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Retention of *Saccharomyces cerevisiae* cell wall proteins through a phosphodiester-linked \( \beta-1,3/\beta-1,6 \)-glucan heteropolymer

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Yeast cell wall proteins, including Cwp1p and \( \alpha \)-agglutinin, could be released by treating the cell wall with either \( \beta-1,3 \) or \( \beta-1,6 \)-glucanases, indicating that both polymers are involved in anchoring cell wall proteins. It was shown immunologically that both \( \beta-1,3 \) and \( \beta-1,6 \)-glucan were linked to yeast cell wall proteins, including Cwp1p and \( \alpha \)-agglutinin. It was further shown that \( \beta-1,3 \)-glucan was linked to the wall protein through a \( \beta-1,6 \)-glucan moiety. The \( \beta-1,6 \)-glucan moiety could be removed from Cwp1p and other cell wall proteins by cleaving phosphodiester bridges either enzymatically using phosphodiesterases or chemically using ice-cold aqueous hydrofluoric acid. These observations are consistent with the notion that cell wall proteins in *Saccharomyces cerevisiae* are linked to a \( \beta-1,3/\beta-1,6 \)-glucan heteropolymer through a phosphodiester linkage and that this polymer is responsible for anchoring cell wall proteins. It is proposed that this polymer is identical to the alkali-soluble \( \beta-1,3/\beta-1,6 \)-glucan heteropolymer characterized by Fleet and Manners (1976, 1977).

**Key words:** \( \alpha \)-agglutinin/*Candida albicans*/CWP1/fungal wall/GPI-anchor/mannoproteins

Introduction

The cell wall of the yeast *Saccharomyces cerevisiae* consists of complex polymers of glucose (\( \beta-1,3 \)-glucan, \( \beta-1,6 \)-glucan), chitin, glycoproteins, and lipids (Fleet, 1991; Kollár et al., 1995). Some glycoproteins are non-covalently linked to the cell wall as demonstrated by their extractability with hot SDS, but the bulk of the wall proteins can only be liberated from the wall by \( \beta-1,3 \)-glucanase digestion, suggesting that they are tightly bound to the \( \beta-1,3 \)-glucan skeleton of the cell wall (Valentin et al., 1984; Zlotnik et al., 1984; Frevert and Ballou, 1985; Van Rinsi et al., 1991; Klis, 1994). Most glucanase-extractable wall proteins carry large \( N \)-linked side-chains consisting of mannose residues, and/or short, linear \( O \)-mannosyl chains. In addition, these wall mannoproteins probably carry a glycosyl phosphatidylinositol (GPI) derived structure, since, to date, all genes that code for glucanase-extractable cell wall proteins have been found to contain a GPI anchor addition sequence (Lipke et al., 1989; Kondo and Inouye, 1991; Roy et al., 1991; Teunissen et al., 1993; De Nobel and Lipke, 1994; Shimo et al., 1995; Van der Vaart et al., 1995). As for the precursors of the pheromone-inducible cell wall protein \( \alpha \)-agglutinin, the addition of a GPI anchor has been biochemically confirmed (Wojciechowicz et al., 1993; Lu et al., 1994). Biochemical studies further showed that the mature cell wall form of \( \alpha \)-agglutinin had a modified GPI anchor lacking at least the inositol and the fatty acid components (Lu et al., 1994). Cell wall anchorage was accompanied by addition of \( \beta-1,6 \)-glucan (Lu et al., 1995).

The mechanism by which proteins are retained in the cell wall is largely unknown. Several studies, however, have shown that the glucanase-extractable wall proteins of *S. cerevisiae* and *Candida albicans* possess a \( \beta-1,6 \)-glucan-containing moiety, which has been proposed to couple the proteins to the \( \beta-1,3 \)-glucan framework (Tkacz, 1984; Van Rinsi et al., 1991; Montijn et al., 1994; Kapteyn et al., 1994, 1995b; Van Berkel et al., 1994; Lu et al., 1995; Van der Vaart et al., 1995). This idea was supported by the identification of protein-bound \( \beta-1,6 \)- and \( \beta-1,3 \)-glucan in cell walls of *C. albicans* (Kapteyn et al., 1995b). These protein-bound glucan polymers were suggested to be related to the alkali-soluble \( \beta-1,3 \)-glucan-\( \beta-1,6 \)-glucan wall fraction of *C. albicans* studied by Bishop et al. (1960) and Yu et al. (1967). A comparable alkali-soluble glucan heteropolymer has been identified in *S. cerevisiae* (Fleet and Manners, 1976, 1977). This glucan heteropolymer was found to be composed of two distinct domains, the largest one containing about 1350 \( \beta-1,3 \)-linked glucose residues, and the smallest one containing approximately 150 \( \beta-1,6 \)-linked glucose residues and some mannose residues (Figure 6). This raises the question whether this alkali-soluble glucan heteropolymer is *in vivo* protein-linked and responsible for retaining proteins in the cell wall.

Here, for the first time we show immunologically that *S. cerevisiae* cell wall mannoproteins form a complex with a heteropolymer of \( \beta-1,6 \) - and \( \beta-1,3 \)-glucan. Evidence is presented that the \( \beta-1,6 \)-glucan moiety connects Cwp1p, \( \alpha \)-agglutinin, and other cell wall mannoproteins with the \( \beta-1,3 \)-glucan part of this complex. The data further indicate that the attachment of the \( \beta \)-glucan complex is most likely responsible for anchoring the proteins into the cell wall. In addition, the \( \beta-1,6 \)-glucosyl moiety is shown to be phosphodiester-linked to protein, which is consistent with the hypothesis of a GPI anchor derived structure as attachment site for \( \beta-1,6 \)-glucan (De Nobel and Lipke, 1994).

**Results**

\( \beta-1,3/\beta-1,6 \)-glucosylated cell wall proteins

Laminarinase, a \( \beta-1,3 \)-glucanase preparation with some \( \beta-1,6 \)-glucanase and \( \alpha \)-mannanase activities, and Quantazyme, a pure \( \beta-1,3 \)-glucanase, were found to liberate about 75 and 60% of SDS-resistant cell wall proteins of *S. cerevisiae*.
iae mnn9, respectively (Table I). As demonstrated before by SDS–PAGE and Western blot analysis (Montijn et al., 1994), laminarinase digestion of yeast cell walls resulted in a well-defined set of β-1,6-glucosylated bands that were characterized by apparent molecular masses of about 245, 135, 105, and 60 kDa, respectively (Figure 1, lane 1). The 60 kDa-band has recently been identified as Cwp1p (Van der Vaart et al., 1995). This was confirmed here, since only this band was recognized by the anti-Cwp1p antiserum (Figure 2, lane 1). After treatment with a purified endo-β-1,6-glucanase (De La Cruz et al., 1995), the laminarinase-liberated bands were slightly reduced in size, having apparent molecular masses of 235, 130, 100, and 58 kDa, respectively, and were less reactive with the β-1,6-glucan antiserum (Figure 1, lane 2). This indicated that the endo-β-1,6-glucanase removed part of, but not the entire β-1,6-glucan epitope from the proteins. Most likely, the 58 kDa-band was the partially deglucosylated form of Cwp1p, since it was the only band that was strongly stained when probed with the anti-Cwp1p antiserum (Figure 2, lane 2). None of the laminarinase-released bands were recognized by the β-1,3-glucan antiserum (data not shown). In contrast, Quantazyme liberated a high-molecular-mass, polydisperse smear, that could be stained with silver (Figure 3A, lane 1), and reacted with both the β-1,6- and β-1,3-glucan antiserum (Figure 1, lanes 4 and 6). Upon pronase treatment, this β-1,6- and β-1,3-glucosylated material was not observed anymore (data not shown), demonstrating that both glucan polymers were protein-linked. The relatively weak immunoreactivity of the Quantazyme-released smear with the anti-Cwp1p antiserum (Figure 2, lane 3) indicated that Cwp1p released by this enzyme had a high, and heterogeneous molecular mass, presumably because it was associated with β-1,6- and β-1,3-glucan.

In the following experiment, a highly purified endo-β-1,6-glucanase was used to investigate whether the β-1,3-glucan epitope was attached to Cwp1p and other proteins through the β-1,6-glucosyl moiety. If this was the case, it was expected that upon incubation with the endo-β-1,6-glucanase the Quantazyme-released wall proteins would lose their antigenicity towards the β-1,3-glucan antiserum, would drop in size, and would, possibly, retain part of their β-1,6-glucan epitope, as observed with laminarinase. Indeed, treatment with β-1,6-glucanase led to the disap-

![Fig. 1](image1)

**Fig. 1.** Western analysis of the laminarinase- (lanes 1 and 2), endo-β-1,6-glucanase- (lane 3), and Quantazyme-released (lanes 4–7) wall proteins of mnn9 cells using the affinity-purified β-1,6-glucan antiserum (β-1,6-glucan Pabs; lanes 1–5) and β-1,3-glucan antiserum (β-1,3-glucan Pabs; lanes 6 and 7). Lanes 1, 3, 4, 6, immunodetection before endo-β-1,6-glucanase treatment. Lanes 2, 5, 7, after endo-β-1,6-glucanase treatment. The sizes of standard molecular mass markers are indicated. The glucanases did not react with the antisera (data not shown).
Anchoring mechanism of *Saccharomyces cerevisiae* cell wall proteins

Fig. 2. Western analysis of laminarinase- (lanes 1 and 2), Quantzyme- (lanes 3 and 4), endo-β-1,6-glucanase- (lane 5), and aqueous HF-released wall proteins with the anti-Cwp1p antiserum. Lanes 1, 3, 5, and 6, immunodetection before endo-β-1,6-glucanase treatment. Lanes 2 and 4, after endo-β-1,6-glucanase treatment. The molecular sizes of the different forms of Cwp1p are indicated.

The β-1,6-glucan moiety is phosphodiester-linked to the proteins

The laminarinase-released cell wall proteins were treated with ice-cold aqueous HF (50%) to determine whether their β-1,6-glucan side chains were phosphodiester-linked. This treatment is routinely used to cleave phosphodiester bridges and does not lead to significant protein degradation or breakdown of N- and O-chains (Mort and Lamport, 1977; Müller et al., 1992; Kapteyn et al., 1995b). As reported for *C. albicans* (Kapteyn et al., 1995b), after aqueous HF-treatment for 72 h on ice, binding of the cell wall proteins to β-1,6-glucan antiserum was strongly diminished (Figure 4A, lane 2). Interestingly, aqueous HF treatment of SDS-extracted cell walls also resulted in the release of the known set of yeast cell wall proteins (Figure 3B, lane 5), including Cwp1p (Figure 2, lane 6). About 50% of total SDS-resistant
cell wall proteins was extracted by this method (Table I). The HF-extracted proteins did not react with the β-1,6-glucan antiserum (data not shown), and, accordingly, they had relatively lower molecular masses than the β-1,6-glucanase-released proteins (Figure 3B, compare lanes 4 and 5). This also held true for Cwp1p (Figure 2, compare lanes 5 and 6). The difference in molecular mass between endo-β-1,6-glucanase- and HF-released Cwp1p was about 9 kDa. Consequently, the experiments with aqueous HF suggest that the β-1,6-glucan chains are linked to the proteins through phosphodiester bonds. However, the most convincing evidence for this type of linkage came from studies in which the laminarinase-released wall proteins were incubated with the phosphodiesterases PDE I and II. PDE II was found to remove the β-1,6-glucan epitope from the laminarinase-released proteins (Figure 4B, lane 5), although some epitope was still present after treatment for 48 h. Accordingly, after PDE II treatment, laminarinase-released Cwp1p was about 13 kDa smaller in size (Figure 4D, lane 11). PDE I was only able to remove the epitope from the 220 kDa band (Figure 4B, lane 4). Apparently, the phosphodiester linkages between the other proteins and their β-1,6-glucan epitope were less accessible to PDE I than to PDE II. The effect of the phosphodiesterase-inhibitors theophylline and IBMX on PDE II activity was also studied. Both compounds clearly inhibited the cleavage of the phosphodiester bonds between the wall proteins and β-1,6-glucan by PDE II (Figure 4C), confirming that PDE II activity was indeed responsible for the effects observed. Taken together, these data demonstrate that the β-1,6-glucan containing chains are phosphodiester-linked to the wall proteins.

RetentionPolicy of α-agglutinin in the cell wall
Laminarinase-released α-agglutinin, a pheromone-inducible cell wall protein, has recently been shown to have a β-1,6-glucan moiety (Lu et al., 1995). It was therefore investigated whether α-agglutinin was retained in the cell wall by a similar mechanism as the constitutively expressed wall proteins, such as Cwp1p. It was found that, like the other wall proteins, α-agglutinin could also be liberated by Quantazyme, endo-β-1,6-glucanase, and aqueous HF (Figure 5). Quantazyme-released α-agglutinin was visualized by fluorography as a very faint and high-molecular-mass smear (Figure 5, lane 1), that strongly reacted with the β-1,6-glucan antiserum (Figure 5, lane 5). The β-1,6-glucanase-released form of α-agglutinin had a molecular size of about 230 kDa (Figure 5, lane 2), and reacted relatively weakly with the β-1,6-glucan antiserum (Figure 5, lane 6), indicating that almost its entire glucosyl moiety had been removed by the endoglucanase. These results show that the glucan moiety contributes considerably to the molecular mass of Quantazyme-released α-agglutinin. The HF-extracted form did not have a β-1,6-glucan epitope (Figure 5, lane 7) and was about 8 kDa smaller in size than β-1,6-glucanase-released α-agglutinin (Figure 5, lanes 2 and 3). The aqueous HF-extracted form of α-agglutinin
Fig. 4. The effect of ice-cold aqueous HF and phosphodiesterases PDE I and II on reactivities of the laminarinase-released wall proteins from \textit{mnn9} cells with the affinity-purified $\beta$-1,6-glucan antiserum (lanes 1–9) and the anti-Cwp1p antiserum (lanes 10 and 11). (A) Lane 1, control; lane 2, after treatment with aqueous HF. (B) Lane 3, control; lanes 4 and 5, after incubation with PDE I and PDE II, respectively. (C) lane 6, control; lanes 7, 8, and 9, after incubation with PDE II in the absence (lane 7) and presence of IBMX (lane 8) or theophylline (lane 9). (D) Lane 10, control; lane 11; after incubation with PDE II

Fig. 5. Characterization of immunoprecipitated $[^{35}\text{S}]$-labeled $\alpha$-agglutinin, which was released with Quantzyme (lanes 1 and 5), endo-$\beta$-1,6-glucanase (lanes 2 and 6), and aqueous HF (3, 4, 7) from SDS-extracted walls of \textit{mnn9} cells, by fluorography (lanes 1–4), and by Western analysis with the affinity-purified $\beta$-1,6-glucan antiserum (lanes 5–7). Lane 4, after treatment with Endo H. The molecular size of the different forms of $\alpha$-agglutinin is indicated. In this experiment 3–15% gradient gels were used.
was found to be sensitive to Endo H (Figure 5, lanes 3 and 4), confirming earlier reports that the $N$-chains were not released by aqueous HF (Kapteyn et al., 1994; 1995b). The data presented indicate that also in the case of a pheromone-inducible cell wall protein a phosphodiester-linked $\beta$-1,6-glucosyl moiety, probably as part of a larger $\beta$-1,3-$\beta$-1,6-glucan heteropolymer, is responsible for retaining it in the cell wall.

Discussion

Recently, laminarinase-extractable yeast cell wall proteins such as $\alpha$-agglutinin, Cwplp, and Tip1p have been shown to have a $\beta$-1,6-glucan moiety (Lu et al., 1995; Van der Vaart et al., 1995). In this report we show that these and other, not yet fully characterized, yeast cell wall proteins could also be released by Quantayzyme, a pure $\beta$-1,3-glucanase preparation in contrast to laminarinase, which is a mixture of $\beta$-1,3- and $\beta$-1,6-glucanase activities. The Quantayzyme-liberated $\beta$-1,6-glucosylated wall proteins, including Cwp1p and $\alpha$-agglutinin, had much higher molecular masses, most likely because they also carried a $\beta$-1,3-glucan epitope (Figs. 1, 2, 5). Comparable results were obtained with C. albicans (Kapteyn et al., 1995b). Presumably, Quantayzyme was hindered by $\beta$-1,6 branch points in the $\beta$-1,3-glucan cell wall polymers, thereby leaving some undigested $\beta$-1,3-glucan attached to the $\beta$-1,6-glucan moiety of the proteins. Since an endo-$\beta$-1,6-glucanase could remove the $\beta$-1,3-glucan epitope, it was concluded that $\beta$-1,3-glucan was attached to protein through the $\beta$-1,6-glucan moiety (Figure 1). The presence of a protein-bound $\beta$-1,3-$\beta$-1,6-glucan heteropolymer in cell walls of S. cerevisiae (Figure 1) and C. albicans (Kapteyn et al., 1995b) suggests that this polymer might be responsible for retaining cell wall proteins. This hypothesis was also sustained by the observation that the $\beta$-1,3-glucanase-extractable wall proteins, including Cwp1p and $\alpha$-agglutinin, could also be freed by endo-$\beta$-1,6-glucanase digestion (Figs. 1, 2, 3A, 5). This enzyme released approximately 90% of all SDS-resistant cell wall proteins, indicating that the attachment of a $\beta$-1,3-$\beta$-1,6-glucan heteropolymer represents a general mechanism for anchoring cell wall proteins. In accordance with this, several kre mutants, which have defects in $\beta$-1,6-glucan synthesis, were found to secrete more $\alpha$-agglutinin and other putative cell wall proteins into the growth medium than the wild-type strain (Lu et al., 1995). Moreover, it was found that krel cwh41 double disruptants showed a 75% reduction in the cell wall $\beta$-1,6-glucan level than the wild-type strain (Lu et al., 1995).

To date, little information has been obtained about the exact carbohydrate composition and structure of the protein-bound $\beta$-1,6-glucosyl moiety. Montijn et al. (1994) found that it consisted of some N-acetylglucosamine or glucosamine, about equal amounts of glucose and mannose, and did not form part of either $O$- or $N$-glycosidic side chains. Other evidence against $N$-linkage of $\beta$-1,6-glucan came from studies showing that some cell wall proteins do not have any $N$-glycosidic side chains at all (Roy et al., 1991; Van der Vaart et al., 1995). Moreover, $\alpha$-agglutinin is efficiently made and anchored in the presence of tunicamycin, an inhibitor of $N$-glycosylation (Terrance, 1983; Hasegawa and Yanagishima, 1984). Accordingly, spheroplasts of C. albicans regenerating in the presence of tunicamycin were still able to synthesize $\beta$-1,6-glucosylated cell wall proteins (Kapteyn et al., 1995a). Several other studies pointed to the involvement of GPI-anchors in the attachment of $\beta$-1,6-glucan to the wall proteins. First, a secretory reporter protein extended with the GPI-anchor addition sequence of the cell wall protein $\alpha$-agglutinin became immobilized in the cell wall of S. cerevisiae and contained $\beta$-1,6-glucan, whereas the nonextended and secreted form was not glucosylated (Van Berkel et al., 1994). Second, in contrast to intact $\alpha$-agglutinin, a C-terminal truncated form, which was not GPI-anchored and was secreted into the medium, did not contain $\beta$-1,6-glucan (Lu et al., 1995). Third, S. cerevisiae cwh6, a calcofluor white hyper-sensitive cell wall mutant, that showed a strongly reduced retention of $\alpha$-agglutinin and other putative cell wall mannoproteins, was found to be mutated in a gene involved in GPI-anchor synthesis (Vossen et al., 1995). Fourth, as demonstrated here in S. cerevisiae (Figure 4A) and in C. albicans (Kapteyn et al., 1994, 1995b), the $\beta$-1,6-glucosyl moieties could be removed from the proteins by treatment with ice-cold aqueous HF, a method commonly used to cleave phosphodiester bridges in GPI-anchors (Ferguson, 1992; Müller et al., 1992). Removal of the glucosyl moiety was also achieved by phosphodiesterases (Figure 4B), confirming the involvement of a phosphodiester linkage. Consistently, aqueous HF-extracted cell wall proteins, such as Cwp1p (data not shown) and $\alpha$-agglutinin (Figure 5, lane 7), appeared to be non-$\beta$-glucosylated. Another line of evidence for the GPI-anchor as attachment site for $\beta$-1,6-glucan came from a preliminary compositional analysis of the aqueous HF-released $\beta$-1,6-glucan side chains, which were partially purified by DEAE-Trisacryl anion exchange chromatography. After hydrolysis of the aqueous HF-released material in trifluoroacetic acid, monosaccharides were identified by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) using known sugars as references (Van Rinsum et al., 1991; Montijn et al., 1994). The aqueous HF-released $\beta$-1,6-glucan side chains were shown to contain mannose, glucose, small amounts of glucosamine, and, presumably, galactose (unpublished results). Apart from glucose, the identified carbohydrates are known constituents of GPI-anchors from parasitic protozoa (McConville and Ferguson, 1993) and, interestingly, from a $c$AMP-binding cell surface protein of S. cerevisiae (Müller et al., 1992). Taken together, the data might implicate that the $\beta$-1,3/$\beta$-1,6-glucan heteropolymer is linked through its $\beta$-1,6-glucan moiety to a GPI anchor-derived structure (Figure 6). Further studies, however, are required to elucidate the exact linkage between the $\beta$-1,3/$\beta$-1,6-glucan heteropolymer and wall proteins, and to establish the hypothesis that the glucan heteropolymer is indeed linked to protein through the GPI-anchor.
**Materials and methods**

**Materials**
Phenylmethanesulfonyl fluoride, bovine serum albumin (BSA), 3-isobutyl-1-methylxanthine (IBMX), pronase E, mollusc laminarinase, bovine intestinal mucosa phosphodiesterase 1 (PDE I), and calf spleen phosphodiesterase II (PDE II) were purchased from Sigma. Endo-N-acetylglucosaminidase H (Endo H), leupeptin, and pepstatin were purchased from Boehringer Mannheim. Quantzyme α-L was from Quantum Biologicals Inc. Aqueous hydrofluoric acid (HF) was from Aldrich Chemical Co., Inc. Theophylline was obtained from BDH Chemicals Ltd. β-Mercaptoethanol was purchased from Fluka AG. DEAE-Trisacryl was supplied by Pharmacia Biotech Inc. Bio-Gel P6 was obtained from Bio-Rad Laboratories. BCA-protein assay reagent and goat-anti-rabbit IgG/horseradish peroxidase were from Pierce. Enhanced chemiluminescence (ECL) detection reagents was obtained from Amersham International. TRANSL S-LABEL was purchased from ICN Pharmaceuticals, Inc. Synthetic α-factor was kindly donated by Dr. Fred Naider (College of Staten Island, Staten Island, NY).

**Yeast strain and growth**

*S. cerevisiae* LB 347-1C (mmn9; MATα) was obtained from Dr. L. Ballou (Department of Biochemistry, University of California, Berkeley, CA). This mutant lacks mannoproteins with the highly extended N-chains, characteristic for wild type cells, but synthesizes, instead, proteins with short N-chains (Manα1-6GlcNAc). Cells were grown at 28°C in YPD medium (1% (w/v) yeast extract (Life Technologies, Inc.), 1% (w/v) Bacto-peptone (Difco), and 3% (w/v) glucose).

**Isolation of cell wall proteins**

Cell walls isolated from early exponential-phase cells (Van Rinsum et al., 1991) were extracted twice for 5 min at 100°C in 50 mM Tris-HCl, pH 8.0, containing 2% (w/v) SDS, 100 mM EDTA, and 40 mM β-mercaptoethanol (Schreuder et al., 1993; Kapteyn et al., 1994). Subsequently, SDS-extracted walls were washed five times with water and digested with mollusc laminarinase (α-L-glucanase preparation containing small amounts of β-1,6-glucanase and α-mannanase) (0.5 μg g⁻¹ (wet weight) of walls) to liberate the SDS-resistant wall proteins (Montijn et al., 1994). These proteins were partially purified by DEAE-Trisacryl anion exchange chromatography, desalted by gel filtration on Bio-Gel P-6, lyophilized, and stored at −20°C. SDS-resistant wall proteins were also obtained by digesting cell walls with Quantzyme, a pure recombinant α-L-glucanase (600 μg g⁻¹ (wet weight) of walls), as described before (Kapteyn et al., 1995). Moreover, SDS-extracted cell walls were digested at 37°C for 20 h with pure endo-β-1,3-glucanase II (10 μg g⁻¹ (wet weight) of walls) isolated from *Trichoderma harzianum* (De La Cruz et al., 1995) in 100 mM sodium acetate (pH 5.5). In some experiments, SDS-extracted walls (100 mg wet weight) were also treated with ice-cold aqueous HF (50% v/v; 10 μl) for 17 h. Subsequently, these walls were dried with a flow of nitrogen, washed three times with 90% (v/v) ice-cold methanol, and taken up in the sample buffer described by Laemmli (1970). Metabolic labeling of cells with [35S]methionine and induction of α-agglutinin synthesis with synthetic α-factor were according to Lu et al. (1994; 1995). After SDS-extraction of the labeled cells, Quantzyme- and endo-β-1,6-glucanase-released α-agglutinin were immunoprecipitated as described (Wojciechowicz et al., 1993; Lu et al., 1995). Labeled SDS-extracted cells were also treated with ice-cold aqueous HF as mentioned above. Upon this treatment, methanol-washed cells were again extracted with SDS, and α-agglutinin was immunoprecipitated from this extract according to Lu et al. (1994).

**Aqueous HF treatment of wall proteins**
Lyophilized laminarinase-released wall proteins were treated with ice-cold aqueous HF (50% v/v) according to a modified procedure described by Ferguson (1992). After aqueous HF treatment for 72 h, the proteins were dried under a constant nitrogen flow, washed with 90% (v/v) ice-cold methanol, and taken up in sample buffer.

**Enzymatic treatments of wall proteins**
To analyze the nature of the Quantzyme-released cell wall material, it was digested for 24 h at 37°C with pronase E in 50 mM Tris-HCl (pH 8.2), containing 2 mM CaCl₂. Laminarinase-extracted wall proteins were treated for 48 h at 37°C with phosphodiesterase PDE I (0.16 U) in 0.5 M Tris-HCl (pH 8.9). Similarly, PDE II digestion (0.16 U) was carried out in 50 mM potassium phosphate buffer, pH 6.0. In some experiments, the phosphodiesterase inhibitors theophylline and IBMX were added at a final concentration of 9 mM. Laminarinase-released proteins were also digested for 20 h at 37°C with endo-β-1,6-glucanase (0.4 U) in 100 mM sodium acetate (pH 5.5). Endo-β-1,6-glucanase digests of Quantzyme-released proteins were carried out under similar experimental conditions. Immunoprecipitated α-agglutinin released by aqueous HF was treated with Endo H as described (Lu et al., 1995).

**Analysis of glucanase-extractable cell wall proteins**
Cell wall proteins were separated by electrophoresis on linear gradient (2.2–20%) polyacrylamide gels (PAGE), and visualized by silver staining (De Nobel et al., 1989) or blotted electrophoretically onto an Immobilon polyvinylidene difluoride membrane for Western (immunoblot) analysis (Montijn et al., 1994). The membranes were blocked with 5% (v/v) milk powder in phosphate-buffered saline (PBS) and incubated with affinity-purified polyclonal antibodies directed against β-1,3-glucan or β-1,6-glucan. Both glucan antisera were used in a dilution of 1:5000 in PBS, containing 3% (v/v) BSA (Montijn et al., 1994; Kapteyn et al., 1995). The membranes were also incubated with a polyclonal antiserum directed against the known cell wall protein Cwp1p (Shimo et al., 1995). In this case, a serum dilution of 1:2500 in PBS, containing 3% (v/v) BSA was used.
used. Moreover, prior to the blocking step, the membranes were treated for 30 min with 50 mM periodic acid, 100 mM sodium acetate (pH 4.5), to enhance binding of the anti-Cwp1p antisera (Schreuder et al., 1993). The β-1,6-glucan specific antibodies were raised in rabbits by using a conjugate of β-1,6-p-glucan from Trichoderma harzianum and BSA as antigen (Montijn et al., 1994). This antisera was purified by affinity chromatography on a pustulan-Epoxy-Sepharose 6B column (Lu et al., 1995). Similarly, the β-1,3-glucan-specific antibodies were raised by using a conjugate of BSA and laminarin (β-1,3-glucan) which was precipitated in 0.25 M NaClO4 at pH 5.5 for 12 min. The β-1,3-glucan antisera was purified on a laminar-in-Epoxy-Sepharose 6B column. Binding of the antiserum was determined with goat-anti-rabbit IgG-peroxidase using ECL detection reagents. Immuno precipitated [32P]-labeled α-agglutinin was analysed on SDS-PAGE (3–15%) gels which were processed for immunoblotting with the β-1,6-glucan antisera (Lu et al., 1995). Subsequently, the blots were submitted to fluorography (Lu et al., 1994, 1995).

Analytical methods

The efficacy of laminarinase, Quantazyme, endo-β-1,6-glucanase, and aqueous HF to release proteins from SDS-extracted cell walls was determined by measuring the protein content of the cell walls after treatment with these agents. To this end, the resulting cell wall residues were extracted twice in SDS-extraction buffer without β-mercaptoethanol for 5 min at 100°C, rinsed five times with water, and heated in 1 N NaOH at 100°C for 10 min. Subsequently, the protein concentration in the alkaline extract was determined with the BCA-protein assay reagent with BSA as a reference protein.

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