View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by idUS. Depósito de Investigación Universidad de Sevilla

JOURNAL OF BACTERIOLOGY, Dec. 1995, p. 6937–6945
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology
Vol. 177, No. 23

A Novel Endo-β-1,3-Glucanase, BGN13.1, Involved in the Mycoparasitism of *Trichoderma harzianum*

JESÚS DE LA CRUZ,[1] JOSÉ A. PINTOR-TORO,[2] TAHÍA BENÍTEZ,[3] ANTONIO LLOBELL,[1]* AND LUIS C. ROMERO[2]

Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Cientificas y Universidad de Sevilla,[1] Instituto de Recursos Naturales y Agrobiología, Consejo Superior de Investigaciones Cientificas,[2] and Departamento Genética, Universidad de Sevilla,[3] Seville, Spain

Received 20 June 1995/Accepted 25 September 1995

The mycoparasitic fungus *Trichoderma harzianum* CECT 2413 produces at least three extracellular β-1,3-glucanases. The most basic of these extracellular enzymes, named BGN13.1, was expressed when either fungal cell wall polymers or autoclaved mycelia from different fungi were used as the carbon source. BGN13.1 was purified to electrophoretic homogeneity and was biochemically characterized. The enzyme was specific for β-1,3 linkages and has an endolytic mode of action. A synthetic oligonucleotide primer based on the sequence of an internal peptide was designed to clone the cDNA corresponding to BGN13.1. The deduced amino acid sequence predicted a molecular mass of 78 kDa for the mature protein. Analysis of the amino acid sequence indicates that the enzyme contains three regions, one N-terminal leader sequence; another, nondefined sequence; and one cysteine-rich C-terminal sequence. Sequence comparison shows that this β-1,3-glucanase, first described for filamentous fungi, belongs to a family different from that of its previously described bacterial, yeast, and plant counterparts. Enzymatic-activity, protein, and mRNA data indicated that *bgn13.1* is repressed by glucose and induced by either fungal cell wall polymers or autoclaved yeast cells and mycelia. Finally, experimental evidence showed that the enzyme hydrolyzes yeast and fungal cell walls.

β-1,3-Glucanases are widely distributed among bacteria, fungi, and higher plants (49), and many of them have been purified and characterized (10, 26, 40, 63, 73). They are classified as exo-β-1,3-glucanases (β-1,3-glucan glucanohydrolase [EC 3.2.1.58]) and endo-β-1,3-glucanases (β-1,3-glucan glucanohydrolase [EC 3.2.1.6 or EC 3.2.1.39]). The corresponding β-1,3-glucanase genes from the aforementioned organisms have also been isolated and characterized, with the exception of genes from filamentous fungi (29, 56, 58, 61, 70, 73). The physiological functions of β-1,3-glucanases depend on their source. In plants, these enzymes are thought to be a type of defense system against fungal pathogens (25, 37), although roles in cell differentiation have also been suggested (6, 20, 42). In bacteria, which lack β-1,3-glucan, a nutritional role has been assigned (73). In fungi, β-1,3-glucanases seem to have different functions. First, a physiological role in morphogenetic-morpholytic processes during fungal development and differentiation has been indicated (47). Second, β-1,3-glucanases have been related to the mobilization of β-glucans under conditions of carbon and energy source exhaustion, functioning as autolytic enzymes (48, 62). β-1,3-Glucanases are also involved in fungal pathogen-plant interactions, degrading callose (β-D-1,3-glucan) in the host's vascular tissues during pathogen attack (56). Finally, a nutritional role in saprophytes and mycoparasites has been suggested (9, 35, 59).

Soilborne fungi of the genus *Trichoderma* have been especially studied for both their cellulolytic activity (38) and their antagonistic properties against fungal plant pathogens (46). The degradation and further assimilation of phytopathogenic fungi, namely, mycoparasitism, has been proposed as the major mechanism accounting for their antagonism against plant pathogens (19). A number of *Trichoderma* isolates produce hydrolytic enzymes such as chitinases, β-1,3- and β-1,6-glucanases, and proteases when grown on laminarin (β-1,3-glucan), chitin, or fungal cell walls as the carbon source (14, 19). This observation, together with the fact that chitin and β-1,3-glucan are the main structural components of fungal cell walls (47), suggests that chitinases and β-1,3-glucanases produced by some *Trichoderma* spp. are the key enzymes in the lysis of cell walls during their mycoparasitic action against phytopathogenic fungi (12, 13, 19, 58).

In this study, we report the purification, molecular cloning, and characterization of an endo-β-1,3-glucanase, namely, BGN13.1, from the mycoparasitic fungus *Trichoderma harzianum*, which has been described as an agent of biological control of phytopathogenic fungi (11). Expression of the gene and enzyme secretion occur when *T. harzianum* grows in fungal cell wall components, and these processes are specifically induced by growing the fungus in autoclaved mycelia from different fungi. These conditions have been described as simulating mycoparasitism (24, 34). Also, on the basis of sequence comparison, BGN13.1 may be classified as a new class of β-1,3-glucanase enzyme. A possible role of this enzyme in mycoparasitism is discussed.

MATERIALS AND METHODS

Fungal material and growth conditions. *T. harzianum* CECT 2413 (Spanish Type Culture Collection, Valencia, Spain) was used in this study. Potato dextrose agar was used for the maintenance of the culture. Production of the enzyme was carried out under the two-step culture conditions previously described (14).

Autoclaved cells from *Saccharomyces cerevisiae* (baker's yeast; La Cinta Roja, Seville, Spain) or mycelia from *Botrytis cinerea* CECT 2100, *Giberella fujikuroi* IMI 58289 (Imperial Mycological Institute, Kew, Surrey, United Kingdom), *Phytophthora syringae* CECT 2351, *Rhizoctonia solani* CECT 2815, or *T. harzianum* CECT 2413 were used as carbon sources. Mycelia from the different filamentous fungi were obtained as described elsewhere (33). Mycelia were lyophilized and added to *T. harzianum* second-step cultures.

β-1,3-Glucanase assay and protein determination. β-1,3-Glucanase activity was determined by measuring the amount of reducing sugar released from

* Corresponding author. Mailing address: Instituto de Bioquímica Vegetal y Fotosintesis, CSIC y Universidad de Sevilla, Apartado 1113, E-41080 Seville, Spain. Phone: (34) (5) 4557080. Fax: (34) (5) 4620154.

Downloaded from http://jb.asm.org/ on July 25, 2016 by USE/BCTA.GEN UNIVERSITARIA

laminarin (Sigma Chemical Co., St. Louis, Mo.). The standard assay mixture (1 ml) contained enzyme solution properly diluted, 4 mg of laminarin, and 50 mM potassium acetate buffer, pH 5.5. The reactions were run for 30 min at 37°C and then stopped by boiling for 5 min, and the reducing-sugar content was determined by the procedure of Somogyi (60) and Nelson (43). Enzyme and substrate blanks were also included. A unit of β-1,3-glucanase activity is defined as the amount of enzyme catalyzing the release of 1 μmol of glucose equivalent per min.

Protein concentration was measured by the method of Bradford, with ovalbumin as the standard (5). For mycelium growth determination, total protein was measured as described elsewhere (14, 36).

Enzyme purification. (i) Step 1. Unless otherwise indicated, all enzyme purification steps were carried out at 4°C. Supernatant fluids (200 ml) from 48-h *T. harzianum* chitin cultures were obtained by filtering the media through Whatman no. 1 paper and centrifuging at 10,000 × *g* for 10 min. The supernatant was then precipitated with $(NH_4)_2SO_4$ (80% saturation) at 4°C for 2 h. The precipitate was recovered by centrifugation (12,000 × *g* for 20 min at 4°C), dissolved in the minimal amount of distilled water, and dialyzed extensively against 2 liters of 50 mM potassium acetate buffer, pH 5.5.

(ii) Step 2. Dialyzed aliquots were then adsorbed to pustulan (β-1,6-glucan; Calbiochem, La Jolla, Calif.) as described elsewhere (13). Adsorbed fractions were washed three times with 1 M NaCl–70 mM phosphate buffer, pH 6.0; resuspended in 50 mM potassium acetate buffer, pH 5.5, containing 1 mM sodium azide and 1 mM phenylmethylsulfonyl fluoride; and incubated at 37°C overnight. Clarified solutions, after pustulan digestion, were dialyzed extensively against 2 liters of 25 mM imidazole-HCl buffer, pH 7.4.

(iii) Step 3. The dialyzed solution (named pustulan digestion) was subjected to chromatofocusing (pH 7.4 to 4) on a Polybuffer exchanger column, PBE 94 (1 by 20 cm; Pharmacia, Uppsala, Sweden), equilibrated in 25 mM imidazole-HCl buffer, pH 7.4. Protein elution and gradient conditions were exactly as described elsewhere (13). Most active fractions were pooled and concentrated to approximately 0.5 ml on Centricon 10 concentrators (Amicon, Beverley, Mass.).

(iv) Step 4. The concentrated pool was filtered through a Sephacryl S200 HR column (1.6 by 40 cm; Pharmacia) equilibrated with 1 M NaCl–100 mM potassium acetate buffer, pH 5.5. Elution was performed in the same buffer at a flow rate of 7 ml/h, collecting fractions of 0.65 ml. Most active fractions were pooled, washed, and concentrated in 50 mM potassium acetate buffer, pH 5.5, on Centricon 10 concentrators and finally stored at −20°C.

Basic chromatofocusing. Basic chromatofocusing was performed by using a column (1 by 20 cm) of PBE 94 resin equilibrated in 25 mM ethanolamine-NaOH, pH 9.4. The column was eluted at a flow rate of 9 ml/h with Polybuffer 96, which had been diluted eightfold with water and adjusted to pH 9.4 with NaOH. The chromatofocusing was monitored by measuring the pH of the eluted fractions.

Gel electrophoresis. Electrophoresis under denaturing conditions (sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) was performed by the method of Laemmli (31) with 4% acrylamide in the stacking gel and 12% acrylamide in the separating gel. Gels were stained with Coomassie R-250 brilliant blue. Low-molecular-mass standard proteins (Bio-Rad, Hercules, Calif.) were used for molecular mass determination.

Glycoprotein stain assays were done in the gels by using either the periodic acid-Schiff reagent procedure (Sigma) or the silver nitrate procedure (18). Extracellular yeast invertase (Sigma) was used as a glycoprotein positive control. Also, for estimating possible mobility changes, deglycosylation reactions prior to SDS-PAGE were performed with endo-β-*N*-acetylglucosaminidase) H (endo-H) (Boehringer, Mannheim, Germany) (70) or with α-mannosidase (74). The presence of carbohydrates in the protein was also estimated by the binding of purified β-1,3-glucanase to various amounts of concanavalin A-Sepharose 4B (Pharmacia) (54).

Isoelectrofocusing was performed at a pI range of 3.5 to 10 as described elsewhere (51). Proteins were visualized by Coomassie staining. β-1,3-Glucanase activity was detected after isoelectrofocusing by using Ampholine PAG plates, pH 3.5 to 9.5 (Pharmacia), in a Multiphor system (Pharmacia) according to the manufacturer's instructions. The activity was assayed by using the laminarin–2,3,5-triphenyltetrazolium chloride method previously described (45). The reagents were purchased from Sigma.

Western blots (immunoblots) were incubated with antiserum against *T. harzianum* β-1,6-glucanase II (13) or antiserum for tobacco β-1,3-glucanase anti-PR2 or tobacco basic β-1,3-glucanase (26). Immunological detection of the antigen-antibody complexes was carried out as described previously (13). Antibodies against tobacco β-1,3-glucanases were a generous gift from B. Fritig, Institut de Biologie Moléculaire des Plantes, Strasbourg, France.

N-terminal and internal amino acid peptide sequencing. N-terminal and internal amino acid peptide sequencing was performed by using an Applied Biosystem 470 A Gas Phase Protein Sequencer, equipped with a 120 A on-line phenylthiohydantoin analyzer, as described elsewhere (4).

Kinetic parameters, temperature optimum, and thermal stability. Michaelis-Menten constants were determined from Lineweaver-Burk representations of data obtained by measuring the initial rate of laminarin hydrolysis under the assay conditions described above and using a range of 20 to 0.5 mg of laminarin per ml. The effect of δ-gluconolactone (Sigma) on the activity of the purified enzyme was measured as described previously (44).

The temperature optimum and thermal stability were determined as described elsewhere (13), by adding laminarin as the substrate. The inactivation temperature was defined as the one at which the specific activity was reduced by 50% under the conditions described above.

Substrate specificity and analysis of β-1,3-glucanase reaction products. The spectrum of substrates hydrolyzed by the enzyme was tested with other α- and β-glucans at a final concentration of 5 mg/ml. Reaction products were determined by the standard assay described above.

β-1,3-Glucanase reaction products were also determined by high-performance liquid chromatography (HPLC) on an HPX 42-A column (Bio-Rad) at 60°C as described elsewhere (13).

Hydrolysis of fungal cell walls. Lytic activity by the β-1,3-glucanase on fungal cell walls was monitored as described previously (13, 65).

Extraction and blotting analysis of DNA and RNA. Total DNA from *T. harzianum* was prepared as described elsewhere (41) and analyzed by Southern blotting under conditions described previously (32).

Total RNA was isolated according to the method of Wadsworth et al. (71). RNA was separated in 1% agarose–formaldehyde gels by standard protocols (53, 71). During Northern (RNA) analysis, the amount of RNA loaded onto the gel was monitored by methylene blue staining of the membranes. Equal amounts of RNA were loaded into the wells.

PCR conditions and screening of the cDNA library. A cDNA library on λgt11-*Sfi-Not* was constructed from poly(A)$^+$ RNA isolated from *T. harzianum* grown for 33 h on a chitin medium as a second-step culture (32). Total DNA was isolated from this library and used as a template for PCR using as a sense primer a degenerated oligonucleotide [5′-AATACICA(A/G)AATGGITATACIG GIGG-3′] corresponding to an internal sequenced peptide of the protein (p12) and as an antisense primer a commercial λgt11 reverse primer. The amplification protocol used was as follows: 95°C, 1 min; 57°C, 1.5 min; 72°C, 2.0 min. This standard cycle was repeated 35 times and was followed by incubation at 72°C for 5 min. The single amplified band was purified from 0.8% agarose gels, subcloned into pGEM-T vector (Promega), and analyzed by sequencing.

Approximately 4 × 10^4 PFU from the λgt11-*Sfi-Not* cDNA library was screened by plaque hybridization, with the ^{32}P-labeled PCR-amplified cDNA fragment described above used as a probe. Hybridizations were carried out by a standard protocol previously described (32).

DNA sequencing. Nucleotide sequencing was performed by the dideoxynucleotide chain termination method (55) with a Pharmacia ALF automatic sequencing system. Computer analyses of sequencing data were performed by using the University of Wisconsin Genetics Computer Group programs (15).

Nucleotide sequence accession number. The nucleotide sequence reported in this article has been submitted to the GenBank/EMBL Data Bank under accession number X84085.

RESULTS

Enzyme production. Enzyme production was attempted under two-step culture conditions. In the first step, minimal medium containing 10% glucose (repression conditions) was used to grow the mycelia; in the second step, minimal medium containing the selected carbon source (induction conditions) was used and the levels of extracellular β-1,3-glucanase activity were measured. Table 1 shows the β-1,3-glucanase-specific activity found after 48 h of induction. The β-1,3-glucanase activity was detected in abundance in chitin, pustulan, and autoclaved yeast cells and in filamentous fungal mycelia used as carbon sources. Activity, though less abundant, was also detected when 0.1 and 2% glucose, 1% laminarin, and chitin supplemented with 2% glucose were used; finally, at a concentration of 10% glucose there was no detection of the activity. Results, therefore, suggest repression at a high glucose concentration, basal activity at a lower glucose concentration, and induction in the presence of fungal cell wall polymer sources, yeast cells, or fungal mycelia.

In order to test whether the induction detected in these experiments is due to the presence of different β-1,3-glucanase isoenzymes, enzymatic detection on isoelectrofocusing gels was carried out with ammonium sulfate-concentrated supernatants of the previous cultures after 48 h of induction. As Fig. 1 shows, no β-1,3-glucanase isoforms were detected in the presence of 2 and 10% glucose. Under starvation conditions (0.1% glucose), a slight basic band was occasionally detected; in the presence of different fungal cell wall polysaccharides, the basic isoenzyme and at least three additional acidic β-1,3-glucanases

Downloaded from http://jb.asm.org/ on July 25, 2016 by USE/BCTA.GEN UNIVERSITARIA

TABLE 1. Extracellular β-1,3-glucanase activity production by *T. harzianum* pregrown in 10% glucose and incubated for 48 h with different carbon sources

Carbon source[a] (% [wt/vol])	β-1,3-Glucanase activity (mU/mg of protein)
Glc (0.1)	10
Glc (2)	75
Glc (10)	BD[b]
Chit (1)	640
Chit (1) + Glc (2)	50
Lam (1)	78
Pust (1)	340
Yeast (1)	1,000
Bot (1)	800
Gib (1)	790
Rhizo (1)	785
Phyto (1)	700
Tricho (1)	670

[a] Glc, glucose; Chit, chitin; Lam, laminarin; Pust, pustulan; Yeast, autoclaved cells from *S. cerevisiae*; Bot, autoclaved mycelia from *B. cinerea*; Gib, autoclaved mycelia from *G. fujikuroi*; Rhizo, autoclaved mycelia from *R. solani*; Phyto, autoclaved mycelia from *Phytophthora citrophthora*; Tricho, autoclaved mycelia from *T. harzianum.*

[b] BD, below limit of detection (<0.5 mU/mg of protein).

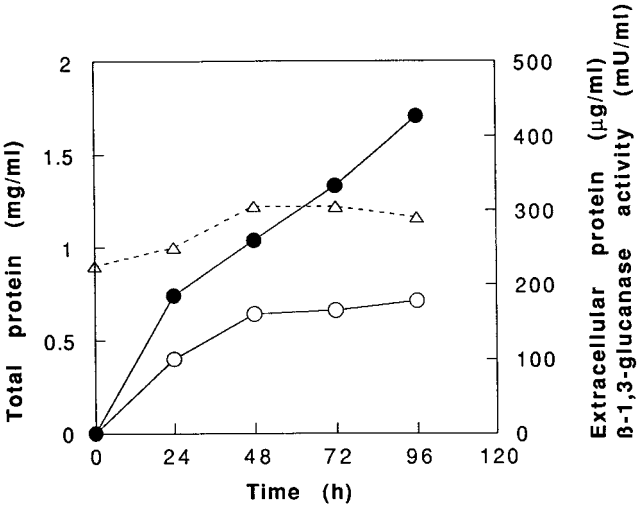

FIG. 2. Analysis of extracellular production of β-1,3-glucanase activity from *T. harzianum* growing in chitin. Shown is a time course curve of β-1,3-glucanase activity produced by *T. harzianum* during growth in 1.5% chitin as the sole carbon source. ●, β-1,3-glucanase activity; ○, extracellular protein; △, growth expressed as total protein.

were found. Finally, in the presence of either autoclaved yeast cells or different autoclaved filamentous fungi the basic isoform was preferentially induced. Thus, the measured β-1,3-glucanase activity is due to at least four proteins, the most basic enzyme being specifically induced under conditions that could simulate mycoparasitism.

Commercially available chitin was a good inducer of β-1,3-glucanase activity (Table 1). Figure 2 shows mycelial growth and β-1,3-glucanase production during the 96 h of induction in chitin as the carbon source. The appearance of extracellular β-1,3-glucanase activity was time dependent but was not related to mycelial growth. In addition, three peaks of β-1,3-glucanase activity (pI of >7.4, pI of 6.2, and pI of 5.3, respectively) were resolved when concentrated extracellular samples were fractionated by acidic chromatofocusing (Fig. 3). Results indicated that in this case the measured β-1,3-glucanase activity is due to at least three proteins, the most basic being also the most active one. For these reasons, further studies were focused on the basic β-1,3-glucanase isoform, namely, BGN13.1 (β-glucanase-1,3 first isoform).

Purification of BGN13.1 and size determination. Purification of BGN13.1 was based on the affinity of β-glucanases on particulated β-glucans. The supernatants of chitin-supplemented cultures (200 ml) were concentrated by 80% saturation ammonium sulfate, and concentrated proteins were then subjected to pustulan adsorption and further digestion. Most of the recovered enzymatic activity in the pustulan digestion was β-1,6-glucanase, as expected with an affinity adsorption, but 7% of the total β-1,3-glucanase activity was also present. Acidic chromatofocusing separated the pustulan digestion into two fractions, a nonadsorbed fraction which retained 10 to 15% β-1,6-glucanase activity and the total β-1,3-glucanase activity and a second fraction as a peak at pH 5.8, which contains the rest of the β-1,6-glucanase activity (13). Active fractions of the nonadsorbed volume were pooled, concentrated by using Centricon 10 concentrators, and finally purified by gel filtration on a Sephacryl S-200 HR chromatography column. Figure 4 shows the elution pattern for β-1,3-glucanase and β-1,6-glucanase found by S-200 HR chromatography, and Table 2 summarizes the purification procedure. The final purified prepa-

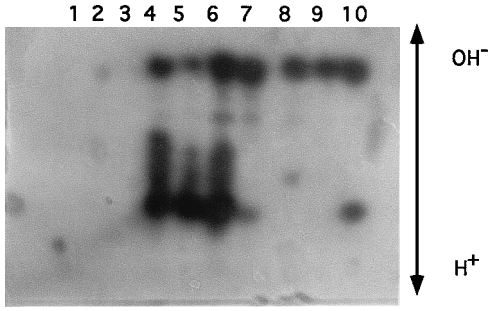

FIG. 1. β-1,3-Glucanase isoenzymes on isoelectrofocusing gels. Carbon sources used were as follows: lane 1, glucose, 0.1%; lane 2, glucose, 2%; lane 3, glucose, 10%; lane 4, chitin, 1%; lane 5, laminarin, 1%; lane 6, pustulan, 1%; lane 7, autoclaved yeast cells, 1%; lane 8, autoclaved mycelia from *B. cinerea*, 1%; lane 9, autoclaved mycelia from *G. fujikuroi*, 1%; lane 10, autoclaved mycelia from *T. harzianum*, 1%. Activity was developed as indicated in Materials and Methods.

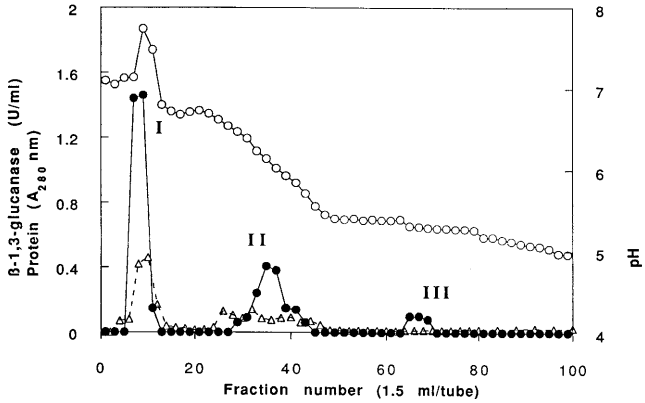

FIG. 3. Chromatofocusing analysis of extracellular β-1,3-glucanase isoenzymes from *T. harzianum* growing with chitin. An 80% $(NH_4)_2SO_4$ fraction from a 48-h chitin *T. harzianum* culture fluid was loaded onto the column. ●, β-1,3-glucanase activity; △, protein measured as A_{280}; ○, pH.

Downloaded from http://jb.asm.org/ on July 25, 2016 by USE/BCTA.GEN UNIVERSITARIA

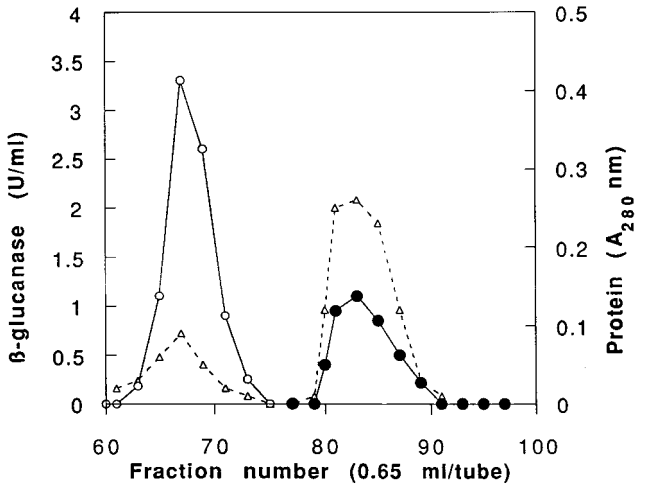

FIG. 4. Gel filtration chromatography through a Sephacryl S-200 HR column. ●, β-1,3-glucanase activity; ○, β-1,6-glucanase activity; △, protein measured as A_{280}.

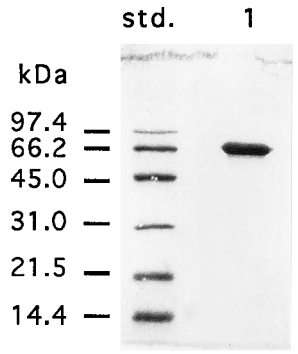

FIG. 5. SDS-PAGE of the purified endo-β-1,3-glucanase. std., molecular mass standards (10 μg); 1, purified β-1,3-glucanase (10 μg). Proteins were stained with Coomassie blue. Numbers on the left refer to molecular masses of protein standards.

ration migrated as a single band on SDS-PAGE, indicating a homogeneous protein with an apparent molecular mass of 66 kDa (Fig. 5). When the molecular mass was calculated by gel filtration, after calibration of the Sephacryl S-200 HR column, the value obtained was 5 to 10 kDa.

Biochemical analysis of the purified BGN13.1. The isoelectric points of the pure enzyme, determined by both electrofocusing and basic chromatofocusing (pH range, 9.4 to 6), were estimated to be pH 7.7 and pH 8.0, respectively. Data were confirmed after enzymatic determination in isoelectrofocusing gels, since the pI of the purified enzyme was a basic one and around pI 8.0 (data not shown).

Purified BGN13.1, subjected to SDS-PAGE, was not stained by the silver nitrate carbohydrate procedure (18) or by the Schiff's reagent (results not shown). endo-H or α-mannosidase treatment also failed to alter the apparent size on SDS gels (results not shown). Also, the enzyme was not retained by concanavalin A-Sepharose at concentrations from 0 to 1.4 mg/ml (results not shown). Positive controls with highly glycosylated extracellular yeast invertase were included for the experiments described in this section.

The optimum temperature for the enzymatic activity was 40°C. After a 30-min preincubation at various temperatures, the inactivation temperature was 55°C. The K_m value with laminarin as the substrate was 3.3 mg/ml. At the same time, a V_{max} of 75 μmol of product per min · mg of protein was calculated.

The purified β-1,3-glucanase was not detected by immunoblotting with either an antiserum against plant acidic extracellular β-1,3-glucanase or basic vacuolar β-1,3-glucanase (data not shown). Furthermore, there was no cross-reaction when anti-*T. harzianum* β-1,6-glucanase II antiserum was used.

N-terminal and internal amino acid sequences. The NH_2-terminal sequence, determined with a confidence level up to 11 residues, is shown in Table 3. Four internal peptide sequences are also shown.

Substrate specificity and hydrolytic action. As shown in Table 4, the enzyme specifically hydrolyzed molecules containing the β-1,3-glucoside bond, including laminarin and periodate-oxidized laminarin, pachyman, and yeast glucan. A slight activity was also found with pustulan (a β-1,6-glucan), probably because of β-1,3 bonds in the pustulan branch points. Slight hydrolysis was observed with the assayed filamentous fungus or yeast purified cell walls. In addition, no clearing activity for the β-1,3-glucanase protein alone, measured as described in Materials and Methods, was observed in agar plates containing fungal cell walls (data not shown). However, when the enzyme was combined with *T. harzianum* β-1,6-glucanases I and II, activity was detected in agar plates containing yeast cell walls (13).

The mode of action of BGN13.1 toward laminarin was examined by measuring the rate of glucose production versus that of reducing-sugar production at different times and also analyzing the corresponding hydrolysis products by HPLC (Fig. 6). Incubation of laminarin with the enzyme resulted in the production of larger amounts of reducing sugars than of glucose (data not shown). After HPLC analysis, a series of oligosaccharides was detected, laminaritetraose, laminaribiose, and glucose being the final products of hydrolysis. When the enzyme was incubated with laminaribiose, no further hydrolysis was obtained (results not shown). In addition, no inhibition was observed with δ-gluconolactone (1 to 20 mM), which has been described as a potent inhibitor of exo-β-glucanases at low concentrations (44). Thus, all these results indicate an endo-type mode of action for the purified enzyme.

Molecular cloning of the *bgn13.1* gene. The molecular cloning of the β-1,3-glucanase *bgn13.1* gene was attempted by PCR techniques, on the basis of the amino acid sequence of an

TABLE 2. Purification of a β-1,3-glucanase from *T. harzianum*

Step	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (fold)
80% $(NH_4)_2SO_4$	18.0	121	428	3.5	100	1
Pustulan digestion	9.0	4.5	29	6.4	7	2
Chromatofocusing eluate	0.4	0.6	10	16.6	2.3	5
Sephacryl S-200 HR eluate	0.4	0.05	2	40.0	0.5	12

Downloaded from http://jb.asm.org/ on July 25, 2016 by USE/BCTA.GEN UNIVERSITARIA

TABLE 3. NH_2-terminal and peptide sequences from BGN3.1

Peptide	Sequence[a]
N terminal	ATSFYYFNMDHV
p5	NPTYGDVTSSNTRPSALAPG
p12	LGXGTFINTQNGYTGGXTPGGGDVXP
p13	FNTDTILMGDPTN
p14	XVGEA(M)ATITGNGNFFK

[a] The most probable amino acids are in parentheses. X, unknown amino acid.

internal peptide of the protein. For PCRs, DNA from a unidirectional cDNA library in λgt11-*Sfi-Not* vector was used as the template and a degenerate oligonucleotide, corresponding to the internal sequence NTQNGYTGG (p12 [Table 3]), and the λgt11 reverse primers were used. Under the conditions described in Materials and Methods, a single 250-bp fragment was repetitively amplified and was further subcloned and sequenced. The amplified DNA was the C-terminal region of the β-1,3-glucanase protein, corresponding mostly to the 3′ untranslated region of the gene. This PCR-amplified band was used as a probe for screening the aforementioned cDNA library. Upon screening around 40,000 plaques, several positive clones were selected and isolated and the one containing the longest insert was chosen for further characterization. The cDNA insert of this phage (named *bgn13.1*) was cloned in the *Eco*RI-*Not*I sites of the pBluescript SKII (+) vector (Promega) and then was sequenced.

Sequence analysis and homologies with other β-glucanases. Nucleotide and amino acid sequences are shown in Fig. 7. The cloned cDNA has a size of 2,484 bp, of which 32 bp corresponds to the 5′ untranslated region, 164 bp corresponds to the 3′ untranslated region, and 2,288 bp corresponds to the unique open reading frame present in the cDNA. Translation of the open reading frame indicates that the *bgn13.1* gene product (BGN13.1) has 762 amino acids with a predicted molecular mass of 81,246 Da. Comparison of the deduced sequence with the N-terminal amino acid sequence from the isolated β-1,3-glucanase protein (Table 3) indicates that the mature protein starts at residue 34, immediately preceded by the basic residues Lys and Arg, which have been described as the recognition sequence of KEX2-like endoproteinases (22). Therefore, the mature β-1,3-glucanase contains 728 amino acid residues and has a predicted molecular mass of 77,972 Da and an estimated isoelectric point of 7.3. The predicted protein contains the five peptides sequenced from the purified protein, demonstrating that the *bgn13.1* gene codes for the β-1,3-glucanase described above (Fig. 7 and Table 3).

TABLE 4. Substrate specificity of the purified β-1,3-glucanase from *T. harzianum*

Substrate[a]	Main linkage type (monomer)	β-1,3-Glucanase activity[b] (%)
Laminarin	β-1,3 (Glc)	100
IO_4-oxidized laminarin	β-1,3 (Glc)	100
Glucan (*S. cerevisiae*)	β-1,3 (Glc)	97
Pachyman	β-1,3 (Glc)	46
Pustulan	β-1,3:β-1,6 (Glc)	5
Carboxymethyl cellulose	β-1,4 (Glc)	0
Colloidal chitin	β-1,4 (GlcNAc)	0
Glycol chitosan	β-1,4 (GlcN)	0
Nigeran	α1,3:α-1,4 (Glc)	0
Soluble starch	α-1,4:α-1,6 (Glc)	0
Dextran	α-1,6 (Glc)	0
B. cinerea cell walls	Glucan:chitin	1
G. fujikuroi cell walls	Glucan:chitin	1
P. syringae cell walls	Glucan:cellulose	4
S. cerevisiae cell walls	Glucan	2

[a] All substrates were used at a concentration of 5 mg/ml.
[b] 100% activity corresponds to 28 U/mg of protein.

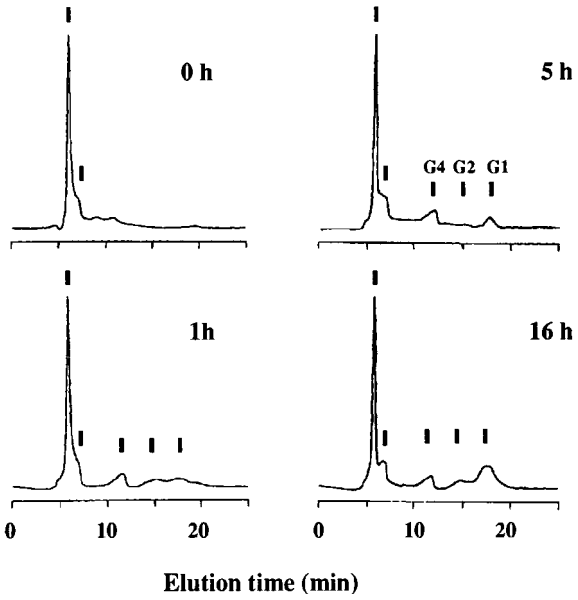

FIG. 6. HPLC analysis of β-1,3-glucanase action on laminarin. Laminarin (5 mg/ml) was incubated as described in Materials and Methods with 2 μg of the purified enzyme, for the indicated times. G1, G2, and G4 indicate glucose oligomers; numbers indicate the degree of polymerization.

Comparison of the nucleotide and amino acid sequences of BGN13.1 with sequences present in the databases does not show any significant homology to any α- or β-glucanases or other related protein families. This result clearly indicates that BGN13.1 represents a new class of β-1,3-glucanase. In fact, alignment of BGN13.1 with known β-1,3-glucanase sequences from bacteria, yeast strains, and plants shows that BGN13.1 has none of the conserved domains present in the other proteins. A phylogenetic tree created with the algorithms DISTANCE and GROWTREE by comparison of 63 glucanase sequences indicates that BGN13.1 is taxonomically far from sequences of bacterial, yeast, or plant β-1,3-glucanase families; that it is not related to either the β-glucosidase (BGL1 [3]) or the β-1,6-glucanase II (BGN16.2 [33]) genes from *Trichoderma* spp., and that it may more likely be related to *Trichoderma* cellulases (data not shown).

Analysis of the protein clearly distinguishes three different regions. The first 33 amino acids mark a hydrophobic sequence and correspond to the leader peptide responsible for secretion and possible KEX2-mediated activation of the protein. It had been demonstrated that several secreted enzymes are synthesized in an inactive form and are activated prior to secretion by a KEX2 endoproteinase (22). Although the presence of KEX2-like proteinase in *T. harzianum* has not been demonstrated, other secreted enzymes, like CHIT42 chitinase (23), BGN16.2 β-1,6-glucanase (33), and PRB1 protease (24), as well as XYN2 xylanase from *T. reesei* (67), contain a similar leader peptide ending in the pair Lys-Arg. The region between amino acids 33 and 520 has no apparent structural relevance, and so it could contain the catalytic site. Even though the protein contains several Glu residues, the flanked conserved

Downloaded from http://jb.asm.org/ on July 25, 2016 by USE/BCTA.GEN UNIVERSITARIA

```
1    GTTGCTGTCGTGTTGATCATTGACCGGCATCATGTTGAAGCTCACGGCGCTCGTTGCGCT   60
                                  M  L  K  L  T  A  L  V  A  L
61   CTTGCTGGGCGCGGCGTCAGCTACGCCGACTCCTAGCCCTCCTGCCAGCGATGAGGGCAT  120
     L  L  G  A  A  S  A  T  P  T  P  S  P  P  A  S  D  E  G  I
121  CACCAAGCGTGCCACCAGCTTCTATTACCCTAACATGGACCATGTTAATGCGCCCAGGGG  180
     T  K  R  A  T  S  F  Y  Y  P  N  M  D  H  V  N  A  P  R  G
181  TTTCGCTCCTGACTTGGATGGCGACTTCAATTACCCAATCTATCAGACTGTCAACGCAGG  240
     F  A  P  D  L  D  G  D  F  N  Y  P  I  Y  Q  T  V  N  A  G
241  AGATGGAAATGCTCTCCAGAATGCTATCACCACTGATGGAAAGGGTGGCTCTCGTCACCC  300
     D  G  N  A  L  Q  N  A  I  T  T  D  G  K  G  G  S  R  H  P
301  ACAGTGGTTTGCTTCACAGCCACGAGTTGTTTACATTCCCCCGGGAACATATACTATCTC  360
     Q  W  F  A  S  Q  P  R  V  V  Y  I  P  P  G  T  Y  T  I  S
361  CAAGACTCTGAGATTCAACACTGATACCATTTTAATGGGTGACCCAACTAATCCTCCCAT  420
     K  T  L  R  F  N  T  D  T  I  L  M  G  D  P  T  N  P  P  I
421  TATTAAGGCTGCTGCCGGTTTCTCAGGCGATCAGACTCTTATCAGTGCTCAAGACCCCTC  480
     I  K  A  A  A  G  F  S  G  D  Q  T  L  I  S  A  Q  D  P  S
481  CACCAACGAGAAGGGAGAGCTTTCTTTCGCCGTAGCTATTAAGAACGTGGTATTGGACAC  540
     T  N  E  K  G  E  L  S  F  A  V  A  I  K  N  V  V  L  D  T
541  TACCGCTATACCAGGTGGAAATTCATTTACTGCCCTATGGTGGGGTGTTGCTCAAGCTGC  600
     T  A  I  P  G  G  N  S  F  T  A  L  W  W  G  V  A  Q  A  A
601  GCATCTGCAGAATGTACGCATTACTATGAGTTCCTCTTCGGGTGGAAACGGCCATACCGG  660
     H  L  Q  N  V  R  I  T  M  S  S  S  S  G  G  N  G  H  T  G
661  CATCCGGATGGGTCGCGGCTCAACACTCGGCCTCGCCGACGTTCGCGTTGAACGCGGCCA  720
     I  R  M  G  R  G  S  T  L  G  L  A  D  V  R  V  E  R  G  Q
721  GAACGGTATTTGGATTGATGGACATCAACAAGCATCATTTCACAACATTTATTTCTTTCA  780
     N  G  I  W  I  D  G  H  Q  Q  A  S  F  H  N  I  Y  F  F  Q
781  AAATACTATAGGCATGCTCATCAGCAGTGGCAATACCTTCAGCATTTTCTCGTCGACCTT  840
     N  T  I  G  M  L  I  S  S  G  N  T  F  S  I  F  S  S  T  F
841  CGACACCTGTGGAACCGCATTTCCAACACTGGCCGGTTCACCCTGGATTGCCCTGATTGA  900
     D  T  C  G  T  A  F  P  T  L  A  G  S  P  W  I  A  L  I  D
901  CGCAAAGTCAATTAACTCTGGTGTTACCTTTACGACCAATCAATTTCCTTCATTTATGAT  960
     A  K  S  I  N  S  G  V  T  F  T  T  N  Q  F  P  S  F  M  I
961  TGAGAATCTGACTAAAGATAATGGCACACCTGTCGTTGTTGTCCGAGGCTCAACTTTGGT 1020
     E  N  L  T  K  D  N  G  T  P  V  V  V  V  R  G  S  T  L  V
1021 TGGCGCTTCCAGCCATGTCAACACTTACTCTTACGGCAACACCGTGGGCAGAAACCCTAC 1080
     G  A  S  S  H  V  N  T  Y  S  Y  G  N  T  V  G  R  N  P  T
1081 TTACGGCGATGTTACGTCTAGTAACACGAGACCTAGTGCTCTTGCTCCTGGTGGTCGTTA 1140
     Y  G  D  V  T  S  S  N  T  R  P  S  A  L  A  P  G  G  R  Y
1141 CCCTTATGTGGCTGCCCCAACTTATGGAGATTTGCCCATCTCGAGCTTCCTCAACGTCAA 1200
     P  Y  V  A  P  P  T  Y  G  D  L  P  I  S  S  F  L  N  V  K
1201 GGACCCAGCGCAGAATGGAAACCGTCAAGTTAAGGGGGATAACACAATCAATGAAGCAGA 1260
     D  P  A  Q  N  G  N  R  Q  V  K  G  D  N  T  I  N  E  A  D
1261 CACACTTAATGCCATCCTGGAACTTGCAGCAAGCCAGAATAAGGTTGCTTATTTTCCTTT 1320
     T  L  N  A  I  L  E  L  A  A  S  Q  N  K  V  A  Y  F  P  F
1321 TGGCAAGTACCGGGTGGATTCCACGCTTTTCATCCCTAAGGGTTCCCGTATCGTGGGTGA 1380
     G  K  Y  R  V  D  S  T  L  F  I  P  K  G  S  R  I  V  G  E
1381 GGCTTGGGCCACCATCACCGGCAACGGCAACTTTTTCAAGAACGAAAACAGCCCACAACC 1440
     A  W  A  T  I  T  G  N  G  N  F  F  K  N  E  N  S  P  Q  P
1441 CGTTGTCTCAGTTGGCCGTGCAGGCGATGTTGGAATTGCACAGCTGCAAGATCTAAGAGT 1500
     V  V  S  V  G  R  A  G  D  V  G  I  A  Q  L  Q  D  L  R  V
1501 CACGACTAACGATGTGCTCCCCGGCGCTATCCTCGTACAGTTCAATATGGCTGGCAATAA 1560
     T  T  N  D  V  L  P  G  A  I  L  V  Q  F  N  M  A  G  N  N
1561 CCCTGGTGATGTTGCTCTTTGGAACTCTTTGGTCACCGTTGGTGGCACACGAGGTGCTCA 1620
     P  G  D  V  A  L  W  N  S  L  V  T  V  G  G  T  R  G  A  Q
1621 AGCCTTGGCTAATGCTTGTACCAACAATAGCAATGAACGTAAGGGTGCTTTCATTGGTAT 1680
     A  L  A  N  A  C  T  N  N  S  N  E  C  K  G  A  F  I  G  I
1681 CCACGTGGCGAAGGGATCATCTCCTTACATTCAAAACGTTTGGGAACTTGGGTTGCGGGA 1740
     H  V  A  K  G  S  S  P  Y  I  Q  N  V  W  E  L  G  L  R  D
1741 TCACATCGCTGAGAACTTCAGTGGCGGCACCTCCCATCGCAGGGAAAGGTGGAATTTTGG 1800
     H  I  A  E  N  F  S  G  G  T  S  H  R  R  E  R  W  N  F  G
1801 TCCAATCCGACGAAACGCAACCTGTCTTTATCCCATAGGAAGTGGGCATTGGTGGTTGTA 1860
     P  I  R  R  N  A  T  C  L  Y  P  I  G  S  G  H  W  W  L  Y
1861 CCAACTCAATCTTCACAATGCCGCCAACGTTGTTGTGTCTCTGCTTCAGGCGGAGACCAA 1920
     Q  L  N  L  H  N  A  A  N  V  V  V  S  L  L  Q  A  E  T  N
1921 CTACCATCAAGGCGCCAACACGCAGCAGATTCCTCCCGCTCCTTGGGTTGCAAATGTTGG 1980
     Y  H  Q  G  A  N  T  Q  Q  I  P  P  A  P  W  V  A  N  V  G
1981 CACTTGGGGCGATCCTGATTTCTCTTGGTGCAACGGTGGCGATAAACGATGCCGTATGGG 2040
     T  W  G  D  P  D  F  S  W  C  N  G  G  D  K  R  C  R  M  G
2041 CCCTGCAAACTTCATCAACGGAGGTTCCAACATCTACACATATGCCTCCGCCGGCATGGGC 2100
     P  A  N  F  I  N  G  G  S  N  I  Y  T  Y  A  S  A  A  W  A
2101 TTTCTTCAGCGGCCCTGGCCAGGGTTGCGCCTCAATTCGAATGTCAACAAACCATCCACTG 2160
     F  F  S  G  P  G  Q  G  C  A  Q  F  E  C  Q  Q  T  I  H  W
2161 GATTGCCAGCACCCCAAGCAACCTTCAGGCTTTTGGACTCTGCTCCAAGGATTCCGTCAA 2220
     I  A  S  T  P  S  N  L  Q  A  F  G  L  C  S  K  D  S  V  N
2221 CACACTGCGTCTGGGCGACGGCACATTTATCAACACCCAGAATGGATACACTGGCGGCTG 2280
     T  L  R  L  G  D  G  T  F  I  N  T  Q  N  G  Y  T  G  G  W
2281 GACTCCCGGAGGTGGTGACGTTGCCCGTTATACTACTTAATCGACGTTTCGAATGAGCTC 2340
     T  P  G  G  G  D  V  A  R  Y  T  T  *
2341 AACATCTTGAGGCCAGAAGGTAGTGTATGAAGTGCCCATATATAAGCGCGCTATGGAGAT 2400
2401 GGCTGCGCAAAAAAGCCCTCCAATTGTACATATCTATTTAAATGCCAATATCAATATATG 2460
2461 CACATAAAAAAAAAAAAAAAAAAA
```

FIG. 7. Nucleotide and predicted amino acid sequences of the *bgn13.1* cDNA clone. Available peptide sequences from the purified protein are underlined. Cysteine residues are marked by circles. Mature BGN13.1 starts at the sequence ATS after the boxed KEX2 recognition sequence.

residues around the Glu catalytic site described for the β-glucanase studied (8) are absent in BGN13.1. The third region is localized at the C-terminal end and extends from residue 520 to the end of the protein. This region contains eight of the nine cysteine residues present in the protein.

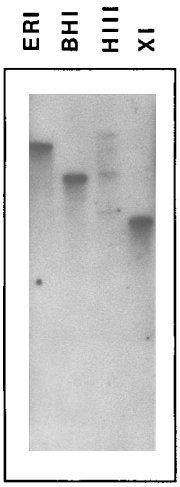

FIG. 8. Southern blot analysis of the *bgn13.1* gene. The experiment was performed by using 15 μg of chromosomal DNA digested with *Eco*RI (ERI), *Bam*HI (BHI), *Hin*dIII (HIII), or *Xba*I (XI). High- and low-stringency conditions show the same band pattern.

Finally, according to DNA-Southern blot hybridizations carried out at high and low stringencies there appears to be only one copy of the *bgn13.1* gene in the *T. harzianum* genome, and no other related sequences were detected (Fig. 8).

Expression pattern of *bgn13.1*. Northern analysis was performed to establish whether the induction of both activity and protein could also be found at the RNA level. As Fig. 9 shows, after 48 h of induction the mRNA levels were below the detection limit when glucose and pustulan were used as carbon sources and the mRNA was detected when laminarin and chitin were used. Also, mRNA accumulation was observed when autoclaved yeast cells or mycelia were used. Thus, these data are in agreement with those obtained when enzymatic activity and protein were determined (Table 1 and Fig. 1) and clearly indicate that *bgn13.1* is controlled at the transcriptional level. Therefore, *bgn13.1* is subjected to both repression by glucose and induction by fungal cell wall polymers, yeast cells, or fungal mycelia. However, when pustulan and laminarin were used as carbon sources, a lack of correlation between the activity and the mRNA levels was observed, suggesting that regulation at the translational level may also occur. This phenomenon has also been observed with a β-1,6-glucanase (BGN16.2) from the same microorganism (33) and could be

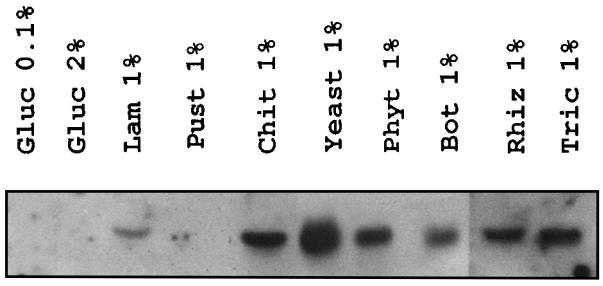

FIG. 9. Northern blot analysis. Carbon sources used were as follows: glucose (Gluc), 0.1 or 2%; laminarin (Lam), 1%; pustulan (Pust), 1%; chitin (Chit), 1%; yeast autoclaved cells (Yeast), 1%; autoclaved mycelia from *P. syringae* (Phyt), 1%; autoclaved mycelia from *B. cinerea* (Bot), 1%; autoclaved mycelia from *R. solani* (Rhiz), 1%; autoclaved mycelia from *T. harzianum* (Tric), 1%. Equal amounts of RNA were loaded into the wells and tested by methylene blue staining.

Downloaded from http://jb.asm.org/ on July 25, 2016 by USE/BCTA.GEN UNIVERSITARIA

explained by the high level of stability of these enzymes in the culture media. The enzymatic activity at 48 h in pustulan would be due to an earlier increase of mRNA levels, further repressed by glucose produced by action of the enzyme over its substrate.

DISCUSSION

T. harzianum CECT 2413 is a mycoparasitic fungus that secretes at least three enzymes with β-1,3-glucanase activity in a chitin-containing medium. The function for these fungal enzymes is unknown, but they might have a nutritional role with β-glucans as substrates (62), a direct role in mycoparasitism (9), or morphogenetic functions in growth and development (47). We report herein the following: first, the expression patterns of these enzymes in different carbon sources (Table 1 and Fig. 1), and second, the purification, molecular cloning, and characterization of the β-1,3-glucanase protein, named BGN13.1, corresponding to the major extracellular activity from supernatants of *T. harzianum* growing in chitin as the sole carbon source.

By the enzymatic detection of β-1,3-glucanase on isoelectrofocusing gels (45), we have shown the different conditions of induction of β-1,3-glucanase isoenzymes from *T. harzianum* CECT 2413. Acidic and basic β-1,3-glucanases were induced in the presence of various polysaccharides, but the basic enzyme (BGN13.1) was specifically induced by autoclaved yeast cell walls or filamentous fungi (Fig. 1). Preliminary results obtained with the mycoparasitic fungus *Stachybotrys elegans* have also shown different induction conditions for some extracellular chitinase and β-1,3-glucanase proteins (68). Although transcription of the *bgn13.1* gene correlated with the data obtained from enzymatic assays (Table 1, Fig. 1, and Fig. 8), the regulation appears to be at either the transcriptional or the translational level. In addition, β-1,3-glucanases from *T. harzianum* were produced coordinately with β-1,6-glucanases, proteases, and chitinases, even though the medium with chitin does not contain β-glucan or protein as a carbon source (14, 24). In *Trichoderma longibrachiatum*, the β-1,3-glucanase activity is constitutively produced and depends on the fungal growth (66), which suggests a morphogenetic role. In other fungi, such as *B. cinerea* (62), *Penicillium italicum* (55), *Penicillium oxalicum* (10), and *Sclerotium glucanicum* (48), a derepression of the β-1,3-glucanase activity in starvation is associated with the mobilization of β-glucans and involved in the autolysis of cell walls. However, this is not the case in *T. harzianum* (Table 1, Fig. 1, and Fig. 8). A coordinated induction of chitinases, β-1,3- and β-1,6-glucanases, and proteases may occur in *T. harzianum* when mycoparasitic action is established, as it occurs in plants for chitinases and β-1,3-glucanases during defense responses against pathogens (72). As previously discussed (13, 14, 16), a coordinated induction of the enzymes by an isolated polysaccharide would be of biological relevance, since the substrates of these enzymes are present simultaneously in fungal cell walls (47).

The purification method included specific adsorption to pustulan (β-1,6-glucan) and digestion, followed by chromatofocusing and gel filtration. This procedure is novel, quick, and easy, and even if the yields seem to be poor, it is good enough to purify protein for an extensive biochemical characterization. In addition, the process allows purification of two other proteins with β-1,6-glucanase activity. Data from SDS-PAGE and S-200 HR gel filtration indicated apparent molecular masses of 66 and 5 to 10 kDa, respectively. This result indicated an affinity for Sephacryl support that has previously been observed for a number of extracellular enzymes, including other fungal cell wall hydrolases (12, 13, 50, 66). In addition, the molecular mass predicted from the amino acid sequence is 77.9 kDa. Disparity between calculated and deduced molecular weights has also been observed for the endo-β-1,3-glucanase from *S. cerevisiae* (BGL2), which shows an apparent size of 29 kDa by SDS-PAGE and a predicted one of 33.5 kDa from the amino acid sequence. In this last case, it has been demonstrated that the difference in the molecular size is due to anomalous migration of the protein in the gel rather than to posttranslational processing of the polypeptide chain (40).

The comparison of the amino acid sequence of BGN13.1 with those of BGL2 and other β-glucanases did not show any significant homology. Comparison between sequences of yeast and plant β-glucanases showed that relevant regions of the deduced primary structure are highly conserved, especially around the glutamine residues involved in the catalysis. The boxes VXEXGWP and EXXXGXF located at the nucleophilic and acidic catalytic sites, respectively, are not present in BGN13.1 (8, 27). Likewise, the box EXDXE present in the bacterial counterpart is also absent in BGN13.1. Therefore, the primary structure of BGN13.1 showed two significant differences with regard to other β-glucanases: (i) BGN13.1 lacks the conserved regions present in the studied β-glucanases, and (ii) BGN13.1 contains a cysteine-rich motif absent in other known β-glucanases, except for the β-1,3-glucanase from the bacterium *Oerskovia xanthineolytica*. Deletion of the cysteine-rich carboxyl-terminal region in the bacterial protein does not affect the enzymatic activity but abolishes the capability to interact with the yeast cell wall and therefore exert its lytic activity (58). A similar Cys-rich motif has also been found in other protein families which are able to bind specific membrane components, like the saccharide-binding plant lectins (e.g., wheat germ agglutinin); the lipid transfer proteins of animals, fungi, and plants; and class I chitinases (7, 17). The Cys-rich domain of BGN13.1 may function by interacting with other components of fungal cell walls. In fact, BGN13.1 is the only β-1,3-glucanase from *T. harzianum* able to interact with polysaccharides which are not substrates for the enzyme (like pustulan). Since the enzymatic activity of BGN13.1 is specific only for β-1,3 linkages, this putative cell wall binding domain may have functional relevance in the mycoparasitism of *T. harzianum* by recognizing a cell wall component of the target fungi. This hypothesis is currently being studied in our laboratories.

The unique structure of the basic β-glucanase from *T. harzianum* was confirmed by the fact that sera raised against β-1,3- and β-1,6-glucanases from plants (26) and fungi (13), respectively, did not cross-react with any of the β-1,3-glucanase enzymes from *T. harzianum*. Furthermore, extracellular hydrolases of fungi are normally glycosylated (76), as are cellulases from *Trichoderma reesei* (30, 52). Our results, including those obtained with specific carbohydrate stains, endo-H or α-mannosidase treatments, and concanavalin A-binding assays, indicated that either the purified BGN13.1 is not glycosylated or the glycosylation level is so low as to be not detected under the conditions used. These results, however, are similar to those found with other extracellular β-glucanases and chitinases from *T. harzianum* (12, 13).

The measured K_m for laminarin (3.3 mg/ml) of the purified enzyme was substantially higher than those reported for other β-1,3-glucanases (21, 54, 66, 69), with the exception of one β-1,3-glucanase of the fungus *S. glucanicum* (48) and that of *Rhizopus chinensis* (75). The enzyme purified in this work was specific for β-1,3 linkages in polysaccharides (Table 4), hydrolyzing laminarin and yeast glucan with the same efficiency and pachyman less efficiently. The slight activity found with pustulan (linear β-1,6-glucan) could be related to the binding of this

Downloaded from http://jb.asm.org/ on July 25, 2016 by USE/BCTA.GEN UNIVERSITARIA

enzyme to this substrate and the presence of some β-1,3 linkages in it. Unlike the exo-β-1,3-glucanases previously described for *T. harzianum* (28) and *Trichoderma reesei* (2), the enzyme purified in this work behaves as an endo-splitting hydrolase as judged from the liberation of more reducing sugars than of glucose from laminarin, the presence of activity on periodate-oxidized laminarin (unable to be hydrolyzed for exo-β-1,3-glucanases), the noninhibitory effect of δ-gluconolactone on the enzymatic activity, the inability to hydrolyze substrates such as laminaribiose, and the pattern of reaction products detected by HPLC. In contrast with some fungal endo-β-1,3-glucanases that degrade β-1,3-glucans to generate oligosaccharides of high molecular mass and that are classified as larger-oligosaccharide-producing types of enzyme (1), the endo-β-1,3-glucanase characterized in this work hydrolyzes β-1,3-glucans in an endo manner with glucose, laminaribiose, and laminaritetraose as end products and therefore, as are other bacterial and fungal endo-β-1,3-glucanases, is classified as a smaller-oligosaccharide-producing type (39, 64).

Finally, the enzyme alone releases reducing sugars but is unable to form hydrolytic haloes when incubated with purified cell walls of several filamentous fungi, including phytopathogenic ones. Furthermore, when it was combined with other fungal cell wall hydrolases from *T. harzianum*, such as β-1,6-glucanases and chitinases (13), not only did the enzyme give clearing activity on fungal cell walls but also an inhibition of the growth of phytopathogenic fungi was observed. Similar results have been found with *T. harzianum* P1 and also with other organisms. A synergistic, inhibitory effect for chitinases, *N*-acetyl-β-glucosaminidases, and β-glucanolytic enzymes from strain P1 of *T. harzianum* has been described (34, 35). In plants, a combination of β-1,3-glucanases with chitinases is essential for observation of inhibitory effects against some phytopathogenic fungi (37). Also, in the lyticase from the bacterium *O. xanthineolytica* a β-1,3-glucanase and a protease activity act in combination in yeast cell lysis (57).

In conclusion, because of the specific induction of this enzyme under simulated mycoparasitic conditions, as well as its antifungal action when combined with other fungal cell wall-degrading enzymes from *T. harzianum*, a contribution of this β-1,3-glucanase during the first steps of mycoparasitism could be expected, although the results did not discard the possibility of the implication of this β-1,3-glucanase in other, unknown saprophytic roles. At present, our work is focused on the overexpression of this enzyme in transgenic *T. harzianum*, which could result in the improvement of this fungus as a biocontrol agent.

ACKNOWLEDGMENTS

We thank F. Dominguez for his helpful assistance during all biochemical experimental work. We also thank E. Martinez-Force for his help with HPLC experiments and R. Rhett for proofreading the manuscript. We thank R. Geremia and G. Bauw at the Laboratorium voor Genetika (Gent, Belgium) for the N-terminal and peptide amino acid determinations.

J.C. is a recipient of a fellowship from the Ministerio de Educación y Ciencia. This work was supported by grant BIO91-1078 from CICYT (Spain) and grant TS3-CT92-0140 from the European Community.

REFERENCES

1. **Bacon, J. C., A. H. Gordon, D. Jones, I. F. Taylor, and D. M. Webley.** 1970. The separation of β-glucanases produced by *Citophaga johnsonii* and their role in the lysis of yeast cell walls. Biochem. J. **120:**67–71.
2. **Bamforth, C. W.** 1980. The adaptability, purification and properties of an exo-β-1,3-glucanase from *Trichoderma reesei*. Biochem. J. **191:**863–866.
3. **Barnett, C. C., R. M. Berka, and T. Flowler.** 1991. Cloning and amplification of the gene encoding an extracellular β-glucosidase from *Trichoderma reesei*: evidence for improved rates of saccharification of cellulosic substrates. Bio/Technology **9:**562–567.
4. **Bauw, G., H. Holm Rasmussen, M. Van den Bulcke, J. Van Damme, M. Puype, B. Gesser, J. E. Celis, and J. Vandekerckhove.** 1990. Two-dimensional gel electrophoresis, protein electroblotting and microsequencing: a direct link between proteins and genes. Electrophoresis **11:**528–536.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
6. **Bucciaglia, P. A., and A. G. Smith.** 1994. Cloning and characterization of *Tag1*, a tobacco anther β-1,3-glucanase expressed during tetrad dissolution. Plant Mol. Biol. **24:**903–914.
7. **Chasan, R.** 1991. Lipid transfer proteins: moving molecules? Plant Cell **3:**842–843.
8. **Chen, L., G. B. Fincher, and P. B. Hoj.** 1993. Evolution of polysaccharide hydrolase substrate specificity. Catalytic amino acids are conserved in barley 1,3-1,4- and 1,3-β-glucanases. J. Biol. Chem. **268:**13318–13326.
9. **Chet, I.** 1987. *Trichoderma*—applications, mode of action and potential as a biocontrol agent of soilborne plant pathogenic fungi, p. 137–160. *In* I. Chet (ed.), Innovative approaches to plant diseases. John Wiley and Sons, New York.
10. **Copa-Patiño, J., F. Reyes, and M. I. Pérez-Leblic.** 1989. Purification and properties of a 1,3-β-glucanase from *Penicillium oxalicum* autolysates. FEMS Microbiol. Lett. **65:**285–292.
11. **Dawson, C., C. Belloch, M. D. García-López, and F. Uruburu.** 1990. Catalogue of strains, Spanish Type Culture Collection, 3rd ed., p. 263–274. University of Valencia, Valencia, Spain.
12. **de la Cruz, J., A. Hidalgo-Gallego, J. M. Lora, T. Benítez, J. A. Pintor-Toro, and A. Llobell.** 1992. Isolation and characterization of three chitinases from *Trichoderma harzianum*. Eur. J. Biochem. **206:**856–867.
13. **de la Cruz, J., J. A. Pintor-Toro, T. Benítez, and A. Llobell.** 1995. Purification and characterization of an endo-β-1,6-glucanase from *Trichoderma harzianum* related to its mycoparasitism. J. Bacteriol. **177:**1864–1871.
14. **de la Cruz, J., M. Rey, J. M. Lora, A. Hidalgo-Gallego, F. Domínguez, J. A. Pintor-Toro, A. Llobell, and T. Benítez.** 1993. Carbon source control on β-glucanase, chitobiase and chitinase from *Trichoderma harzianum*. Arch. Microbiol. **159:**316–322.
15. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
16. **di Pietro, A., M. Lorito, C. K. Hayes, R. M. Broadway, and G. E. Harman.** 1993. Endochitinase from *Gliocladium virens*: isolation, characterization, and synergistic antifungal activity in combination with gliotoxin. Phytopathology **83:**308–313.
17. **Drenth, J., B. W. Low, J. S. Richardson, and C. S. Wright.** 1980. The toxin-agglutinin fold. A new group of small protein structures organized around a four-disulfide core. J. Biol. Chem. **255:**2652–2655.
18. **Dubray, G., and G. Bezard.** 1982. A highly sensitive periodic acid-silver stain for 1,2-diol groups of glycoproteins and polysaccharides in polyacrylamide gels. Anal. Biochem. **119:**325–329.
19. **Elad, Y., I. Chet, and Y. Henis.** 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Can. J. Microbiol. **28:**719–725.
20. **Fincher, G. B.** 1989. Molecular and cellular biology associated with endosperm mobilization in germinating cereal grains. Annu. Rev. Plant Physiol. Plant Mol. Biol. **40:**305–346.
21. **Fleet, G. H., and H. J. Phaff.** 1974. Glucanases in *Schizosaccharomyces*. Isolation and properties of the cell wall-associated β-1,3-glucanases. J. Biol. Chem. **249:**1717–1728.
22. **Fuller, R. S., A. J. Brake, and J. Thorner.** 1989. Intracellular targeting and structural conservation of a prohormone-processing endoprotease. Science **246:**482–485.
23. **Garcia, I., J. M. Lora, J. de la Cruz, T. Benitez, A. Llobell, and J. A. Pintor-Toro.** 1994. Cloning and characterization of a chitinase (CHIT42) cDNA from the mycoparasitic fungus *Trichoderma harzianum*. Curr. Genet. **27:**83–89.
24. **Geremia, R., G. H. Goldman, D. Jacobs, W. Ardiles, S. B. Vila, M. Van Montagu, and A. Herrera-Estrella.** 1993. Molecular characterization of the proteinase-encoding gene, *prb1*, related to mycoparasitism by *Trichoderma harzianum*. Mol. Microbiol. **8:**603–613.
25. **Grenier, J., C. Potvin, and A. Asselin.** 1993. Barley pathogenesis-related proteins with fungal cell wall lytic activity inhibit the growth of yeast. Plant Physiol. **103:**1277–1283.
26. **Kauffmann, S., M. Legrand, P. Geoffrey, and B. Fritig.** 1987. Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have β-1,3-glucanase activity. EMBO J. **11:**3209–3212.
27. **Keitel, T., O. Simon, R. Borris, and U. Heinemann.** 1993. Molecular and active-site structure of a *Bacillus* 1,3-1,4-D-β-glucanase. Proc. Natl. Acad. Sci. USA **90:**5287–5291.
28. **Kitamoto, Y., R. Kono, A. Shimotori, N. Mori, and Y. Ichikawa.** 1987. Purification and some properties of an exo-β-1,3-glucanase from *Trichoderma harzianum*. Agric. Biol. Chem. **51:**3385–3386.
29. **Klebl, F., and W. Tanner.** 1989. Molecular cloning of a cell wall exo-β-1,3-glucanase from *Saccharomyces cerevisiae*. J. Bacteriol. **171:**6259–6264.
30. **Knowles, J., P. Lehtovaara, and T. Teeri.** 1987. Cellulase families and their

Downloaded from http://jb.asm.org/ on July 25, 2016 by USE/BCTA.GEN UNIVERSITARIA

genes. Trends Biotechnol. **5:**255–261.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
32. **Lora, J. M., J. de la Cruz, T. Benitez, A. Llobell, and J. A. Pintor-Toro.** 1994. A putative catabolite-repressed cell wall protein from the mycoparasitic fungus *Trichoderma harzianum*. Mol. Gen. Genet. **242:**461–466.
33. **Lora, J. M., J. de la Cruz, T. Benitez, A. Llobell, and J. A. Pintor-Toro.** 1995. Molecular characterization and heterologous expression of an endo-β-1,6-glucanase gene from the mycoparasitic fungus *Trichoderma harzianum*. Mol. Gen. Genet. **247:**639–645.
34. **Lorito, M., G. E. Harman, C. K. Hayes, R. M. Brodway, A. Tronsmo, S. L. Woo, and A. di Pietro.** 1993. Chitinolytic enzymes produced by *Trichoderma harzianum*; antifungal activity of purified endochitinase and chitobiosidase. Phytopathology **83:**302–307.
35. **Lorito, M., C. K. Hayes, A. di Pietro, S. L. Woo, and G. F. Harman.** 1994. Purification, characterization, and synergistic activity of a glucan 1,3-β-glucosidase and an N-acetyl-β-glucosaminidase from *Trichoderma harzianum*. Phytopathology **84:**398–405.
36. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. L. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
37. **Mauch, F., B. Mauch-Mani, and T. Boller.** 1988. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. **88:**936–942.
38. **Montenecourt, B. S.** 1983. *Trichoderma reesei* cellulases. Trends Biotechnol. **1:**156–161.
39. **Mori, H., S. Yamamoto, and S. Nagasaki.** 1977. Multiple forms of the lytic glucanase of *Flavobacterium dormitator* var. *glucanolyticae* and the properties of the main component enzyme. Agric. Biol. Chem. **41:**611–613.
40. **Mrsa, V., F. Klebl, and W. Tanner.** 1993. Purification and characterization of the *Saccharomyces cerevisiae BGL2* gene product, a cell wall endo-β-1,3-glucanase. J. Bacteriol. **175:**2102–2106.
41. **Murray, M. G., and W. F. Thomson.** 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. **8:**4321–4325.
42. **Neale, A. D., J. A. Wahleithner, M. Lund, H. T. Bonnet, A. Kelly, D. Ry Meeks-Wagner, W. J. Peacock, and E. S. Dennis.** 1990. Chitinase, β-1,3-glucanase, osmotin, and extensin are expressed in tobacco explants during flower formation. Plant Cell **2:**673–684.
43. **Nelson, N. J.** 1955. Colorimetric analysis of sugars. Methods Enzymol. **3:**85–86.
44. **Notario, V., T. G. Villa, and J. R. Villanueva.** 1976. Purification of an exo-β-glucanase from cell-free extracts of *Candida utilis*. Biochem. J. **159:** 555–562.
45. **Pan, S., X. Ye, and J. Kuc.** 1989. Direct detection of β-1,3-glucanase isoenzymes on polyacrylamide electrophoresis and isoelectrofocusing gels. Anal. Biochem. **182:**136–140.
46. **Papavizas, G. C.** 1985. *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. Annu. Rev. Phytopathol. **23:**23–54.
47. **Peberdy, J. F.** 1990. Fungal cell wall—a review, p. 5–24. *In* P. J. Kuhn, A. P. J. Trinci, M. J. Jung, M. W. Goosey, and L. G. Copping (ed.), Biochemistry of cell walls and membranes in fungi. Springer-Verlag, Heidelberg, Germany.
48. **Rapp, P.** 1992. Formation, separation and characterization of three β-1,3-glucanases from *Sclerotium glucanicum*. Biochim. Biophys. Acta **1117:**7–14.
49. **Reese, E. T., and M. Mandels.** 1959. β-D-1,3-Glucanases in fungi. Can. J. Microbiol. **5:**173–185.
50. **Ridout, C. J., J. R. Coley-Smith, and J. M. Lynch.** 1988. Fractionation of extracellular enzymes from a mycoparasitic strain of *Trichoderma harzianum*. Enzyme Microb. Technol. **10:**180–187.
51. **Robertson, E. F., H. K. Dannelly, P. J. Malloy, and H. C. Reeve.** 1987. Rapid isoelectrofocusing in a vertical polyacrylamide minigel system. Anal. Biochem. **167:**290–294.
52. **Saloheimo, A., B. Henrissat, A. M. Hoffrén, O. Teleman, and M. Penttilä.** 1994. A novel, small endoglucanase gene, *egl5*, from *Trichoderma reesei* isolated by expression in yeast. Mol. Microbiol. **13:**219–228.
53. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
54. **Sánchez, M., C. Nombela, J. R. Villanueva, and T. Santos.** 1982. Purification and partial characterization of a developmentally regulated 1,3-β-glucanase from *Penicillium italicum*. J. Gen. Microbiol. **128:**2047–2053.
55. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
56. **Schaeffer, H. J., J. Leykan, and J. D. Walton.** 1994. Cloning and targeted gene disruption of *EXG1*, encoding exo-β-1,3-glucanase, in the phytopathogenic fungus *Cochliobolus carbonum*. Appl. Environ. Microbiol. **60:**594–598.
57. **Scott, J. H., and R. Schekman.** 1980. Lyticase: endoglucanase and protease activities that act together in yeast cell lysis. J. Bacteriol. **142:**414–423.
58. **Shen, S., P. Chrétien, L. Bastien, and S. N. Slilaty.** 1991. Primary structure of the glucanase gene from *Oerskovia xanthineolytica*. Expression and purification of the enzyme from *Escherichia coli*. J. Biol. Chem. **266:**1058–1063.
59. **Sivan, A., and I. Chet.** 1989. Degradation of fungal cell walls by lytic enzymes from *Trichoderma harzianum*. J. Gen. Microbiol. **135:**675–682.
60. **Somogyi, M.** 1952. Notes in sugar determination. J. Biol. Chem. **195:**19–23.
61. **Sperisen, C., J. Ryahs, and F. Meins.** 1991. Comparison of cloned genes provides evidences for intergenomic exchange of DNA in the evolution of a tobacco glucan endo-1,3-β-glucosidase family. Proc. Natl. Acad. Sci. USA **88:**1820–1824.
62. **Stahmann, K. P., P. Pielken, K. L. Schimz, and H. Sahm.** 1992. Degradation of extracellular β-(1,3)-(1,6)-D-glucan by *Botrytis cinerea*. Appl. Environ. Microbiol. **58:**3347–3354.
63. **Stahmann, K. P., K. L. Schimz, and H. Sahm.** 1993. Purification and characterization of four extracellular 1,3-β-glucanases of *Botrytis cinerea*. J. Gen. Microbiol. **139:**2833–2840.
64. **Takahushi, M., M. Komuro, and S. I. Soutome.** 1978. Purification and properties of β-1,3-glucanase from *Lentinus lepideus*. J. Ferment. Technol. **56:**499–505.
65. **Tanaka, H., and H. J. Phaff.** 1965. Enzymatic hydrolysis of the yeast cell walls. I. Isolation of wall-decomposing organisms and separation and purification of lytic enzymes. J. Bacteriol. **89:**1570–1580.
66. **Tangarone, B., J. C. Royer, and J. P. Nakas.** 1989. Purification and characterization of an endo-1,3-β-D-glucanase from *Trichoderma longibrachiatum*. Appl. Environ. Microbiol. **55:**177–184.
67. **Torronen, A., R. L. Mach, R. Messner, R. Gonzalez, N. Kalkkinen, A. Harkki, and C. Kubicek.** 1992. The two major xylanases from *Trichoderma reesei*: characterization of both enzymes and genes. Bio/Technology **10:**1461–1465.
68. **Tweddell, R., S. Jabaji-Hare, and P. M. Charest.** 1994. Production of chitinases and β-1,3-glucanases by *Stachybotrys elegans*, a mycoparasite of *Rhizoctonia solani*. Appl. Environ. Microbiol. **60:**489–495.
69. **Usui, T., K. Tonaki, A. Totsuka, and M. Oguchi.** 1985. Purification of endo-β-D-1,3-glucanase lysing yeast cell walls from *Rhizoctonia solani*. Biochim. Biophys. Acta **840:**255–263.
70. **Vázquez de Aldana, C. R., J. Correa, P. San Segundo, A. Bueno, A. R. Nebreda, E. Mendez, and F. del Rey.** 1991. Nucleotide sequence of the exo-β-1,3-glucanase-encoding gene, *EXG1*, of the yeast *Saccharomyces cerevisiae*. Gene **97:**173–182.
71. **Wadsworth, G. J., M. G. Redinbaugh, and J. G. Scandalios.** 1988. A procedure for the small-scale isolation of plant RNA suitable for RNA blot analysis. Anal. Biochem. **172:**279–283.
72. **Ward, E. R., S. J. Uknes, S. C. Willians, S. S. Dincher, D. L. Wiederhold, D. C. Alexander, P. Ahl-Goy, J. P. Metraux, and J. A. Ryals.** 1991. Coordinate gene activity in response to agents that induce systematic acquired resistance. Plant Cell **3:**1085–1094.
73. **Watanabe, T., N. Kasahara, K. Aida, and H. Tanaka.** 1992. Three N-terminal domains of β-1,3-glucanase A1 are involved in binding to insoluble β-1,3-glucan J. Bacteriol. **174:**186–190.
74. **Williamson, G., N. J. Belshaw, and M. P. Williamson.** 1992. O-glycosylation in *Aspergillus* glucoamylase. Conformation and role in binding. Biochem. J. **282:**423–428.
75. **Yamamoto, S., and S. Nagasaki.** 1975. Purification, crystallization, and properties of an endo-β-1,3-glucanase from *Rhizopus chinensis* R-69. Agric. Biol. Chem. **39:**2163–2169.
76. **Yoshizumi, H., and T. Ashikari.** 1987. Expression, glycosylation and secretion of fungal hydrolases in yeast. Trends Biotechnol. **5:**277–281.

Downloaded from http://jb.asm.org/ on July 25, 2016 by USE/BCTA.GEN UNIVERSITARIA