FISSION YEAST CELL WALL ANALYSIS

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Abbreviations: B-BG, branched β(1,3)-glucan; CW, Calcofluor white; GS, glucan synthase; IEM, Immunoelectron microscopy; L-BG, linear β(1,3)-glucan; TEM, Transmission electron microscopy.

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Abstract

*Schizosaccharomyces pombe* cell wall is a rigid exoskeletal structure mainly composed of interlinked glucose polysaccharides and galactomannoproteins. It is essential for survival of the fission yeast, prevents cells from bursting from the internal turgor pressure and protects them from mechanical injuries. Additionally, the cell wall determines the cell shape and, therefore, a better knowledge of the cell wall structure and composition could provide valuable data in *S. pombe* morphogenetic studies. Here we provide information about this structure and the current reliable methods for rapid analysis of the cell wall polymers by specific enzymatic and chemical degradations of purified cell walls.

I. Introduction

The fission yeast cell wall represents around 20% of the cell dry weight. Although rigid, this structure has some elasticity and is remodeled in a strictly regulated manner since it must adapt to the environment and permit growth and the morphological changes that occur during the life cycle. When observed by transmission electron microscopy, the *S. pombe* wall shows a three-layer organization, with two electron-dense layers in the outer and inner sides, which are formed mainly by galactomannoproteins representing 9–14% of total wall (Fig. 1A-D) (Horisberger et al. 1978). The electron-transparent core layer is formed by α-glucan (28-32%) and three types of β-glucans: linear and branched β(1,3)-linked glucans (50-54%) and β(1,6)-linked glucans, (5-10 %) (Fig. 1F) (Manners and Meyer 1977; Humbel et al. 2001; Magnelli et al. 2002; Bush et al. 1974; Kopecka et al. 1995). These components form crystalline fibers that provide rigidity to the cell wall (Sugawara et al. 2003). Budding yeast wall lacks linear β(1,3)-glucan and α(1,3)-glucan polysaccharides and consists of branched β(1,3) and β(1,6)-glucan, chitin, and mannoproteins with no galactose residues (Lesage and Bussey 2006) (Orlean 2012).

II. Molecular structure of fission yeast cell wall

2.1. β-glucans

The β-glucans are polysaccharides composed of D-glucose monomers linked by β(1,3) or β(1,6) bonds. Traditionally, these β-glucans have been classified by the type of
bonding of the main chain in two types: β(1,3)-glucan with branches attached via β(1,6) bonds (14% of the total glucose) and β(1,6) glucan with branches linked via β(1,3) bonds (75% of the total glucose) (Bush et al. 1974; Manners and Meyer 1977). The β(1,3)-glucan is formed by two types of glucans, linear β(1,3)-glucan (L-BG) and branched β(1,3)-glucan (B-BG) (Manners and Meyer 1977; Humbel et al. 2001).

a) **L-BG** consists of β-(1,3)-linked glucose units forming linear chains arranged in a conformation of single-helix with a small proportion of triple-helix structures (Saito et al. 1990; Young and Jacobs 1998; Young et al. 2000; Cortés et al. 2007). It is the polysaccharide responsible for the primary septum structure observed by electron microscopy (Fig. 1D, E and G). This structure is similar to those found in plants, which is also formed by an L-BG named callose (Verma and Hong 2001; Chen and Kim 2009), or in *Saccharomyces cerevisiae*, which is formed by chitin. The single-helix conformation of L-BG makes the primary septum more susceptible to degradation by β(1,3)-glucanases (Pelosi et al. 2003). L-BG is recognized with higher affinity than the rest of *S. pombe* wall polysaccharides by the fluorochrome Calcofluor White (CW), and is responsible for the strong labeling of the septum with this compound (Cortés et al. 2007).

b) **B-BG** is the most abundant cell wall polymer. It is made of β(1,3)-linked glucose units forming linear chains with branching β-(1,6)-bound glucose units forming filamentous structures. Unlike L-BG, B-BG chains form closed triple-helix structures (Kopecka and Kreger 1986; Saito et al. 1990; Gawronski et al. 1999). Studies on protoplast cell wall regeneration have shown that β-(1,3)-glucan polysaccharide is first deposited on the plasma membrane, generating a network of microfibrils that extend to cover the whole surface; this network constitutes the frame on which the other polymers are deposited. As the protoplast grows, β-(1,3)-glucan allows the restoration of the initial cylindrical form of fission yeast (Osumi 1998; Humbel et al. 2001). B-BG is essential for cell integrity and for secondary septum formation (Fig. 1D and G). In addition, B-BG plays a crucial role in cytokinesis, linking the cell wall to the plasma membrane and contractile actomyosin ring, which is needed to couple the septum synthesis with the membrane and ring progression.
c) **β(1,6)-glucan** is a highly branched polysaccharide formed by a main chain of β(1,6)-linked glucose and 75% of β(1,3)-linked branches. This percentage of branches is much higher than that of the β(1,6)-glucan of other yeasts like *S. cerevisiae* or *Candida albicans*. Because of the abundance of both types of glucose links it is also called diglucan and represents 5-10% of the wall (Sugawara et al. 2004; Magnelli et al. 2005; Lesage and Bussey 2006).

2. 2. **α-glucan**

This polysaccharide is absent in the budding yeast and in *C. albicans*, but is present in the wall of filamentous and dimorphic fungi (Bush et al. 1974; Edwards et al. 2011). *S. pombe* α-glucan is a linear polysaccharide of approximately 260 glucose residues, formed by a tandem of two linear chains, each consisting of about 120 residues of D-glucose bound by α-(1,3) linkages and 10 α-(1,4) linked residues (7%) located at the reducing end of each chain (Grun et al. 2005). It has an essential role in the maintenance of cell shape, as treatment with α-glucanases in addition to β-glucanases is required to produce round protoplasts (Alfa et al. 1993). Like B-BG, this polysaccharide is essential for cell integrity and for secondary septum formation (Fig. 1D and G)(Cortes et al. 2012; Muñoz et al. 2013). It also plays an essential role in the primary septum adhesion strength needed to support the physical force of the internal turgor pressure during cell separation (Cortes et al. 2012).

2. 3. **Galactomannan**

This polysaccharide is part of the cell wall glycoproteins covalently bound via N- and O-glycosidic linkages. It consists of a linear backbone of α-(1,6) linked D-mannose units with branches formed by α(1,2)- or α(1,3)-linked D-mannose and a terminal D-galactose residue in the non-reducing ends. A small amount of galactose not located at the terminal position has also been found (Bush et al. 1974; Horisberger et al. 1978). Most proteins found in the cell wall are water- or detergent-soluble and can be secreted to the medium. Few cell wall proteins are covalently linked to the polysaccharides and can be divided into two groups, proteins covalently attached to β(1,3)-glucan (Pir proteins) through a glutamine residue, an alkali-labile linkage that can be extracted by a mild alkali treatment; and proteins covalently attached by a GPI anchor to the β(1,6)-glucan that can be removed by glucaenanase treatment (de Groot et al. 2005; Ecker et al.
S. pombe genome contains 33 hypothetical GPI proteins (de Groot et al. 2003). Only two PIR-type proteins, with alkali-sensitive bonds to the wall, have been described in S. pombe: Psu1, similar to proteins belonging to S. cerevisiae SUN family; and Asl1, which is related to proteins from Aspergillus fumigatus and Ustilago maydis (de Groot et al. 2007).

2.4. Cell wall organization

Analysis by scanning electron microscopy of wall regeneration in protoplasts showed that the cell wall is initiated by creating a network of fibers, which in turn, are grouped into bundles interconnected and surrounded by amorphous particles. β-(1,3) glucan is the primary component of the fiber structure (Osumi et al. 1998). However, α-(1,3)-glucan is also required to form the rigid structure, since mutants defective in the synthesis of α-(1,3)-glucan, while maintaining the fibrillar structure, are unable to develop shape (Horisberger and Rouvet-Vauthey 1985; Osumi 1998). Immunelectron microscopy using colloidal gold-labeled lectins or antibodies specific against galactomannan or different β-glucans respectively, have helped to define the organization of the different polysaccharides in the S. pombe cell wall and septum (Fig. 1C-G) (Horisberger and Rouvet-Vauthey 1985; Osumi 1998; Humbel et al. 2001). Lectins specifically localize the galactomannan to the outer and inner sides of the cell wall (Fig. 1F). L-BG presents an almost exclusive localization in primary septum (Humbel et al. 2001), although a small amount is also observed in the cell wall (Cortés et al. 2007). B-BG is located in both primary and secondary septum, as well as in the whole cell wall. Branched β-(1,6)-glucan forms a layer in both the secondary septum and cell wall, closer to the outer surface of galactomannoproteins (Fig. 1F) (Humbel et al. 2001). This would agree with the proposed function for β-(1,6)-glucan connecting the exterior surface proteins with the remaining cell wall polysaccharides (Sugawara et al. 2004). Although it has not been detected yet by immuneelectron microscopy, it is believed that α-(1,3)-glucan is localized with the B-BG in the less electron-dense region of the cell wall and in both primary and secondary septum (Fig. 1F-G) (Cortes et al. 2012).

2.5. Cell wall biosynthetic and remodeling enzymes
The biosynthesis of β-(1,3)-glucan is catalyzed by an enzyme complex well conserved in fungi and plants called β-(1,3)-glucan synthase (GS) (EC 2.4.1.34, UDP-glucose:1,3-β-D-glucan 3-β-D-glucosyltransferase) which is associated to the inner side of the plasma membrane (Shematek and Cabib 1980). This complex uses UDP-glucose as substrate, and in vitro synthesizes de novo linear chains of 60-80 glucoses, which is a considerably smaller length compared to that of the wall glucan chains. In all fungi and plants the GS complex is composed of at least two subunits: an integral membrane protein corresponding to the catalytic subunit, and Rho1 GTPase, which acts as the regulatory subunit (Arellano et al. 1996). The first GS catalytic subunit identified in S. pombe was Cps1, later renamed Bgs1, which is very similar to the catalytic subunits of S. cerevisiae (Ishiguro et al. 1997). bgs1+ was first cloned by complementation of the cps1-12 mutant hypersensitive to a spindle poison (Ishiguro et al. 1997). Later, it was also isolated by complementation of mutants defective in septum formation, and was involved in the Wee1-dependent septation checkpoint (Le Goff et al. 1999; Liu et al. 2000b; Liu et al. 1999). Bgs1 is responsible for the synthesis of the L-BG that forms the primary septum (Fig. 1E) (Cortés et al. 2007). Three more genes bgs2+, bgs3+ and bgs4+ have been found in the S. pombe genome. bgs2+ expression is induced during sporulation. Bgs2 localizes to the ascospore periphery and is required for ascospore wall maturation and survival (Liu et al. 2000a; Martín et al. 2000). Bgs3 and Bgs4, are like Bgs1, essential in vegetative cells and localized to the growing poles and septum (Cortés et al. 2002; Liu et al. 2002; Martín et al. 2003; Cortés et al. 2005). The role of Bgs3 is currently unknown. Genetics studies have shown that bgs4+, cwg1+, orb11+, and pbr1+ are the same gen. Bgs4 is essential and its depletion promotes cell lysis mainly at the cell separation onset. Bgs4 is responsible for B-BG synthesis, the major GS activity and the in vivo and in vitro resistances to specific GS antifungal drugs (Ribas et al. 1991; Castro et al. 1995; Cortés et al. 2005; Martins et al. 2011).

Ags1, also named Mok1, is a putative α-glucan synthase whose activity has not been detected in vitro. Ags1 is essential for cell integrity (Hoschstenbach et al. 1998; Katayama et al. 1999). Ags1 is detected in dividing and growing areas and confers the essential septum strength needed for proper cell abscission (Katayama et al. 1999; Cortes et al. 2012). S. pombe contains four
additional *ags1* paralogues (*mok1*–*mok14*), which are induced during sexual differentiation (García et al. 2006).

Although the biosynthetic enzymes of the main polymers have been described, a comprehensive understanding of the interactions between *S. pombe* cell wall components is still missing. The L-BG, B-BG, or α(1,3)-glucan synthesized remain disorganized until they are cross-linked to other polysaccharides and proteins. Four of the fission yeast GPI proteins (Gas1, Gas2, Gas4, and Gas5) are β(1,3)-glucanosyl-transferases related to *S. cerevisiae* Gas1, which is involved in the maturation and branching of β(1,3)-glucan, and therefore is essential for the cell wall assembly and proper maintenance of the cell. *S. pombe* Gas1 is essential during vegetative growth and Gas4 during sporulation. Gas2 and Gas5 seem to play a minor role in cell wall construction (de Medina-Redondo et al. 2008; de Medina-Redondo et al. 2010).

### III. Choosing an appropriate protocol for cell wall analysis

Different methods can be used to detect the cell wall structure or composition. Some of them do not need precise polymer quantification. Among them, those more commonly used are briefly described below.

#### 3.1. Non-quantitative methods for analysis of cell wall polysaccharides

#### 3.1.1. Electron microscopy

These techniques are numerous including A) *Transmission electron microscopy (TEM)* that allows observation of the three-layered cell wall structure with different electron densities (Figure 1A-D) (Osumi et al. 1998). B) *Scanning electron microscopic (SEM)*, useful to observe the cell surface with high fidelity (Osumi et al. 1995). This technique can be coupled with lectins to detect a cell wall surface completely filled with particles specific for the mannan carbohydrate of glycoproteins (Osumi et al. 1995). C) *Atomic force microscopy (AMF)*, used to measure the mechanical properties of the cell wall macromolecules (Dufrene 2010). D) *Cryoscanning and cryosectioning electron microscopy*, useful to analyze fractured and coated cell samples, allowing the observation of the ultrastructure of both external and internal cell
components (Osumi et al. 2006). E) Immunoelectron microscopy (IEM) which uses specific lectins or antibodies in combination with secondary antibodies conjugated to colloidal gold particles to locate the different types of polysaccharides in the septum and cell wall structures (Figure 1E) (Humbel et al. 2001).

3.1.2. Microscopy with specific fluorochrome

Direct observation of living cells is an important method for cell wall studies. Fluorochromes and fluorescence-conjugated lectins can be used for cell wall fluorescence microscopy analysis. The most commonly used are: Calcofluor white (CW), also called Blankophor (Blankophor GmbH & Co.) or Fluorescent Brightener 28 (Sigma-Aldrich), which specifically stains the cell wall L-BG; Concanavalin A, a lectin that binds mannose residues; lectin from Bandeiraea simplicifolia, which recognizes specifically terminal galactose residues; and wheat germ agglutinin (WGA), which recognizes N-acetylglucosamine residues from glycoproteins (since there is no chitin in S. pombe).

3.1.3. Cell Sensitivity to enzymatic degradations or to cell wall synthesis inhibitors

This method can be used when alteration of the cell wall is suspected. It provides a rough estimate of the cell wall state (Calonge et al. 2000; Martins et al. 2011). It can also be used in screenings for mutations affecting the cell wall. Enzymatic complexes degrading β-glucans (Zymolyase, Kitalase) or the entire cell wall (Glucanex) are used. Cell wall synthesis inhibitors such as Calcofluor white, 2-deoxy-D-glucose, echinocandins (caspofungin, micafungin, anidulafungin, pneumocandin or aculeacin), papulacandins or acidic terpenoids (enfumafungin) are also used (Martins et al. 2011).

3.1.4. Analysis of cell wall proteins

The SDS-soluble cell wall proteins are extracted by hot 2% SDS–40 mM mercaptoethanol treatment (15 min 100°C) and concentrated by cold acetone precipitation. The proteins covalently attached to β(1,3)-glucan can be purified by treatment with 30 mM NaOH at 4°C for 16 h. The GPI attached proteins are released by degradation of the GPI anchor by treatment with undiluted pyridine hydrofluoride at 24°C for 16 h. Both groups of covalently attached proteins can be released by treatment with recombinant endo-β(1,3)-glucanase. Then, the proteins are
analyzed by SDS-PAGE or Western blot. As wall glycoproteins are heavily glycosylated and this may interfere with the electrophoresis analysis, the N-linked oligosaccharides can be removed by treatment with endoglycosidase H. Additionally, the cell wall proteins can be biotin-labeled directly in the cell prior to cell wall purification, and visualized in the Western blot with streptavidin-horseradish peroxidase conjugate (Mrsa et al. 1997; Mrsa and Tanner 1999).

Galactomannan can be specifically analyzed by incorporation of radioactive orthophosphate into N-mannosylated glycoproteins. The phosphate is bound as mannose-6-phosphate to both SDS-soluble and covalently linked cell wall mannanproteins. The phosphorylated cell wall oligosaccharides can be characterized after hydrolysis in trifluoroacetic acid by Quaternary aminoethyl (QAE)-Sephadex A50 chromatography, Bio-Gel P2 chromatography, high-performance anionic-exchange chromatography (HPAEC), electrospray ionization tandem mass spectrometry (ESI-MS-MS), and methylation analysis with gas chromatography-mass spectrometry (Mrsa et al. 1999).

3.2. Radioactive labeling and quantitative analysis of cell wall polysaccharides

Different methods can be used to analyze the cell wall polysaccharide composition. All of them require a separation of the wall from the rest of cell components. Some methods consist of combinations of chemical degradations and analytical techniques that permit the determination of the degradation products. These methods usually require more effort but provide information about the type of bonds between the monosaccharides forming the polymers. Common chemical degradations include alkali solubilization, acid hydrolysis, periodate oxidation, Smith degradation, borohydride reduction, β-elimination, carboxymethylation, and permethylation. Common analytical techniques include determination of reducing sugars, of total sugars, of glucose, of glucosamine, methylation analysis, gas-liquid chromatography, mass spectrometry, paper chromatography, gel filtration (size-exclusion) chromatography, thin-layer chromatography (TLC), affinity chromatography, high-performance ion-exchange chromatography (HPLC and HPAEC), nuclear magnetic resonance (NMR) spectroscopy, X-ray diffraction, and pulse amperometry (Manners et al. 1973a; Manners et al. 1973b; Bush et al.
The current method has been adapted for a simple, accurate and rapid analysis of wall polysaccharides based on $^{14}$C-glucose labeling and fractionation of cell wall polysaccharides using specific chemical and enzymatic procedures.

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FIGURE LEGEND

Figure 1. (A,B,C,D) TEM images of the fission yeast. Whole cells (A and B); details of the three-layered cell wall (C), and the septum (D) are shown. Images originally published in J. Cell Biol. 198: 637-656 (Cortes et al., 2012). (E) IEM showing L-BG distribution in the primary septum. Image originally published in Mol Microbiol 65: 201-217 (Cortes et al., 2007). F) Scheme of the cell wall organization. G) Scheme of the septum. PS, primary septum; SS, secondary septum.