

Chapter

4

**Spore-wall α -glucan of fission yeast is not assembled
from building blocks but rather consists of
a single (1 \rightarrow 3)- α -glucan polymer**

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Abstract

Under conditions of nitrogen starvation, diploid fission-yeast cells undergo meiosis followed by sporulation. During sporulation, individual nuclei are surrounded by a double layer of membranes, called the forespore membranes. In between these membranes, the spore wall is formed. In contrast to vegetative growth where the existing cell wall is extended, spore walls are formed *de novo*. Although it is known that spore walls of fission yeast contain chitin and β -glucan, the presence of α -glucan has not been demonstrated. Here, we identify (1 \rightarrow 3)- α -glucan as a major constituent of fission-yeast spore walls. Upon extracting spore walls with dimethyl sulfoxide, we obtained, in addition to an amylose-like polysaccharide, two distinct linear (1 \rightarrow 3)- α -glucan populations consisting of approximately 480 and 680 glucose residues, respectively. Importantly, their chemical structures differed fundamentally from (1 \rightarrow 3)- α -glucan from vegetative cell walls in that it was not composed of two covalently-linked building blocks (dimeric structure), but instead consisted of a monomer of (1 \rightarrow 3)-linked α -glucose residues. This finding is consistent with DNA-microarray analyses that showed that the synthase for cell-wall α -glucan, Ags1p, is downregulated during sporulation, whereas Ags1p homologs are upregulated. Taken together, we propose that an α -glucan synthase other than Ags1p is responsible for the *de novo* biosynthesis of α -glucan in spore walls.

Introduction

Fission yeast, *Schizosaccharomyces pombe* (Lindner), undergoes a haplontic life cycle when grown under nutrient-rich conditions. Under conditions of nutritional stress (i.e., nitrogen starvation), sexual differentiation occurs in which haploid cells of opposite mating types fuse, forming diploid cells that undergo meiosis and sporulation. Three major meiotic phases can be distinguished. During the early, premeiotic S phase, chromosome pairing and recombination takes place. During the middle phase, the first (M I) and second (M II) meiotic division occur, forming four haploid nuclei within the cell wall of the original cell, called the ascus. During the late phase, each nucleus is surrounded by a double layer of membranes, called the forespore membranes. In between these membrane layers, the spore wall is formed. This spore wall, like the cell wall of haploid cells, consists mainly of polysaccharides and glycoproteins. The spore wall ensures structural integrity, regulates cell permeability, and resists environmental stresses. However, in contrast to vegetative growth, in which the cell wall extends, spore walls are created entirely *de novo*.

In *Saccharomyces cerevisiae*, cell-wall as well as spore-wall architecture are well-studied. In vegetatively-grown *S. cerevisiae* cells, cell-walls are composed of (1 \rightarrow 6)- β -glucan and (1 \rightarrow 3)- β -glucan layers followed by a layer of mannoproteins (Klis *et al.*, 1997; Klis, 1994), whereas spore walls contain two additional surface layers, namely a carbohydrate layer

consisting of chitosan and an outermost layer consisting of dityrosine polymers (Briza *et al.* 1988; Briza *et al.* 1986). This implies that the biochemistry of spore-wall formation and also the genes involved differ from those of cell-wall formation. In fission yeast, meiotic expression of 99.3% of the approximately 4,900 known genes was followed using DNA microarrays (Mata *et al.*, 2002). Mata and co-workers demonstrated that during meiosis and sporulation more than 50% of the genome is regulated. More specifically, they found that 1,033 genes were upregulated at least four-fold. Among these are several genes with known or putative functions in spore-wall construction. For instance, a gene encoding a (1→3)-β-glucan synthase, *bgs2⁺*, which is dispensable for cell-wall formation in vegetatively growing cells, is essential for the assembly of spore walls (Liu *et al.*, 2000; Martin *et al.*, 2000). Furthermore, two chitin synthase homologs *chs1⁺* and *chs2⁺* were identified in fission yeast, of which *chs1⁺* is required for proper spore maturation (Arellano *et al.*, 2000). The function of *chs2⁺* remains unknown, but is probably also required for sporulation as this gene is strongly upregulated during meiosis (Mata *et al.*, 2002).

α-Glucan is a major polysaccharide constituent in the fission-yeast cell wall and its synthase has recently been identified (Hochstenbach *et al.*, 1998). This α-glucan is composed of two building blocks, each comprising a (1→3)-α-glucan segment of approximately 135 residues with a small number of (1→4)-linked α-glucose residues attached to its reducing end (see Chapter 2). Importantly, the gene encoding the α-glucan synthase, *ags1⁺*, is downregulated during meiosis, whereas four homologous genes (*mok11⁺*, *mok12⁺*, *mok13⁺*, and *mok14⁺*) are upregulated (Mata *et al.*, 2002). Together with the fact that these genes could be deleted without forming a noticeable phenotype in the vegetative growth phase (Katayama *et al.*, 1999), this indicates that the genes do not possess an essential function during vegetative growth but may be involved in sporulation. However, thus far, no (1→3)-α-glucan was identified in spore walls of fission yeast.

Here, we identify two different populations of (1→3)-α-glucan in fission-yeast spore walls and describe their chemical structures. By using high-performance size-exclusion chromatography in combination with chemical analyses and NMR spectroscopy, we show that both spore-wall (1→3)-α-glucans are composed of single, linear (1→3)-α-glucan polymers. The significant differences in chemical structures between α-glucan of cell walls and of spore walls together with the regulation patterns of genes encoding putative α-glucan synthases suggest that an α-glucan synthase different from Ags1p may be involved in its biosynthesis.

Results

Composition of spore walls

To determine the chemical composition of spore walls, we physically lysed fission-yeast spores and collected spore walls by centrifugation. Quantitative monosaccharide analysis of whole spore walls revealed the presence of glucose, mannose and galactose in the molar

ratio of 92.2 : 4.8 : 3.0 in addition to traces of *N*-acetylglucosamine. Cell walls were then dried completely and extracted with dimethyl sulfoxide (DMSO). The molecular mass distribution of the extract was determined by high-performance size-exclusion chromatography (HPSEC). The size-exclusion profile showed two overlapping peaks with a number-average molecular mass (M_n) of 22.2 kDa and a polydispersity (M_w/M_n) of 5.87 (**Fig. 1A**, **Table I**). The heterogeneous nature of the spore walls was also apparent in the 1D $^1\text{H-NMR}$ spectrum, which showed two H-1 α signals at 5.146 ppm ($^3J_{1,2}$ 4.0 Hz) and 5.093 ($^3J_{1,2}$ 3.7 Hz), and a H-1 β signal at 4.544 ppm ($^3J_{1,2}$ 7.8 Hz) in a relative abundance of 4:1:1 (**Fig. 2A**). Based on the monosaccharide composition, we infer that these signals correspond to glucose residues, indicating that the DMSO extract of fission-yeast spore walls contains three major polysaccharides, namely two α -glucans and one β -glucan.

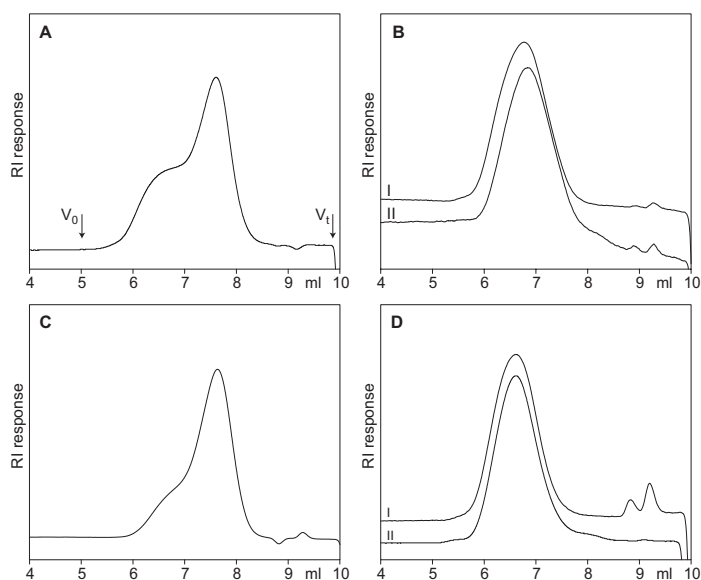


Fig. 1. Molecular mass distributions of whole spore polysaccharides (**A**), **P I** ((1→3)- α -glucan) untreated (**I**) and Smith degraded (**II**) (**B**), **P II** ((1→4)- α -glucan) (**C**), and **P III** ((1→3)- α -glucan) untreated (**I**) and Smith degraded (**II**) (**D**). Dried polysaccharide preparations were dissolved in DMSO containing 3 mM sodium nitrate and their molecular mass distributions were analyzed by HPSEC on a calibrated mixed-bed column.

Table I. Molecular-mass averages and polydispersities of spore components

	M_n (kDa)	M_w (kDa)	M_w/M_n	DP_n
Whole spore walls	22.2	130.1	6.45	137
P I	77.8	196.3	2.52	480
P I , Smith degraded	55.9	139.4	2.50	345
P II	16.5	55.7	3.36	102
P III	109.7	256.4	2.33	677
P III , Smith degraded	96.2	200.5	2.08	594

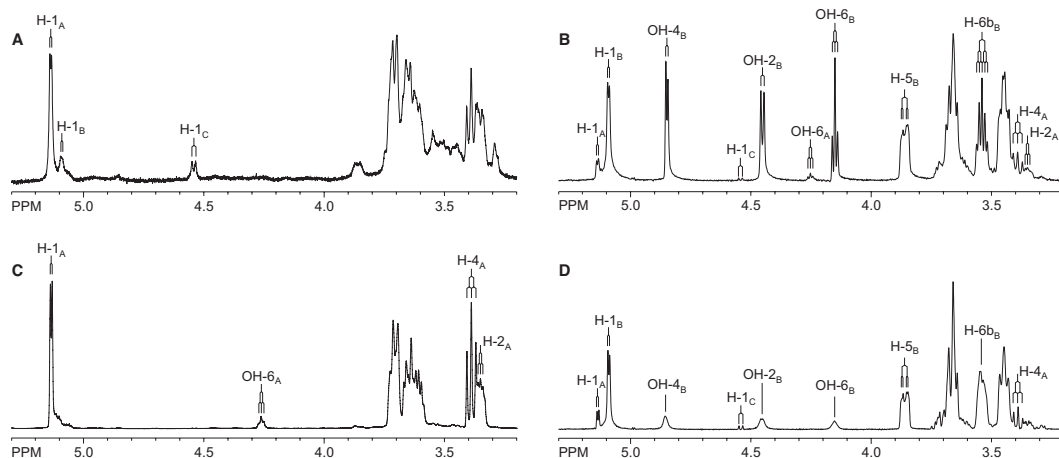


Fig. 2. The DMSO extract of spore polysaccharides consists of two α -glucans and one β -glucan. $^1\text{H-NMR}$ spectra of whole spore polysaccharides (A), P I (B), P II (C), and P III (D). Constituent A represents (1 \rightarrow 4)-linked α -Glc_p, B represents (1 \rightarrow 3)-linked α -Glc_p, and C represents (1 \rightarrow 3)-linked β -Glc_p (see text for assignment). The numbers refer to the corresponding carbon atom as assigned by 2D NMR spectroscopy. Samples were dissolved in DMSO- d_6 and spectra were recorded at 500.08 MHz at 80 °C.

Fractionation of spore walls

To investigate the chemical structures of the α -glucans, we separated the major spore wall components into three different fractions (Fig. 3, denoted P I, P II, and P III). Spore walls were digested with Zymolyase, which solubilized approximately 80% of the β -glucan as was shown by $^1\text{H NMR}$ (data not shown). Monosaccharide analysis of the Zymolyase-resistant material showed that glucose was the only monosaccharide constituent, indicating that in addition to (1 \rightarrow 3)- β -glucan, also the mannose, galactose, and *N*-acetylglucosamine constituents were liberated. Since the enzyme used to hydrolyze (1 \rightarrow 3)- β -glucan also contains proteases, we suppose that the monosaccharides other than glucose constituted protein-linked glycans. Spore walls were then dissolved in DMSO and two polysaccharide fractions were obtained by precipitation. A first fraction precipitated after addition of ethanol to a final volume of 40%, yielding P I, which comprised 30% (w/w) of the Zymolyase-resistant cell wall. A second fraction was obtained by precipitation at 60% of ethanol (P II, 56% (w/w) of the Zymolyase-resistant cell wall). The homogeneity of the preparations was investigated by $^1\text{H-NMR}$ spectroscopy. The extraction procedure was repeated for P I until a near homogeneous preparation was obtained. The DMSO-insoluble residue could be dissolved completely in 1 M sodium hydroxide, and a polysaccharide fraction precipitated upon neutralization that was collected by centrifugation (P III, 14% (w/w) of the Zymolyase-resistant cell wall). Addition of three volumes of ethanol to the supernatant did not result in precipitation of additional material, indicating that the supernatant was devoid of polysaccharides.

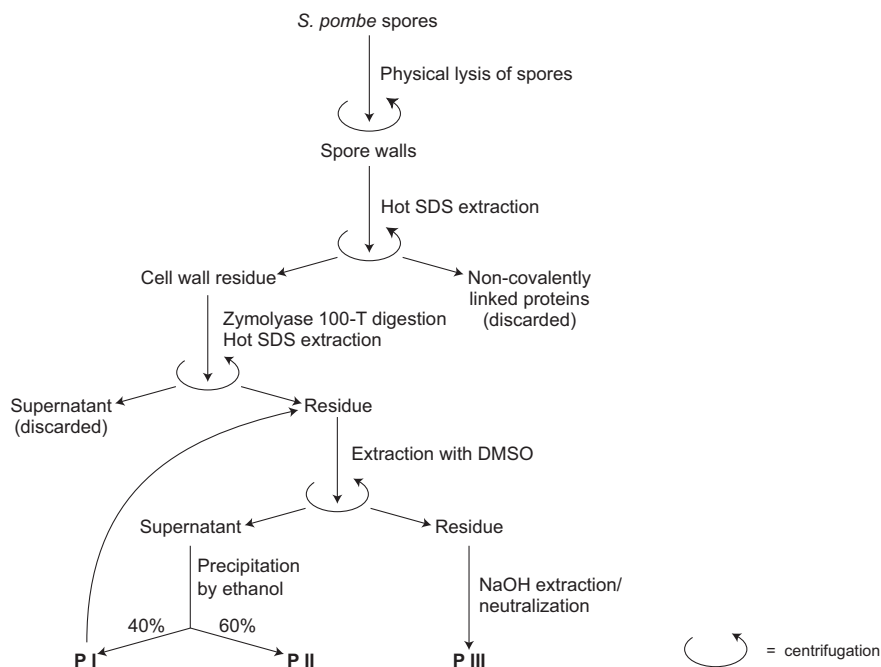


Fig. 3. Schematic representation for the isolation and fractionation of spore α -glucans.

P I consists mainly of (1 \rightarrow 3)- α -D-glucan

To characterize the **P I** fraction, monosaccharide analysis together with the determination of the absolute configuration showed that **P I** consisted entirely of D-glucose. Linkage analysis demonstrated the presence of (1 \rightarrow 3)-linked residues as a main constituent together with 12.6% (1 \rightarrow 4)-linkages (**Table II**). Triple substituted residues were not observed, indicating that branching points are not present.

Table II. Linkage analysis of spore α -glucans

Residue	Molar amounts (%)		
	P I	P II	P III
Glc p -(1 \rightarrow	-	1.1	-
\rightarrow 3)-Glc p -(1 \rightarrow	87.4	1.6	91.6
\rightarrow 4)-Glc p -(1 \rightarrow	12.6	97.3	8.4

In the 1D $^1\text{H-NMR}$ spectrum of **P I** (**Fig. 2B**) H-1 α signals at 5.138 ppm (denoted H-1 $_A$) and 5.092 ppm (denoted H-1 $_B$) are present in the relative intensity of 1:10, in addition to a low-intensity H-1 β signal at 4.544 ppm (denoted H-1 $_C$), indicating the presence of three constituents denoted A, B, and C. For the major constituent B, proton signals at 4.849, 4.453, and 4.139 ppm were observed, which did not produce cross-peaks in the 2D $^1\text{H-}^{13}\text{C}$ HSQC spectrum (**Fig. 4A**), demonstrating that these signals represent slowly-exchanging

hydroxyl groups. By using 2D ^1H - ^1H TOCSY (**Fig. 4B**), the hydroxyl signals could be assigned to OH-4_B, OH-2_B, and OH-6_B, respectively. 2D NMR spectroscopy (TOCSY and HSQC) allowed the assignment of all proton and carbon resonances of constituent B (**Table III**). The low-field shift of C-3_B at 83.0 ppm together with the absence of an OH-3_B and the small $^3J_{1,2}$ coupling constant demonstrate that the major constituent of **P I** consists of (1→3)-linked α -glucopyranose residues.

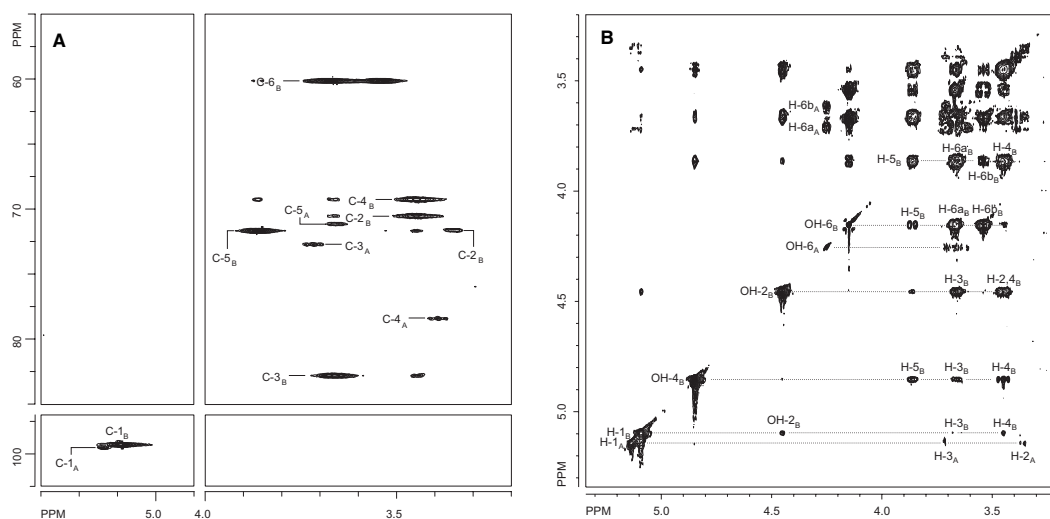


Fig. 4. Fraction **P I** consists of (1→3)-linked α -glucopyranose residues with some (1→4)-linked residues. ^1H - ^{13}C HSQC spectrum (**A**) and ^1H - ^1H TOCSY spectrum (50 ms mixing time) (**B**). The major constituent (denoted B) represents (1→3)-linked α -Glc_p, whereas the minor constituent (denoted A) represents (1→4)-linked α -Glc_p. C-1_B means a cross-peak between H-1 and C-1 of constituent B in the HSQC spectrum. Cross-peaks belonging to the same scalar coupling network are indicated along a dotted line starting from the corresponding diagonal peak in the TOCSY spectrum. Note that the down-field shifts of C-3_B and C-4_A are typical for (1→3)-linked and (1→4)-linked residues, respectively. Note also that OH-2_B, OH-4_B, OH-6_B, and OH-6_A give cross-peaks in the TOCSY spectrum, whereas these cross-peaks are absent in the HSQC spectrum. Samples were dissolved in DMSO-*d*₆ and spectra were recorded at 500.08 MHz at 80 °C.

The constituent of medium intensity, A, has an anomeric signal at 5.138 ppm (**Fig. 2B**, denoted H-1_A). The low-field shift together with the small $^3J_{1,2}$ coupling constant of 3.9 Hz indicates an α -anomeric configuration. The assignments of proton and carbon signals as obtained by the TOCSY and HSQC spectra are displayed in **Table III**. The low-field chemical shift of C-4_A at 78.5 ppm demonstrates that constituent A represents (1→4)-linked α -D-glucopyranose residues (**Fig. 5**). ^1H and ^{13}C chemical shifts are in good agreement with experimental data obtained from a (1→4)- α -glucan, amylose (not shown).

Table III. Proton and carbon chemical shifts of **P I**

Constituent A ((1→4)- α -Glc _p)				Constituent B ((1→3)- α -Glc _p)			
Proton	PPM	Carbon	PPM	Proton	PPM	Carbon	PPM
H-1	5.138	C-1	99.6	H-1	5.092	C-1	99.4
H-2	3.353	C-2	71.7	H-2	3.449	C-2	70.7
H-3	3.715	C-3	72.8	H-3	3.660	C-3	83.0
H-4	3.390	C-4	78.5	H-4	3.449	C-4	69.3
H-5	3.660	C-5	71.2	H-5	3.863	C-5	71.7
H-6a	4.252	C-6	n.d.	H-6a	3.671	C-6	60.2
H-6b	3.608			H-6b	3.540		
OH-2	n.d.			OH-2	4.453		
OH-4	n.d.			OH-4	4.849		
OH-6	4.253			OH-6	4.139		

n.d. = not detected

Constituent **C** is present at very low intensity and only the anomeric proton was observed in the $^1\text{H-NMR}$ spectrum (**Fig. 2B**). Due to its low abundance, cross-peaks were not observed in the TOCSY and HSQC spectra, making allocation difficult. Nevertheless, this signal was observed also in the $^1\text{H-NMR}$ spectrum of whole spore walls (**Fig. 2A**) and its intensity decreased fourfold by Zymolyase digestion. Therefore, we assume that constituent **C** represents a (1→3)- β -glucan. Indeed, NMR analysis of a (1→3)- β -glucan (laminaran) demonstrated that the anomeric proton has a chemical shift and coupling constant similar to constituent **C** (not shown).

Together, our results indicate that **P I** comprises a mixture of polysaccharides consisting mainly of a linear (1→3)- α -D-glucan, with approximately 10% (1→4)-linked α -D-glucose residues. Based on our results, we cannot exclude the possibility that the (1→4)-linked glucose residues form a contaminating component, namely of **P II** (see below). In addition, small amounts of (1→3)-linked β -glucose residues are present, indicating (1→3)- β -glucan contamination.

To determine the molecular mass distribution of **P I**, the preparation was dried, dissolved in DMSO and analyzed by high-performance size-exclusion chromatography (HPSEC) on a calibrated column. **P I** eluted as a single peak at the same volume as the high molecular mass population in **Fig. 1A** (**Fig. 1B**, profile I). The polysaccharide had an Mn of 77.8 kDa, which corresponds to a number-average degree of polymerization (DP_n) of 480. The polydispersity of 2.52 was significantly lower than that of whole spore extracts, which was 5.87.

We wondered whether the (1→4)-linked glucose residues are covalently linked to the (1→3)-linked main constituent, and if so, whether they are distributed randomly throughout the polysaccharide or whether they are located at specific regions. To pursue this, we selectively removed the (1→4)-linked residues by periodate oxidation followed by mild acid hydrolysis (Smith degradation), and analyzed the remaining (1→3)- α -glucan

by HPSEC. In the case of a random distribution, a rapid decrease in molecular mass together with an increased polydispersity may be expected. However, the Smith degraded polysaccharide eluted as a Gaussian-curved peak with an M_n of 55.9 kDa (DP_n 378), which is a decrease of approximately 28% compared to the native sample. Importantly, the polydispersity (2.50) remained unchanged (**Fig. 1C**, profile II; **Table I**). These results indicate that the (1→3)-linked main constituent is not interspersed with (1→4)-linkages, but that the (1→4)-linked residues either form a contaminating component or are located near or at one of the termini of the (1→3)-linked main chain.

P II comprises two types of amylose

Similar to fraction **P I**, we determined the chemical structure of fraction **P II**. Monosaccharide analysis together with the determination of the absolute configuration demonstrated D-glucose as the sole monosaccharide constituent of **P II**. Linkage analysis showed (1→4)-linked glucopyranose residues to be the main constituent in addition to small amounts (~1%) of (1→3)-linked residues and terminal Glc. Triple substituted residues indicating branching could not be detected, and therefore we assume that **P II** consists of a linear polysaccharide (**Table II**).

The 1D $^1\text{H-NMR}$ spectrum (**Fig. 2C**) showed a H-1 α signal at 5.146 ppm. A signal of low intensity was observed at 4.263 ppm that represents OH-6, because it is a slowly-exchanging triplet, does not form cross-peaks in the HSQC spectrum (**Fig. 5A**), and forms cross-peaks with the H-6a/b protons in the TOCSY spectrum (**Fig. 5B**). Proton and carbon chemical shifts were assigned using TOCSY and HSQC experiments (**Table IV**) The down-field shift of C-4 at 78.5 ppm indicates 4-substitution, which is consistent with the chemical analysis data. From these data we conclude that **P II** consists of a linear (1→4)- α -D-glucan. The absence of 6-substituted and 4,6-disubstituted residues indicates that the (1→4)- α -D-glucan represents amylose rather than glycogen.

Table IV. Proton and carbon chemical shifts of **P II** ((1→4)- α -Glc p)

Proton	PPM	Carbon	PPM
H-1	5.146	C-1	99.7
H-2	3.35	C-2	71.7
H-3	3.714	C-3	72.9
H-4	3.39	C-4	78.5
H-5	3.652	C-5	71.2
H-6a	3.701	C-6	60.2
H-6b	3.612		
OH-2	n.d.		
OH-4	n.d.		
OH-6	4.263		

n.d. = not detected

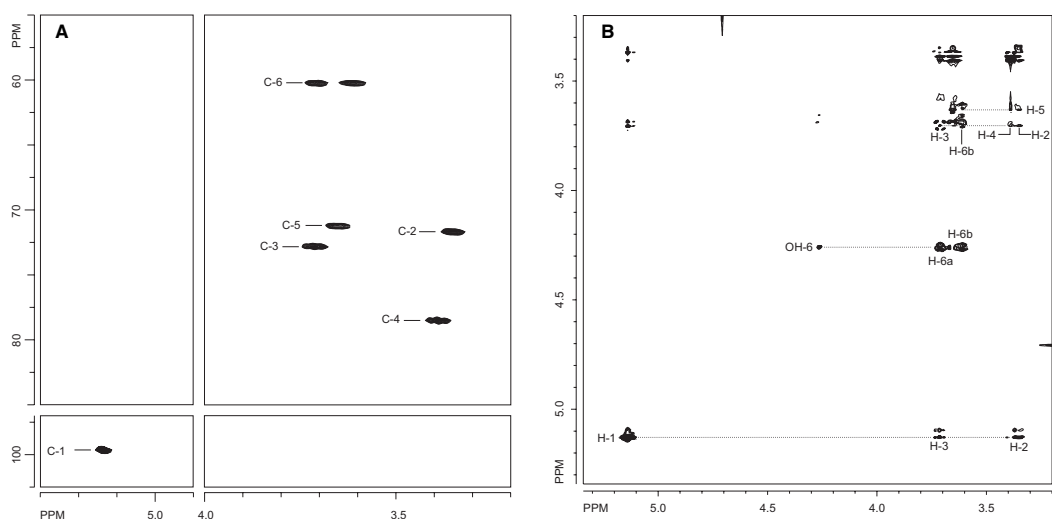


Fig. 5. Fraction **P II** consists of (1→4)-linked α -glucopyranose residues. ^1H - ^{13}C HSQC spectrum (A) and ^1H - ^1H TOCSY spectrum (50 ms mixing time) (B). C-1 means a cross-peak between H-1 and C-1 in the HSQC spectrum. Cross-peaks belonging to the same scalar coupling network are indicated along a dotted line starting from the corresponding diagonal peak in the TOCSY spectrum. Note that the down-field shifts of C-4 typify 4-substituted residues. Note also that OH-6 gives cross-peaks in the TOCSY spectrum, whereas these cross-peaks are absent in the HSCQ spectrum. Samples were dissolved in DMSO-d_6 and spectra were recorded at 500.08 MHz at 80 °C.

The HPSEC profile of **P II** showed two overlapping peaks with a combined M_n of 16.5 kDa, which corresponds to a DP of 102 (**Fig. 1C**, **Table I**). The profile was very similar to that of whole spore wall extracts, albeit with a lower polydispersity, namely 3.30 rather than 5.87. Since chemical analysis and NMR data demonstrated that (1→4)- α -D-glucan was virtually the sole component of **P II** (apart from traces of (1→3)-linked glucose residues), we conclude that both peaks represent amylose. Taken together, the results demonstrate that **P II** comprises two different amylose populations.

P III contains a second (1→3)- α -glucan fraction

After extraction of the DMSO-insoluble residue with cold sodium hydroxide solution, fraction **P III** was obtained by neutralization (14% w/w of total spore carbohydrates). This fraction, to our surprise, was readily soluble in DMSO, and could therefore be analyzed by using the same methods as for fractions **P I** and **P II**. Monosaccharide analysis gave glucose as only monosaccharide constituent. These glucose residues were mainly (1→3)-linked (91.6%) in addition to 8.4% (1→4)-linked residues (**Table II**). The 1D ^1H -NMR spectrum of **P III** was virtually indistinguishable from **P I** in that both spectra contained signals corresponding to (1→4)-linked and (1→3)-linked α -Glc p , and (1→3)-linked β -Glc p (**Fig. 2D**). HPSEC analysis showed that **P III** consisted of a single population of polymers with an M_n of 109.7 kDa (DP 677) and a polydispersity of 2.33 (**Fig. 1D**, profile I; **Table I**), demonstrating that the average degree of polymerization of **P III** was significantly higher

than that of **P I**, which was approximately 480. These results indicate that **P III** comprises a second population of α -glucans consisting mainly of (1→3)-linked α -glucose residues. Removal of the (1→4)-linked residues by Smith degradation did not significantly affect the molecular mass distribution of **P III** (**Fig. 1D**, profile II), demonstrating that in the case of a covalent linkage between the (1→3)- α -glucan backbone and the (1→4)-linked residues, the latter must be located at the reducing or non-reducing end.

Discussion

Spore α -glucan

In this study, we elucidated the chemical structures of the major types of water-insoluble α -glucan from isolated walls of *S. pombe* spores. We showed that the DMSO-extract of the wall contains (1→3)- α -D-glucan (**P I**) and (1→4)- α -D-glucan (**P II**). As amylose is known to accumulate in spore walls, it is very well possible that spore-wall preparations become contaminated with amylose-containing granula of cytoplasmic origin. We assume therefore that the presence of (1→4)- α -glucan (**P II**) in our preparations is due to cytoplasmic contamination. The DMSO-insoluble material, accounting for approximately 14% (w/w) of the Zymolyase-resistant spore wall fraction, contains a second (1→3)- α -glucan that could be obtained via alkaline extraction (**P III**). Structural characterization of the α -glucans revealed that both (1→3)- α -D-glucan fractions, **P I** and **P III**, are linear polysaccharides consisting mainly of (1→3)-linked α -D-Glcp residues in addition to approximately 10% (1→4)-linkages. The two fractions have similar chemical composition, but differ in degree of polymerization, namely approximately 480 for **P I** and 680 for **P III**. Despite their different solubility characteristics, we cannot exclude that **P I** and **P III** initially form one population and that the differences in solubility may be attributed to their molecular masses or crystallinity.

In addition to the (1→3)-linked Glcp, both fractions contain a small percentage of (1→4)-linked Glcp residues that may originate from contamination by **P II**, which is a linear (1→4)- α -glucan. Alternatively, in the case of a covalent linkage between the (1→3)- α -glucan and the (1→4)-linked residues, these residues must be located at or near the reducing or non-reducing end of the α -glucan because removing the (1→4) linkages by Smith degradation did not result in a strong decrease of the molecular mass of the α -glucan. This means that the chemical structures of spore-wall (1→3)- α -glucans are significantly different from (1→3)- α -glucan of vegetatively-grown cells, which broke in two halves after Smith degradation, indicating that it is composed of two similarly-sized (1→3)- α -glucan segments that were covalently-linked via a number of (1→4)-linked α -glucose residues (see Chapter 2).

Homologs of Ags1p may synthesize spore-wall (1→3)- α -glucan

The differences in the chemical structures of α -glucan from vegetatively-grown cells and

from spores suggest that the biosynthesis of (1→3)- α -glucan in vegetatively growing cells and in sporulating cells is different. We speculate that during sporulation an α -glucan synthase different from Ags1p may be involved in (1→3)- α -glucan synthesis. This is consistent with the findings of Mata *et al.* (2002) who showed by DNA-microarray analysis that *ags1⁺* is downregulated during meiosis, whereas the *ags1⁺* homologs *mok11⁺*, *mok12⁺*, *mok13⁺*, and *mok14⁺* are upregulated, suggesting that one (or more) of the *mok⁺* homologs may be essential for (1→3)- α -glucan biosynthesis during sporulation. The Mok proteins also have a similar multidomain structure as found in Ags1p. The only exception is Mok14p in which the extracellular domain is missing. Previously we proposed a mechanism of cell-wall α -glucan biosynthesis, in which the extracellular domain may function as a transglycosylase that couples two α -glucan building blocks (Chapter 2). Considering the chemical structure of spore-wall α -glucan, the question regarding the function of the extracellular domain of the Mokp homologs arose. We wondered whether the catalytic site in these homologs is conserved. Svensson (1994) showed that in fungal and plant α -amylases, three regions are involved in hydrolytic activity. In *Aspergillus oryzae* α -glucosidase (TAKA), the first region includes Glu₂₃₀, which is regarded as a general acid catalyst and constitutes one of the three invariant carboxylic acid residues at the catalytic site. The other regions include Asp₂₀₆, that acts as a nucleophile, and Asp₂₉₇. Sequence alignment of TAKA with Ags1p showed that the three regions were conserved. Importantly, these regions were also conserved in Mok11p, Mok12p, and Mok13p. Presently, we have no explanation for the different mechanisms of α -glucan biosynthesis in vegetatively growing cells and in sporulating cells, and whether the extracellular domain is catalytically active in the Mokp homologs remains to be investigated.

Materials and Methods

Isolation of spore walls and α -glucans

Diploid cells of strain FH045 (*h⁺/h⁻ ade6-210/ade6-216*) were grown at 28 °C in 1 L of a chemically defined medium, EMM, in the presence of a high concentration of nitrogen (10 g/L of ammonium chloride) to prevent sporulation (Moreno *et al.*, 1991). Then, the cells were washed and incubated at 28 °C in EMM without ammonium chloride to induce sporulation. After four days, the cell culture was centrifuged at 3300 × g. To digest (1→3)- β -glucan of contaminating ascus walls, the cell pellet was resuspended in 5 mM sodium azide, 40 mM 2-mercaptoethanol, 50 mM citrate-phosphate buffer, pH 5.3 containing 45 mg of Zymolyase-100T (Seikagaku). After an overnight-incubation in a rotary shaker at 200 rpm at 37 °C, spores were collected by centrifugation at 3300 × g for 15 min and resuspended in water. Then, the cell suspension was layered on top of 5 mM sodium azide, 8% sucrose, 90% Percoll and centrifuged at 10,000 × g. Spores were able to enter the sucrose-Percoll layer and pellet, whereas cell debris (including asci remnants) remained in the water fraction. The pellet fraction was resuspended and subjected to mechanical

breakage by addition of glass beads (0.45 mm diameter) using a Mikro-Dismembrator (B. Braun Biotech International). Subsequently, spore walls were extracted with SDS and treated with Zymolyase as described (Chapter 2).

To isolate α -glucans, Zymolyase-resistant spore walls were lyophilized and further dried *in vacuo* over phosphorus pentoxide, and were dissolved in DMSO to a concentration of approximately 4 mg/ml by heating at 80 °C. DMSO-soluble polysaccharides were obtained by centrifugation at 10,000 \times g for 10 min. This extraction procedure was repeated twice. α -Glucans were precipitated from the combined supernatants by addition of ethanol to a final concentration of 40% (fraction **P I**) or 60% (fraction **P II**) with intermediate collection of the fractions by centrifugation at 10,000 \times g for 10 min. Fraction **P I** was washed twice with ethanol and three times with water, whereas fraction **P II** was washed three times with ethanol. Fraction **P I** was further purified by repeating the dissolution-precipitation procedure. Fraction **P III** was obtained by suspending the dried DMSO-insoluble spore-wall fraction in 1 M NaOH for 10 min at RT. The supernatant was collected by centrifugation at 16,000 \times g for 10 min and fraction **P III** was obtained by addition of 1 M acetic acid to pH 6 at 4 °C. The precipitate was collected by centrifugation at 10,000 \times g for 10 min and the pellet was washed thoroughly with water.

Monosaccharide analysis and linkage analysis

For monosaccharide analysis, samples were subjected to methanolysis [1.0 M methanolic HCl, 24 h, 85 °C], followed by trimethylsilylation [5:1:1 pyridine/chlorotrimethylsilane/hexamethyldisilazane, 30 min, RT], and were analyzed by GLC and GLC-IE-MS (Kamerling and Vliegenthart, 1989). Absolute configurations were determined by butanolysis (Gerwig *et al.*, 1978).

For linkage analysis, per-*O*-methylation was performed using the method of Hakomori (1964). Then, per-*O*-methylated polysaccharides were hydrolyzed in aqueous 90% (v/v) formic acid [1 h, 100 °C], followed by evaporation and treatment with 2 M trifluoroacetic acid [1 h, 120 °C]. Samples were reduced with excess NaBD₄ in 0.5 M NH₄OH for 90 min at RT, followed by acetylation with acetic acid anhydride [3 h, 120 °C].

GLC analyses were performed on a WCOT CP-SIL 5CB fused-silica capillary column (25 m \times 0.32 mm) (Chrompack) using a CP 9002 gas chromatograph (Chrompack) and a temperature program of 140-240 °C at 4 °C/min. GLC-EI-MS of partially methylated alditol acetates was carried out on an MD800/8060 system (Fisons Instruments, Manchester, UK) equipped with a WCOT CP-SIL 5CB fused-silica capillary column (25 m \times 0.25 mm) (Chrompack), using the same temperature program.

Size-exclusion chromatography

The HPSEC system consisted of a Delta 600 pump (Waters) with a DRI 2410 refractive index detector (Waters). For the mobile phase, sodium nitrate was added to DMSO to a final concentration of 3 mM to reduce aggregation of polymers and to eliminate ionic

strength effects (Chuang, 1990). It was delivered at a flow-rate of 1.0 ml per min. A single PLgel 5 μm MIXED-C column (300 \times 7.5 mm) (Polymer Laboratories) was connected in series with a PLgel 10 μm guard column (50 \times 7.5 mm). Both columns were thermostated at 80 $^{\circ}\text{C}$. The system was calibrated using pullulan narrow standards (Standard P-82, Shodex) plus maltohexaose and glucose. Samples were lyophilized and then further dried overnight *in vacuo* over phosphorus pentoxide, dissolved in the mobile phase to a concentration of 2 mg/ml, and filtered through 0.45 μm PTFE filters. Injection volumes of 100 μl were used. Data were processed using Millenium³² software (Waters). Number-average molecular mass (M_n) and weight-average molecular mass (M_w) were determined by dividing each HPSEC profile into a number of virtual time slices, n_i , each corresponding to a certain molecular mass M_i that is obtained by calibrating the column. From these values, M_n and M_w can be calculated according to:

$$M_n = \frac{\sum_i n_i M_i}{\sum_i n_i} \quad \text{and} \quad M_w = \frac{\sum_i n_i M_i^2}{\sum_i n_i M_i} .$$

The polydispersity is given by the ratio of the weight to the number-average molecular mass (M_w/M_n) and is a measure of the breadth of the molecular mass distribution.

Nuclear magnetic resonance

All NMR spectra were recorded on a DRX500 spectrometer (Bruker). Samples were dissolved in 600 μl of 99.6% DMSO-*d*₆ and were analyzed at 80 $^{\circ}\text{C}$. Residual water signals were suppressed by applying a WEFT pulse sequence in 1D ^1H NMR experiments (Hård *et al.*, 1992). 10 ms and 50 ms 2D ^1H - ^1H total correlation spectroscopy (TOCSY) was carried out in the phase-sensitive mode using the States-TPPI method and the MLEV-17 sequence for mixing. The sensitivity enhanced 2D ^1H - ^{13}C heteronuclear single quantum coherence (HSQC) experiment used Echo/Antiecho gradient selection with decoupling. Chemical shifts are expressed in ppm relative to internal DMSO (^1H , 2.505 ppm; ^{13}C , 39.6 ppm). Data were processed using in-house developed software.

Smith degradation

For Smith degradation (Smith & Montgomery, 1956), polysaccharide samples were suspended in 15 mM sodium periodate at a concentration of approximately 2 mg per ml. The mixture was placed in the dark at 4 $^{\circ}\text{C}$ under continuous mixing. After 48 h, the reaction was stopped by adding ethylene glycol to a final concentration of 350 mM. Oxidized polysaccharides were reduced with an excess of NaBH_4 during 24 h. Then, excess borohydride was removed by the addition of acetic acid. The product was washed three times with water and hydrolyzed in 100 mM hydrochloric acid at RT during 8 h.

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