Purification, Characterization, and Synergistic Activity of a Glucan 1,3-β-Glucosidase and an *N*-Acetyl-β-Glucosaminidase from *Trichoderma harzianum*

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ABSTRACT

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A glucan 1,3-β-glucosidase (EC 3.2.1.58) and an *N*-acetyl-β-glucosaminidase (EC 3.2.1.30) were purified to homogeneity from the culture filtrate of *Trichoderma harzianum* strain P1. The molecular masses and the pIs were 78 kDa and 6.2, respectively, for the glucan 1,3-β-glucosidase and 72 kDa and 4.6, respectively, for the *N*-acetyl-β-glucosaminidase. The glucan 1,3-β-glucosidase and the *N*-acetyl-β-glucosaminidase were tested against *Botrytis cinerea*, and their antifungal activity was compared with that obtained for an endochitinase and a chitin 1,4-β-chitobiosidase also purified from *T. harzianum* strain P1. The four cell wall-degrading enzymes were also tested as mixtures containing two, three, or all four proteins in all possible combinations. A synergistic, inhibitory effect was detected on both spore germination and germ tube elongation of *B. cinerea* when two, three, or four enzymes were applied together. The highest level of antifungal activity was obtained when a solution containing four different cell wall-degrading enzymes was used. ED_{50} (50% effective dose) values were as low as 1.6 μg ml^{-1} for inhibition of conidial germination and 1.7 μg ml^{-1} for inhibition of germ tube elongation of the surviving spores.

Additional keywords: biocontrol, biological control, fungitoxic compounds, mycoparasitism, synergism.

The ability of *Trichoderma* spp. to control different plant pathogens under a variety of conditions has been reported by a number of authors in the last 60 yr (5). Strains of *Trichoderma* spp. have been genetically manipulated to increase their biocontrol capability or to elucidate the process of biocontrol (10). Mycoparasitism, which may require the production of cell wall-degrading enzymes, has been proposed as a major mechanism of action (5). *Trichoderma* spp. are known to be efficient producers of polysaccharide lyases, proteases, and lipases, all of which may be used for degradation of cell walls of target fungi (4,11). Although the final proof is still lacking, evidence for involvement of chitinolytic enzymes in the biocontrol ability of these fungi has been suggested (17). Biocontrol strains of *T. harzianum* Rifai produce a complex array of chitinolytic and glucanolytic enzymes, all of which may be involved in the mycoparasitic activity (11, 25,32). Therefore, purified enzymes are required in order to determine their specific roles in biological control. Some of these enzymes have been purified and partially characterized (11,15,31). A β-1,3-glucanase has been purified from *T. harzianum* (15), but its role in mycoparasitism has not been investigated. Ulhoa and Peberdy (31) have purified an *N*-acetyl-β-D-chitobiase (an *N*-acetyl-β-glucosaminidase according to the terminology used in this paper) from *T. harzianum*. However, full chemical and biological characterization of this enzyme has not been reported. Finally, the availability of an increasing number of individual proteins permits detailed studies on this complex of enzymes and on their possible roles in biocontrol.

In the present study, we report the production of β-1,3-glucanases and *N*-acetyl-β-glucosaminidase by *T. harzianum* strain P1 grown in liquid culture and the purification and partial characterization of a glucan 1,3-β-glucosidase and an *N*-acetyl-β-glucosaminidase. In a previous report, we showed the ability of two different chitinolytic enzymes from *T. harzianum* to inhibit spore germination and germ tube elongation of a variety of fungi (17). This paper, a continuation of that study, expands the number of enzymes tested by including the *N*-acetyl-glucosaminidase and the glucan β-1,3-glucosidase purified from the same strain of *T. harzianum*. In addition, we show evidence that the synergistic antifungal interaction reported for two chitinolytic enzymes (17) is dramatically improved when one or two more fungal cell wall-degrading enzymes, with different substrate specificities, are added to the mixture. The involvement of the enzyme complex of *T. harzianum* in the mycoparasitic process and the possibility of directly using these proteins in biocontrol applications are also discussed.

MATERIALS AND METHODS

Fungal strains and culture conditions. *T. harzianum* strain P1 (ATCC 74058) is an iprodione-resistant strain with biocontrol activity against *Botrytis cinerea* Pers:Fr. and other plant-pathogenic fungi in vivo (28,29). For the production of chitinolytic and glucanolytic enzymes, the fungus was grown in 250-ml Erlen-

meyer flasks containing 100 ml of either Richard's modified medium (RM) (11) or SMCS, a synthetic medium with colloidal chitin and sucrose as carbon sources composed of 680 mg of KH_2PO_4, 870 mg of K_2HPO_4, 200 mg of KCl, 1 g of NH_4NO_3, 200 mg of $CaCl_2$, 200 mg of $MgSO_4 \cdot 7H_2O$, 2 mg of $FeSO_4$, 2 mg of $ZnSO_4$, 2 mg of $MnSO_4$, 42 g of moist purified colloidal chitin (34), and 5 g of sucrose in 1 L of distilled water adjusted to pH 6.0. The flasks were inoculated with conidia (10^7/ml) freshly grown on potato-dextrose agar (Difco Laboratories, Detroit, MI) plates. The flasks were then incubated at 25 C for 5 days on a rotary shaker at 200 rpm. For both glucan 1,3-β-glucosidase (EC 3.2.1.58 [3]; hereafter designated glucosidase) and *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30 [3]; hereafter designated NAGase), the culture filtrates were harvested as described elsewhere (11). Both enzymes were produced in both media. Glucosidase was purified from culture filtrates obtained from SMCS because this enzyme was produced at higher levels in SMCS than in RM. NAGase was purified from culture filtrate obtained from RM since this enzyme was contained in a less complex mixture when produced in RM than in SMCS.

For bioassays, *B. cinerea* strain 26 was isolated from grapes and used as a model test fungus throughout this study, because it was originally used for developing the bioassay system and it was one of the strains most sensitive to the enzymes produced by *T. harzianum* strain P1. Conidia of *B. cinerea* were produced at 25 C on potato-dextrose agar; harvested; suspended in water; filtered through sterile Kimwipes, if necessary, to remove hyphal fragments; and adjusted to 10^4 to 10^6 conidia per milliliter.

Enzyme assays. Glucosidase activity was determined by measuring the amount of reducing sugar released from laminarin (Sigma Chemical Co., St. Louis, MO). The standard assay contained 250 μl of enzyme solution and 250 μl of a 0.1% solution of laminarin in 50 mM potassium phosphate buffer, pH 6.7. After incubation at 30 C for 6 h, the reducing sugar content was determined by the method of Ashwell (1) modified according to Hayes (12). Appropriate controls without either the enzyme or the substrate were run simultaneously. The quantity of reducing sugar released was calculated from glucose standards (1, 5, 10, 15, 30, and 50 μg) included in the assay. One nanokatal corresponds to the release of 1 nmol glucose equivalent per second under the above conditions. Glucopyranosidase activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucopyranoside (Sigma). Fifty microliters of enzyme solution and 50 μl of a 0.03% (w/v) *p*-nitrophenyl β-D-glucopyranoside solution in 50 mM potassium phosphate buffer, pH 6.7, were mixed in a microtiter plate well and incubated at 37 C for 1, 6, and 24 h. Absorption was measured at 405 nm, and the quantity of *p*-nitrophenol released was calculated as described elsewhere (11).

Chitinolytic enzyme nomenclature and assays were the same as those described by Harman et al (11). NAGase and chitin 1,4-β-chitobiosidase (hereafter designated chitobiosidase) activities were determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma) and *p*-nitrophenyl β-D-*N,N'*-diacetylchitobiose (Sigma), respectively. Endochitinase activity was determined by measuring the percent reduction in turbidity of a suspension of colloidal chitin (11). One nanokatal of NAGase or chitobiosidase activity corresponds to the release of 1 nmol of *p*-nitrophenol per second under the above conditions, whereas one unit of endochitinase activity corresponds to a 5% reduction in turbidity of a suspension of colloidal chitin.

Enzyme purification. All procedures were carried out at 4 C, except for concentration steps, which were done at room temperatures. The culture filtrate was transferred to dialysis tubing (6,000–8,000 Da cutoff) and concentrated 30- to 40-fold by placing the tubing in 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 HR (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 by reverse gravitational flow at a rate of 140 μl h^{-1}. Fractions containing activity of the enzyme of interest were pooled as described in Results. The pooled set was concentrated as described above and dialyzed overnight against a 20-fold volume of 25 mM Tris-CH_3COOH buffer, pH 8.0, or 25 mM imidazole-HCl buffer, pH 7, for the purification of the glucosidase or the NAGase, respectively. The sample was then applied to a chromatofocusing column (1 × 30 cm) packed with PBE 94 (Pharmacia LKB) and equilibrated with the same buffer used for dialysis. For the glucosidase, the column was eluted at a flow rate of 50 ml h^{-1} with Polybuffer 96 (Pharmacia LKB), diluted 1:13 with water, and adjusted to pH 7.0 with CH_3COOH according to the manufacturer's directions. For the NAGase, the column was eluted at the same flow rate with Polybuffer 74 (Pharmacia LKB) diluted 1:8 with water. Fractions of interest were pooled as described in Results, dialyzed first against a 20-fold volume of 1 M NaCl and then against a 40-fold volume of distilled water to remove Polybuffer, and concentrated to a volume of 2 μl in a colloidion bag system (10,000 Da cutoff; UH 100/1, Schleicher & Schuell Inc., Keene, NH). For the final purification of the NAGase, the sample was applied to a Rotofor apparatus (BioRad Laboratories, Richmond, CA) according to the manufacturer's direction. Bio-Lyte 3/5 ampholyte was employed at 10% (v/v) of the total volume of the sample, and the peak fractions were collected, dialyzed, and concentrated as described above. Numerous similar purifications were conducted for glucosidase and NAGase, and the elution profiles presented in the figures are typical examples of results obtained.

Endochitinase and chitobiosidase were purified as described elsewhere (11).

PAGE and protein determination. Polyacrylamide gel electrophoresis (PAGE) was employed to assess the purity of the enzyme preparations and to partially characterize the purified proteins. Electrophoresis was performed in a PhastGel system (Pharmacia LKB) according to the manufacturer's directions. PAGE and sodium dodecyl sulfate (SDS)-PAGE were conducted with 20% discontinuous homogeneous gels (Pharmacia LKB), while isoelectric focusing (IEF) employed gels with pH values of 3–9. Gels were stained with Coomassie blue. For SDS-PAGE and IEF, the protein standards provided by Pharmacia or Sigma were used. Molecular weights and pIs of the *T. harzianum* glucosidase and NAGase were estimated by linear regression analysis of standard markers versus distance migrated. NAGase activity was visualized as fluorescent bands on polyacrylamide gels by using 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide as described by Tronsmo and Harman (30). Quantitative protein determination was performed with the micro BCA assay (Pierce, Rockford, IL) with soybean trypsin inhibitor (Sigma) as a protein standard. Enzyme solutions were kept at 4 C and utilized for the bioassays within 2 wk or concentrated to dryness in a SpeedVac apparatus (Savant Instruments, Farmingdale, NY) and stored at −20 C until use.

Enzyme characterization. Glucosidase and NAGase activities at various pH levels were determined in a 50 mM citric acid-potassium phosphate buffer mixture at pH 3.0–9.0 with the enzyme assays described above. For this purpose, both enzymes and substrates were dissolved in the buffer at different pH values, and the final pH value of each reaction mixture was determined. The effect of temperature on enzyme activity was tested in 50 mM potassium phosphate buffer, pH 6.7, and by performing the enzyme assays described above at temperatures of 20–100 C. Amino acid composition was determined with the Picotag amino acid analysis procedure of Heinrikson and Meredith (13); amino acid sequences were determined on a gas phase protein sequencer (Applied Biosystems, Inc., Foster City, CA) as described by Hunkapiller et al (14). Polyclonal antibodies were produced in rabbit against the pure glucosidase, following the method described by Goding (9). The affinity of the purified NAGase for polyclonal antibodies produced against the glucosidase, a pure endochitinase (11), or a pure chitobiosidase from the same strain of *T. harzianum*

TABLE 1. Purification of a glucan 1,3-β-glucosidase and an *N*-acetyl-β-glucosaminidase from 1 L of culture filtrate of *Trichoderma harzianum*[a]

Enzyme Step	Total protein (mg)	Enzyme activity (nkatal)	Specific activity (nkatal mg^{-1})	Purification (fold)	Yield (%)
Glucan 1,3-β-glucosidase	450.0	1,937.6	4.3	1.0	100
Dialyzed culture filtrate[b]	109.5	600.6	5.5	1.2	31
Sephacryl S-300 HR	1.4	135.6	96.8	22.5	7
Chromatofocusing					
N-acetyl-β-glucosaminidase					
Culture filtrate	1,850	2,413.4	1.3	1.0	100
Dialyzed culture filtrate[c]	850	2,125.5	2.5	1.9	88
Sephacryl S-300 HR	17.6	74.3	4.2	3.2	3
Chromatofocusing	4.0	24.3	6.0	4.6	1
Isoelectric focusing	1.5	17.4	11.8	9.0	0.7

[a] The fungus was grown in chitin containing synthetic medium for 5 days at 25 C for the purification of the glucan 1,3-β-glucosidase and in Richard's modified medium (11) for 5 days at 25 C for the purification of the *N*-acetyl-β-glucosaminidase.
[b] Crude culture filtrate before dialysis is not considered since it contained free glucose interfering with the enzyme assay.
[c] Specific activity against *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide. The specific activity against *p*-nitrophenyl β-D-*N,N'*-diacetylchitobiose after isoelectric focusing was 1.5 nkatal mg^{-1}.

was determined with an enzyme-linked immunosorbent assay protocol (12). This protocol was also used to determine the affinity of the pure *T. harzianum* glucosidase for polyclonal antibodies produced against either the pure endochitinase or chitobiosidase from the same strain of *T. harzianum*.

Assay for antifungal activity and analyses of the data. Homogeneously pure endochitinase (40 kDa), chitobiosidase (40 kDa), NAGase (72 kDa), and glucosidase (78 kDa) were used for bioassays for antifungal activity as described by Lorito et al (17). Briefly, the final reaction mixture contained 20 μl of 3× potato-dextrose broth (Difco), 20 μl of a suspension of conidia of *B. cinerea* containing 1×10^6 spores per milliliter, and 20 μl of a solution of the enzyme(s) to be tested (sterile water was used for the control). The enzyme solutions to be tested were made at three times the final concentration desired in the bioassay and contained either a single enzyme or a mixture of equal parts of two, three, or four different enzymes. Reaction mixtures were made in sterile Eppendorf tubes, and the final pH was 5.0–5.5. Tubes were incubated at 25 C. After 24–30 h, the percentage of germinating conidia was determined (percentage of the first 100 spores seen on a microscope slide) and the lengths of 20 germ tubes were measured and averaged. All the enzyme treatments and the controls were performed simultaneously in a single experiment. Each experiment was repeated on two separate days and contained three treatment replicates each time. The treatment replicates were then combined, and data from the two experiments were kept separate for the statistical analyses. For each enzyme(s) treatment, dosage response curves were obtained by probit analysis of the data. The lower and the upper 95% fiducial limits for 95% probability and ED_{50} (50% effective dose) values for each enzyme and each combination of enzymes were also obtained by probit analysis. This method of presenting data was chosen because it summarizes entire probit analyses from a number of determinations in a single figure. According to Richer (22), the following formula was used to determine an antifungal synergistic effect between two or more lytic enzymes: if synergism exists, $E_0(xA + yB + \ldots + nN) > E_0(x + y + \ldots + n)A > E_0(x + y + \ldots + n)B, \ldots > E_0(x + y + \ldots + n)N$, where E_0 is the percentage of inhibition; *A, B, . . . , N* are the compounds tested; and *x, y, . . . , n* are the concentrations of each component in the mixture.

RESULTS

Purification of glucosidase and NAGase from *T. harzianum*. The results of each purification step are summarized in Table 1. Purification of *T. harzianum* glucosidase with activity against laminarin was performed from culture filtrates obtained after 5 days of growth in SMCS containing chitin. Several peaks containing enzyme activity were detected following gel filtration (Fig. 1A). The fractions containing enzyme activities of interest were

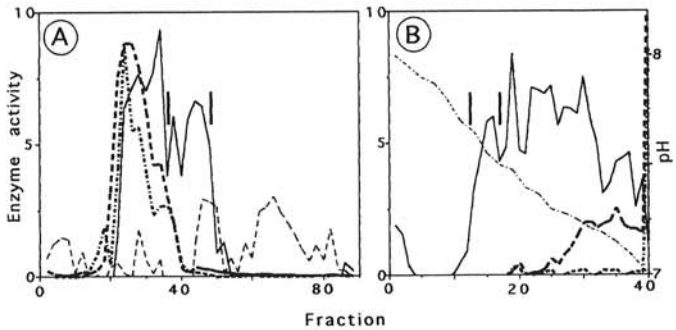

Fig. 1. Purification of a glucan 1,3-β-glucosidase from *Trichoderma harzianum* strain P1 by **A,** gel filtration chromatography followed by **B,** chromatofocusing. Glucosidase (glucan 1,3-β-glucosidase) (—), NAGase (*N*-acetyl-β-glucosaminidase) (- - -), endochitinase (- - -), and chitobiosidase (chitin 1,4-β-chitobiosidase [11]) (- · - · -) activities are in units as follows: nanokatals per milliliter of enzyme for NAGase and chitobiosidase activities, nanokatals per milliliter of enzyme times 1,000 for glucosidase activity, while one enzyme unit for endochitinase is the amount of enzyme required to reduce the turbidity of a chitin suspension by 5%. - · - · - = pH; vertical bars = fractions pooled for further purification steps.

pooled as shown in Figure 1A and applied to a chromatofocusing column, pH 7–8. After chromatofocusing, several peaks containing glucosidase activity were detected in the eluted fractions (Fig. 1B). The fractions of the first activity peak were pooled, dialyzed, and concentrated. The solution contained a pure glucosidase as shown by a single protein band from IEF as well as from native and SDS-PAGE (Fig. 2A and data not shown).

For the NAGase, a major activity peak was detected after gel filtration chromatography when *T. harzianum* was grown for 5 days in RM containing chitin (Fig. 3A). The fractions containing enzyme activities of interest were pooled as shown in Figure 3A and subjected to chromatofocusing, pH 4–7. A broad peak containing NAGase and chitobiosidase activities was detected after chromatofocusing (Fig. 3B). This peak was separated from a glucosidase peak present in earlier fractions (Fig. 3B). Under the conditions used, the Polybuffer formed a precipitate in the reducing group assay used for detection of glucosidase activity, and this caused a significant background value for all the fractions (Fig. 3B). Peak fractions containing chitinolytic enzyme activity were pooled, and the mixture was further fractionated by a Rotofor cell. After IEF, a major peak containing NAGase and a lower level of chitobiosidase activity was separated from the major chitobiosidase peak (Fig. 3C). The fractions exhibiting NAGase activity were pooled, dialyzed, and concentrated. The solution contained a single protein band from IEF as well as from native and SDS-PAGE (Fig. 2B and data not shown). Although the fractions selected after gel filtration did not include

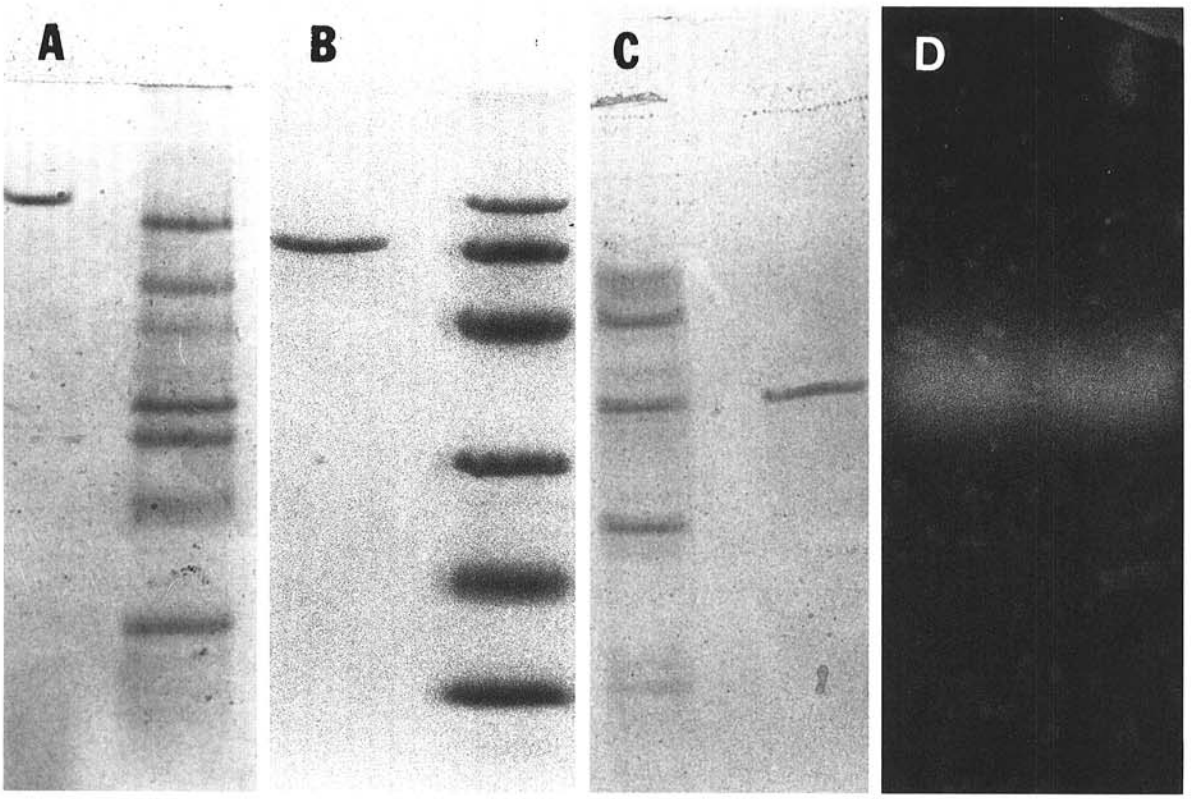

Fig. 2. Polyacrylamide gel electrophoresis (PAGE) and sodium dodecyl sulfate (SDS)-PAGE of a purified glucosidase (glucan 1,3-β-glucosidase) and NAGase (*N*-acetyl-β-glucosaminidase) both from *Trichoderma harzianum* strain P1. **A,** SDS-PAGE: lane 1, purified glucosidase; lane 2, standard molecular weight proteins 66, 45, 36, 29, 24, 20.1, and 14.2 kDa (markers from Sigma). **B,** SDS-PAGE: lane 1, purified NAGase; lane 2, standard molecular weight proteins 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa (markers from Pharmacia LKB). **C,** PAGE: lane 1, crude culture filtrate from *T. harzianum* strain P1 concentrated about 20-fold; lane 2, purified NAGase. **D,** PAGE: lane 1, crude culture filtrate from *T. harzianum* strain P1 concentrated about 20-fold; lane 2, purified NAGase. A polyacrylamide concentration of 20% was used. Proteins bands were detected by staining with Coomassie blue (**A, B, and C**) or by visualization under UV light (**D**) of NAGase activity, as described by Tronsmo and Harman (30).

the majority of the NAGase activity peak (Fig. 3A), the purified NAGase corresponded to the major protein band present in the culture filtrate with NAGase activity (Fig. 3C and D). The major chitobiosidase peak separated by IEF contained an electrophoretically pure chitobiosidase (11).

Characterization of the glucosidase and the NAGase from *T. harzianum*. The purified glucosidase from *T. harzianum* consisted of a single polypeptide with a molecular weight of 78 kDa (Fig. 2A) and a pI of 6.2, as calculated from migration after SDS-PAGE and IEF, respectively. The enzyme cleaved laminarin but was incapable of releasing *p*-nitrophenol from *p*-nitrophenyl β-D-glucopyranoside (data not shown). Glucosidase was active in citric acid-phosphate buffer over a pH range of 4.0 to 7.5 (Fig. 4A). The enzyme displayed optimal activity between pH 4.5 and 5.5. The optimum temperature for enzyme activity was 40 C, and no activity was detected at 60 C (Fig. 4B). The specific activity of the glucosidase under standard assay conditions was calculated to be 96.8 nkat mg^{-1}. The first 20 N terminal amino acids of the glucosidase from *T. harzianum* were alanine, threonine, serine, tryptophan, asparagine or proline, leucine, proline, asparagine, isoleucine, alanine, arginine, asparagine, glycine, asparagine, valine, proline, phenylalanine, alanine, serine, and glutamine or alanine. The amino acid composition of glucosidase was aspartic acid, 14%; glutamic acid, 7.2%; serine, 9%; glycine, 11.7%; histidine, 1.1%; arginine, 2.5%; threonine, 8.6%; alanine, 8.7%; proline, 4%; tyrosine, 5.6%; valine, 6.9%; methionine, 1.3%; cysteine, 0.7%; isoleucine, 6.3%; leucine, 5.2%; phenylalanine, 4.2%; and lysine, 3%. The composition appeared to be similar to that of a recently purified endochitinase from the same *T. harzianum* strain (11). However, the enzyme showed no affinity to polyclonal antibodies prepared against the previously characterized endochitinase or chitobiosidase from *T. harzianum*.

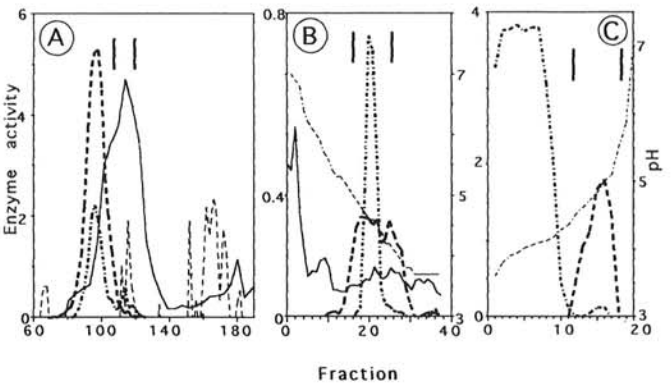

Fig. 3. Purification of a NAGase (*N*-acetyl-β-glucosaminidase) from *Trichoderma harzianum* strain P1 by **A,** gel filtration chromatography followed by **B,** chromatofocusing and **C,** isoelectric focusing. Glucosidase (glucan 1,3-β-glucosidase) (—), NAGase (- - -), endochitinase (- - -), and chitobiosidase (chitin 1,4-β-chitobiosidase [11]) (- · - · -) activities are in units as follows: nanokatals per milliliter of enzyme for NAGase and chitobiosidase activities, nanokatals per milliliter of enzyme times 1,000 for glucosidase activity, while one enzyme unit for endochitinase is the amount of enzyme required to reduce the turbidity of a chitin suspension by 5%. - · - · - = pH; vertical bars = fractions pooled for further purification steps.

The purified NAGase from *T. harzianum* was a single protein with a molecular weight of 72 kDa (Fig. 2B) and a pI of 4.6 as determined by SDS-PAGE and IEF. Under the standard assay conditions used, the enzyme cleaved *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (monomeric substrate) with a reproducible, calculated specific activity of 11.8 nkat mg^{-1}. The purified protein

also showed a low level of enzymatic activity against *p*-nitrophenyl β-D-*N,N'*-diacetylchitobiose (dimeric substrate) (Fig. 3A and C) with a specific activity of 1.5 nkat mg^{-1}. However, the release of *p*-nitrophenol occurred much more rapidly from the monomeric than from the dimeric substrate; i.e., there was a lag phase before *p*-nitrophenol was released from the monomeric substrate (Fig. 5). NAGase was active in citric acid-phosphate buffer over pHs of 4–7 (Fig. 4A); the optimum for activity was pH 5.0–5.5. The NAGase was active under the standard assay conditions over temperatures of 25–100 C, and the optimal temperature was 60 C (Fig. 4B). The enzyme showed no affinity to polyclonal antibodies prepared against purified glucanase, endochitinase, or chitobiosidase from *T. harzianum*.

Antifungal activity of chitinolytic and glucanolytic enzymes from *T. harzianum*. Under the condition used, the average value of spore germination of *B. cinerea* was 70% and the average germ tube length 425 mm in the absence of enzymes (control). Spore germination and germ tube elongation of *B. cinerea* were inhibited by endochitinase, chitobiosidase, NAGase, and glucosidase from *T. harzianum* when used individually (Fig. 6). ED_{50} values were 47.5–125 μg ml^{-1} (Table 2). The most effective enzyme was the endochitinase with ED_{50} values of 47.5 and 53 μg ml^{-1} for spore germination and germ tube elongation, respectively (17). For all the enzymes, 200–600 μg ml^{-1} were needed for complete inhibition when the proteins were tested individually (Fig. 6). In most cases, a great improvement in antifungal activity was detected when two, three, or all four enzymes were mixed in equal parts and assayed in all possible combinations (Figs. 6 and 7, Table 2). Usually, the antifungal and the synergistic effect increased as the number of different enzymes in the mixture increased (Table 2). The highest level of inhibition was obtained with a mixture containing all four cell wall-degrading enzymes; the ED_{50} value was as low as 1.6 μg ml^{-1} for spore germination (Table 2). The fungicidal interactions between different cell wall-degrading enzymes from *T. harzianum* were synergistic in nature. In all cases, the E_0 values in the Richer's formula for combinations of enzymes were higher than for individual enzymes. This was also true if the lower and the upper 95% fiducial limits obtained by probit analysis were used in Richer's formula (22) (data not shown). For instance, the E_0 values in the Richer's formula (percentages of inhibition of spore germination) with $x = y = \ldots = n$ and $x + y + \ldots + n = 10$ μg ml^{-1} were 8 for endochitinase, 1 for chitobiosidase, 2 for NAGase, 3 for glucosidase, 12–87.5 for combinations of two enzymes, 75–87.5 for combinations of three enzymes, and 98 for the combination of four enzymes. Among the two-enzyme combinations, a lower level of synergism was detected between chitobiosidase and NAGase; the E_0 value for $x = y = 5$ μg ml^{-1} was 12 for the combination of these two enzymes. Conversely, the mixture endochitinase + glucosidase was the most effective among the two-enzyme combinations; the E_0 value for $x = y = 5$ μg ml^{-1} was 90 for the combination of these two enzymes. Among the three-enzyme combinations,

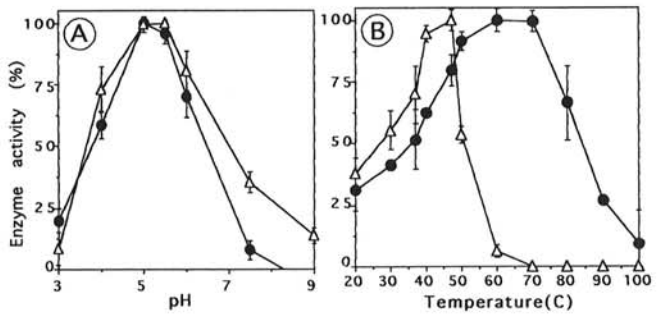

Fig. 4. Effect of **A,** pH and **B,** temperature on NAGase (*N*-acetyl-β-glucosaminidase) (●) and glucosidase (glucan 1,3-β-glucosidase) (△) activities. Purified enzymes were assayed against laminarin (glucosidase) or *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (NAGase) as described in Materials and Methods. Assays were performed in triplicate and results averaged, and the entire experiment was repeated. Values presented are averages of the two experiments. The error bars represent standard deviations of the means of the two experiments.

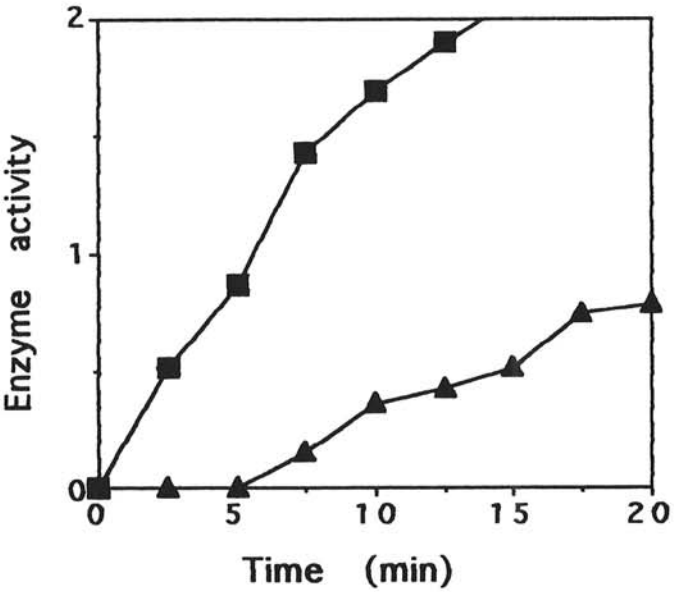

Fig. 5. Time course of enzyme activity of purified NAGase (*N*-acetyl-β-glucosaminidase) from *Trichoderma harzianum* against monomeric (*p*-nitrophenyl *N*-acetyl-β-D-glucosaminide) (■) or dimeric (*p*-nitrophenyl-β-D-*N,N'*-diacetylchitobiose) (▲) synthetic substrates (Sigma). Enzyme activity = nanokatals per milliliter of enzyme.

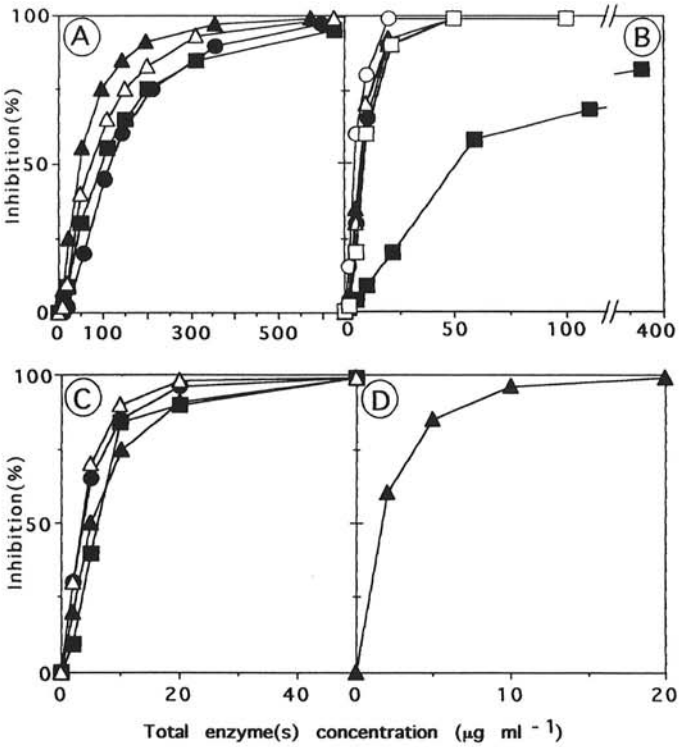

Fig. 6. Effect of chitinolytic and glucanolytic enzymes from *Trichoderma harzianum* strain P1 on spore germination of *Botrytis cinerea*. **A,** Single enzyme treatment with endochitinase (▲), chitobiosidase (chitin 1,4-β-chitobiosidase) (●), NAGase (*N*-acetyl-β-glucosaminidase) (△), and glucosidase (glucan 1,3-β-glucosidase) (■); **B,** dual enzyme treatment with endochitinase + chitobiosidase (●), endochitinase + NAGase (□), endochitinase + glucosidase (○), chitobiosidase + NAGase (■), chitobiosidase + glucosidase (△), and NAGase + glucosidase (▲); **C,** triple enzyme treatment with endochitinase + chitobiosidase + NAGase (■), endochitinase + chitobiosidase + glucosidase (△), endochitinase + NAGase + glucosidase (●), and chitobiosidase + NAGase + glucosidase (▲); **D,** quadruple enzyme treatment with endochitinase + chitobiosidase + NAGase + glucosidase (▲). Mixtures of enzymes contained equal parts of each component.

the endochitinase + chitobiosidase + glucosidase mixture showed the highest inhibitory effect; the E_0 value for $x = y = \ldots = n = 3.3$ $\mu g\ ml^{-1}$ was 87.5 for the combination of these three enzymes.

Morphological effects produced by chitinolytic and glucanolytic enzymes from *T. harzianum* included lysis of germ tubes, spores, and mycelium, as well as hyphal distortion, heavy vacuolization, and swelling of the hyphae of *B. cinerea* (data not shown) (17).

DISCUSSION

Glucans and chitin are the major constituents of fungal cell walls (2), and the important roles of glucosidases and chitinolytic enzymes in the degradation of fungal cell walls during mycoparasitism by *T. harzianum* have been previously suggested (17,23,25). In the present study, we have examined the production of glucosidase and NAGase in liquid culture by an effective biocontrol strain of *T. harzianum*. *T. harzianum* strain P1 produced a number of extracellular glucosidases with activity against laminarin (Fig. 1) when grown in synthetic medium containing colloidal chitin and sucrose as carbon sources. The fact that glucosidases were produced in the absence of exogenously added laminarin indicates that β-1,3-glucan is not required for enzyme induction. This has also been reported for the β-1,3-glucanases and xylanases from *T. longibrachiatum* (24,27) and for β-1,3-glucanases from the plant pathogen *Cochliobolus carbonum* (33). A possible explanation is that glucanase is produced constitutively, perhaps because it may be involved in fungal growth and differentiation (2).

Alternatively, a simultaneous induction with chitinolytic enzymes may occur (11). Concurrent induction of chitinases and glucosidases has been described in plants as a response to infection by microbial pathogens (19), and it has been shown that the two classes of enzymes exhibit synergistic activity against growth of several fungi (20). In addition, evidence for a synergistic antifungal effect of either exo- or endochitinases in combination with glucosidase, all from the same strain of *T. harzianum,* is presented in this paper. Since the substrates of both classes of enzymes frequently occur together in nature, e.g., in fungal cell walls (2), a combined induction of glucosidases and chitinases may be ecologically relevant.

A glucan 1,3-β-glucosidase from *T. harzianum* was purified to homogeneity by gel filtration and chromatofocusing. This enzyme was purified from a slightly different medium than that used for the purification of NAGase, although in other experiments the glucosidase was also produced (Fig. 3) and purified (data not shown) from RM. SMCS was chosen for the production of glucosidase because a higher production of enzyme was obtained from this medium than from RM (data not shown). The molecular weight of the glucosidase was estimated to be 78 kDa. It was similar to those of the β-1,3-glucanases from *T. longibrachiatum*, 70 kDa (27), and from *C. carbonum*, 73 kDa (33), but considerably larger than those of an exo-β-1,3-glucanase from *T. harzianum*, 31.5 kDa (15), and of plant β-1,3-glucanases (6,19). The pI of the purified enzyme, 7.2, was the same as that of the β-1,3-glucanase from *T. longibrachiatum* but higher than that of the *C. carbonum* enzyme (27,33). The optimal pH for enzyme activity was similar to that found for other β-1,3-glucanases from fungi (27,33) and plants (6,19).

An *N*-acetyl-β-glucosaminidase was purified to homogeneity by column gel filtration, chromatofocusing, and IEF. The optimal

TABLE 2. Inhibition of spore germination and germ tube elongation of *Botrytis cinerea* by chitinolytic and glucanolytic enzymes from *Trichoderma harzianum* strain P1

Enzyme(s)[a]	ED_{50} value[b]	
	Spore germination ($\mu g\ ml^{-1}$)	Germ tube elongation ($\mu g\ ml^{-1}$)
Endoc	47.5 (45–49)	53 (49–55)
Chitob	117 (112–121)	125 (115–133)
NAG	51 (49.9–52.8)	70 (63–75)
Glucos	94.5 (90–99)	90 (86–96)
Endoc + Chitob	7.8 (7.5–8.2)	19 (16.5–22)
Endoc + NAG	8.8 (8.5–9.2)	9.5 (8–11)
Endoc + Glucos	4.5 (4.3–4.7)	3.5 (2.8–4)
Chitob + NAG	44 (41–47)	48 (44–51)
Chitob + Glucos	6.4 (6.2–6.6)	9 (8–9.9)
NAG + Glucos	7.1 (6.8–7.5)	8 (7.2–9)
Endoc + Chitob + NAG	6.7 (6.3–7)	6 (5.4–6.8)
Endoc + Chitob + Glucos	3.2 (3–3.4)	2.5 (2–3)
Endoc + NAG + Glucos	3.5 (3.3–3.7)	5 (4.2–5.5)
Chitob + NAG + Glucos	5.1 (4.8–5.4)	7 (5.8–7.9)
Endoc + Chitob + NAG + Glucos	1.6 (1.5–1.7)	1.7 (1.5–2)

[a] Endoc = endochitinase; Chitob = chitobiosidase (chitin 1,4-β-chitobiosidase); Glucos = glucosidase (glucan 1,3-β-glucosidase); NAG = NAGase (*N*-acetyl-β-glucosaminidase). Mixtures of enzymes contained equal parts of each component.

[b] Fifty percent effective dose. The lower and upper 95% fiducial limits for 95% probability are in parentheses.

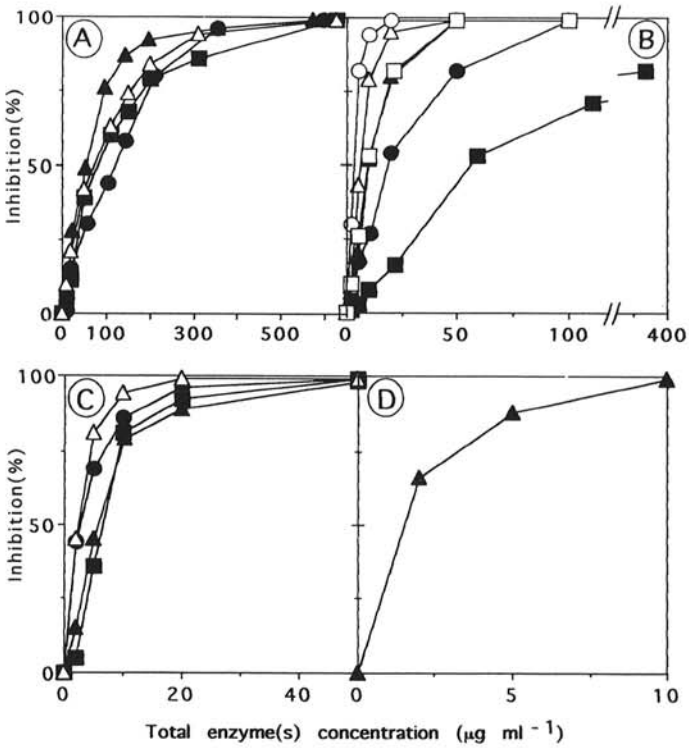

Fig. 7. Effect of chitinolytic and glucanolytic enzymes from *Trichoderma harzianum* strain P1 on germ tube elongation of *Botrytis cinerea*. **A,** Single enzyme treatment with endochitinase (▲), chitobiosidase (chitin 1,4-β-chitobiosidase) (●), NAGase (*N*-acetyl-β-glucosaminidase) (△), and glucosidase (glucan 1,3-β-glucosidase) (■); **B,** dual enzyme treatment with endochitinase + chitobiosidase (●), endochitinase + NAGase (□), endochitinase + glucosidase (○), chitobiosidase + NAGase (■), chitobiosidase + glucosidase (△), and NAGase + glucosidase (▲); **C,** triple enzyme treatment with endochitinase + chitobiosidase + NAGase (■), endochitinase + chitobiosidase + glucosidase (△), endochitinase + NAGase + glucosidase (●), and chitobiosidase + NAGase + glucosidase (▲); **D,** quadruple enzyme treatment with endochitinase + chitobiosidase + NAGase + glucosidase (▲). Mixtures of enzymes contained equal parts of each component.

pH of 5–6 was similar to that found for another NAGase purified from *T. harzianum* (32) (an *N*-acetyl-β-D-chitobiase according to the terminology used by these authors) and slightly lower than that reported for a similar enzyme from *Candida albicans* and *Aeromonas hydrophila* (26,35). However, its molecular weight (72 kDa) and optimal temperature (60 C) were higher than those of the protein (64 kDa and 50 C, respectively) obtained by Ulhoa and Peberdy (32). The NAGase purified in this work was quite resistant to heat inactivation, retaining about 70, 25, and 10% of activity after 15 min at 80, 90, and 100 C, respectively. This finding may be of practical utility for direct utilization of the enzyme in biocontrol or other purposes. The pI of the purified enzyme (4.6) was similar to that of another NAGase partially purified and characterized from *T. harzianum* (23) but lower than that of the *A. hydrophila* enzyme (35).

The NAGase from *T. harzianum* cleaved either the monomeric substrate (*p*-nitrophenyl *N*-acetyl-β-D-glucosaminide) or the dimeric substrate (*p*-nitrophenyl β-D-*N,N'*-diacetylchitobiose), but the specific activity was approximately eightfold higher with the monomeric substrate. We also found that enzyme activity against the monomeric substrate was clearly detectable within 20 s of incubation, while 5 min was needed before any activity could be detected with the dimeric substrate (Fig. 5). These findings indicate that the *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide is a better substrate for quantification of NAGase activity. These results can be explained by considering that NAGase hydrolyzes "terminal nonreducing *N*-acetyl-β-D-glucosamine residues from chitobiose or higher analogues" (3), whereas the *p*-nitrophenol group is bound on the reducing end of the molecule of the dimeric substrate. Therefore, the first products of hydrolysis of the dimeric substrate will be *N*-acetyl-β-D-glucosamine and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (corresponding to the monomeric substrate), and a second cleavage of the latter will be necessary for the release of the *p*-nitrophenol. Obviously, in the case of the monomeric substrate, any enzymatic reaction will release *p*-nitrophenol, and, therefore, NAGase activity will be more rapidly detectable than with the dimeric substrate.

The purified NAGase described in this paper was the major enzyme with NAGase activity produced by *T. harzianum* strain P1 under the growth condition used (Fig. 2). The final amount of purified enzyme represented a small percentage of the total content of NAGase in the culture filtrate, since only a minor part of the large NAGase peak was selected after gel filtration (Fig. 3A). However, when the whole NAGase peak was used, final purification of the enzyme was difficult, since contaminating proteins contained in this peak were difficult to remove (data not shown).

Synergism among fungal cell wall-degrading enzymes and between lytic enzymes and fungicides or biocontrol bacteria has been reported (16–18,21). Two different chitinolytic enzymes from *T. harzianum* interacted synergistically to inhibit chitin-containing fungi (17) or to degrade isolated cell walls of *B. cinerea* (7). Previous studies have shown that mycolytic enzymes were able to enhance fungicidal activity of a number of pesticides, such as nikkomycin and polyoxin B (21), while more recently we demonstrated that the same cell wall-degrading enzymes used in this work were synergistic with flusilazole, miconazole, captan, benomyl, and the natural fungicide gliotoxin (18). We suggested that cell wall-degrading enzymes affect the biological properties of the toxins by digesting part of the fungal cell wall and, therefore, increasing the uptake of the drugs (18). Further, endochitinase and chitobiosidase from *T. harzianum* strain P1 were able to synergistically enhance the antifungal activity of a biocontrol strain of *Enterobacter cloacae* with a concomitant stimulation of bacterial growth and bacterial attachment to hyphae of target fungi (16). Finally, synergism was observed between chitinolytic and glucanolytic enzymes obtained from peas (20). Similarly, the results of this study demonstrate that chitinolytic and glucanolytic enzymes from *T. harzianum* are able to interact synergistically in the inhibition of spore germination and hyphal elongation of *B. cinerea*. All these findings suggest that fungal cell wall-degrading enzymes may be able to interact with a variety of biotic and abiotic agents and that effective combinations may be found and successfully used in biocontrol.

Strain P1 of *T. harzianum* produces a complex mixture of chitinolytic and glucanolytic enzymes, as do other similar fungi (8,11,23), and four of these proteins were purified and tested for biological activity in vitro. All the enzymes assayed were effective against a variety of chitin-containing fungi (17) or against the chitin-containing fungus *B. cinerea,* selected in this study as a model test organism. In a previous report, combining endochitinase and chitobiosidase reduced the ED_{50} from 41–152 μg ml^{-1} for a single enzyme to 10 μg ml^{-1} for the mixture of enzymes (17). These values are slightly different from values reported in this paper because they were obtained by a polynomial regression instead of probit analysis.

These results extend the work described earlier for two enzymes (17) to additional proteins and demonstrate that the levels of synergy with various combinations of enzymes is greater than that which we described earlier. In this work, we were able to further increase the effectiveness of a given total amount of protein by mixing equal parts of three chitinolytic enzymes and a glucosidase, all from *T. harzianum* strain P1. The ED_{50} value for spore germination, obtained by statistical analysis, against *B. cinerea* was as low as 1.6 μg ml^{-1}. This indicates that the level of toxicity of enzyme combinations may approach that of chemical pesticides. In addition, these enzymes may be more environmentally safe than the chemical counterparts. The present work suggests that synergy between enzymes is a general phenomenon and is not just restricted to endochitinase and chitobiosidase. These results suggest that cell wall-degrading enzymes from *T. harzianum* strain P1 are more effective than similar enzymes from other sources (17).

The antifungal activity of all the enzymes tested strongly suggests the involvement of extracellular enzymes in the biocontrol mechanism of *T. harzianum* strain P1. It is conceivable that this complex of antifungal proteins, produced in the presence of a chitinous substrate, is directly utilized for mycoparasitism of other fungi or for surviving in an antagonistic environment. The level of synergistic interaction between cell wall-degrading enzymes from *T. harzianum* seemed to increase when additional enzymes with different substrate specificities were combined and tested. This suggests that most, if not all, of the lytic enzymes produced may be utilized by *T. harzianum* against other fungi and that the level of effectiveness of the whole enzymatic complex may be substantially higher than indicated by this work. The lowest level of synergism was observed for the combination of the two exochitinases (chitobiosidase and NAGase), while the presence of the endochitinase or the glucosidase was required for higher antifungal activity. This finding seems to be in agreement with results reported by De La Cruz et al (7), who found that a proper combination of different chitinolytic enzymes, from another strain of *T. harzianum,* was needed for an efficient degradation of isolated fungal cell walls. This also suggests that the role of each protein in the antifungal, enzymatic complex of *T. harzianum* is different and that different enzymes with different or complementary modes of action may be required for maximal efficacy.

Chitinolytic and glucanolytic enzymes from *T. harzianum* are 1) inhibitory to other fungi, 2) able to interact synergistically to achieve a high level of antifungal activity, 3) stable and active after drying and storage and at natural biological conditions of temperature and pH, and 4) not likely to be toxic to plants and higher vertebrates, since these organisms contain little or none of the polymers upon which these enzymes act. The biological and biochemical properties of these cell wall-degrading enzymes suggest that these proteins or the genes encoding for them may be successfully utilized in biological control of some plant diseases. In addition, the ability to improve the efficacy of antifungal agents may allow the utilization of genes and gene products in a variety of biocontrol strategies. For instance, transgenic plants may be developed that are able to express fungal genes and produce active fungal enzymes, thereby showing an acceptable level of resistance to some plant pests. However, if necessary, increased levels of plant resistance to fungal attacks may be achieved by taking ad-

vantage of enzyme synergism with common drugs, which would result in the application of very low doses of pesticides.

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