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Purification and Characterization of an Endo-β-1,6-Glucanase from *Trichoderma harzianum* That Is Related to Its Mycoparasitism

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The enzymes from *Trichoderma* species that degrade fungal cell walls have been suggested to play an important role in mycoparasitic action against fungal plant pathogens. The mycoparasite *Trichoderma harzianum* produces at least two extracellular β-1,6-glucanases, among other hydrolases, when it is grown on chitin as the sole carbon source. One of these extracellular enzymes was purified to homogeneity after adsorption to its substrate, pustulan, chromatofocusing, and, finally, gel filtration. The apparent molecular mass was 43,000, and the isoelectric point was 5.8. The first 15 amino acids from the N terminus of the purified protein have been sequenced. The enzyme was specific for β-1,6 linkages and showed an endolytic mode of action on pustulan. Further characterization indicated that the enzyme by itself releases soluble sugars and produces hydrolytic halli on yeast cell walls. When combined with other *T. harzianum* cell wall-degrading enzymes such as β-1,3-glucanases and chitinases, it hydrolyzes filamentous fungal cell walls. The enzyme acts cooperatively with the latter enzymes, inhibiting the growth of the fungi tested. Antibodies against the purified protein also indicated that the two identified β-1,6-glucanases are not immunologically related and are probably encoded by two different genes.

Some species of *Trichoderma* have been described as biological control agents against several fungal plant pathogens (31). The degradation and further assimilation of phytopathogenic fungi, namely, mycoparasitism, has been proposed as the major mechanism accounting for the antagonistic activity of *Trichoderma* species against fungal pathogens (8). From recent work, it appears that *Trichoderma* mycoparasitism is a complex process involving several successive steps (9). Initially, the mycoparasite grows directly towards its host and often coils around it or attaches to it by forming hook-like structures and apressoria (15). Following these interactions, *Trichoderma* spp. sometimes penetrate the host mycelium, apparently by partially degrading its cell walls (14). Finally, it is assumed that *Trichoderma* spp. utilize the intracellular contents of the host (14).

Chitin and β-1,3-glucan are the main structural components of fungal cells walls, except those from members of the class *Oomycetes*, which contain β-1,3-glucan and cellulose (2). Thus, chitinases (EC 3.2.1.14) and β-1,3-glucanases (EC 3.2.1.39), proteins secreted by *Trichoderma* spp., have been suggested as the key enzymes in the lysis of phytopathogenic fungal cell walls during mycoparasitic action (11, 16, 42). However, other cell wall-degrading enzymes, including those hydrolyzing minor polymers (proteins, β-1,6-glucans, α-1,3-glucans, etc.), may be involved in the effective and complete degradation of mycelial or conidial walls of phytopathogenic fungi by *Trichoderma* spp. A subtilisin-type serine proteinase induced by chitin has already been described in a mycoparasitic strain of *Trichoderma harzianum* (19). Also, β-1,6-glucanases (EC 3.2.1.75) have been shown to lyse yeast and fungal cell walls in filamentous fungi (41, 51) and bacteria (39), but unfortunately there is almost no information about the purification and characterization of β-1,6-glucanases (20, 39, 41, 51), and to the best of our knowledge, neither antibodies nor a sequence of a β-1,6-glucanase has ever been reported.

Chitinases and β-1,3-glucanases have also been reported to be pathogenesis-related proteins in plants and proposed to have a major role in the defense reactions against pathogens (5, 7). Plants lack β-1,6-glucans (47), and β-1,6-glucanases have never been described in these organisms.

In this paper, we report the identification of two β-1,6-glucanase activities from a mycoparasitic strain of *T. harzianum*, described as an agent of biological control (10). The major β-1,6-glucanase activity has been purified and characterized. The probable role of this activity in the mycoparasitic action of *T. harzianum* is also discussed.

MATERIALS AND METHODS

Materials and reagents. Pustulan (from *Umbilicaria papullosa*) and pachyman (from *Poria cocos*) were purchased from Calbiochem (La Jolla, Calif.). Aniline blue (methyl blue), calcofluor (fluorescent brightener 28), carboxymethyl-cellulose, cellooligosaccharides, chitin (from crab shells), dextran (from *Leuconostoc mesenteroides*), gentibiose, glucose, glycol-chitosan, horseradish peroxidase-conjugated anti-mouse immunoglobulin G, laminarin (from *Laminaria digitata*), MeUmb-Glc (4-methylumbelliferyl β-1,4-D-glucoside), nigeran (from *Aspergillus nidulans*), N*p*-$(Glc)_2$ (*p*-nitrophenyl β-1,6-D-gentibioside), N*p*-$(GlcNAc)_2$ (*p*-nitrophenyl-*N*,*N*′-diacetyl chitobiose), N*p*-GlcNAc (*p*-nitrophenyl-*N*-acetyl β-D-glucosaminide), phenylmethylsulfonyl fluoride (PMSF), and soluble starch were purchased from Sigma Chemical Co. (St. Louis, Mo.). Endo-H (endo-β-*N*-acetylglucosaminidase H) and α-mannosidase were purchased from Boehringer (Mannheim, Germany). Chemicals for electrophoresis and protein assay dye reagents were purchased from Bio-Rad (Richmond, Calif.). Chemicals for column chromatography, ampholites, and pI standard proteins were supplied by Pharmacia-LKB (Uppsala, Sweden). Novozym 234 was purchased from Novo Industries. Fungal cell walls were prepared after cell breakage with a Braun MSK homogenizer and washing, by the method of Fleet and Phaff (17). Yeast glucan

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was prepared from baker's yeast as described by Rombouts and Phaff (39). All other chemicals were of analytical grade.

Organisms and growth conditions. *T. harzianum* CECT 2413 was obtained from the Spanish Type Culture Collection (Burjassot, Valencia, Spain). Glucose-potato agar was used to maintain the cultures. For the production and purification of the β-1,6-glucanase, *T. harzianum* was grown as described by De la Cruz et al. (11). First, conidia were inoculated in Czapek medium supplemented with 10% (wt/vol) glucose and incubated for 4 days at 28°C and 200 rpm (repression conditions). Then the mycelia were washed extensively with 2% (wt/vol) $MgCl_2$ and water and transferred to fresh Czapek medium containing 1.5% (wt/vol) chitin and 70 mM phosphate-KOH buffer, pH 6.0 (induction conditions).

In some experiments, *Trichoderma koningii* CECT 2412, *Trichoderma reesei* CECT 2414, and *Trichoderma viride* CECT 2423 were also used; growth conditions were as before, but in these cases, 0.1% and 2% (wt/vol) glucose, 1% (wt/vol) chitin, washed autoclaved cells of *Saccharomyces cerevisiae* (La Cinta Roja, Spain), and washed autoclaved mycelia of *Botrytis cinerea* CECT 2100 or of *Phytophthora syringae* CECT 2351 were used as carbon sources under induction conditions.

Enzyme assay. β-1,6-Glucanase activity was determined by measuring the amount of reducing sugars released from pustulan. Pustulan was dissolved in hot water or buffer and, after cooling, remained in solution without precipitation. The standard assay (1 ml) contained the enzyme preparation appropriately diluted, 4 mg of pustulan, and 50 mM potassium acetate buffer, pH 5.5. After 30 min to 1 h of incubation at 37°C, the reaction was stopped by boiling for 5 min, and the reducing sugar content was determined by the procedure of Somogyi (43) and Nelson (30). Standards of glucose, as well as enzyme and substrate blanks, were also included. For the β-1,3-glucanase assay, pustulan was replaced by laminarin as the reaction substrate. An enzymatic unit was defined as the amount of enzyme that catalyzes the release of reducing sugar groups equivalent to 1 μmol of glucose per min, under the described assay conditions.

Mycelial growth and protein determination. Mycelial growth was monitored by measuring total protein by the method of Lowry et al. or (27) as described in De la Cruz et al. (12) or by measuring mycelial dry weight. Bovine serum albumin was used as a protein standard. Extracellular protein was determined by the Bradford method (6), using ovalbumin as a protein standard.

Purification of β-1,6-glucanase. (i) Ammonium sulphate precipitation. Unless otherwise indicated, all steps were performed at 0 to 4°C. *T. harzianum* cultures grown for 48 h in Czapek medium with 1.5% chitin were filtered through Whatman no. 1 filter paper and centrifuged at 6,000 × *g* for 10 min. The supernatant (about 200 ml) was precipitated with ammonium sulfate (80% saturation). The precipitate was recovered by centrifugation at 12,000 × *g* for 20 min, resuspended in a minimal amount of distilled water, and dialyzed against 50 mM potassium acetate buffer, pH 5.5. The dialyzed fraction, labelled crude enzyme, had a final volume of about 20 ml.

(ii) Pustulan adsorption-digestion. Dialyzed aliquots of 3 ml were adsorbed to 0.6 ml of 1-mg/ml ethanol-precipitated pustulan for 20 min with magnetic stirring and pelleted by centrifugation at 12,000 × *g* for 10 min. The supernatants (nonadsorbed pustulan fraction) were reincubated with ethanol-precipitated pustulan, and the process was repeated twice. All the pellets were washed three times with 3 ml of 70 mM phosphate-KOH buffer, pH 6.0, containing 1 M NaCl and resuspended in 50 mM potassium acetate buffer, pH 5.5, with 1 mM phenylmethylsulfonyl fluoride and 1 mM sodium azide. These samples were incubated overnight at 37°C, and the clarified solutions obtained after pustulan digestion were centrifuged at 12,000 × *g* for 10 min. The supernatants (5 to 10 ml) were dialyzed against 25 mM imidazole-HCl buffer, pH 7.4.

(iii) Chromatofocusing. The dialyzed solution was subjected to chromatofocusing on a Polybuffer exchanger column (1 by 20 cm) equilibrated in 25 mM imidazole-HCl buffer, pH 7.4. Proteins were eluted at a flow rate of 9 ml/h through a pH gradient formed by eightfold diluted Polybuffer adjusted to pH 4.0 with HCl. Fractions (1.5 ml each) were collected and assayed for β-1,6-glucanase by using pustulan as substrate, as described above. The most active fractions were pooled and concentrated to approximately 0.5 ml on Centricon 10 concentrators (Amicon, Danver, Mass.).

(iv) Gel filtration. The concentrated pool was applied to a Sephacryl S-200 HR column (1.6 by 40 cm) equilibrated in 100 mM potassium acetate buffer, pH 5.5, with 100 mM KCl and was eluted with the same buffer at a rate of 7 ml/h. Fractions (0.65 ml each) were assayed for β-1,6-glucanase activity as described above. Active fractions were pooled, concentrated, and washed in 50 mM potassium acetate buffer, pH 5.5, on Centricon 10 concentrators (final volume, about 0.5 ml) and finally stored at 4°C, under which conditions enzyme activity remained unchanged for at least 1 month. This protein solution was used for both β-1,6-glucanase characterization and generation of anti-β-1,6-glucanase antibodies.

Preparation of antibodies. Polyclonal antibodies were raised by intraperitoneally injecting 75 μg of purified enzyme into female mice (BALB/c) in complete Freund's adjuvant. At 1-week intervals, the mice received two additional injections with the same amount of protein in incomplete Freund's adjuvant. Blood samples were taken twice at 2-week intervals after the last injection; blood samples were centrifuged, and the supernatants (sera) were stored at −20°C.

Gel electrophoresis and immunoblotting. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (24) in 12% acrylamide gels. The proteins were stained with Coomassie R-250 brilliant blue. Low-molecular-mass standard proteins (Bio-Rad) were used for molecular mass determination.

Detection of β-1,6-glucanase activity in agar replicas of the SDS-polyacrylamide gels was done as described by Beguin for *T. reesei* cellulases (4), replacing cellulose with 0.1% pustulan. Before use of the replica, enzymes were renatured by the casein-EDTA procedure described by McGrew and Green (29).

Glycoprotein stain assays were carried out in the gels by either the periodic acid-Schiff reagent procedure (Sigma) or the silver nitrate procedure of Dubray and Brezard (13). Extracellular yeast invertase (Sigma) was used as a glycoprotein positive control. Before SDS-PAGE, and to remove the carbohydrates linked to the protein, standard deglycosylation reactions were performed with endo-H or α-mannosidase, as described by Vazquez de Aldana et al. (48) and Williamson et al. (49), respectively. Both β-1,6-glucanase and deglycosidase enzyme blanks were used. As before, extracellular invertase was used as a positive control.

Isoelectrofocusing was performed as described by Robertson et al. (38) at a pI range of 3.5 to 10. Proteins were visualized by Coomassie staining.

After SDS-PAGE, proteins were electrotransferred onto nitrocellulose sheets as described by Towbin et al. (46). Blocking with 5% skim milk; incubation with mouse anti-β-1,6-glucanase (1:5,000) and peroxidase-conjugated anti-mouse immunoglobulin G (1:1,000) as first and secondary antibodies, respectively; and washes were performed as described by Kombrink et al. (23).

Protein microsequencing. To determine the NH_2-terminal amino acid sequence, the purified β-1,6-glucanase was separated by SDS-PAGE as described before and electroblotted to a polyvinylidene difluoride membrane (Immobilon-P; Millipore) as described by Bauw et al. (3). The membranes were stained with amido black. The amino acid sequence analysis was performed with a 470-A gas-phase sequencer equipped with a 120-A, on-line, phenylthiohydantoin amino acid analyzer (Applied Biosystems), as described also by Bauw et al. (3).

Kinetic parameters. Michaelis-Menten constants were determined according to the Lineweaver-Burk representation of data obtained by measuring the initial rate of pustulan hydrolysis under the assay conditions described above, and using pustulan in the range of 20 to 0.5 mg/ml.

Temperature optimum and stability. The temperature optimum was determined by performing the standard assay within the temperature range of 20 to 70°C.

The inactivation temperature was also determined by incubating the purified β-1,6-glucanase for 30 min at temperatures from 20 to 80°C in 50 mM potassium acetate buffer, pH 5.5, and then measuring the remaining activity at 37°C by adding pustulan as the assay substrate. The inactivation temperature was defined as that temperature at which the specific activity was reduced by 50%, under the conditions described above.

Substrate specificity and lytic activity on fungal cell walls. In addition to pustulan, the activity of the purified β-1,6-glucanase was also tested on other polymers at a final concentration of 5 mg/ml. When glucans were used, the reaction products were detected as reducing sugar groups and measured as described before. Chitinase and chitosanase products were detected as described by De la Cruz et al. (11).

Activity on *p*-nitrophenyl-β-glucosides (final concentration, 1 mM) was measured as described by Roberts and Selitrennikoff (37). When the substrate was MeUmb-Glc (final concentration, 1 mM), the procedure followed was as described by Robbins et al. (36).

To monitor cell wall hydrolytic activity, 4 mg of lyophilized *B. cinerea* CECT 2100, *Gibberella fujikuroi* IMI 58289 (Imperial Mycological Institute, Kew, United Kingdom), *P. syringae* CECT 2351, or *S. cerevisiae* cell walls were incubated, as in the standard assay, with the purified β-1,6-glucanase either alone or in combination with either the three chitinases present in *T. harzianum* and described elsewhere (11), the other two β-glucanases identified in this work (see Results, "Enzyme purification"), or all the enzymes together. The released reducing sugars were measured as previously described. Lytic activity on fungal cell walls was also monitored by observing clearing in agar plates containing those fungal cell walls (final concentration, 1 mg/ml) in 50 mM potassium acetate buffer, pH 5.5, by using a modification of the cup-plate technique reported by Tanaka and Phaff (44). After 48 h of incubation at 37°C, the plates were stained with 0.005% (wt/vol) aniline blue in 150 mM potassium phosphate buffer, pH 8.6, for 10 min at room temperature, washed once with distilled water, and stained with 0.01% (wt/vol) calcofluor in 500 mM Tris-HCl, pH 8.9, for 5 min at room temperature. The plates were faded with distilled water at room temperature several times, the hydrolytic halli were observed under long-wave ultraviolet light, and the hallus radia were measured. In all cases, enzyme and substrate blanks were included.

Analysis of β-1,6-glucanase reaction products. Pustulan (5 mg/ml) or gentiobiose (2 mg/ml) was incubated with 2 μg of the purified β-1,6-glucanase for periods of time ranging from 10 min to 16 h, in distilled water under the standard assay conditions. Substrate blanks were included in parallel. Following hydrolysis, the reactions were stopped by boiling, and the products were analyzed by high-performance liquid chromatography (HPLC). HPLC analyses were performed with an HPX-42A column (Bio-Rad) maintained at 60°C. Water was used as the eluant at a flow rate of 0.6 ml/min. Hydrolysis products were detected on the basis of their A_{195} and identified by comparison with glucose, gentibiose, and cellulose oligosaccharide (from $n = 2$ to $n = 5$) standards.

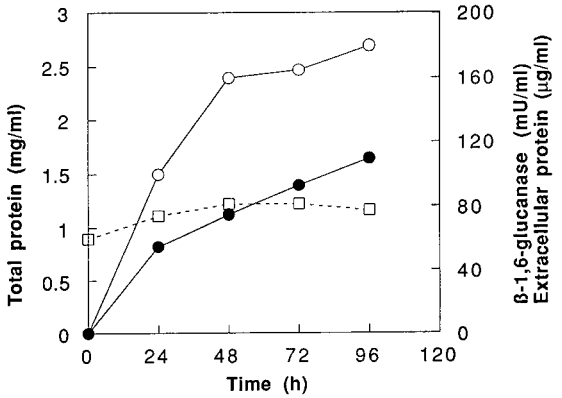

FIG. 1. Extracellular production of β-1,6-glucanase activity. Time course curve of β-1,6-glucanase activity produced by *T. harzianum* during growth in 1.5% chitin as the sole carbon source. ●, β-1,6-glucanase activity; ○, extracellular protein; □, growth, expressed as total protein.

β-1,6-Glucanase antifungal activity. The antifungal activity of the purified β-1,6-glucanase, alone or in combination with other *T. harzianum* fungal cell wall-degrading enzymes, was tested by using a modification of the bioassay in microtiter dishes described by Woloshuk et al. (50). Briefly, 96-well microtiter dishes were prepared by pipetting into wells 150 μl of Czapek medium supplemented with 2% (wt/vol) glucose as the carbon source and 2% (wt/vol) agar. A spore suspension (ca. 1,000 spores in 50 μl of distilled water) of either of the phytopathogenic fungi tested was added to the wells. After 10 to 16 h of incubation at 22°C, protein solutions in 50 mM potassium acetate buffer, pH 5.5, appropriately diluted, were added. As controls, 50 mM potassium acetate buffer, pH 5.5, was added to the wells. The microtiter dishes were incubated at 22°C again for 24 to 48 h, and growth was determined spectrophotometrically by measuring the turbidity at 690 nm in a microtiter reader (Labsystems EMS reader MF).

RESULTS

Enzyme production. β-1,6-Glucanase was produced with chitin as the carbon source after inoculation of mycelia previously grown for 96 h in glucose-supplemented minimal medium. The time course of β-1,6-glucanase production is illustrated in Fig. 1. Under the conditions used, the enzyme production was not related to mycelial growth (indicated as total protein) and increased continuously with time following a curve similar to that of extracellular protein.

When samples taken after 48 h were concentrated by ammonium sulphate precipitation, as indicated in Materials and Methods, and the crude enzyme was subjected to chromatofocusing, two peaks of β-1,6-glucanase activity were resolved (data not shown). This result suggests that the measured β-1,6-glucanase activity consists of at least two β-1,6-glucanase proteins with different pIs and also, as will be seen, with different molecular masses and immunological properties.

Enzyme purification. Protein from 48-h culture supernatants of chitin-supplemented medium was routinely used for β-1,6-glucanase protein purification. The purification procedure consisted of an adsorption-digestion step followed by chromatofocusing and, as a final step, gel filtration chromatography.

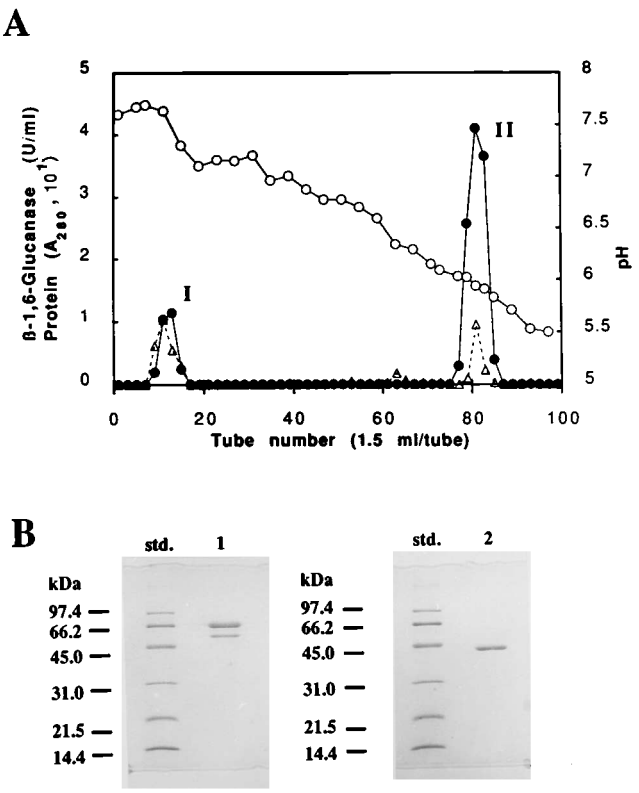

FIG. 2. Purification of the β-1,6-glucanase by chromatofocusing. (A) Elution profile of chromatofocusing on a Polybuffer exchanger column. ●, β-1,6-glucanase activity; ○, pH; △, protein at A_{280}. (B) SDS-PAGE analysis of 10 μg of protein from the pooled peaks with β-1,6-glucanase activity. The proteins were stained with Coomassie blue. Std, 2 μg of each standard protein; lane 1, pooled peak I; lane 2, pooled peak II. The numbers on the left are molecular masses of protein standards.

The elution pattern of the chromatofocusing is shown in Fig. 2A. As can be seen, two peaks equivalent to those previously obtained with ammonium sulphate (see "Enzyme production") were observed. Analysis of the pool corresponding to the more basic peak (Fig. 2A, peak I) by SDS-PAGE revealed two major protein bands with molecular masses of 66 and 51 kDa (lane 1). When these bands were resolved and identified further by basic chromatofocusing (pH, 9.4 to 6.0), SDS-PAGE activity staining, or Sephacryl S-200 HR chromatography, they turned out to be a β-1,3-glucanase and a β-1,6-glucanase, respectively (12a). These proteins were named, arbitrarily, β-1,3-glucanase-I and β-1,6-glucanase-I, respectively. The second peak revealed a major band with a molecular mass of 43 kDa (Fig. 2B, lane 2). Active fractions of this second peak were pooled, concentrated on Centricon 10 concentrators, and finally purified by gel filtration on a Sephacryl S-200 HR column. Table 1 summarizes the steps involved in a typical purification, as well

TABLE 1. Purification of a β-1,6-glucanase from *T. harzianum*

Step	Volume (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
Recovery of crude enzyme	16.0	64.0	62.2	0.97	100	1
Pustulan digestion	9.0	5.1	18.3	3.58	30	4
Chromatofocusing elution	0.45	0.16	11.6	72.5	18	75
Sephacryl S-200 HR elution	0.42	0.10	7.7	77.0	12	80

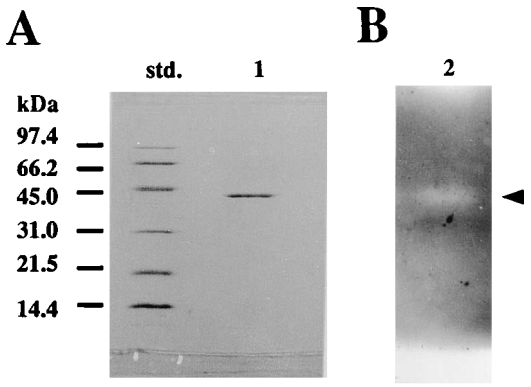

FIG. 3. SDS-PAGE analysis after the final step of β-1,6-glucanase purification. (A) Coomassie blue staining. Std, 2 μg of each standard protein; lane 1, 10 μg of purified β-1,6-glucanase. (B) β-1,6-glucanase activity detected on the agar replica of the polyacrylamide gel. Lane 2, 10 μg of purified β-1,6-glucanase (indicated by the arrowhead). The numbers on the left are molecular masses of protein standards.

as the final specific activity and yield of the purified protein. In addition, the purified β-1,6-glucanase (named, arbitrarily, β-1,6-glucanase-II) yielded a single band of protein and β-1,6-glucanase activity (Fig. 3).

Molecular and kinetic properties of purified β-1,6-glucanase. The molecular mass of the purified β-1,6-glucanase was estimated to be approximately 43 kDa by SDS-PAGE. However, when the molecular mass was determined by S-200 HR gel filtration, the value obtained was 20 to 25 kDa. An increase in the concentration of KCl from 100 to 1,000 mM in the elution buffer did not change the apparent molecular mass of 20 to 25 kDa displayed by the enzyme.

The isoelectric point, determined both by chromatofocusing and by electrofocusing, was acidic, at approximately 5.8.

The optimal temperature was determined by varying the incubation temperature at pH 5.5. The enzyme was most active at 50°C. Heat inactivation arises at temperatures equal to or greater than 50°C. Thus, pustulan seemed to stabilize the enzyme, because in its presence optimal activity was at 50°C and thermal inactivation occurred at 50°C in the absence of the substrate. The enzyme activity was measured at different substrate concentrations. A K_m of 2.4 mg of pustulan per ml and a V_{max} of 224 μmol of product/min · mg of protein were calculated.

No evidence was found for the presence of carbohydrates in the purified β-1,6-glucanase. Staining with periodic acid-Schiff or silver nitrate reagents after SDS-PAGE was negative (data not shown). Endo-H and α-mannosidase treatments were also negative, as indicated by the same electrophoretical mobilities for both intact and treated β-1,6-glucanase enzymes (data not shown). Finally, the NH_2-terminal sequence of the β-1,6-glucanase, determined with confidence up to 15 residues, was FEPALASGKTIKRGV.

Substrate specificity and action pattern. Table 2 shows the activity detected when different substrates with high and low molecular weights were used. As can be seen, the enzyme specifically hydrolyzed molecules containing the β-1,6 glucosidic bond, including *S. cerevisiae* glucan and the *p*-nitrophenyl derivate N*p*-$(Glc)_2$.

Figure 4 shows the hydrolysis products from pustulan generated by the purified enzyme at different times. Incubation of pustulan with the enzyme resulted in the production of a complete series of large oligosaccharides, which were later degraded to smaller ones, leading to gentibiose (disaccharide) as the major final product. Gentibiose was not split by the enzyme (data not shown). Thus, the action pattern on pustulan indicates an endo-type mechanism for the purified β-1,6-glucanase.

TABLE 2. Substrate specificity of the purified β-1,6-glucanase

Substrate	Main linkage type (monomer)	Activity (U/mg)
Laminarin	β-1,3 (Glc)	<0.1
Pachyman	β-1,3 (Glc)	<0.1
Glucan (*S. cerevisiae*)	β-1,3:β-1,6 (Glc)	31
Carboxymethyl cellulose	β-1,4 (Glc)	<0.1
Colloidal chitin	β-1,4 (GlcNAc)	<0.1
Glycol chitosan	β-1,4 (GlcN)	<0.1
Pustulan	β-1,6 (Glc)	86
Nigeran	α-1,3:α-1,4 (Glc)	<0.1
Soluble starch	α-1,4:α-1,6 (Glc)	<0.1
Dextran	α-1,6 (Glc)	<0.1
N*p*-$(Glc)_2$	β-1,6 (Glc)	0.2
N*p*-$(GlcNAc)_2$	β-1,4 (Glc)	<0.01
N*p*-GlcNAc	β-1,4 (Glc)	<0.01
MeUmb-Glc	β-1,4 (Glc)	<0.001

Evaluation of the biological significance of the purified β-1,6-glucanase. To establish the possible role of the purified enzyme in *Trichoderma* mycoparasitism, four sets of experiments were carried out.

In the first set, the presence of β-1,6-glucanase activity in different *Trichoderma* isolates described as mycoparasites (*T. harzianum* and *T. viride*) and nonmycoparasites (*T. koningii* and *T. reesei*) (10) was studied. At 48, 72, or 96 h of culture, β-1,6-glucanase activity was almost absent in 0.1 and 2% glucose in all the *Trichoderma* strains. In the presence of autoclaved fungal mycelia, there was an increase in all cases. However, with chitin, only in the mycoparasites was there an increase in enzyme activity similar to that induced with the autoclaved mycelia. Besides, enzyme activity was in all cases higher in mycoparasites compared with that in the nonmycoparasites (data not shown). From immunoblotting experiments with antibodies against β-1,6-glucanase-II, a correlation be-

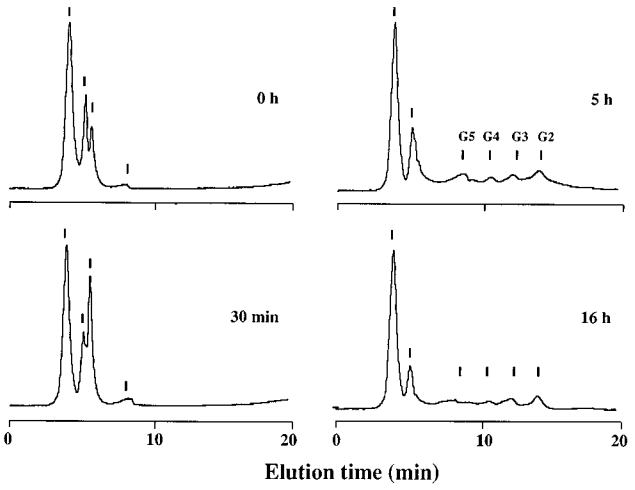

FIG. 4. HPLC analysis of β-1,6-glucanase action on pustulan. Pustulan (5 mg/ml) was incubated, as described in Materials and Methods, with 2 μg of the purified enzyme, for the times indicated. G_n, glucose oligomers (n = degree of polymerization).

TABLE 3. Clearing activity of different fungal cell wall-degrading enzymes from *T. harzianum*

Enzyme(s)[a]	Clearing activity on cell walls from[b]:			
	B. cinerea	*G. fujikuroi*	*P. syringae*	*S. cerevisiae*
β-1,6-II	0	0	0	2
β-1,3-I–β-1,6-I	0	0	0	0
CHITs	2	2	0	2
β-1,6-II + β-1,3-I–β-1,6-I	0	0	0	3
β-1,6-II + CHITs	4	7	3	3
β-1,6-II + β-1,3-I–β-1,6-I + CHITs	4	7	3	3

[a] β-1,6-II, β-1,6-glucanase-II; β-1,3-I–β-1,6-I, β-1,3-glucanase-I–β-1,6-glucanase-I; CHITs, chitinases. Two micrograms of protein was used in the assays. In combinations, the same amounts of each enzyme were used.

[b] Activity was determined as described in Materials and Methods and is expressed in mm of hallus radia. Data are the averages of four independent experiments.

tween the levels of β-1,6-glucanase activity and the amount of the purified β-1,6-glucanase-II protein was observed (data not shown).

In the second set of experiments, the fungal cell wall-degrading activity was studied with the purified protein alone or in combination with the other β-glucanases herein described and/or the chitinases described elsewhere (11). The hydrolytic activity (measured as released reducing sugars) detected using fungal cell walls from *B. cinerea*, *G. fujikuroi*, *P. syringae*, and *S. cerevisiae* indicates that the enzyme alone was active only against yeast cell walls (3.8 nmol of glucose equivalent per h) and was less efficient against the cell wall from *P. syringae* (1.4 nmol glucose equivalent per h). Additive effects on the lytic action against all the fungal cell walls tested were observed when β-1,6-glucanase-II was combined with other enzymes (data not shown).

In the third set, clearing activity in agar plates was assayed as described in Materials and Methods. Table 3 shows that β-1,6-glucanase-II alone and the combination of β-1,3-glucanase-I and β-1,6-glucanase-I did not produce clearing halli on the filamentous fungal cell walls tested. However, combinations of these enzymes with the above-mentioned chitinases or all the enzymes altogether produced clearing halli, indicating a cooperative effect of the enzymes in the degradation of the filamentous fungal cell walls.

Finally, when antifungal activity was tested, the results were similar to those previously obtained for clearing activity assays: growth of the tested fungi was slightly inhibited by the purified enzymes alone, but cooperative effects were detected when the enzymes were used in combination (Fig. 5).

Serological relationships between *T. harzianum* β-glucanases. Antiserum against the purified β-1,6-glucanase was raised in mice and used in immunoblotting experiments, the results of which are presented in Fig. 6. The serum was assayed for cross-reactivity with the β-glucanases identified in *T. harzianum* growing in chitin-supplemented medium, as well as the enzymes belonging to Novozym 234, a commercial source of cell wall hydrolytic enzymes from *T. harzianum*. As can be seen, the antiserum recognized only a 43-kDa band corresponding to the purified protein. Preimmune mice serum did not show an immunoreaction (data not shown).

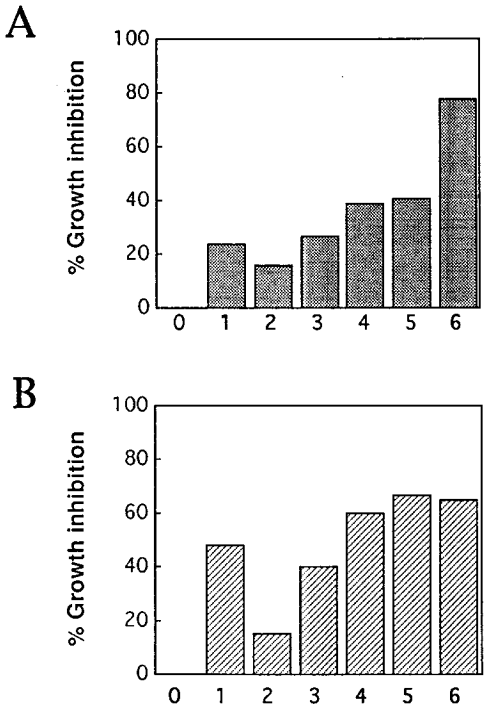

FIG. 5. Antifungal activity on *B. cinerea* and *G. fujikuroi* by β-glucanases and chitinases from *T. harzianum*. Spores of *B. cinerea* (A) and *G. fujikuroi* (B) were grown in a volume of 150 μl per microplate well for 10 to 16 h. Afterwards, enzymes in 50 μl were added to the wells. Percent inhibition shown. Each bar represents the average of four independent measurements with a relative standard deviation of less than 5%. Bars: 0, 50 mM potassium acetate buffer, pH 5.5; 1, 5 μg of protein of the purified β-1,6-glucanase-II; 2, 5 μg of protein of the β-1,3-glucanase-I–β-1,6-glucanase-I; 3, 5 μg of protein of the three chitinases; 4, 5 μg of protein containing β-1,6-glucanase-II:β-1,3-glucanase-I–β-1,6-glucanase-I (1:1); 5, 5 μg of protein containing β-1,6-glucanase-II:chitinases (1:1); 6, 5 μg of protein containing β-1,6-glucanase-II:β-1,3-glucanase-I–β-1,6-glucanase-I:chitinases (1:1:1).

DISCUSSION

The mycoparasitic action of *Trichoderma* species has been proposed as the major mechanism accounting for their antagonistic activity against phytopathogenic fungi, their lytic activity being mainly due to the β-1,3-glucanase and chitinase enzymes (31, 42). We have previously reported finding three extracellular chitinases in a mycoparasitic strain of *T. harzianum*, and a major role for the 42-kDa chitinase in mycoparasitism has been proposed (11). Because of the complexity of the fungal cell walls, we are also interested in those hydrolytic enzymes with the ability to degrade other nonabundant cell wall polysaccharides, which might, together with chitinases and β-1,3-glucanases, contribute to an effective degradation of phytopathogenic fungal cell walls. With this aim, two β-1,6-glucanases of *T. harzianum* already present in cultures containing chitin as the sole carbon source have been described and the major β-1,6-glucanase activity has been purified and characterized.

β-1,6-Glucanases in microorganisms have seldom been described (20, 33, 34, 39–41, 51). In *S. cerevisiae* extracellular exo-β-1,3-glucanases (EC 3.2.1.58) also displaying β-glucosidase and exo-β-1,6-glucanase activities have been reported (40, 48). An exo-β-1,3-glucanase that hydrolyzes both β-1,3 linkages and, less efficiently, β-1,6 linkages in *T. reesei* has also been described (1). In *T. harzianum*, the β-1,6-glucanase activity was secreted into the growth medium containing chitin,

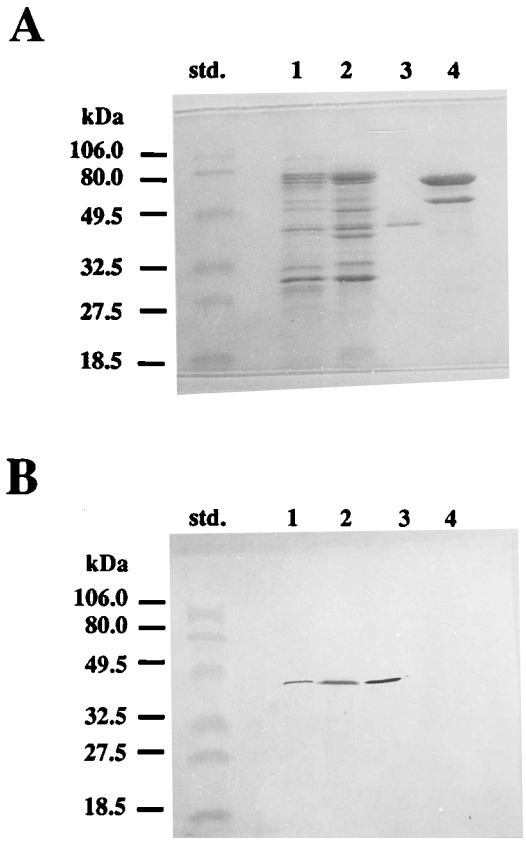

FIG. 6. Immunoblotting analysis of *T. harzianum* β-1,6-glucanases. Samples were separated by SDS-PAGE and stained with Coomassie blue (A) or electrophoretically transferred onto nitrocellulose sheets and immunodetected with the sera against the purified β-1,6-glucanase (B). Std, 2 μg of each prestained standard protein; lanes 1, 20 μg of protein from a crude enzyme; lanes 2, 20 μg of protein from Novozym 234; lanes 3, 5 μg of protein of the purified β-1,6-glucanase; lanes 4, 20 μg of protein from peak I pool (Fig. 2) containing a β-1,3- and a second β-1,6-glucanase. The numbers on the left are molecular masses.

pustulan, nigeran, fungal cell walls (12), or autoclaved mycelia as the sole carbon source. The data also suggested transcriptional induction in pustulan and catabolite repression by glucose (26). In this work, a simple, quick, and novel method has been developed for β-1,6-glucanase purification from extracellular preparations of *T. harzianum*, based on the combination of affinity adsorption and digestion followed by chromatofocusing and gel filtration. This method is also extremely specific, since one β-1,6-glucanase (β-1,6-glucanase-II) was totally purified (Fig. 2B, lane 2; Fig. 3), whereas a β-1,3-glucanase (β-1,3-glucanase-I) and a second β-1,6-glucanase (β-1,6-glucanase-I) showed a high degree of purification (Fig. 2B, lane 1). Further attempts are in progress to purify to homogeneity the two latter enzymes and to study the affinity of the β-1,3-glucanase for β-1,6-glucan. Preliminary results indicate that this enzyme is an endo-β-1,3-glucanase and is different from the above-mentioned exo-β-1,3-glucanase in *T. reesei* (12a). To our knowledge, this is the first report of purification of β-1,6-glucanase from the genus *Trichoderma.*

The purified β-1,6-glucanase has been biochemically characterized. Data from SDS-PAGE indicated an apparent molecular mass of 43 kDa. However, S-200 HR gel filtration analysis yielded a value of 20 to 25 kDa. Protease cleavage during the purification procedure did not account for this lower estimation since, once eluted for gel filtration, the protein recovered its molecular mass of 43 kDa after SDS-PAGE analysis (Fig. 3A). Instead, this result could be due to an affinity for Sephacryl support, an α-1,6-allyl dextran cross-linked with *N,N'*-methylene bisacrylamide. Others reports on fungal cell wall hydrolases that displayed affinity for Sephacryl supports, among them, the 42-kDa chitinase from *T. harzianum* (11) and a β-1,3-glucanase from *Trichoderma longibrachiatum* (45), have been described.

As would be expected for extracellular proteins, the purified β-1,6-glucanase was probably monomeric. In this sense, the enzyme maintained its activity when tested after SDS-PAGE and renaturation on agar replicas containing pustulan (Fig. 3B). This is the first time that any β-1,6-glucanase activity has been directly analyzed after SDS-PAGE. Also, this approach is useful because the molecular weight can be estimated after electrophoresis, and as previously discussed for bacterial cell wall hydrolases in a *Bacillus* sp. (32), the enzyme production can be easily studied by changing the growth conditions.

Extracellular hydrolases of fungi are normally glycosylated (52). For instance, cellulases, the best-characterized extracellular cell wall hydrolases in *Trichoderma* spp., are glycosylated (22). Our results indicate that either the purified β-1,6-glucanase is not glycosylated or that the level of glycosylation is so low as not to be detected under the conditions used. Results of NH_2-terminal sequence analysis for other β-1,6-glucanases have not yet been reported and are pertinent. Unfortunately, neither significant homology nor additional information about the sequence was detected when it was compared with the sequences present in the GenBank(GenProt)-Protein and SwissProt databases.

The enzyme purified in this work was specific for β-1,6 linkages in polysaccharides (Table 2), exhibited an endolytic activity, and lacked β-glucosidase activity since it was not able to split the disaccharide gentibiose. Therefore, the mode of action of the purified endo-β-1,6-glucanase is similar to that previously suggested for the filamentous fungus *Rhizopus chinensis* (51), and it is different from that found in *S. cerevisiae* or *Schizosaccharomyces pombe*, which have nonspecific β-1,3-glucanase, β-1,6-glucanase, and β-glucosidase activities, with an exolytic mode of action (40).

With regard to its biological significance, the following data strongly support the implication of the β-1,6-glucanase activity and specifically of the β-1,6-glucanase-II protein in *Trichoderma* mycoparasitism. First, β-1,6-glucanase activity levels are higher in mycoparasitic than in nonmycoparasitic *Trichoderma* strains, with either chitin, autoclaved yeast cell walls, or phytopathogenic fungal mycelia. Results also suggest a different regulation of the β-1,6-glucanase activity in the mycoparasitic strains, which could be induced by conditions described as simulating mycoparasitism (mycelia as carbon sources) (19). Because of the presence of, at least, two isoenzymes with β-1,6-glucanase activity, the induction of the β-1,6-glucanase-II protein was tested with specific antibodies, and a correlation between levels of β-1,6-glucanase activity and β-1,6-glucanase-II protein was observed. Secondly, the release of reducing sugars and the clearing hallus formation (Table 3) from *S. cerevisiae* and *P. syringae* cell walls (fungi with β-glucans as the main components of their cell walls; [2]) indicate that the enzyme alone is able to hydrolize β-1,6-glucans present in these cell walls. When other fungi were used, this activity could not be detected. This result would probably be due to differences in the accessibility of the enzyme to the β-1,6-glucans in the different fungal cell walls. The coordinated activity of hydrolases able to degrade different polymers would lead to an effective breakdown of fungal cell walls, and additive and/or synergistic effects could be expected when different

combinations of lytic enzymes act together, even when no effects are detected with each enzyme alone. Synergistic effects between fungal cell wall hydrolases have previously been reported; a cooperative action by several exo- and endo-β-1,3-glucanases during the degradation of cell wall glucans of *Sclerotinia sclerotiorum* by *T. viride* has been suggested (21). In order to demonstrate this result in our system, combinations of the purified β-1,6-glucanase-II with other cell wall-degrading enzymes were made and the release of reducing sugars and the clearing activity on different fungal cell walls were assayed. A cooperative action of the different hydrolases acting together was detected (data not shown for the release of reducing sugars; Table 3). Finally, the antifungal activity of the β-1,6-glucanase-II enzyme and other hydrolases from *T. harzianum* was tested. An effective inhibition of *B. cinerea* and *G. fujikuroi* was mediated by β-1,6-glucanase-II alone and strengthened by the combination of this enzyme with other fungal cell wall hydrolases (Fig. 5). These results are in agreement with data from experiments in which some plant chitinases, with antifungal activity, inhibited the growth of only some phytopathogenic fungi when combined with β-1,3-glucanase (25, 28). In other parasitic systems, such as that used by entomopathogenic fungi, a conjunction of proteases with chitinases is required during the penetration of insect cuticles (32).

Finally, when antibodies against the purified proteins were assayed by Western blotting (immunoblotting), they did not exhibit cross-reactivity with other β-glucanases already present in extracellular crude preparations and in the enzymatic components of Novozym 234 (Fig. 6). These results also indicated that the 43-kDa purified protein was not a proteolytic extracellular major product of 51 kDa of the β-1,6-glucanase protein. So, the two β-1,6-glucanases are different and are probably encoded by different genes. Different genes have also been suggested to code for the three chitinases of *T. harzianum* (11, 18) and demonstrated to code for the cellulases of *T. reesei* (22). At the present time, we have cloned and molecularly characterized the gene corresponding to β-1,6-glucanase-II, named *bgn 16.2* (26) and that corresponding to the 42-kDa chitinase from *T. harzianum* (18). The introduction of these genes into a biological antagonist strain which strongly expressed them could be useful in the development of a transgenic *T. harzianum* with enhanced mycoparasitic activity.

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