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Differential Patterns of Activity Displayed by Two Exo-β-1,3-Glucanases Associated with the *Aspergillus fumigatus* Cell Wall

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Two exo-β-1,3-glucanases (herein designated exoG-I and exoG-II) were isolated from the cell wall autolysate of the filamentous fungus *Aspergillus fumigatus* and purified by ion-exchange, hydrophobic-interaction, and gel filtration chromatographies. Molecular masses estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and gel filtration chromatography were 82 kDa for the monomeric exoG-I and 230 kDa for the dimeric exoG-II. exoG-I and exoG-II were glycosylated, and N glycans accounted, respectively, for 2 and 44 kDa. Their pH optimum is 5.0. Their optimum temperatures are 55°C for exoG-I and 65°C for exoG-II. By a sensitive colorimetric method and high-performance anion-exchange chromatography for product analysis, two patterns of exo-β-1,3-glucanase activities were found. The 230-kDa exoG-II enzyme acts on *p*-nitrophenyl-β-D-glucoside, β-1,6-glucan, and β-1,3-glucan. This activity, which retains the anomeric configuration of glucose released, presented a multichain pattern of attack of the glucan chains and a decrease in the maximum initial velocity (V_m) with the increasing size of the substrate. In contrast, the 82-kDa exoG-I, which inverts the anomeric configuration of the glucose released, hydrolyzed exclusively the β-1,3-glucan chain with a minimal substrate size of 4 glucose residues. This enzyme presented a repetitive-attack pattern, characterized by an increase in V_m with an increase in substrate size and by a degradation of the glucan chain until it reached laminaritetraose, the limit substrate size. The 82-kDa exoG-I and 230-kDa exoG-II enzymes correspond to a β-1,3-glucan-glucohydrolase (EC 3.2.1.58) and to a β-D-glucoside-glucohydrolase (EC 3.2.1.21), respectively. The occurrence and functions of these two classes of exo-β-1,3-glucanases in other fungal species are discussed.

Exo-β-1,3-glucanases are widely distributed among yeast and filamentous fungi (28, 33). These enzymes are able to release glucose units from the nonreducing end of the β-1,3-glucan chain. Fungal exo-β-1,3-glucanases present different specificities. For example, some activities are able to degrade the β-1,6-glucan (28) whereas others are blocked by a β-1,6 branch point on linear β-1,3-glucan (23, 33). These properties distinguish two classes of exo-β-1,3-glucanases: β-1,3-glucan-glucohydrolase (EC 3.2.1.58) and β-D-glucoside-glucohydrolase (EC 3.2.1.21). Yeast and filamentous fungi produce extracellular and cell wall-associated exo-β-glucanases (13, 36). Secretion of β-glucanases is dependent on the carbon source (38) or the glucose limitation of the culture medium (35). Genetic studies have suggested that the function of such extracellular exo-β-glucanases is to release glucose residues used as the carbon source (47). In contrast, preliminary studies have shown that different exo-β-glucanases are required at different stages of the development of fungi (44, 45) and may have a specific function in the organization of the cell wall. In *Saccharomyces cerevisiae*, the production of β-glucanases is growth associated and cell cycle regulated (3, 5, 6, 15, 19), suggesting that their activities are required at very specific stages during morphogenesis (2, 53). However, the disruption of exo-β-1,3-glucanase genes in *S. cerevisiae* is not lethal (25, 47). Their role in cell wall morphogenesis remains unknown (20).

Few cell wall-associated exo-β-1,3-glucanases have been isolated from filamentous fungi (4, 30, 46). This paper presents the characteristics of two exo-β-1,3-glucanase activities isolated from the cell wall of *Aspergillus fumigatus* (herein designated exoG-I and exoG-II).

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MATERIALS AND METHODS

Substrates and inhibitors. *p*-Nitrophenol-β-D-glucoside, laminarin from *Laminaria digitata*, cellopentaose, carboxymethyl (CM) cellulose, castanospermine, deoxynojirimycin, and glucono-δ-lactone were from Sigma. Curdlan was from Serva. Pustulan was from Calbiochem. Schizophyllan was from Kaken Pharmaceutical Co. Reduced laminari-oligosaccharides were obtained by hydrolysis of curdlan with trifluoroacetic acid and reduction with BH_4Na as described previously (11). Gentio-oligosaccharides were obtained from pustulan by the same acid hydrolysis procedure. Pustulan hydrolysis products, curdlan, and laminarin were reduced by $NaBH_4$ in 50 mM NaOH and then dialyzed against water and freeze dried. Reduced curdlan, which is a water-insoluble polymer, was heated for 20 min at 65°C to obtain a hydrated gel. Reduced laminari-oligosaccharide containing 10 glucose residues and an intrachain β-1,6 linkage at the sixth linkage from the reducing end (bG10r) was obtained by incubating reduced laminarihexaose (G6r) with a glucanosyl transferase homolog purified from *A. fumigatus* (9) to *Candida albicans* (10). The bG10r was purified in the same manner as the laminari-oligosaccharides (11). The alkali-insoluble fraction from *A. fumigatus* cell walls was prepared as described previously (7).

β-Glucanase assays. The *p*-nitrophenol-β-D-glucosidase (pNPGase) activities were detected by measuring the amount of *p*-nitrophenol liberated from *p*-nitrophenol-β-D-glucoside (pNP-Glc) with a calibration curve at 405 nm. The enzymatic reaction was performed at 37°C for 30 min in 165 μl of 120 mM sodium acetate, pH 5.6, containing 0.8 mg of pNP-Glc. β-1,3-Glucanase activities were detected with reduced laminarin. The enzymatic reaction was performed at 37°C for 30 min in 100 mM sodium acetate buffer, pH 5.6, containing 0.64 mg of reduced laminarin per ml. The activities were determined by measuring the amount of reducing end liberated from the reduced substrate by the *p*-hydroxybenzoic acid–hydrazide reagent method (34). One unit is defined as the amount of enzyme required to release 1 μmol of glucose from reduced laminarin per min.

Characterization of the β-1,3-glucanase activities. The specificity of β-glucanase activity was studied by measuring the amount of reducing sugars liberated from reduced glucans with β-1,3 linkages, i.e., laminari-oligosaccharides (0.16 mM or 0.64 mg/ml), laminarin (0.64 mg/ml), and curdlan (0.64 mg/ml), or β-1,6 linkages, i.e., gentio-oligosaccharides (0.64 mg/ml), and from several nonreduced polysaccharides, e.g., CM-cellulose (0.64 mg/ml) and schizophyllan (0.64 mg/ml),

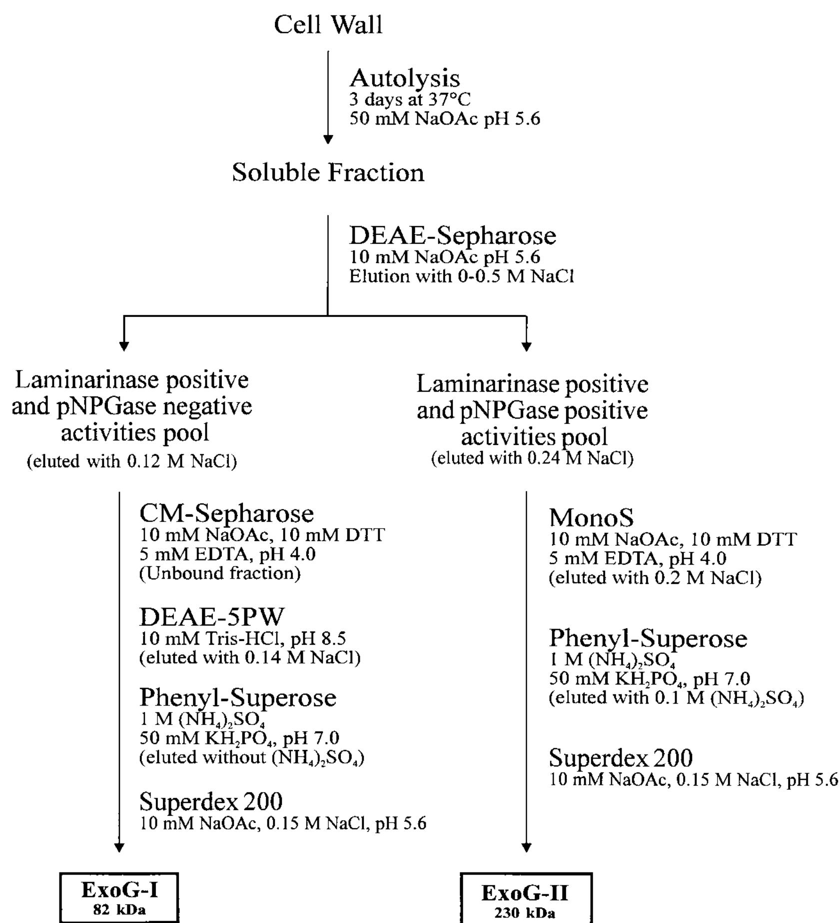

FIG. 1. Diagram of chromatographic steps for the isolation of the exo-β-1,3-glucanases exoG-I and exoG-II produced by *A. fumigatus*. (Column stabilization and elution conditions are indicated under each chromatographic step.)

in a 100 mM sodium acetate buffer at pH 5.0, by the colorimetric method described above.

Products released from reduced laminari-oligosaccharides by glucanase activities were analyzed by high-performance anion-exchange (HPAE) chromatography with a pulsed electrochemical detector and an anion-exchange column (Carbo-PAC PA1, 4.6 by 250 mm; Dionex) under the conditions of a flow rate of 1 ml/min and the following gradient: 0 to 2 min with 98% buffer A (50 mM NaOH)–2% buffer B (500 mM sodium acetate in 50 mM NaOH) (isocratic), 2 to 15 min with 75% buffer A–25% buffer B (linear), and 15 to 45 min with 60% buffer A–40% buffer B (linear). Laminari-oligosaccharide standards were obtained from Seikagaku. For high-performance liquid chromatography (HPLC) analysis, enzymes (10 μl containing 20 and 430 ng of protein for exoG-I and exoG-II, respectively) were incubated in 50 μl of reaction mixture containing 2 mM substrate and 50 mM sodium acetate buffer, pH 5.0, at 37°C.

The influences of parameters were tested in a 250-μl mixture containing 0.06 or 0.43 μg of protein for exoG-I or exoG-II, respectively. The effect of pH on activities was measured at 37°C over the pH range 3 to 7.5 in 150 mM imidazole–citric acid buffer for 10 min. pH stability was determined by assaying the residual activity after a preincubation of enzymes over the pH range 2 to 11 in 150 mM buffer (imidazole-citric acid, pH 2 to 8; glycine-sodium hydroxide, pH 8 to 11) for 1 h. The effect of temperature on activities was measured at pH 5.0 in 100 mM sodium acetate over a temperature range of 4 to 80°C during 5 and 10 min of incubation. Thermal stability was determined by measuring the activities remaining after preincubation of the enzyme at various temperatures (4 to 75°C) for 1 h in 100 mM sodium acetate at pH 5.0. Kinetic studies were performed at 37°C and at pH 5.0. For K_m determination, pNP-Glc, G5r, G10r, G18r, and reduced laminarin were used over the concentration ranges 4.2 to 60 μM for exoG-I activity and 40 to 400 μM for exoG-II activity. The effects of chemical compounds ($HgCl_2$, $KMnO_4$, $ZnSO_4$, $CuCl_2$, $FeCl_3$ and $MgCl_2$, *N*-bromosuccinimide, *p*-hydroxymercuribenzoic acid, carbodiimide, iodoacetic acid, and succinic anhydride) on β-glucanase activities were observed by incubation of enzymes with 1 mM compound with reduced laminarin at 0.64 mg/ml for exoG-I and 10 mg/ml for exoG-II. β-D-Glucosidase inhibitors, such as glucono-δ-lactone, deoxynojirimycin, and castanospermine, were tested on β-glucanase activities at the concentration of 0.4 mM. The type of inhibition and K_i constants were determined with reduced laminarin as the substrate with the same concentration ranges as described for K_m determinations. For the exoG-I activity, deoxynojirimycin was used at 1.4 and 2 μM and castanospermine was used at 50 and 100 μM. For exoG-II, deoxynojirimycin and castanospermine were used at 1 and 2 μM and glucono-δ-lactone was used at 2 and 4 μM.

For the measure of the activity on the alkali-insoluble fraction from *A. fumigatus* cell walls, 800 μg of dried cell wall fraction suspended in 400 μl of 100 mM sodium acetate (NaOAc), pH 5.0, was incubated with 0.11 U of exoG-I or 0.3 U of exoG-II at 37°C. Fifty-microliter samples were taken at regular intervals from the mixture for the quantification of glucose release by the *p*-hydroxybenzoic acid–hydrazide colorimetric method.

Enzyme production and chromatographic purification. *A. fumigatus* CBS 143-89 was cultivated for 48 h in a 15-liter fermentor (Chemap) at 25°C as described previously (21). Preparations of the cell wall autolysate were done according to the method of Hartland et al. (11). After cell wall autolysis, enzyme activities were purified by several successive chromatographic steps as shown in Fig. 1. Types of chromatography, loading, and elution conditions were the following. (i) For DEAE-Sepharose anion-exchange chromatography, the crude extract obtained by autolysis of cell walls was loaded onto a column (4 by 18 cm) of DEAE-Sepharose Fast-Flow (Pharmacia) equilibrated in 10 mM sodium acetate, pH 5.6. Proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in the starting buffer (2 liters) at a flow rate of 240 ml/h. (ii) For CM-Sepharose cation-exchange chromatography, fractions from the DEAE-Sepharose step with laminarinase activities (without pNPGase activity) were pooled, dialyzed against 10 mM sodium acetate–5 mM EDTA–10 mM dithiothreitol (DTT) at pH 4.0, and applied to a CM-Sepharose Fast-Flow column (1.4 by 30 cm; Pharmacia). Proteins were eluted with a linear gradient of 0 to 0.15 M EDTA (pH 8) in the starting buffer (300 ml) at a flow rate of 30 ml/h. (iii) For DEAE-5PW anion-exchange chromatography, unbound-laminarinase-positive fractions from the CM-Sepharose step were dialyzed against 10 mM Tris-HCl, pH 8.5, and applied to a DEAE-5PW column (75 by 8 mm; Tosohaas). Proteins were eluted at a flow

rate of 0.75 ml/min with the following gradient of NaCl in the starting buffer: 0 to 250 mM NaCl over 45 min (linear) and 250 to 500 mM NaCl over 15 min. (iv) For phenyl-Superose chromatography, fractions containing the laminarinase and/or pNPGase activities after dialysis against 50 mM potassium phosphate (pH 7.0) were adjusted to 1 M ammonium sulfate and applied to a column of phenyl-Superose (HR 5/5; Pharmacia). Proteins were eluted at a flow rate of 0.3 ml/min with a reverse gradient of ammonium sulfate of 1 to 0 M over 60 min. (v) For MonoS cation-exchange chromatography, pNPGase-positive fractions from the DEAE-Sepharose step were pooled, dialyzed against 10 mM sodium acetate–5 mM EDTA–10 mM DTT, pH 4.0, and applied to a MonoS column (HR 5/5; Pharmacia). Proteins were eluted at a flow rate of 0.8 ml/min with a gradient of NaCl in the starting buffer of 0 to 300 mM over 50 min, 300 to 500 mM over 10 min, and 500 to 1,000 mM over 5 min. (vi) For gel filtration chromatography, samples were applied to a Superdex 200 column (30 by 1 cm; Pharmacia), which was equilibrated in 10 mM sodium acetate (pH 5.6) containing 150 mM NaCl, at the flow rate of 0.2 ml/min.

Quantification of exoG-I and exoG-II activities was estimated over time during growth. Measurements of growth kinetics were followed by measurements of mycelia (dried weight) as follows: mycelia from 10-ml aliquots of culture medium were filtered through filter paper, repeatedly washed with water, and weighed after 24 h at 80°C. The production of exoG-I and exoG-II activities over culture time was quantified after cell wall autolysis of 500 ml of culture medium and after the DEAE-Sepharose and CM-Sepharose chromatographic steps as described above.

Molecular mass determination and glycan analysis. Amounts of protein in samples were estimated by the Bradford assay (1) with bovine serum albumin as the standard and were analyzed qualitatively by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (18) with a 7.5 or a 10% separating gel and a 4% stacking gel. Proteins were detected by silver staining or by Coomassie blue staining. The presence of O- and/or N-linked glycan was detected on Western blots with concanavalin A (ConA) coupled to peroxidase (Sigma). After Western blot transfer, the nitrocellulose membrane was saturated with 1% bovine serum albumin in TBS (10 mM Tris, 500 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM $CaCl_2$ [pH 7.2], 0.5% Tween 20) for 1 h. Then, the membrane was incubated in the presence of a 1:100 dilution in TBS of ConA-peroxidase conjugated with or without 300 mM *methyl*-α-D-mannopyranoside for 2 h at room temperature. After three washings with TBS, ConA-peroxidase decoration was visualized after addition of 5 ml 0.4% diaminobenzidine and 5 μl of H_2O_2. De-N glycosylation was done with the recombinant endo-*N*-glycosidase F from *Flavobacterium meningosepticum* (Oxford Glycosystems) according to the manufacturer's instructions. The efficiency of enzymatic deglycosylation was monitored by SDS-PAGE. The molecular masses of the native enzymes were estimated by gel filtration chromatography on a Superdex 200 column as described above. Molecular mass protein standards (thyroglobulin, ferritin, catalase, lactate dehydrogenase, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, chymotrypsinogen, and RNase) were from Pharmacia.

^{1}H NMR spectroscopy. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 35°C on a Varian Unity 500 spectrometer operating at a proton frequency of 500 MHz and equipped with a 5-mm-diameter triple pulsed-field gradient gel probe. Spectra were referenced to external trimethylsilyl-3-propionic acid-*d*4 2,2,3,3-sodium salt. exoG-I and exoG-II enzymes were dialyzed against water and then concentrated under vacuum. Residues were dissolved in 50 mM CD_3COONa (Spectrometrie Spin et Techniques, Paris, France) in D_2O (99.95% D; Solvants Documentations Synthèses, Peypin, France), pH 5.5, and then concentrated again under vacuum and redissolved in a 50 mM CD_3COONa solution. Reduced laminarioctaose (11 mg) was dissolved in D_2O, dried under vacuum to remove exchangeable protons, and finally redissolved in D_2O. Experiments were performed with 5 mg of G8r in 750 μl of 50 mM CD_3COONa–D_2O, pH 5.5, containing 0.3 U of exoG-I or 1.5 U of exoG-II. The spectrum of the substrate (at time zero) was recorded without the presence of enzyme. After addition of enzyme, spectra were periodically collected. Substrate and enzyme solutions were equilibrated at 35°C prior to NMR measurements.

RESULTS

Enzyme purification. After 48 h of culture, a 15-liter fermentor produced an average of 600 g (wet weight) of washed broken cell walls. Cell wall autolysis at 37°C during 3 days solubilized 250 to 300 mg of total protein containing β-glucosidase and laminarinase activities. Two exo-β-1,3-glucanases activities were purified following several successive chromatographic steps (Fig. 1). The first purification step, DEAE-Sepharose chromatography, separated two pools of laminarinase activities, one of which contained a pNPGase. The first exo-β-1,3-glucanase activity (exoG-I) was purified from the laminarinase-positive and pNPGase-negative peak eluted at 120 mM NaCl. The second exo-β-1,3-glucanase (exoG-II) was isolated from the pNPGase-positive peak eluted at 240 mM NaCl.

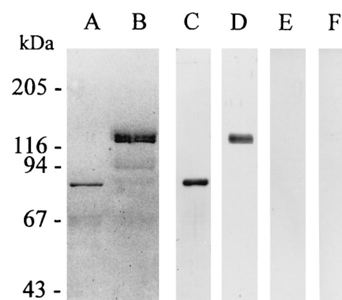

FIG. 2. Analysis of the 82-kDa exoG-I and 230-kDa exoG-II enzymes of *A. fumigatus* by SDS-PAGE with a 7.5% separating gel. (A, C, and E) exoG-I enzyme; (B, D, and F) exoG-II enzyme; (A and B) Coomassie blue staining (0.5 μg/lane); (C and D) analysis by the ConA-peroxidase conjugate method without *methyl*-α-D-mannoside (0.1 μg/lane); (E and F) analysis by the ConA-peroxidase conjugate method with 300 mM *methyl*-α-D-mannoside (0.1 μg/lane).

Purification of 82-kDa exoG-I. The exoG-I activity did not bind to the CM-Sepharose column at pH 4.0 (Fig. 1), and this step allowed us to separate it from other laminarinase activities. The CM-Sepharose unbound fraction was fractionated through the DEAE-5PW column at pH 8.5. The laminarinase activity was eluted at 140 mM NaCl. This activity was further purified by a hydrophobic-interaction chromatographic step. In the presence of 1 M $(NH_4)_2SO_4$, exoG-I strongly bound to the phenyl-Superose column; it was eluted by 50 mM phosphate buffer in the absence of $(NH_4)_2SO_4$. These steps permitted us to obtain a single protein with a molecular mass of 82 kDa as estimated by SDS-PAGE (Fig. 2). Analysis by gel filtration on a Superdex 200 column showed that exoG-I had an apparent molecular mass of 80 kDa, suggesting that this enzyme is a monomeric polypeptide (Fig. 3A).

Purification of 230-kDa exoG-II. The pNPGase-positive fraction of the DEAE-Sepharose step was applied to a MonoS column. At pH 4.0, 200 mM NaCl eluted the exoG-II activity from the cation-exchange column. The laminarinase activity bound to phenyl-Superose and was eluted by 50 mM phosphate buffer containing 100 mM $(NH_4)_2SO_4$. SDS-PAGE analysis showed that this exoG-II enzyme had an apparent molecular mass of 120 kDa (Fig. 2). By gel filtration chromatography on a Superdex 200 column, this enzyme was eluted with an apparent molecular mass of 230 kDa (Fig. 3B), suggesting that the exoG-II enzyme contained two subunits of 120 kDa.

Glycan analysis. The 82-kDa exoG-I and 230-kDa exoG-II glucanases reacted with a ConA-peroxidase conjugate on a nitrocellulose blot after SDS-PAGE. The reaction was inhibited by 0.3 M *methyl*-α-D-mannoside, indicating that both enzymes contain α-mannan chains (Fig. 2). To determine whether both enzymes contained N-linked carbohydrate, these proteins were digested with the endo-*N*-glycosidase F. The digested exoG-I and exoG-II enzymes ran on SDS-polyacrylamide gels as 80- and 98-kDa proteins (data not shown), respectively, indicating that exoG-I and each subunit of exoG-II contained about 2 and 22 kDa of N glycans, respectively.

Kinetics of exoglucanase production. Production of exoG-I and exoG-II activities, estimated after cell wall autolysis, was roughly parallel to fungal growth (Fig. 4). However, exoG-I activity was detected much earlier than exoG-II activity. Thereafter, the rate of exoG-II activity increased rapidly to become higher than the level of exoG-I activity.

Influence of pH and temperature on exoG-I and exoG-II activities. With reduced laminarin as the substrate, exoG-I and

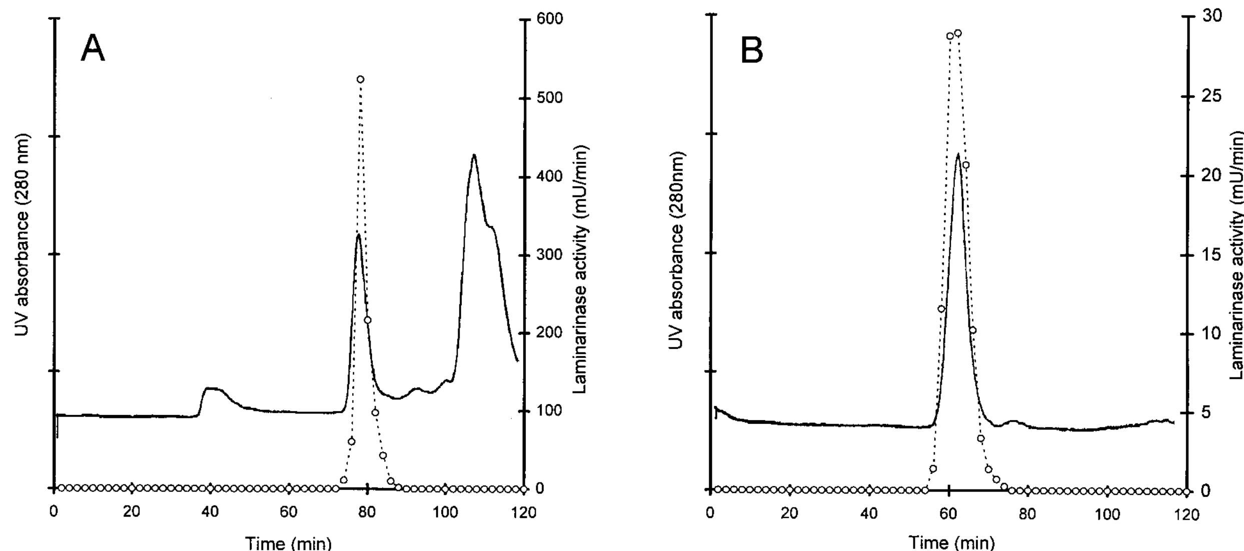

FIG. 3. Results of gel filtration chromatography on a Superdex 200 column of exoG-I (A) and exoG-II (B) enzyme preparations. The column was equilibrated in 10 mM NaOAc–150 mM NaCl, pH 5.6. The continuous lines correspond to UV absorbance at 280 nm and the discontinuous lines correspond to laminarinase activity.

exoG-II activities presented the same pH optimum of 5.0 and optimum temperatures of 55 and 65°C, respectively (Fig. 5). Incubation of the enzymes for 1 h at a pH range of 3 to 8 did not modify the activity of either exoglucanase. Preincubation of enzymes for 1 h at different temperatures showed that enzyme stability was highly reduced at temperatures higher than the optimum temperature.

Specificities of the glucan linkages hydrolyzed by exoG-I and exoG-II activities. HPAE chromatography-pulsed electrochemical detection (PED) analysis showed that both β-glucanase activities released glucose residues from laminarin (data not shown). Various oligosaccharides and glucans containing β-1,3, β-1,6, or β-1,4 linkages were tested as putative substrates for the exo-β-glucanase activities (Table 1). Both β-glucanase activities degraded reduced laminarin and reduced laminari-oligosaccharides (soluble β-1,3 glucan). In contrast, curdlan, an insoluble β-1,3-glucan, was resistant to these exo-β-glucanase activities. Cellopentaose and CM-cellulose

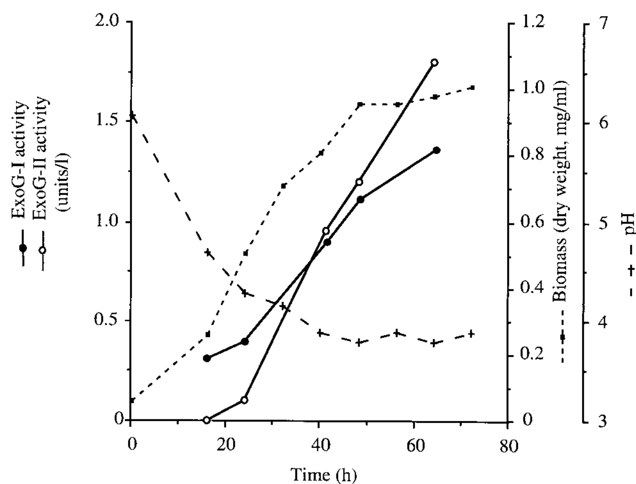

FIG. 4. Production of exoG-I and exoG-II activities during growth of *A. fumigatus* in a glucose-mycopeptone medium at 25°C.

(soluble β-1,4 glucan) were not degraded by either preparation. The exoG-I enzyme did not degrade reduced gentio-oligosaccharides (β-1,6-linkage type). In contrast, exoG-II was able to cleave soluble β-1,6-glucan. Schizophyllan (soluble β-1,3-glucan with β-1,6-glucosyl side branches) was not degraded by exoG-II but was slightly hydrolyzed by exoG-I.

The results presented in Table 1 showed that the specificities of exoG-I and exoG-II were different. exoG-I did not degrade pNP-Glc and was specific for the β-1,3-linkage type, and the presence of a β-1,6-linked branched glucose residue on a β-1,3-glucan chain only slowed down its action. In addition, the longer the chain of β-1,3-glucan is, the higher exoG-I's activity is. The exoG-II enzyme degraded pNP-Glc and cleaved β-1,3 and β-1,6 linkages but was unable to hydrolyze schizophyllan. Laminari-oligosaccharides are hydrolyzed more rapidly by exoG-II than laminarin is. At the same substrate concentration, exoG-II degraded reduced laminarin and laminari-oligosaccharides more rapidly than it degraded pNP-Glc.

Analysis of products released by exoG-I and exoG-II activities. Figure 6 presents the HPAE chromatographic analysis of the products liberated from the degradation of a reduced laminarioctaose by the two exo-β-1,3-glucanase activities of *A. fumigatus*. This HPLC system separated reduced and nonreduced laminari-oligosaccharides. Chromatographic data showed that both enzymes produced glucose residues and reduced laminari-oligosaccharides in the absence of any nonreduced laminari-oligosaccharide, indicating that these enzymes degraded the substrate from the nonreducing end. The production of the glucose residue by exoG-I activity was concomitant with the main accumulation of reduced laminaritetraose without the release of the intermediately sized oligosaccharides, such as G5r, G6r, and G7r. In contrast, the accumulation of glucose residues by the 230-kDa exoG-II activity was accompanied by the liberation of all of the reduced laminari-oligosaccharides with intermediate sizes from G7r to G2r. These two patterns of degradation confirmed that the patterns of action of the two enzymes were different, as shown in Table 1. The results suggest that the exoG-I enzyme degraded G8r until the substrate hydrolyzed reached the size of 4 glucose units linked by β-1,3 linkages and then moved to another substrate molecule. In

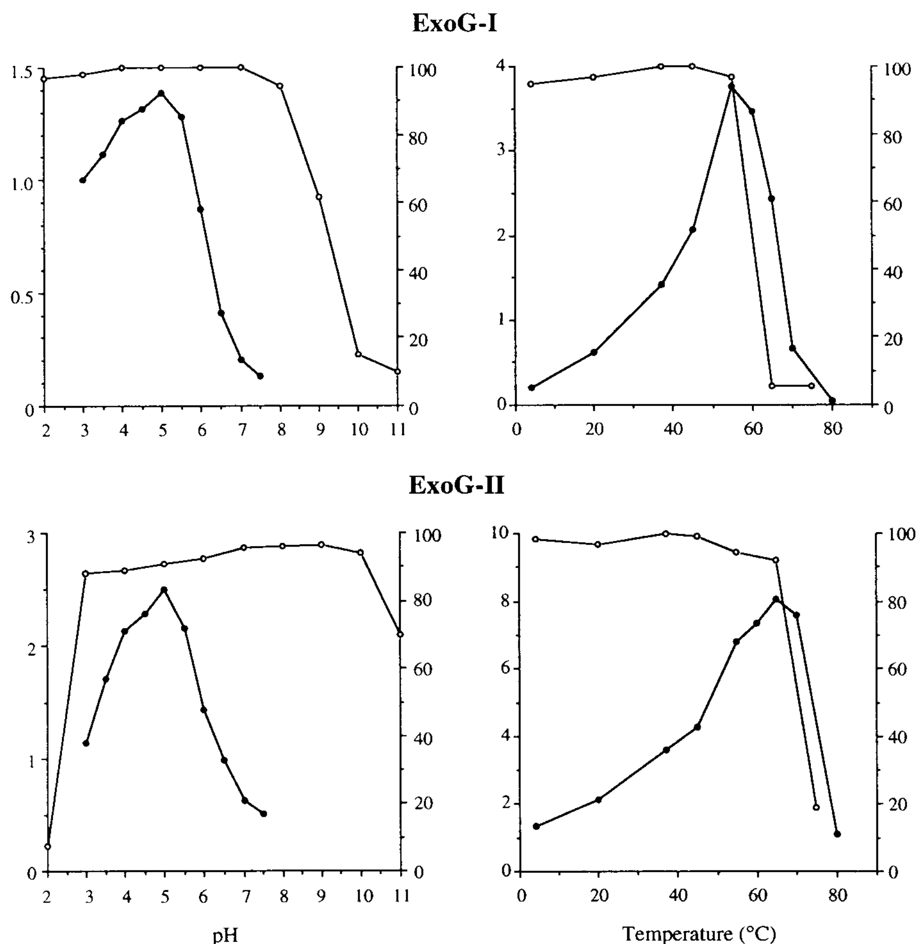

FIG. 5. Effects of pH and temperature on the activities and stability of the exoG-I and exoG-II enzymes of *A. fumigatus*. ●, Laminarinase activity (mU/min); ○, stability at pH 5.0 or at 37°C for 1 h (percentage of initial activity).

contrast, exoG-II did not remain bound to a substrate molecule and moved to another substrate molecule after releasing one residue from the substrate. This may explain the random distribution of the sizes of the substrates hydrolyzed.

TABLE 1. Relative rates of hydrolysis of β-D-glucans, oligosaccharides, and pNP-Glc by exoG-I and exoG-II activities purified from *A. fumigatus* cell walls

Substrate	Relative rate of hydrolysis (%)[a] of:	
	exoG-I	exoG-II
Reduced laminarin	100	100
Reduced laminaritriose	0	50
Reduced laminaritetraose	3	139
Reduced laminaripentaose	10	140
Reduced laminarihexaose	15	172
Reduced laminaridecaose	37	211
Reduced gentio-oligosaccharides	0	124
Cellopentaose	0	0
CM-cellulose	0	0
Curdlan	0	0
pNP-Glc	0	14
Schizophyllan	9	0

[a] In comparison with rates of reduced laminarin degradation after a 10-min enzyme incubation.

Hydrolysis of schizophyllan, which contains a β-1,6-branched glucose residue every 3 glucose residues of a β-1,3-glucan chain, by exoG-I produced glucose and gentiobiose (Fig. 7). A branched reduced laminaridecaose (bG10r) containing an intrachain β-1,6 linkage at the sixth linkage from the reducing end was also hydrolyzed by exoG-I (Fig. 8). In contrast with the results shown in Fig. 6, bG9r, bG8r and bG7r were accumulated with G4r. The accumulation of bG7r indicated that a glucose residue linked by β-1,6 linkages on the terminal nonreducing end of a linear β-1,3-glucan was slowly cleaved by exoG-I. The relatively high levels of liberation of intermediate-sized oligosaccharides bG8r and bG9r indicated that the exoG-I enzyme did not remain bound to the substrate and released only 1 glucose residue before moving to another substrate molecule. The presence of a β-1,6 side-branched glucose or an intrachain β-1,6 linkage in a β-1,3-glucan chain did not prevent exoG-I's action but modified its pattern of degradation, indicating a reduced affinity for the substrate.

Enzyme kinetics. Enzymatic analysis with different concentrations of various substrates (pNP-Glc, G4r, G5r, G10r, G18r, and reduced laminarin) showed that all reactions followed Michaelis-Menten kinetics (data not shown). Using the Lineweaver-Burk double-inverse plot, calculated K_m values for reduced laminarin, based on a molecular mass of 5.3 kDa (degree of polymerization, 32) (8), were 2.3 and 380 μM for exoG-I and exoG-II, respectively (Table 2). This result showed

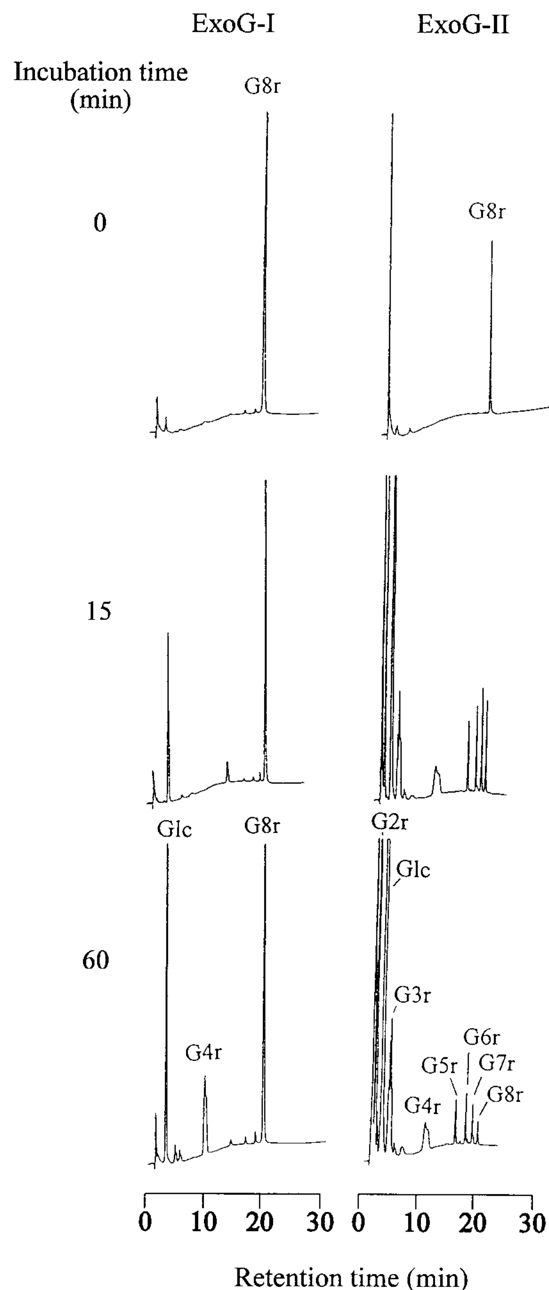

FIG. 6. HPLC analysis of oligosaccharides liberated by exo-β-1,3-glucanase activities from reduced laminarioctaose (HPAE chromatography-PED). Glc, glucose residue; G*n*r, reduced oligosaccharides containing the indicated numbers of β-1,3-linked glucose residues.

that exoG-I, in contrast to exoG-II, had a high affinity for this substrate. K_m values with reduced laminari-oligosaccharides decreased with the increase of the substrate size, suggesting that the affinities of exoG-I and exoG-II activities increased with the size of the β-1,3-glucan.

Effect of metal ions and inhibitors on the exo-β-1,3-glucanase activities. With reduced laminarin as a substrate, a range of metal ions were examined for their effect on exo-β-1,3-glucanases of *A. fumigatus*. As shown in Table 3, divalent metal ions such as Zn^{2+}, Cu^{2+}, Hg^{2+}, and Mg^{2+}, did not inhibit exoG-I and exoG-II activities. In contrast, both activities were totally inhibited by 1 mM $KMnO_4$ and 1 mM *N*-bromosuccinimide whereas a slight inhibition of exoG-II activity was observed with *p*-hydroxymercuribenzoic acid and $FeCl_3$. Deoxynojirimycin, castanospermine, and glucono-δ-lactone acted as competitive inhibitors of exoG-II. In contrast, glucono-δ-lactone and castanospermine had no or little effect on exoG-I whereas deoxynojirimycin was a very potent competitive inhibitor.

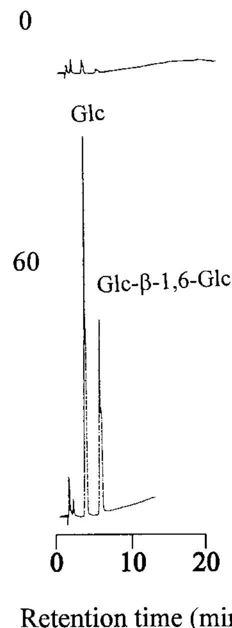

FIG. 7. HPLC analysis of oligosaccharides liberated by exoG-I activity from schizophyllan (a β-1,3-glucan chain with a β-1,6 glucosyl side branch every 3 glucose residues; degree of polymerization > 400) (HPAE chromatography-PED). Glc, glucose residue; Glc-β-1,6-Glc, gentiobiose.

Anomeric configuration of the glucose produced by exoG-I and exoG-II. The one-dimensional ^1H NMR spectrum of the oligosaccharide G8r without enzyme (at time zero) contained three signals in the anomeric region: a doublet at 4.75 ppm corresponding to the glucose residue of the nonreducing end, a doublet at 4.79 ppm corresponding to five glucose residues linked by β-1,3 linkages, and a signal at 4.67 ppm corresponding to the residual water (Fig. 9). The doublet corresponding to the glucose residue linked to the glucitol group was under the water signal (11). The integration of the anomeric signals confirmed the length of the reduced laminarioctaose. The addition of 0.3 U of 82-kDa exoG-I to the substrate (Fig. 9A) led to the appearance of a small doublet in the anomeric region at 5.22 ppm. The coupling constant of this signal was 3.7 Hz. This signal corresponded to the α-anomer of the free glucose released from the oligosaccharide during hydrolysis. Another signal appeared later during the reaction at 4.64 ppm with a coupling constant of 7.9 Hz. It corresponded to the β-anomer of the free-glucose residue. This second peak was due to the anomeric equilibrium in solution between the α and β forms of free-glucose residues. The preponderance of the α-anomer at the beginning of the reaction indicated that the 82-kDa enzyme inverted the configuration of glucose released from reduced

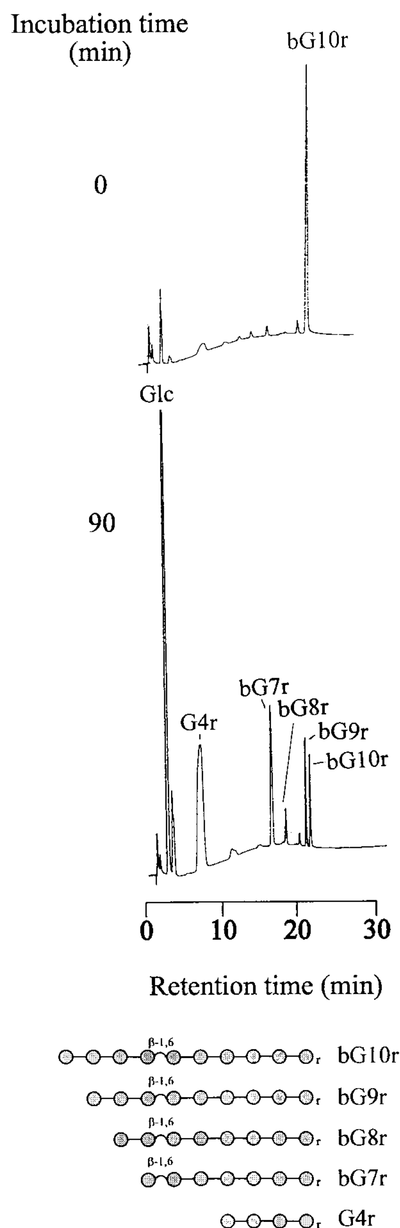

FIG. 8. HPLC analysis of oligosaccharides liberated by exoG-I activity from reduced branched laminaridecaose (HPAE chromatography-PED). Glc, glucose residue; G*n*r, reduced oligosaccharides containing the indicated numbers of β-1,3-linked glucose residues; bG*n*r, reduced laminari-oligosaccharides containing the indicated numbers of glucose residues with an intrachain β-1,6 linkage at the sixth linkage from the reducing end.

TABLE 3. Effects of metal ions and inhibitors on exo-β-1,3-glucanase activities of *A. fumigatus*

Compound or competitive inhibitor	Activity (%) or K_i (μM)[a] of: exoG-I	exoG-II
Compounds		
None	100	100
$KMnO_4$	0	0
$ZnSO_4$	110	98
$CuCl_2$	112	97
$HgCl_2$	104	100
$FeCl_3$	99	93
$MgCl_2$	103	103
N-Bromosuccinimide	0	0
p-HMB[b]	94	88
Carbodiimide	106	103
Iodoacetic acid	104	104
Succinic anhydride	105	102
Competitive inhibitors		
Glucono-δ-lactone	—[c]	21
Deoxynojirimycin	0.5	0.8
Castanospermine	230	3.2

[a] Activities are expressed as relative percentages of the activities of the respective enzymes with metal ions or inhibitors. For competitive inhibitors, K_is are given.
[b] *p*-HMB, *para*-hydroxymercuribenzoic acid.
[c] —, no effect.

laminarioctaose. After 8 h of reaction at 35°C, the α/β ratio was 29/71. During the reaction, we also observed a diminution of the signal at 4.79 ppm, which corresponds to the diminution of the oligosaccharide length, to obtain a mixture of G4r and G3r.

The addition of 1.5 U of the 230-kDa activity to the substrate (Fig. 9B) led to the appearance of a strong doublet at 4.64 ppm, with a coupling constant of 7.9 Hz. This signal corresponds to the β-anomer of free glucose. A small doublet was also present at 5.22 ppm, with a coupling constant of 3.7 Hz, which corresponds to the α-anomer of free glucose. After 29 min of reaction, the ratio of α to β was 6/94 and then this ratio shifted progressively toward the equilibrium ratio of 29/71 under these conditions. This result indicated that the 230-kDa exoG-II enzyme catalyzed hydrolysis of laminarioctaose with the retention of the anomeric configuration of the glucose product released. After 1 h, the anomeric signals at 4.75 and 4.79 ppm disappeared, indicating the complete degradation of the substrate by the enzyme.

Activity on the *A. fumigatus* cell wall. exoG-I and exoG-II were able to release glucose residues from the β-1,3-glucans containing the alkali-insoluble fraction of the *A. fumigatus* cell

TABLE 2. K_m and V_m values of exo-β-1,3-glucanase activities purified from *A. fumigatus* cell walls[a]

Substrate	exoG-I K_m μM	exoG-I K_m mg/ml	exoG-I V_m (mU/μg of protein)	exoG-II K_m μM	exoG-II K_m mg/ml	exoG-II V_m (mU/μg of protein)
Reduced laminarin	2.3	0.012	173	380	2.0	79
pNP-Glc	NA	NA	NA	4,700	1.4	98
Reduced laminaritetraose	NA	NA	NA	820	0.55	231
Reduced laminaripentaose	ND	ND	ND	510	0.42	157
Reduced laminaridecaose	4.9	0.008	86	390	0.64	91
Reduced laminarioctadecaose	3.3	0.010	116	ND	ND	ND

[a] ND, not determined; NA, not appropriate.

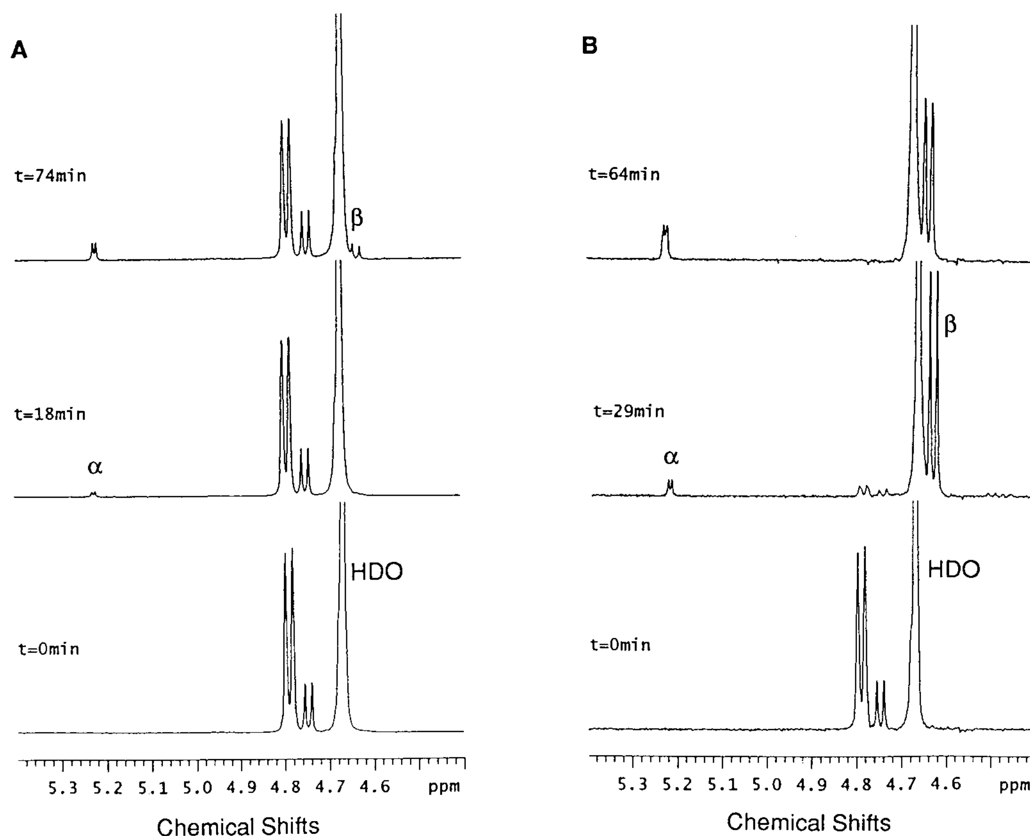

FIG. 9. Partial ^{1}H NMR spectra of anomeric protons of reduced laminarioctaose and hydrolysis products released from it by the action of the 82-kDa exoG-I (A) and 230-kDa exoG-II (B) enzymes. The reference spectrum (time zero [t=0]) was recorded before addition of enzyme, and the other spectra were recorded at times after addition of enzyme (exoG-I, 0.3 U; exoG-II, 1.5 U). Spectra were acquired with 32 accumulations for exoG-I and 1 accumulation for exoG-II.

wall (Fig. 10). After 30 min of incubation, exoG-II released an amount of glucose corresponding to 0.5% (wt/wt) of the fraction and then did not act further on the cell wall fraction. exoG-I was more active than exoG-II, and after 4 h of incubation, it released 5% of the cell wall fraction. Fifty percent of the alkali-insoluble fraction was β-1,3-glucan, which corresponds to the total β-1,3-glucan amount of the cell wall and which is associated with chitin (25%) (12) and galactomannan (25%) (unpublished results), indicating that exoG-I was able to degrade 10% of total β-1,3-glucans in the cell wall.

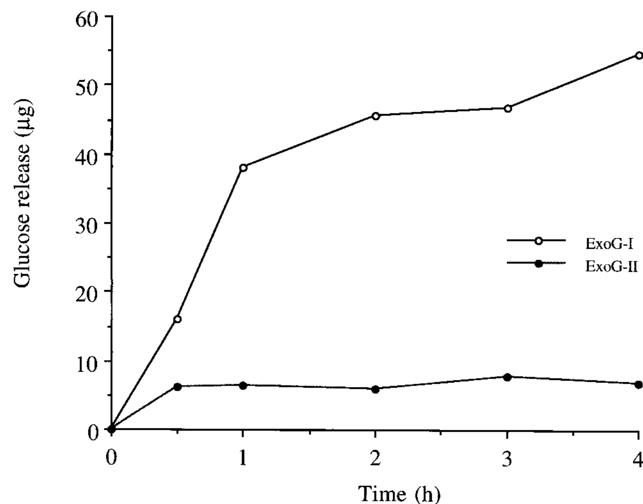

FIG. 10. ExoG-I and exoG-II activities on the alkali-insoluble cell wall fraction of *A. fumigatus*. Dried cell wall fractions (800 μg) containing 400 μg of glucose in 400 μl were incubated with 0.11 U of exoG-I or 0.3 U of exoG-II at 37°C.

DISCUSSION

The two exo-β-1,3-glucanases, isolated from the cell wall of *A. fumigatus* and described in this study as exoG-I and exoG-II, are N glycosylated, have an acidic pH optimum, and are stable under a wide range of pHs and temperatures (Fig. 2 and 4). These properties are common to most fungal β-1,3-glucanases (23, 33, 50). SDS-PAGE and gel filtration chromatography indicated that exoG-I is an 82-kDa monomeric polypeptide and that exoG-II is a 230-kDa dimeric polypeptide containing two 120-kDa subunits.

Two different types of activity were noticed for the two *A. fumigatus* exo-β-1,3-glucanases reported here. The 230-kDa exoG-II enzyme was able to degrade β-1,3- and β-1,6-glucan chains and pNP-Glc (Table 1). Degradation of a β-1,3-glucan by exoG-II resulted in the progressive accumulation of intermediates and was inhibited by structural analogs of glucose, which are classical competitive inhibitors of β-glucosidases (39). The 82-kDa exoG-I enzyme was specific to the β-1,3-glucan chain and did not act on pNP-Glc, laminaribiose, or laminaritriose. The anomeric configuration of glucose released by the exo-β-1,3-glucanases of *A. fumigatus*, analyzed by ^{1}H

NMR (Fig. 9), also showed that the catalytic mechanisms of the enzyme reaction are different (22, 48, 50). Results from substrate specificities (Table 1) and the anomeric configuration of glucose released (Fig. 9) indicated that 82-kDa exoG-I corresponds to a 1,3-β-D-glucan-glucohydrolase (EC 3.2.1.58) and that 230-kDa exoG-II corresponds to a β-D-glucoside-glucohydrolase (EC 3.2.1.21) (33). The molecular mass and enzymatic characterization of the latter enzyme indicated that it did not correspond to the secreted β-glucosidases already described for *A. fumigatus* (42, 43).

Most fungal exo-β-1,3-glucanases are able to degrade β-1,3-*gluco*-oligosaccharides and pNP-Glc and correspond to β-glucosidase activities. Few fungal exo-β-1,3-glucanases like *A. fumigatus* exoG-I are not able to degrade this synthetic substrate (17, 24, 31, 36, 49). The exoG-I enzyme degraded the glucan chain to a limit substrate size of 4 glucose residues, suggesting that the active site of this enzyme recognizes 5 linked β-1,3 glucose residues. The high specificity of exoG-I for β-1,3-glucan was in agreement with its lower sensitivities to castanospermine and glucono-δ-lactone. In addition, a single β-1,6 intrachain linkage in a β-1,3-glucan partially blocked the recognition and the catalytic effect of this activity, indicating the high affinity of this enzyme for β-1,3-linked glucan. However, the presence of a β-1,6-branched glucose residue on a β-1,3-glucan did not prevent the degradation, suggesting that the free hydroxyl group of carbon 6 is not involved in the recognition site. This property was also common to fungal exo-β-glucanases cleaving specifically a β-1,3-glucan chain (24, 31, 49, 52). A third exo-β-1,3-glucanase was isolated from the cell wall autolysate of *A. fumigatus* (data not shown). SDS-PAGE and gel filtration analysis indicated that this activity corresponded to a 43-kDa protein. This third exo-β-1,3-glucanase presented enzymatic properties similar to those of 82-kDa exoG-I, with an optimum pH of 4.5; an optimum temperature of 55°C; a K_m of 2.5 μM with reduced laminarin; competitive inhibition by deoxynojirimycin (K_i, 58 μM); similar hydrolysis of laminarin, G8r, bG10r, and schizophyllan; and the absence of reactivity with pNP-Glc, laminaribiose, and laminaritriose (data not shown). This enzyme, which constitutes a second specific exo-β-1,3-glucanase produced by *A. fumigatus* accounted for only less than 5% of the total laminarinase activity found in the cell wall and was not further characterized.

Two main patterns of enzymatic attack by exo- and endohydrolases have been discussed: the multichain single attack and the single-chain repetitive attack. In the multichain-attack theory, once the enzyme has cleaved a glucan chain it moves to another glucan chain. In the repetitive-attack theory, the same glucan chain is cleaved several times before the enzyme-bound substrate complex is disassociated (40, 41). The release of reduced glucans of all intermediate sizes by 230-kDa exoG-II indicated that exoG-II cleaves the substrate once at random and then moves to other substrate molecules as in the multichain-attack theory. In contrast, the exoG-I enzyme produced only glucose residues and a tetrasaccharide without any products of an intermediate size. These data indicated that this enzyme remained bound to the same glucan chain and cleaved the same substrate repetitively until the limit substrate size was obtained and then moved to another glucan chain as in the single-chain repetitive attack. The comparison of the maximum initial velocity (V_m) obtained with β-1,3-glucans of various sizes suggests also the existence of these two patterns (50). As shown for α-amylase activity, the increase in exoG-I's V_m with the increase in size of the substrate chain favors the repetitive-attack theory (51). Indeed, the calculated V_ms for exoG-I and exoG-II with G4r, G5r, G10r, G18r, and reduced laminarin as substrates (Table 2) showed opposite patterns for exoG-I and exoG-II. These results suggested that (i) the number of times cleavage by exoG-I occurred increased with the length of the β-1,3-glucan chain and (ii) at high concentrations of substrate, the exoG-II enzyme recognized more easily the nonreducing end of the smaller β-1,3-glucan chain. To date, patterns of enzymatic attack of fungal exo-β-1,3-glucanase have not been investigated. However, a comparison of the specificities of the glucan linkages hydrolyzed as well as the size of the substrate suggested that *Sporotrichum dimorphosporum* (formerly *Basidiomycete* sp. strain QM806 [26, 27]), *Porodisculus pendulus* (14), *C. albicans* (24), *Trichoderma harzianum* (17), *Rhizoctonia solani* (31), *Sclerotium glucanicum* (36), *Aspergillus saitoi* (16), and *Botrytis cinerea* (49) secrete activities similar to those of the 82-kDa exoG-I enzyme of *A. fumigatus* with a repetitive attack.

Numerous fungal exo- and endo-β-1,3-glucanases are cell wall associated. Because of their cellular location as well as their ability to degrade β-1,3-glucan, which is the main component of the fungal cell wall, a role in hyphal morphogenesis has been postulated for these enzymes (29, 30, 32, 37, 53). In *A. fumigatus*, in addition to the 3 exo-β-1,3-glucanases mentioned above, one cell wall-associated endo-β-1,3-glucanase of 74 kDa was purified (7). This endo-glucanase and exoG-I accounted for 13 and 83% of the total specific β-1,3-glucanase activity, whatever stage of fungal growth was considered (data not shown). The presence of the exo-β-1,3-glucanase in an amount higher than that of endo-β-1,3-glucanase seems a general condition among fungi (3, 4, 36, 49). In *A. fumigatus*, exoG-II had a very limited activity on cell wall β-1,3-glucans (Fig. 10) and was produced later than exoG-I (Fig. 4) and 74-kDa endo-β-1,3-glucanase during mycelium growth (not shown). The exoG-II enzyme, able to degrade β-1,3, β-1,6-glucans and pNP-Glc, is similar to many exo-β-glucanases (28, 33) and probably does not play a role in the organization of β-1,3-glucan in the cell wall (20, 25, 47). In contrast to exoG-II, exoG-I and the 74-kDa endo-β-1,3-glucanase were able to hydrolyze β-1,3-glucans of the cell wall. In addition, exoG-I as well as the 74-kDa endo-β-1,3-glucanase seems constitutively expressed and specifically acts on β-1,3-glucan. These observations suggest a possible involvement of these enzymes during wall expansion. However, the reason of the formation of the undegraded tetrasaccharidic limit chain by exoG-I during cell wall morphogenesis remains unknown. Disruption of the structural gene encoding these enzymes is under way and should give an indication of the respective functions of these activities during cell wall morphogenesis. Until now, no gene encoding an exo-β-1,3-glucanase similar to exoG-I has been disrupted.

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