Biochemical aspects of the cell wall of Fusarium oxysporum
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Citation for published version (APA):
Schoffelmeer, E. A. M. (1999). Biochemical aspects of the cell wall of Fusarium oxysporum

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CHAPTER 3

The molecular architecture of the cell wall of *Fusarium oxysporum*. Linkages between cell wall proteins and other cell wall components

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to be submitted

ABSTRACT

Purified hyphal cell walls of the tomato pathogen *Fusarium oxysporum* f.sp. *lycopersici* were treated with β-1,3-glucanase to release covalently bound cell wall proteins (CWPs). This released about 30% of the CWPs. In general, CWPs released by β-1,3-glucanase were highly glycosylated and linked to both β-1,3-glucan and β-1,6-glucan. Both the β-1,3-glucan and β-1,6-glucan epitopes were sensitive to aqueous hydrofluoric acid, which specifically cleaves phosphodiester bridges. These observations suggest that the cell walls of the mycelial fungus *Fusarium oxysporum* may contain a similar structural unit as found in the budding yeast *Saccharomyces cerevisiae*, namely CWP-(-P-)-β1,6-glucan-β1,3-glucan. Treatment of cell walls with aqueous hydrofluoric acid released up to 50% of all CWPs, suggesting that some CWPs are linked through a phosphodiester bridge to another structural cell wall component that is resistant to β-1,3-glucanase digestion such as chitin. Finally, the linkage of the remaining 50% of the CWPs to the skeletal network is discussed.
INTRODUCTION

The tomato plant, which is an agriculturally important crop, is a host for *Fusarium oxysporum* f.sp. *lycopersici*. Successful colonization leads to wilting and, finally, death of the plant. During infection and subsequent growth within the plant there is an intimate contact between host tissues and the fungal cell wall. As cell wall glycoproteins (CWPs) are exposed at the outside of the cell wall, they are likely candidates for potential interactions between plant and fungus. This study was set up to characterize cell wall proteins of *Fusarium oxysporum* and the way they are linked to other cell wall components.

The cell wall of *Fusarium* has been studied in a limited number of species (Sjuikins et al. 1965; Barran et al., 1975; Sivan and Chet, 1989, Barbosa and Kemmelmeijer, 1993 and Schoffelmeer et al. 1999). It is composed of a limited number of components: polymers of glucose containing β-1,3- and β-1,6-linkages (β-glucan) or α-1,3-linkages (α-glucan), polymers of (N-acetyl-)glucosamine (chitin and chitosan) and proteins. Cell wall proteins are generally heavily glycosylated indicating that they follow the secretory pathway. In this pathway modification of proteins may occur by trimming of signal peptides, addition and maturation of glycan chains, formation of disulfide bridges and addition of a glycosyl phosphatidylinositol (GPI) anchor. Glycan chains can either be attached to asparagine residues that comply with the sequence Asn-X-Thr/Ser (N-glycan chains) or to serine and threonine residues (O-glycan chains). The presence of O-glycan chains on *Fusarium* cell wall proteins has been confirmed. Chemical analysis of these chains show that they are elaborate structures composed of glucose, mannose, galactose and glucuronic acid (Jikibara et al., 1991).

Incorporation of cell wall proteins into the cell wall has been studied extensively in the yeast *Saccharomyces cerevisiae* (Van Rinsum et al., 1991; Montijn et al., 1994; Kapteyn et al. 1995a and Kollár et al. 1997), the human pathogen *Candida albicans* (Kapteyn et al. 1995b), and some filamentous fungi (Brul et al. 1997, Cao et al. 1998). By extraction with hot SDS some fungal cell wall proteins can be released. Other cell wall proteins are extractable by β-1,3-glucanase suggesting that these proteins are linked to β-1,3-glucan (Kapteyn et al. 1997, Kapteyn 1999). Studies of glucanase-extractable cell wall proteins in *Saccharomyces cerevisiae* show that they are attached to a heteropolymer of β-1,3-/β-1,6-glucan. β-1,6-Glucan mediates the linkage between cell wall proteins and β-1,3-glucan which is a major structural cell wall component (Kapteyn et al. 1996; Kollár et al. 1997, Smits et al. 1999). β-1,6-Glucan itself is indirectly linked to the cell wall protein via the mannan chain of a GPI-anchor. GPI-anchor addition is required for membrane association and cell surface localization of various proteins in mammalian cells and surfaces of some protozoa and yeasts (Takeda and Kinoshita 1995, Carrington et al. 1998, Lu et al. 1994 and Guadiz et al. 1998). For cell wall anchoring of
proteins into yeast cell walls the presence of a GPI attachment site at the C-terminal part of a protein is essential (van Berkel et al. 1994; Shimoi et al. 1995; Lu et al. 1995 and van der Vaart 1997). However, it has been shown that cell wall association of these GPI-anchored proteins requires cleavage from the cell membrane, preceding its linkage into the cell wall. For the α-agglutinin present in the cell wall of S. cerevisiae a plasma membrane associated form, a periplasmatic intermediate and a cell wall-anchored form have been identified (Lu et al. 1994).

Here we present evidence that the cell wall of Fusarium oxysporum may contain a similar structural unit (cell wall protein-(P-)→β1,6-glucan→β1,3-glucan) as has been identified in the budding yeast Saccharomyces cerevisiae. In addition, we present evidence for the existence of alternative linkage of cell wall proteins to skeletal cell wall components.

MATERIAL AND METHODS

Fungal strain and growth. Race 2 of Fusarium oxysporum f.sp. lycopersici (isolate 007, VCG 0030si) was cultured on potato dextrose agar (Difco) plates. Conidia were collected from these plates by washing the aerial hyphae with sterile water. Hyphal fragments were removed by filtration through glass-wool to obtain a conidia suspension which was added to B5 medium (de Wit and Flach, 1979) at a final concentration of 1×10^5 conidia ml^-1. Liquid cultures were shaken on a reciprocal shaker for three days at 24 °C.

Isolation of hyphal cell walls. Mycelium was obtained by filtering three-day-old cultures through one layer of Miracloth (CalBiochem). The mycelium was washed twice with deionized water, and collected by filtration through Miracloth. Subsequently, the mycelium was collected on an 18-μm filter (Sartorius). From this mycelial mat, 1.4 g fresh weight was added to a glass tube containing 10 ml buffer (10 mM Tris-HCl, pH 7.5 and 1 mM PMSF) and 10 g glass beads (diameter, 0.5 mm). Disruption of mycelium was performed by sequential shaking of the tube with a Vortex (1 min at maximum speed), a Griffin shaker (30 min at half-maximum speed in a cold room at 5 °C) and finally again on a Vortex (1 min at maximum speed). As observed by microscopy, this procedure rendered hyphae free of cytoplasm. Glass beads were separated from the homogenate and cell walls were collected by centrifugation. Subsequently, the cell walls were washed three times with an ice-cold 1 M NaCl solution and three times with ice-cold deionized water both containing 1 mM PMSF. Finally, the cell walls were stored at -20 °C until needed.

Treatment of cell walls with β-1,3-glucanase and hydrofluoric acid. Non-covalently bound proteins were removed from the cell walls by boiling for 5 min in a 50 mM Tris-HCl (pH 7.8) buffer supplemented with 2% SDS, 100 mM NaEDTA, 40 mM β-mercaptoethanol and 1 mM PMSF (Elorza et al., 1985). Cell walls were collected by centrifugation, extracted a second time, and washed six times with deionized water supplemented with 1 mM PMSF. Cell wall proteins (CWPs) were released from SDS-extracted cell walls by Quantazyme, a recombinant β-1,3-glucanase (Quantum Biotechnologies inc., Montréal) or ice-cold aqueous hydrofluoric acid (HF) (50% (v/v)). Digestion of cell walls with Quantazyme (1200 U g^-1 wet weight) was performed at 37 °C in 50 mM Tris-HCl, pH 7.5, supplemented with 1 mM PMSF and 40 mM...
DTT for 24 h. Ice-cold aqueous HF was added to lyophilized SDS-extracted cell walls and incubated for three days on ice in a cold-room. Insoluble cell wall components were removed by centrifugation (Eppendorf centrifuge, 14,000 rpm; for 30 min at 4 °C). Solubilized glycoproteins were precipitated by adding nine volumes of ice-cold 100 % methanol and were recovered by centrifugation (Eppendorf centrifuge, 14,000 rpm, for 30 min at 4 °C). The resulting pellet was washed three times with ice-cold 90 % (v/v) methanol and thereafter resuspended in water and finally lyophilized. Quantification of CWPs that had been released by β-1,3-glucanases and aqueous HF was performed by determining the remaining CWPs in the treated cell walls. Added enzymes and aqueous HF were removed by washing the cell walls several times in water. Thereafter they were lyophilized and boiled for 10 min in 1 M NaOH to release CWPs. CWPs were quantified with the BCA-protein assay reagent (Sigma) using bovine serum albumin as a reference protein (Smith et al. 1985).

**Deglycosylation of proteins.** Deglycosylation of cell wall glycoproteins was performed on CWPs that had been released by Quantazyme (Sojar and Bahl 1987). Released CWPs were dialyzed overnight against ultra pure water (Millipore) and subsequently lyophilized. Each sample was chilled on dry-ice and treated with trifluoromethanesulfonic acid for 1 h. Samples were neutralized by addition of 60 % (v/v) pyridine. To this mixture 200  μl 10 mM Tris-HCl buffer (pH 8.0) were added to increase the volume of the samples. The samples were dialyzed against ultra pure water (Millipore) and subsequently lyophilized.

**Gel electrophoresis and immunoblotting.** CWPs were separated by gel-electrophoresis on gradient polyacrylamide gels (2.2-20%). These gels were loaded with protein samples equivalent to 8 mg of fresh weight cell walls taken up in 4 x sample buffer consisting of 0.5 M Tris-HCl, pH 6.8, glycerol 10 % (v/v), SDS 10 % (w/v), β-mercaptoethanol 5 % (v/v) and, bromphenol blue, 0.05 % (w/v). Proteins were either visualized by silver staining (De Nobel et al. 1989) or by staining with periodic acid-Schiff (PAS) reagent (Sigma). Western analysis was initiated by transferring the proteins electrophoretically (Bio-Rad, Mini Protean II) to an Immobilon polyvinylidene-difluoride (PVDF) membrane (Millipore). The membranes were blocked with 5 % (w/v) milk powder dissolved in phosphate-buffered saline (PBS) for analysis with the β-1,6-glucan antiserum. For the analysis with β-1,3-glucan antiserum PVDF membranes were blocked in PBS containing 1 % gelatine (Montijn et al.1994; Kapteyn et al. 1996). β-1,6-Glucan and β-1,3-glucan antisera were used in a dilution of 1 : 5000 and 1 : 50,000, respectively, in PBS containing 5 % milk powder. Binding of the antiserum to cell wall proteins was determined with goat-anti-rabbit IgG-peroxidase using ECL detection reagents (Amersham). For Western blot-analysis with peroxidase-labelled Concanavalin A (ConA) (Sigma), a mannose-binding lectin, the membranes were blocked in PBS containing 3 % (w/v) BSA. ConA-peroxidase was added to a final concentration of 1 μg.ml⁻¹ in PBS supplemented with 2.5 mM MnSO₄.H₂O, 2.5 mM CaCl and 3 % (w/v) BSA. Specificity of ConA for mannose was demonstrated by addition of α-methyl mannoside to prevent binding of ConA to its ligand.
RESULTS

General characterization of β-1,3-glucanase-extractable cell wall proteins

We have shown earlier that the cell wall of *F. oxysporum* contains proteins that are resistant to extraction with hot SDS (Schoffelmeer et al., 1999), indicating that they are covalently linked to other cell wall polymers. In order to investigate how these proteins are linked to the skeletal framework of the wall, SDS-extracted cell walls were first treated with Quantzyme, a recombinant β-1,3-glucanase. This treatment released 31% of the SDS-resistant cell wall proteins (CWPs) (Table 1), indicating that β-1,3-glucan is involved in linking part of the SDS-resistant CWPs to the cell wall framework. The CWPs released by β-1,3-glucanase were analyzed by SDS-PAGE followed by staining with either silver, periodic acid-Schiff reagent (PAS) or ConA. Silver staining of β-1,3-glucanase-extractable proteins revealed several minor low-molecular-weight bands and a major smear around 220 kDa (Fig. 1, lane 1). This smear points to the presence of protein molecules that are glycosylated to a different degree. The presence of glycosylated proteins was confirmed by PAS (Fig. 1, lane 2). A similar staining pattern as obtained with PAS was seen with peroxidase-linked ConA (Fig. 1, lane 3), indicating that mannose residues are part of the glycan moiety of the proteins involved. To determine the size of the protein moiety of the 220-kDa smear, β-1,3-glucanase-extractable CWPs were chemically deglycosylated with trifluoromethanesulfonic acid (TMSF). This treatment is known to remove O- as well as N-glycan chains from glycoproteins. Silver staining showed loss of the 220 kDa smear. Instead, a diffuse band of about 26 kDa was found (Fig 1, lane 4). When PAS staining was carried out, the 26-kDa band was not detected, indicating that the protein molecules had indeed lost their glycan moiety (Fig. 1, lane 5). This suggests that the bulk of β-1,3-glucanase-extractable CWPs consist of a protein moiety of around 26 kDa that is extensively glycosylated.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>μg mg⁻¹ F.W</th>
<th>Amount of protein retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4.0 ± 0.5</td>
<td>100</td>
</tr>
<tr>
<td>β-1,3-Glucanase</td>
<td>2.8 ± 0.3</td>
<td>69</td>
</tr>
<tr>
<td>HF</td>
<td>2.1 ± 0.2</td>
<td>52</td>
</tr>
</tbody>
</table>

Table 1. Retention of proteins in SDS-extracted cell walls of *Fusarium oxysporum* upon treatment with β-1,3-glucanase or ice-cold aqueous hydrofluoric acid (HF). F.W. = fresh weight. Means ± standard deviation are presented (n = 3).
β-1,3-Glucanase-extractable cell wall proteins are linked to a β-1,6-/β-1,3-glucan heteropolymer through a phosphodiester bond

The most abundant structural unit in the cell wall of the budding yeast *Saccharomyces cerevisiae* consists of a GPI-CWP linked through a remnant of its GPI anchor to a β-1,6-glucan molecule which in turn is linked to the β-1,3-glucan framework of the cell wall (Van Der Vaart *et al.* 1996, Kollar *et al.* 1997, Kapteyn *et al.* 1999). The remnant of the GPI anchor includes a phosphodiester bond linking ethanolamine to the carboxyterminus of a cell wall protein (Van Der Vaart *et al.* 1996, Kollar *et al.* 1997, Kapteyn *et al.* 1999). To gain more insight in how β-1,3-glucanase-extractable CWP s in *Fusarium* might be linked to the skeletal framework, they were investigated by Western analysis. Antisera were used that specifically recognize β-1,3- or β-1,6-glucan (Montijn *et al.* 1994). Using the β-1,3-glucan antiserum, three minor low molecular weight bands and a large smear (> 70 kDa) (Fig. 1, lane 6) were detected. Analysis of the β-1,3-glucanase-released CWPs by the β-1,6-glucan antiserum revealed only a large smear (> 70 kDa) whereas the low molecular weight bands did not react (Fig. 1, lane 7). This suggests that the large smear (> 70 kDa) represents one or possibly more CWPs which are physically connected to a heteropolymer of β-1,6-/β-1,3-glucan, whereas the low molecular weight CWPs contain predominantly or exclusively β-1,3-glucan. Whether β-1,3-glucan is linked to the β-1,3-glucanase-released CWPs via a phosphodiester bond was
further investigated by treating these CWPs with ice-cold aqueous HF, which specifically splits phosphodiester bonds. This resulted in the loss of both the β-1,6-glucan and β-1,3-glucan epitopes (data not shown). In a time course experiment, the smear detected by the β-1,3-glucan antiserum (Fig. 2, lane 1) was progressively reduced in size upon treatment with aqueous HF, indicating a loss of β-1,3-glucan (Fig. 2, lane 1 to 4). PAS staining of these aqueous HF-treated CWPs showed that upon progressive treatment two fuzzy bands of approximately 87 and 43 kDa (indicated by arrows) emerged and that after three days the reaction was largely complete (Fig. 2, lanes 5 to 8). The 87 and 43 kDa bands were not detected by the β-1,3-glucan antiserum, indicating that the β-1,3-glucan moiety was lost.

These data suggest that a phosphodiester bond mediate the linkage between these CWPs and β-1,3-glucan. Consistent with these results, treatment of isolated cell walls with aqueous HF resulted in the release of about half of all cell wall proteins (Table 1), confirming that many cell wall proteins are linked to the β-1,3-glucan framework through a phosphodiester bond.

**DISCUSSION**

Analysis of cell wall proteins (CWPs) and their incorporation into the cell wall of mycelial fungi is in the early phases (Brul et al. 1997, Cao et al. 1998). Most of our knowledge is derived from the unicellular yeast Saccharomyces cerevisiae. The most abundant structural unit found in this fungus consists of GPI-CWPs linked through a remnant...
of their GPI-anchor to a β-1,6-glucan molecule which in turn is linked to β-1,3-glucan (CWP-GPI remnant → β1,6-glucan → β1,3-glucan) (Kapteyn et al. 1999 a, Smits et al. 1999). Importantly, the GPI anchor remnant contains a phosphodiester bridge that links ethanolamine to the carboxyterminus of the cell wall protein and that can be specifically cleaved by aqueous hydrofluoric acid. Consistent with this, it has been shown for these fungi that CWPs can be released by β-1,3-glucanases and aqueous hydrofluoric acid (Kapteyn et al. 1996). Therefore, similar treatments were utilized to release CWPs from the *Fusarium oxysporum* cell wall. Here we show that about 30% of the SDS-resistant CWPs were extractable by Quantazyme, a recombinant β-1,3-glucanase (Table 1). As these proteins were recognized by both β-1,3-glucan and β-1,6-glucan antisera and as these epitopes were sensitive to treatment with hydrofluoric acid (Figures 1 and 2), this suggests that at least about one third of all CWPs in *Fusarium oxysporum* are GPI-CWPs linked through an phosphodiester bridge in their GPI remnant to a β-1,6-β-1,3-glucan heteropolymer. However, Iwahara et al. (1992) have shown that O-linked glycan side-chains of *Fusarium* glycoproteins possess internal phosphodiester bridges. We can therefore not exclude the possibility that some *Fusarium* CWPs are linked to the β-1,3-glucan framework through an O-linked side-chain.

Interestingly, some minor, low-molecular-weight, β-1,3-glucanase-released cell wall proteins do not react with the β-1,6-glucan antiserum, but are recognized by the β-1,3-glucan antiserum (Fig. 1). This indicates that they are directly linked to β-1,3-glucan without an interconnecting β-1,6-glucan molecule. Similar proteins have been identified in the cell wall of *Saccharomyces cerevisiae* (Kapteyn et al. 1999 b), where they form part of the structural unit Pir-CWP-β-1,3-glucan.

About two thirds of all CWPs are resistant to β-1,3-glucanase digestion. This raises the question how they might be linked to the cell wall. Kapteyn et al. (1997) has demonstrated that β-1,3-glucanase-resistant CWPs in the wall of *S. cerevisiae* are linked via β-1,6-glucan to chitin (CWP-GPI remnant → β1,6-glucan→ chitin). As *Fusarium* cell walls contain in comparison to *S. cerevisiae* a much larger amount of chitin (Schoffelmeer et al, in press), it is conceivable that β-1,3-glucanase-resistant CWPs of *Fusarium* are also linked through a β-1,6-glucan molecule to chitin. Indeed, aqueous HF was more efficient than Quantazyme in releasing CWPs and released about half of all CWPs. This is consistent with the presence of GPI-CWPs that are linked through β-1,6-glucan to chitin as found in *Saccharomyces cerevisiae*. If indeed a similar structural unit is present in *Fusarium* cell wall, it seems possible that all three structural units identified so far in *Saccharomyces cerevisiae* are also present in the mycelial fungus *Fusarium oxysporum*.

The remaining 50% of all CWPs were resistant to aqueous HF raising the question how these proteins are linked to the skeletal framework of the cell wall. Possibly, these CWPs are
directly linked to chitin in an as yet unknown way. Alternatively, as *Fusarium* walls contain α-1,3-glucan (Schoffelmeer et al, in press) it is conceivable that some CWPs might be directly linked to α-1,3-glucan.

**ACKNOWLEDGEMENT**

We thank Dr R. Montijn for the antisera directed against β-1,3- and β-1,6-glucan.

**REFERENCES**


albicans. Journal of Bacteriology. 177;3788-3792.
Kapteyn, J.C., van den Ende, H. and Klis, F.M. (1999 a) The contribution of cell wall proteins to the organization of the yeast cell wall. (Biochimica et Biophysica Acta 1426:373-385