Endochitinase from *Gliocladium virens*: Isolation, Characterization, and Synergistic Antifungal Activity in Combination with Gliotoxin

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ABSTRACT

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The culture filtrate from the biocontrol agent *Gliocladium virens* strain 41 grown in chitin-containing medium was strongly inhibitory to mycelial growth of different plant-pathogenic fungi. The antibiotic gliotoxin was isolated from the culture liquid. The culture filtrate also contained different types of chitinolytic enzyme activities, including endochitinase, chitin 1,4-β-chitobiosidase, and glucan N-acetyl-β-D-glucosaminidase, as well as glucan 1,3-β-glucosidase activity. An endochitinase was purified to homogeneity. The enzyme had a molecular weight of approximately 41,000 Da and a pI of 7.8. The optimal range for enzyme activity was pH 4–6. The inhibitory effect of pure endochitinase and gliotoxin on the germination of conidia and germ tube elongation of *Botrytis cinerea* was tested in vitro. When applied alone at concentrations of 150 μg ml^{-1}, the endo-

chitinase inhibited spore germination of the test fungus and caused cell wall damage, resulting in bursting of hyphal tips. The ED$_{50}$ value of gliotoxin was 1.25 μg ml^{-1}. When gliotoxin and the endochitinase were applied together, a synergistic inhibitory effect was observed. Addition of 25 or 50 μg ml^{-1} of endochitinase reduced the ED$_{50}$ of gliotoxin to 0.75 μg ml^{-1} and 0.5 μg ml^{-1}, respectively. These enzyme concentrations applied alone showed no inhibitory effect. Furthermore, applied individually, 0.75 μg ml^{-1} of gliotoxin and 75 μg ml^{-1} of endochitinase caused no inhibition and 20% inhibition, respectively, whereas the combined application resulted in 95% inhibition. Synergistic antifungal activity of endochitinase and gliotoxin may play a role in biological control by *G. virens*.

Additional keywords: fungitoxic compounds, mycoparasitism.

Gliocladium virens J. H. Miller, J. E. Giddens, & A. A. Foster is one of the most promising and studied fungal biocontrol agents. It has been successfully used against a wide range of plant pathogens, including *Pythium ultimum* (8), *Rhizoctonia solani* (8,9,14), and *Phytophthora cactorum* (25). Production of antibiotic metabolites, such as gliotoxin and gliovirin, by *G. virens* has been investigated extensively (9–11,15,30,31) and has been reported as the main biocontrol mechanism (9,10,15). Mycoparasitism, involving production of cell wall degrading enzymes, has been proposed as an alternative mode of action (8,10) but has received considerably less attention. High levels of chitinolytic and β-1,3-glucanolytic activities in culture filtrates of *G. virens* (21) and *Gliocladium roseum* (20) have been reported, but there have been no reports on the purification of chitinolytic enzymes or β-1,3-glucanases from *Gliocladium*. In this article, we report the purification and characterization of an endochitinase from a biocontrol strain of *G. virens*. In addition, the in vitro antifungal activity of the pure endochitinase and its synergistic effect in combination with gliotoxin are described.

MATERIALS AND METHODS

Fungal strains and culture condition. *G. virens* strain 41 (ATCC 20906) was originally isolated from an *Aphanomyces*-suppressive soil at Mt. Morris, NY. This strain showed good biocontrol ability against *P. cactorum*, the cause of root and crown rot of apple (25). It also showed activity against *Phytophthora* diseases on other crops and a range of other plant-pathogenic fungi (G. E.

Harman and W. F. Wilcox, *unpublished data*). Conidia of the fungus were stored in 30% glycerol at −20 C and used to inoculate potato-dextrose agar (PDA) plates (Difco Laboratories, Detroit, MI). For enzyme production, the fungus was grown in 250-ml Erlenmeyer flasks containing 100 ml of a synthetic medium (SMCS) with colloidal chitin and sucrose as carbon sources. SMCS contained 680 mg of KH$_2$PO$_4$, 870 mg of K$_2$HPO$_4$, 200 mg of KCl, 1 g of NH$_4$NO$_3$, 200 mg of CaCl$_2$, 200 mg of MgSO$_4$·7 H$_2$O, 2 mg of FeSO$_4$, 2 mg of ZnSO$_4$, 2 mg of MnSO$_4$, 42 g of moist purified colloidal chitin (29), and 5 g of sucrose in 1 L of distilled water, final pH 6. The flasks were inoculated with conidia collected from freshly grown PDA plates. The medium, with 10^7 conidia per milliliter, was incubated at 25 C for 5 days on a rotary shaker at 200 rpm. The culture filtrate was harvested as described elsewhere (5). For the inhibition assay on plates, as well as for production and isolation of gliotoxin, *G. virens* was grown either in SMCS or in 1% malt extract broth (Difco). Cultures were inoculated, incubated, and harvested under the same conditions as described above, except that incubation time was 7 days. For the inhibition assay on petri plates, *Phytophthora megasperma*, *P. ultimum*, *Rhizoctonia solani*, and *Fusarium solani* were grown on PDA. For the assay of antifungal activity, *Botrytis cinerea* strain 26 (isolated from grapes and kindly supplied by R. Pearson, Cornell University) was grown on PDA at 25 C. After 7 days, conidia were collected, suspended in sterile water containing 30% glycerol to a density of 10^5 conidia per milliliter, and stored at −20 C until use.

Inhibition assay on plates and isolation of gliotoxin. For determination of production of fungitoxic compounds by *G. virens* strain 41, an inhibition assay on plates against different fungi was carried out as described earlier (4). Briefly, culture filtrate

from *G. virens* was collected, passed through a 0.45-μm filter to sterilize the solution, mixed with malt extract agar at 60 C, and poured into petri dishes. Control plates contained culture medium that had not been inoculated with *G. virens*. The plates were allowed to solidify and then were inoculated with different test fungi. Radial growth was recorded daily and results were expressed as percentage of growth inhibition compared with that on the control plates. The fungitoxic metabolite present in the culture filtrate of *G. virens* was isolated using a bioassay on thin-layer chromatography (TLC) plates (4). Briefly, culture filtrates were extracted with ethyl acetate, and the extract was concentrated and applied to TLC plates. Plates were developed in methylene chloride and methanol (95:5) and air-dried. Subsequently, the plates were sprayed with V8 (20% v/v) and glucose solution (0.5% w/v) containing sporangia of *P. ultimum* and incubated in a moist chamber for 2 days at 20 C. Inhibition zones on the plates, corresponding to fungitoxic metabolites, were visualized by immersing the plates in a 4% carbon suspension, resulting in black staining of the mycelium and white spots where mycelial growth was absent. Gliotoxin (Sigma Chemical Co., St. Louis, MO) was run as a standard on the same plate. The metabolite causing the inhibition zones was visible under UV light at 254 nm. The silica gel containing the compound of interest was scraped from the plates, and the metabolite was dissolved in ethyl acetate. Final identification of gliotoxin (kindly performed by C. R. Howell, USDA, Agricultural Research Service, College Station, TX) was carried out by comparative high-performance liquid chromatography analysis with pure gliotoxin.

Enzyme assays. The nomenclature for chitinolytic enzymes proposed by Harman et al (5) was used throughout this work. Activities of *N*-acetyl-β-D-glucosaminidase (hereafter designated as glucosaminidase), chitin 1,4-β-chitobiosidase (hereafter designated as chitobiosidase), and endochitinase were assayed as described elsewhere (5). Briefly, glucosaminidase and chitobiosidase activities were determined by measuring the release of nitrophenol from *p*-nitrophenyl-β-D-*N*-acetylglucosaminide and *p*-nitrophenyl-β-D-*N,N'*-diacetylchitobiose, respectively (5,22). Absorption was measured at 410 nm. One nkatal corresponds to the release of 1 nmol nitrophenol per second, under the conditions given above. Endochitinase activity was measured at 510 nm as the reduction of turbidity (3,5) of a 1% suspension of moist purified colloidal chitin (29). The suspension was prepared in 100 mM sodium acetate buffer, pH 5.5, after 24 h of incubation at 30 C. For calculation of specific activity, one unit was defined as the amount of enzyme required to obtain 5% turbidity reduction under the above conditions. Glucan 1,3-β-glucosidase activity was determined by measuring the amount of reducing sugar released from laminarin (Sigma). The standard assay contained 250 μl of enzyme solution and 250 μl of a 0.1% laminarin solution in 50 mM potassium phosphate buffer, pH 6.7. After incubation at 30 C for 6 h, the reducing sugar content was determined using the method of Ashwell (1) modified according to Hayes (6). Appropriate controls, without enzyme or substrate, were run simultaneously. The quantity of reducing sugar was calculated from the glucose standards included in the assay. One nkatal corresponds to the release of 1 nmol glucose-equivalent per second, under the conditions given above.

Enzyme purification. Enzyme purification procedures were similar to those described for a *Trichoderma* endochitinase (5). The culture filtrate was transferred into dialysis tubing (6,000–8,000 Da cut-off) and concentrated 30- to 40-fold by placing the tubing in solid polyethylene glycol (35,000 M_r; Fluka Chemika-Biochemika, Buchs, Switzerland). The concentrate was dialyzed overnight against 50 mM potassium phosphate buffer, pH 6.7 (5 L of buffer per liter of culture filtrate), and applied to a gel-filtration column (5 × 60 cm) containing Sephacryl S-300 (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with 50 mM potassium phosphate buffer, pH 6.7, containing 200 mM NaCl. Fractions were eluted with the same buffer using reverse gravitational flow at 140 ml h^{-1}. Fractions containing activity of the enzyme of interest were pooled as described in the results. Each pooled set was concentrated as described above and dialyzed

overnight against a 20-fold volume of 25 mM ethanolamine-HCl buffer, pH 8.7. The sample was applied to a chromatofocusing column (1 × 30 cm) packed with PBE 94 (Pharmacia LKB) and equilibrated with the same buffer used for dialysis. The column was eluted at a flow rate of 50 ml h^{-1} with Polybuffer 96 (Pharmacia LKB) diluted 1:10 and adjusted to pH 7.0 with HCl according to the manufacturer's directions. Fractions containing endochitinase activity were dialyzed first against a 20-fold volume of 1 M NaCl and then against a 40-fold volume of distilled water to remove Polybuffer. They were concentrated to a volume of 2 ml in a colloidion bag system (10,000 Da; UH 100/1, Schleicher & Schuell Inc., Keene, NH). A Rotofor isoelectric focusing (IEF) cell (Bio-Rad, Richmond, CA) was loaded with 35 ml of distilled water containing 2% Bio-Lyte ampholytes, pH 3–10 (Bio-Rad), and run at 12 W constant power at 4 C. After a 1-h prefocusing run, the sample was applied to the Rotofor cell compartments 15 and 16 (pH 8.0–8.5), and the run was continued under the same conditions for 5 h. The fractions were collected and assayed for endochitinase activity. The active fractions were pooled, dialyzed against 1 M NaCl and distilled water as described above, and concentrated to dryness in a SpeedVac apparatus (Savant Instruments, Farmingdale, NY). The enzyme was stored at −20 C and reconstituted in an appropriate volume of sterile distilled water for use.

Polyacrylamide gel electrophoresis and protein determination. Polyacrylamide gel electrophoresis (PAGE) was employed to assess purity of enzyme preparations and to partially characterize the purified proteins. Electrophoresis was performed in a Phast-System (Pharmacia LKB) according to the manufacturer's directions. SDS (12) and native PAGE were conducted using 20% discontinuous homogeneous gels (Pharmacia LKB). IEF gels had a range of pH 3–9. Gels were stained either with Coomassie blue, following the standard protocols supplied with the PhastSystem, or with AgNO₃, following the procedure of Oakley (18) as modified by Hayes (6). SDS-PAGE and IEF used the protein standards provided by Sigma and Pharmacia, respectively. Molecular weight and pI of the *Gliocladium* endochitinase were estimated either from a regression equation of the log of molecular weight of the standard proteins versus distance migrated or from a regression of pI of standard proteins versus distance migrated. Endochitinase activity of the protein bands in the native and IEF gels was visualized by overlaying the gels immediately after the run with a 0.03% 4-methylumbelliferyl-β-D-*N,N'N''*-triacetylchitotriose (Sigma) solution in 100 mM sodium acetate buffer, pH 5.5, containing 1% low-melting-point agarose (FMC Bio Products, Rockland, ME). Fluorescent activity bands were detected at 254 nm (5). Quantitative protein determination was performed using the micro BCA assay (Pierce, Rockford, IL) according to manufacturer's directions, with soybean trypsin inhibitor (Sigma) as a protein standard.

Enzyme characterization. Endochitinase activity, as a function of pH, was determined in a 50 mM citric acid and K₃PO₄ buffer mixture at pH 2.7–8.5 using the turbidity reduction assay described above. The effect of temperature on endochitinase activity was tested in 100 mM sodium acetate buffer, pH 5.5, at 20–60 C. Amino acid composition was determined at the analytical and synthesis facility of the Cornell biotechnology program, using the picotag amino acid analysis procedure of Heinrikson and Meredith (7). Affinity of the pure *G. virens* endochitinase for a polyclonal antibody produced against a pure *Trichoderma harzianum* endochitinase (5) was determined using enzyme-linked immunosorbent assay (6) for native protein and endochitinase denatured with 4 M urea.

Assay for antifungal activity. A microscopic bioassay described elsewhere (13) was used to determine antifungal activity of the purified *G. virens* endochitinase, alone and in combination with gliotoxin. Briefly, 30 μl of a conidial suspension of *B. cinerea* was aliquoted into sterile Eppendorf tubes. Ten microliters of a 6× PDB solution and 20 μl of sterile distilled water containing the pure endochitinase and/or gliotoxin (Sigma) at 3× the desired final concentration were added and mixed. The tubes were incubated at 25 C. The final solution was pH 5. For some exper-

iments the pH was adjusted to 6.7 by adding 6 μl of 500 mM sterile potassium phosphate buffer to provide a final concentration of 50 mM potassium phosphate buffer. The control tubes contained distilled water or potassium phosphate buffer instead of the enzyme-toxin solution. After 24 h, three 20-μl samples from each tube were transferred to a microscope slide and analyzed under a phase-contrast light microscope (Balplan, Bausch & Lomb, Rochester, NY). Percent of spore germination (the first 100 conidia randomly considered) was determined and compared with that of the control. Experiments were conducted at least twice.

RESULTS

Production of gliotoxin by G. virens strain 41 in liquid culture. The culture filtrate of *G. virens* strain 41 grown in SMCS or in 1% malt extract broth was strongly inhibitory against *P. ultimum, P. megasperma, B. cinerea,* and *R. solani* when incorporated in 0.5% malt extract agar plates (data not shown). Extraction of the filtrate with ethyl acetate and analysis of the extract with a bioassay on TLC plates showed the presence of only one metabolite inhibitory to *P. ultimum*. Comparison of the inhibition zones on TLC plates to those caused by pure gliotoxin suggested that the fungitoxic compound in the culture filtrate was gliotoxin. This was confirmed by recovering the active band from the silica gel, purifying the compound by recrystallization in hexane, and comparing high-performance liquid chromatography analysis with pure gliotoxin. The quantity of gliotoxin in the SMCS culture filtrate was 5–10 mg L^{-1}, assessed by size comparison of the spots on the TLC plates to known quantities of pure gliotoxin.

Production and separation of chitinolytic enzymes and glucan 1,3-β-glucosidases by G. virens in liquid culture. Production of extracellular chitinolytic enzymes and glucan 1,3-β-glucosidases by *G. virens* was determined after 5-days growth in chitin-containing SMCS medium. The crude culture filtrate contained endochitinase, chitobiosidase, and glucosaminidase activities, as well as glucan 1,3-β-glucosidase (laminarinase) activity. The filtrate was concentrated and dialyzed against 50 mM potassium phosphate buffer, applied to a gel-filtration column, and eluted with 1,500 ml of 50 mM potassium phosphate buffer containing 200 mM NaCl. A first peak between fractions 70 and 120 contained high levels of chitobiosidase and glucosaminidase activities (Fig. 1A). A second peak with endochitinase, glucan 1,3-β-glucosidase, and chitobiosidase activities was detected in fractions 120–140. Fractions 140–160 contained endochitinase activity. Proteins in this region were apparently not separated on the basis of molecular weight but adsorbed to the gel matrix because they eluted at greater than the total column volume. The fractions 140–160 showing only endochitinase activity (Fig. 1A) were pooled and subjected to further purification.

Purification of G. virens endochitinase. The fractions containing endochitinase activity were concentrated, dialyzed, and applied to a chromatofocusing column. A sharp peak at pH 8 containing endochitinase activity was detected in the eluted fractions (Fig.
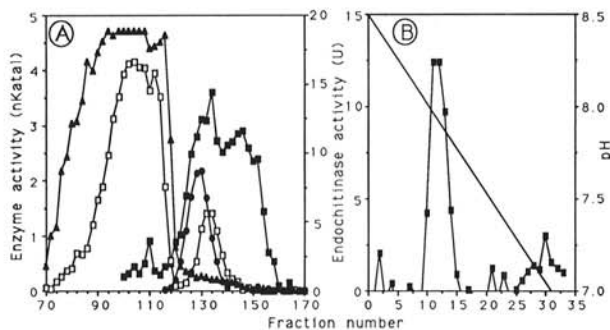
1B). The peak fractions were pooled, dialyzed to remove Poly-buffer, and applied to a Rotofor cell after a 1-h prefocusing run with an ampholyte solution, pH 3–9. After 5 h, the fractions were recovered and endochitinase activity was determined. The peak fractions contained homogeneous endochitinase as shown by the presence of a single protein band on SDS-PAGE (Fig. 2) and on native PAGE (not shown). A single fluorescent activity band, observed following overlay of the native gel with the methyl-umbelliferyl substrate, corresponded to the position of the single protein band detected with the Coomassie blue or AgNO$_3$ stains.

The results of each purification step are summarized in Table 1. The endochitinase was purified 105-fold with a recovery of 8%. The quantity of endochitinase produced in the original culture filtrate was calculated to be at least 10 mg L^{-1}.

Characterization of the endochitinase. The purified endochitinase from *G. virens* consists of a single protein with a molecular weight of approximately 41,000 and a pI of 7.8 as calculated from migration during SDS-PAGE (Fig. 2) and IEF, respectively.
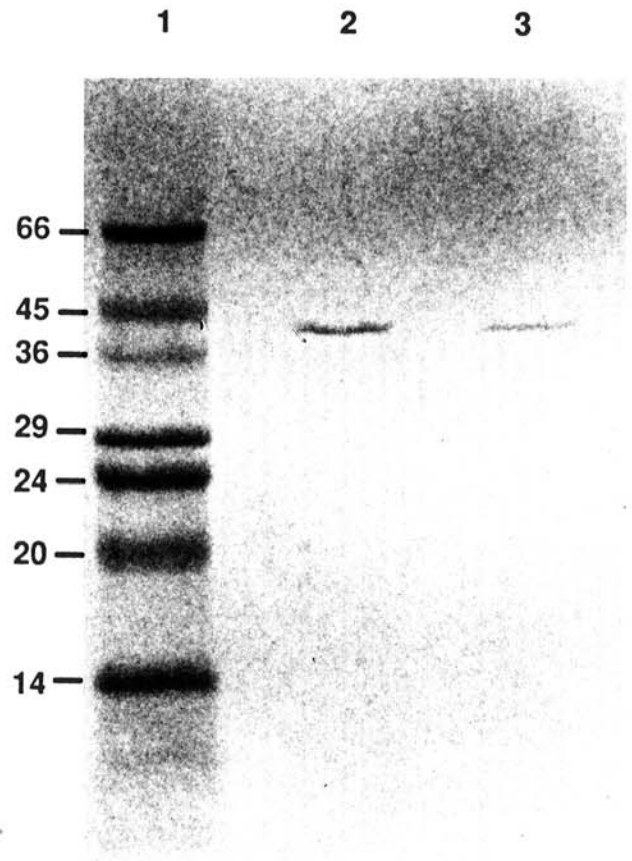

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified endochitinase from *Gliocladium virens* using a 20% polyacrylamide concentration. Coomassie blue stain. Lanes 1 and 2, purified endochitinase, 1 and 2 μg of protein, respectively. Lane 3, standard molecular weight proteins.

Fig. 1. **A,** Gel-filtration chromatography on Sephacryl S-300 of dialyzed culture filtrate from *Gliocladium virens* grown in chitin-containing medium. **B,** Chromatofocusing on PBE 94 of endochitinase from the same strain. ■ = Endochitinase, □ = chitobiosidase, ▲ = glucosaminidase, and ● = 10 × glucan 1,3-β-glucosidase activities.

TABLE 1. Purification of *Gliocladium virens* endochitinase from 1 L of culture filtrate[a]

Step	Total protein (mg)	Enzyme activity (units)[b]	Specific activity (units mg^{-1})	Purification (fold)	Yield (%)
Crude filtrate	1,065.0[c]	10,400	9.7	1.0	100
Dialysis	192.0	9,066	47.2	4.8	87
Sephacryl S-300	7.7	2,849	371.0	38.2	27
Chromatofocusing	3.2	1,984	620.3	63.9	19
Rotofor cell	0.8	815	1,018.5	105.0	8

[a] Fungus grown in chitin-containing synthetic medium for 5 days at 25 C.
[b] One unit = amount of enzyme required for 5% turbidity reduction.
[c] Data from one representative purification run.

The enzyme was active in a citric acid and K_3PO_4 buffer over pH 3.5–7.0. In the pH 4.0–6.0 range, the endochitinase showed 90–100% activity, with a maximum at pH 4.5. The optimum temperature for endochitinase activity at pH 5.5 was 30–37 C, and the activity dropped sharply at temperatures above 40 C, possibly due to heat inactivation of the enzyme. The specific activity of the endochitinase at 30 C was 1 unit per microgram under the assay conditions used. The denatured *G. virens* endochitinase showed a strong affinity to a polyclonal antibody produced against the *T. harzianum* endochitinase (5), whereas the native protein had a significantly lower affinity.

Antifungal activity of the endochitinase alone and in combination with gliotoxin. Germination of conidia of the test fungus *B. cinerea* was inhibited by the *G. virens* endochitinase at concentrations greater than 75 µg ml^{-1}. Adding 200 µg ml^{-1} of endochitinase resulted in approximately 50% inhibition of spore germination, at both pH 7.6 and 5. Moreover, bursting of spores or hyphal tips of *B. cinerea* was observed under the light microscope (Fig. 3). Application of gliotoxin inhibited germination and heavy hyphal vacuolization of the target fungus. There was an ED_{50} of 1.25 µg ml^{-1} for spore germination. When the endochitinase and gliotoxin were applied together, even low concentrations of both components were sufficient to completely inhibit spore germination of *B. cinerea* (Fig. 4). Applied alone, 0.75 µg ml^{-1} of gliotoxin or 75 µg ml^{-1} of endochitinase had no or only a very low inhibitory effect. Their combination resulted in 95% inhibition of spore germination. Even as low an amount of endochitinase as 25 µg ml^{-1} increased the effect of 1 µg of gliotoxin per milliliter from lower than 20 to over 80% inhibition. On the other hand, 100 µg of endochitinase per milliliter dramatically increased the inhibitory activity of 0.5 µg of gliotoxin per milliliter from 0 to 90% (Fig. 4).

DISCUSSION

G. virens produced different types of chitinolytic enzymes and glucan 1,3-β-glucosidases in chitin-containing medium. Types of chitinolytic enzymes produced included an endochitinase and an unknown number of glucosaminidases and chitobiosidases. The variety of chitinolytic enzymes produced indicates a complex functional and regulatory pattern for these enzymes. Multiple functions of these enzymes may include release of nutrients from chitinous substrates, a direct role in mycoparasitism and competition, and regulatory functions in the fungal growth process (2). A similar diversified profile of chitinolytic enzymes has been observed in the closely related genus *Trichoderma* (5).

An endochitinase was purified to homogeneity from the culture filtrate of *G. virens*. The M_r of the enzyme was approximately 41,000, which is similar to that of a chitinolytic enzyme from *T. harzianum* (40,000) (28) and of an endochitinase from another strain of *T. harzianum* (40,000) (5). However, the *G. virens* endochitinase has a pI of 7.8, which was considerably higher than

that of the *T. harzianum* endochitinase (5). The optimum range for enzyme activity was pH 4–6, which is comparable to those of other fungal and plant chitinases (5,16,28). The endochitinase from *G. virens* is similar to that from *T. harzianum* as indicated by the strong affinity of the *G. virens* enzyme to a polyclonal antibody raised against the *T. harzianum* chitinase (5). However, the *G. virens* endochitinase was less active against *B. cinerea* than the endochitinase from *T. harzianum* (13).

Interestingly, glucan 1,3-β-glucosidases were produced concurrently with the chitinolytic enzymes, even though the growth medium contained no β-1,3-glucan as carbon source. There has been evidence that, in the closely related genus *Trichoderma*, glucan 1,3-β-glucosidases are produced constitutively, even in the absence of substrate (26). Alternatively, a combined induction of the two classes of enzymes may occur in fungi as it does in plants (16). Since the substrates of both enzyme classes frequently occur together in nature (e.g., in fungal cell walls), a concurrent induction of both enzymes would be of biological relevance. Moreover, there has been evidence of an interactive antifungal effect of glucan 1,3-β-glucosidases and chitinolytic enzymes in plants (17), and the same may be true for fungal lytic enzymes.

The role of the *Gliocladium* spp. and *Trichoderma* spp. lytic enzymes in mycoparasitism has been frequently suggested (20,24). *B. cinerea*, the test fungus used in our bioassay, has been reported previously to be parasitized by *Gliocladium catenulatum* (23). Our data demonstrate that a single purified endochitinase from *G. virens* inhibited spore germination of *B. cinerea* in vitro at concentrations higher than 100 µg ml^{-1}. The inhibitory activity of the *Gliocladium* endochitinase applied alone was significantly lower than that of a purified *T. harzianum* endochitinase (13). However, at concentrations of 150 µg of enzyme per milliliter, the *G. virens* endochitinase caused bursting of spores and hyphal tips of *B. cinerea*. Similar effects have been observed on *Sclerotium rolfsii* with a chitinase from *Serratia marcescens* (19). These results indicate that different microbial chitinolytic enzymes have different biocontrol mechanisms affecting the cell walls of target fungi. The *G. virens* endochitinase seems to weaken the cell wall
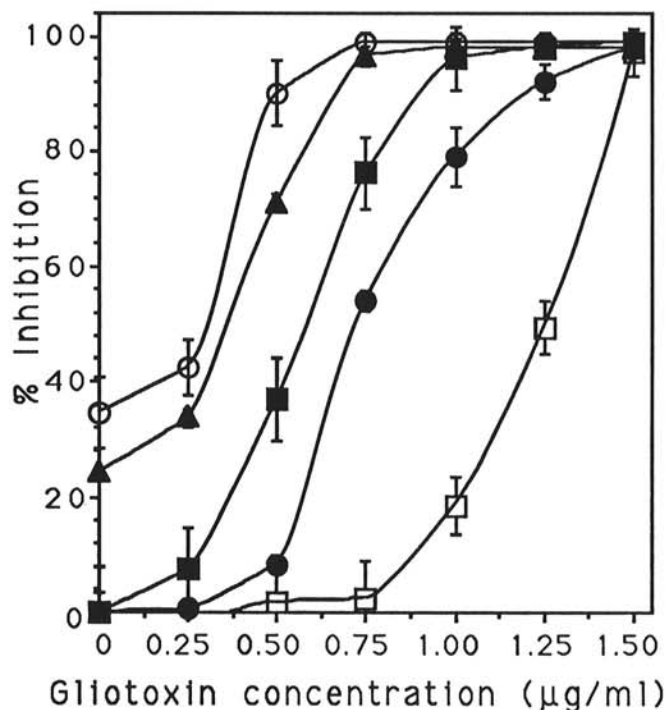
Fig. 3. Effect of 150 µg of *Gliocladium virens* endochitinase per milliliter on *Botrytis cinerea* including bursting of spores and hyphal tips. Bar = 7 µm.

Fig. 4. Inhibition of conidial germination of *Botrytis cinerea* by combinations of different concentrations of gliotoxin and pure endochitinase from *G. virens*. Endochitinase concentrations: □ = 0 µg ml^{-1}, ● = 25 µg ml^{-1}, ■ = 50 µg ml^{-1}, ▲ = 75 µg ml^{-1}, and ○ = 100 µg ml^{-1}. Each point represents the means of three replicates, with 100 conidia per replicate. Error bars indicate SD. Experiment performed twice with similar results.
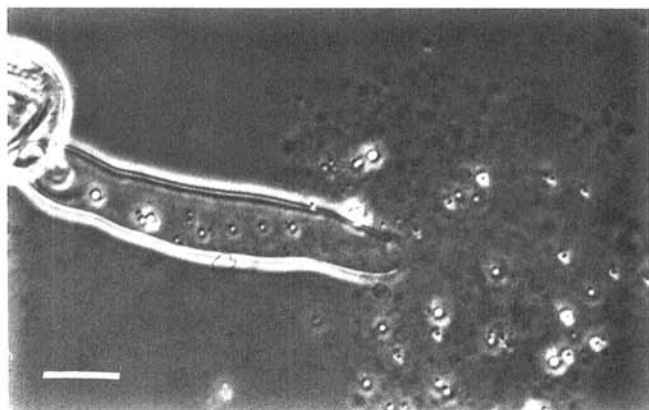
structure of the target fungus, thereby facilitating the diffusion of fungitoxic metabolites. On the other hand, the inhibitory effect of the *T. harzianum* endochitinase is probably directly due to degradation of the host cell wall.

The results of this study emphasize the major role of antibiotic metabolites in biological control by *G. virens* that was proposed previously (9,10,15,21). Roberts and Lumsden (21) found that gliotoxin was the only compound in culture filtrates of *G. virens* that was inhibitory to *P. ultimum*. They concluded that lytic enzymes from *G. virens* probably did not play a major role in biological control of *P. ultimum*. However, they did not exclude the occurrence of a subtle interactive mechanism between the two components under natural conditions (21). Our study confirmed the finding that gliotoxin was the only extractable inhibitory metabolite from *G. virens* capable of inhibiting mycelial growth of *P. ultimum*. We found a clear synergistic interaction in vitro between the endochitinase and gliotoxin. Addition of 75 mg of endochitinase per liter reduced by 50% the quantity of gliotoxin required for complete inhibition of *B. cinerea* spore germination. A model for synergism between gliotoxin and cell wall degrading enzymes has been proposed earlier by Jones and Hancock (11). These authors found that the toxicity of gliotoxin is directly related to the diffusion rate of the metabolite through the cell wall of the target organism. Therefore, gliotoxin is affected by the cell wall structure and its chemical composition. They suggested that cell wall degradation by lytic enzymes may allow a more rapid diffusion of gliotoxin to its presumed action site on the cytoplasmic membrane (11). In our study, the single endochitinase from *G. virens* caused visible damage to the cell wall of *B. cinerea* and enhanced the inhibitory effect of gliotoxin. It is likely that this synergistic effect will be even more pronounced when the host cell wall is exposed to the entire set of lytic enzymes produced by this fungus. The fact that the endochitinase exhibits optimum activity under acidic conditions may be of importance for its synergistic interaction with gliotoxin because acidic conditions also enhance both production and chemical stability of gliotoxin (15,30).

In the bioassay used in this study, on a per-weight basis, gliotoxin was approximately 100 times more active against spore germination of *B. cinerea* than the *G. virens* endochitinase. It seems, therefore, that the synergistic effect will occur only at low gliotoxin concentrations because at higher concentrations the metabolite alone is strongly inhibitory to fungal growth. Moreover, fairly high concentrations of lytic enzymes were required for in vitro inhibition of target fungi. Low gliotoxin concentrations and biologically significant levels of chitinolytic enzymes may be present when biocontrol occurs. In the related genus *Trichoderma*, it has been shown that high levels of chitinolytic enzymes are induced only in presence of chitin (27). In situ, there may be local induction of very high enzyme concentrations in close proximity to such chitinous substrates as hyphae of another fungus but only a low constitutive level of chitinolytic enzymes in absence of the substrate. Furthermore, our results show that, in combination with gliotoxin, even rather low levels of the endochitinase are sufficient for inhibition of the target fungus. In addition, conditions of low gliotoxin concentrations may frequently be associated with growth of *G. virens* in soil because the level of gliotoxin production is strongly limited by the quantity of nutrients available to the fungus (15,31). Rapid biological and physical inactivation of the metabolite and adsorption to soil particles may further reduce the concentration of active gliotoxin in soil. Therefore, the synergistic effect of cell wall degrading enzymes produced in close proximity to the target organism may be important in lowering the critical concentration of gliotoxin necessary for inhibition. The present study indicates that synergistic interaction between lytic enzymes and fungitoxic metabolites may play a role in biological control by *G. virens*.

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