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The study of intracellular and secreted high-molecular-weight protease(s) of *Trichoderma* spp., and their responses to conidiation stimuli

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Abstract
We continued the study of high-molecular-weight proteases (HMWP) using several strains of the genus *Trichoderma*, and other filamentous fungi (*Botrytis cinerea*, *Aspergillus niger*, *Fusarium culmorum* and *Penicillium purpurogenum*) in this work. We found that five *Trichoderma* strains secreted HMWP into the media after induction with bovine serum albumin. *B. cinerea* and *F. culmorum* secreted proteases without the presence of inducer, while *A. niger* or *P. purpurogenum* did not secrete proteolytic activity (PA). The activity of HMWP in *Trichoderma spp.* represents the predominant part of cellular PA, according to zymogram patterns. This observation allowed the study of its physiological role(s) independent from the secretion. In the study of conidiation, we found that the illumination significantly stimulates the PA in *Trichoderma* strains. In *T. atroviride IMI 206040* strain, we demonstrated that this stimulation is dependent on the BLR1 and BLR2 receptors. No stimulation of PA was observed when the mechanical injury was used as an elicitor of conidiation. Compounds used as inhibitors or activators of conidiation exerted no congruent effects on both PA and conidiation. These results do not favour the direct role of HMWP in the conidiation. Probably, its activity may be involved in the process of the activation of metabolism during vegetative growth, differentiation, and aging-related processes.

Keywords
*Trichoderma* spp.; high-molecular-weight proteases; conidiation; the influence of abiotic factors.

Introduction
*Trichoderma spp.* mostly known as cellulase producers (Kubicek and Penttilä 1998; for review), are frequently used as biocontrol agents (Hjelljord and Tronsmo 1998; Markovich
and Kononova 2003; Harman et al. 2004; Benitez et al. 2004) due to their mycoparasitic properties. Various aspects of mycoparasitism have been studied, beginning with the role of lectins (Inbar and Chet 1992), through the role of peptaibol antibiotics (Schirmböck et al. 1994, Reithner et al. 2007), ABC transporters (Ruocco et al. 2009), or novel variant of hydrophobin (Ruocco et al. 2015). In the course of these studies, several novel proteases and/or protease genes were identified (Geremia et al. 1993; Olmedo-Monfi et al. 2002; Kredics et al. 2004; De Marco and Felix 2002; Flores et al. 1997; Yedidia et al. 1999; Uchikoba et al. 2001; Viterbo et al. 2002; Markovich and Kononova 2003; Pozo et al. 2004; Suarez et al. 2004; Dienes et al. 2007). The *Trichoderma harzianum* secretome (Grinyer et al. 2004, 2005) or proteome induced by fungal cell walls was characterized. The secretome contained a protease among other hydrolases (Suarez et al. 2005). It is important to note that the isolated and characterized proteases were of low molecular weight (~30 kDa).

Proteases were found to influence the intracellular traffic of proteins and their secretion. Intracellular proteases negatively affected heterologously expressed proteins in *Trichoderma reesei* (Nevalainen and Peterson 2014). Landowski et al. (2015, 2016) disrupted several protease genes without damaging underlying physiological processes, and significantly improved the yield of heterologously expressed protein. Braaksma et al. (2009) found that extracellular proteases affected the production of secreted heterologously expressed proteins as well.

Secreted fungal proteases participate in the interaction between plants and phytopathogenic fungi. Such proteases were produced by *Sclerotinia sclerotiorum* (Poussereau et al. 2001a, b; Billon-Grand et al. 2002; Bueno et al. 2012; Li et al. 2004; Sexton et al. 2006), *Botrytis cinerea* (Movahedi and Heale 1990; Rolland et al. 2008, 2009; ten Have et al. 2004), *Phytophtora infestans* (Paris and Lamattina 1999) and other phytopathogenic fungi, such as *Glomerella cingulata* (Clark et al. 1997). It is interesting that the interaction of...
Trichoderma asperellum with cucumber plants stimulate the secretion of fungal proteases (Viterbo et al. 2004). Pathogenesis of Candida albicans infections also involves secretion of proteases (MacDonald and Odds 1980; Hube and Naglik 2001). Thus, low-molecular-weight proteases seem to be involved in many processes of fungal physiology.

The processes of protease secretion triggered by proteinaceous inducers in Saccharomyces cerevisiae (Kurucová et al. 2009) and Trichoderma atroviride (Šimkovič et al. 2008, 2012) has been studied in our laboratory. Secreted proteases in both organisms found in the early stages of mycelial growth were predominantly high-molecular-weight proteases (HMWPs) (~170-200 kDa). Interestingly, their properties were dependent on the protein inducer. Our results with S. cerevisiae confirmed Seredyński et al. (2016). It should be noted that Farnell et al. (2012) found that secreted proteases of Aspergillus flavus were also dependent on the choice of protein inducer, but authors did not show data about their molecular weight.

Here, we present additional information about HMWP involvement in T. atroviride physiology. Also, we attempted to find out whether the HMWP secretion is limited to other strains of Trichoderma genus or other genera of filamentous fungi and to identify roles that HMWP plays in the physiology of Trichoderma spp.

Materials and methods

Strains

Totally 16 fungal strains were studied in this work. Trichoderma atroviride CCM F-534, Botrytis cinerea CCM F-16, Aspergillus niger CCM F-330, Fusarium culmorum CCM F-21 and Penicillium purpurogenum CCM F-257 were from the Czech Collection of Microorganisms, Masaryk University, Brno, Czech Republic. Trichoderma atroviride CCM 8369 (formerly I-2) provided by Dr. V. Farkaš, Institute of Chemistry, Slovak Academy of
Sciences, Bratislava, Slovakia. *Trichoderma atroviride* ATCC 74058 (formerly P1), and *Trichoderma reesei* QM6a were provided by Dr. S. Zeilinger, Institute of Microbiology, University of Innsbruck, Innsbruck, Austria. *Trichoderma hamatum* LU593 and *Trichoderma atroviride* LU132 were provided by Dr. J. Steyert, Bio-Protection Research Centre, Lincoln University, Lincoln, New Zealand. *Trichoderma atroviride* IMI 206040, \( \Delta \text{blr1} \) and \( \Delta \text{blr2} \) *Trichoderma atroviride* (IMI 206040); *Trichoderma reesei* QM9414, \( \Delta \text{blr1} \) and \( \Delta \text{blr2} \) *Trichoderma reesei* (QM 9414) were provided by Dr. M. Schmoll, Austrian Institute of Technology GmbH, Wien, Austria. *T. hamatum* IP2 was from the Collection of Institute of Biochemistry and Microbiology, Slovak University of Technology, Bratislava, Slovakia.

**Maintenance and cultivation of fungal strains**

Fungi were routinely cultivated and maintained either on the solid Czapek-Dox (CzD) medium (g L\(^{-1}\): 30 sucrose, 2 NaNO\(_3\), 0.5 MgSO\(_4\), 0.5 KCl, 1 K\(_2\)HPO\(_4\), 0.01 FeSO\(_4\), 20 agar, pH 6.5) supplemented with 0.5% (w/v) yeast extract (YE) or Potato dextrose agar (PDA) medium (Biolife, Italy) under continuous illumination at 25 °C.

In experiments with aerial mycelia, these were grown on the agarized CzD medium (CzDA) (in 9-cm diameter Petri dishes) covered with the sterile cellophane sheet and inoculated with 25-50 \( \times \) 10\(^4\) conidia. Cultures were incubated at 25 °C under different light regimens; circadian (12 h: 12 h of light and dark period) or permanent illumination. Petri plates with fungal cultures were exposed to LED light (Osram, 2100 lm) placed 15 cm away from plates. Plates that served as dark controls were packed in two layers of aluminum wraps and placed close to illuminated plates. When the effect of pH on growth and conidiation of *T. atroviride* F-534 was tested, the pH of media was adjusted by 100 mM Tris-maleate buffer (with pH from 3.0 to 10.0).
Preparation of conidial suspension

Two-week-old aerial cultures grown under light-dark cycles at 25 °C were used as a source for conidial suspensions. Conidial suspensions were prepared by adding 10 mL of 0.1% (v/v) sterile Tween 80 to the culture plates and conidia were scraped out from the agar surface. The resulting suspension was filtered through a sterile cloth in order to remove remnants of mycelia. Conidial suspensions were concentrated to a smaller volume by spinning down at 1000g for 10 min and aspiring the necessary volume of supernatant. The concentration of conidia was determined employing the hemocytometer. Conidial concentration was adjusted to 2 x 10^9 conidia per mL. This suspension was diluted with the equal volume of sterile 50% (v/v) glycerol and stored at -20 °C.

Submerged cultivation

Submerged cultivation of fungi was performed in the CzD medium containing 0.05% (w/v) YE. Conidial suspension was inoculated to CzD medium to a final concentration of 2.5 x 10^6 conidia per mL of liquid medium. Submerged cultivations were performed in 150 mL of cultivation medium (in 500 mL of flat bottomed flasks) using rotary shaker (at speed of 240 r/min) at 27 °C in the dark. When a protein inducer or other polymeric substrate was added (to the final concentration of 0.1 or 0.5% (w/v)), it was sterilized together with CzD medium. Biomass concentration was determined during the cultivation after aseptic withdrawal of 2 mL aliquots. These aliquots as samples were used for both biomass concentration and the enzyme analyses, or zymography.

Measurement of biomass concentration

During cultivation, 2 mL aliquots of submerged cultures were withdrawn (at times as indicated in figures) and filtered through pre-weighed glass microfiber filter papers (pore
141 diameters of 1-2 µm, Fisher Scientific). Membranes with collected mycelia were washed two
142 times with 4 mL deionized water (dH₂O) and dried to a constant weight (70 °C for 5 h).
143 Biomass concentration (referred to as the dry mycelial mass (DRM)) was expressed in
144 milligram per millilitre of submerged culture. Two experiments each with the submerged
145 culture of different origin were performed in duplicate. Data are expressed as average ±
146 standard deviation.

147 The disintegration of submerged and aerial mycelia
148 Submerged mycelia was washed by a surplus of dH₂O on the polyamide fabric with pore size
149 of 43 µm (Tempex, Slovakia) and suspended in the homogenization medium (50 mM Tris-
150 HCl buffer, pH 7.4, 0.7 M KCl) to obtain a thick suspension. Glass beads were added (half
151 volume of mycelial suspension) and the suspension was pre-cooled on ice. The pre-cooled
152 suspension was vortexed (at maximal speed of 2500 r/min) in 15 cycles per 60 s, interrupted
153 by 60 s period of chilling in an ice-water mixture. Disintegrated mycelia were fractionated by
154 differential centrifugation (Avanti J-30I, Beckman Coulter). First, cell debris was removed in
155 two centrifugations at 7000 g for 15 min, and the resulting supernatant was centrifuged at
156 15000 g for 20 min. Final supernatant was used for experiments.
157 Aerial mycelia were grown up on the agarized media covered with cellophane sheet.
158 Mycelia were rinsed down from cellophane sheet, washed with an excess of dH₂O and
159 disintegrated as described above.

160 Isolation of cell walls
161 Cell walls of *Aspergillus flavus* used as an inducer for the synthesis of HMWP were isolated
162 according to the method of Ren and West (1992).
Protein determination

Protein concentration was measured according to the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Protease activity (PA) determination

PA in cell-free culture filtrates and homogenates prepared from mycelia was determined using of chromolytic substrate azocasein (Sigma, St. Louis, Missouri, U.S.A.). PA assessed in duplicate by measuring the release of trichloroacetic-acid (TCA) soluble peptides from 0.25% (w/v) azocasein in 50 mM Tris-HCl buffer (pH 7.5) at 37 °C for 4 h. Fifty μg of proteins were taken for the measurement of PA. The 0.5 mL-reaction was terminated by the addition of 0.4 mL of 10% (w/v) trichloroacetic acid and then centrifuged at 10000 g for 10 min. After centrifugation, 850 μL of the supernatant was collected and mixed with 320 μL of 1 M NaOH. PA was determined by reading the absorbance of the resulting solution at 440 nm. Control assay without enzyme was used as a blank. One arbitrary unit of PA (U) was defined as the amount of enzyme required to produce a change of one absorbance unit at 440 nm per hour under the assay conditions. Specific PA is expressed in units of activity per one milligram of dried mycelial mass or per one milligram of protein. Values are presented as the mean +/- standard deviation of two independent measurements.

Electrophoretic analysis of proteins

Proteins were analyzed by SDS PAGE according to Laemmli (1970) and detected by staining with Coomassie Brilliant Blue R-250 according to standard procedure.

Gelatin zymography
Zymography analysis was performed on 8.5% or 10.0% (w/v) SDS-PAGE (Laemmli 1970) containing 2 mg mL⁻¹ gelatin as proteinase substrate. Samples, containing approximately 20 µg of protein, were mixed in Laemmli solubilizing solution without reducing agents and loaded to the polyacrylamide gels with gelatin. Electrophoresis was performed at 28 mA and 4 °C for 1 h. Then, in order to remove SDS, the gels were soaked with gentle agitation for 2 x 30 min at room temperature in 50 mM Tris-HCl buffer (pH 7.5), containing 2.5% (v/v) Triton X-100. The gels were shortly washed by dH₂O and placed in the enzyme incubation buffer for 4 h at 37 °C (50 mM Tris-HCl, pH 7.4, 5 mM CaCl₂ and 20 mM NaCl). Finally, the gels were stained for 2 h with Coomassie Brilliant Blue R-250 (0.25%; w/v) dissolved in the mixture of methanol:H₂O:acetic acid (45:45:10; v/v) and then destained in methanol:H₂O:acetic acid mixture (10:80:10; v/v) for at least 8 h at room temperature on a rotary shaker to visualize the PAs as clear bands (areas of digestion) against the blue background of stained substrate.

Chemicals

Azocasein; BSA; sodium dodecyl sulfate (SDS); Trizma base; Tween 80; N,N,N’,N’-tetramethylene diamine (TEMED); acrylamide; ammonium persulfate; dithiothreitol (DTT); ovalbumin (OVA); Coomassie Brilliant Blue G-250; Folin-Ciocalteu reagent; TCA; protein standards (6.5-200 kDa; 36-200 kDa), pre-stained protein standards (26.6-180 kDa); caffeine; atropine; N-acetyl-L-cysteine (NAC); dibutyryl cAMP (dBeAMP); 8-bromo-cAMP (BrCAMP) and ethylene glycol-bis(2-aminoethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA) were purchased from Sigma Aldrich (St. Luis, MO, USA). 3-isobutyl-1-methylxanthine (IBMX) was provided by MP Biomedicals (Santa Anna, USA). Coomassie Brilliant Blue R-250 was purchased from Merck Millipore (Darmstadt, Germany), 2-(N-morpholine) ethane sulfonic acid (Mes), from Serva (Heidelberg, Germany). All other chemicals were of analytical reagent purity and purchased from common commercial sources.
Results

Intra- and inter-species differences in the protein induced protease secretion

Our observations of the protein-induced HMWP secretion were until now performed with *T. atroviride* F-534 as a model (Šimkovič et al. 2008, 2012). It is essential to know, whether this phenomenon is present in this strain only, or it is present ubiquitously in the genus of *Trichoderma*, or other filamentous fungi. In the first instance, we tested the presence of inducible HMWP secretion in several *Trichoderma* species. Following strains were used for the study: *T. atroviride* CCM F-534, *T. reesei* QM6a, *T. atroviride* ATCC 74058; *T. hamatum* IP2; *T. atroviride* CCM 8369, and mutant Δblr1 *T. atroviride* (IMI 206040). These strains were different in growth characteristics (such as growth rate, lag phase, biomass yield) under cultivation conditions and in a response to the addition of protein inducer (0.1% BSA) (Fig. 1A, B). In some strains, BSA suppressed the growth (*T. atroviride* CCM F-534) while in others (*T. atroviride* IMI 206040 and its mutant Δblr1) stimulated it. The parallel measurements of secreted PA also showed differences between strains (Fig. 1C, D). The time course of the PA secretion was a complex one. Regardless of the presence of protein inducer, the secretion started on the first day of cultivation, albeit both total and specific activities were one order of magnitude lower without inducer. Without inducer, mycelia produced low PA within first 10 d of cultivation. Later, the PA disappeared from the cultivation medium in all cultures, except *T. atroviride* F-534 and *T. reesei* QM6a. These strains increased the PA secretion abruptly (after 7 and 15 d of cultivation, respectively) and kept it on the high level throughout the whole experiments (up to 30 d). The character of this PA has not been analyzed yet. The presence of protein inducer (BSA) dramatically accelerated the secretion rate and the secreted PA (Fig. 1D). A high PA was observed after 1 d cultivation with BSA, and the maximal activity was observed mostly during the exponential growth (between 2nd
and 3rd day). The amount of PA was different by a factor 12 between the most (\textit{T. reesei} QM6a) and least (\textit{T. atroviride} ATCC 74058) productive strains. Later, the activity decreased in most strains. Only \textit{T. atroviride} F-534 exhibited a dramatic increase of PA after 10 d of cultivation, which was transient and the activity disappeared after about subsequent 10 d. Several strains released some PA at the end of the experiment (after 30 d). Samples of cultivation media from the 3-day cultivation were analyzed using the gelatin zymography (Fig. 2). Both low-molecular-weight proteases (LMWPs) and HMWPs were detected regardless of the presence of protein inducer, although some irregularities were observed. First, in some strains (\textit{T. atroviride} F-534 or \textit{T. hamatum} IP2) the protease activities were secreted without inducer. In \textit{T. reesei} QM6a and \textit{T. hamatum} IP2, also LMWPs were detected. BSA induced the secretion of HMW protease in all strains, and its highest activity was being secreted by \textit{T. reesei} QM6a. All proteases displayed autoproteolytic patterns, which blurred the determination of the molecular weight. Some strains (\textit{T. atroviride} F-534, \textit{T. reesei} QM6a) produced concomitantly also LMWPs.

It is apparent that the significant parts of the PA are represented by HMWPs (Mr 66-200 kDa). No detectable LMWP activity was secreted without proteinaceous inducer (BSA) except \textit{T. reesei} QM6a strain, while both HMWPs and LMWPs (29 kDa) were induced by BSA in \textit{T. atroviride} F-534, \textit{T. reesei} QM6a, \textit{T. atroviride} CCM 8369 and \textit{T. hamatum} IP2 strains. Thus, the secretion of HMWP seems to be an inherent property of \textit{Trichoderma} spp. Similar experiments probed their secretion in other filamentous fungi (\textit{A. niger, B. cinerea, F. culmorum}, and \textit{P. purpurogenum}) (Fig. 3). Of these strains, \textit{A. niger} and \textit{P. purpurogenum} failed to secrete PA even in the presence of protein inducer, while \textit{B. cinerea} and \textit{F. culmorum} secreted it, although both time course and overall activities were dissimilar to those observed in \textit{Trichoderma} spp. In \textit{B. cinerea}, the maximum of PA without inducer was observed between 2-4 d of cultivation, and the activity rapidly disappeared onwards. The
second peak of activity was secreted between 7-10 d of cultivation, and the activity in part disappeared onwards. At the end of cultivation (day 32) another release of activity was observed. Protein inducer did not dramatically affect the time course; its effect was rather modulatory. *F. culmorum* secreted PA in two transient peaks (2 and 10 d) but not at day 32.

Protein inducer suppressed both activities (Fig. 3 A, B).

Thus, the results obtained with *A. niger*, *B. cinerea*; *F. culmorum* and *P. purpurogenum* were dramatically different from those obtained for *T. atroviride* F-534 strain and will be analyzed later. Those obtained with *T. atroviride* were corroborated by measurements of PAs in both medium and in mycelial homogenate using the chromolytic substrate, and by zymography.

Inducer stimulated the PA in both media and in the mycelial homogenate (Table I). These results demonstrate that the tested strain possesses high PA in mycelia, in addition to that released through the secretion pathway, and the intracellular PA equally responds to the presence of protein inducer. In order to find out components of the total PA, both media and homogenates were analyzed by zymography (Fig. 4).

Zymogram shows that the PA in both media and homogenates contain two main components with similar patterns consisting of HMWP and LMWP (Fig. 4). It is essential that the HMWP activity represents almost complete PA in homogenates. This observation enabled us to study changes in PA elicited by physiological stimuli directly in mycelial homogenates (see below).

*The protease secretion by T. atroviride induced by complex substrates*

In experiments presented here or previously published (Šimkovič et al. 2008, 2012) we used purified protein as inducers of PA. We know, however, that *T. atroviride* can utilize also very complex substrates, such as plant remnants (Maťaťa et al. 2016). In order to confirm that only proteins serve as inducers of PA, we tested several synthetic, purified, or semi-purified materials, which fungi may meet during their saprophytic way of life. Besides purified...
proteins (BSA, OVA), we tested chemically pure polysaccharides (acetyl cellulose (AC),
carboxymethyl cellulose (CMC), microcrystalline cellulose (CMC), oat xylan (XY), chitosan
(CHI), and cell walls (CW) isolated from Aspergillus flavus. Growth characteristics of
T. atroviride F-534 in control and with added polymers and complex substrates showed that
this fungus could utilize all substrates, although with different extent (Fig. 5A). The highest
growth rate was supported by isolated CW, with concomitant rapid onset of the stationary
phase. This stimulation was transient and led to the rapid decrease of the cell mass. CMC only
barely stimulated the growth of the fungus. The dry mass of mycelia decreased after 10-12 d
of cultivation, i.e., at the end of the stationary phase. This decrease was accompanied by the
decrease in the filterability of mycelia, probably, due to its fragmentation.

Measurements of secreted PAs showed that by tested substrates influence them in a complex
manner (Fig. 5B). As expected (Šimkovič et al. 2008, 2012), protein substrates (BSA and
OVA) induced the secretion as the first, with a maximum in about 3 d and minimum after 7 d.
Later, the PA was kept on some minimum level until the end of the experiment (22 d).
Polysaccharidic substrates did not induce PA during the first week of cultivation when the PA
attained virtually zero values except a small amount induced by XY. The PA increased
dramatically in the medium at the end of the stationary phase (after 7-10 d cultivation). The
highest specific PA was released in the presence of polysaccharidic substrates, and in control
with some delay. The presence of inducer proteins suppressed this late release of PA. It is
worth to mention that each culture passes the state where the early release of PA is minimal,
and the late phase of the protease release still does not occur (Fig. 5B).
The late release of PA was surprising, and it was not observed before with any fungus. In
order to obtain additional information about this activity, the zymography of media withdrawn
at the 17th day of cultivation were performed (Fig. 6). Zymographic patterns were unique for
each substrate, and the spectrum of proteolytic spots ranged from 30 to more than 200 kDa,
with various intensities. LMWPs were found mainly in the presence of polysaccharidic substrates (XY, CMC, MC, CHI) (and in control) but their intensities were low. HMWPs (66 to 220 kDa) were prominent in CW, CHI and XY substrates. Zones in BSA, OVA, CHI, and XY revealed the presence of a diffuse band at about 220 kDa. Possible CMC and MC zones are overlapped by a CHI-evoked proteases. A dominant zone induced by CW was about 70 kDa. It should be noted that patterns of BSA-induced proteases were similar after 3 and 17 d (Fig. 2 and Fig. 6) albeit slightly shifted to lower molecular weights.

These results show that the extracellular protein-induced secretion (of HMWP) is not the universal feature of filamentous fungi we tested but the intracellular presence of this protease seems to be characteristic one. In mycelial homogenates, the HMWP represents the major PA with some contribution of LMWP. This finding enabled us to study responses of HMWP activity to external stimuli by measuring the PA in mycelial homogenates only.

The effect of illumination on the PA in T. atroviride F-534.

The appearance/induction of HMWP secretion in the presence of non-proteinaceous polymers suggested that their release need not to have a purely nutrition-linked character. Instead, it may be a part of a systemic response to external stimuli, such as starvation, or nutritional disbalance. Therefore, we tested the role of other stimuli in triggering this response. In Trichoderma spp., it is the light pulse, which also has a systemic effect on the physiology and morphogenesis, and is accompanied by a stimulation of energetic metabolism (Betina and Farkaš 1998; Tisch and Schmoll 2010). Therefore, we tested the effect of light in three regimens: dark, circadian light, and permanent light. In these experiments, however, intracellular PA was measured instead of the secreted one, and the measurements started after 3 d of cultivation, when a sufficient mass of mycelia was present in the culture. It was found that the illumination stimulated the specific activity of protease as compared to dark control.
The stimulation was dependent on the age of the culture being maximal on day 3, and was followed by a slow decrease. The extent of the stimulation seems to be proportional to the duration of illumination, as the stimulation by permanent light was higher than that by circadian light.

These changes were confirmed by zymographic analysis (Fig. 7B). It is apparent that both HMWPs and LMWPs are formed in mycelia, which, however, appear in a complex way. In the dark, the autoproteolysis seems to be suppressed, and discrete bands are prominent. Bands have the molecular weights ~31, 66 and 120 kDa in addition to the HMWPs with the molecular weight ≥200 kDa, with a faint autoproteolytic smear. The illumination caused the increase of the total PA and changed also proteolytic patterns. First, on day 3, the autoproteolysis visible as smear was more pronounced and blurred discrete bands. All LMWPs (30 kDa) disappeared at day 3 but transiently appeared at the day 4. Bands of HMWPs disappeared monotonously after day 3 and their patterns did not change much except that one discrete band appeared within the smear at day 4 in the sample illuminated in the circadian regimen.

After the cultivation in the dark, no conidia were formed and fluffy mycelia covered all the surface of the plate (Fig. 7C). The permanent illumination triggered the formation of conidia after 3 d. Conidia also covered the surface of the plate after 5 d (Fig. 7C).

The effect of illumination on the PA in other Trichoderma isolates.

In order to understand the role of HMWPs in Trichoderma spp. in the first instance, we tested the effect of light on the PA in several Trichoderma spp. Experiments were designed as described in the Fig. 7 and all fungi were cultivated similarly. For these experiments, following Trichoderma strains were used: *T. atroviride* ATCC 74058, *T. atroviride* LU132, *T. hamatum* LU593 and *T. reesei* QM6a. After 5 d cultivation in the dark, these strains grew
without conidiation, and the cultivation under permanent light led to the conidiation, although
the pigmentation of conidia was different, e.g. *T. hamatum* formed only white conidia, and
*T. reesei* yellow conidia (not shown).

Measurements of intracellular PA showed that the level of its stimulation by the light is
different in strains and is not universal. Apparent stimulatory effect of the light on PA was
observed in *T. atroviride* LU132, and with some reservation in *T. atroviride* ATCC 74058
strain, while in *T. hamatum* LU593 and *T. reesei* QM6a it was present only on the 3rd day of
growth. The PA in these strains changed with time regardless of the illumination without
showing some regularities (Fig. 8).

In order to explain the stimulation of the PA by light, we, therefore, performed experiments
with mutant strains with deleted BLR genes, which have disrupted (blue) light-induced
conidiation pathway. These mutants were derived from the *T. atroviride* IMI 206040 strain.
These mutants (Δblr1 and Δblr2) did not conidiate upon pulse illumination (Fig. 9A). The
parental strain conidiated under identical conditions and the conidiation was dependent on the
cultivation medium, the fungus preferred PDA medium rather than CzDA with YA for
expressing conidiation. PA in mycelial homogenates was stimulated by light in the parental
strain but not in Δblr1, and Δblr2 mutants (Fig. 9B). Only small differences between Δblr1
and Δblr2 mutants were observed. The dynamics of appearance of PA was similar as in strains
shown previously (Fig. 7A, 8). These observations suggest that photoreceptors BLR1 and
BLR2 participate on the control of expression of genes encoding protease(s) in *T. atroviride*
spp. exposed to the light.

*Effects of inhibitors and activators of conidiation on the mycelial PA*

In order to get some insight into the intracellular signalling pathway preceding the synthesis
and secretion of protease activities in the context of illumination, effects of inhibitors of
selected processes on the PA was tested (Table 2), in parallel with their effects on conidiation (Fig. 10). First, we tested compounds modulating cAMP levels, which were suggested/found to participate in the light-induced conidiation (Sulová and Farkaš 1991; Nemčovič and Farkaš 1998; Steyaert et al. 2010a). Two derivatives of cAMP, BrcAMP and dBcAMP, and two phosphodiesterase inhibitors, IBMX (Sulová and Farkaš 1991) and caffeine (Tisch et al. 2011), were used. Together with external ATP (Medina-Castellanos et al. 2014) they could be considered as activators of conidiation. Other compounds tested in this experiment were inhibitors of conidiation: EGTA (Kryštofová et al. 1996), DTT (Sulová and Farkaš 1991), N-acetyl cysteine (Hernández-Oñate et al. 2012) and atropine (Casas-Flores et al. 2006). In these experiments, fungus was cultured on the plates covered with cellophane sheets in the dark, until the colony reached the end of the cellophane sheet. Then (usually after 3 d), cultures were transferred to fresh media containing tested compounds or the vehicle (controls). This transfer was performed under red illumination. The cultivation was continued for another three days in the dark, except positive control, which was cultivated under constant light, and was used as a reference for the maximal formation of conidia.

Effects of most tested compounds on the conidiation were mostly weak albeit discernible. Only EGTA completely blocked both growth and conidiation. The presence of caffeine, atropine, NAC, IBMX had only marginal stimulatory effect on conidiation as compared with the dark control. Also, BrcAMP and dBcAMP only weakly stimulated conidiation in the dark with the formation of yellow-greenish conidia. However, ATP and DTT stimulated the conidiation to the extent comparable with this induced by the light pulse (Fig. 10). These compounds also modified PA in homogenates (Table 2).

Measurements of PA in homogenates from fungi identically treated with these compounds showed some effects which did not correlate with the effect on the conidiation (Table 2). For example, NAC strongly inhibited PA with the concomitant activation of conidiation but
extracellular ATP with similar stimulatory effect on conidiation slightly suppressed the PA. On the other hand, DTT, which could be considered as antioxidant similar to NAC, had no inhibitory effect on the PA. Effects of other compounds, such as those modifying cAMP signalling pathway are not congruent with the notion that conidiation and the activation of protease are closely linked.

In order to further characterize mechanisms which lead to the production of protease(s), PA was measured after mechanical damage (with scalpel under red illumination) of dark-cultivated mycelia. This treatment was found to stimulate conidiation (Steyaert et al. 2010a). We were able to reproduce this phenomenon (Fig. 11). Measurements of PA in mycelial homogenates performed for 7 days did not reveal differences between damaged conidiating mycelia and the intact non-conidiating dark controls in two *T. atroviride* strains (F-534 and ATCC 74058)(Fig. 11) except that the activity moderately decreased in time.

Extracellular pH is known to regulate both expression of cellulase genes and conidiation in the genus *Trichoderma* (Steyaert et al. 2010b, He et al. 2014). Changes in extracellular pH are expected to affect both surface-growth and conidiation. This notion was tested with *T. atroviride* F-534 strain. The radial growth was maximal at neutral pH (i.e., pH 7). Alkaline pH (8.5 or 10.0) strongly suppressed growth, while growth at acidic pH (5.5 or 4 or 3) was affected moderately, mainly at pH 3. Concomitantly, conidiation in the dark or under permanent illumination, were affected (Fig. 12A). Intracellular protease activities measured after 3-5 d cultivation were also affected by extracellular pH. Alkaline pH (8.5 or 10.0) strongly suppressed PA in illuminated cultures while the suppression by acidic pH was less pronounced (Fig. 12B). It is worth of mentioning that the PAs in the dark were less affected by ambient pH. These results suggest that ambient pH has dramatic effect on both growth and conidiation of *T. atroviride* F-534 strain. The light-induced PAs follow the growth and
conidiation patterns (Fig. 12B). Maximal PA was observed in the pH range between 4 and 7, lesser one at acidic pH (3-5.5).

Discussion

In our previous studies (Šimkovič et al. 2008, 2012), we characterized the secretion of HMWP(s) induced by proteinaceous inducer(s). In the first part of this work we extended these results from *T. atroviride* F-534 to other *Trichoderma* strains, but not to other fungal species (Fig. 1). These results confirmed the observation by Larcher et al. (1996) using *Scedosporium apiospermum* as a model, which authors left unnoticed. If we take into account that the first observation of HMWP was made with yeasts (Kurucová et al. 2009), which was later confirmed and elaborated by other authors (Seredyński et al. 2016), it emerges that the secretion of HMWPs upon induction with proteins in *Trichoderma* spp. may not be unique among fungi, although some exceptions exist, which we have shown above (Fig. 1).

Interspecies differences in protease secretion in the study involving tens of isolates were observed recently by Rodarte et al. (2011). These authors, however, did not attempt to identify the HMWP(s) in analysed isolates.

Common features of this process may be overshadowed by differences in properties of secreted protease(s) as deduced from the zymogram patterns (Fig. 2,4,6,7) (Šimkovič et al. 2012). At present, we cannot explain these differences, as the only structural information we have, was obtained from *T. atroviride* F-534 HMWP enzyme after its partial purification and MS analysis (unpublished).

The secretion of polysaccharide hydrolases (e.g. cellulases, xylanases or chitinases) induced by external stimuli is typical for *Trichoderma* spp. (Kubicek et al. 1998, 1993; Kulkarni et al. 1999; Ulhoa and Peberdy 1991). Therefore, it was justified to test whether the secretion of proteinases is a part of molecular machinery involved in the secretion of polysaccharidases.
This possibility does not seem to be true as pure polysaccharides did not induce the secretion of HMWP during the early phase of fungal growth (Fig. 6A, B). It was interesting, however, that a PA appeared in media after long-term (more than 10 d) cultivation. According to the zymographic analysis, mostly a HMWP activity observed, with a small contribution of LMWP activity (Fig. 6C). Thus, the secretion of HMWP seems to operate not only during the phase of rapid growth but also during later phases of mycelial growth, such as the deterioration of mycelia (autophagy) and/or conidia formation in submerged medium.

If the HMWP has to be secreted into the medium, it should be present in some molecular form also in the mycelium. This is the case as can be seen from the Fig. 4 and Table 1. It is feasible that the measurement of mycelial HMWP activity should be equivalent to that of secreted HMWP. Furthermore, the inspection of zymograms shows that the HMWP activity is the predominant PA in both mycelia and cultivation media. Therefore, we used the measurement of mycelial PA as an indicator of changes in PA induced by stimuli we tested in this work.

Light (or illumination), known as a potent stimulus eliciting conidiation and metabolic changes in *Trichoderma* (Betina and Farkaš 1998, Tisch and Schmoll 2010), was able to stimulate HMWP activity (Fig. 7, 8). The effect of light could be attributed to the general activation of metabolism that leads to the conidia formation. It seems to involve transduction mechanisms known to participate in the light-induced processes (Carreras-Villaseñor et al. 2012) as strains of *T. atroviride* IMI 206040 with deleted BLR genes lost the ability to activate the HMWP synthesis (Fig. 9). On the other hand, other mechanism of eliciting conidiation-mechanical damage of mycelia, which is mediated by different mechanisms (Carreras-Villaseñor et al. 2012, Hernández-Oñate et al. 2012), did not change the PA (Fig. 11). This observation could be explained by a notion that HMWP participates rather in the general activation of metabolism induced by the light pulse than in specific processes of conidia formation. The pH dependence of both conidiation and PA (Fig. 12) are also in
accordance with this notion, the conidiation was equally stimulated by neutral pH. This is in accord with results of Steyaert et al. (2010b) who observed the parallel activation of growth and conidiation at neutral pH. Recently, Stappler et al. (2017) observed the putative protease inhibitor, CLF1, which was unequally expressed in course of T. reesei QM6a growth under dark and permanent light and involved in regulation of PA secreted. However the existence of similar protease inhibitor in the cells of Trichoderma atroviride has not been proven so far.

The inspection of zymograms in this and previous works reveals a conspicuous feature. The major part of, if not all, PA represents HMWP only (Fig. 2, 4, 7), although there are hundreds of protease-encoding genes in fungal genomes (Budak et al. 2014, Muszewska et al. 2017). Albeit most of them were identified by an in silico analysis, and have to be identified experimentally (López-Otín and Bond 2008), the rare appearance of other proteases in zymograms is really intriguing. The reason of this observation may be quite technical, due to low sensitivity of zymography, or low specific activity (turnover) of most proteases as compared to HMWP. In fact, we found that the HMWP has a very high specific activity as the zymogram spots could be seen (without identification of corresponding band) in SDS-PAGE stained by a standard procedure(s). The partial purification (accompanied with a loss of activity) enabled us to compare the activity with corresponding protein with standard proteases. We found that the activity of purified protease was higher than that of crystalline trypsin under optimal conditions (unpublished). The high specific activity of HMWP may also be connected to the high autoproteolytic activity which is the cause of smears in zymograms. Nevertheless, it seems that HMWP, as a major protease seen in zymograms, may play a role of "master" protease, which may be involved in physiological processes we analysed in this work.

Despite of the suggestion that HMWP plays some role in the vegetative growth, light-induced response, and aging, we could not provide satisfying information, which could go over the
phenomenological description. Experiments with the aim to purify this enzyme and elucidating its structure, we perform in our laboratory, may bring relevant information in the future.
Acknowledgement

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Conflict of interest

Authors declare that they do not have any conflict of interest.
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Reithner, B., Schuhmacher, R., Stoppacher, N., Pucher, M., Brunner, K., and Zeilinger, S. 2007. Signaling via the *Trichoderma atroviride* mitogen-activated protein kinase Tmk 1


Table 1. PA in cell-free medium and cellular homogenate prepared from the 3 d old submerged culture of *T. atroviride* F-534 cultivated in CzD medium in the absence or presence of BSA.

<table>
<thead>
<tr>
<th>Addition of protein inducer</th>
<th>Medium PA (U mL⁻¹)*</th>
<th>Homogenate PA (U mL⁻¹)*</th>
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</thead>
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<tr>
<td>without BSA</td>
<td>0.24±0.01</td>
<td>0.34±0.03</td>
</tr>
<tr>
<td>with BSA</td>
<td>1.31±0.04</td>
<td>3.31±0.08</td>
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</tbody>
</table>

*Values are expressed in units per milliliter of cell-free medium or homogenate.
Table 2. Effects of modulators of conidiation on the mycelial PA in homogenate of surface-cultivated mycelium of *T. atroviride* F-534. All cultures were grown in the darkness. Results are expressed as a percentage of the PA measured in the control experiment (culture grown in the absence of any compound). Compounds and their concentrations are identical as those shown in the Fig. 10.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect</th>
<th>Conidiation</th>
<th>PA (U (mg PROT)^{-1})</th>
<th>(% of control)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>Control</td>
<td>None</td>
<td>1.8±0.1</td>
<td>100</td>
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<tr>
<td>BrcAMP</td>
<td>cAMP analogue</td>
<td>Minor stimulation</td>
<td>1.9±0.1</td>
<td>106</td>
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<tr>
<td>dBcAMP</td>
<td>cAMP analogue</td>
<td>Minor stimulation</td>
<td>1.2±0.2</td>
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<td>IBMX</td>
<td>PDE inhibitor</td>
<td>No effect</td>
<td>2.4±0.1</td>
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<tr>
<td>Caffeine</td>
<td>PDE inhibitor</td>
<td>Minor stimulation</td>
<td>2.7±0.3</td>
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<td>DTT</td>
<td>Antioxidant</td>
<td>Stimulation</td>
<td>1.9±0.1</td>
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<td>NAC</td>
<td>Antioxidant</td>
<td>Minor stimulation</td>
<td>0.28±0.08</td>
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<td>Atropine</td>
<td>Acetyl choline receptor</td>
<td>Minor stimulation</td>
<td>2.5±0.2</td>
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<td>EGTA</td>
<td>Me^{2+} chelator</td>
<td>Complete inhibition</td>
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<tr>
<td>ATP</td>
<td>Energy donor</td>
<td>Stimulation</td>
<td>1.7±0.3</td>
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**Figure 1.** Growth curves of *Trichoderma* strains during the submerged cultivation in CzD medium in the absence (A) or presence (B) of 0.1% (w/v) BSA and the time course of spontaneous (C) and BSA-induced (D) secretion of proteolytic activity (PA). Strains used:

- ■ *T. atroviride* CCM F-534 (F-534);
- ● *T. reesei* QM6a (QM6a);
- ▲ *T. atroviride* ATCC 74058 (ATCC 74058);
- ▼ *T. hamatum* IP2 (IP2);
- ◆ *T. atroviride* CCM 8369 (CCM 8369), and ▲Δblr1 mutant of *T. atroviride* IMI 206040 (Δblr1). Specific PA is expressed as U per mg of dry mycelial mass (U (mg DRM)^{-1}).

**Figure 2.** Protease analysis by zymography of cell-free cultivation media obtained from the 3 d old submerged cultures being grown in the absence and presence of 0.1% BSA as inducer. Ten µl samples of media were subjected to 10% polyacrylamide gels containing 0.2% (w/v) of gelatin in a non-reducing condition. The following culture media were used: *T. atroviride* CCM F-534 (1); *T. reesei* QM6a (2); *T. atroviride* ATCC 74058 (3); Δblr1 mutant of *T. atroviride* IMI 206040 (4); *T. atroviride* CCM 8369 (5) and *T. hamatum* IP2 (6).

**Figure 3.** Secretion of PA of filamentous fungi cultivated in CzD medium in the absence (A) and in the presence (B) of 0.5% BSA. Strains:

- ■ *P. purpurogenum*;
- ● *A. niger*;
- ▲ *B. cinerea*;
- ▼ *F. culmorum*.

**Figure 4.** Zymographic analysis of cell-free medium and cellular homogenate of *T. atroviride* F-534 after 3 d submerged cultivation in the CzD medium without (-) or with (+) BSA. The electrophoresis was done on a 10% SDS-polyacrylamide gel containing 0.2% (w/v) gelatin under non-reducing conditions. M-molecular weight markers.
Figure 5. Growth (A) and secretion of PA (B) of *T. atroviride* F-534 during submerged cultivation in CzD medium enriched by 0.05% (w/v) YE and 0.5% (w/v) polymeric substrates: ■ control experiment (no addition); ● BSA (bovine serum albumin); ▲ OVA (ovalbumin); ▼ AC (acetyl cellulose); ◆ CMC (carboxymethyl cellulose); ▲ MC (microcrystalline cellulose); ▶ CHI (chitosan); ● XY (xylan); ● CW (cell wall of *A. flavus*).

Figure 6. Gelatin zymography of secreted proteases from the experiment shown in the Fig. 5. Samples were withdrawn at the day 17<sup>th</sup> (except in sample 4) and the remnants of mycelia were removed by centrifugation. The electrophoresis was done on a 10% SDS-polyacrylamide gel and co-polymerized with 0.2% (w/v) gelatin under non-reducing conditions. M-molecular weight markers.

Figure 7. Changes in specific protease activities in the cell-free homogenates of *T. atroviride* F-534 surface mycelia cultivated in various regimens of illumination by white light (A) and their zymographic analysis after protein separation (20 µg proteins per lane) by electrophoresis on the 8.5% SDS-PAGE containing 0.2% (w/v) gelatin (B). (C) The effect of illumination on the growth and conidiation of the surface cultivated *T. atroviride* F-534. Specific PA is expressed as U per mg of sample protein (U (mg PROT)<sup>-1</sup>).

Figure 8. Comparison of specific activities of protease(s) in different illumination regimens in four *Trichoderma* strains: *T. atroviride* ATCC 74058 (dark gray), *T. atroviride* LU132 (gray), *T. reesei* QM6a (light gray) and *T. hamatum* LU593 (white). Bars displayed with gray background represent the control cultures cultivated in dark, while aerial cultures exposed to...
continuous light during cultivation are depicted by the bars with white background. The experimental setup was identical with this shown in the Figure 7.

**Figure 9.** Macroscopic changes in surface cultures: parental strain of *T. atroviride* IMI 206040 (WT) cultivated on the CzDA and PDA medium and its mutants, Δblr1 and Δblr2, grown on the CzDA medium (A). Specific PAs measured in cell-free mycelial homogenates prepared from parental *T. atroviride* IMI 206040 strain and its mutants, Δblr1 or Δblr2 (B). These strains were cultivated on the standard CzDA medium in the dark or under continuous illumination for the time indicated in the Figure.

**Figure 10.** Effects of known modulators of conidiation on the conidiation in the dark-grown culture of *T. atroviride* F-534. Fungus was cultivated on the solid CzD medium covered with cellophane disks for 3 d in the dark. Cellophane disks were transferred aseptically and under red illumination, onto fresh media containing tested compound, and plates were cultivated in the dark for other 3 d. Tested compounds were added to the medium kept at ~50°C and thoroughly mixed. Their concentrations were: 100 µM ATP, 1 mM DTT, 10 mM atropine, 2 mM caffeine, 60 mM NAC, 15 mM EGTA, 200 µM IBMX, 200 µM Br2cAMP a 20 µM dBcAMP. The dark control without additions was treated identically. The positive control was illuminated with white light continuously or for 15 min immediately after the disk transfer onto CzD medium.

**Figure 11.** The effect of mechanical damage on the growth and conidiation of *T. atroviride* F-534 (A) and ATCC 74058 (B) and its relation to PA production. Cultures were cultivated in the dark and were cut with scalpel under red illumination at the end of 3rd day. Petri plates with cultures used to take the pictures were excluded from the experiment.
Figure 12. The effect of ambient pH on growth and conidiation of *T. atroviride* F-534 cultivated on the CzDA medium in the dark and under permanent illumination with white light (A). The dependence of specific PA in *T. atroviride* F-534 on both ambient pH and illumination (dark or permanent illumination) (B). Bars displayed with the gray background represent the control cultures cultivated in the dark.
Fig. 1.
Fig. 2.

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1. T. atroviride F-534
2. T. reesei QM6a
3. T. atroviride ATCC 74058
4. T. atroviride Δblr1
5. T. atroviride CCM 8369
6. T. hamatum IP2
Fig. 3.
Fig. 4.
Fig. 5.
Fig. 6.
Fig. 7.
Fig. 8.
Fig. 9.
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Fig. 11.
Fig. 12.