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EXTRACELLULAR PROTEASES OF *TRICHODERMA* SPECIES - A REVIEW*

L. KREDICS[1], ZSUZSANNA ANTAL[1], A. SZEKERES[2], L. HATVANI[2], L. MANCZINGER[2], C. VÁGVÖLGYI[2] AND ELISABETH NAGY[1, 3]

[1]Hungarian Academy of Sciences and University of Szeged, Microbiological Research Group, P.O. Box 533, H-6701 Szeged, Hungary, [2]Department of Microbiology, Faculty of Sciences, University of Szeged, P.O. Box 533, H-6701 Szeged, Hungary, [3]Department of Clinical Microbiology, Faculty of Medicine, University of Szeged, Somogyi Béla tér 1, H-6725 Szeged, Hungary

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* In memoriam Prof. Lajos Ferenczy

Abstract

Cellulolytic, xylanolytic, chitinolytic and β-1,3-glucanolytic enzyme systems of species belonging to the filamentous fungal genus *Trichoderma* have been investigated in details and are well characterised. The ability of *Trichoderma* strains to produce extracellular proteases has also been known for a long time, however, the proteolytic enzyme system is relatively unknown in this genus. Fortunately, in the recent years more and more attention is focused on the research in this field. The role of *Trichoderma* proteases in the biological control of plant pathogenic fungi and nematodes has been demonstrated, and it is also suspected that they may be important for the competitive saprophytic ability of green mould isolates and may represent potential virulence factors of *Trichoderma* strains as emerging fungal pathogens of clinical importance.

The aim of this review is to summarize the information available about the extracellular proteases of *Trichoderma*. Numerous studies are available about the extracellular proteolytic enzyme profiles of *Trichoderma* strains and about the effect of abiotic environmental factors on protease activities. A number of protease enzymes have been purified to homogeneity and some protease encoding genes have been cloned and characterized. These results will be reviewed and the role of *Trichoderma* proteases in biological control as well as their advantages and disadvantages in biotechnology will be discussed.

Introduction

Proteases are subdivided into two major groups: exopeptidases cleaving the peptide bond proximal to the amino or carboxy terminal of the substrate, and endopeptidases cleaving peptide bonds distant from the termini [1]. According to the functional group present at the

active site, proteases are further classified into four major groups: serine proteases, aspartyl proteases, cysteine proteases and metalloproteases. Based on the pH optimal for their functioning, proteolytic enzymes can be characterised as alkaline, neutral or acidic proteases.

Trichoderma species are asexual filamentous fungi with teleomorphs belonging to the Hypocreales order of the Ascomycota kingdom. Members of this genus are well-known as cellulase producers of biotechnological importance [2] and as antagonists of plant pathogenic fungi with biocontrol potential [3]. Proposed mechanisms of biocontrol are including mycoparasitism by the action of cell-wall degrading enzymes, production of antibiotics, competition for the substrate, rhizosphere competence and induction of the defense responses in plants [4]. The possible role of proteases in the antagonism of *Trichoderma* strains has been proposed already in 1969 by Rodriguez-Kabana [5], who suggested that increased proteolytic activities of *T. viride* in mixed-culture soil may have accounted for a decline in the enzymatic activities of *Sclerotium rolfsii*. The presence of a proteolytic system with maximal activity at pH 6.0 was later demonstrated in these mixed cultures [6]. Elad *et al.* [7] proved that the hydrolytic enzymes produced by *Botrytis cinerea* were partially deactivated by protease activities of *T. harzianum,* and demonstrated that the protease-containing culture liquid of *Trichoderma* reduced germination and germ tube length of the pathogen, suggesting the involvement of proteases in biocontrol. Besides deactivation of the plant pathogens' enzymes, proteases may be involved in competition for protein substrates as well as in the mycoparasitic process by degrading the protein components of the host cell wall. Sivan and Chet [8] reported that the treatment of *Fusarium* hyphae with proteolytic enzymes increased their susceptibility to chitinases and β-1,3-glucanases of *T. harzianum*. The involvement of extracellular chitinolytic and β-1,3-glucanolytic enzyme systems of *Trichoderma* in mycoparasitism was investigated in details [9, 10], while the extracellular proteolytic enzyme system remained relatively unknown in the case of this genus. Fortunately, in the recent years

more and more attention is focused on the investigation of *Trichoderma* proteases and their potential role in biocontrol and other processes.

Extracellular protease profiles of *Trichoderma* strains

A series of data is available about the extracellular proteolytic enzyme profiles of *Trichoderma* strains. Ridout *et al.* [11] used polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF), gel filtration and chromatofocusing for the fractionation of extracellular enzymes, including proteases from a mycoparasitic strain of *T. harzianum.* Using activity stains after PAGE in 6% gels, four active bands could be detected at pH 4.0, and one of these bands was also active at pH 9.0. Several protease enzymes were detected by activity staining of IEF gels at pH 9.0. By the use of gel filtration, two peaks of protease activity were found at pH 4.0, corresponding to enzymes with molecular weights of 65 and 23 kDa, while a large number of protease enzymes was separated by chromatofocusing in both the range pH 7–4 and pH 9–6 [11].

Delgado-Jarana *et al.* [12] detected several acidic, neutral and basic extracellular proteases by IEF in the case of *T. harzianum*. The acidic proteases were found to be pH-regulated, and nitrogen sources such as yeast extract, peptone and casein induced their production. Alkaline and neutral proteases seemed to be induced only by lactose and chitin, carbon starvation, and some organic nitrogen sources such as casein.

Antal *et al.* [13] examined and compared the extracellular enzyme profiles of mycoparasitic *T. aureoviride*, *T. harzianum* and *T. viride* strains by Sephadex G150 gel filtration chromatography. The profiles of trypsin-like and chymotrypsin-like proteases were found to be similar between the strains, and chromatographic profiles suggested that both

systems consist of more isoenzymes. In the case of a *T. viride* strain, at least six proteases were detected under inductive conditions by gel filtration chromatography [14]. The supernatants derived from cultures of a *T. harzianum* strain induced by heat-inactivated *Bacillus subtilis* cells were fractionated on a Sephadex G-150 column and the detected enzyme profiles proved to be complex including at least 3 trypsin-like (approx. 5, 13 and 19 kDa in size) and 6 chymotrypsin-like proteases (between 12 and 43 kDa) [15]. According to this study, *Trichoderma* strains may be able to degrade bacterial cells, and proteases are suggested to play a role in this process.

Williams *et al.* [16] demonstrated that growth on mushroom cell walls *in vitro* resulted in rapid production of trypsin- and chymoelastase-like proteases by *Trichoderma* isolates belonging to the groups Th2 and Th4 aggressive to *Agaricus bisporus*. Several protease isoenzymes were detected by IEF both on *A. bisporus* cell walls and on wheat straw, and aggressive isolates produced a dominant protease isoform (pI 6.22) on mushroom cell walls. This study suggests that proteases may play an important role both in mycoparasitism and extensive saprophytic growth, which seem to be among the main components of aggressiveness.

Supernatants from induced liquid cultures of six clinical *T. longibrachiatum* isolates were screened for proteolytic enzyme activities with 11 different chromogenic *p*-nitroanilide substrates [17]. The production of trypsin-like, chymotrypsin-like and chymoelastase-like protease activities was common among the examined strains. Separation of trypsin- and chymotrypsin-like activities by column chromatography revealed that both systems are complex consisting of several isoenzymes. It was suggested that extracellular proteolytic enzymes may represent potential virulence factors of *Trichoderma* strains as emerging human pathogens.

In a recent paper [18], nine different protease alleles with a wide range of molecular weights were detected by SDS-PAGE in the case of 17 biocontrol strains of *Trichoderma*. A great variability could be detected between the individual strains, and the protease isoenzyme profiles along with profiles of other cell wall-degrading isoenzymes proved to be applicable for taxonomic investigations.

Data are available also about the influence of abiotic environmental factors on the extracellular proteolytic activities of *Trichoderma* strains. The effect of low temperature on the production and activity of extracellular enzyme systems, including proteases, was examined in the case of cold tolerant *Trichoderma* isolates [19]. Results showed that trypsin- and chymotrypsin-like activities were produced at 10 °C and remained highly active even at 5 °C, and most of the strains could antagonize the phytopathogens *R. solani* and *F. oxysporum* f. sp. *dianthi* in dual culture tests at low temperatures. *In vitro* water activity (a_w) and pH-dependence of the extracellular enzyme activities of five cold tolerant *Trichoderma* strains was also examined [20]. Maximal activities for trypsin-like and chymotrypsin-like proteases were measured at a_w 0.950, which is lower than the values optimal for mycelial growth. I*n vitro* protease activities were detected even at the a_w value of 0.860, where mycelial growth has already ceased. Both protease systems were active under a wide range of pH, even at alkalic values, where mycelial growth was already inhibited (pH 8.0-9.0). Optimal pH values were at pH 6.0 for trypsin-like protease and between pH 6.0-7.0 for chymotrypsin-like protease activities. The high activity of both proteases measured in the wide pH range between 5.0-9.0 suggests the presence of isoenzymes with different pH optima [20]. Similar pH profiles were reported for the protease activities of clinical *T. longibrachiatum* isolates [17]. The effect of a 1 mM concentration of 10 metal ions on the *in vitro* activities of extracellular enzymes, including trypsin-like and chymotrypsin-like proteases was examined by Kredics *et al.* [21] in the case of six cold tolerant *Trichoderma* isolates. Both of the

examined protease activities were strongly inhibited by mercury and slightly by aluminium, copper and lead. Copper inhibited chymotrypsin-like activities to a larger extent than trypsin-like proteases. The other examined heavy metals, nickel, cobalt, cadmium, zinc, manganese and iron did not influence the *in vitro* enzyme activities of proteases to the same extent as they inhibited mycelial growth [21].

These studies indicate that *Trichoderma* strains possess a complex proteolytic system consisting of a large set of enzymes displaying different types of activities, and that the proteases of *Trichoderma* can remain active even under environmental conditions that are unfavorable for mycelial growth.

Purification and properties of extracellular proteases from *Trichoderma*

The most important characteristics of proteases purified from *Trichoderma* strains are summarized in Table I. The first report about the purification of a *Trichoderma* protease has been published by Stepanov *et al.* [22], who prepared and tested new biospecific sorbents based on cyclopeptide antibiotics for affinity chromatography of proteolytic enzymes. Purification strategies incorporating this method were succesfully applied for the isolation of carboxylic proteases from *T. viride* and *T. lignorum* [23], as well as of subtilisin-like serine proteases from *T. lignorum* and *T. koningii* [24]. PRB1, a subtilisin-like alkaline serine protease of *T. atroviride* was purified and biochemically characterized by Geremia *et al.* [25]. Another subtilisin-like serine protease with a temperature optimum of 40 ºC was found in the case of *T. harzianum* [26]. Trypsin-like serine proteases were purified from *T. viride* [27] and *T. harzianum* (PRA1) [28]. The PRA1 protease of *T. harzianum,* purified by chromatofocusing and gel filtration, was shown to have a nematicidal effect: its preparations

significantly reduced the number of hatched eggs of the root-knot nematode *Meloidogyne incognita* [28]. A *T. koningii* alkaline serine protease with large proportion of carbohydrates and a temperature optimum of 50 ºC was purified to apparent homogeneity by ion exchange, gel permeation and affinity chromatography [29]. A 18.8 kDa alkaline protease produced by *T. harzianum* was purified by precipitation with ammonium sulphate followed by hydrophobic chromatography; the purified enzyme substantially affected the cell wall of the phytopathogenic fungus *Crinipellis perniciosa*, suggesting that it may be actually involved in the antagonistic process between the two fungi [30].

Acidic aspartyl proteases produced under cellulase-inducing conditions were partially or completely purified from *T. reesei*. Gel filtration and ion exchange chromatography steps were used by Haab *et al.* [31] for the partial purification of a pepstatin insensitive, *N*-chlorosuccinimide sensitive aspartyl protease. Pepstatin-sensitive proteases with similar pI (4.3 – 4.8) were partially purified by Dunne [32], while Pitts *et al.* performed the complete purification, crystallization and X-ray analysis of trichodermapepsin, a pepstatin sensitive aspartyl protease [33, 34]. Another pepsin-like aspartyl protease inhibited by pepstatin and *N*-diazo-acetyl-L-phenylalanine was purified by Eneyskaya *et al.* [35]. A pH-dependent aspartyl protease from *T. viride* was also isolated and characterized [36].

Cloning and characterization of genes coding for *Trichoderma* proteases

The first report about the cloning of a *Trichoderma* protease gene has been published in 1993 [25]. In the last few years, increased attention has been paid on the investigation of the genetic background of the proteolytic system of *Trichoderma* strains. Table II shows some properties of *Trichoderma* proteases predicted based on the sequences of their cloned genes.

The gene coding for a subtilisin-like serine protease of *T. atroviride*, PRB1, has been identified by Geremia *et al.* [25]. The promoter sequence of *prb1* possess potential AreA and CreA sites for nitrogen and carbon regulation, respectively, and four putative mycoparasitic response elements (MYREs) were also described [37]. Olmedo-Monfil *et al.* [38] suggested that either one or both of MYRE boxes 1 and 2 might be required for the induction of *prb1* during mycoparasitism. The gene was shown to be active when the fungus was grown in media containing chitin or *R. solani* cell walls [25]. High-level expression was detected in dual cultures even if contact with *R. solani* was prevented by using cellophane membranes, demonstrating that the induction of *prb1* is contact-independent [37]. Results of this study showed that a heat and protease resistant, diffusible molecule produced by the host is the signal that triggers the expression of the gene. *Prb1* was found to be repressed by glucose [25] and it is subject to nitrogen catabolite repression [38]. It was demonstrated, that induction of transcription of the gene by *R. solani* cell walls and by osmotic stress requires release from a repressed condition which is determined by nitrogen availability, and the response of *prb1* to nutrient limitation depends on the activation of conserved mitogen activated protein kinase (MAPK) pathways [38].

The *prb*1 gene was cloned from *T. hamatum* as well (Steyaert, J.M. *et al*., unpublished, GenBank Accession Number: AY258899), and *tvsp1,* the homologue of *prb1* in *T. virens,* was also isolated recently [39]. The promoter of the *T. virens* gene possess potential AreA and CreA sites, as well as a PacC site for pH regulation and four MYREs almost identical to those described for *prb1*. Two potential *N*-glycosilation, and two *O*-glycosilation sites were present in the predicted polypeptide sequence, and multiple phosphorylation sites were also suggested. Expression of *tvsp1* could be induced by cell walls of plant pathogenic fungi in liquid cultures. Northern analysis revealed no transcript from mycelia incubated in medium containing either glucose or sucrose as a carbon source [39]. In contrast to *prb1*, a

mitogen-activated protein kinase was found to be a negative element in the expression of *tvsp1* under nitrogen limitation or simulated mycoparasitism [40].

Another recent study reports about the cloning of a further serine protease gene, *pra1* from *T. harzianum,* which is coding for the enzyme PRA1 [28]. Three possible *O*-glycosilation sites could be identified in the putative mature protein. Expression of this gene is also induced by fungal cell walls, subject to nitrogen and carbon derepression and affected by pH in the culture media.

The gene coding for an aspartyl protease from *T. harzianum, papA* was isolated and characterized by Delgado-Jarana *et al.* [41]. The promoter sequence contained potential AreA and PacC sites, but no potential CreA sites. Expression of the *papA* gene proved to be pH regulated, repressed by ammonium, glucose and glycerol and induced by organic nitrogen sources. The deduced amino acid sequence contained no potential post-translational modification signals, in contrast to that of a homologous gene isolated from *T. asperellum* [42], where three potential sites for *N*-glycosilation were found. Dual plate confrontation assays with *R. solani* revealed a fourfold mRNA induction of the *T. asperellum papA* in presence of the pathogen before actual physical contact, suggesting that aspartyl proteases may also be involved in mycoparasitism. Furthermore, this gene was induced in response to plant roots attachment, indicating its possible role in plant colonization of *Trichoderma* strains as opportunistic plant symbionts. The isolation of *papB,* a further aspartyl protease gene from *T. asperellum* was also reported in this study [42], however, PAPB is suggested to be an intracellular enzyme. The cloning of the gene encoding for the aspartyl protease purified from *T. reesei* by Pitts *et al.* [33] was also performed [43].

Extracellular proteases of *Trichoderma* and efficiency of biocontrol

Although Mischke [44] reported that the specific activity of proteases produced by *Trichoderma* strains do not correlate with their known biocontrol ability, other studies indicate the opposite. Transformation systems were developed for increasing the copy number of the *T. atroviride prb1* gene [45, 46]. Transformants exhibited increased control of *R. solani*, suggesting that *prb1* is a mycoparasitism-related gene [45]. A transformant containing multiple copies of *prb1* displayed improved biocontrol activity against *Meloidogyne javanica*, indicating that this protease may be important also for the biological control of nematodes [47]. Overexpression of *tvsp1* in *T. virens* also resulted in an increased biocontrol activity against *R. solani*. Results of these studies suggest that the overexpression of protease encoding genes is a powerful tool for strain improvement. However, Flores *et al.* [45] reported that transformants with extremely high protease levels were not the best biocontrol agents. As the partial or full proteolysis of other proteins important in the mycoparasitic process can not be ruled out under conditions of extremely elevated protease production, the isolation of mutants with only a moderate increase in extracellular enzyme concentrations was suggested as a preferable tool for improving the biocontrol capabilities of mycoparasitic *Trichoderma* strains [48]. Zaldivar *et al.* [49] isolated a mutant by *N*-methyl-*N*-nitro-*N*-nitrosoguanidine treatment from *T. aureoviride.* The mutant displayed enhanced production of lytic enzymes including proteases: a banding pattern of protease activities more complex than that of the wild type strain could be observed in native PAGE. The method of UV-mutagenesis with the selection for *p*-fluorophenyl-alanine resistant or colony morphology mutants was used by Szekeres *et al.* [50] for the isolation of protease overproducing strains from *T. harzianum*. Certain mutants were better producers of extracellular trypsin- and chymotrypsin-like proteases with manifold levels of the activities of the wild type strain. The

increase in the proteolytic activities of these mutants was low when compared to transformants overexpressing the proteinase gene *prb1* [45], but they proved to be much better antagonists of plant pathogens than the parental strain. Recently it was also demonstrated that certain extracellular trypsin-like protease izoenzymes are produced in a larger degree in the presence of copper, suggesting that the antagonistic abilities of *Trichoderma* strains could be enhanced by adding certain sublethal amounts of $CuSO_4$ [51]. Consequently, an appropriate level of crop protection could be ensured within the frames of integrated pest management by the application of reduced amounts of copper-containing fungicides in combination with biocontrol *Trichoderma* strains.

Advantages and disadvantages of *Trichoderma* proteases in biotechnology

Proteases have a large variety of applications in the detergent, leather, dairy, baking and pharmaceutical industries [1], therefore it is very important to screen for potential microbial sources of these enzymes. *Trichoderma* species seem to be promising candidates, although only a few reports are available about the possible application of their proteases in biotechnology. Robbins *et al.* [52] patented a meat tenderization technique based on a *T. reesei* aspartyl protease, which has proteolytic properties similar to the animal protease, cathepsin D. The enzyme acts selectively upon the myofibrillar proteins of meat producing a desirable uniform texture. The insolubilization of a *T. koningii* alkaline serine protease by crosslinking with glutaraldehyde resulted in an enzyme preparation stable over a wide range of temperature and pH and resistant to inhibition by detergents, suggesting its applicability in the detergent industry [53]. Triveni *et al.* [54] used proteases of *T. koningii* produced in solid-state fermentation of wheat bran for the clarification of xanthan gum. The preparation

succesfully lysed *Xanthomonas campestris* cells present in xanthan fermentation broth. A trypsin-like serine protease purified from *T. viride* has been suggested to represent a promising alternative for animal trypsin in the food industry and in medical products [27].

The presence of extracellular proteases of *Trichoderma* may be a disadvantage in fermentation processes aiming the production of other extracellular enzymes, as they can reduce or eliminate the activity of the desired product. Nakayama [55] was the first who demonstrated the presence of acidic, neutral and alkaline proteases in a commercial *Trichoderma* cellulase product. An endocellulase component of this product was subjected to partial proteolysis with a homologous protease preparation, which resulted in a modified cellulase with very similar chromatographic patterns to those of cellulase subfractions without proteolytic treatment [56]. Later, truncated forms of endoglucanases [57, 58] and cellobiohydrolases [59] have been isolated from *T. reesei* and it was demonstrated that proteolysis at late culture stages may contribute to the multiplicity of cellulolytic enzymes. The high levels of protease in the extracellular culture fluid of a proteolytic selectant of *T. reesei* correlated with the appearance of proteolytic cellulase degradation products [31]. A study on the mechanisms regulating post-secretory limited proteolysis of cellobiohydrolase and α-galactosidase of *T. reesei* revealed that a purified acidic protease cleaved both enzymes into the same proteolytic fragments, that had been isolated from the culture medium, and the enzymatic degradation was dependent on the degree of glycosylation of the secreted enzymes and pH [35]. When an endochitinase gene of *T. harzianum* was overexpressed in *T. reesei*, the amounts of the produced enzyme decreased remarkably with the decrease in the culture pH at the late stages of cultivation [60]. However, mRNA levels were still high at these time points, suggesting that the endochitinase was sensitive to an acidic protease, which occurred concominantly with the change in the culture pH. Delgado-Jarana *et al.* [12] overexpressed a homologous β-1,6-glucanase in *T. harzianum* and found that low pH resulted in the

degradation of the product due to the induction of aspartyl proteases other than *papA*. This problem could be overcome by buffering the medium to avoid the production of these pH-induced enzymes, or by adding their potential substrates, e.g. yeast extract, peptone or casein in order to protect the β-1,6-glucanase from proteolysis. Another possibility could be the application of *Trichoderma* strains with low levels of protease production. Low protease mutants have been isolated from *T. reesei* by classical mutagenesis [43, 61] and by gene disruption [62].

Conclusions

Studies examining the proteolytic enzyme profiles of *Trichoderma* strains revealed that the protease system of *Trichoderma* is complex containing a large set of enzymes. The proteases of *Trichoderma* can remain active even under environmental conditions unfavorable for mycelial growth, suggesting the possibility of strain improvement for better stress tolerance properties. Certain components of the protease system, including carboxylic proteases, subtilisin-like, trypsin-like and chymotrypsin-like serine proteases as well as aspartyl proteases were purified and characterised, and genes of some serine and aspartyl proteases were also isolated. It was demonstrated, that *Trichoderma* proteases are involved in the mycoparasitic action, nematicidal activity and plant colonization. Certain proteases appeared to be associated also with the aggressiveness of *Trichoderma* groups towards the commercial mushroom *A. bisporus*, which seems to be based mainly on competitive saprophytic ability. It has also been suggested that proteases may represent potential virulence factors of *T. longibrachiatum* strains as emerging fungal pathogens of clinical importance. In conclusion, it seems to be obvious, that *Trichoderma* strains evolved the ability of

extracellular protease production to increase their survival advantage as competitive saprophytic and parasitic organisms.

Only a few *Trichoderma* proteases have been examined until now for their potential applicability for commercial purposes. However, there is an emerging need for microbial protease sources in biotechnology, and members of the genus *Trichoderma* seem to be promising candidates. On the other hand, if a *Trichoderma*-based biotechnology process is aimed at the production of other enzymes or proteins, it has to be considered during the selection of the producer strain and fermentation conditions that acidic *Trichoderma* proteases may have negative effects on the activity and yield of the desired product.

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References

1. Rao, M.B., Tanksale, A.M., Ghatge, M.S., Deshpande, V.V.: Molecular and biotechnological aspects of microbial proteases. Microbiol Mol Biol Rev **62,** 597−635 (1998).

2. Kubicek, C.P., Eveleigh, D.E., Esterbauer, H., Steiner, W., Kubicek-Pranz, E.M. (eds): *Trichoderma reesei* cellulases: biodiversity, genetics, physiology and applications. Royal Society of Chemistry, Cambridge (1990).

3. Harman, G.E.: Myths and dogmas of biocontrol: Changes in perceptions derived from research on *Trichoderma harzianum* T-22. Plant Dis **84,** 377−393 (2000).

4. Howell, C.R.: Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: The history and evolution of current concepts. Plant Dis **87,** 4−10 (2003).

5. Rodriguez-Kabana, R.: Enzymatic interactions of *Sclerotium rolfsii* and *Trichodema viride* in mixed soil culture. Phytopathology **59,** 910–921 (1969).

6. Rodriguez-Kabana, R., Kelley, W.D., Curl, E.A.: Proteolytic activity of *Trichoderma viride* in mixed cultures with *Sclerotium rolfsii* in soil. Can J Microbiol **24,** 487–490 (1978).

7. Elad, Y., Kapat, A.: The role of *Trichoderma harzianum* protease in the biocontrol of *Botrytis cinerea*. Eur J Plant Pathol **105,** 177−189 (1999).

8. Sivan, A., Chet, I.: Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum*. J Gen Microbiol **135,** 675−682 (1989).

9. Kubicek, C.P., Mach, R.L., Peterbauer, C.K., Lorito, M.: *Trichoderma*: from genes to biocontrol. J Plant Pathol **83(S2),** 11−23 (2001).

10. Vazquez-Garciduenas, S., Leal-Morales, C.A., Herrera-Estrella, A.: Analysis of the β-1,3-glucanolytic system of the biocontrol agent *Trichoderma harzianum*. Appl Environ Microbiol **64,** 1442−1446 (1998).

11. Ridout, C.J., Coley-Smith, J.R., Lynch, J.M.: Fractionation of extracellular enzymes from a mycoparasitic strain of *Trichoderma harzianum*. Enzyme Microb Technol **10,** 180−187 (1988).

12. Delgado-Jarana, J., Pintor-Toro, J.A., Benítez, T.: Overproduction of β-1,6-glucanase in *Trichoderma harzianum* is controlled by extracellular acidic proteases and pH. Biochim Biophys Acta **1481,** 289–296 (2000).

13. Antal, Z., Kredics, L., Manczinger, L., Ferenczy, L.: Extracellular enzyme profiles of mycoparasitic *Trichoderma* strains. IOBC/wprs Bull **24(3),** 337−340 (2001).

14. Manczinger, L., Antal, Z., Schoop, A., Kredics, L.: Characterization of the extracellular enzyme systems of *Trichoderma viride* AH124. Acta Biol Hung **52,** 223−229 (2001).

15. Manczinger, L., Molnár, A., Kredics, L., Antal, Z.: Production of bacteriolytic enzymes by mycoparasitic *Trichoderma* strains. World J Microbiol Biotechnol **18,** 147−150 (2002).

16. Williams, J., Clarkson, J.M., Mills, P.R., Cooper, R.M.: Saprotrophic and mycoparasitic components of aggressiveness of *Trichoderma harzianum* groups toward the commercial mushroom *Agaricus bisporus*. Appl Environ Microbiol **69,** 4192−4199 (2003).

17. Kredics, L., Antal, Z., Szekeres, A., Manczinger, L., Kevei, F., Nagy, E.: Production of extracellular proteases by human pathogenic *Trichoderma longibrachiatum* strains. Acta Microbiol Immunol Hung **52,** 223−229 (2004).

18. Sanz, L., Montero, M., Grondona, I., Vizcaino, J.A., Llobell, A., Hermosa, R., Monte, E.: Cell wall-degrading isoenzyme profiles of *Trichoderma* biocontrol strains show correlation with rDNA taxonomic species. Curr Genet **46,** 277–286 (2004).

19. Antal, Z., Manczinger, L., Szakács, G., Tengerdy, R.P., Ferenczy, L.: Colony growth, *in vitro* antagonism and secretion of extracellular enzymes in cold-tolerant strains of *Trichoderma* species. Mycol Res **104,** 545–549 (2000).

20. Kredics, L., Manczinger, L., Antal, Z., Pénzes, Z., Szekeres, A., Kevei, F., Nagy, E.: *In vitro* water activity and pH dependence of mycelial growth and extracellular enzyme activities of *Trichoderma* strains with biocontrol potential. J Appl Microbiol **96,** 491–498 (2004).

21. Kredics, L., Dóczi, I., Antal, Z., Manczinger, L.: Effect of heavy metals on growth and extracellular enzyme activities of mycoparasitic *Trichoderma* strains. Bull Environ Contam Toxicol **66,** 249–254 (2001).

22. Stepanov, V.M., Rudenskaya, G.N., Gaida, A.V., Osterman, A.L.: Affinity chromatography of proteolytic enzymes on silica-based biospecific sorbents. J Biochem Biophys Methods **5,** 177–186 (1981).

23. Gaida, A.V., Osterman, A.L., Rudenskaia, G.N., Stepanov, V.M.: Carboxylic proteinases from the microscopic fungi *Trichoderma viride* and *Trichoderma lignorum*. Biokhimiia **46,** 181–189 (1981).

24. Gaida, A.V., Rudenskaia, G.N., Stepanov, V.M.: Isolation and comparative properties of serine proteinases of the microscopic fungi *Trichoderma lignorum* and *Trichoderma koningii*. Biokhimiia **46,** 2064–2073 (1981).

25. Geremia, R., Goldman, G.H., Jacobs, D., Ardiles, W., Vila, S.B., Van-Montagu, M., Herrera-Estrella, A.: Molecular characterization of the proteinase-encoding gene *prb*1, related to mycoparasitism by *Trichoderma harzianum.* Mol Microbiol **8,** 603–613 (1993).

26. Dunaevsky, Y.E., Gruban, T.N., Beliakova, G.A., Belozersky, M.A.: Enzymes secreted by filamentous fungi: Regulation of secretion and purification of an extracellular protease of *Trichoderma harzianum*. Biochemistry (Moscow) **65,** 723–727 (2000).

27. Uchikoba, T., Mase, T., Arima, K., Yonezawa, H., Kaneda, M.: Isolation and characterization of a trypsin-like protease from *Trichoderma viride.* Biol Chem **382,** 1509–1513 (2001).

28. Suarez, B., Rey, M., Castillo, P., Monte, E., Llobell, A.: Isolation and characterization of PRA1, a trypsin-like protease from the biocontrol agent *Trichoderma harzianum* CECT 2413 displaying nematicidal activity. Appl Microbiol Biotechnol **65,** 46–55 (2004).

29. Manonmani, H.K., Joseph, R.: Purification and properties of an extracellular proteinase of *Trichoderma koningii*. Enzyme Microb Technol **15,** 624–628 (1993).

30. De Marco, J.L., Felix, C.R.: Characterization of a protease produced by a *Trichoderma harzianum* isolate which controls cocoa plant witches' broom disease. BMC Biochem **3,** http://www.biomedcentral.com/1471-2091/3/3 (2002).

31. Haab, D., Hagspiel, K., Szakmary, K., Kubicek, C.P.: Formation of the extracellular proteases from *Trichoderma reesei* QM 9414 involved in cellulase degradation. J Biotechnol **16,** 187−198 (1990).

32. Dunne, C.P.: Relationship between extracellular proteases and the cellulase complex of *Trichoderma reesei*. In: Chibata, I., Fukui, S., Wingard, L.B. (Eds.): Enzyme Engineering, Vol. 6, Plenum Press, New York, pp. 355−356 (1982).

33. Pitts, J.E.: Crystallization by centrifugation. Nature **355,** 117 (1992).

34. Pitts, J.E., Crawford, M., Nugent, P.G., Western, R., Cooper, J.B., Mäntylä, A., Fagerström, R., Nevalainen, H.: The three dimensional X-ray crystal structure of the aspartic proteinase native to *Trichoderma reesei* complexed with renin inhibitor CP-80794. Adv Exp Med Biol **362,** 543−547 (1995).

35. Eneyskaya, E.V., Kulminskaya, A.A., Savelev, A.N., Saveleva, N.V., Shabalin, K.A., Neustroev, K.N.: Acid protease from *Trichoderma reesei*: limited proteolysis of fungal carbohydrases. Appl Microbiol Biotechnol **52,** 226−231 (1999).

36. Simankova, A.N., Mirgorodskaya, O.A., Saveleva, N.V., Savelev, A.N., Kerner, R., Rijpstorff, P., Alexandrov, S.L.: Specificity of aspartic protease from fungus *Trichoderma viride* in the hydrolysis of oligopeptides. Bioorg Khim **24,** 822−830 (1998).

37. Cortes, C., Gutierrez, A., Olmedo, V., Inbar, J., Chet, I., Herrera-Estrella, A.: The expression of genes involved in parasitism by *Trichoderma harzianum* is triggered by a diffusible factor. Mol Gen Genet **260,** 218−225 (1998).

38. Olmedo-Monfil, V., Mendoza-Mendoza, A., Gomez, I., Cortes, C., Herrera-Estrella, A.: Multiple environmental signals determine the transcriptional activation of the mycoparasitism related gene *prb*1 in *Trichoderma atroviride*. Mol Gen Genom **267,** 703−712 (2002).

39. Pozo, M.J., Baek, J.M., Garcia, J.M., Kenerley, C.M.: Functional analysis of *tvsp*1, a serine protease-encoding gene in the biocontrol agent *Trichoderma virens*. Fungal Genet Biol **41,** 336–348 (2004).

40. Mendoza-Mendoza, A., Pozo, M., Grzegorski, D., Martinez, P., Garcia, J.M., Olmedo-Monfil, V., Cortes, C., Kenerley, C., Herrera-Estrella, A.: Enhanced biocontrol activity of *Trichoderma* through inactivation of a mitogen-activated protein kinase. Proc Natl Acad Sci USA **100,** 15965–15970 (2003).

41. Delgado-Jarana, J., Rincón, A.M., Benítez, T.: Aspartyl protease from *Trichoderma harzianum* CECT 2413: cloning and characterization. Microbiology (UK) **148,** 1305–1315 (2002).

42. Viterbo, A., Harel, M., Chet, I.: Isolation of two aspartyl proteases from *Trichoderma asperellum* expressed during colonization of cucumber roots. FEMS Microbiol Lett **238,** 151–158 (2004).

43. Mäntylä, A., Saarelainen, R., Fagerström, R., Suominen, P., Nevalainen, H.: Cloning of the aspartic protease gene from *Trichoderma reesei*. In: Abstracts of the 2nd European Conference on Fungal Genetics; Lunteren, The Netherlands, abstract B52 (1994).

44. Mischke, S.: Evaluation of chromogenic substrates for measurement of protease production by biocontrol strains of *Trichoderma*. Microbios **87,** 175−183 (1996).

45. Flores, A., Chet, I., Herrera-Estrella, A.: Improved biocontrol activity of *Trichoderma harzianum* by over-expression of the proteinase-encoding gene *prb*1. Curr Genet **31,** 30−37 (1997).

46. Goldman, M.H.S., Goldman, G.H.: *Trichoderma harzianum* transformant has high extracellular alkaline proteinase expression during specific mycoparasitic interactions. Gen Mol Biol **21,** 329−333 (1998).

47. Sharon, E., Bar-Eyal, M., Chet, I., Herrera-Estrella, A., Kleifeld, O., Spiegel, Y: Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathology **91,** 687–693 (2001).

48. Rey, M., Delgado-Jarana, J. and Benítez, T.: Improved antifungal activity of a mutant of *Trichoderma harzianum* CECT 2413 which produces more extracellular proteins. Appl Microbiol Biotechnol **55,** 604–608 (2001).

49. Zaldivar, M., Velasquez, J.C. Contreras, I. Perez, L.M.: *Trichoderma aureoviride* 7-121, a mutant with enhanced production of lytic enzymes: Its potential use in waste cellulose degradation and/or biocontrol. Electr J Biotechnol **4(3),** 160−168 (2001).

50. Szekeres, A., Kredics, L., Antal, Z., Kevei, F., Manczinger, L.: Isolation and characterization of protease overproducing mutants of *Trichoderma harzianum*. FEMS Microbiol Lett **233,** 215−222 (2004).

51. Kredics, L., Hatvani, L., Antal, Z., Szekeres, A., Manczinger, L., Nagy, E.: Protease overproduction in the presence of copper by a *Trichoderma harzianum* strain with biocontrol potential. IOBC/wprs Bull in press (2005).

52. Robbins, F.M., Allen, A.L., Walker, J.E., Cohen, S.H.: Meat tenderization with a proteolytic enzyme from *Trichoderma reesei*. US Patent No. 4600589, 1986

53. Manonmani, H.K., Joseph, R.: Preparation and properties of insolubilized proteinase of *Trichoderma koningii.* Process Biochem **28,** 325−329.

54. Triveni, R., Shamala, T.R.: Clarification of xanthan gum with extracellular enzymes secreted by *Trichoderma koningii*. Process Biochem **34,** 49−53 (1999).

55. Nakayama, M.: The possible proteolytic modification of cellulase components in the aqueous solutions of a crude enzyme preparation from *Trichoderma viride*. Memoirs Osaka Kyoiku Univ Ser III Nat Sci Appl Sci **24,** 127−143 (1975).

56. Nakayama, M., Tomita, Y., Suzuki, H., Nisizawa, K.: Partial proteolysis of some cellulase components from *Trichoderma viride* and the substrate specificity of the modified products. J Biochem **79,** 955−966 (1976).

57. Shoemaker, S.P., Brown, R.D.: Characterization of endo-1,4-β-D-glucanases purified from *Trichoderma viride*. Biochim Biophys Acta **523,** 147−161 (1978).

58. Stahlberg, J., Johansson, G., Pettersson, G.: A binding-site-deficient catalytically active core protein of endoglucanase III from the culture filtrate of *Trichoderma reesei*. Eur J Biochem **173,** 179−184 (1988).

59. Hagspiel, K., Haab, D., Kubicek, C.P.: Protease activity and proteolytic modification of cellulases from a *Trichoderma reesei* QM9414 selectant. Appl Microbiol Biotechnol **32,** 61−67 (1989).

60. Margolles-Clark, E., Hayes, C.K., Harman, G.E., Penttila, M.: Improved production of *Trichoderma harzianum* endochitinase by expression in *Trichoderma reesei*. Appl Environ Microbiol **62,** 2145−2151 (1996).

61. Janas, P.: Production of extracellular enzymes by low-protease mutants of *Trichoderma reesei.* Acta Sci Pol Technol Alim **2,** 103−114 (2003).

62. Mäntylä, A., Paloheimo, M., Suominen, P.: Industrial mutants and recombinant strains of *Trichoderma reesei*. In: Harman, G.E., Kubicek, C.P. (Eds): *Trichoderma* and *Gliocladium*, vol. 2, Taylor and Francis, London, 1998.

Addresses of the authors:

Corresponding author: Dr. László Kredics, Hungarian Academy of Sciences and University of Szeged, Microbiological Research Group, P.O. Box 533, H-6701 Szeged, Hungary, Fax: 06 62 544 823, E-mail: kredics@bio.u-szeged.hu

Dr. Zsuzsanna Antal: same address as above

András Szekeres, Lóránt Hatvani, Dr. László Manczinger, Dr. Csaba Vágvölgyi: Department of Microbiology, Faculty of Sciences, University of Szeged, P.O. Box 533, H-6701 Szeged, Hungary, Fax: 06 62 544 823

Prof. Dr. Elisabeth Nagy: Department of Clinical Microbiology, Faculty of Medicine, University of Szeged, Somogyi Béla tér 1, H-6725 Szeged, Hungary

Table I.

Properties of extracellular proteases purified from *Trichoderma* strains

Organism	Type of protease	Molecular weight (kDa)	Isoelectric point	pH optimum (Examined range)	Inhibitors	Reference
T. viride	carboxilic protease	32	4.3	2.3 (3.0-6.0)	pepstatin diazoacetyl-D,L-norvaline methylester	[23]
T. lignorum	carboxilic protease	32	4.5	2.8 (3.0-6.0)	*N*-diazoacetyl-N'-2,4-dinitro-phenylethylenediamine	
T. lignorum	subtilisin-like serine protease	21	6.8	10.5 (4.0-11.0)	phenyl-methylsulfonyl fluoride	[24]
T. koningii	subtilisin-like serine protease	21	6.7	10.5 (4.0-11.0)	diphenylcarbamoylchloride	
T. atroviride	subtilisin-like serine protease	31	9.2	8.0-9.5 (NR)	phenyl-methylsulfonyl fluoride	[25]
T. harzianum	subtilisin-like serine protease	73	5.35	7.5 and 10.0 (6.0-11.0)	NR	[26]
T. viride	trypsin-like serine protease	25	7.3	7.0-8.0 (3.0-12.0)	diisopropyl fluorophosphate *N*-tosyl-L-lysine chloromethylketone aprotinin antipain	[27]
T. harzianum	trypsin-like serine protease	28	4.8	NR	phenyl-methylsulfonyl fluoride	[28]
T. koningii	alkaline serine proteinase	85	9.0	10.0 (4.0-11.0)	phenyl-methylsulfonyl fluoride benzamidine *N*-bromo-succinimide	[29]
T. reesei	pepsin-like aspartyl protease	42.5	4.3	3.0-4.0 (NR)	*N*-chlorosuccinimide	[31]
T. reesei	aspartyl protease	32	NR	2.8 (2.5-5.5)	pepstatin *N*-diazo-acetyl-L-phenylalanine	[35]

NR: not reported

Table II.

Properties of *Trichoderma* proteases predicted based on sequences of their cloned genes

Organism	Type of protease	Gene	Length of ORF (bp)	Length of deduced amino acid sequence	Length of putative signal peptide	Length of putative propeptide	Predicted properties of the mature protein			Reference
							Length	Calculated molecular weight (kDa)	Calculated isoelectric point	
T. harzianum	chymotrypsin-like serine protease	*prb1*	1227	409 aa	20 aa	100 aa	289 aa	29.0	9.2	[25]
T. virens	subtilisin-like serine protease	*tvsp1*	1368	409 aa	20 aa	100 aa	289 aa	29.0	8.98	[39]
T. harzianum	trypsin-like serine protease	*pra1*	774	258 aa	20 aa	9 aa	229 aa	25.0	4.91	[28]
T. harzianum	aspartyl protease	*papA*	1212	404 aa	20 aa	32 aa	352 aa	36.7	4.35	[41]
T. asperellum	aspartyl protease	*papA*	1297	405 aa	20 aa	31 aa	354 aa	NR	5.45	[42]

NR: not reported