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ORIGINAL PAPER

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Characterization of a chitinase and an endo-β-1,3-glucanase from *Trichoderma harzianum* Rifai T24 involved in control of the phytopathogen *Sclerotium rolfsii*

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Abstract Of 24 *Trichoderma* isolates, *T. harzianum* Rifai (T24) showed a potential for control of the phytopathogenic basidiomycete *Sclerotium rolfsii*. When T24 was grown on different carbon sources, growth inhibition of *S. rolfsii* by the T24 culture filtrate correlated with the activity of extracellular chitinase and β-1,3-glucanase. The 43-kilodalton (kDa) chitinase and the 74-kDa β-1,3-glucanase were purified from the T24 culture filtrate in two and three steps, respectively, using ammonium sulphate precipitation followed by hydrophobic interaction chromatography (phenyl-Sepharose) and gel filtration (β-1,3-glucanase). K_m and K_{cat} were 3.8 g l^{-1} and 0.71 s^{-1} for the chitinase (chitin) and 1.1 g l^{-1} and 52 s^{-1} for the β-1,3-glucanase (laminarin). The chitinase showed higher activity on chitin than on less-acetylated substrate analogues (chitosan), while the β-1,3-glucanase was specific for β-1,3-linkages in polysaccharides. Both enzymes were stable at 30°C, while at 60°C the chitinase and the β-1,3-glucanase were rapidly inactivated, showing half-lives of 15 and 20 min, respectively. The enzymes inhibited growth of *S. rolfsii* in an additive manner showing a promising ED_{50} (50% effective dose) value of 2.7 µg/ml.

Introduction

The plant pathogen basidiomycete *Sclerotium rolfsii* causes stem and pod rots, which are major constraints to peanut production in many regions. For example, in the United States, southern blight of peanuts is a problem in all peanut-producing states and has to be controlled primarily by the use of fungicides. Furthermore, the fungus causes disease in over 500 plant species. Recently, the fungus has also been found in Europe on different hosts, including juglans and sunflowers (Belisario and Corazza 1996; Infantino et al. 1997). Mycoparasitic fungi have been shown to have a potential for the control of plant diseases caused by *S. rolfsii* (Maoa et al. 2000). Lectins of *S. rolfsii* were found to be the recognition signal initiating the coiling process in *Trichoderma harzianum* (Inbar and Chet 1995) (Fig. 1), while it has been suggested that constitutive carbohydrolases of *T. harzianum* release oligosaccharides from *Rhizoctonia solani* cell walls, which subsequently induce production of large amounts of cell wall-degrading enzymes by *T. harzianum* (Kullnig et al. 2000).

Chitinases and β-1,3-glucanases produced by some *Trichoderma* species are the key enzymes in the lysis of cell walls during their mycoparasitic action against phytopathogenic fungi (Cruz et al. 1992, 1995; Shen et al. 1991). The lytic activity of several *Trichoderma* species on cell walls of phytopathogenic fungi has been correlatd with the degree of biological control of these pathogens in vivo (Papavizas 1985), and chitinolytic and glucano-

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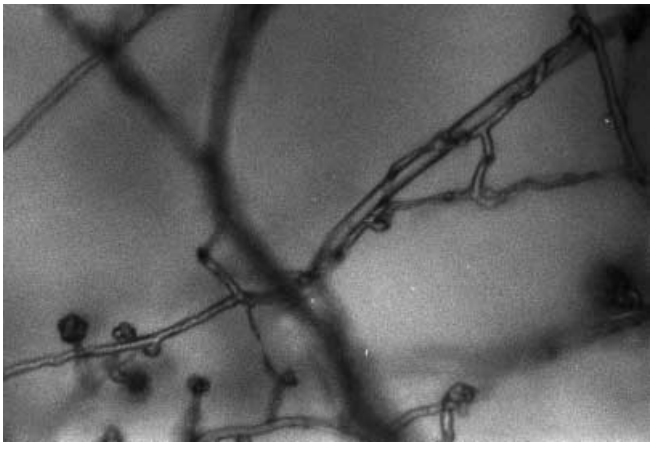

Fig. 1 Coiling formed by *Trichoderma harzianum* T24 during mycoparasitism of *Sclerotium rolfsii*

lytic enzymes from *T. harzianum* P1 interacted synergistically in the inhibition of spore germination and hyphal elongation of *Botrytis cinera* (Lorito et al. 1994). Apart from mycoparasitic fungi, chitinases are widely distributed in nature and play important roles in the degradation of chitin, a structural polysaccharide present in different organisms, mainly arthropods and fungi (Cabib 1987). The physiological function of chitinases depends on their source. In plants, which lack chitin, the enzymes are thought to be a defence system against fungal pathogens (Bowles 1990). In fungi, chitinases seem to play a physiological role in cell division and differentiation, as well as a nutritional role (Papavizas 1985).

As with chitinases, some plants have developed a β-1,3-glucanase defence system against fungal pathogens (Grenier et al. 1993; Mauch et al.1988), although involvement of these enzymes in cell differentiation has also been suggested (Bucciaglia and Smith 1994). In bacteria, which usually lack β-1,3-glucan, a nutritional role has been assigned to β-1,3-glucanases (Watanabe et al. 1992). In fungi, β-1,3-glucanases seem to have different functions. First, a physiological role in morphogenetic/morpholytic processes during fungal development and differentiation has been indicated. Second, β-1,3-glucanases have been related to the mobilization of β-1,3-glucans under conditions of carbon and energy source exhaustion, functioning as autolytic enzymes (Rapp 1992; Stahmann et al. 1992). Finally, a nutritional role in saprophytes and mycoparasites has been suggested (Chet 1987; Lorito et al. 1994; Sivan and Chet 1989).

Interestingly, both the spectrum of chitinases and β-1,3-glucanases produced and the ability to antagonize plant pathogens varies significantly within the *T. harzianum* species (El-Katatny et al. 2000; Ghisalberti et al. 1990). In this study, a chitinase and an endo-β-1,3-glucanase from a newly isolated *T. harzianum* strain (T24) have been characterized. Previously, this strain was selected from 24 *Trichoderma* isolates due to its potential in biocontrol (El-Katatny et al. 2000), which correlated with chitinase and β-1,3-glucanase activity in the culture filtrate. For the first time we show that a combination of the 74-kilodalton (kDa) β-1,3-glucanase and the 43-kDa chitinase from *T. harzianum* T24 can be used as a highly efficient agent for the control of *S. rolfsii*. Since in the future, chemical pesticides may be replaced by such enzyme preparations with similar or even lower ED_{50} (50% effective dose) values (Lorito et al. 1994), we have also determined the stability of the isolated enzymes.

Materials and methods

Microorganisms and cultivation

Trichoderma harzianum Rifai T24 (identified by CBS and deposited as BT 2324 at the Graz University of Technology culture collection) and *Sclerotium rolfsii* (ATCC 200224) were obtained from the Botany Department, Faculty of Science, University of Minia, Egypt. Potato dextrose agar (Biolife) was used to maintain cultures of the isolates. The minimal synthetic medium used for the cultivation of *T. harzianum* contained the following components (in grams per litre): chitin, 5.0; corn steep solid, 5.0; $MgSO_4$ $7H_2O$, 0.2; K_2HPO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; $FeSO_4$ $7H_2O$, 0.002; $MnSO_4$, 0.002 and $ZnSO_4$, 0.002. The pH was maintained at 6.3 (50 mM phosphate buffer).

Preparation of dried mycelium of *S. rolfsii*

Dried mycelium of *S. rolfsii* was prepared by the method described previously (Ridout et al. 1986). Erlenmeyer flasks (250-ml) containing 100 ml of potato dextrose broth were incubated with 1-cm^2 discs of potato dextrose agar of actively growing mycelium of *S. rolfsii* and incubated at 30°C for 7 days. The mycelium was then collected by filtration through Whatman no.1 filter paper, washed with distilled water, and homogenized in distilled water using a laboratory homogenizer. The suspension was washed three times with distilled water and the mycelium was stored in a lyophilized state until further use.

Enzyme activity assays

Chitinase activity was assayed using the colorimetric method described previously (Molano et al. 1977), with minor modifications. The assay mixture contained 1 ml of 0.5% chitin (Sigma, suspended in 50 mM acetate buffer pH 5.2) and 1 ml of enzyme solution. The mixture was incubated for 7 h at 37°C with shaking, and the reaction was stopped by centrifugation (7,000 *g*) for 10 min and by addition of 1 ml of dinitrosalicylate (DNS) reagent. β-1,3-Glucanase was assayed similarly by incubating 500 µl of 5.0% (w/v) laminarin in 50 mM acetate buffer (pH 4.8) with 200 µl enzyme solution at 45°C for 30 min and determination of the reducing sugars with DNS. The amount of reducing sugars released was calculated from standard curves recorded for *N*-acetylglucosamine and glucose, and chitinase and β-1,3-glucanase activities were expressed in pkat (pmol s^{-1}) and nkat (nmol s^{-1}), respectively.

Enzyme purification

T. harzianum was grown for 5 days in chitin-containing medium. The culture broth was filtered through MSI MAGNA nylon disc filters (0.45 µm, 50 mm), centrifuged (2,500 *g* for 20 min), and used for the isolation of both chitinase and β-1,3-glucanase. Unless otherwise indicated, all purification steps were carried out at 4°C. Proteins were precipitated from the supernatant with ammonium sulphate (75% saturation) and collected by centrifugation (2,500 *g* for 20 min). The pellet was dissolved in distilled water and further purified by ultrafiltration (MACROSEP centrifugal concentrator, MWCO 30-kDa, Pall Filtron MA, USA). Aliquots (1-ml) of the samples containing 2 M ammonium sulphate were loaded onto a phenyl-Sepharose column (Pharmacia, 1.6×6.5 cm) equilibrated with 50 mM phosphate buffer (pH 6.5) containing 2 M ammonium sulphate. The column was washed with one column volume of the same buffer and proteins were eluted with a linear gradient of 2.0–0.0 M of ammonium sulphate at a flow rate of 3 ml min^{-1} (5-ml fractions). Pooled fractions showing β-1,3-glucanase activity were concentrated by ultrafiltration (MWCO 30-kDa, Pall Filtron MA) and 500-µl aliquots were loaded onto a Superdex gel filtration column (Pharmacia, 1×30 cm) previously equilibrated with 50 mM phosphate buffer (pH 6) containing 0.2 M sodium chloride. Proteins were eluted with the same buffer at a flow rate of 0.5 ml min^{-1}. Fractions of 1 ml were collected and monitored for β-1,3-glucanase activity. All chromatographic purification steps were performed with a Pharmacia ÄKTA (Amersham Pharmacia Biotech, Uppsala, Sweden) system and proteins were monitored with an UV detector at 280 nm.

Protein analysis

Protein concentrations were determined by the method of Lowry, using bovine serum albumin as a standard. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a mini-Protean electrophoresis cell from Bio-Rad.

Proteins were stained with Coomassie brilliant blue R-250 and low-molecular-mass standard proteins (Bio-Rad) were used to determine the molecular mass of the enzymes. Isoelectric focusing (IEF) was carried out using IEF Ready Gels from Bio-Rad which were run according to the application guide, at 100 V for 1 h, 250 V for 1 h, and 500 V for 30 min. Phosphoric acid (7 mM) was used as anode buffer and 20 mM lysine and 20 mM arginine as cathode buffer. The IEF staining solution was 27% isopropanol, 10% acetic acid, 0.04% Coomassie blue R250, and 0.05% crocein scarlet, and the gels were destained using 40% methanol and 10% acetic acid.

Enzyme stability

The effect of pH on the enzyme activity was determined using the procedures described for the standard assay and varying the pH of the reaction mixtures in increments of 0.5 pH units using 50 mM buffer (sodium citrate for pH 3–4, sodium acetate for pH 4.5–5.5, and phosphate buffer for pH 6–7). The effect of temperature on enzyme activity was determined under standard assay conditions (50 mM acetate buffer, pH 4.5) at temperatures between 30°C and 80°C. Half-lives of the enzymes were determined by incubating 5-ml samples (92.2 pkat ml^{-1} chitinase or 44.5 nkat ml^{-1} glucanase of pure enzyme) at 35°C with 5 ml of 50 mM of various buffer, adjusted to different pH values as described above (pH 3, 4.5, 6, 7) or at various temperatures (25, 30, 40, 50, 60°C) with 5 ml of 50 mmol acetate buffer (pH 4.5). Samples were taken at various time intervals and the remaining activity was measured using the standard assay. To evaluate the effect of various substances on enzyme activity, samples were pre-incubated with a range of compounds at concentrations indicated below for 5 min at 37°C for chitinase and 45°C for β-1,3-glucanase. Thereafter the remaining activity was assayed as described above.

Substrate specificity

To establish the specificity of the purified enzymes, pure chitin, practical grade chitin, chitosan (deacetylated chitin), cellulose, cellobiose, *S. rolfsii* (dried mycelium), laminarin, pullulan, amylose, *p*-nitrophenyl-β-D-glucopyranoside and locust bean gum galactomannan (Sigma), ivory nut mannan (Megazyme, Sydney, Australia), pustulan, and birch wood xylan (Roth, Karlsruhe, Germany) were used as substrates. In each case, degradation was monitored by measuring the release of reducing sugars. The hydrolysis products of laminarin by the endoglucanase were analysed using thin-layer chromatography silica plates (Merck, Darmstadt, Germany) with water, 1-propanol, and nitromethane (2:7:1) as the eluent. Different concentrations of chitin or laminarin were used to determine the kinetic parameters of the chitinase and β-1,3-glucanase, respectively. K_m and V_{max} were calculated by non-linear analysis using the program "Origin", version 4.10 (Microcal, Northampton, USA).

Antifungal activity of enzyme preparations

Agar plates (potato dextrose agar, 10% v/v) were prepared with culture filtrates, concentrated samples (ammonium sulphate or ultrafiltration), purified enzymes (chitinase or β-1,3-glucanase), and with water as a control. All enzyme preparations were sterilized by filtration (through MSI MAGNA nylon disc filters, 0.22 µm, 25 mm). The phytopathogenic fungus *S. rolfsii* was inoculated in the centre of agar plates using 5-mm mycelial discs and incubated at 30°C, for 3 days. The radial diameter of the colonies was measured at right angles every day, for six replicate plates per treatment, and the percentage of inhibition was calculated.

Results

The chitinase from *T. harzianum* T24 grown in liquid medium containing chitin was purified 27-fold to electrophoretic homogeneity in two steps using ammonium sulphate precipitation and hydrophobic interaction chromatography. SDS-PAGE showed that the enzyme migrated as a single band with an estimated molecular mass of 43 kDa (Table 1). The β-1,3-glucanase from *T. harzianum* T24 has been purified 91-fold using ammonium sulphate precipitation, hydrophobic interaction chromatography (phenyl-Sepharose), and gel filtration (Superdex-75), showing a molecular mass of 74 kDa (Table 1).

Table 1 Biochemical properties of the *Trichoderma harzianum* T24 chitinase and β-1,3-glucanase (*kDa* kilodaltons)

	Chitinase	β-1,3-Glucanase
Molecular weight (kDa)	43	74
pH optimum	4.5	4.5
Temperature optimum (°C)	40	60
Temperature stability – half-life (h)		
pH 4.5/40°C	18	>24
pH 4.5/50°C	42 min	38 min
pH 4.5/60°C	15 min	20 min
pI	4.0	6.2
Specific activity (nkat mg^{-1})	16.6	1.6
K_m (g l^{-1})	3.8	1.1
V_{max}	49.3 (pkat ml^{-1})	9.1 (nkat ml^{-1})
K_{cat} (s^{-1})	0.71	52

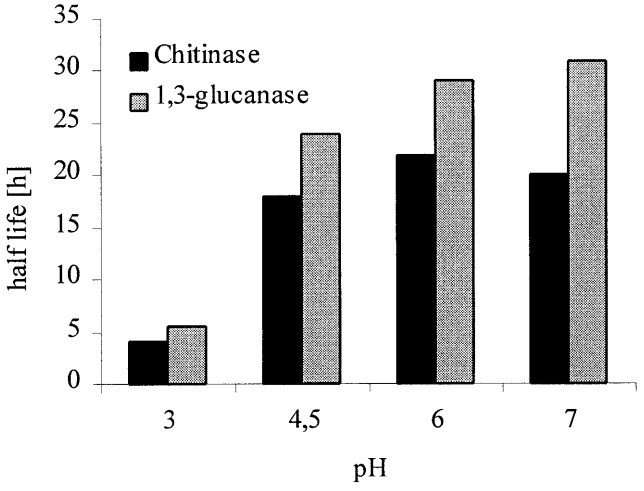

Fig. 2 pH stabilities of the chitinase and β-1,3-glucanase from *T. harzianum*

The optimal pH for the T24 chitinase was pH 4.5 and the enzyme showed 75% of its maximum activity between pH 4 and pH 5. The enzyme was quite stable in a wide pH range between 4.5 and 7, whereas at pH 3 (30°C) the half-life was only 4 h (Fig. 2). The pH optimum of the β-1,3-glucanase from *T. harzianum* was the same as for the chitinase (pH 4.5); however, the activity peak was relatively wider, and about 75% of the optimum activity was measured between pH 3 and pH 6. The β-1,3-glucanase showed a slightly higher pH stability (half-life) than the chitinase (Fig. 2).

The temperature optimum of the T24 chitinase was 40°C, while the temperature optimum of the T24 β-1,3-

Table 2 Effect of various compounds on chitinase and β-1,3-glucanase from *T. harzianum*

Items	Concentration	Relative activity(%)[a]	
		Chitinase	β-1,3-Glucanase
None	–	100.0	100.0
$CaCl_2.2H_2O$	5 mM	100.0	97.3
$CuCl_2.2H_2O$	5 mM	123.8	100.4
$FeSO_4.7H_2O$	5 mM	52.0	79.0
KCl	5 mM	88.0	100.0
$MnCl_2.4H_2O$	5 mM	133.3	132.0
$NiCl_2.6H_2O$	5 mM	104.9	98.7
NaN_3 (sodium azide)	5 mM	114.0	97.2
$ZnSO_4.7H_2O$	5 mM	98.0	98.7
EDTA	5 mM	88.8	91.7
EDTA	10 mM	82.0	91.7
$HgCl_2$	5 mM	10.0	6.6
SDS	5 mM	76.0	86.2
$HgSO_4$	5 mM	15.0	7.3
Urea	5 mM	133.0	86.2
Cycloheximide	5 mM	113.0	97.2
Hydrazinum sulphate	5 mM	105.0	106.4
LiCl	5 mM	79.0	95.4
Glucono-δ-lactone	5 mM	100.0	100.0
Glucono-δ-lactone	10 mM	100.0	97.2

[a] 100% activity corresponds to 39.1 pkat ml^{-1} (chitinase) and 8.5 nkat ml^{-1} (β-1,3-glucanase)

Table 3 Substrate specificity of chitinase and β-1,3-glucanase from *T. harzianum* (*d.m.* dried mycelium, *SDS* sodium dodecyl sulphate, *GlcNA* *N*-acetylglucosamine)

Substrate[a]	Linkage(s)	Monomer	Relative activity(%)[b]	
			Chitinase	β-1,3-Glucanase
Chitin	β-1,4-	GlcNAc	100	0.0
Chitin (practical grade)	β-1,4-	GlcNAc	47	0.0
Chitosan (deacetylated chitin)	β-1,4-	GlcN	30	0.0
Cellulose	β-1,4-	Glucose	0.0	0.0
Cellobiose	β-1,4-	Glucose	0.0	0.0
Rhizoctonia solani (d.m.)	β-1,4-	GlcNAc	52	9.9
Sclerotium rolfsii (d.m.)	β-1,4-	GlcNAc	57	8.7
Laminarin	β-1,3-	Glucose	0.0	100
Pustulan	β-1,6-	Glucose	0.0	5.8
Xylan	β-1,4-	Xylose	0.0	3.0
Pullulan	α-1,4–α-1,6	Glucose	0.0	4.1
Amylose	α-1,4-	Glucose	0.0	4.5
Mannan	β-1,4-	Mannose	0.0	4.7
Galactomannan	β-1,4-	Mannose, Galactose	0.0	5.8

[a] All substrates were used at a concentration of 0.5%
[b] 100% activity corresponds to 39.1 pkat ml^{-1} (chitinase) and 8.5 nkat ml^{-1} (β-1,3-glucanase)

glucanase activity was in the range from 50°C to 60°C. Although the β-1,3-glucanase produced by *T. harzianum* T24 was quite stable below 50°C, the enzyme lost 40% of the initial activity after 30 min incubation at 50°C, and more than 70% at 60°C after the same time. The chitinase from *T. harzianum* T24 was strongly inhibited by Hg^{2+} and slightly inhibited by Fe^{2+} (Table 2), while EDTA and SDS had no significant inhibitory effect on the T24 β-1,3-glucanase activity. The T24 β-1,3-glucanase was strongly inhibited by Hg^{2+}, while no inhibition was observed with 5 or 10 mM glucono-δ-lactone.

Substrate specificity and control of *S. rolfsii*

The chitinase from *T. harzianum* T24 showed the highest activity towards chitin and to a lesser extent towards practical grade chitin (Table 3). Obviously removal of lipid and protein components during purification of chitin enhances accessibility of the substrate to the enzyme. There was no activity detected against any other non-chitinaceous substrate, indicating that the enzyme is highly specific for linear polymers of N-acetylglucosamine. Interestingly, activity on chitosan was only 30% of that measured for chitin. The purified T24 β-1,3-glucanase enzyme was specific for β-1,3-linkages in polysaccharides, hydrolysing laminarin and dried mycelium of phytopathogenic fungi (Table 3). Only slight activity was found with pustulan (linear β-1,6-glucan), which could be related to the presence of some β-1,3-linkages in branching points (Cruz et al. 1995). The β-1,3-glucanase seemed to be endo-acting, as the enzyme released mainly dimers, trimers, and higher oligomers from laminarin and showed no reaction against *p*-nitrophenyl-β-D-glu-

copyranoside (data not shown). K_m and K_{cat} of the *T. harzianum* T24 chitinase and β-1,3-glucanase were determined for the hydrolysis of chitin and laminarin, respectively. K_m and K_{cat} were 3.8 g l^{-1} and 0.71 s^{-1} for chitinase and 1.1 g l^{-1} and 52 s^{-1} for β-1,3-glucanase, respectively.

T. harzianum T24 was able to overgrow *S. rolfsii* in dual culture and coiling was observed (Fig. 1). We found that culture filtrates of *T. harzianum* T24 at a 1:10 dilution inhibited growth of *S. rolfsii* by 24.5±0.9 %. A combination of the pure chitinase (15.3±0.7% inhibition) and β-1,3-glucanase (5.5±0.4 % inhibition) from *T. harzianum* T24, used in the same ratio as they are present in the culture filtrate, inhibited growth of *S. rolfsii* by 20.0±0.8%. Partial inhibition of 7.0±0.6% was observed, when chitinase or β-1,3-glucanase activities had been removed from the culture filtrate by ultrafiltration (10-kDa MWCO).

Discussion

Previously, of 24 *Trichoderma* isolates, a strain identified as *T. harzianum* Rifai (T24) showed potential for biocontrol of various phytopathogenic fungi, including *S. rolfsii*. Inhibition of *S. rolfsii* correlated with both chitinase and β-1,3-glucanase activities in the culture filtrate of *T. harzianum* T24, suggesting the involvement of these enzymes in the biocontrol process (El-Katatny et al. 2000). In this study, the major chitinase and β-1,3-glucanase from T24 were purified and characterized to elucidate the role of these enzymes in the biocontrol process.

The molecular mass of the T24 chitinase of 43 kDa was similar to the molecular masses of other *T. harzianum* strains of 46 kDa (Lima et al. 1997), 40 kDa (Ulhoa and Peberdy 1992), and 42 kDa (Cruz et al. 1995; Haran et al. 1995), while other chitinases from *T. harzianum* showed molecular masses of 37 kDa and 33 kDa (Cruz et al. 1992) and 31, 33, and 52 kDa (Haran et al. 1995; Inbar and Chet 1995). The β-1,3-glucanase purified from *T. harzianum* T24 had a molecular mass of 74 kDa. Molecular masses of β-1,3-glucanases from other *T. harzianum* strains were in a similar range of 78 kDa (Lorito et al. 1994) and 66 kDa (Cruz et al. 1995), while a smaller 36-kDa endo-β-1,3-glucanase (Noronha and Ulhoa 1996) and a 31-kDa exo-β-1,3-glucanase have been isolated from *T. harzianum* (Kitamoto et al. 1987). The molecular masses of fungal β-1,3-glucanases appear to vary considerably, not only between organisms, but also within the same species (Pitson et al. 1993).

There are few data available in the literature about the stability (half-life) of chitinases and β-1,3-glucanases, and rather pH and temperature optima, which depend on the assay conditions, are reported. The pH optimum of the T24 chitinase of 4.5 was in agreement with the pH optima of 4.0 and 4.5 for previously described chitinolytic enzymes of *T. harzianum* strain P1 (Harman et al. 1993) and strain 39.1 (Ulhoa and Peberdy 1992), respectively. It was slightly higher than the pH optimum reported previously (Deane et al. 1998) for another *T. harzianum* strain, which was pH 3.5. Generally, most fungal chitinolytic enzymes have pH optima between 4.0 and 7.0, with the exception of those found in the microsomal fractions of *Candida albicans* (Dickinson et al. 1989) and cytosolic chitinolytic enzymes of *Saccharomyces cerevisiae* (Kuranda and Robbins 1991), which have pH optima of 8.0 and 2.5, respectively. The pH stability of the T24 chitinase was in agreement with data reported for other chitinolytic enzymes, which were stable between 4.0 and 8.0 pH (Cabib 1987). Another chitinase from *T. harzianum* was stable over a wide range of pH, maintaining over 80% and 50% of its activity from pH 2.0 to 5.5 and from pH 2.0 to 8.0, respectively (Deane et al. 1998). The optimal activity of fungal β-1,3-glucanases is usually measured in the range between pH 4.0 and pH 6.0, exceptions are enzymes from *Phytophthora infestans* and *Polyporus* species, which are most active around pH 7.0 (Pitson et al. 1993). The pH optimum of the T24 β-1,3-glucanase was similar to that found for endo-β-1,3-glucanases from a variety of organisms (Manners and Wilson 1974; Takahashi et al. 1978; Totani et al. 1983). The temperature optima of the T24 chitinase (40°C) and the T24 β-1,3-glucanase (60°C) were in agreement with those of other chitinases from other *T. harzianum* (Deane et al. 1998) and β-1,3-glucanases from *Bacillus circulans* (Aono et al. 1995).

Strong inhibition of both the *T. harzianum* T24 chitinase and the β-1,3-glucanase by Hg^{2+} suggests that sulfhydryl groups are involved in the catalytic reaction, as reported for chitinolytic enzymes from other sources (Lynn 1990; Yabuki et al. 1986) and for β-1,3-glucanases from *T. harzianum* (Kitamoto et al. 1987), *T. longibrachiatum* (Tangarone et al. 1989), and *R. solani* (Totsuka and Usui 1986). Interestingly, no inhibition was observed with 5 or 10 mM glucono-δ-lactone, which is known as an inhibitor of exo-β-1,3-glucanase at low concentration (Notario et al. 1976).

Little information is available in the literature on the kinetic properties of fungal chitinolytic enzymes. Previously, the K_m value of a *T. harzianum* chitinase for the soluble substrate chitotriose was calculated to be 0.53 g l^{-1} (Deane et al. 1998), while the T24 chitinase showed a K_m of 3.8 g l^{-1} on chitin. The K_m of the T24 β-1,3-glucanase (1.1 g l^{-1}) was lower than the K_m values (2.1 g l^{-1}) of the exo-β-1,3-glucanases from another *T. harzianum* strain (Kitamoto et al. 1987) and higher than those found for endo-β-1,3-glucanases from *Rhizopus arrizus* QM 1032 (0.31 g l^{-1}), *T. longibrachiatum* (0.02 g l^{-1}) and *Schizophyllum commune* ATCC 38548 (0.28 g l^{-1}) (Clark et al. 1978; Prokop et al. 1994; Tangarone et al. 1989). However, it was in agreement with the K_m value of another endo-β-1,3-glucanase (1.2 g l^{-1}) from *T. harzianum* (Noronha and Ulhoa 1996).

It has been suggested that constitutive carbohydrolases of *T. harzianum* release oligosaccharides from *R. solani* cell walls, which subsequently induce production of large amounts of cell wall-degrading enzymes, such

as the 42-kDa endochitinase ech42 by *T. harzianum* (Kullnig et al. 2000). Culture filtrates of *T. harzianum* T24 inhibited growth of *S. rolfsii* by 24.5%, which is in agreement with results for another *T. harzianum* previously found to be suppressive for the white-rot pathogen *S. cepivorum* (Papavizas et al. 1982) under the same experimental conditions (dilution of culture filtrate). In contrast, other authors suggested that *Trichoderma* culture filtrates had only marginally curtailed pathogen growth (Calistru et al. 1997), while metabolites of *Gliocladium virens* completely inhibited the growth of *S. cepivorum* (Jackson et al. 1991). However, both the spectrum of chitinases and β-1,3-glucanases produced and the ability to antagonize plant pathogens varies significantly among the *T. harzianum* species (El-Katatny et al. 2000; Ghisalberti et al. 1990).

The pure chitinase and β-1,3-glucanase from *T. harzianum* T24 inhibited the growth of *S. rolfsii* in an additive manner. Additive and synergistic effects of β-1,3-glucanases with chitinases against some phytopathogenic fungi have been found both for fungal and plant enzymes (Mauch et al. 1988). A synergistic inhibitory effect for chitinases, *N*-acetyl-β-glucosaminidases, and β-glucanolytic enzymes from *T. harzianum* P1 has been described (Lorito et al. 1994). These authors measured a ED_{50} value of 3.5 µg/ml for a combination of chitinase and β-1,3-glucanase from *T. harzianum* P1 in germ tube elongation of *Botrytis cinera*. This value is comparable to a ED_{50} value of 2.7 µg/ml that we measured for a combination of the chitinase and β-1,3-glucanase from *T. harzianum* T24 in the inhibition of *S. rolfsii*. With ED_{50} values in this range, enzyme preparations are approaching values of chemical fungicides (Lorito et al. 1994). Although biocontrol enzymes cannot compete yet with fungicides in terms of their production costs, the enzyme-based alternatives could become more economic once these systems have proven successful in field experiments and are produced in larger amounts.

Inhibition of *S. rolfsii* by chitinase or β-1,3-glucanase from *T. harzianum* T24 was lower than with the culture filtrate, and some partial inhibition was observed when chitinase or β-1,3-glucanase activities had been removed from the culture filtrate by ultrafiltration, indicating that antifungal metabolites other than enzymes also play an important role in antagonism. Previously, it has been shown that cell wall-degrading enzymes and peptaibol antibiotics were co-produced in liquid cultures of *T. harzianum*, indicative of the likely importance of synergism between hydrolytic enzymes and toxic compounds in the antagonistic action of *T. harzianum* against phytopathogenic fungi (Schirmböck et al. 1994). Similarly, a strong synergistic effect was observed on the inhibition of *Pythium* cyst germination by a combination of endo-β-1,3-glucanase and the fungicide Fongarid (Thrane et al. 1997).

In summary, we have shown that both the 43-kDa chitinase and the 74-kDa β-1,3-glucanase of the newly isolated *T. harzianum* T24 inhibit growth of *S. rolfsii*. A combination of these enzymes, which exhibited reasonable thermostability and low ED_{50} values against *S. rolfsii*, seems to offer a promising means of controlling this plant pathogen. Future investigations will focus on the roles of mycotoxic substances secreted by *T. harzianum* T24 and their role in the biocontrol process.

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