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Chapter 11

ALTERING THE FUNGAL CELL WALL INTEGRITY AND PRACTICAL ASPECTS OF THESE MODIFICATIONS

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ABSTRACT

In this chapter we compare the cell wall structure of filamentous fungi and the yeast *Saccharomyces cerevisiae*. The genetic engineering of the cell wall of *Trichoderma*, *Aspergillus*, *Neurospora*, *S. cerevisiae*, *Schizosaccharomyces pombe*, *Hansenula polymorpha*, *Kluyveromyces* and others is discussed and the consequences of direct changes in the synthesis of cell wall components, especially those affecting protein production and secretion by the mutants and their sensitivity to antifungal agents are highlighted. Furthermore, we discuss the close correlation between the cell wall structure and the activity of enzymes engaged in protein glycosylation and in the biosynthesis of glycosylphosphatidy linositol anchors.

We also show problems with stability of the generated changes in the cell wall structure, which are detected and often overcome by cell wall compensatory mechanisms.

INTRODUCTION

Filamentous fungi are an object of interest for biotechnology, since they are able to produce and secrete up to 40 g of protein per liter of culture [1]. A significant amount of reports suggesting that cell wall plays a role as a barrier for protein secretion has been reported (see details below). Thus, it is beneficial for protein secretion a cell wall as permeable as possible. The cell wall engineering should aim for higher cell wall permeability, bearing in mind, that the cell wall defense mechanisms could wreck the results of the

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introduced changes. In addition considering that protein secretion occurs at the mycelium tip, a hypersecretory effect could be obtained by hyperbranching of mycelia coupled with disturbed cell wall synthesis. Affecting the cell wall structure can be achieved by modulating the activity of enzymes in charge of cell wall elaboration. In this chapter we summarize the attempts to perform such strategies to improve proteins secretion, including changes of some other enzymes apparently not connected with the cell wall formation, but successfully altering its structure.

CELL WALL OF FILAMENTOUS FUNGI DIFFERS FROM THE CELL WALL OF S. CEREVISIAE

The *Saccharomyces cerevisiae* cell wall is the most examined structure within the fungi kingdom and is often seen as a representative model for fungal cells, when in fact it differs from the cell walls of filamentous fungi where different carbohydrate residues and polysaccharide structures are plentiful. The major polysaccharides found in the *S. cerevisiae* cell wall are moderately branched β1,3-glucan and highly branched β1,6-glucan, representing 30-45% and 5-10% of the wall mass, respectively [2]. The β1,6-glucan is attached to the non-reducing ends of the β1,3-glucan network. Next, chitin chains, constituting from 1.5 to 6% of the wall mass, may be attached to non-terminal residues of the β1,3-glucan network, although chitin can also be linked glycosidically to the β1,6-glucan in stress conditions [2].

Total hydrolysis of the *S. cerevisiae* cell wall gives glucose and *N*-acetylglucosamine, which come from polysaccharides, and mannose from mannoproteins that represent 30-50% of the yeast cell wall [2]. The cell wall of *Aspergillus niger*, one of the most biotechnologically relevant fungi, contains not only derivatives of glucose, mannose and *N*-acetyl-glucosamine (normally found in the *S. cerevisiae* cell wall), but also D-galactose, D-arabinose, D-galactosamine, and small amounts of L-galactose [3]. The cell wall of the plant pathogen *Fusarium oxysporum*, apart from glucose and *N*-acetyl-glucosamine, also contains mannose, galactose, and uronic acid, which presumably originate from cell wall glycoproteins [4]. Moreover, its cell wall proteins contain higher amounts of uronic acid than the glycol-proteins found in other *Fusarium* species. The *Trichoderma reesei* cell wall carbohydrates are composed of glucose, *N*-acetyl-glucosamine, *N*-acetylgalactosamine, galactose, and mannose [5]. The main polysaccharides found in the cell wall of *S. cerevisiae* and filamentous fungi are chitin and β1,3- and β1,6-glucans [2, 6]. In the *Fusarium* cell wall, β1,3-glucan occasional branching due to single β1,6-linked glucose residues are present [7]. In addition, chitin is partially deacetylated giving chitosan, a polysaccharide composed of β1,4- linked D-glucosamine and N-acetyl-D-glucosamine residues in variable proportions [8]. The acetylation degree of the chitinous component of the cell wall was estimated to be 25-35% [8]. Chitosan is also present in the cell wall of many other fungi, between them *Aspergillus niger*, *Botrytis cinerea*, and *Penicillium hirsutum* [9]. Furthermore, X-ray diffraction analysis has demonstrated distinct fractions of α1,3-glucan in the cell wall of *Fusarium* [4], which are not found in *S. cerevisiae* and *C. albicans*. In some virulent forms of pathogenic dimorphic fungi (*Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*) α1,3-glucan represents up to 35–46% of the total cell wall carbohydrate [10].

The α1,3-glucan has been suggested to replace chitin in the structure of the cell wall when chitin synthesis is compromised in *A. fumigatus* [11]. In some human fungal pathogens

the amount of cell wall α1,3-glucan correlated with their virulence [12, 13]. On the other hand, a 50% reduction in the content of this wall polymer as a result of disruption of two α1,3-glucan synthase-encoding genes, *AGS1* and *AGS2*, did not significantly affect the virulence of *A. fumigatus* [14]. A highly intricate polysaccharide has been found in the cell wall of *T. reesei*. It is composed of an α1,6-mannose chain with monomeric α1,3-mannose branches and longer branches (6-10 residues) containing galactose linked to the mannose core by α1,2- bonds and subsequently elongated with a galactose or a pair of galactose and glucose residues variably bound to each other [15, 16]. A linear β1,3-/1,4-glucan and *N*-acetyl-galactosamine polymer were described within the cell wall of *A. fumigatus*, without detection of β1,6-glucan [16]. Thus far, those polymers have not been described in other fungi [17]. In addition, the *A. fumigatus* cell wall contains a characteristic galactomannan that can be used for diagnosis of human aspergillosis [18].

Determination of the cell wall components and their proportion is additionally complicated by the fact that β1,3-glucan, β1,6-glucan, chitin and mannoproteins are tightly interconnected , e.g., in *S. cerevisiae* β1,6-glucan has some β1,3-linked branches attached to chitin via β1,4- or β1,2-linkages [19]. The fungal cell wall is composed of an internal layer of polysaccharides and an external one of glycoproteins. The *S. cerevisiae* cell wall proteins have been isolated using enzymatic [19] or chemical methods [20, 21] and 19 covalently bound cell wall proteins have been identified using tandem mass spectrometry [22]. The same laboratory identified proteins from the cell wall of *Schizosaccharomyces pombe* and found some of them to be the orthologues to the ones found in *S. cerevisiae*; however, Asl1p had no ortholog in *S. cerevisiae* but was related to *A. fumigatus* and *Ustilago maydis* proteins [23]. Many of these glycoproteins are glycosylphosphatidylinositol (GPI)-modified at the C-terminus and are covalently bound via β1,6-glucan to the β1,3-glucan layer; but in *A. fumigatus* lacking β1,6-glucan, β1,3/1,4-glucan or α1,3-glucan have been proposed as alternative acceptor molecules for GPI-anchored cell wall proteins [17, 24]. In contrast, in *A. niger* and *F. oxysporum* where β1,6-glucan is present, some cell wall proteins covalently linked to this polymer have been identified [2, 4, 25]. There is also another group of proteins in the fungal cell wall which are bound to the β1,3-glucan through a mild-alkali-sensitive ester linkage [20, 26]. It is believed that the protein layer is responsible for the cell wall selective permeability and could play a protective role against cell wall-degrading enzymes [26]. Glucanase-extractable mannoproteins are known to affect cell wall porosity [27, 28]. This brief glance at the fungal cell wall composition and structure uncovers a very complicated puzzle; each element plays a role on its own as well as in concert with the others in the functioning of this structure.

MODIFICATIONS OF THE FUNGAL CELL WALL AND PRACTICAL ASPECTS OF THESE CHANGES

Filamentous fungi are widely exploited in biotechnology for protein production owing to their high secretory ability. On the other hand, their cell wall is still a barrier for maximal protein secretion [5, 29, 30]. Therefore, understanding the consequences of changes engineered in the cell wall structure should facilitate creation of novel strains with a more permeable cell wall. In addition, alterations in the cell wall components occasionally lead to

changes in morphology such us hyperbranched mycelia [31, 32], which, taking into consideration that protein secretion occurs at the tip of the mycelium, could give hyper-secretory strains.

The most straightforward manipulation to alter the fungal cell wall is disruption of genes coding for enzymes responsible for the synthesis of individual cell wall components. Disruption of the chitin synthase gene *chsB* in *A. oryzae* altered its cell wall, as manifested by an increased sensitivity of the strain to Calcofluor White. Additionally, the strain exhibited morphological changes such as hyperbranching of mycelia [31, 32]. The hyperbranching decreased clumping, which improved oxygen transfer to the cells and enhanced flow of metabolites and substrates [31]. Since filamentous fungi are used in fermentation industry, formation of clumps makes aeration of the culture more difficult than for a single-cell culture with the same biomass concentration. Excessive branching was also observed in *A. fumigatus* carrying disruption of *ags1* and *ags2* genes that encode α1,3-glucan synthases [14]. On the other hand, disruption of the *csmA* gene coding for myosin-tailed chitin synthase resulted in different changes than the *chsB* disruption [32]. Cells lacking CsmA had ballooning hyphae, lower tip extension rate and lower branching. Six classes of chitin synthases have been identified in filamentous fungi, and loss of any individual chitin synthase results in different cell wall morphologies [11]. Disruption of *chsD* and *chsE* in *A. nidulans* revealed different functions of chitin synthesized by these two chitin synthases [33]. A *chsE* null mutant did not show morphological or developmental defects while the hyphae and conidia of a *chsD* disruptant swelled extensively, indicating that the chitin synthesized by the *chsD*-encoded enzyme is necessary for cell wall rigidity. Finally, single and combining disruption of the genes *chsB*, *chsC* and *chsD* gave similar effects, suggesting overlapping functions of the encoded enzymes [34]. On the other hand, the phenotypes of individual chitin synthase mutants are probably suppressed by compensatory overexpression of other *chs* genes [11]. There are also some mutations resulting in hyperbranching of mycelia, although not directly connected with the synthesis of cell wall components.

A stimulatory effect of hyperbranching on protein secretion was shown in a *Neurospora crassa mcb* mutant [35], whose protein secretion was increased three- to fivefold [36]. In filamentous fungi, hypha growth results from continuous deposition of new cell wall material at the hyphal apex. It was shown that *mcb* gene encodes the regulatory subunit of cAMP-dependent protein kinase (PKA), and it was suggested that the PKA pathway regulates the polarized deposition of cell wall material for hyphal growth [35]. Furthermore, hyperbranching of mycelia and higher secretion of amylase and alkaline (*alpP*) and neutral (*nptB*) proteases were observed in *A. oryzae* carrying disruption of the *pclA* gene encoding a secretion pathway specific (KEX2-like) endo-protease [37]. Those authors pointed at the fact that chitin synthase B, encoded by the *chsB* gene, contains processing sites for PclA and the abnormal branching phenotype could result from unprocessed dysfunctional chitin synthase B, as reported previously for an *A. oryzae chsB* disruptant [31]. On the other hand, hyperbranching not always promotes protein secretion: the hyperbranched *A. oryzae chsB* mutant secreted the same amount of α-amylase as the wild type strain [31].

Moreover, alteration of the fungal cell wall structure often results in stimulation of cell defence mechanisms and the expected effect could be compounded or simply abolished [38]. To induce this mechanism, sensor proteins present in the cell wall detect and transmit the cell wall status to a signaling pathway comprised of a cascade of MAP kinases [38]. The response to the cell wall dysfunction is conserved in yeast and filamentous fungi, and includes incre-

ments in chitin deposition at the lateral walls and activation of β1,3-glucan synthase [39, 40]. Synthesis of β1,3-glucan is catalysed by a plasma membrane-bound complex of glucan synthase and modified by some glucanases or transglycosidases in the periplasmic space [41]. The β1,3-glucanosyltransferase splits internally a β1,3-glucan molecule and transfers the newly generated reducing end to the non-reducing end of another β1,3-glucan molecule, resulting in the elongation of β1,3-glucan chains [41]. In *A. fumigatus*, β1,3-glucanosyltranferases encoded by *GEL1* and *GEL2* genes were disrupted and the phenotype of the disruptants was studied [42]. Their cell wall contained an increased amount of chitin, a typical compensatory effect known from *S. cerevisiae* cell wall mutants. These compensatory changes in the cell wall make difficult to link the observed phenotypes with disruption of the *GEL* genes. The only modification that correlated with the *GEL2* mutation was a decrease of β1,3-glucan content, associated with an increased amount of β1,3- and β1,6- glucose linkages [42]. Interestingly, the Δ*gel2* mutant secreted more glycoproteins, somewhat similar to the Δ*gas1* mutant. The latter effect is due to the fact that Gas1p plays an important role in the incorporation of β1,3-glucan in the cell wall [43]. Such a finding suggests that also the cell wall of the Δ*gel2* mutant is more permeable [43].

Carbohydrate polymers are the main components of the fungal cell wall however, mannosylated proteins make up about 40% of the yeast cell wall [44]. It has been suggested that defects in protein glycosylation could impair the cell wall integrity by influencing folding and activity of enzymes catalysing synthesis of the cell wall components [45]. *S. cerevisiae stt3* mutants exhibited a 60-70% reduction in the content of cell wall β1,6-glucan [45]. The *STT3* gene encodes an essential subunit of the oligosaccharyl transferase complex (OST), which catalyses the transfer of the oligosaccharide chain from dolichol phosphate (Dol-P) to a protein, during the *N*-linked glycosylation process [45]. Detailed studies indicated that the *stt3* mutation led to synthetic lethality in combination with a mutation in either *KRE5* or *KRE9*, genes involved in the biosynthesis of β1,6-glucan [45]. In the *stt3* mutants Kre5p was underglycosylated and possibly inactive [45]. It was also proposed that mutation in Stt3p led to insufficient synthesis of the glucan primer for β1,6-glucan biosynthesis. Moreover, it has been suggested that *N*-linked sugars of certain secretory proteins, or the cell wall mannoproteins themselves, could serve as the initial acceptors of glucosyl residues to generate β1,6-glucan chains [46]. The *stt3* mutation served as an example of the crucial role of protein *N*-glycosylation in the cell wall integrity process.

Other studies have attested to the importance of *O*-linked glycosylation in the cell wall formation and integrity. Almost all cell wall proteins are heavily *O*-linked mannosylated, and it was suggested that hypo-*O*-linked glycosylation limits their secretion and that the *O*-linked carbohydrates as such could play an important role in the structure of the cell wall [47, 48].

The direct transfer of the mannosyl residue to the OH-groups in proteins is catalysed by protein-*O*-mannosyltransferases. In *S. pombe* protein-*O*-mannosyltransferases are encoded by three *oma* genes. *Oma1* or *oma4* single deletion resulted in abnormal cell wall and septum formation [49]. Ultrastructure analysis showed that the cell wall of the *oma* mutants was thicker than in the wild type strain, and occasionally contained depositions of the cell wall material. Some cells exhibited irregular shape and about 20-30% of septa showed an abnormal structure. Reduced *O*-linked mannosylation of the cell wall sensor proteins could affect the cell wall integrity pathway [50]. *A. nidulans* carrying disruption of *pmtA*, encoding a protein-*O*-mannosyltransferase, exhibited 6% of the wild type activity of protein-*O*-

mannosyltransferase and an altered cell wall composition with elevated skeletal chitin content and a decreased amount of β-glucan, which was manifested as an increased sensitivity to cell wall perturbing agents (Calcofluor White and Congo Red) [51]. A decreased amount of the alkali-insoluble fraction containing β1,3-/1,6-glucan covalently linked to chitin indicated that the cell wall was weakened [51]. These results indicate that PmtA is required for the formation of a normal cell wall in *A. nidulans*. Disruption of *pmtB* or/and *pmtC* genes encoding protein *O*-mannosyltransferases resulted in slightly swollen hyphae with balloon structures and hyperbranching [52].

In *A. nidulans*, *A.awamori*, and *T. reesei* carrying defects in protein-*O*-mannosyltransferase activity the cell wall was weakened, but protein secretion was unaffected. In the *T. reesei pmt1* mutant the cell wall integrity pathway was not stimulated, leaving the cell wall chitin content at the wild type level [53], which suggests a loss of activity of the cell wall sensor proteins caused by their decreased *O*-linked glycosylation.

The changes in the cell wall structure caused by limited glycosylation could also result in elevated protein secretion, as was observed for heterologously expressed human urokinase-type plasminogen activator in a *Hansenula polymorpha opu24* mutant, with a defect in the synthesis of GDP-mannose, a substrate in both *N*- and *O*-linked glycosylation [54]. The *OPU24* gene codes for a guanylyltransferase catalysing the last step in GDP-mannose synthesis. Cells of the *opu2*4 mutant secreted significantly more nominal *N*-linked glycoproteins than the wild type strain, although they were not glycosylated.

Overexpression of *PSA1* (*VIG9/OPU24/MPG1*) gene encoding guanylyltransferase in *Kluyveromyces lactis* caused resistance of the strain to compounds such as SDS, Calcofluor White, and hygromycin B; the latter an *N*-linked glycosylation defect sensing drug [55]. When the *K. lactis* overexpressing *PSA1* strain was transformed with a construction to express human serum albumin, this strain secreted more of the foreign protein than the wild type control cells.

The unlimited supply of GDP-mannose, essential for protein glycosylation and the cell wall assembly resulted also in an increased cell wall porosity, and thus in increased secretion of albumin by the *PSA1*-overexpressing strain. On the other hand, in a *T. reesei mpg1*-overexpressing strain no changes were observed in the amount of secreted proteins [56].

The properties of the fungal cell wall depend also on the cell wall proteins as such. These proteins are engaged in cell flocculation, which has turned out to be advantageous in biotechnological setting. The flocculation phenomenon, found in yeast strains, results from non-sexual aggregation of single cells into multicellular masses caused by interactions between cell wall lectins and cell wall polysaccharides, resulting in sedimentation of the cell aggregates [57].

This allows skipping of the time-consuming steps of cell separation prior to further processing of the secreted products; therefore, there is a rising interest in obtaining industrial strains with a flocculent phenotype. The proteins involved in flocculation appear to be weakly anchored to the cell wall outer layer. Analysis of the proteins in a flocculent strain of *K. bulgaricus* showed them to be galactose-specific lectins, with primary structure homologous to the cytosolic glycolysis enzymes glyceraldehyde-3-phosphate dehydrogenase, enolase and 3-phosphoglycerate mutase [57].

The flocculent phenotype could be engineered in *K. marxianus* by overexpression of the *GAP1* gene coding for glyceraldehyde-3-phosphate dehydrogenase [58]. Aggregation of the

yeast cells occurred as a result of exposure of Gap1 domains on the cell surface, which easily interacted with Gap1 in the cell wall of a neighbouring cell.

An elevated protein secretion of flocculent strain was reported for *S. cerevisiae* NCYC869-A3/pVK1.1 strain overexpressing *A. niger* β-galactosidase [59]; however, the expression level of heterologous proteins is also highly dependent on the genetic background of the host strain [59, 60].

The flocculent phenotype is also connected with mannosylation. Mannose-containing carbohydrates bound to the cell wall proteins act as receptors in flocculation, therefore manipulating the intracellular level of GDP-mannose, a product of the guanylyltransferase (Psa1p/Mpg1p) activity, can alter properties of the cell wall surface due to its influence on the biosynthesis of cell wall mannoproteins and GPI anchors [61]. Paradoxically, repression of the guanylyltransferase encoding gene *PSA1*strongly enhanced cell flocculation [61].

In addition to affecting flocculation, dysfunction of genes encoding enzymes involved in the GPI biosynthesis pathway can also alter the cell wall permeability. The GPI-anchored proteins that decorate the cell wall and have enzymatic activity connected with synthesis of the cell wall components, are responsible for proper assembly and modification of glucan branches, the main structural components determining cell morphology [6, 62-64]. Furthermore, these proteins also participate in filamentation, mating and adhesion to the external matrix [65, 66].

The synthesis and attachment of the GPI anchor involves an enzymatic pathway comprising about 20 proteins [67]. The cell wall and membrane proteins of the secretory pathway are provided with the GPI anchor at their C-terminus in the endoplasmic reticulum. Modulating the level of individual enzymes from the GPI biosynthesis pathway may force secretion of selected proteins for biotechnological purposes. Davydenko et al. [68] observed that a mutation in the yeast gene *GPI14* (*PIG-M*) led to increased secretion of Gas1p, and basing on this phenomenon the authors used GPI14 strain to secrete heterologously expressed α-amylase from *Bacillus amyloliquefaciens*, obtaining eleven and five-fold more of the enzyme after 27 and 54 h post inoculation, respectively [68].

In *A. nidulans*, disruption of *pigP*, the gene coding for a subunit of the first enzyme responsible for GPI synthesis, showed a different pattern of secreted proteins than the wild type strain, in which abundantly secreted alkaline serine protease (ALP) was the dominant protein released into liquid medium [69].

In this case, ALP secretion correlated positively with the impairment in GPI synthesis, even though ALP was not a GPI-anchored protein.

Beside the positive aspects exemplified above, impairment of GPI anchoring may have, however, unpredictable and undesirable side effects including growth impairment. For instance, Li et al. [70] noted that an *A. fumigatus Δpig-a* mutant released to the liquid medium significant quantities of chitinase ChiB, an enzyme responsible for degradation and recycling of fungal chitin during autolysis. Consistent with that, the frequency of cell death of the *pig-a* mutant was enhanced.

CONCLUSION

The above examples amply illustrate the essential role of the cell wall status for protein secretion in fungi. Moreover, it becomes clear that genetic manipulations of enzymes engaged in the cell wall formation often result in phenotypic changes, such as hyperbranching of mycelia, flocculation or increased permeability of the cell wall, desirable traits from the biotechnological point of view. On the other hand, some effects obtained are contrary to expectations and may even compromise the parameters crucial for the strain's performance. Evidently, more thorough understanding of the intricacies of the fungal cell wall biogenesis and functionality is needed.

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