# Chitinolytic Enzymes Produced by *Trichoderma harzianum*: Antifungal Activity of Purified Endochitinase and Chitobiosidase

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## ABSTRACT

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Two chitinolytic enzymes from *Trichoderma harzianum* strain P1 were tested for their antifungal activity in bioassays against nine different fungal species. Spore germination (or cell replication) and germ tube elongation were inhibited for all chitin-containing fungi except *T. harzianum* strain P1. The degree of inhibition was proportional to the level of chitin in the cell wall of the target fungi. For most of the fungi tested, the $ED_{50}$ values for the endochitinase and the chitobiosidase were 35–135 $\mu g\ ml^{-1}$ and 62–180 $\mu g\ ml^{-1}$, respectively. Complete inhibition occurred at 200–300 $\mu g\ ml^{-1}$. Combining the two enzymes resulted in a synergistic increase of antifungal activity. The $ED_{50}$ values for a 1:1 mixture of endochitinase and chitobiosidase were as low as 10 $\mu g\ ml^{-1}$ for *Botrytis cinerea*, 34 $\mu g\ ml^{-1}$ for *Ustilago avenae*, 13 $\mu g\ ml^{-1}$ for *Uncinula necator*, and 30 $\mu g\ ml^{-1}$ for *Fusarium solani*. *T. harzianum* strain P1 was resistant to its own chitinolytic enzymes up to 800 $\mu g\ ml^{-1}$, with an $ED_{50}$ value >1,000 $\mu g\ ml^{-1}$. The chitinolytic enzymes from *T. harzianum* appeared to be biologically more active than enzymes from other sources and more effective against a wider range of fungi. The involvement of these chitinolytic enzymes in biocontrol is also discussed.

*Additional keywords:* biological control, fungitoxic compounds, mycoparasitism.

Fungi in the genus *Trichoderma* are among the most promising biocontrol agents against plant-pathogenic fungi. Specific strains have the ability to control a range of pathogens under a variety of environmental conditions (11,21). Moreover, they may be rhizosphere competent, which allows them to colonize and protect plant roots (1,11,27). Their biological activity can be increased by genetic manipulation (14,15,29).

In spite of extensive research on utilization of these fungi for biocontrol, the mechanisms by which they control plant-pathogenic fungi are not understood. Among the mechanisms proposed is mycoparasitism. It is presumed that this complex process requires the production of enzymes that digest the fungal cell wall (8). *Trichoderma* spp. are known to be efficient producers of polysaccharide lyases, proteases, and lipases, all of which may be involved in cell wall degradation (7,12). Among the enzymes most commonly suggested to be involved in mycoparasitism are those that degrade chitin. Recently, a few chitinolytic enzymes from *T. harzianum* Rifai were purified and characterized (12,32–34). The availability of purified chitinolytic enzymes from *Trichoderma* permits studies on their effects against other fungi. Several questions need to be answered. Are chitinolytic enzymes active in vitro, and, if so, how does their activity compare to that of similar enzymes from other sources? What is the spectrum of activity of these enzymes against plant-pathogenic and non-pathogenic fungi? Are combinations of chitinolytic enzymes more effective than single enzymes? Answers to these questions are important in understanding the role of these enzymes in biocontrol and in determining how best to use these proteins or the encoding gene(s) in pest management strategies.

This article compares the ability of two different types of chitinolytic enzymes from *T. harzianum* to inhibit spore germination and germ tube elongation of a variety of fungi. We also present evidence that these enzymes are more effective in combination than alone.

## MATERIALS AND METHODS

**Enzyme assays.** We used the nomenclature for chitinolytic enzymes proposed by Harman et al (12) and conducted similar detection and assay of enzymes. Briefly, *N*-acetyl-β-D-glucosaminidase (hereafter designated glucosaminidase) and chitin 1,4-β-chitobiosidase (hereafter designated chitobiosidase) activities were quantified by detecting the release of pigmented nitrophenol from *p*-nitrophenyl-β-D-*N*-acetylglucosaminide and *p*-nitrophenyl-β-D-*N,N'*-diacetylchitobiose (Sigma Chemical Co., St. Louis, MO), respectively. Endochitinase activity was measured by the reduction of turbidity of a suspension of colloidal chitin. Alternatively, endochitinase activity was assayed by the release of nitrophenol from *p*-nitrophenyl-β-D-*N,N',N''*-triacetylchitotriose (Sigma). Glucan 1,3-β-glucosidase activity was detected by measuring the release of reducing groups from a 0.1% solution of laminarin in 50 mM phosphate buffer, pH 6.7 (Sigma), as indicated by the reducing sugar assay described by Ashwell (3).

**Enzyme production and purification.** Production and purification of enzymes that degrade fungal cell walls were essentially as described by Harman et al (12). Strain P1 of *T. harzianum* (ATCC 74058) was grown for 4 days on a rotary shaker in Richard's modified medium, which contained 10 g of $KNO_3$, 5 g of $KH_2PO_4$, 2.5 g of $MgSO_4 \cdot 7H_2O$, 2 mg of $FeCl_3$, 1% (w/v) crab shell chitin (Sigma), 1% polyvinylpyrrolidone (Polyclar AT, GAF Corp., Wayne, NJ), 150 ml of V8 juice, and 1,000 ml of $H_2O$ at pH 6.0 (12). The biomass was removed by filtration,

the supernatant dialyzed against 50 mM potassium phosphate buffer (pH 6.7), and enzymes separated by gel filtration chromatography in a chromatography column packed with Sephacryl S-300 (Pharmacia LKB Biotechnology, Uppsala, Sweden), followed by chromatofocusing (12). A single protein with endochitinase activity was obtained. Purity was confirmed by using native and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) (PhastSystem, Pharmacia) (12) and by isoelectric focusing (IEF). The fractions containing chitobiosidase activity were further separated in a Rotofor IEF cell (Bio-Rad, Richmond, CA) (12). Peak fractions containing only chitobiosidase activity were collected, dialyzed against distilled water, and concentrated to dryness in a SpeedVac apparatus (Savant Instruments, Farmingdale, NY). PAGE, followed by staining with Coomassie blue, indicated a single protein band. PAGE, followed by fluorescent staining with 4-methylumbelliferyl-*S*-D-*N,N'*-diacetylchitobioside (Sigma) (12), indicated that this protein had chitobiosidase activity. The protein band exhibiting chitobiosidase activity was identical to the major protein band (40 kDa) reported by Harman et al (12).

Protein concentration in the enzyme preparations was determined using the Micro BCA protein assay (Pierce, Rockford, IL) with trypsin inhibitor from soybean (Sigma) as the standard protein. Enzyme solutions were kept at 4 C and utilized for the bioassays within 2 wk or dried in a SpeedVac apparatus and stored at −20 C until used.

**Microorganisms.** The following strains were tested: *Fusarium graminearum* Schwabe (14) and *Fusarium solani* (Mart.) Sacc. isolated from peas; *Botrytis cinerea* Pers.:Fr. strain 26 isolated from grapes; *T. harzianum* Rifai strain 1295-22 (ATCC 20847), an active biocontrol strain obtained by protoplast fusion (29) (hereafter designated as strain 22); *Pythium ultimum* Trow strain P4 described elsewhere (13); *Ustilago avenae* (Pers.) Rostr; *Uncinula necator* (Schwein.) Burrrill isolated from grapes; and a strain of *Saccharomyces cerevisiae* (Hansen). *T. harzianum* Rifai strain P1 (ATCC 74058), the strain used to produce the various enzymes, was also tested. It was derived from a strain isolated from wood chips and is able to control *B. cinerea* (31) and other fungi in vivo (G. Harman and G. Nash, *unpublished data*). Fungi were grown at 20–25 C on various media. Potato-dextrose agar (Difco Laboratories, Detroit, MI) was used to grow conidia of *Fusarium* spp., *T. harzianum* strains, and *B. cinerea*; sporangia of *P. ultimum* were produced in carrot medium (9) and harvested as described in Harman et al (14). Cells of *S. cerevisiae* were produced in potato-dextrose broth; sporidia of *U. avenae* were produced on the medium described in Köller and Wubben (16); and conidia of *U. necator* were produced on grapes grown aseptically in tissue culture. Cells or spores were suspended in water (0.4 osM mannitol for *U. necator*), filtered through sterile Kimwipes, if necessary to remove hyphal fragments, and adjusted to $10^5$–$10^6$ propagules per milliliter.

**Assay of antifungal activity.** All assays were performed under sterile conditions. Equal volumes of spore suspension, 3× potato-dextrose broth (the medium described by Köller and Wubben [16] was used for *U. avenae*), and enzyme test solution were mixed. The enzyme solution was replaced with sterile distilled water in control samples. In early experiments, these mixtures were applied to wells on sterile microscope slides prepared by heavy marks with a Dixon china marking pen and incubated in a humid chamber over 5% glycerol agar plates. In later experiments, a simplified procedure was used: test suspensions were transferred to a sterile Eppendorf tube and incubated at 20 C for *U. avenae* and at 25 C for the other fungi. For microscopic examination, samples were removed at 22–28 h for *Trichoderma* spp., *Fusarium* spp., *B. cinerea, P. ultimum*, and *U. necator*. The percentage of conidia (sporangia for *P. ultimum*) germinating was determined as the percentage of the first 100 spores randomly found on a microscope slide. In addition, the length of 20 germ tubes was measured and averaged. Abnormal mycelial growth and morphological anomalies such as branching, bursting, appearance of necrotic zones, and lysis of the hyphal tips were also noted and photographed. Furthermore, the effect of the enzyme preparations

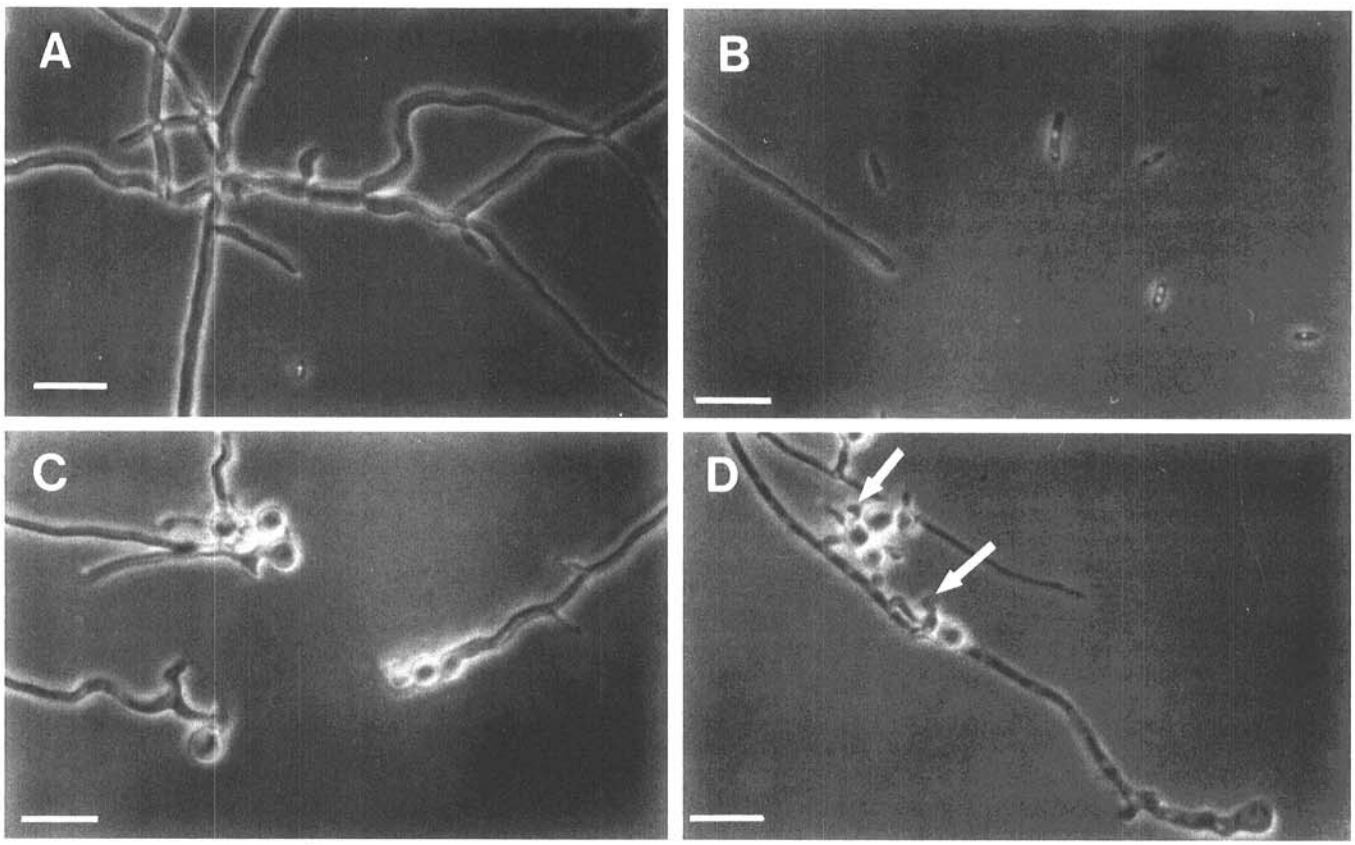


**Fig. 1.** Effect of 100–150 $\mu$g ml$^{-1}$ chitinolytic enzymes from *Trichoderma harzianum* strain P1 on spore germination and germ tube elongation of different fungi. **A,** *Fusarium solani* control. **B,** *F. solani* treated with endochitinase. **C,** *T. harzianum* strain 22 control. **D,** *T. harzianum* strain 22 treated with chitobiosidase. Arrows indicate swelling hyphal tips. Bars = 70 $\mu$m (A and B), 10 $\mu$m (C and D).

on very young mycelium was determined by delaying the addition of the enzyme solution until thalli began to form (30–40 h). For *U. avenae,* which forms monokaryotic, haploid sporidia that replicate in a yeastlike manner (19), and for *S. cerevisiae*, the effect of the enzymes on cell replication was determined after 48 and 24 h, respectively, by counting individual cells in a Petroff-Hausser counting chamber (Thomas Scientific, Philadelphia, PA). For the pure endochitinase obtained from chromatofocusing, an additional control sample containing Polybuffer (Pharmacia) was included in the bioassays. This excluded any interference in the biotests from Polybuffer residues.

**Analysis of data.** The results of each experiment are the average of three replicates, and every experiment was repeated at least twice. The standard deviations were calculated. The values obtained for the control were taken as 0% of inhibition, and all other values were divided by these values and multiplied by 100 to obtain percent of inhibition. To determine the $ED_{50}$ values, the dosage response curves were subjected to regression analysis by using a polynomial regression of third order, with $R^2$ ranging between 0.95 and 0.99. According to Richer (22), the following formula was used to determine an antifungal synergistic effect between the two chitinolytic enzymes: if synergism exists, $E_0(xA+yB) > E_0(x+y)A$, and $> E_0(x+y)B$, where $E_0$ is the percentage of inhibition, $A$ and $B$ are the two components, and $x$ and $y$ are the concentrations of each component in the mixture. The $E_0$ values were calculated by regression analysis of the dosage response curves.

## RESULTS

**Inhibition of spore germination (cell replication) and hyphal elongation by chitinolytic enzymes.** Spore germination (cell replication) of most of the fungi tested was inhibited by the endochitinases (Fig. 1A and B), with $ED_{50}$ values at enzyme concentrations of 35–135 $\mu g\ ml^{-1}$ (Table 1). Complete inhibition occurred at 200–300 $\mu g\ ml^{-1}$ (Fig. 2A). However, the $ED_{50}$ value for *S. cerevisiae* was 535 $\mu g\ ml^{-1}$, whereas the $ED_{50}$ value for *T. harzianum* strain P1 was >1,000 $\mu g\ ml^{-1}$ (Table 1). No inhibition occurred at a concentration as high as 800 $\mu g\ ml^{-1}$. Comparable values were obtained for inhibition of elongation of surviving germ tubes (Fig. 2B and Table 1). Sporangia and germ tubes of the Oomycete *P. ultimum* were unaffected by the endochitinase from *T. harzianum* at a concentration as high as 1,000 $\mu g\ ml^{-1}$ (Table 1).

The chitobiosidase preparation gave levels of inhibition comparable to, or slightly lower than, those obtained with the endochitinase (Table 1). Like the endochitinase, 200–300 $\mu g\ ml^{-1}$ of chitobiosidase produced complete inhibition of spore germination (cell replication) and germ tube elongation of *B. cinerea,*

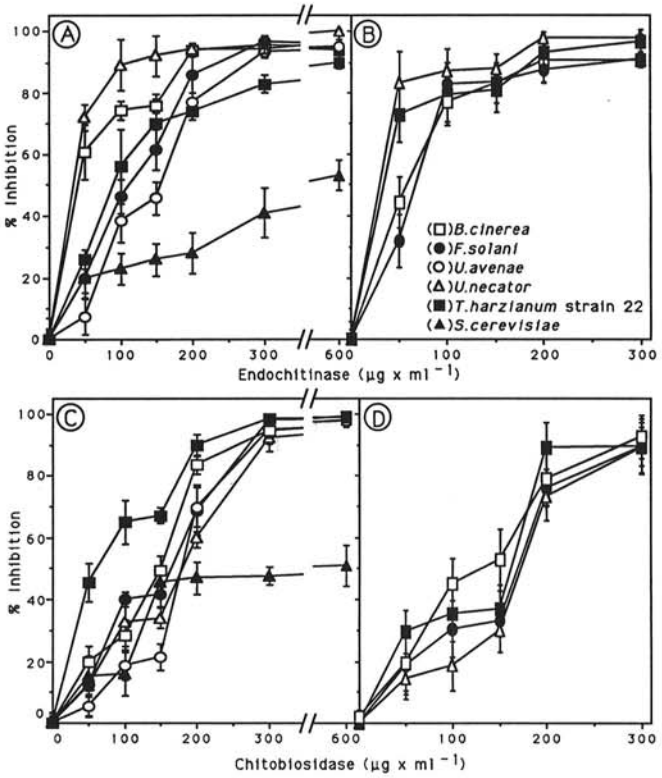


**Fig. 2.** Effect of an endochitinase (**A** and **B**) and a chitobiosidase (**C** and **D**) from *Trichoderma harzianum* strain P1 on different fungi. Dosage response curves for inhibition of spore germination (or cell replication) (**A** and **C**) or germ tube elongation (**B** and **D**) of target fungal species. Error bars indicate standard deviations.

TABLE 1. In vitro[a] inhibitory effect ($ED_{50}$) of chitinolytic enzymes from *Trichoderma harzianum* strain P1 on spore germination or cell replication and germ tube elongation of different fungi

| | $ED_{50}$ for enzymes ($\mu g \times ml^{-1}$)[b] | | | | | |
|---|---|---|---|---|---|---|
| | Chitobiosidase | | Endochitinase | | Chitob+endoc[c] | |
| Fungi | ge/re[d] | elong[e] | ge/re | elong | ge/re | elong |
| *Botrytis cinerea*[f] | 152[g] | 125 | 41 | 58 | 10 | 24 |
| *Fusarium solani*[f] | 165 | 168 | 110 | 67 | 30 | 28 |
| *Ustilago avenae*[h] | 179 | ... | 135 | ... | 34 | ... |
| *Uncinula necator*[i] | 180 | 173 | 35 | 30 | 13 | 10 |
| *Trichoderma harzianum* (22)[f] | 62 | 162 | 90 | 35 | ND[j] | ND[j] |
| *Saccharomyces cerevisiae*[k] | 490 | ... | 535 | ... | >400 | ... |
| *Fusarium graminearum*[f] | 125 | 132 | 100 | 70 | ND[j] | ND[j] |
| *Trichoderma harzianum* (P1)[f] | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |
| *Pythium ultimum*[l] | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 | >1,000 |

[a] Bioassay carried out by mixing equal parts of enzyme solution, spore (or cell) suspension of the test fungus, and the appropriate liquid medium (3×). Samples were incubated for 22–48 h at 20–25 C.
[b] The enzymes were purified from culture filtrate of *T. harzianum* strain P1 as described by Harman et al (12).
[c] 1:1 mixture of chitobiosidase and endochitinase.
[d] Spore germination or cell replication.
[e] Germ tube elongation.
[f] Conidia were obtained from cultures grown on potato-dextrose agar plates.
[g] $ED_{50}$ values obtained by regression analysis of the dosage response curves by using a polynomial regression of third order, with $R^2$ ranging between 0.95 and 0.99. Each regression curve consisted of six to seven concentrations of enzymes, and the inhibition value for each concentration was determinated in triplicate. A separate experiment was conducted to verify these results.
[h] Data represent $ED_{50}$ values for inhibition of sporidia replication. Sporidia produced in the medium described by Köller and Wubben (16).
[i] Conidia produced in tissue culture on grapes.
[j] Not determined.
[k] Data represent $ED_{50}$ values for inhibition of cell replication. Cells produced in potato-dextrose broth.
[l] Sporangia were produced from cultures grown in carrot medium (9).

*F. solani, F. graminearum, U. avenae, U. necator*, and *T. harzianum* strain 22 (Fig. 2C and D). Moreover, when the endochitinase and the chitobiosidase were combined in mixtures containing equal parts of each enzyme and compared with either enzyme alone at the same total concentration, a synergistic interaction between the two chitinolytic enzymes was detected. The enzyme combination resulted in a significant increase in antifungal activity against *B. cinerea, U. avenae, U. necator*, and *F. solani* (Fig. 3 and Table 1). For *B. cinerea,* the $E_0$ values (percentages of inhibition) in the Richer's formula (22) with $x = y = 10$ $\mu$g ml$^{-1}$ were: 25 for the endochitinase, 10 for the chitobiosidase, and 95 for the combination of the two enzymes. Similarly, $E_0$ values calculated for *U. avenae, U. necator*, and *F. solani* indicated the presence of synergism in all cases (data not shown). The combination of enzymes had very little effect against *T. harzianum* strain P1 and was ineffective against *P. ultimum* (Table 1).

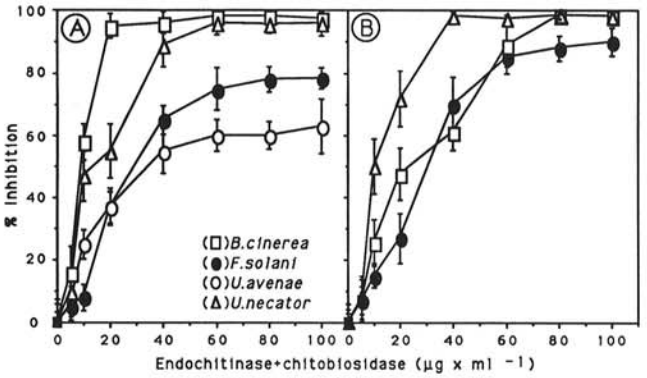


**Fig. 3.** Effect of a mixture (1:1) of an endochitinase and a chitobiosidase from *Trichoderma harzianum* strain P1, on **A,** spore germination and **B,** germ tube elongation of different fungi. Error bars indicate standard deviations.

**Morphological effects of chitinolytic enzymes.** A number of morphological changes were induced by *T. harzianum* strain P1 chitinolytic enzymes. The endochitinase produced a substantial lysis of germ tubes, spores, and mycelium of the target fungi (Fig. 4A). The chitobiosidase primarily gave rise to swollen rather than lysed spores and mycelium (Fig. 4B). In both cases, and when a mixture of the two enzymes was applied, inhibition was accompanied by hyphal distortion, heavy vacuolization and swelling, and lysis of the hyphae (Figs. 1D and 4B–D). With *F. graminearum*, adding low levels of endochitinase gave rise to premature phialogenesis. This particular strain sporulates very poorly in culture, with conidia appearing only after 10–14 days. However, when exposed to the endochitinase, a proliferation of phialides and conidia was produced in only 24–48 h.

Adding chitinolytic enzymes to the medium containing young, growing mycelium of *B. cinerea, Fusarium* spp., and *T. harzianum* strain 22 promptly reduced mycelial growth. This very fast reaction (<30 min) coincided with the appearance of necrotic zones and high mortality of the hyphae (Fig. 4D).

## DISCUSSION

*Trichoderma* spp. are among the most studied fungi for biocontrol of plant diseases. Chitinolytic enzymes have been implicated as factors contributing to the ability of *Trichoderma* spp. to act as biocontrol agents (2,7,23,32). However, definitive evidence is still lacking. Tests for loss of biocontrol ability in mutants deficient in chitinolytic enzyme production, obtained by gene(s) disruption, are needed for definitive proof. However, the isolation of sequences encoding for these enzymes from *Trichoderma* spp. has not been reported. We believe that this study

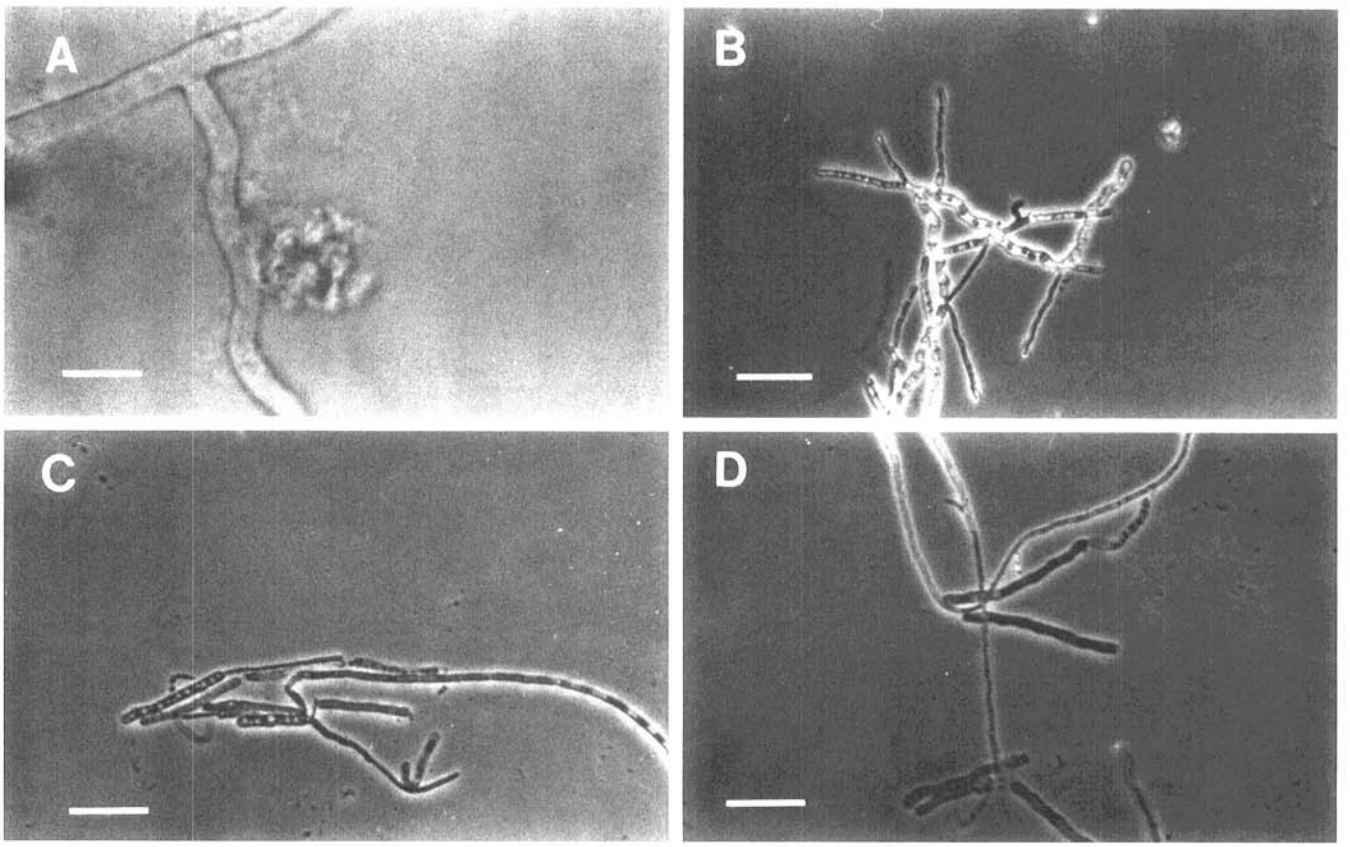


**Fig. 4.** Effects of chitinolytic enzymes from *Trichoderma harzianum* strain P1 on morphology of phytopathogenic fungi. **A,** mycelial lysis of *Botrytis cinerea* in presence of 50 $\mu$g ml$^{-1}$ endochitinase. **B,** swelling, distortion, and vacuolization of *Fusarium solani* hyphae grown in presence of 150 $\mu$g ml$^{-1}$ chitobiosidase. **C,** swelling, distortion, and vacuolization of *F. solani* hyphae grown in presence of 75 $\mu$g ml$^{-1}$ endochitinase + 75 $\mu$g ml$^{-1}$ chitobiosidase. **D,** morphological changes and necrosis on young mycelium of *F. solani* induced by 100 $\mu$g ml$^{-1}$ chitobiosidase. Bars = 12 $\mu$m (A), 100 $\mu$m (B, C, and D).

provided evidence for the involvement of chitinolytic enzymes in the biocontrol mechanism. An endochitinase, purified to homogeneity, strongly inhibited the growth and the development of all the chitin-containing fungi tested in vitro. A chitobiosidase showed similar capabilities, and a combination of the two enzymes resulted in a dramatic increase of the antifungal effect. Recently, another fungal endochitinase, purified from the closely related *Gliocladium virens*, showed antifungal activity against *B. cinerea,* although with lower efficacy (10). Therefore, it seems very likely that this effective and complex arrangement of antifungal enzymes is utilized by these fungi in the antagonistic or mycoparasitic processes.

Chitinolytic enzymes of *Trichoderma* did not inhibit the Oomycete *P. ultimum*. Neither sporangia germination nor mycelial growth was affected, even when the fungus was exposed to 1,000 $\mu g\ ml^{-1}$. This result was consistent with other reports (17,25) and was expected because *P. ultimum* does not contain chitin in its cell wall (4). The low sensitivity of *S. cerevisiae* to chitinolytic enzymes from *T. harzianum* (Fig. 2A and C) was also not surprising because the yeast cell wall contains a low level of chitin (4). Because the antifungal activity of the purified chitinolytic enzymes was roughly proportional to the quantity of chitin in the cell walls of test fungi, the ability of these proteins to inhibit fungal growth is probably a consequence of their chitinolytic activity, not of secondary toxic properties.

*T. harzianum* strain P1 produced high concentrations of a variety of chitinolytic enzymes in liquid culture (12). Clearly, this release of large amounts of enzymes that degrade cell walls must occur without significant damage to the producing fungus. We found that *T. harzianum* strain P1 was resistant to its own chitinolytic enzymes up to concentrations of 800 $\mu g/ml^{-1}$ ($ED_{50}$ >1,000 $\mu g/ml^{-1}$), whereas another strain of the same species was inhibited at 50 $\mu g/ml^{-1}$. Possibly, this finding could be explained by the fact that *T. harzianum* strain P1 produces an inhibitor of its own chitinolytic enzymes in liquid culture (12). However, another possibility is that a biochemical modification of the cell wall composition protects the strain against its own enzymes.

The chitinolytic enzymes from *T. harzianum* appear to be more effective than chitinolytic enzymes from plants or bacteria against plant-pathogenic fungi. Broekaert et al (5) tested the antifungal activity of endochitinases from tobacco, thorn apple, and wheat using a microscopic assay comparable to the one we used. *T. hamatum* and *Phycomyces blakesleeanus* were found to be sensitive at concentrations as low as 8 $\mu g\ ml^{-1}$ and 32 $\mu g\ ml^{-1}$, respectively. However, spore germination and hyphal growth of *B. cinerea* were unaffected even at 320 $\mu g\ ml^{-1}$. Similarly, an endochitinase isolated from *Arabidopsis thaliana* was an efficient inhibitor of *T. reesei* but was inactive against several other chitin-containing fungi (35). A treatment of 400 $\mu g\ ml^{-1}$ of a bean endochitinase was necessary to significantly inhibit *Rhizoctonia solani* (6). In our tests, another Basidiomycete (*U. avenae*), which may have cell-wall chemistry similar to that of *R. solani*, showed an $ED_{50}$ value for *T. harzianum* endochitinase of 135 $\mu g\ ml^{-1}$. An endochitinase from pea was tested against 18 species of fungi, including *F. solani* and *B. cinerea* (17). This enzyme was ineffective at concentrations up to 250 $\mu g\ ml^{-1}$ against all of the fungal strains except a very sensitive strain of *T. viride*. The authors also reported that the combination of endochitinase and $\beta$-1,3-glucanase from pea was required for significant antifungal activity; the fungitoxic effect appeared to be due to lysis of the hyphal tips (17). In our observations, the thallus (Fig. 4A) and the spore cell walls, as well as the hyphal tips, were lysed by *T. harzianum* enzymes. This evidence indicates that differences between *Trichoderma* chitinolytic enzymes and plant enzymes may include sites of action influencing the biological efficacy. Bacterial chitinolytic enzymes may also have a role in biocontrol (20,28,30). Transgenic bacterial strains with enhanced biocontrol ability in vivo were obtained, but they were effective against only one of the pathogens tested (30) or effective exclusively at high temperatures (26). Furthermore, in a comparative study, bacterial chitinolytic enzymes purified from *Serratia marcescens, Pseudomonas stutzeri*, and *Streptomyces griseus* at 50 $\mu g\ ml^{-1}$ were ineffective against *T. reesei* and *P. blakesleenus* (24). Thus, the enzymes from *T. harzianum* seem to be more active than chitinolytic enzymes from other sources, and they affect a wider range of target fungi. From our results, the $ED_{50}$ values for the combined enzymes from *T. harzianum* appear to be at least one order of magnitude lower than corresponding values for chitinolytic enzymes from other sources.

Chitobiosidase from *T. harzianum* strain P1 inhibited growth and spore germination of all the chitin-containing fungi tested (except for the producing strain), even though the concentration was higher than that of the endochitinase. However, combining endochitinase and chitobiosidase resulted in greater inhibition than that of either enzyme used singly. Synergism was assessed using the formula proposed by Richer (22) where the $E_0$ value corresponds to percentage of inhibition. For several fungal species, including *B. cinerea* and *F. solani*, the percentage of inhibition of the combination enzyme mixture was higher than the percentage of inhibition of the single enzyme solutions, showing a significant synergistic interaction between the two chitinolytic enzymes. Similarly, a mixture of chitinolytic enzymes and $\beta$-1,3-glucanases from higher plants was significantly more effective against several phytopathogenic fungi than either of the classes of enzymes used singly (17,24). These results indicate that mixtures of hydrolytic enzymes with complementary modes of action may be required for maximum efficacy, and that correct combinations of enzymes may increase in vitro antifungal activity and approach the toxicity level of some chemical pesticides. Moreover, recent findings in the related species *Gliocladium virens* suggest that hydrolytic enzymes may also act synergistically with fungitoxic metabolites (10).

Chitinolytic enzymes, and the genes encoding them, may be useful for the production of transgenic microorganisms with superior biocontrol capabilities and of transgenic plants with high resistance to plant-pathogenic fungi. The employment of plant genes coding for chitinolytic enzymes to improve pest resistance in transgenic plants has been suggested (24) and recently performed with contrasting results. Transgenic tobacco and canola seedlings were more resistant to *R. solani* than were the control plants (6). In *Nicotiana sylvestris*, however, high levels of chitinases did not increase the resistance to *Cercospora nicotiana* (18). This did not demonstrate that transgenic proteins were enzymatically active, but only that they were serologically related to chitinases (18). Genes from *T. harzianum* coding for chitinolytic enzymes appear to be very attractive for this purpose; their products are highly active in vitro against a wide range of phytopathogenic fungi. Because these enzymes also act synergistically, maximum activity will depend on their correct combination. Once the encoding sequences are isolated, they may be combined under the control of a strong promoter(s) and used to increase the efficacy of defense mechanisms in plants, or they can add new biocontrol capabilities to microbial biocontrol strains that are not chitinolytic enzyme producers. If the results presented in this study can be confirmed in vivo, *T. harzianum* could become a valuable source of biocontrol genes and gene products for the control of chitinous plant pathogens.

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