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Structural characterization of (1→3)-β-D-glucans isolated from blastospore and hyphal forms of *Candida albicans*

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Abstract

Glucans are (1→3)-β-linked linear and branched polymers containing anhydroglucose repeat units. They comprise a major portion of the cell wall of saprophytic and pathogenic fungi. Glucans activate a wide range of innate immune responses. They are also released from the fungal cell wall as exopolymers into the blood of patients with fungal infections. Extensive studies have been done on glucans isolated from saprophytic fungi, such as *Saccharomyces cerevisiae*; however, much less is known about the glucans produced by the polymorphic fungal pathogen *Candida albicans.* We have undertaken an extensive structural characterization and comparison of glucans isolated from *C. albicans* blastospores and hyphae using high-resolution, solution-state proton nuclear magnetic resonance spectroscopy (NMR). In addition, we developed a simple and straightforward method for the production of *Candida* hyphae that resulted in gram quantities of hyphal mass. Also, we compared and contrasted the *Candida* glucans isolated by two different protocols with those isolated from *S. cerevisiae*. Isolation protocols provide high purity glucans with source-based structural differences. Structural details provided by this NMR analysis included the degree of polymerization, molecular weight, degree and type of branching, and structural composition. We observed that *Candida* glucans, derived from blastospores or hyphae, are different compared to those isolated from *S. cerevisiae* with regard to side-chain branching along the backbone and at the reducing terminus. These structural details are an important prerequisite for biomedical studies on the interaction of isolated fungal cell wall glucans with the innate immune system.

Keywords: *Candida albicans*; Blastospore; Hyphae; NMR; Pathogen

1. Introduction

Candida albicans is a polymorphic fungal pathogen that causes life threatening infections in the critically ill and immunocompromised host. During the course of infection *Candida* species undergo a morphogenic transformation from a unicellular blastospore (yeast) form to germ tubes and true hyphae.[1] Hyphae are thought to play an important role in *Candida* tissue invasion and pathogenesis.[2] Despite the medical significance of *Candida* species, there is still much that we do not know about the manner in which this fungal pathogen interacts with the immune system. It is generally accepted that the innate immune system identifies pathogens based on the carbohydrates, lipids and proteins expressed by the organism.[3] These macromolecular structures, which are called pathogen-associated molecular patterns (PAMPs), are ideal recognition molecules for the innate immune system because they are structurally distinct from those expressed on the surface of mammalian cells.[3]

A major cell wall carbohydrate of saprophytic and pathogenic fungi[4–7] is (1→3)-β-linked linear and (1→6)-β-branched glucans (Fig. 1) composed of anhydroglucose repeat units (AGRUs).[8–11] The fact that glucans modulate a wide range of innate host immune responses[12] and that they are found in the blood of

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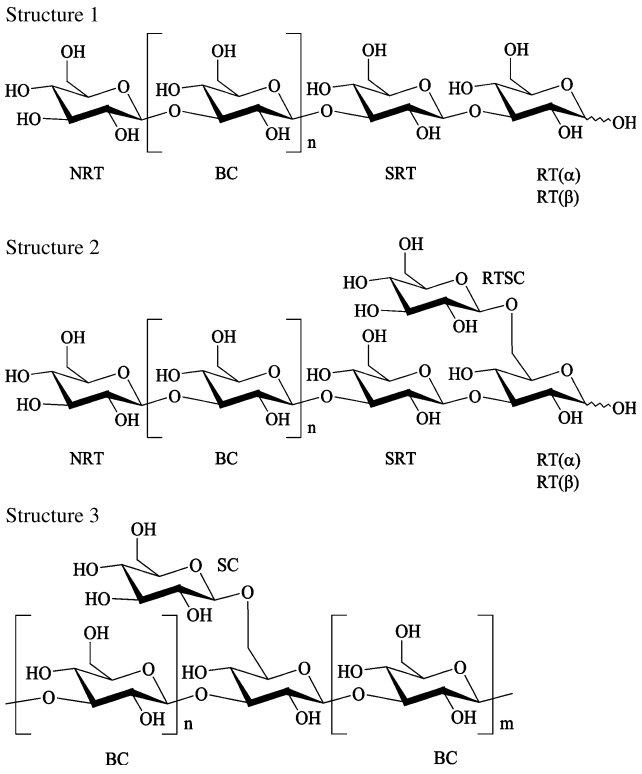

Fig. 1. Proposed structures employed to describe glucan structural features observed in the glucans isolated from *C. albicans* and *S. cerevisiae*. AGRU labels are described in the text.

patients with fungal sepsis[13,14] has prompted speculation that these carbohydrates may be fungal PAMPs.[15]

Extensive structural studies have been conducted on glucans isolated from saprophytes, such as *Saccharomyces cerevisiae*.[8,9] In contrast, little is known about the glucans produced by *C. albicans*, despite the medical significance of this pathogen.[16,17] The studies that have been done on *Candida* glucans have focused on the unicellular yeast or blastospore form.[18] To the best of our knowledge, there has been no extensive analysis of the structure of glucans isolated from *C. albicans* hyphae. Deciphering the structural features of these carbohydrates is an essential prerequisite for understanding how fungal glucans are recognized by the innate immune system.[19–21]

In the present study, we compared and contrasted the structure of (1→3)-β-D-glucans isolated from *C. albicans* blastospore and hyphal forms using solution-state proton nuclear magnetic resonance spectroscopy (NMR). In addition, we compared and contrasted glucan isolated from the saprophytic yeast *S. cerevisiae* with the glucans isolated from *C. albicans*. Our comparisons focused on molecular weight (MW), degree of polymerization (DP), degree of branching (DB), and structural composition of the glucans.

2. Results and discussion

(1→3)-β-D-Glucans are polymers composed of a linear AGRU backbone (Fig. 1, Structure 1). Side-chain AGRUs branch exclusively from the 6-position of the backbone AGRU (Fig. 1, Structures 2 and 3). Kim and co-workers[9] assigned specific proton NMR resonances to AGRU anomeric protons in various structural features of the glucan. We used the assignment of these structural features to describe structural differences in glucans isolated from *C. albicans* blastospores and hyphae as well as glucans isolated from the non-pathogen, *S. cerevisiae*.

In order to isolate glucan from hyphae in sufficient quantities for analysis, it was necessary to culture large quantities of hyphal mass. The methods currently available for induction of hyphal growth in *Candida* are expensive, tedious, and not amenable to large scale production (>1 L).[22] We developed a simple and straightforward method for the production of *Candida* hyphae. This method, which employs Mueller–Hinton broth, can be used to produce gram quantities of hyphal mass. The advantage of this method over previous methods, such as that of Shepherd and Sullivan,[22] is the growth of predominately hyphal forms, with a low percentage of other morphologies. The lyophilized hyphal mass was sufficient for multiple glucan extractions. We speculate that this methodology could easily be applied to the extraction of other hyphal cell wall macromolecules, a possibility that we are currently investigating.

The isolation protocols that we employed have been optimized for the extraction of highly purified glucans from fungal cell walls.[23,24] The structural features of these glucans are influenced, not only by their native conformation in the cell wall, but also by the isolation methodology. By way of example, Mueller and co-workers[24] have reported that the strength of the protic acid employed for glucan extraction influences MW and perhaps the degree of side-chain branching.

For the highly purified glucan isolated from *S. cerevisiae* (Fig. 2), the areas of the indicated NMR resonances are used to characterize the glucan in terms of DP (Eq. (1)) and DB(SC) (Eq. (2)). For this glucan, DP is 198.8 and DB(SC) is 0.37 for a MW (Eq. (3)) of 32,211 and a (1→3)-to-(1→6) linkage ratio of 534-to-1, indicating a very low level of (1→6)-linked AGRU side-chains along the glucan backbone (Structure 3). No evidence for a (1→6)-linked AGRU attached to reducing terminus (RT) is observed (Structure 2). Based on high-performance size-exclusion chromatography (HPSEC) using dimethyl sulfoxide (DMSO) as the mobile phase and multi-angle laser light scattering (MALLS) detection, the MW of this glucan was determined to be 35,000,[25] a value which strongly agrees with the NMR result. It is important to note that this

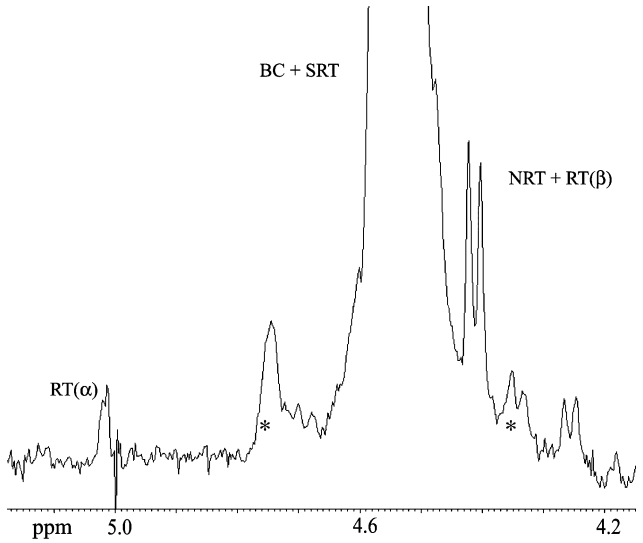

Fig. 2. Anomeric proton region of the NMR spectrum of a glucan isolated from *S. cerevisiae*. Resonance labels are described in the text. Asterisks (*) indicate ^{13}C satellite resonances from the resonance assigned to the BC anomeric protons.

water-insoluble glucan is thought to exist as a stable triple helix in its native state. When dissolved in DMSO, glucans undergo helix–coil transition, i.e., the hydrogen bonds that stabilize the helix are disrupted, resulting in a solution containing individual glucan polymer strands.[25] Consequently, the excellent agreement between the MW as determined by NMR spectroscopy and HPSEC reflects the MW of the individual polymer strands or approximately one-third of the MW in the triple helical form (99,000–105,000 g/mol). Internal standard proton NMR assay indicated that the isolated glucan has 97.7% glucan content. Also interesting is that the level of branching (DB(SC) = 0.37) indicates 1 branch per 2.7 chains on average, or slightly more than 1 branch per glucan triple helix structure. At RT α and β anomers are present at levels of 46 and 54%, respectively.

Glucans isolated from either *C. albicans* blastospore (Fig. 3A and B) or hyphae (Fig. 3C) are different compared to those isolated from *S. cerevisiae* (Fig. 3D) as is evident by examining NMR spectra of the anomeric proton regions. Two different reducing termini, each containing α and β anomers, are evident in the *C. albicans* spectra (Fig. 3A–C). One group of RT ends is consistent with that observed in *S. cerevisiae* (Fig. 1, Structure 1 and Fig. 3D), while the other RT ends have anomeric proton resonance chemical shifts consistent with a (1→6)-linked AGRU (Fig. 1, Structure 2) similar to that observed for the disaccharide gentiobiose.[9] These spectra support the observation that the level of (1→6) linkages in glucan isolated from *C. albicans* is higher than that in *S. cerevisiae*.[6]

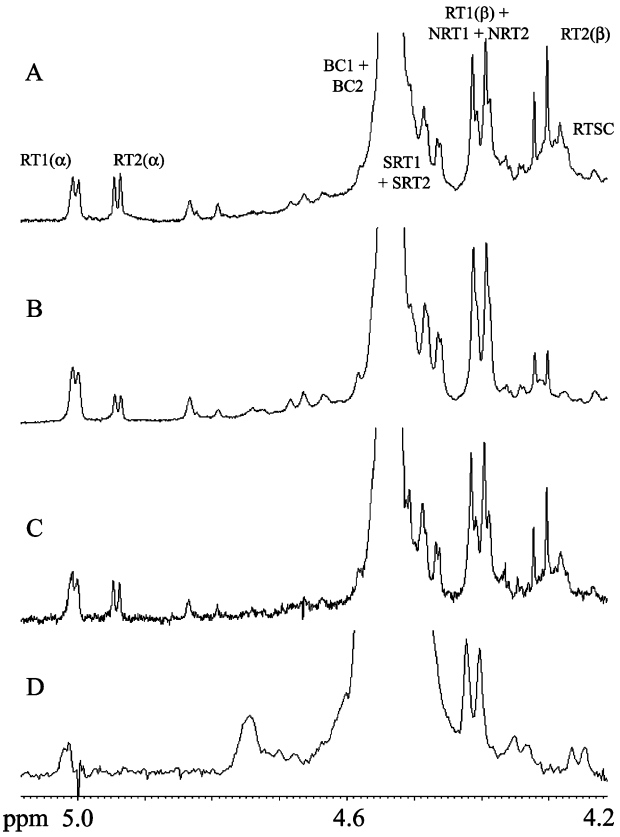

Fig. 3. Comparison of the anomeric proton region of the NMR spectra from blastospore and hyphal glucans isolated from *C. albicans* (A, B and C) and from glucans isolated from *S. cerevisiae* (D): (A) blastospore 1 (HCl extraction method); (B) blastospore 2 (H_3PO_4 extraction method); (C) hyphae (H_3PO_4 extraction method); and (D) glucan from Fig. 2. Resonance labels are described in the text. Numbers in the resonance labels refer to either Structure 1 or 2 (Fig. 1).

Table 1 summarizes the results of the structural characterizations for these glucans. The tabulated data provide insight into structural differences resulting from fungal species, extraction protocol and blastospores versus filamentous hyphae.

The glucans were isolated from *C. albicans* blastospores using either the HCl (Blastospore 1) or H_3PO_4 (Blastospore 2) extraction method, while hyphal glucans were isolated using only the H_3PO_4 extraction method (Fig. 3 and Table 1). Glucans extracted from blastospores by the two methods resulted in different compositions of glucan structures 1 and 2 and higher DP for the H_3PO_4 method as demonstrated previously.[24] The DP of the hyphal glucans was reduced compared to the blastospore glucans also isolated by the H_3PO_4 method.

Considering the entire isolated glucan, the level of (1→3) linkages relative to the RT end group (as in Structure 1), designated RT_{Linear}, was constant for glucans isolated from the two blastospores and the hyphae (as indicated by the molar ratio (1→3)/RT_{Linear}

Table 1
Tabulation of structural features and compositions observed by ^{1}H NMR spectroscopy for the highly pure isolated glucans

Sample [a]	i [b]	$[Conc]_i$ (%)	DP	α/β Anomer ratio (%)	$(1\rightarrow3)/(1\rightarrow6)_{RTSC}$	$(1\rightarrow3)/RT_{Linear}$	$(1\rightarrow3)/(1\rightarrow6)_{SC}$
S. cerevisiae (NaOH–HCl)			198.8	46/54	nd [c]	198	534
C. albicans Blastospore 1 (NaOH–HCl)	1	52	10.4	46/54	20	18	nd
	2	48	10.5	46/54			
C. albicans Blastospore 2 (NaOH–H_3PO_4)	1	80	15.3	46/54	73	18	nd
	2	20	15.2	66/34			
C. albicans Hyphae (NaOH–H_3PO_4)	1	57	11.3	42/58	30	18	nd
	2	43	13.7	41/59			

[a] Glucan extraction method is shown in parenthesis.
[b] Species i designates either Structure 1 or 2 from Fig. 1.
[c] nd, none detected.

in Table 1), while the level of (1→6) linkages decreased about threefold in the blastospore glucans and about 50% in the hyphal glucans (as indicated by the molar ratio $(1\rightarrow3)/(1\rightarrow6)_{RTSC}$ in Table 1) using the H_3PO_4 extraction method compared to blastospore results with the HCl extraction method. There were no (1→6)-linked branch points (Structure 3) observed along the backbone of glucans extracted from *C. albicans* blastospore and hyphal sources (as indicated by the molar ratio $(1\rightarrow3)/(1\rightarrow6)_{SC}$ in Table 1).

The DP of the glucans isolated from *C. albicans* is significantly smaller than DP determined for glucans isolated from *S. cerevisiae*. The increased level of (1→6)-linked side-chains at RT (Structure 2) in the glucans isolated from *C. albicans* possibly reflects an increased ease of hydrolysis of the (1→3) linkage next to the AGRU containing a (1→6)-linked branch point using this glucan isolation protocol. In that case, the original glucan present prior to isolation by this protocol would have a higher DP and contain more (1→6)-linked side-chains than is evident in Table 1. This ease of hydrolysis would then result in glucan polymer chains with lower DP relative to the much higher DP glucan isolated from *S. cerevisiae*, which also contained a very low level of (1→6)-linked branch points.

By applying a ^{1}H NMR approach, we have developed a detailed structural characterization of (1→3)-β-D-glucans derived from *C. albicans* blastospores and hyphae. We have also identified structural differences between glucan derived from a saprophytic fungus versus those derived from a clinically relevant pathogen. Structural data derived from these studies are an essential prerequisite for understanding the structure–activity relationships of isolated fungal cell-wall carbohydrates as we begin to investigate the interactions of blastospore and hyphae carbohydrates with pattern-recognition receptors involved in mediating innate immunity.

3. Materials and methods

3.1. *Candida albicans*

A laboratory control strain of *C. albicans* was employed for these experiments. This strain produced germ tubes and hyphae when incubated in fetal calf serum at 37 °C.

3.2. Growth of *C. albicans* blastospores

We employed brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) for production of a maximal biomass of blastospores. A blood agar plate (BAP) was streaked with a control culture of *C. albicans* and incubated at 37 °C for 48 h. The strain produces two colony types, a raised, irregular bordered colony 'stellate', and a circular, convex colony 'dome'. The 'dome' colony type was used for these experiments. Tubes (5 mL) of brain–heart infusion broth (BHIB) were inoculated with 1 colony each of *C. albicans* from the 48 h BAP and were incubated in a slanted position at 37 °C with agitation at 200 rpm for 18 h. This resulted in a predominance of yeast-form cells and a high turbidity. Flasks (2 L) containing 1 L of BHIB were inoculated with 1 tube each (5 mL) of BHIB-grown *C. albicans.* The flasks were incubated at 37 °C with agitation at 200 rpm (18–24 h). Morphology was confirmed by direct microscopic examination and Gram staining. Blastospores were harvested by centrifugation (3000*g*) and washed 3× with sterile distilled water. The yield averaged 15–20 g/L (wet weight) of *C. albicans* blastospores which were lyophilized to dryness and stored at 4 °C until used for glucan isolation.

3.3. Induction of hyphal growth

Tubes (5 mL) of BHIB were inoculated (1 colony) with *C. albicans* from the 48 h BAP and incubated in a slanted position at 37 °C with agitation at 200 rpm for 7–8 h. This resulted in a predominance of yeast forms and a low turbidity. Flasks (1 L) containing 500 mL of Mueller–Hinton broth were inoculated with 5 mL of the BHIB culture. The flasks were incubated at 37 °C and agitated at 125 rpm for 12 h. The hyphal growth appeared as filamentous masses suspended throughout the medium, which quickly settled out leaving a clear, non-birefringent broth. The clear supernatant was decanted, and the hyphae were washed 3 × with sterile distilled water and harvested by centrifugation (3000*g*). The yield averaged 11 g/L (wet weight) of *Candida* hyphae. Hyphal growth was confirmed by Gram stain (heat-fixed smear) (Fig. 4) and wet-mount phase-contrast microscopy. The hyphal mass was lyophilized to dryness and stored (−20 °C) until used.

3.4. Isolation of glucans

Glucans were isolated by the method of Mueller and co-workers[23] using ethanol containing HCl, and by the modified method[24] using ethanol containing H_3PO_4. Glucan purity was determined by an internal-standard proton NMR method.[11] All glucans were water-insoluble microparticulates. The glucan yield from blastospores was 3.65% of dry weight. The glucan yield from hyphae was 5.1% of dry weight.

Fig. 4. Photomicrograph of the mycelial mass from *C. albicans* produced for the extraction of glucan. The mycelial mass shows predominantly hyphae. The hyphae were Gram stained and examined under light microscopy (200 ×).

3.5. NMR spectroscopy

Spectral data were collected using either a JEOL Model Eclipse+600 or a Model DELTA-400 NMR spectrometer operating at 80 °C in 5 mm OD NMR tubes. For each sample, 10–25 mg of the glucan isolate was dissolved in 1 mL of DMSO-d_6 at 80 °C. A few drops of trifluoroacetic acid-*d* (99.8% deuterated or better from Cambridge Isotope Laboratories) were added to the solution to shift the water resonance downfield.[26] NMR chemical shifts were referenced to the residual DMSO-d_6 proton resonance at 2.50 ppm. Generally, NMR spectral collection and processing parameters were the following: 25 ppm spectral width centered at 7.5 ppm, 32,768 data points, 1024 scans, 15 s relaxation delay, 2.18 s acquisition time, and exponential apodization. The number of scans was varied based upon the sample size.

3.6. Calculations

DP, calculated using Eq. (1), is the number of AGRUs in the linear polymer chain.

$$\mathrm{DP} = \frac{\mathrm{Area(BC + SRT)}}{(\mathrm{Area(RT}(\alpha) + \mathrm{NRT} + \mathrm{RT}(\beta))/2)} + 2 \qquad (1)$$

where, using the terminology of Kim and co-workers,[9] BC is the anomeric proton of the backbone chain AGRUs, SRT is the anomeric proton of the second AGRU connected to the reducing terminus, RT is the anomeric proton of the reducing terminus with α and β anomer stereochemistry indicated as RT(α) and RT(β), respectively, and NRT is the anomeric proton of the non-reducing terminus (Fig. 1, Structure 1). The average integral area from RT(α), RT(β), and NRT resonances, equivalent to two protons, gives the area attributed to one AGRU end group. The integral area of the polymer backbone resonances assigned to BC and SRT provides the polymer length relative to an end group. Two is included in the calculation to account for the AGRU end groups.

Side-chain AGRUs are defined as those attached to the RT (reducing terminus side-chain, RTSC) (Fig. 1, Structure 2) and those attached along the backbone (SC) (Fig. 1, Structure 3), both attached to the 6-position of the AGRU in the linear glucan chain. DB for these two types of side-chains is calculated in a similar manner. DB due to RTSC side-chains, DB(RTSC), is calculated using Eq. (2).

$$\mathrm{DB(RTSC)} = \frac{\mathrm{Area(RTSC)}}{(\mathrm{Area(RT}(\alpha) + \mathrm{NRT} + \mathrm{RT}(\beta))/2)} \qquad (2)$$

where RTSC is the anomeric proton of the side-chain AGRU attached to the RT. DB due to SC side-chains, DB(SC), is calculated in a manner similar to that shown in Eq. (2). Since DB is calculated relative to an end group, total DB, DB(total), from the sum of DB(RTSC) and DB(SC) can be converted to the total branching level per polymer chain containing the number of AGRUs indicated by DP. Side-chains in these calculations are assumed to only be 1 AGRU in length.

MW is calculated using Eq. (3).

$$\mathrm{MW} = (\mathrm{DP} + \mathrm{DB(RTSC)} + (\mathrm{total})^{*}162.14 + 18.02 \quad (3)$$

The MW of an AGRU (162.14) equals the MW of a glucose monomer minus 1 hydrogen and 1 hydroxyl, or 18.02. An AGRU side-chain results from insertion of an AGRU between the hydroxyl oxygen and hydrogen at the 6-position. The value 18.02 is added to the polymer MW since the polymer chain end groups are completed by adding a hydrogen to the AGRU at one end of the chain and a hydroxyl to the AGRU at the other end of the chain.

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