THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 36, Issue of September 8, pp. 27594–27607, 2000

Printed in U.S.A.

Molecular Organization of the Alkali-insoluble Fraction of *Aspergillus fumigatus* Cell Wall*

Received for publication, December 12, 1999, and in revised form, June 22, 2000
Published, JBC Papers in Press, June 26, 2000, DOI 10.1074/jbc.M909975199

Thierry Fontaine‡§, Catherine Simenel¶, Guy Dubreucq‡, Olivier Adam‡, Muriel Delepierre¶, Jérome Lemoine‖, Constantin E. Vorgias, Michel Diaquin‡, and Jean-Paul Latgé‡**

*From the ‡Laboratoire des Aspergillus, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris cedex 15, France, the ¶Laboratoire de Résonance Magnétique Nucléaire, Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris cedex 15, France, the ‖Laboratoire de Chimie Biologique, Université des Sciences et Technologie de Lille Flandres-Artois 59655 Villeneuve d'Ascq cedex, France, and the **University of Athens, Department of Biology, Division of Biochemistry and Molecular Biology GR-15701, Athens, Greece*

Physical and biological properties of the fungal cell wall are determined by the composition and arrangement of the structural polysaccharides. Cell wall polymers of fungi are classically divided into two groups depending on their solubility in hot alkali. We have analyzed the alkali-insoluble fraction of the *Aspergillus fumigatus* cell wall, which is the fraction believed to be responsible for fungal cell wall rigidity. Using enzymatic digestions with recombinant endo-β-1,3-glucanase and chitinase, fractionation by gel filtration, affinity chromatography with immobilized lectins, and high performance liquid chromatography, several fractions that contained specific interpolysaccharide covalent linkages were isolated. Unique features of the *A. fumigatus* cell wall are (i) the absence of β-1,6-glucan and (ii) the presence of a linear β-1,3/1,4-glucan, never previously described in fungi. Galactomannan, chitin, and β-1,3-glucan were also found in the alkali-insoluble fraction. The β-1,3-glucan is a branched polymer with 4% of β-1,6 branch points. Chitin, galactomannan, and the linear β-1,3/1,4-glucan were covalently linked to the nonreducing end of β-1,3-glucan side chains. As in *Saccharomyces cerevisiae*, chitin was linked via a β-1,4 linkage to β-1,3-glucan. The data obtained suggested that the branching of β-1,3-glucan is an early event in the construction of the cell wall, resulting in an increase of potential acceptor sites for chitin, galactomannan, and the linear β-1,3/1,4-glucan.

The fungal cell wall is a physically rigid layer that protects the fungal cell from its environment, mediates cell-cell interaction, and is responsible for the shape of the cell. Despite its central role in growth and survival, the fungal cell wall remains poorly studied and its biosynthesis is insufficiently understood (1, 2).

Cell wall polysaccharides are separated in two groups according to their solubility in hot alkali solution. The structural skeleton of the cell wall is alkali-insoluble. It has been known for a long time that β-1,3-glucan and chitin (linear polymer of β-1,4-*N*-acetylglucosamine) are the main components of the alkali-insoluble fraction. The alkali insolubility of glucan is due to its covalent linkage with chitin (3–5). The covalent bond between the two polysaccharides has been characterized in *Saccharomyces cerevisiae* by Kollar *et al.* (6), who showed that chitin is linked to the nonreducing end of a β-1,3-glucan chain. More recently, the same research group reported that the core of the yeast cell wall is a complex structure with a β-1,6- and β-1,3-glucan to which chitin and some mannoproteins are attached (7). In yeast, cell wall-bound glycoproteins have been described to be covalently linked to β-1,6-glucan (8–10). These proteins are originally GPI-anchored to the membrane (11, 12) and then cleaved to be transferred onto β-1,6-glucan using the sugar moiety of GPI as a bridge (7, 13). Ethanolamine and mannose residues, but not glucosamine and inositol, are in the GPI remnant involved in the protein-glucan linkage (14). Another family of cell wall proteins are directly bound to β-1,3-glucan and released by mild alkali treatments (15). In contrast to yeast, the polymer organization of the cell wall of filamentous fungi has been poorly studied. Basically, it is only known that the alkali insolubility of their cell wall results, like in yeast, from the covalent association between glucan and chitin, with a concentration of chitin (around 10%) that is considerably higher than in yeast (2%) (16).

To better understand the organization of the cell wall components of a filamentous fungus and to gain further insight into the biosynthetic pathways involved in cell wall construction, we have focused our studies toward the chemical characterization of the interpolymer linkages occurring in the structural part of the cell wall, *i.e.* alkali-insoluble fraction of cell wall. The fungal model used is *Aspergillus fumigatus*. Using specific enzymatic digestion and various carbohydrate chemistry methods, we have shown that four polysaccharide components constituted this fraction: β-1,3-glucan was highly branched and was linked to chitin, galactomannan, and a linear β-1,3/1,4-glucan never described before.

EXPERIMENTAL PROCEDURES

Preparation of Cell Walls

A. fumigatus CBS 144–89 was grown in a 15-liter fermenter in a liquid medium containing a 2% glucose and 1% mycopeptone (Biokar Diagnostics) as described previously (17). After 24 h of culture (linear growth phase), the mycelia were collected by filtration, washed extensively with water and disrupted in a 50 mM Tris-HCl, pH 7.5 buffer containing 50 mM EDTA and 1 mM phenylmethylsulfonyl fluoride in a Dyno-mill (W. A. Bachofen AG, Basel, Switzerland) cell homogenizer in the presence of 1-mm-diameter glass beads at 4 °C. The disrupted mycelial suspension was centrifuged (8000 × *g* for 10 min), and the cell wall pellet was washed three times with the same buffer and stored at −20 °C.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed. Tel.: 33-0145688225; Fax: 33-0140613419; E-mail: tfontain@pasteur.fr.

Fractionation of Cell Walls by Alkali and Enzymatic Treatments

Fractionation and digestion steps of the cell wall are summarized in Fig. 1. The disrupted cell wall pellet (50 g of wet weight, equivalent to 7.5 g of dried material) was incubated twice in 200 ml of 1 M NaOH at 65 °C for 30 min. A third NaOH treatment did not release any extra material from the pellet. The alkali-insoluble pellet was washed five times with water and once with 50 mM Tris-HCl, pH 7.4 buffer. The pellet was resuspended in the same buffer supplemented with 5 mM sodium azide (60 ml) and incubated with 180 μl of Quantazyme (50 units/μl, recombinant endo-β-1,3-glucanase; Quantum Industry, Quebec, Canada) at 37 °C for 5 days. Endoglucanase digestion was repeated once. Pooled supernatants (QzSN) were kept frozen. After Quantazyme digestion, the insoluble pellet was treated twice with 1 M NaOH at 65 °C for 30 min both to inactivate Quantazyme and to extract material that had become alkali-soluble after the glucanase treatment (accounting for 8% of the total alkali-insoluble starting material). After washing with water, the insoluble pellet residue was resuspended in 80 ml of 50 mM Tris-HCl, pH 8.0 containing 5 mM sodium azide and incubated at 37 °C for 5 days with 4 ml of recombinant chitinase A (0.5 mg of protein/ml) from *Serratia marcescens* produced in *Escherichia coli* and purified as described previously (18). After centrifugation, the residual pellet was treated again with 1.2 ml of chitinase A in 40 ml of 50 mM Tris-HCl, pH 8.0, for 3 days. After centrifugation, supernatants (ChSN) were pooled. The Quantazyme-chitinase resistant pellet was treated with sodium hydroxide in the same conditions as described above releasing an alkali-soluble fraction, which accounted for 2% of the total alkali-insoluble starting material. The final pellet (FP)[1] was extensively washed with water before freeze drying. QzSN, ChSN, and FP were the three fractions analyzed.

Liquid Chromatography Procedures

Fractionation of Water-soluble Fractions (QzSN and ChSN) Released during Enzymatic Digestions—After concentration under vacuum, the QzSN and ChSN fractions were fractionated by gel filtration on a TSK HW40S column (90 × 1.4 cm, ToyoPearl) eluted with 0.25% (v/v) acetic acid at 0.5 ml/min. The products were detected by refractometry. The excluded fractions, eluting at the void volume of the TSK HW40S column, were run on a Sephadex G100 column (90 × 1.4 cm; Amersham Pharmacia Biotech) eluted with 50 mM sodium acetate, pH 6.0, at 9 ml/h. Polysaccharide sizes were estimated based on dextran standards (Amersham Pharmacia Biotech). All fractions were desalted by gel filtration on a Sephadex G15 column (35 × 2.5 cm; Amersham Pharmacia Biotech) eluted with 20 mM acetic acid at 2 ml/min and freeze dried.

High Performance Anion Exchange Chromatography of Oligosaccharides—Analysis of oligosaccharides was performed by HPAEC with a pulsed electrochemical detector and an anion exchange column (Carbo PAC PA-1, 4.6 × 250 mm, Dionex) using the following gradient at a flow rate of 1 ml/min: 0–2-min isocratic step with a mixture containing 98% of solution A (NaOH 50 mM) and 2% of solution B (NaOAc, 500 mM in NaOH 50 mM), 2–15 min of linear gradient (98% A + 2% B − 60% A + 40% B), 15–35 min of linear gradient (60% A + 40% B − 25% A + 75% B), and 35–37 min of linear gradient (25% A + 75% B − 100% B). The column was stabilized 20 min before injection. Purification of oligosaccharides was performed with a preparative column (Carbo PAC PA-1, 9 × 250 mm, Dionex) at a flow rate of 4 ml/min and the following gradient: 0–2-min isocratic step with a mixture containing 90% of solution A and 10% solution B, 2–5 min of linear gradient (90% A + 10% B − 68% A + 32% B), 5–36 min of linear gradient (68% A + 32% B − 61% A + 39% B), 36–37 min of linear gradient (61% A + 39% B − 100% B). The column was stabilized 20 min before injection. To avoid degradation by peeling, occurring during chromatography in 50 mM alkali solution, laminarioligosaccharides were reduced with $NaBH_4$ (10 mg/ml in 100 mM NH_4OH) overnight and desalted over a Sephadex G15 column.

Carbohydrate Composition

Total hexoses were quantified by the phenol-sulfuric acid procedure using glucose as standard (19). Total hexosamines were quantified by the Johnson procedure using glucosamine as standard (20). Monosaccharides were analyzed by GLC as trimethylsilylated methyl glycosides obtained after methanolysis (0.5 M HCl in dried methanol, 24 h, 80 °C), *N*-reacetylation and trimethylsilylation (21), and/or as alditol acetates obtained after hydrolysis (4 N trifluoroacetic acid, 100 °C, 4 h), reduction, and peracetylation (22). Derivatized monosaccharides were separated and quantified on a DB5 capillary column (25 m × 0.32 mm, SGE) using a Delsi 200 apparatus (carrier gas, 0.7 bar helium; temperature program, 120–180 °C at 2 °C/min and 180–240 °C at 4 °C/min).

Methylation

Fractions were methylated using the lithium methyl sulfinyl carbanion procedure (23) modified by Fontaine *et al.* (24). Methyl ethers were either obtained (i) after hydrolysis (4 N trifluoroacetic acid, 4 h, 100 °C) and analyzed as polyol acetates by GLC-MS (25) or (ii) after methanolysis (0.5 M HCl in dried methanol, 24 h, 80 °C) and analyzed as partially methylated methyl glycosides by GLC-MS (26).

Chemical Degradation of Polysaccharides and Oligosaccharides

Release of galactofuranoside residues from galactomannan was obtained by mild acid hydrolysis with 15 mM HCl at 100 °C for 20 h (17). Periodate oxidation was performed after incubation of 10 mg of material in 2 ml of sodium *meta*-periodate, 100 mM, during 7 days at 4 °C in the dark (27). Excess of reagent was destroyed following addition of 200 μl of ethylene glycol. After dialysis against water (membrane cut-off, 1000 Da) or gel filtration chromatography on Sephadex G15 column as described above, the oxidized product was reduced for 2 h in 100 mM ammonium hydroxide (2 ml) containing 20 mg of $NaBH_4$. Excess of reagent was destroyed by addition of Dowex 50 × 8 (H^+ form) resin beads until a pH of 5–6 was reached. After co-distillations with methanol, Smith degradation was performed with 10% acetic acid at 100 °C for 1 h. Degraded products were then separated by gel filtration chromatography on a TSK HW40S column as described above.

Acetolysis of soluble polymers was performed according to Ferguson (28). Peracetylated products (40 mg) were treated with 10 ml of an acetic acid/acetic anhydride/sulfuric acid solution (10:10:1 v/v/v) at 25 or 37 °C for 3, 5, 7, and 24 h. The reaction was stopped by addition of 40 ml of pyridine and water (1:3 v/v). The peracetylated products were extracted with chloroform and washed with water. Deacetylation was performed in 300 mM NaOH and $NaBH_4$ (10 mg/ml) overnight at room temperature.

Enzymatic Treatments

Hydrolysis with a 74-kDa endo-β-1,3-glucanase (ENG1) purified from an *A. fumigatus* cell wall autolysate was performed as described previously (29). Briefly,1 mg of sample was digested in 500 μl of a 100 mM imidazole-acetic acid, pH 7.0 buffer with 10 μl of the 74-kDa endo-β-1,3-glucanase solution (specific activity, 1.5 μmol glucose equivalents/min/ml) at 37 °C for 24 h.

To remove GlcNAc from the terminal nonreducing end of an oligosaccharide, 5 mg of sample were incubated with 5 μl of β-D-*N*-acetylglucosaminidase from Jack bean (10 units/160 μl, Sigma) in 250 μl of a 250 mM sodium acetate, pH 5.0 buffer at 25 °C for 24 h. After addition of 50 μl of 1 M NaOH, oligosaccharides were treated with $NaBD_4$ as described before.

Transgalactosylation of terminal nonreducing GlcNAc residues was performed using the following procedure: samples containing 5 mg of carbohydrate were incubated in 600 μl of 50 mM Tris-HCl, pH 7.5, containing 1 mM $MnCl_2$ and 5 mM sodium azide, with 120 μl of UDP-Gal (10 mg/200 μl) and 30 μl of galactosyl transferase (1 milliunit/ml; Roche Molecular Biochemicals) at 37 °C during 3 days. A supplementary batch of 12 μl of enzyme and 20 μl of UDP-Gal was added to the sample, which was incubated for another 3 days at 37 °C. The reaction was stopped by passing through two ion exchange columns (3 ml, Dowex 1 × 2 acetate form, Dowex 50 × 2, H^+ form). The eluted products were desalted by gel filtration on a HW40S column as described above.

Lectin Affinity Chromatography

Fractions containing 10 mg of carbohydrate were applied to a column of Concanavalin A-Sepharose (4 ml; Amersham Pharmacia Biotech)

[1] The abbreviations used are: FP, final pellet; TOCSY, total two-dimensional correlated spectroscopy; DQF-COSY, two-dimensional double-quantum-filtered correlated spectroscopy; RELAYH, two-dimensional relayed coherence transfer correlated spectroscopy; geHSQC, pulsed field gradient heteronuclear single quantum coherence experiment with multiplicity editing; gHMBC, multiple bond heteronuclear multiple quantum coherence experiment with gradient selection; gHSQC-TOCSY, HSQC with TOCSY and gradient selection; MALDI-TOF, matrix-assisted desorption/ionization-time of flight; HPAEC, high performance anion exchange chromatography; GLC, gas liquid chromatography; GLC-MS, GLC-mass spectrometry; dp, degree of polymerization; ConA, concanavalin A; GPI, glycosylphosphatidylinositol.

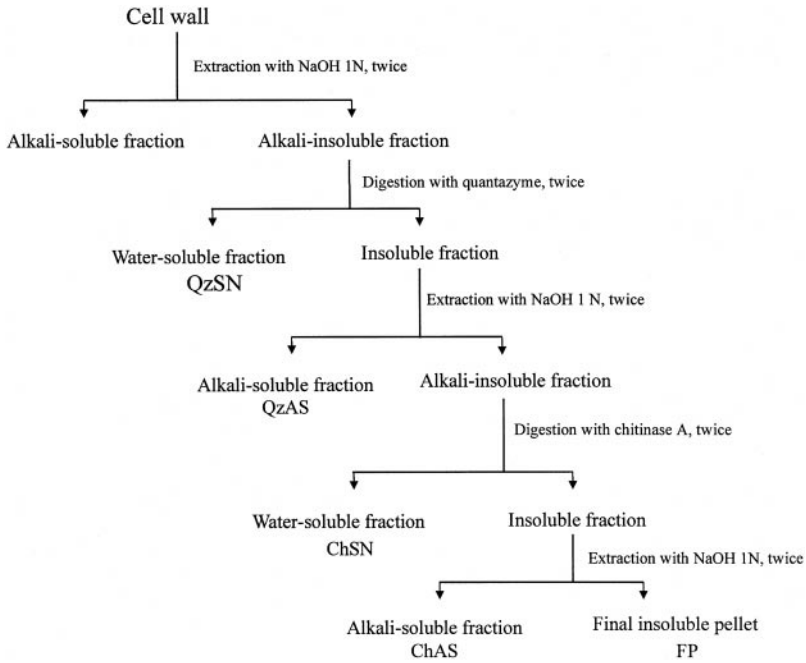

FIG. 1. **Fractionation scheme of *A. fumigatus* cell wall using alkali and enzymatic treatments.**

equilibrated in 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. After washing with the same buffer, bound products were eluted with 200 mM α-methyl-mannoside in the Tris buffer. Removal of salts from collected fractions (500 μl volume) were performed by gel filtration on a Sephadex G15 column as described above.

Transgalactosylated samples were applied to a column of *Erythrina cristagalli* lectin-agarose (4 ml, Vector) equilibrated in 50 mM HEPES, pH 7.5, containing 100 mM KCl, and 2 mM $MgCl_2$. After washing with the same buffer, bound products were eluted with 200 mM lactose in the HEPES buffer (7). Fractions of 500-μl volume were collected and desalted by gel filtration on a TSK HW40S column as described above.

Nuclear Magnetic Resonance Spectroscopy

Samples were deuterium exchanged by freeze drying solution in D_2O and then dissolved in 99.95% D_2O (Solvants Documentation Synthèse, Peypin, France). The polysaccharide concentrations were approximately 3, 40, and 15 mg/ml for QzSN II, QzSN I_B, and ChSN II, respectively. All NMR spectra were collected between 31 and 40 °C on a Varian Unity spectrometer operating at a proton frequency of 500 MHz and a ^{13}C frequency of 125 MHz and equipped with a z-gradient triple resonance (^1H, ^{15}N, ^{13}C) probe. The temperature was chosen for each sample to avoid superposition of the HOD signal with anomeric protons signals. Spectra were collected at 31, 40, and 35 °C for QzSN II (fractions c, d, e, and f), QzSN I_B, and ChSN II, respectively. Spectra were referenced to external trimethylsilyl-3 propionic acid-d_4 2,2,3,3-sodium salt. The NMR signals were assigned by ^1H homonuclear experiments (DQF-COSY (30), RELAYH with single- and two-step relayed coherence transfer using τ delays of 60 ms (31) and TOCSY with mixing times of 80–120 ms (32)) and ^1H-^{13}C heteronuclear NMR experiments (geHSQC leading to one-bond ^1H-^{13}C correlations and identification of methylenic carbons (33) and gHSQC-TOCSY with long mixing times of 80 and 120 ms (33, 34)). This last experiment gave all the ^{13}C resonances of the same residue from the anomeric proton. Linkage assignments were made using two-dimensional nuclear Overhauser effect spectroscopy experiments with mixing time of 400 ms for QzSN II (35), off-resonance rotating frame two-dimensional nuclear overhauser effect spectroscopy with a mixing time of 200 ms (36), and gHMBC experiments with a delay of 60 ms for others samples (33). All two-dimensional data, except for RELAYH and gHMBC, were collected in the phase sensitive mode using the States-Haberkorn method (37). Coupling constants were measured from one-dimensional spectrum recorded with a digital resolution of 0.30 Hz/point or from the DQF-COSY experiment with a digital resolution of 0.75 Hz/point after a zero filling.

Mass Spectrometry

Matrix-assisted desorption ionization/time of flight (MALDI-TOF) mass spectrometry. Mass spectra were measured on a reflectron-type Vision 2000 time of flight mass spectrometer (Finnigan MAT, Bremen, Germany). Samples were mounted on a x, y movable stage allowing irradiation of selected sample areas. A nitrogen laser with an emission wavelength of 337 nm and 3-ns pulse duration was used. The spectrum was recorded in the positive ion mode and accelerated to an energy of 10 keV before entering the flight tube. Ions were post-accelerated for detection to an energy of 10 keV. Samples were prepared by mixing directly on the target 1 μl of oligosaccharide solution (about 25 pmol) and 1 μl of 2.5-dihydroxybenzoic acid matrix solution (12 mg/ml dissolved in CH_3OH/H_2O (80:20 v/v)).

Gas Chromatography-Mass Spectrometry

GLC-MS analysis were recorded using a Automass II 30 quadripolar mass spectrometer interfaced with a Carlo Erba 8000 Top gas chromatograph (Finnigan, Argenteuil, France). Electron ionization spectra were recorded using an ionization energy of 70 eV. Positive chemical ionization spectra were obtained at 70 eV using ammonia as reagent gas. Gas chromatograph was equipped with a CP-Sil 5CB/MS capillary column (25 m × 0.32 mm, Chrompack) gas vector, and helium was at the flow rate of 2 ml/min; the column temperature was 100–240 °C at 5 °C/min.

RESULTS

Fractionation and General Composition of the Cell Wall

NaOH treatment of 7.5 g of cell wall dried material (equivalent to 50 g of wet weight) resulted in the production of 3 g of alkali-insoluble fraction (40% of the wall dry weight). Treatment of this fraction by sequential incubation with recombinant β-1,3-glucanase (Quantazyme) and chitinase, alternated with alkali treatment, made soluble 90% of the alkali-insoluble starting material. (Fig. 1 and Table I). Further purification of the soluble fractions by gel filtration chromatography on TSK-HW40S and Sephadex G100 columns resulted in the separation of nine fractions of different molecular mass (Figs. 2 and 3). The amounts of material of each fraction, expressed as percentages of original dry weight, and their sugar compositions are shown in Table I. Three out of the four fractions released by Quantazyme contained only glucose, whereas the high molecular weight fraction (QZSN I_A) contained glucose associated with galactose and mannose. In a similar way, low molecular weight fractions ChSN III and ChSN IV released by chitinase were exclusively composed of GlcNAc, whereas fractions ChSN I_A, ChSN I_B, and ChSN II contained GlcNAc associated with various amounts of glucose, galactose, and mannose. The chemical analysis of the different fractions resulted in the identifi-

TABLE I
Monosaccharide composition of the fractions obtained by NaOH and enzymatic treatments of the alkali-insoluble fraction of A. fumigatus cell wall

Fraction	% (weight)	Monosaccharide molar ratio				
		Man	Glc	Gal	GlcNAc	GalNAc
QzSN I_A	16.4	1.1	1	1.4		
QzSN I_B	18.7		1			
QzSN II	4.8		1			
Qz SN III	12.3		1			
ChSN I_A	5.7	0.9	1	0.9	0.01	
ChSN I_B	6.2	0.03	1	0.04	0.02	
ChSN II	0.6	0.02	1	0.04	0.06	
ChSN III	0.2				1	
ChSN IV	14.2				1	
QzAS	8.3	0.5	1	0.6	0.15	0.05
ChAS	1.8	0.34	1	0.36	0.06	0.12
FP	11	0.36	1	0.39	1.35	2.7

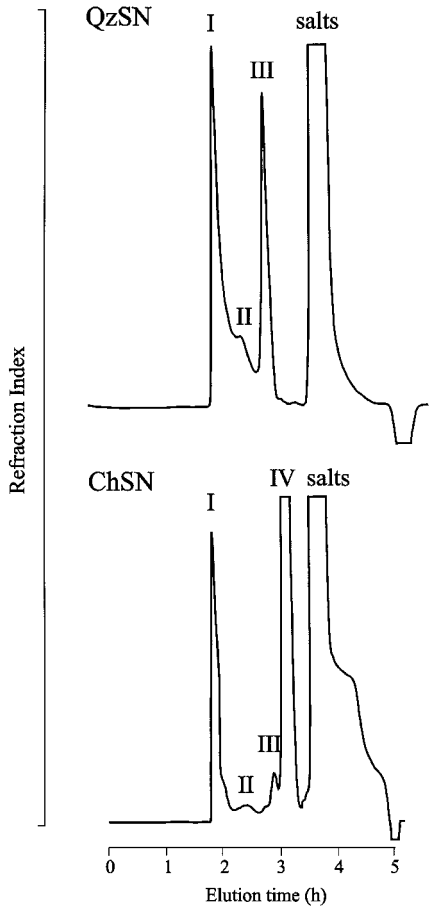

FIG. 2. **Gel filtration chromatography on a TSK HW40S column of the products released by Quantazyme (QzSN) and chitinase (ChSN) digestion of the alkali-insoluble cell wall fraction.** The column (90 × 1.4 cm) was eluted with 0.25% (v/v) acetic acid at 0.5 ml/min. Products (20 mg) were applied to the column and detected by refractometry.

cation of the chemical linkages occurring between the different polysaccharides of the alkali insoluble fraction.

Chemical Characterization of the Water-soluble Products Released by Quantazyme

Fraction QzSN III: Degradation Products Obtained with Endo-β-1,3-glucanase—HPAEC, methylation, and MALDI-TOF mass spectrometry analysis showed that QzSN III corresponded to laminaripentaose, which is the product of hydrolysis of β-1,3-glucan by Quantazyme (data not shown)

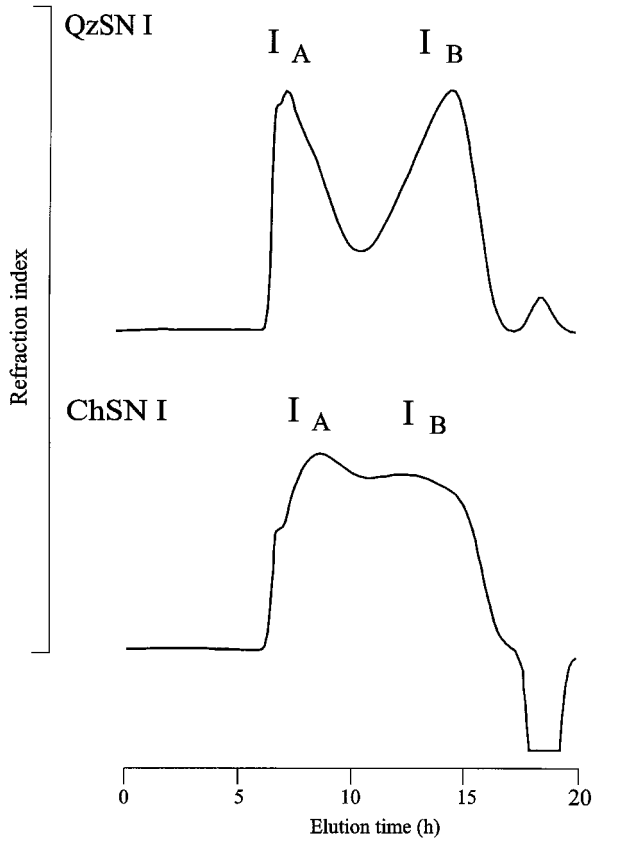

FIG. 3. **Gel filtration chromatography on a Sephadex G100 column of the QzSN I and ChSN I fractions, which eluted at the void volume of the TSK HW40S column (Fig. 2).** The column (90 × 1.4 cm) was eluted with 50 mM sodium acetate, pH 6.0, at 0.5 ml/h. Products (50 mg) were applied to the column and detected by refractometry.

Fraction QzSN II: Mixture of Branched Laminarioligosaccharides—QzSN II (1.5–2.5 kDa) contained a mixture of laminarioligosaccharides that had been reduced with $NaBH_4$ before separation by HPAEC (Fig. 4). MALDI-TOF mass spectrometry analysis indicated that the degree of polymerization (dp) of oligosaccharide varied from 9 to 15 (Fig. 4). Products with the same *M*r gave two peaks on HPAEC analysis, suggesting they were chemically organized differently. Analysis of these oligosaccharides was performed by ^1H NMR spectroscopy on two couples of oligosaccharides with 10 and 11 glucose residues (QzSN IIc, QzSN IId, QzSNIIe, and QzSN IIf). The one-dimensional ^1H NMR spectra of QzSN IId and QzSN IIf oligosaccharides were similar to a linear β-1,3-glucan with a glucitol residue at the reducing end as described previously (Ref. 38 and data not shown). The one-dimensional spectrum of the QzSN IIe oligosaccharide contained five doublets and two close-lying doublets in the anomeric region between 4.5 and 4.8 ppm (Table II). These chemical shifts and the coupling constants $^3J_{1,2}$ of these doublets were in good agreement with those published for a linear β-1,3-glucan containing a β-1,6 linkage (39, 40). Each signal corresponded to one anomeric proton, except for the signals at 4.76 and 4.80 ppm, which accounted for two and four protons, respectively. The two close-lying doublets corresponding to one proton were due to different populations of conformers. Because glucitol did not give any signal in this part of the spectrum, the NMR results indicated the presence of 11 glucose units/QZSNIIe molecule, in agreement with the MALDI-TOF data. Because of severe overlap of

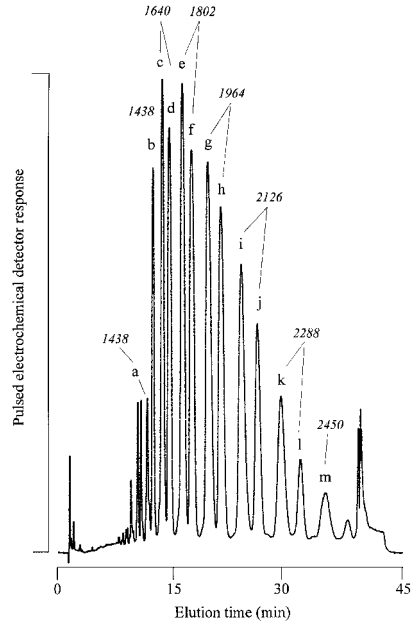

FIG. 4. **HPLC of the QzSN II fraction on an anion exchange CarboPAC PA1 column (Dionex, 9 × 250 mm).** Flow rate of 4 ml/min; 0–2-min isocratic step with a mixture containing 90% of solution A and 10% solution B, 2–5 min of linear gradient (90% A + 10% B − 68% A + 32% B), 5–36 min of linear gradient (68% A + 32% B − 61% A + 39% B), 36–37 min of linear gradient (61% A + 39% B − 100% B). To avoid degradation by peeling, laminarioligosaccharides were treated, prior to HPAEC, with $NaBH_4$ (10 mg/ml in 100 mM NH_4OH) overnight and then desalted. Oligosaccharides were detected with a pulsed electrochemical detector. Collected oligosaccharides are labeled *a* to *m* to increasing time retention. *Numbers* written in *italics* correspond to molecular masses obtained by MALDI-TOF mass spectrometer analysis.

other proton resonances, even in the DQF-COSY experiment, RELAYH experiments were performed to identify H3 resonances for further sequential assignment and linkage determination. From the chemical shifts reported in Table II, it can be seen that four units (labeled F) are undistinguishable by their proton resonances, as expected for a linear β-1,3-glucan chain. Chemical shifts of H3 protons are comprised between 3.75 and 3.80 ppm, except for the two C units with H3 resonances at 3.53 ppm, typical value for β-Glc unsubstituted in C3 corresponding to nonreducing end units. These results, which showed the presence of two glucose residues at a nonreducing end of the QzSN IIe molecule, indicated that the oligosaccharide QzSN IIe was branched. This was confirmed by the analysis of the D residue, which was substituted in C6, inducing large resonance shifts at low field for protons H6 and H6′ of 0.14 and 0.29 ppm, respectively, and smaller shifts up to the H4 as described previously (39). The position of H3 at 3.80 ppm in the D unit implied that it is substituted in C3; therefore, the D residue is a branching point in the oligosaccharide. The two-dimensional nuclear Overhauser effect spectroscopy experiment showed that all residues were linked via a β-1,3 linkage, except for residue A, which is connected to the D unit via a β-1,6 linkage. For each anomeric proton, intra-residue NOE was observed with H2 and also with H3 and H5 as expected for a β anomer. An inter-residue NOE is observed with the proton of the neighboring unit where glycosidic linkage occurred. Complete sequential assignment was not possible because of the drastic overlap of H3 resonances for most units (δ comprised between 3.75 and 3.80 ppm for A, B, D, E, and F). For the same reason, the β-1,6 linkage could not be localized in the sequence by NMR. The structure of QzSN IIe identified from the NMR data is as follows.

Glcβ1–3(Glcβ1–3)$_{z-6}$Glcβ1–3(Glcβ1–3)$_y$-Glcβ1–3-glucitol
/
Glcβ1–3-(Glcβ1–3)$_x$Glcβ1–3-Glcβ1

STRUCTURE 1

where x, y, and z are between 0 and 4, and $x + y + z = 4$.

The one-dimensional ^{1}H NMR spectrum of the QzSN IIc oligosaccharide is identical to that of QzSN IIe except for the signal intensities that correspond to a branched β-1,3-glucan decasaccharide (data not shown). In addition, GLC-MS of the methyl ethers obtained after methylation of all oligosaccharides recovered by HPLC (dp 9–14) showed that half of the oligosaccharides with a given molecular weight were linear β-1,3-glucan, whereas the other half were branched β-1,3-glucan with a single 1,6-glucose linkage as indicated by a ratio 2:1:1 corresponding to glucose units at the nonreducing end (2,3,4,6-Glc), disubstituted glucose units (2,4-Glc), and glucose units at the reducing end (1,2,4,5,6-Glc), respectively (data not shown).

Acetolysis degradation, which preferentially cleaves 1,6-glycosidic bonds, was performed on QzSN IIe. Degraded products were analyzed by HPAEC and MALDI-TOF mass spectrometry. A kinetic analysis showed that acetolysis released preferentially two laminarioligosaccharides: pentaose and hexaose from the QzSN IIe undecaoligosaccharide (Fig. 5). These results suggested that QzSN IIe contained one major oligosaccharide structure: a laminarihexaose branched with a laminaripentaose. However, like for the ^{1}H NMR and methylation data, the exact position of the branch point could not be identified with this technique. NMR, GLC-MS, and acetolysis data suggested that the structure of QzSN IIe as follows.

Glcβ1–3(Glcβ1–3)$_{z-6}$Glcβ1–3(Glcβ1–3)$_y$-Glcβ1–3-glucitol
/
Glcβ1–3-(Glcβ1–3)$_x$Glcβ1–3-Glcβ1

STRUCTURE 2

where $x = 2$ or 3, y and z are between 0 and 2, and $x + y + z = 4$.

Fraction QzSN I_B: 1-Laminarioligosaccharides of dp > 15 Are Also Branched—Fraction QzSN I_B of 2–5 kDa isolated after gel filtration on a Sephadex G100 column was composed of glucose residues (Fig. 3 and Table I). MALDI-TOF mass spectrometry and HPAEC showed that this fraction contained a mixture of oligosaccharides of dp 11–25, the major species having a dp of 16–18 (data not shown). After reduction, methylation analysis produced five types of methyl ether of glucose units: 1,2,4,5,6-Glc, 2,3,4,6-Glc, 2,4,6-Glc, 2,3,6-Glc, and 2,4-Glc[2] with a molar ratio of 0.4:1.2:13.3:0.8:1. The presence of β-1,6 linkages in the β-1,3-glucan indicated by the methylation analysis was confirmed by ^{1}H and ^{13}C NMR analysis. The one-dimensional spectrum of the QzSN I_B, obtained by ^{1}H NMR, contained seven doublets in the anomeric region between 4.5 and 4.8 ppm (Table III). These chemical shifts and coupling constant $^3J_{1,2}$ were identical to the ones obtained with QzSN IIe (Tables II and III) except for (i) two signals at 4.67 and 5.24 ppm that corresponded to the α and β anomer of the glucose at the reducing end (whereas QzSN IIe was reduced before NMR analysis) and (ii) one signal at 4.76 ppm, which corresponded to 4-*O* substituted glucose (in agreement also with the methylation data). ^{13}C resonances were assigned from the geHSQC and gHSQC-TOCSY experiments (34) (Table III).

[2] Nomenclature of methyl ethers of monosaccharides is as follows: 2,4,6-Glc is 2,4,6-tri-*O*-methyl-3-*O*-acetyl-glucoside, 2,4-Glc is 2,4-di-*O*-methyl-3,6-*O*-acetyl-glucoside, etc.

TABLE II

Proton chemical shifts in ppm (in upper line) and coupling constants in Hz (in lower line) of the undecasaccharide QzSN IIe

The nonequivalent geminal proton resonating at lower field is denoted H′. The glucose residues were labeled A to F in order of increasing chemical shift of their anomeric protons.

Residues	H1	H1′	H2	H3	H4	H5	H6	H6′
3glucitol	3.68	3.79	4.03	4.06	3.66	3.91	3.655	3.86
A 3Glcβ1–6	4.56 $^3J_{1,2}$ = 7.9		3.52	3.75	3.52	3.52	3.75	3.93
B 3Glcβ1–3glucitol	4.68 $^3J_{1,2}$ = 7.9		3.59	3.79	3.53	3.52	3.75	3.93
C Glcβ1–3	4.76 $^3J_{1,2}$ = 7.9		3.37	3.53	3.415	3.49	3.72	3.92
D 3,6Glcβ1–3	4.77 $^3J_{1,2}$ = 7.9		3.58	3.80	3.61	3.70	3.90	4.22
E 3Glcβ1–3A	4.77 $^3J_{1,2}$ = 7.9		3.56	3.79	3.53	3.52	3.75	3.93
F 3Glcβ1–3	4.80 $^3J_{1,2}$ = 7.9		3.56	3.79	3.53	3.52	3.75	3.93

All the methylenic carbon signals, easily identified in the geHSQC experiment, had typical chemical shift values of β-1,3-glucan (between 63.3 and 63.5 ppm) except for $C6_D$ signals that resonate at 71.6 ppm. The large downfield shift of $C6_D$ was compatible with a β-1,6 linkage. The $C3_D$ resonance at 87.0 ppm was typical of β-1,3-Glc. These ^1H and ^{13}C chemical shifts indicated that the D unit was 3-*O*- and 6-*O*-disubstituted. Resonance values lying between 105.0 and 105.5 ppm were in agreement with β-1,3 glucan containing a β-1,6 linkage, except for two anomeric carbons $C1_B$ at 98.4 ppm and $C1_G$ at 94.7 ppm, which corresponded, respectively, to the β and α Glc moieties of the reducing end, respectively (39). The gHMBC experiment confirmed that all the units are β-1,3 linked except for the A residue, which was β-1,6 linked with the D residue. Further confirmation that QzSN I_B contained a mixture of branched oligosaccharides was obtained by an acetolysis assay. In a way similar to QzSN IIe, acetolysis of QzSN I_B released a large number of linear β-1,3 linked oligosaccharides of variable size (dp 2–20 and higher; Fig. 5).

In conclusion, data obtained by ^1H and ^{13}C NMR, methylation analysis, and HPAEC separation of QzSN I_B submitted to acetolysis were all in agreement indicating that this fraction contained β-1,6 branched β-1,3-glucans, but it was of higher M_r than QsSN II with oligosaccharides of dp higher than 15.

Fraction QzSN I_B 2: Identification of a New β-1,3/1,4-Glucan—Methylation analysis of QzSN I_B showed the presence of 2,3,6-Glc and 2,4,6-Glc in the molar ratio 1:16.6. The glucan containing 4-*O*-substituted glucose was also recovered from mycelium grown in a chemically defined medium in flasks without cotton plugs, indicating that its presence was not due to cellulose contamination but was an intrinsic component of the cell wall. The β-1,3/1,4-glucan was released from the alkali-insoluble fraction of the cell wall by quantazyme treatment, suggesting that it was bound to the β-1,3/1,6-glucan. To separate the glucan containing 4-linked glucose from the branched β-1,3/1,6-glucan, QzSN I_B was incubated with the 74-kDa endo-β-1,3-glucanase from *A. fumigatus* (ENG1). ENG1 has the ability to cleave laminari-oligosaccharides of shorter dp than Quantazyme (29). As a consequence, ENG1 was able to cleave a branched β-1,3/1,6-glucan with short β-1,3-glucan side chains, so that Quantazyme would not be able to cleave. Indeed, ENG1 was able to cleave QzSN I_B resulting from the action of Quantazyme. The ENG1-digested product was borohydride-reduced and submitted to gel filtration chromatogra-

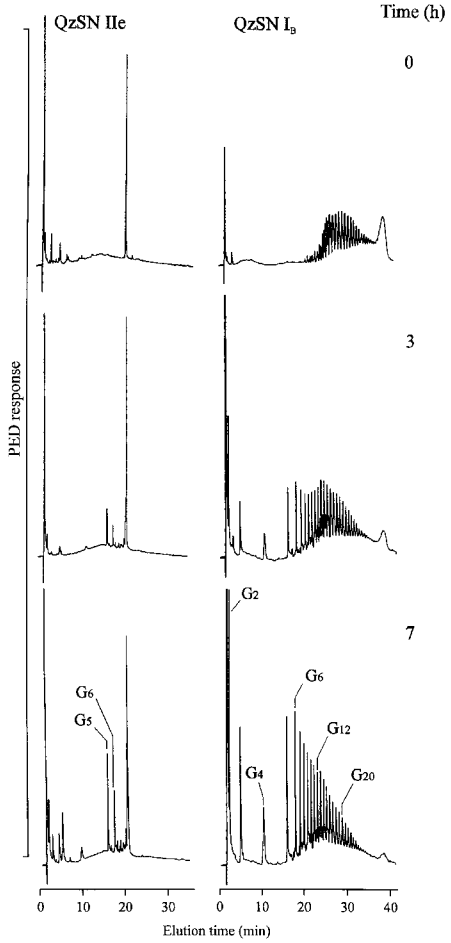

FIG. 5. **Acetolysis of QzSN IIe and QzSN I_B fractions.** Peracetylated products were treated with an acetic acid/acetic anhydride/sulfuric acid solution (10:10:1 v/v/v) at 25 °C for 3 and 7 h. The reaction was stopped by addition of pyridine and water. The peracetylated products were extracted with chloroform and washed with water. Deacetylation was performed in 300 mM NaOH and $NaBH_4$ (10 mg/ml) overnight at room temperature. Analysis of oligosaccharides was performed using HPLC with a pulsed electrochemical detector and an anion exchange column (CarboPAC PA-1, 4.6 × 250 mm, Dionex). Flow rate; 1 ml/min; 0–2 min isocratic step with a mixture containing 98% of solution A (NaOH 50 mM) and 2% of solution B (NaOAc, 500 mM in NaOH 50 mM), 2–15 min of linear gradient (98% A + 2% B − 60% A + 40% B), 15–35 min of linear gradient (60% A + 40% B − 25% A + 75% B), and 35–37 min of linear gradient (25% A + 75% B − 100% B).

TABLE III

^1H and ^{13}C chemical shifts in ppm of QzSN I_B (in upper and lower line, respectively)

The homonuclear proton coupling constant in Hz are written in the middle. The nonequivalent geminal proton resonating at lower field is denoted H′. The glucose residues were labeled A to G in order of increasing chemical shift of their anomeric protons.

Residues	H1 $^3J_{H1,H2}$ C1	H2 $^3J_{H2,H3}$ C2	H3 $^3J_{H3,H4}$ C3	H4 $^3J_{H4,H5}$ C4	H5 $^3J_{H5,H6}$ C5	H6 $^3J_{H6,H6'}$ C6	H6′ $^3J_{H5,H6'}$
A	4.55	3.52	3.75	3.52	3.52	3.75	3.92
3Glcβ1–6	7.8	11.0					
	105.2	75.6	87.2	70.8	78.2	63.5	
B	4.67	3.44	3.74	3.52	3.50	3.74	3.90
3Glcβ	8.0	9.6					
reducing end	98.4	76.5	87.4	70.9	78.3	63.5	
C	4.74	3.36	3.53	3.41	3.49	3.72	3.92
Glcβ1–3	8.1	9.5	9.1	9.4	5.9	12.4	2.3
nonreducing end	105.5	76.2	78.3	72.3	78.7	63.4	
D	4.75	3.57	3.79	3.59	3.70	3.89	4.22
3_6Glcβ1–3	7.9	9.9			5.5	11.3	2.3
	105.5	75.9	87.0	70.8	77.1	71.6	
E	4.76	3.40	3.67	3.67	3.62	3.82	3.98
4Glcβ1-	7.9					11.6	
	105.3	75.9	76.9	81.3	77.5	62.7	
F	4.79	3.56	3.78	3.53	3.52	3.74	3.92
3Glcβ1–3	8.1	10.2			5.0	12.0	3.2
	105.3	75.9	87.0	70.8	78.3	63.4	
G	5.24	3.72	3.92	3.52	3.87	3.79	3.84
3Glcα	3.8						
reducing end	94.7	73.8	85.2	70.8	73.9	63.3	

phy on the TSK HW40S column (Fig. 6). Gel permeation profile showed laminari-oligosaccharides of low dp (mainly 2 and 3), branched β-1,3/1,6 laminari-oligosaccharides (dp 6–9, fraction b), and a fraction a of high M_r that was further analyzed. Methylation analysis showed that a polymer with an equimolar ratio of glucose residues substituted in position 3 and substituted in position 4 was recovered in the fraction a. ^1H and ^{13}C NMR data of this fraction are presented in Table IV and Fig. 7. Two main doublets were observed in the anomeric region at 4.53 and 4.77 with a $^3J_{1,2}$ coupling constant value of 7.9 Hz typical of β-linked units. Integration of these two signals shows that A and B residues were in the ratio 1:1. H3 and H4 resonances determined with RELAYH and TOCSY experiments showed that residue A was substituted in position 3 and residue B in position 4. The gGHMBC experiment showed interglycosidic couplings between H1A and C4B and between H1B and C3A, indicating that A was linked to B in position 4 and B was linked to A in position 3 (Fig. 7). Degradation of this fraction by periodic oxidation and Smith degradation yielded a monosaccharide glycoside isolated from the TSK HW40S column as a dp 2 (Fig. 6). GLC analysis indicated that it was composed of glucose and erythritol. Erythritol residue was produced by cleavage of glucose residue substituted in position 4 by sodium periodate. GLC-MS analysis using the chemical ionization mode showed that the permethylated compound had a M_r of 382, corresponding to an hexose plus erythritol. Analysis with electronic impact mode after methanolysis indicated that the glucose residue was bound to carbon 2 of erythritol. NMR and methylation data indicated that the linear β-1,3/1,4-glucan has the following repeating unit: [3Glcβ1–4Glcβ1].

Fraction QzSN I_A 1: Linkage between β-1,3-Glucan and Galactomannan—This fraction had a molecular mass of 40 kDa and contained galactose, mannose, and glucose residues in similar amounts (Table I). Methylation analysis of this fraction showed that methyl ethers of galactose and mannose residues corresponded to the galactomannan polymer, which is composed of a core chain of α-mannose residues with short side chain of β-1,5-galactofuranose residues, as described previously by Latgé *et al*. (Ref. 17 and Table V). Acetolysis experi-

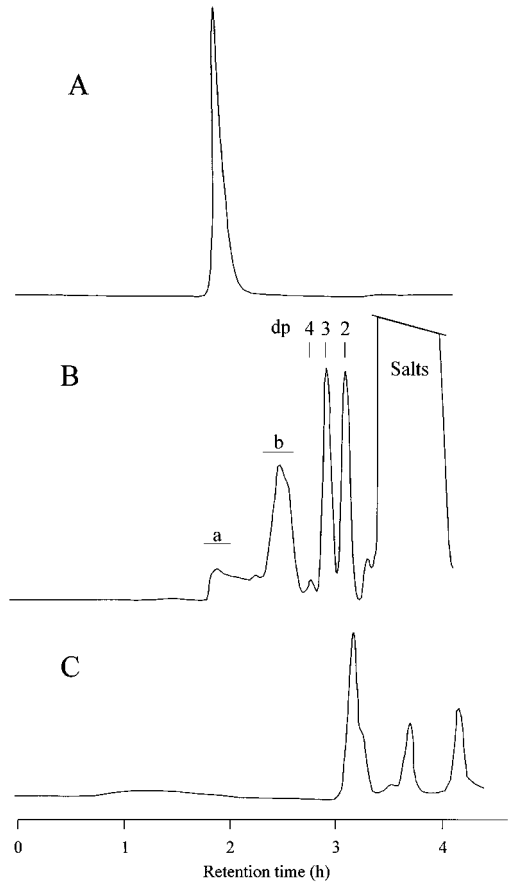

FIG. 6. **Gel filtration chromatography on a TSK HW40S column of QzSN I_B fraction after periodic oxidation or enzymatic digestion with the 74-kDa endo-β-1,3-glucanase ENG1 of *A. fumigatus*.** A, QzSN I_B without treatment. *B*, QzSN I_B after enzymatic digestion with ENG1. *C*, QzSN I_B fraction a after periodate oxidation and Smith degradation. A TSK HW40S column (90 × 1.4 cm) was eluted with 0.25% (v/v) acetic acid solution at 0.5 ml/min. Products were applied to the column and monitored by refractometry. dp was established with malto-oligosaccharides as standard.

ments preferentially released a tetrasaccharide consistent with the presence of a repeating mannose unit [6Manα1–2Manα1–2Manα1–2Manα1] (data not shown). As indicated by methylation analysis, two of the α-1,2 linked mannose residues were substituted in position 3 or 6 and were branching point for the galactofuran side chain (Table V). Because galactomannan binds to Concanavalin A, the galactomannan containing molecules were purified by affinity chromatography on a ConA-Sepharose column. The fraction bound to the ConA-Sepharose and released with 0.2 M α-methylmannoside accounted for 80% of QzSN I_A. It was composed of mannose, galactose, and glucose residues in a molar ratio of 2.5:2.7:1. This result indicated that the galactomannan polymer was covalently bound to the glucan moiety. The polysaccharide bound to ConA-Sepharose was reduced with $NaBH_4$, hydrolyzed, and derivatized. GLC analysis showed the absence of mannitol and the presence of glucitol, indicating that a glucose residue was located at the reducing end (data not shown). This fraction was sequentially submitted to mild acid hydrolysis to remove galactofuran side chains and to periodate oxidation to degrade the mannan moiety The products were separated by gel filtration chromatography on TSK HW40S column (Fig. 8). Fraction b contained typical products of degradation by periodate oxidation. Fraction a was composed of a mixture of oligosaccharides with sizes varying between 2 and 11 residues. Only glucose and arabitol were detected by GLC analysis in the fraction a with a glucose/arabitol ratio of 2.9. Arabitol was produced by periodate oxidation, which cleaves glucose residues at the reducing end between carbon 1 and 2, indicating that the β-1,3-glucan chain was at the reducing end. This result was in agreement with the composition analysis performed in the reduced undegraded QzSN I_A. MALDI-TOF mass spectrometry analysis of the fraction a showed the presence of a series of oligosaccharides containing 1 arabitol residue plus an increasing number of glucose residues (Fig. 9). The major oligosaccharides contained 2–5 glucose units. Methylation of the fraction a showed the presence of several methyl ethers: 1,3,4,5-arabitol, 2,3,4,6-Glc, 2,4,6-Glc, and 2,4-Glc in the ratio of 0.4:1:3.2:0.2, indicating that this fraction was composed of short β-1,3 linked glucan chains with some β-1,6 branch points. The predominance of glucan chains with 2–5 glucose units constituting the glucan moiety of QzSN I_A resistant to hydrolysis by Quantazyme suggested that QzSN I_A fraction was composed of a galactomannan chain linked to the nonreducing end of a short β-1,3-glucan chain as follows.

TABLE IV

^1H and ^{13}C chemical shifts in ppm of QzSN IB after digestion with the 74-kDa endoglucanase and fractionation on a HW40S column

The nonequivalent geminal proton resonating at lower field is denoted H′.

Residues	H1 C1	H2 C2	H3 C3	H4 C4	H5 C5	H6 C6	H6′
A	4.53	3.52	3.76	3.52	3.51	3.75	3.92
3Glcβ1–4	105.0	75.6	86.6	70.6	78.2	63.2	
B	4.77	3.40	3.67	3.67	3.61	3.80	3.98
4Glcβ1–3	105.2	75.8	76.8	81.3	77.5	62.6	

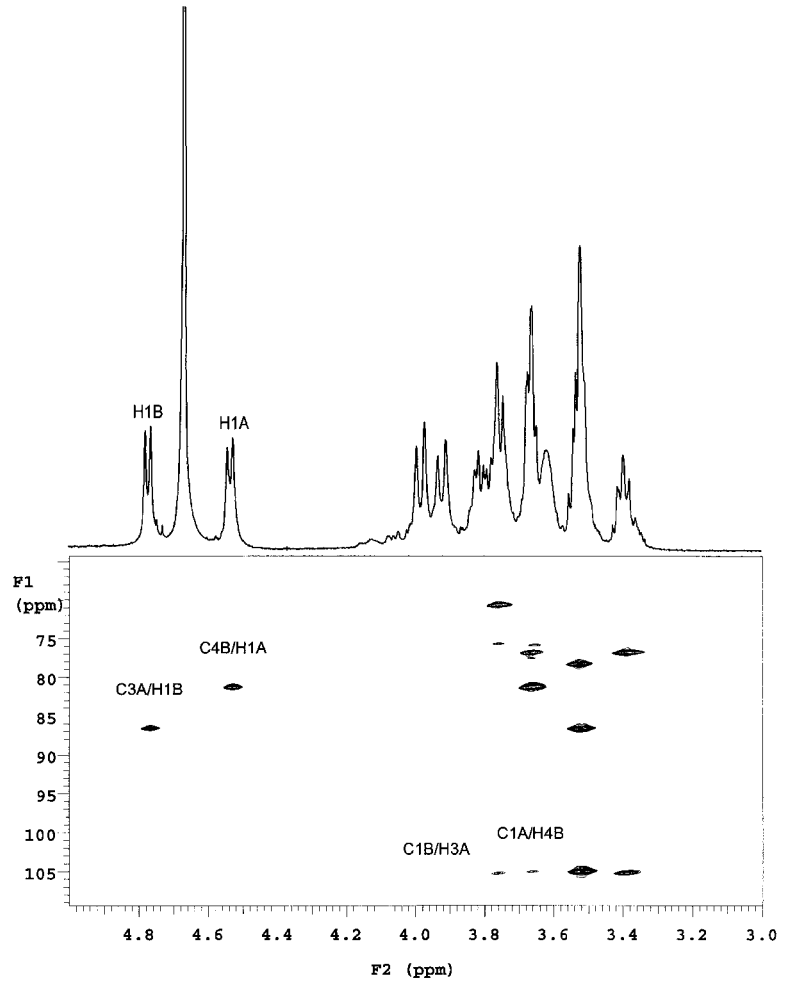

FIG. 7. **Two-dimensional gHMBC NMR spectrum of fraction a purified by gel filtration on TSK HW40S column after ENG1 digestion of the QzSN I_B fraction.** Only chemical shifts of H1 and C1 were presented on the spectrum.

TABLE V
Ratios of methyl ethers obtained after methanolysis of permethylated fractions from the most complex polysaccharide structure of the AIS fraction of A. fumigatus
Ratios were estimated for one 3,4-Man residue.

Methyl ethers	QzSN I_A	ChSN I_A	FP
2,3,5,6-Gal	1.8	1.5	2.1
2,3,6-Gal	4.3	3.2	13.8
2,3,4,6-Man	0.1	0.1	0.6
3,4,6-Man	2	1.6	2.9
2,3,4-Man	0.8	0.7	4
3,4-Man	1	1	1
4,6-Man	0.4	0.3	1.4
2,3,4,6-Glc	0.2	0.1	2.1
2,4,6-Glc	7.1	3.8	50.6
2,3,6-Glc	0.9	2.3	21
2,4-Glc	0.5	0.2	4.1
2,6-Glc			6.7
3,4,6-GlcNAc		traces	
3,6-GlcNAc			57
3,6-GalNAc			81

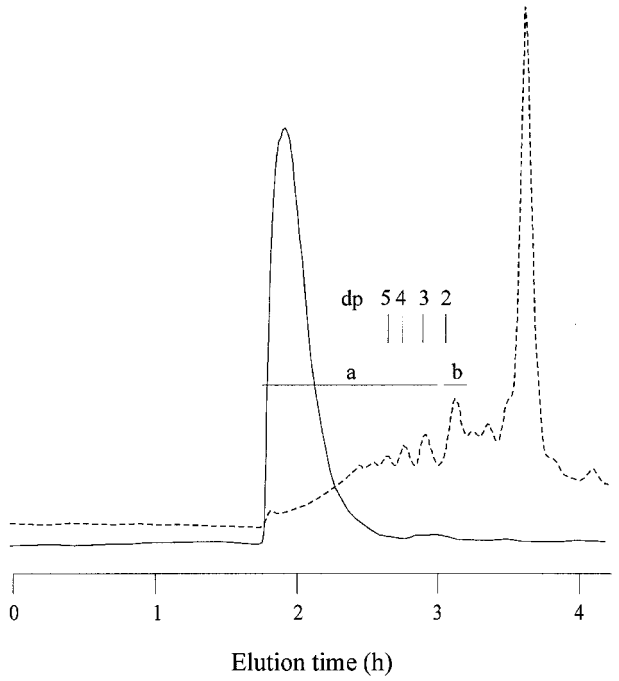

FIG. 8. **Analysis by gel filtration chromatography on a TSK HW40S column of oligosaccharides resistant after periodic oxidation of the fraction of QzSN I_A binding to ConA-Sepharose column.** *Continuous line*, product after mild acid hydrolysis (15 mM HCl, 100 °C 24 h); *broken line*, product after mild acid hydrolysis, periodate, and Smith degradation. TSK HW40S column (90 × 1.4 cm) was equilibrated with 0.25% (v/v) acetic acid solution at 0.5 ml/min. Products were applied to the column and detected by refractometry. dp was established with malto-oligosaccharides as standard.

Man-Man-Man-Man-Man-Man-Man-Man-Man-Glc-Glc-Glc-Glc-Glc-
| | |
$[Gal]_x$ $[Gal]_x$ $[Gal]_x$

STRUCTURE 3

where Man is α-mannose, Gal is β-galactofuranose, and Glc is β-1,3-glucose.

Acetolysis degradation of the fraction bound to ConA-Sepharose resulted in the release of laminarioligosaccharides free from mannose residues. As acetolysis cleaves preferentially 1,6 glycosidic linkage, these data suggested, although indirectly, that the mannan was linked through a 1,6 linkage to the nonreducing end of the glucan chain.

Fraction QzSN I_A 2: Linkage between Linear β-1,3/1,4-Glucan and β-1,3-Glucan—The unbound ConA-Sepharose fraction contained only glucose residues. Methylation and GLC-MS analysis revealed the presence of five methyl ethers: 1,2,4,5,6-Glc, 2,3,4,6-Glc, 2,4,6-Glc, 2,3,6-Glc, and 2,4-Glc in the molar ratio of 0.1:0.8:16.4:6.5:1. The unbound ConA-Sepharose fraction was treated with ENG1. The TSK HW40S gel filtration pattern was similar to the one obtained after ENG1 treatment of the fraction QzSN I_B and shown in Fig. 6 (data not shown). The fraction obtained at the void volume was submitted to periodic oxidation. A single disaccharide peak was obtained. GLC-MS analysis showed that this disaccharide was composed of glucose linked to erythritol. These results indicated that the unbound ConA-Sepharose fraction contained both branched β-1,3/1,6-glucan and a linear β-1,3/1,4-glucan. According to the methylation data, the linear β-1,3/1,4-glucan represents 52% of the unbound ConA-Sepharose fraction.

When periodate oxidation and Smith degradation, degrading the β-1,3/1,4-glucan, was performed on the unbound ConA fraction, without previous ENG1 enzymatic digestion, small laminarioligosaccharides of dp 2–5 were released. This short size of the laminarioligosaccharides linked to the β-1,3/1,4-glucan, resulted from the Quantazyme digestion of the alkali-insoluble fraction of the cell wall, and indicated that the linear β-1,3/1,4-glucan was linked to β-1,3-glucan chains.

Characterization of Water-soluble Products Released by Chitinase

Fractions ChSN III and ChSN IV: Products of Degradation of Chitinase—Fractions ChSN III and ChSN IV contained only *N*-acetylglucosamine residues (GlcNAc). MALDI-TOF mass spectrometry showed that ChSN IV corresponded to *N*-acetylchitobiose and ChSN III to a mixture of *N*-acetylchitotriose and *N*-acetylchitotetraose (data not shown).

Fraction ChSN II: Linkage between Chitin and Glucan—Fraction ChSN II (molecular mass, 1–2 kDa) was composed of glucose and GlcNAc residues in a molar ratio of 16:1 (Table I). Because of the low amount of material recovered, this fraction was analyzed *in toto* without further purification. MALDI-TOF mass spectrometry showed that ChSN II contained a mixture of oligosaccharides of dp 7–13. After 9 h of chitinase digestion, half of the oligosaccharides contained one GlcNAc residue (Fig. 10). If chitinase incubation was prolonged for 24–72 h, the amount of oligosaccharides containing the GlcNAc residue gradually decreased over time (data not shown), indicating that GlcNAc residues bound to glucan chain were hydrolyzed by the chitinase treatment. Methylation was performed after reduction with $NaBD_4$ of ChSN II treated with chitinase for 24 h. GLC analysis of methyl ethers showed the presence of 2,3,4,6-Glc, 2,4,6-Glc, 2,3,6-Glc, 2,4-Glc, and 3,4,6-*O*-methyl 2-*N*-methyl 1,5-*O*-acetyl 2-*N*-acetyl glucosaminitol in a molar ratio of 0.7:10:1.6:0.4:0.6. A 24-h incubation of ChSN II with β-D-glucosaminidase removed 70% of the GlcNAc residues and 4-*O*-substituted glucose residues, indicating that GlcNAc was located at the nonreducing end of the oligosaccharide (data not shown). These methylation data suggested that the GlcNAc residue was linked to the nonreducing end of the β-1,3-glucan oligosaccharide via a β-1,4 glycosidic linkage.

The one-dimensional ^1H NMR spectrum of the ChSN II (after 24 h of chitinase digestion) fraction contained five doublets in the anomeric region between 4.5 and 4.8 ppm (Table VI). Chemical shifts of 4.52, 4.75, and 4.80 for residues A, C, and E and the associated coupling constants values $^3J_{1,2}$ for these doublets were similar to those obtained with QzSN II and QzSN I_B and indicated the presence of a linear β-1,3-glucan containing a β-1,6 branching point. H3 resonances, determined

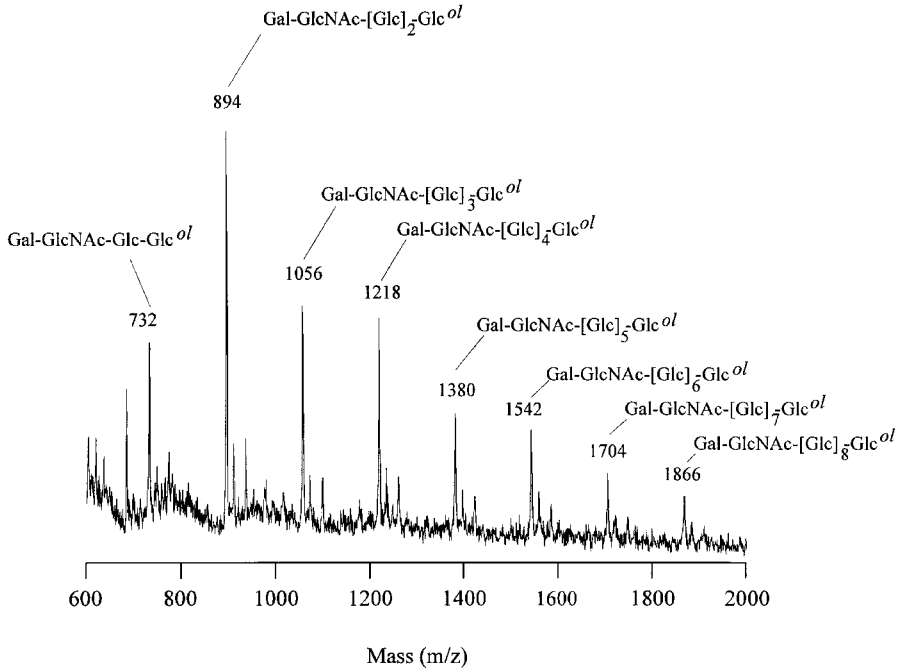

FIG. 9. **MALDI-TOF mass spectra of oligosaccharides resistant to periodate oxidation of the fraction of QzSN I_A binding to ConA-Sepharose column and fractionated on the TSK HW40S column (fraction a of Fig.** 8). Mass spectra were recorded in the positive ion mode and accelerated to an energy of 5 keV before entering the flight tube. Samples were prepared by mixing directly on the target 1 μl of oligosaccharide solution (about 25 pmol) and 1 μl of 2.5-dihydroxybenzoic acid matrix solution (12 mg/ml dissolved in CH_3OH/H_2O (80:20 v/v)). Mass (*m/z*) correspond to the oligosaccharide mass plus sodium.

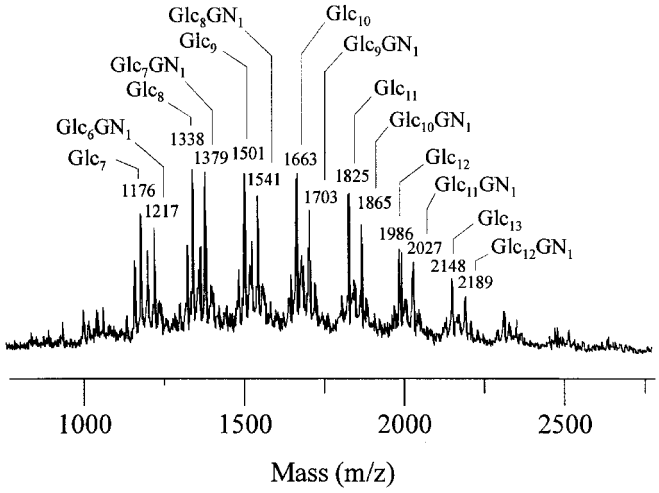

FIG. 10. **MALDI-TOF mass spectra of oligosaccharides of the ChSN II fraction (chitinase digestion of 9 h at 37 °C) purified by gel filtration on a TSK HW40S column.** Mass spectra were recorded in the positive ion mode and accelerated to an energy of 5 keV before entering the flight tube. Samples were prepared by mixing directly on the target 1 μl of oligosaccharide solution (about 25 pmol) and 1 μl of 2.5-dihydroxybenzoic acid matrix solution (12 mg/ml dissolved in CH_3OH/H_2O (80:20 v/v)). Mass (*m/z*) correspond to the oligosaccharide mass plus sodium.

with RELAYH experiments and ^{13}C resonances, assigned from the geHSQC and gHSQC-TOCSY experiments confirmed the presence of a β-1,3-glucan chain with β-1,6 linkage (Glc A, C, and E; Table VI) (34). The B residue was unambiguously identified to be a GlcNAc residue from the gHMBC experiment through the observed long range couplings between the carbon of the carbonyl group at 177.1 ppm and the proton chemical shift of the CH_3 group at 2.06 ppm on the one hand and the ring H2 proton at 3.74 ppm on the other hand. Proton chemical shift values were typical of a nonreducing end GlcNAc residue (41). Sequence analysis, obtained from the gHMBC experiment, showed that all the units are β-1,3 linked except for the nonreducing terminal GlcNAc residue, which is β-1,4-linked with the D unit and for the A residue, which is β-1,6 linked with the reducing-end residue. Specific anomeric ^1H and ^{13}C signals were not identified at the reducing end. Moreover, methylation and GLC-MS analysis showed the presence of an unknown methyl ether. Methylation and GLC-MS analysis of reducing ends in QzSN I_B treated with hot NaOH showed that the modification of the reducing end was due to a peeling reaction, which was sequential and stopped at a 1,6 branching point. From these data, it can be deduced that GlcNAc was linked to β-1,3-glucan side chains through a 1,4 linkage according to the following structure.

GlcNAcβ1–4Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1

STRUCTURE 4

Fraction ChSN I_B: Complex Glucan Structures Are Also Linked to Chitin—ChSN I_B had a molecular mass of 5–10 kDa (Fig. 3) and was mainly composed of glucose with a ratio Glc: GlcNAc of 50. (Table I). Methyl ethers of glucose residues obtained after methylation were 1,2,4,5,6-Glc, 2,3,4,6-Glc, 2,4,6-Glc, 2,3,6-Glc, and 2,4-Glc in a molar ratio of 0.3:0.9:14: 10:1. This fraction was characterized by the presence of β-1,3-glucan with a high amount of 4-*O*-substituted glucose residues and was analyzed as described previously for QzSN I_B, using hydrolysis by endo-β-1,3-glucanase (ENG1), gel permeation fractionation, NMR, periodate oxidation, and GLC-MS (data not shown). Results were very reminiscent of the ones obtained with the QzSN I_B fraction and indicated that the glucan structures of ChSN I_B were very similar to the glucan structures of QzSN I_B: (i) The β-1,3-glucan molecule was branched through β-1,6 linkages; (ii) the molecule containing the 4-*O*-substituted glucose had the following repeating unit [Glcβ1–4Glcβ-1,3] and was linked to the nonreducing end of the β-1,3-glucan side chain.

The only chemical difference between QzSN I_B and ChSN I_B was the presence of GlcNAc residues in ChSN I_B. To identify the linkages and the position of GlcNAc residues to the sugar core, transgalactosylation of GlcNAc residues was performed. Acetolysis of ChSN I_B released a mixture of oligosaccharides of variable size with a maximal dp of 10 (data not shown). The mixture of oligosaccharides was incubated with galactosyltransferase and UDP-galactose and then applied to a *E. cristagalli* lectin-agarose column. The fraction retarded on the

affinity column was analyzed by GLC and MALDI-TOF mass spectrometry. The Glc/GlcNAc ratio was 6, and all oligosaccharides recovered contained the sequence Galβ1–4GlcNAc, indicating that all GlcNAc residues were bound in a β anomeric configuration to the branched β-1,3/1,6-glucan. The size of the side chains containing one GlcNAc residue varied from 2 to 8 residues (Fig. 11). These chemical data showed that all GlcNAc residues were bound to the nonreducing end of the branched β-1,3/1,6-glucan as in ChSN II, in agreement with the following structure.

Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1–3Glcβ1–3$_6$Glcβ1–3Glcβ1–3
Glcβ1–3Glcβ1
GlcNAcβ1–4Glcβ1–3[Glcβ1–3]$_n$Glcβ1

STRUCTURE 5

where n is between 0 and 7.

Fraction ChSN I_A: Chitin, β-1,3/1,4-Glucan and Galactomannan Are Linked to Different β-1,3-Glucan Branches—ChSN I_A had a molecular mass of 40 kDa (Fig. 3) and contained galactose, mannose, and glucose in equivalent amounts with the presence of traces of GlcNAc residues in a Glc/GlcNAc ratio of 100 (Table I). Methylation analysis showed that the fraction released by the chitinase treatment was originally composed of galactomannan, β-1,3/1,4-glucan and chitin, as shown by the presence of GlcNAc in this fraction resulting from the action of chitinase (Table V). The main question addressed in the analysis of ChSN I_A was the identification of the linkage between chitin, galactomannan, and glucan. To address this question, CHSN I_A was incubated with the 74-kDa endo-β-1,3-glucanase, ENG1. Degradation products were separated by gel filtration chromatography on a TSK HW40S column (Fig. 12). Monosaccharide composition showed that GlcNAc residues were only found in the tetraoligosaccharide peak (dp 4) and in fraction b containing oligosaccharides of dp 5–10, whereas galactose and mannose associated to glucose residues were in fraction a excluded at the void volume. MALDI-TOF analysis of fractions b and dp 4 showed that GlcNAc residues were covalently linked to the glucan fragment (Fig. 13). Methylation analysis of fraction b showed the presence of four methyl ethers: 1,2,4,5,6-Glc, 2,4,5,6-Glc, 2,4,6-Glc, and 2,4-Glc in a molar ratio of 0.7:2:2.9:1, respectively, indicating that this fraction consisted of a highly branched β-1,3/1,6-glucan with short chains of linear β-1,3-glucans, which explained its resistance to the 74-kDa endo-β-1,3-glucanase (see above). These data suggested that ChSN I_A had a structure similar to that of QzSN I_A with the presence of galactomannan and the β-1,3/1,4-glucan associated to β-1,3-glucan and also similar to ChSN I_B or ChSN II where GlcNAc residue was linked to branched β-1,3-glucan. This result indicated that GlcNAc, β-1,3/1,4-glucan and galactomannan were covalently associated to three different β-1,3-glucan side chains of the branched β-1,3/1,6 core glucan.

Characterization of the Quantazyme/Chitinase-resistant Pellet—The residual insoluble pellet (FP) was very rich in glucosamine and galactosamine, because hexosamine accounted for 80% of this material (Table I). The insolubility of this residual pellet made the analysis of this fraction difficult, and only partial information was obtained on the chemical organization of FP. The most striking features were the following: (i) the glucan moiety was a branched β-1,3/1,6 polymer similar in composition to the one analyzed in QzSN I_A and ChSN I_A; (ii) treatment of FP with the 74-kDa endoglucanase (ENG1) solubilized 15% of FP. Gel permeation and MALDI-TOF analysis showed that the soluble oligosaccharides had a composition similar to those released by ENG1 from ChSN I_A with a dp of 2–10 and the presence of GlcNAc linked to some of the laminarioligosaccharides (data not shown); (iii) D-*N*-acetylgalactosamine residues, which is the major monosaccharide of FP, was substituted in position 4 and should correspond to a poly-*N*-acetylgalactosamine polymer.

TABLE VI
^{1}H and ^{13}C chemical shifts in ppm of ChsSN II (in upper and lower lines respectively)

The nonequivalent geminal proton resonating at lower field is denoted H′. The glucose residues were labeled A to E in order of increasing chemical shift of their anomeric protons.

Residues	H1 C1	H2 C2	H3 C3	H4 C4	H5 C5	H6 C6	H6′
A	4.52	3.52	3.76	3.50	3.52	3.75	3.92
3Glcβ1–6	105.3	75.6	86.9	70.8	78.2	63.4	
B	4.57	3.74	3.57	3.47	3.52	3.75	3.93
GlcNAcβ1–4	104.2	58.3	76.2	72.4	78.5	63.3	
C	4.75	3.36	3.53	3.41	3.48	3.73	3.92
Glcβ1–3	105.6	76.1	78.3	72.3	78.3	63.3	
D	4.77	3.38	3.68	3.58	3.57	3.65	3.84
β1–4Glcβ1–3	105.2	75.8	77.0	82.0	77.3	62.7	
E	4.78	3.55	3.78	3.52	3.51	3.74	3.92
3Glcβ1–3	105.2	75.9	86.9	70.8	78.3	63.3	
X				3.87	3.79	3.74	4.09
reducing end				71.4	76.1	73.6	

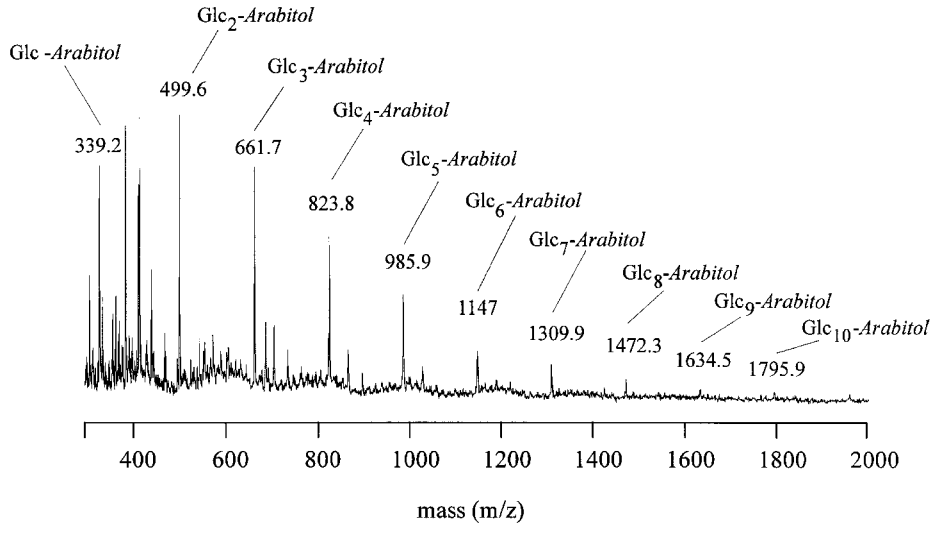

FIG. 11. **MALDI-TOF mass spectra of oligosaccharides of the fraction retarded on *E. cristagalli* lectin-agarose chromatography, after transgalactosylation of ChSN I_B.** Mass spectra were recorded in the positive ion mode and accelerated to an energy of 5 keV before entering the flight tube. Samples were prepared by mixing directly on the target 1 μl of oligosaccharide solution (about 25 pmol) and 1 μl of 2.5-dihydroxybenzoic acid matrix solution (12 mg/ml dissolved in CH_3OH/H_2O (80:20 v/v)). Mass (*m/z*) correspond to the oligosaccharide mass plus sodium.

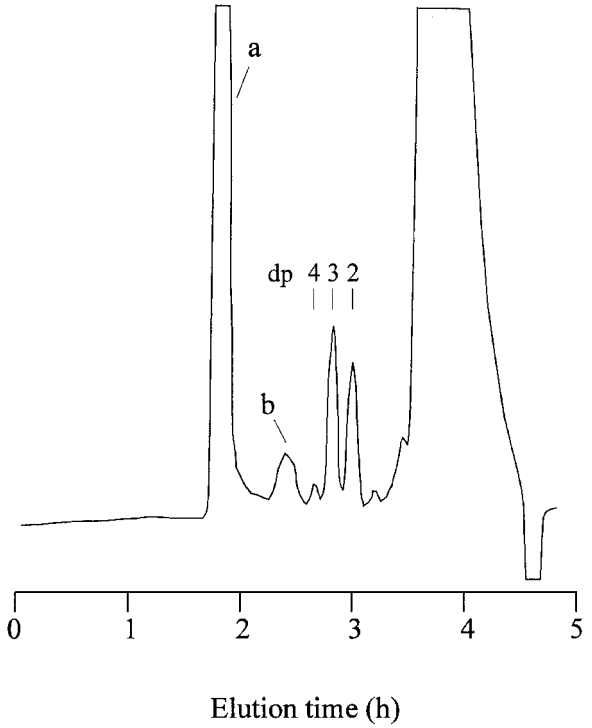

FIG. 12. **Gel filtration chromatography on a TSK HW40S column of products released after 74-kDa endo-β-1–3-glucanase digestion of the ChSN I_A fraction.** TSK HW40S column (90 × 1.4 cm) was eluted with 0.25% (v/v) acetic acid solution at 0.5 ml/min. The eluate was monitored by refractometry. dp was established with malto-oligosaccharides as standard.

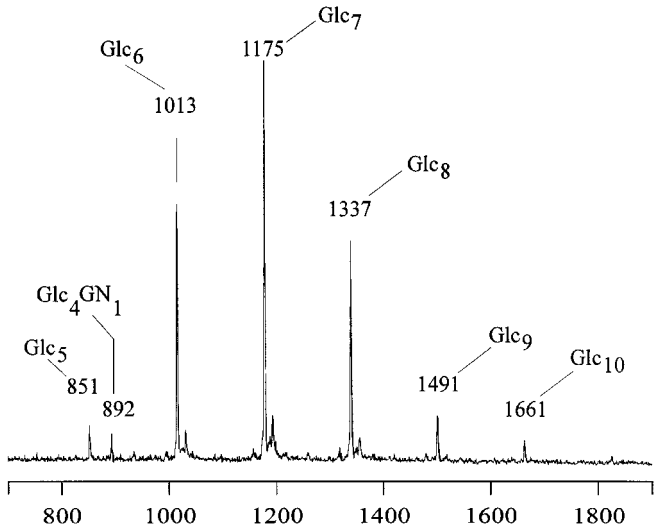

FIG. 13. **MALDI-TOF mass spectra of oligosaccharides of the fraction b of Fig. 12 isolated by gel filtration on a TSK HW40S column after 74-kDa endo-β-1,3-glucanase digestion of the ChSN I_A fraction.** Spectra were recorded in the positive ion mode and accelerated to an energy of 5 keV before entering the flight tube. Samples were prepared by mixing directly on the target 1 μl of oligosaccharide solution (about 25 pmol) and 1 μl of 2.5-dihydroxybenzoic acid matrix solution (12 mg/ml dissolved in CH_3OH/H_2O (80:20 v/v)). Masses (*m/z*) correspond to the oligosaccharide mass plus sodium.

DISCUSSION

Cell wall biosynthesis is a key process in the formation, growth, and morphogenesis of fungal cells. Despite its essential function in fungal development, cell wall structure and biosynthesis, especially polymer cross-linking, remain poorly studied. In this study, a fractionation procedure using alkali extraction and enzymatic digestions of the cell wall, followed by a physicochemical analysis of the soluble polysaccharides, was followed to understand the general organization of the polysaccharides (β-glucans, chitin, and galactomannan) in the alkali-insoluble fraction of the *A. fumigatus* cell wall. Care has been taken to use exclusively recombinant β-1,3-glucanase and chitinase, which are free of any contaminating enzymes. Most previous reported studies in cell wall chemistry, including the study of Kollar *et al*. (7) have used commercial enzymes such as laminarinase or zymolyase, which also contain protease and exo-β-glucanase activities, or chitinase, which is contaminated by chitobiase and β-glucanase activities. Such contaminating activities can mislead the identification of sugar linkages in the cell wall.

From the data presented in this paper, a tentative scheme of the organization of the β-1,3-glucan and its covalently associated polymers found in the cell wall of *A. fumigatus* can be presented (Fig. 14). β-1,3-Glucan is the main component of the alkali-insoluble fraction of *A. fumigatus* cell wall, and it is highly branched with β-1,6 linkages (4% of branch points), constituting a three-dimensional network with a large number of nonreducing ends. Chitin, galactomannan, and β-1,3/1,4-glucan are covalently anchored onto these nonreducing ends, producing a large heteropolymer complex. Chitinase digestion (without previous Quantazyme treatment) degraded 80–90% of the total amount of the chitin of the alkali-insoluble fraction. Chitinase treatment only released GlcNAc and did not release other water-soluble polymers such as β-glucan or galactomannan (data not shown), indicating that these components are not directly linked to chitin. Based on Fig. 14, our current hypothesis of the chronological events occurring in the biosynthesis of the *A. fumigatus* polysaccharide network is as follows: (i) biosynthesis of the individual polysaccharides (β-1,3-glucan, β-1,3/1–4 glucan, chitin, and galactomannan); (ii) branching of β-1,3-glucan, increasing the number of acceptor sites; and (iii) covalent addition of chitin, galactomannan, and β-1,3/1,4-glucan to glucan branches.

The first class of biosynthetic events, synthesis of β-1,3-glucan and chitin, is the only one known in *A. fumigatus* (42–44). Synthesis of β-1,3-glucan in *A. fumigatus,* like in yeast, is under the control of a transmembrane glucan-synthase complex, using UDP-glucose as substrate to extrude linear glucan chain in the periplasmic space (45). Two components of this complex, the regulatory GTPase Rho1 and the putative catalytic subunit FKS have been characterized at the molecular level in yeast (46–50) and recently in *A. fumigatus*.[3] The enzymes responsible for the second and third classes of biosynthetic events (branching and cross-linking) are totally unknown. Two glucanosyltransferases have been recently characterized in *A. fumigatus,* but none of them could synthesize the β-1,3/1,6 branched glucan of the cell wall. The first glucanosyltransferase, homologous to Bgl2p in *S. cerevisiae* and *Candida albicans*, is able to construct a β-1,6 linkage inside a β-1,3-glucan chain (39, 51, 52). However, this enzyme (i) requires a free reducing end to act on the β-1,3-glucan chain and (ii) does not make a branched glucan but makes a kinked linear glucan. Moreover, disruption of the gene encoding this protein in *A. fumigatus* and homologous yeast genes did not result in a phenotype distinct from the parental strain, suggesting that this glucanosyltransferase does not play a role in cell wall construction (40, 53, 54). In contrast, the second glucanosyltransferase identified in *A. fumigatus* (Gelp) plays a major role in fungal morphogenesis. This transferase is responsible for the elongation of β-1,3-glucan chains (38). Molecular characteriza-

[3] A. Beauvais and P. Mol, personal communication.

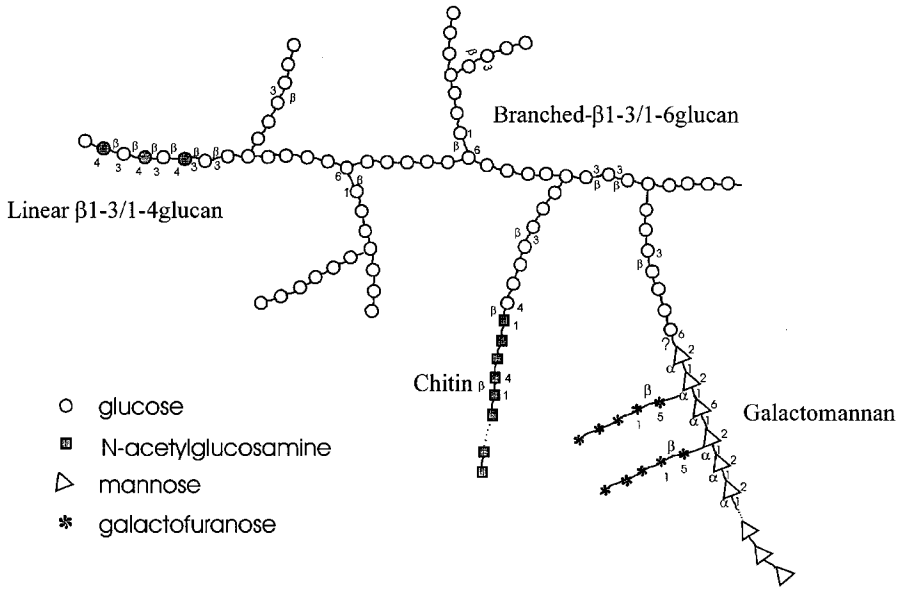

FIG. 14. **Tentative representation of the polysaccharide organization in the structural alkali-insoluble core of the *A. fumigatus* cell wall.**

tion of this enzyme in our laboratory showed that it was homologous to *S. cerevisiae* Gasp, *C. albicans* Phrp, and *Candida maltosa* Epdp.[4] Phenotypic analysis of the yeast and *A. fumigatus* mutants suggested that this enzyme activity was involved in hyphal growth, cell separation, and cell wall construction (55–59).[4] However, the exact biological function of these proteins remains unclear. It has been only shown that the transfer activity requires nonreducing ends of β-1,3-glucan chains, similar to the ones produced during branching of glucan. A putative role for this enzyme could be the elongation of the β-1,3-glucan side chains of this β-1,3/1,6 branched glucan, reinforcing the primary role of the branching of this glucan in the construction of the cell wall.

The only other fungal cell wall that has been studied in detail is the one of *S. cerevisiae*. Comparison of polysaccharide structures showed that these two fungal species present striking similarities: (i) the β-glucan ore of *S. cerevisiae* is branched via β-1,6 linkages. The β-1,3/β-1,6-glucan fraction represents 85% of the total cell wall glucans and contains 3% of branch points (60) and (ii) the alkali-insoluble fraction is a complex structure associating chitin to β-1,3-glucan via a β-1,4-linkage (6). This cross-linking is essential for the alkali insolubility of β-glucan in yeast as well as in filamentous fungi (3, 4, 5).

However, these two fungi present important differences in their cell wall composition: (i) β-1,6-glucans have a central role in *S. cerevisiae*, where they interconnect proteins, chitin, and β-1,3-glucans (7, 13). In *A. fumigatus*, no β-1,6-glucan has been identified. (ii) Two new polysaccharides have been identified in *A. fumigatus*: A poly-*N*-acetylgalactosamine polymer and a linear β-1,3/1,4-glucan. A poly-*N*-acetylgalactosamine polymer has been already described in *Aspergillus parasiticus* (61, 62). In *A. fumigatus*, the galactosamine polymer was also found in the culture filtrate (data not shown) where it became rapidly insoluble.[5] We have no evidence that this polymer is covalently associated to other cell wall polymers. Its presence in FP could be only due to its co-precipitation with the alkali-insoluble material. A linear β-1,3/1,4-glucan never described before in fungi was released with Quantazyme and/or chitinase from the alkali-insoluble cell wall fraction *in A. fumigatus*. This β-1,3/1,4-glucan from *A. fumigatus* has a structure close to vegetal and lichen glucan. Indeed, lichenin, isolated from *Cetraria islandica*, barley, and oat β-glucans are β-1,3/1,4-glucans constituted with β-1,4-glucooligosaccharides of dp 2 or 3 mainly, joined by a single β-1,3-linkage (63–65). Methylation data from the total soluble fraction released by Quantazyme suggested that it represented 10% of total β-glucan. The identification and characterization of this β-1,3/1–4-glucan could explained the presence of 4-*O*-substituted β linked glucose residue reported 30 years ago in the cell wall of Ascomycete species (66). (iii) Galactomannan with galactofuran side chains has been only described in *Penicillium* and *Aspergillus* species. Among these filamentous fungi, this polymer has been previously isolated from a culture filtrate or from the alkali-soluble fraction of the mycelial wall (17, 67, 68). It is the first time that galactomannan was described to be covalently associated to β-1,3-glucan in fungi.

In yeast, cell wall bound glycoproteins have been described to be covalently linked to β-1,3-glucan trough a β-1,6-glucan moiety (8, 9, 10). These proteins are originally GPI-anchored to the membrane (11, 12) and then cleaved to be transferred onto β-1,6-glucan using the sugar moiety of GPI as a bridge (13, 7). Another family of cell wall proteins are directly bound to β-1,3-glucan through an unknown linkage (15). In our study, the use of hot NaOH to prepare the alkali -insoluble fraction of *A. fumigatus* cell wall destroyed all proteins putatively associated to the cell wall and did not allow to study the covalent incorporation of proteins in the *A. fumigatus* cell wall. The presence of such proteins is being presently investigated.

Despite the differences observed in the composition of the structural polysaccharides of *S. cerevisiae* and *A. fumigatus*, branching of β-1,3-glucan appears as a central event for the cell wall construction. Recent analysis of *C. albicans* cell wall also indicated the presence of branched β-1,3/1,6-glucan and the linkage of the β-1,6-glucan to the nonreducing end of the β-1,3-glucan chain.[6] The enzymatic activity responsible for the branching of β-1,3-glucan has not yet been identified and is presently under study in our laboratory since the understanding of this biosynthetic step in the cell wall construction may lead to the discovery of new antifungal drugs for the treatment of life-threatening invasive aspergillosis.

[4] Mouyna, I., Fontaine, T., Vai, M., Monod, M., Fonzi, W. A., Diaquin, M., Popolo, L., Hartland, R. P., and Latgé, J. P. (2000) *J. Biol. Chem.* **275,** 14882–14889.

[5] N. Viseux, personal communication.

[6] G. Dubreucq, unpublished results.

REFERENCES

1. Cabib, E., Roberts, R., and Bowers, B. (1982) *Ann. Rev. Biochem.* **51,** 763–793
2. Fleet, G. H. (1991) in *The Yeast*, 2nd Ed. (Rose, A. H., and Harrisson, J. S., eds), pp. 199–277, Academic Press, N. Y.
3. Sietsma, J. H., and Wessels, J. G. H. (1979) *J. Gen. Microbiol.* **114,** 99–108
4. Mol, P. C., and Wessels, J. H. G. (1987) *FEMS Microbiol. Lett.* **41,** 95–99
5. Hartland, R. P., Vermeulen, C. A., Klis, F. M., Sietsma, J. H., and Wessels J. H. G. (1994) *Yeast* **10,** 1591–1599
6. Kollar, R., Petrakova, E., Ashwell, G., Robbins, P. W., and Cabib, E. (1995) *J. Biol. Chem.* **270,** 1170–1178
7. Kollar, R., Reinhold, B. B., Petrakova, E., Yeh, H. J. C., Ashwell, G., Drgonova, J., Kapteyn, J. C., Klis, F. M., and Cabib, E. (1997) *J. Biol. Chem.* **272,** 17762–17775
8. Montijn, R. C., van Rinsum, J., van Schagen, F. A., and Klis, F. M. (1994) *J. Biol. Chem.* **269,** 19338–19342
9. Kapteyn, J. C., Montijn, R. C., Dijkgraaf, G. J. P., and Klis, F. M. (1994) *Eur. J. Cell Biol.* **65,** 402–407
10. van der Vaart, J. M., Caro, L. H. P., Chapman, J. W., Klis, F. M., and Verrips, C. T. (1995) *J. Bacteriol.* **177,** 3104–3110
11. Lu, C. F., Montijn, R. C., Brown, J. L., Klis, F. M., Kurjan, J., Bussey, H., and Lipke, P. N. (1995) *J. Cell Biol.* **128,** 333–340
12. Vossen, J. H., Muller, W. H., Lipke, P. N., and Klis, F. M. (1997) *J. Bacteriol.* **179,** 2202–2209
13. Kapteyn, J. C., Montijn, R. C., Vink, E., de la Cruz, J., Llobell, A., Douwes, J. E., Shimoi, H., Lipke, P. N., and Klis, F. M. (1996) *Glycobiology* **6,** 337–345
14. Fujii, T., Shimoi, H., and Iimura, Y. (1999) *Biochim. Biophys. Acta* **1427,** 133–144
15. Msra, V., Seidl, T., Gentzsch, M., and Tanner, W. (1997) *Yeast* **13,** 1145–1154
16. Aronson, J. (1981) in *Biology of Conidial Fungi* (Cole, G. T., and Kendrick, B., eds) pp. 459–507, Academic Press, N. Y.
17. Latgé, J. P., Kobayashi, H., Debeaupuis, J. P., Diaquin, M., Sarfati, J., Wieruszeski, J. M., Parra, E., Bouchara, J. P., and Fournet, B. (1994) *Infect. Immun.* **62,** 5424–5433
18. Vorgias, C. E., Tews, I., Perrakis, A., Wilson, K. J., and Oppenheim, A. B. (1993) in *Chitin Enzymology* (Muzzarelli, R. A. A., ed) pp. 417–422, Eur. Chitin Soc., Ancona, Italy
19. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956) *Anal. Chem.* **28,** 350–356
20. Johnson, A. R. (1971) *Anal. Biochem.* **44,** 628–635
21. Kamerling, J. P., Gerwig, G. J., Vliegenthart, J. F. G., and Clamp, J. R. (1975) *Biochem. J.* **151,** 491–495
22. Sawardeker, J. S., Sloneker, J. H., and Jeanes, A. (1967) *Anal. Chem.* **37,** 1602–1604
23. Paz Parente, J., Cardon, P., Leroy, Y., Montreuil, J., Fournet, B., and Ricart, G. (1985) *Carbohydr. Res.* **141,** 41–47
24. Fontaine, T., Talmont, F., Dutton, G. G. S., and Fournet, B. (1991) *Anal. Biochem.* **199,** 154–161
25. Björndal, H., Hellerqvist, C. G., Lindberg, B., and Svensson, S. (1970) *Angew. Chem. Int. Edit.* **9,** 610–619
26. Fournet, B., Strecker, G., Leroy, Y., and Montreuil, J. (1981) *Anal. Biochem.* **116,** 489–502
27. Goldstein, I. J., Hay, G. W., Lewis, B. A., and Smith, F. (1965) *Methods Carbohydr. Chem.* **5,** 361–370
28. Ferguson, M. A. J. (1993) in *Glycobiology: A Practical Approach* (Fukuda, M., and Kobata, A., ed) pp. 349–383, IRL Press, Oxford, U. K.
29. Fontaine, T., Hartland, R. P., Beauvais, A., Diaquin, M., and Latgé, J.-P. (1997) *Eur. J. Biochem.* **243,** 315–321
30. Rance, M., Sorensen, O. W., Bodenhausen, G., Wagner, G., Ernst, R. R., and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.* **117,** 479–485
31. Wagner, G. (1983) *J. Magn. Reson.* **55,** 151–156
32. Griesinger, C., Otting, G., Wüthrich, K., and Ernst, R. R. (1988) *J. Am. Chem. Soc.* **110,** 7870–7872
33. Wilker, W., Leibfritz, D., Kerssebaum, R., and Bermel, W. (1993) *Magn. Reson. Chem.* **31,** 287–292
34. Delay, C., Gavin, J. A., Aumelas, A., Bonnet, P.-A., and Roumestan, C. (1997) *Carbohydr. Res.* **302,** 67–78
35. Macura, S., Huang, Y., Suter, D., and Ernst, R. R. (1981) *J. Magn. Reson.* **43,** 259–281
36. Desvaux, H., Berthault, P., Bilirakis, N., and Goldman, M. (1994) *J. Magn. Reson.* **A 108,** 219–229
37. States, D. J., Haberkorn, R. A., and Ruben, D. J. (1982) *J. Magn. Reson.* **48,** 286–292
38. Hartland, R. P., Fontaine, T., Debeaupuis, J.-P., Simenel, C., Delepierre, M., and Latgé J.-P. (1996) *J. Biol. Chem.* **271,** 26843–26849
39. Yu, L., Goldman, R., Sullivan, P., Walker, G. F., and Fesik, S. W. (1993) *J. Biomol. NMR* **3,** 429–441
40. Mouyna, I., Hartland, R. P., Fontaine, T., Diaquin, M., Simenel, C., Delepierre, M., Henrissat, B., and Latgé, J.-P. (1998) *Microbiology* **144,** 3171–3180
41. Boyd, J., Porteous, R., Soffe, N., and Delepierre, M. (1985) *Carbohydr. Res.* **139,** 35–46
42. Beauvais, A., Drake, R., Ng, K., Diaquin, M., and Latgé J.-P. (1993) *J. Gen. Microbiol.* **139,** 3071–3078
43. Mellado, E., Aufauvre-Brown, A., Specht, C. A., Robbins, P. W., and Holden, D. W. (1995) *Mol. Gen. Genet.* **246,** 353–359
44. Aufauvre-Brown, A., Mellado, E., Gow, N. A. R., and Holden, D. W. (1997) *Fungal Genet. Biol*. **21,** 141–152
45. Shematek, E. M., Braatz, J. A., and Cabib, E. (1980) *J. Biol. Chem.* **255,** 888–894
46. Douglas, C. M., Foor, F., Marrinan, J. A., Morin, N., Nielsen, J. B., Dahl, A. M., Mazur, P., Baginsky, W., Li, W., El-Sherbeini, M., Clemas, J. A., Mandala, S. M., Frommer, B. R., and Kurtz, M. B. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 12907–12911
47. Mazur, P., Morin, N., Baginsky, W., El-Sherbeini, M., Clemas, J. A., Nielsen, J. B., and Foor, F. (1995) *Mol. Cell. Biol.* **15,** 5671–5681
48. Drgonova, J., Drgon, T., Tanaka, K., Kollar, R., Chen, G. C., Ford, R. A., Chan C. S. M., Takai, Y., and Cabib, E. (1996) *Science* **272,** 277–279
49. Mazur, P., and Baginsky, W. (1996) *J. Biol. Chem.* **271,** 14604–14609
50. Qodata, H., Python, C. P., Inoue, S. B., Arisawa, M., Anraku, Y., Zheng, Y., Watanabe, T., Levin, D. E., and Ohya, Y. (1996) *Science* **272,** 279–281
51. Hartland, R. P., Emerson, G. W., and Sullivan, P. A. (1991) *Proc. R. Soc. Lond. B. Biol. Sc.* **246,** 155–160
52. Goldman, R. C., Sullivan, P. A., Zakula, D., and Capobianco J. O. (1995) *Eur. J. Biochem.* **227,** 372–378
53. Klebl, F., and Tanner, W (1989) *J. Bacteriol.* **171,** 6259–6264
54. Sarthy, A. V., McGonigal, T., Coen, M., Frost D. J., Meulbroek, J. A., and Goldman, R. C. (1997) *Microbiology* **143,** 367–376
55. Saporito-Irwin, S. M., Birse, C. E., Sypherd, P. S., and Fonzi, W. A. (1995) *Mol. Cell. Biol.* **15,** 601–613
56. Muhlschlegel, F. A., and Fonzi, W. A. (1997) *Mol. Cell. Biol.* **17,** 5960–5967
57. Nakazawa, T., Horiuchi, H., Ohta, A., and Takagi, M. (1998) *J. Bacteriol.* **180,** 2079–2086
58. Popolo, L., Vaï, M., Gatti, E., Porello, S., Bonfante, P., Balestrini, R., and Alberghina, L. (1993) *J. Bacteriol.* **175,** 1879–1885
59. Popolo, L., and Vaï M. (1999) *Biochim. Biophys. Acta* **1426,** 385–400
60. Manners, D. J., Masson, A. J., and Patterson J. C. (1973) *Biochem. J.* **135,** 19–30
61. Araki, Y., Takada, H., Fudjii, N., and Ito, E. (1979) *Eur. J. Biochem.* **102,** 35–42
62. Takada, H., Araki, Y., and Ito, E. (1981) *J. Biochem.* **89,** 1265–1274
63. Parrish, F. W., Perlin, A. S., and Reese, E. T. (1960) *Can. J. Chem.* **38,** 2094–2104
64. Perlin, A. S., and Suzuki, S. (1962) *Can. J. Chem.* **40,** 50–56
65. Buliga, G. S., Brant, D. A., and Fincher, G. B. (1986) *Carbohydr. Res.* **157,** 139–156
66. Bartnicki-Garcia, S. (1968) *Annu. Rev. Microbiol.* **22,** 87–105
67. Barreto-Berger, E., Gorin, P. A. J., and Travassos L. R. (1981) *Carbohydr. Res.* **95,** 205–218
68. Hearn, V. M., and Sietsma, J. H. (1994) *Microbiology* **140,** 789–795