C. glabrata cell wall (1→3,1→6)-β-D-glucan side chains have a unique structure

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Abstract

The yeast cell wall is composed of several different components including mannan, mannoprotein, chitin and glucan. Glucan in the ascomycete *C. glabrata* predominantly exists as polymers of (1→3)-β-D-glucan with sidechain attachments containing (1→6)-linked glucosyl repeat units. Despite extensive physicochemical characterization, the average structures of (1→3,1→6)-β-D-glucans isolated from different natural sources have been difficult to unravel. Of considerable interest is the nature of the side chains in these polysaccharides. In addition to the location of these side chains along the main backbone of the polymer, knowledge about the average length of the side chains is also of importance. For example, recognition of (1→3,1→6)-β-D-glucans by pattern recognition receptors in the innate immune system is influenced by side chain structure. (1→3,1→6)-β-D-glucan was isolated from *C. glabrata* HLS122 (*sce2*). Cells were grown in YPD at 30°C for 24 hr, harvested and lyophilized. The lyophilized material was then subjected to a modified glucan extraction method. We accomplished the detailed structural characterization of the (1→6)-β-linked side chains using ^{1}H and ^{13}C 1D and 2D NMR techniques on an 800 MHz NMR. We confirmed the presence of (1→6)-β-linked side chains containing more than 1 glycosyl repeat unit. We were also able to establish the average length and relative position of the (1→6)-β-linked side chains along the (1→3)-linked polymer backbone. Comparison of results from 4 strains demonstrates differences in side chain structures obtained as a function of protic acid concentration. By understanding these side chain structural details of fungal cell wall glucans, we can gain a greater appreciation of fungal cell wall composition and structure and how cell wall glucan structure relates to the biology of this opportunistic pathogen.

Introduction

Fungal cells are surrounded by a cell wall that generally is composed of four main components, chitin, glucan, mannan and mannoprotein. The cell wall plays an important function of helping the cell to maintain its shape and viability in response to osmotic challenge. (1→3)-β-D-glucan is a major component of the cell wall and, in regards to fungal pathogenesis, is recognized by the host cell receptor Dectin-1 (Brown and Gordon, 2003). (1→3)-β-D-glucan is generally arrayed as a polymer in a triple helix, which helps in maintaining the rigidity of the cell wall. Additionally, (1→3)-β-D-glucan polymers have (1→6)-β-D-glucan side chain branches, which have not been well characterized structurally. Tada and coworkers (Tada et al, 2009) characterized the structure of Grifolan-LE, a (1→3,1→6)-β-D-glucan polymer containing a (1→3)-linked backbone chain and side chains composed of a single (1→6)-β-D-glucan repeat unit attached to the backbone chain every 3 backbone repeat units on average. Grifolan-LE was isolated from *Grifola frondosa*. In this current study, we have isolated glucan from several strains of *Candida glabrata*, an opportunistic fungal pathogen. Using a modification of a standard glucan extraction procedure, we have isolated a (1→3,1→6)-β-D-glucan that contains a unique poly-(1→6)-linked side chain structure. Based on extensive ^{1}H and ^{13}C 1D and homonuclear (COSY, NOESY, and TOCSY) and heteronuclear (HSQC, HSQC-NOESY, HSQC-TOCSY, and HMBC) 2D NMR studies, we have found that the glucan isolated from *C. glabrata* contains a (1→3)-β-D-glucan polymer backbone with (1→6)-β-D-glucan-containing sidechain branches that are composed of multiple glucosyl repeat units. Additionally, we have extended our structural investigation to include a molecular modeling study of what the sidechain structure conformation may be in the organism. The structure of the sidechain appears to have a hook-like, bent-curve structure, which may allow for bonding to a parallel (1→3)-β-D-glucan polymer, or serve as a point of attachment for a glycophosphoinositol-linked protein. Another possibility is that this hook-like structure plays a different role in the fungal cell wall structure, one that allows for physically grabbing another (1→3)-β-D-glucan polymer, much in the same manner that a Velcro hook grabs a Velcro fastener strand. Based on these results, we propose a new model to explain the plasticity of the cell wall architecture that allows for a rigid cell wall that exhibits flexibility as the cell grows.

Materials & Methods

Strains and growth conditions. *C. glabrata* strains (**Table 1**) were routinely cultured on YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) at 30°C.

Glucan isolation. Strains were cultured in 1L of YPD at 30°C for 18 hr with agitation. Cells were harvested and then lyophilized. The lyophilized cells were extracted using modifications of the methods of Williams (Williams et al, 1991). In method 1 the protic acid concentration was 1 N, while in method 2 the protic acid concentration was 2 N. All other aspects of the method were as previously reported by our group (Müller et al, 1997).

NMR. Proton and ^{13}C NMR spectra were collected on a Bruker Avance II 800 NMR spectrometer using a 5mm TCI inverse cryoprobe operating at 343°K. For ^{1}H NMR spectra, ~25 mg of glucan was solvated in 950 mL DMSO-d_6 (Sigma-Aldrich, "100") with 40 µL trifluoroacetic acid-d (Cambridge Isotope Laboratories, 99.8+% deuterated) to shift the exchangeable proton resonances downfield. For ^{13}C NMR spectra, a second sample was prepared using ~15 mg of glucan dissolved in 600 mL DMSO-d_6 with no added trifluoroacetic acid. Proton and ^{13}C 1D and COSY, NOESY, TOCSY, HSQC, HSQC-TOCSY, HSQC-NOESY, and HMBC 2D NMR spectra were obtained in this study. For 1D NMR experiments, chemical shift referencing used absolute referencing based on the ^{2}H DMSO-d_6 lock signal and ^{1}H and ^{13}C gyromagnetic ratios. For 2D NMR experiments, chemical shift referencing was accomplished relative to the anomeric resonance of the (1→3)-linked backbone repeat unit at 4.526 ppm for ^{1}H and 102.70 ppm for ^{13}C. 1D NMR spectra were collected and processed as follows: for ^{1}H NMR, 64 90° scans with 8 dummy scans, acquisition of 65,536 real points zero-filled once to 65,536 complex points, 10.5 ppm sweep width, centered at 3.85 ppm with a total acquisition time of 3.9 s, exponential apodization with 0.3 Hz broadening, and 3 s pulse delay for a total interpulse period of 6.9 s; for ^{13}C NMR, 16,384 30° scans with 8 dummy scans, acquisition of 65,536 real points zero-filled once to 65,536 complex points, 65 ppm sweep width, centered at 80 ppm with a total acquisition time of 2.5 s, exponential apodization with 3 Hz broadening, and 2 s pulse delay for a total interpulse period of 4.5 s. 2D NMR spectra were collected and processed as follows: for homonuclear NMR experiments, 512 x 256 point matrix was zero-filled to 2048 x 1024 points, 32 scans per row with 16 dummy scans, 3 ppm sweep width centered at 3.85 ppm, cosine-squared apodization in both dimensions, and 2 sec pulse delay; for heteronuclear NMR experiments, 512 x 512 point matrix was zero-filled to 1024 x 1024 points, 64 scans per row with an initial 64 dummy scans, 3 ppm sweep width centered at 3.85 ppm for the f1 dimension and 80 ppm sweep width centered at 65 ppm for the f2 dimension, cosine-squared apodization in both dimensions, and 1.5 s pulse delay except for HMBC which was zero-filled to 4096 x 1024 points and sine apodized. Adiabatic ^{13}C decoupling was used during the t_2 acquisition period of the heteronuclear experiments, except for the HMBC experiment, and shaped inversion pulses were used on the ^{13}C channel in inverse heteronuclear experiments. NMR spectra were processed using TOPSPIN 2.0 running on the Avance II 800 NMR and TOPSPIN running on Windows XP operating system under VMWare on a Macintosh MacBook Pro.

Molecular Modeling. Chemical structures were drawn in a linear format using CS ChemDraw Ultra® (version 6.0.1; Cambridge Soft Corporation; Cambridge, MA). The structures were then copied into CS Chem3D Ultra®. For each polysaccharide, a molecular mechanics (MM) minimization was conducted using a root-mean-square (RMS) of 0.005. Next, molecular geometries were generated in the Gaussian Z-matrix style via the CS MOPAC application. For each compound, an Austin-Model-1 (AM1) semi-empirical calculation (closed shell, tight convergence criteria) was conducted using a Gaussian® software packet (Gaussian, Inc.; Carnegie, PA) (Frisch et al, 2004). *Ab initio* geometry optimizations were subsequently performed using Gaussian 03 at the Hartree-Fock level of theory using the STO-3G (Hehre et al, 1969; Collins et al, 1976) basis set. Geometry optimizations using implicit solvation were performed with the Onsager method (Onsager, 1936) using the dielectric constant of water (78.39) and the solute radius; calculated with the more accurate molecular volume computation (Volume_Tight). These calculations were performed with a Microway AMD (Microway Technology, Plymouth, MA) dual 64 bit 2.0 GHz central processing unit (CPU) with 4GB RAM running Fedora Core 3 and an Aspen Systems 30 CPU cluster (Wheatridge, CO): each blade containing two 32-bit Xeon 3.2 GHz CPUs with 4GB RAM running Red Hat Linux.

Strain Name	Genotype
ATCC2001	Wild type from ATCC
HLS122	*his3::URA3 ura3 trp1 ace2::HIS3 (pCgACT-14 [TRP1])*
Anp1	*his3::URA3 ura3 trp1 anp1::HIS3 (pCgACT-14 [TRP1])*
Mnn2	*his3::URA3 ura3 trp1 mnn2::HIS3 (pCgACT-14 [TRP1])*
Mnn11	*his3::URA3 ura3 trp1 mnn11::HIS3 (pCgACT-14 [TRP1])*

Table 1. Genotypes of strains used in this study.

Proton Assignment	(1-3)-β-Linked Backbone Chain	Br	SC1	SC Internal(1-6)-Linked Side Chain	SC SNRT (1-6)	SC NRT (1-6)	NRT (1-3)
H1	4.526	4.361	4.255	4.274	4.274	4.374	4.390
H2	3.309	3.242	3.033	3.037	3.037	3.102	3.095
H3	3.485	3.395	3.192	3.193	3.193	3.228	3.220
H4	3.274	3.324	3.153	3.153	3.165	3.152	3.152
H5	3.272	3.229	3.320	3.320	3.331	3.247	3.247
H6	3.726	3.999	4.010	4.010	4.031	3.721	3.721
H6'	3.484	3.637	3.612	3.612	3.663	3.462	3.462

Table 2. Proton NMR Chemical Shifts (in ppm) for Various Structural Features Characterized in the Glucan Isolated from *C. glabrata*.

Carbon Assignment	(1-3)-β-Linked Backbone Chain	Br	SC1	SC Internal (1-6)-Linked Side Chain	SC SNRT (1-6)	SC NRT (1-6)	NRT (1-3)
C1	102.70	102.43	103.10	102.96	102.96	103.66	103.48
C2	72.54	71.99	73.60	73.16	73.16	76.47	76.57
C3	85.97	87.14	76.32	76.32	76.32	75.89	75.95
C4	68.15	67.90	69.74	69.74	69.79	69.79	69.79
C5	76.08	75.88	75.13	75.23	75.22	76.48	76.48
C6	60.63	67.86	68.24	68.17	68.21	60.82	60.84

Table 3. ^{13}C NMR Chemical Shifts (in ppm) for Various Structural Features Characterized in the Glucan Isolated from *C. glabrata*.

Isolation Method	Strain	SCn	Br Freq.
1A	2001 (wt)	4.0	22.2
1A	HLS122	4.2	20.3
1A	Mnn11	4.1	21.8
1B	2001 (wt)	4.4	19.7
1B	HLS122	4.7	20.7
1B	Mnn11	4.1	21.4
2	2001 (wt)	2.5	40.7
2	Anp1	1.8	43.0
2	Mnn2	2.0	30.8
2	Mnn11	2.1	30.5

Table 4. Comparison of average side chain lengths, SCn, and branching frequencies, Br Freq, for several strains of *C glabrata* isolated by 2 different methods.

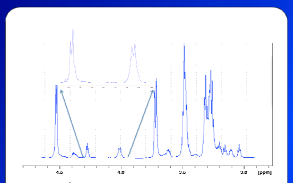

Figure 1. 1D ^{1}H NMR spectrum of the (1→3,1→6)-β-D-glucan isolated from *C. glabrata* (strain HLS122). The spectral region from 4.33 to 3.94 ppm is expanded.

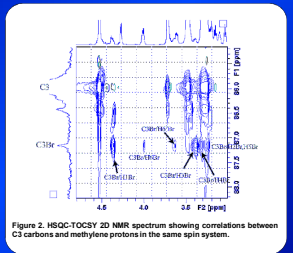

Figure 2. HSQC-TOCSY 2D NMR spectrum showing correlations between C3 carbons and methylene protons in the same spin system.

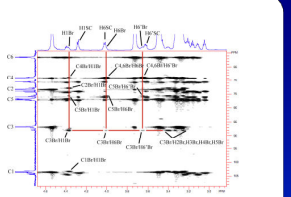

Figure 3. HSQC-TOCSY spectrum indicating assignments and correlations for protons and carbons in the branch point repeat unit based upon correlations initially identified for C3 (Figure 2).

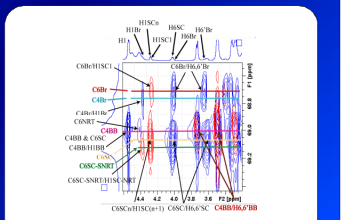

Figure 4. Overlay of the 2D HSQC-TOCSY (blue) and HMBC (red) NMR spectra expanded around the C4 spectral region to show the correlation (C6Br/H1SC1) across the glycosidic link between C6Br of the branchpoint repeat unit and the anomeric proton (H1SC1) of the first (1-6)-linked repeat unit in the side chain as well as correlations across other (1-6)-linked side chain glycosidic linkages.

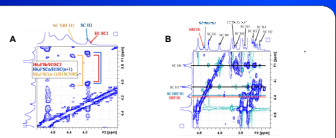

Figure 5. (A) The three different (1-6)-linked glycosidic bonds from the side chain are detailed in the NOESY 2D NMR spectrum for SC1, SC Internal, and SC NRT glucosyl groups associated with H1 SC1, SC H1 and SC NRT H1. (B) Similarity of the structures of the glycosyl group associated with SC_NRT H1 (blue line) and NRT H1 (red line) is indicated in the TOCSY 2D NMR spectrum.

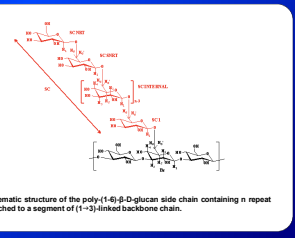

Figure 6. Schematic structure of the poly-(1-6)-β-D-glucan side chain containing n repeat units and attached to a segment of (1→3)-linked backbone chain.

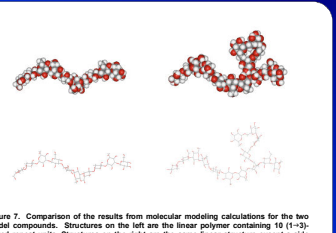

Figure 7. Comparison of the results from molecular modeling calculations for the two model compounds. Structures on the left are the linear polymer containing 10 (1→3)-linked repeat units. Structures on the right are the same linear structure except a side chain containing 4 (1-6)-linked repeat units branches from the third repeat unit.

Results and Discussion

Tada and coworkers (Tada et al, 2009) recently presented the complete structural characterization of Grifolan-LE isolated from *Grifola frondosa*. Grifolan-LE is a purified (1→3)-β-D-glucan with single (1→6)-linked glucosyl side chain branches every third backbone chain repeat unit. They assigned specific ^{1}H NMR resonances to the anomeric proton and to one of the methylene protons of the (1→6)-linkages of the side chain repeat unit. In the ^{1}H NMR spectrum, the resonances for the anomeric proton and methylene ^{1}H were of equal integrated area, consistent with a side chain structure containing only a single repeat unit. The ^{1}H NMR spectrum of the glucan isolated from *C. glabrata* (**Figure 1**) resembles the NMR spectrum of Grifolan-LE except that the integral areas of the anomeric proton and one of the methylene protons involved in the side chain (1→6)-linkages are not of equal area.

The NMR spectrum (**Figure 1**) shows 5 major resonances that were previously assigned to glucosyl repeat units of a (1→3)-linked β-D-glucan polymer chain (Ensley et al, 1994). These assignments, confirmed by COSY 2D NMR (data not shown), are summarized in **Table 1**.

The branchpoint glucosyl repeat unit in the (1→3)-linked polymer chain is unique in that it is the only repeat unit in either the backbone chain or the side chain that is substituted in 1-, 3- and 6-positions. Using the spectral region of the C3 carbon in the HSQC-TOCSY 2D NMR spectrum (**Figure 2**), only the carbon resonating at 87.14 ppm (C3Br) exhibits long-range correlations to equivalent methylene protons at 4.00 (C3Br/H6Br) and 3.64 (C3Br/H6'Br) ppm. Other protons correlated to the C3Br resonance include protons assignable to the branchpoint repeat unit (**Table 1**). Proton and ^{13}C NMR resonance assignments are based upon results from HSQC-TOCSY and HSQC 2D NMR experiments. The HSQC-TOCSY spectrum provides information about all protons that are dipolar coupled to a specific carbon all in the same repeat unit with the strength of these correlations being dependent upon the distance of the individual, dipolar-coupled protons from the individual carbon atom in the spin system. Since the TOCSY spin lock time is short, only 4 protons correlate to each carbon around the glucosyl residue in this study. Therefore, the correlation to H1Br observed from C3Br (C3Br/H1Br) in the HSQC-TOCSY 2D NMR spectrum is important for establishing C1 through C4 in the branchpoint repeat unit while the correlations to H6Br/H6'Br observed from C3Br are important for establishing C3 through C6 in the branchpoint repeat unit (**Figure 3**).

The correlation from the methylene carbon of the branchpoint repeat unit (C6Br) and the anomeric proton (H1) of the first side chain repeat unit (SC1) across the (1→6)-linked glycosidic bond is established in the HMBC 2D NMR spectrum (C6Br/H1SC1, **Figure 4**). The anomeric proton of SC1 correlates with H2 in the COSY 2D spectrum (data not shown), and then H2 correlates with H3 and the remainder of the glycosyl protons H4 through H6/H6' in the HSQC-TOCSY 2D NMR spectrum (**Figure 4**). The methylene protons in SC1 correlate with the anomeric proton of the first side chain internal repeat unit (**Figure 4**), which coincides with correlations between all of the internal repeat unit protons and the correlation across the (1→6)-linked glycosidic bond of the internal repeat units (C6SCn/H1SC(n+1)) in **Figure 4**).

Three types of (1→6)-linkages across glycosidic bonds are evident in the NOESY 2D NMR spectrum (**Figure 5A**). These (1→6)-linked glycosidic bonds for SC1 (H6,6'Br/H1SC1) and SC INTERNAL (H6,6'SCn/H1SC(n+1)) have been identified. The remaining (1→6)-linkage exhibits H6,H6' correlations to an anomeric proton with chemical shifts typical of a (1→6)-linkage. The anomeric proton in this linkage has a chemical shift characteristic of the anomeric proton of a non-reducing terminus (NRT H1). TOCSY spectra of the NRT glycosyl repeat units (SC NRT H1 and NRT H1 in **Figure 5B**) are characteristic of mono-substituted glucosyl repeat units. Therefore this third (1→6)-linkage is assigned to the glycosidic linkage between the second to the last glycosyl repeat unit (SC SNRT) and the non-reducing terminus of the (1→6)-linked side chain (SC NRT). These data complete the assignment of all repeat units and glycosidic linkages in the (1→6)-linked side chain of this glucan isolated from *C glabrata* and support the side chain structure shown in **Figure 6**.

The anomeric proton for the α-conformer of the reducing terminus (α-RT) is assigned to 4.99 ppm while the chemical shift of the β-RT anomeric proton is overlapped by resonances from the anomeric protons of Br, SC NRT and NRT. Anomeric proton resonances for α- and β-glucose are observed at 4.93 and 4.30 ppm, respectively. Anomeric protons for the α- and β-conformers of SRT are observed at 4.46 and 4.49 ppm, respectively. These anomeric proton resonance assignments are in agreement with previous assignments by Kim and coworkers (Kim et al, 2000) and in general agreement with assignments by Størseth and coworkers (Størseth et al, 2006).

Interestingly, the integrated areas of the 4.01 and 4.27 ppm resonances are not equal in the glucan isolate from *C. glabrata* (**Inset in Figure 1**). The integrated area of the H6 multiplet resonance at 4.01 ppm is larger than the area of the H1 multiplet resonance at 4.27 ppm. Integration of these two resonances gives an area ratio of 0.787:1 for resonances H1 and H6, respectively, not the 1:1 ratio reported for Grifolan-LE with the single (1→6)-linked glucosyl repeat unit in the side chain (Tada et al, 2009). The difference in areas of the two resonances represents the integral area assignable to the anomeric proton H1 in SC NRT. The ratio suggests that there are on average 4.7 (1→6)-linked repeat units in the average side chain which is attached to the polymer backbone on average every 21 repeat units along the backbone chain.

In addition, the H1 and H6 resonances from the (1→6)-linkage also exhibit several inflection points (**Inset in Figure 1**). Størseth and coworkers (Størseth et al, 2006) suggest that these multiple inflection points reflect multiple side chain lengths. Monteiro and coworkers (Monteiro et al, 2000) show that a (1→6)-β-D-glucan isolated from *Actinobacillus suis* exhibits multiple conformations about the (1→6)-linked glycosidic bond resulting in additional resonances in the ^{1}H NMR spectrum. Similar NMR spectrum was obtained for a (1→6)-β-D-glucan isolated from *Malassezia sympodialis* (Kruppa et al, 2009). Therefore, it is reasonable to consider that, while the multiple inflection points in the H1 and H6 resonances may result from different side chain lengths, they may also result from different conformations observed for the (1→6)-linked glycosidic bond.

Several other aspects of *C. glabrata* isolated under different conditions have also been examined by ^{1}H NMR. Isolation method 1A and 1B represent two isolations with reduced protic acid concentration. Method 2 represents isolation with a higher concentration of protic acid. All other parameters of the extraction were held constant. At the higher acid concentration (Method 2), the (1→6)-linked side chains are 2 repeat units in length on average while the branchpoint frequency differs for the 4 strains examined (**Table 2**). For 2001 (wild type) and anp1 strains, the branch frequency is about one-third larger than for strains Mnn2 and Mnn11. At the lower acid concentration (Methods 1A and 1B), the average chain length is about twice the length of that from Method 2 and the average branching frequency is constant at 20 repeat units.

Adams and coworkers (Adams et al, 2008) have discussed the importance of these side chain structures relative to interaction of (1→3,1→6)-β-D-glucans with Dectin-1, the primary pattern recognition receptor (PRR) for these glucans (Brown et al, 2003). To better understand how these glucans with longer side chains may interact with Dectin-1, theoretical optimized molecular geometries were investigated for a model compound containing 10 (1→3)-linked repeat units in the polymer backbone and 4 (1→6)-linked repeat units in the side chain branching at the third repeat unit in the polymer chain. Compared to a linear polymer containing 10 (1→3)-linked repeat units (**Figure 7**), the calculations reveal that the branchpoint repeat unit takes on a skewed conformation. Also, the calculation converted the initial linear side chain to a curled structure with added H-bonding. This side chain structural proposal is interesting in that it may offer insight into how these side chains aid in not only the rigidity and structure, but also the plasticity of the fungal cell wall. The curled side chain may provide a mechanism for interaction of the side chain with other glucan chains within the cell wall by wrapping around other polymer chains, thus not requiring covalent bonding between the side chain repeat units and other chains in the polymer. The wrapped side chain might then enable greater flexibility, or plasticity, within the cell wall structure. Specifically, the curled side chain may be sufficiently flexible to allow the fungal cell to change its shape/volume as required for response to various environmental stressors.

Conclusions

A detailed 1D and 2D ^{1}H and ^{13}C NMR structural characterization and molecular modeling study of the side chain poly-(1→6)-linked glucosyl repeat units of (1→3,1→6)-β-D-glucan from *C. glabrata* has lead to a new proposal for the fungal cell wall structure that supports the cell wall's plasticity and strength. Curl in the side chain structure suggests that these side chains may connect neighboring glucan polymer chains in a manner that allows flexibility, or plasticity, in the cell walls, not previously appreciated.

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