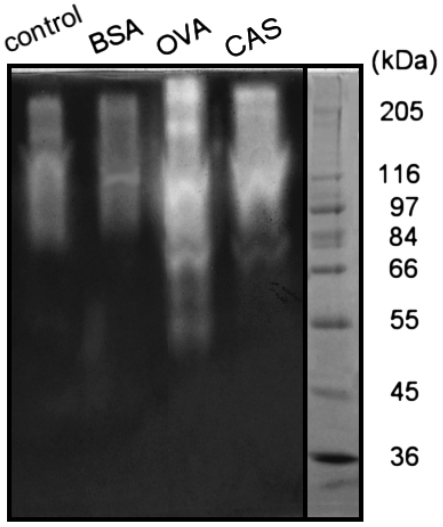

Fig. 7. Gelatin zymography of proteases secreted by *T. viride* in the presence of purified protein inducers. Zymography was performed under nonreducing conditions on 7% polyacrylamide gel copolymerized with 0.2% gelatin.

Although various proteases and their secretion by *Trichoderma* genus, mainly under mycoparasitic conditions, have been wide studied in details, we have tried to show, that the secretion of proteases in *T. viride* is not only phenomenon triggered by nutrition status of fungus and/or starvation, but it is specific reaction of the fungus induced by recognition of some protein(s) in the environment too.

Our results presented above suggest that filamentous fungus of *T. viride* cultivated in the presence of purified proteins secretes proteases at the beginning of the exponentially state of growth, although secretion in the control without inducer started almost in the stationary phase. It was shown that, even if purified proteins are in excess in the growth medium, they do not have the principal effect on fungal biomass. Neither starvation nor cleavage of proteins has effect on the growth of fungus. Then only differences in their nutritive value (i.e., composition of proteins) may explain the quantitative differences in the induction of extracellular proteolytic activity. Chromatography purification of proteases revealed some molecular properties of secreted proteases. Proteolytic activity profile of proteases obtained during ion-exchange chromatography is dependent on the type of protein inducer. On the other hand, protease molecules are similar in size how was shown by gel filtration. These observations indicate that secreted proteases are different in primary structure but similar in size.

Moreover, differences in catalytic properties of secreted proteases have been confirmed by the measurement of substrate specificity, temperature- a pH- dependence and sensitivity to protease inhibitors. Although from these results is not possible to determine accurate type of proteases it indicates the existence of several proteases with different catalytic properties in dependence on the type of protein inducer used during cultivation. Proteases are secreted as high-molecular weight proteins, which are cleaved to shorter enzymes in the medium. Mycelium is able to recognize the presence of protein as well as the quality (e.g., primary structure) of protein.

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References

Archer DB, Peberdy JF (1997) Crit. Rev. Biotechnol. 17: 273-306.

Bradford MM (1976) Anal. Biochem. 72: 248-254.

Cortes C, Gutierrez A, Olmedo V, Inbar J, Chet I, Herrera-Estrella A (1998) Mol. Gen. Genet. 260: 218-225.

De la Cruz J, Rey M, Lora JM, Hidalgo-Gallego A, Dominguez F, Pintor-Toro JA, Llobell A, Benitez T (1993) Arch. Microbiol. 159: 316-322.

Eneyskaya EV, Kulminskaya AA, Savelev AN, Saveleva NV, Shabalin KA, Neustroev KN (1 999) Appl. Microbiol. Biotechnol. 52: 226-231.

Delgado-Jarana J, Rincón AM, Benítez T (2002) Microbiology 148: 1305-1315.

Denison S (2000) Fungal Genet. Biol. 29: 61-71.

Djonovic S, Pozo MJ, Dangott LJ, Howell CR, Kenerley CM (2006) Mol. Plant. Microbe Interact. 19: 838-853.

Dunaevsky YE, Gruban TN, Beliakova GA, Belozersky MA (2006) Mikrobiologiia 75: 747-751.

Geremia RA, Goldman GH, Jacobs D, Ardiles W, Vila SB, Van Montagu M, Herrera-Estrella A (1993) Mol. Microbiol. 8: 603-613.

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004) Nat. Microbiol. Rev. 2: 43-56.

Kredics L, Antal Z, Szekeres A, Manczinger L, Kevei F, Nagy E (2004) Acta Microbiol. Immunol. Hung. 52: 223-229.

Laemmli UK (1970) Nature 227: 680-685

Manczinger L, Molnár A, Kredics L, Antal Z (2002) World J. Microbiol. Biotechnol. 18: 147-150.

Manonmani HK, Joseph R (1993) Enzyme Microb. Technol. 15: 624-628.

McIntyre M, Berry DR, McNeil B (2000) Appl. Micorbiol. Biotechnol. 53: 235-242.

Suarez B, Rey M, Castillo P, Monte E, Llobell A (2004) Appl. Microbiol. Biotechnol. 65: 46-55.

McIntyre M, Muller C, Dynesen J, Nielsen J (2001) Adv. Biochem. Eng. Biotechnol. 73: 103-128.

Olmedo-Monfil V, Mendoza-Mendoza A, Gomez I, Cortes C, Herrera-Estrella A (2002) Mol. Gen. Genom. 267: 703-712.

Paoletti M, Clave C, Begueret J (1998) Gene 210: 45-52.

Paterson IC, Charnley AK, Cooper RM, Clarkson JM (1994) Microbiology 140: 185-189.

Pitts JE, Crawford, M, Nugent PG, Western R, Cooper JB, Mäntylä A, Fagerström R, Nevalainen H (1995) Adv. Exp. Med. Biol. 362: 543-547.

Pozo MJ, Baek JM, Garcia JM, Kenerley CM (2004) Fungal Genet. Biol. 41: 336-348.

Schirmböck M, Lorito M, Wang YL, Hayes CK, Arisan-Atac, I, Scala F, Harman, G, Kubicek C (1994) Appl. Environ. Microbiol. 60: 4364-4370.

Simankova AN, Mirgorodskaya OA, Saveleva NV, Savelev AN, Kerner R, Rijpstorff P, Alexandrov SL (1998) Bioorg. Khim. 24: 822-830.

Sivan CJ, Chet I (1989) J. Gen. Microbiol. 135: 675-682.

Small CL, Bidochka MJ (2005) Mycol. Res. 109: 307-313.

Stepanov VM, Rudenskaya GN, Gaida AV, Osterman AL (1981) J. Biochem. Biophys. Methods 5: 177-186.

StLeger RJ, Joshi L, Roberts DW (1997) Microbiology 143: 1983-1992.

Tibbet M, Sanders FE, Cairney JW, Leake JR (1999) Mycol. Res. 103: 707-714.

Uchikoba T, Mase T, Arima K, Yonezawa H, Kaneda M (2001) Biol. Chem. 382: 1509-1513.

Viterbo A, Harel M, Chet I (2004) FEMS Microbiol. Lett. 238: 151-158.

Wosten HA, Bohlmann R, Eckerskorn C, Lottspeich F, Bolker M, Kahmann R (1996) EMBO J. 15: 4274-4281.

Yedidia I, Benhamou N, Chet I (1999) Appl. Environ. Microbiol. 65: 1061-1070.