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

Kapteijn, J.C.; Montijn, R.C.; Vink, E.; de la Cruz, J.; Llobell, A.; Douwes, J.E.; Shimoi, H.; Lipke, P.N.; Klis, F.M.

DOI
10.1093/glycob/6.3.337

Publication date
1996

Published in
Glycobiology

Citation for published version (APA):
Kapteijn, J. C., Montijn, R. C., Vink, E., de la Cruz, J., Llobell, A., Douwes, J. E., Shimoi, H., Lipke, P. N., & Klis, F. M. (1996). Retention of Saccharomyces cerevisiae cell wall proteins through a phosphodiester-linked beta-1,3-/beta-1,6-glucan heteropolymer. *Glycobiology*, *6*(3), 337-345. https://doi.org/10.1093/glycob/6.3.337

Glycobiology vol. 6 no. 3 pp. 337–345, 1996

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

Johan C. Kapteyn[5], Roy C. Montijn, Edwin Vink, Jesús de la Cruz[1], Antonio Llobell[1], Jeroen E. Douwes[2], Hitoshi Shimoi[3], Peter N. Lipke[4] and Frans M. Klis

Institute of Molecular Cell Biology, BioCentrum Amsterdam, University of Amsterdam, The Netherlands, [1]Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas, Universidad de Sevilla, 41080-Seville, Spain, [2]Department of Epidemiology and Public Health, Agricultural University Wageningen, Wageningen, The Netherlands, [3]National Research Institute of Brewing 8511-14, Misonou, Saijo-cho, Higashihiroshima-shi, 739 Japan, and [4]Department of Biological Sciences and Institute for Biomolecular Structure and Function, Hunter College of the City University of New York, New York 10021, USA

[5]To whom correspondence should be addressed

Yeast cell wall proteins, including Cwp1p and α-agglutinin, could be released by treating the cell wall with either β-1,3- or β-1,6-glucanases, indicating that both polymers are involved in anchoring cell wall proteins. It was shown immunologically that both β-1,3- and β-1,6-glucan were linked to yeast cell wall proteins, including Cwp1p and α-agglutinin. It was further shown that β-1,3-glucan was linked to the wall protein through a β-1,6-glucan moiety. The β-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 β-1,3-/β-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 β-1,3-/β-1,6-glucan heteropolymer characterized by Fleet and Manners (1976, 1977).

Key words: α-agglutinin/*Candida albicans*/CWP1/fungal wall/GPI-anchor/mannoproteins

Introduction

The cell wall of the yeast *Saccharomyces cerevisiae* consists of complex polymers of glucose (β-1,3-glucan, β-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 β-1,3-glucanase digestion, suggesting that they are tightly bound to the β-1,3-glucan skeleton of the cell wall (Valentin *et al.*, 1984; Zlotnik *et al.*, 1984; Frevert and Ballou, 1985; Van Rinsum *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; Shimoi *et al.*, 1995; Van der Vaart *et al.*, 1995). As for the precursors of the pheromone-inducible cell wall protein α-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 α-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 β-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 β-1,6-glucan-containing moiety, which has been proposed to couple the proteins to the β-1,3-glucan framework (Tkacz, 1984; Van Rinsum *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 β-1,6- and β-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 β-1,3-glucan-β-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 β-1,3-linked glucose residues, and the smallest one containing approximately 150 β-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 β-1,6- and β-1,3-glucan. Evidence is presented that the β-1,6-glucan moiety connects Cwp1p, α-agglutinin, and other cell wall mannoproteins with the β-1,3-glucan part of this complex. The data further indicate that the attachment of the β-glucan complex is most likely responsible for anchoring the proteins into the cell wall. In addition, the β-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 β-1,6-glucan (De Nobel and Lipke, 1994).

Results

β-1,3- /β-1,6-glucosylated cell wall proteins

Laminarinase, a β-1,3-glucanase preparation with some β-1,6-glucanase and α-mannanase activities, and Quantazyme, a pure β-1,3-glucanase, were found to liberate about 75 and 60% of SDS-resistant cell wall proteins of *S.cerevis-*

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.

Table I. Release of wall proteins from SDS-extracted isolated walls of *Saccharomyces cerevisiae mnn9* cells; after the different treatments the remaining protein in the cell walls was determined

Treatment	Protein content (μg mg^{-1} wet weight of walls)	Percentage (%) of proteins released
Control	0.50	0
Laminarinase	0.13	74
Quantazyme	0.21	58
Endo-β-1,6-glucanase	0.07	86
Aqueous HF	0.26	48

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-

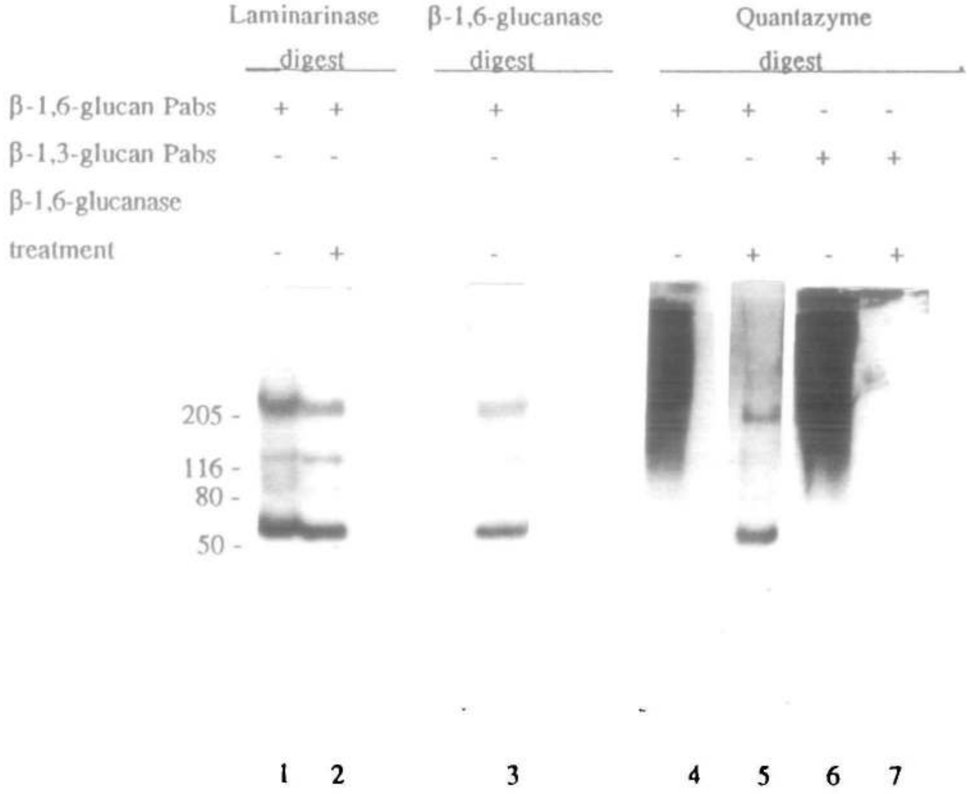

Fig. 1. Western analysis of the laminarinase- (lanes 1 and 2), endo-β-1,6-glucanase- (lane 3), and Quantazyme-released (lanes 4–7) wall proteins of *mnn9* cells using the affinity-purified β-1,6-glucan antiserum (β-1,6-glucan Pabs; lanes 1–5) and β-1,3-glucan antiserum (β-1,3-glucan Pabs; lanes 6 and 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).

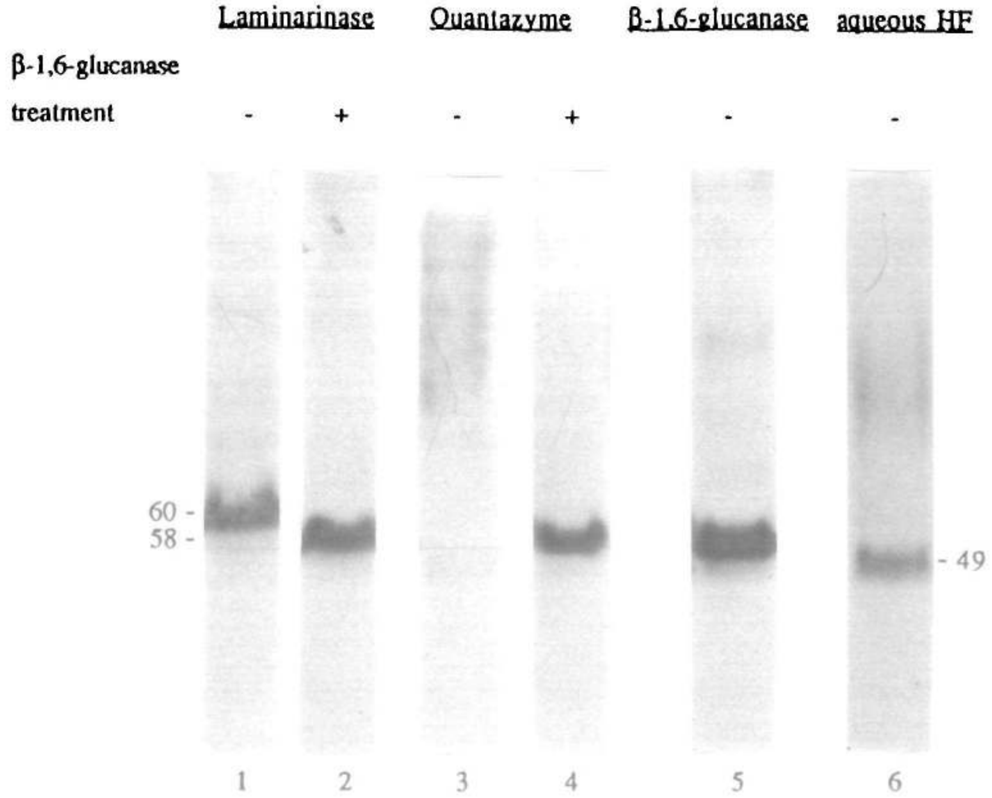

Fig. 2. Western analysis of laminarinase- (lanes 1 and 2), Quantazyme- (lanes 3 and 4), endo-β-1,6-glucanase- (lane 5), and aqueous HF-released wall proteins with the anti-Cwp1p antiserum. Lanes 1, 3, 5, and 6, immunodetection before endo-β-1,6-glucanase treatment. Lanes 2 and 4, after endo-β-1,6-glucanase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

pearance of the Quantazyme-released β-1,6- and β-1,3-glucosylated smear, containing Cwp1p, and to the appearance of four major silver-stainable bands with apparent molecular masses of about 235, 130, 100, and 58 kDa (Figure 3A, lane 2), that did not react with the β-1,3-glucan antiserum (Figure 1, lane 7). The 58 kDa-band was again found to be reactive with the anti-Cwp1p antiserum (Figure 2, lane 4), whereas the β-1,6-glucan antiserum only recognized the 230 and 58 kDa-bands (Figure 1, lane 5). Possibly, due to the endo-β-1,6-glucanase treatment, the β-1,6-glucan epitopes attached to the 130 and 100 kDa bands were too small to be recognized by the 1,6-glucan antiserum. Consequently, the data demonstrate that the β-1,3-glucan epitope is linked through a β-1,6-glucan moiety to Cwp1p and other wall proteins. The β-1,6-glucosylated bands found after endo-β-1,6-glucanase treatment of the Quantazyme digests, the lowest one representing Cwp1p, had similar molecular masses as the laminarinase-released ones when these were treated with the β-1,6-glucanase (Figure 1, compare lanes 2 and 5; Figure 2, compare lanes 2 and 4). This finding indicates that both β-1,3-glucanase preparations release the same set of wall proteins, but differ in their ability to digest the β-glucan moiety of these proteins.

Assuming that the protein-bound β-1,6-/β-1,3-glucan heteropolymer is responsible for anchoring these cell wall proteins, one should expect that digestion with β-1,6-glucanase alone should be enough to release cell wall proteins. Indeed, this enzyme was found to free wall proteins with similar molecular masses as those released by Quantazyme followed by treatment with the β-1,6-glucanase (Figure 3A, compare lanes 2 and 3). Cwp1p was also released (Figure 2, lane 5). Approximately 90% of total SDS-resistant cell wall proteins were solubilized by the endo-β-1,6-glucanase (Table I). As expected, the endo-β-1,6-glucanase-liberated proteins, including Cwp1p, reacted less strongly with the β-1,6-glucan antiserum and were 2 to 10 kDa smaller in size than the laminarinase-released ones (Figure 1, compare lanes 1 and 3; Figure 2, lanes 1 and 5). The data clearly demonstrate that the β-1,6-glucan is essential for the immobilization of Cwp1p and other proteins in the cell wall.

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

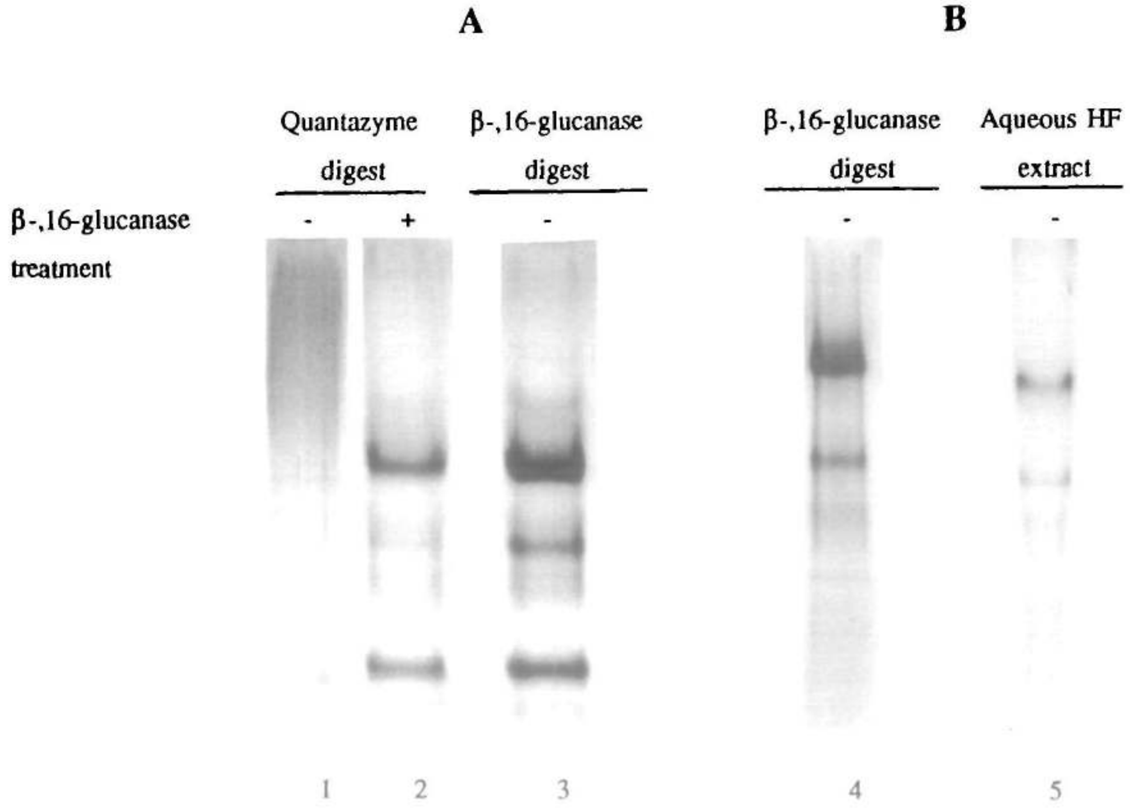

Fig. 3. Characterization of the cell wall proteins released by Quantazyme, endo-β-1,6-glucanase, and aqueous HF from *mnn9* cells by silver staining (**A**) Lanes 1 and 2, Quantazyme-released proteins, before and after treatment with endo-β-1,6-glucanase, respectively; lane 3, endo-β-1,6-glucanase-released wall proteins. (**B**) comparison of the endo-β-1,6-glucanase-released wall proteins (lane 4) with those released by aqueous HF (lane 5).

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.

Retention 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

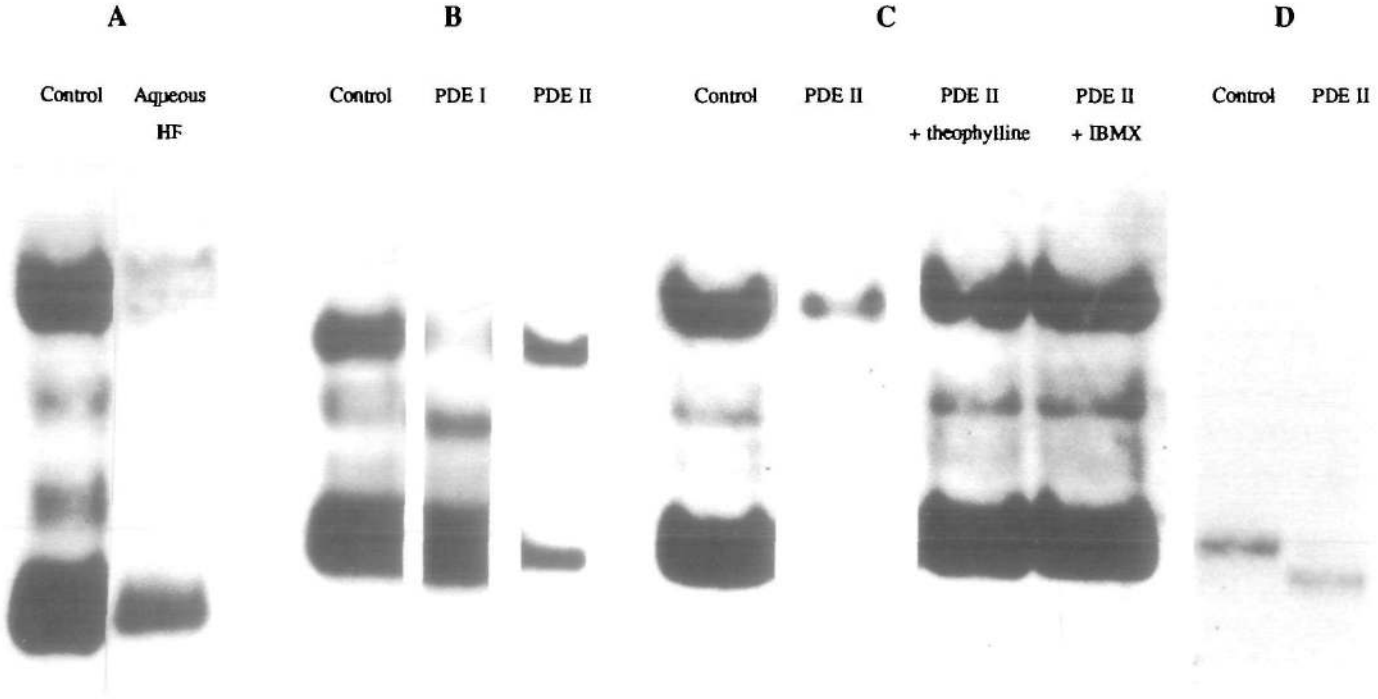

Fig. 4. The effect of ice-cold aqueous HF and phosphodiesterases PDE I and II on reactivities of the laminarinase-released wall proteins from *mnn9* cells with the affinity-purified β-1,6-glucan antiserum (lanes 1–9) and the anti-Cwp1p antiserum (lanes 10 and 11). (**A**) Lane 1, control; lane 2, after 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

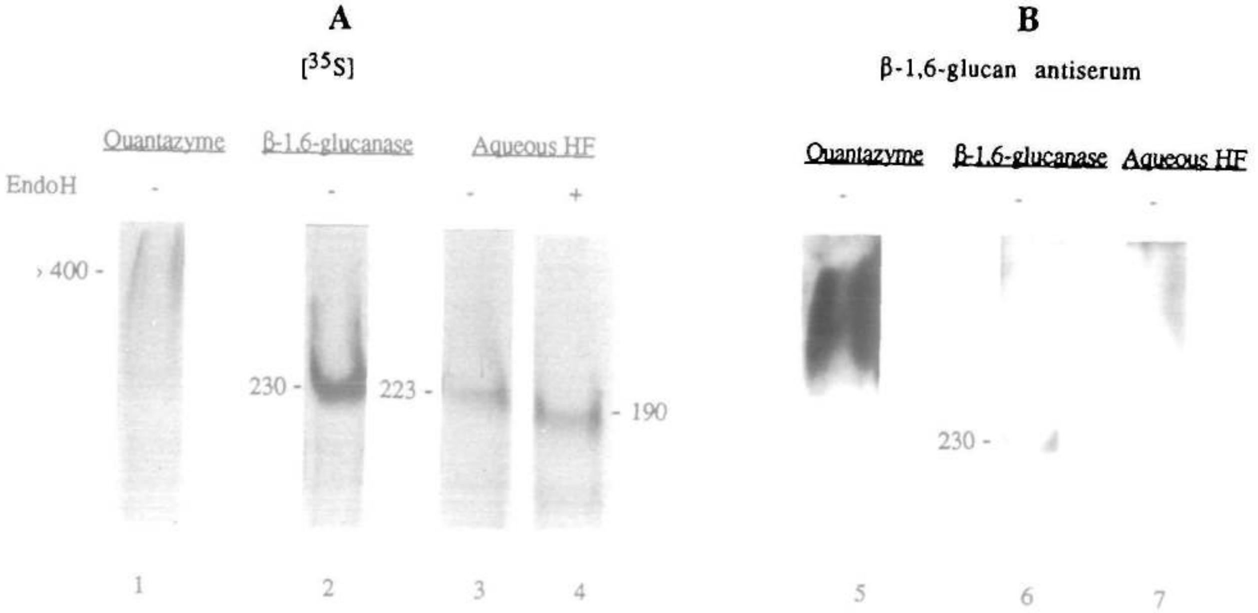

Fig. 5. Characterization of immunoprecipitated [^{35}S]-labeled α-agglutinin, which was released with Quantazyme (lanes 1 and 5), endo-β-1,6-glucanase (lanes 2 and 6), and aqueous HF (3, 4, 7) from SDS-extracted walls of *mnn9* cells, by fluorography (lanes 1–4), and by Western analysis with the affinity-purified β-1,6-glucan antiserum (lanes 5–7). Lane 4, after treatment with Endo H. The molecular size of the different forms of α-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 β-1,6-glucosyl moiety, probably as part of a larger β-1,3-/β-1,6-glucan heteropolymer, is responsible for retaining it in the cell wall.

Discussion

Recently, laminarinase-extractable yeast cell wall proteins such as α-agglutinin, Cwp1p, and Tip1p have been shown to have a β-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 Quantazyme, a pure β-1,3-glucanase preparation in contrast to laminarinase, which is a mixture of β-1,3- and β-1,6-glucanase activities. The Quantazyme-liberated β-1,6-glucosylated wall proteins, including Cwp1p and α-agglutinin, had much higher molecular masses, most likely because they also carried a β-1,3-glucan epitope (Figs. 1, 2, 5). Comparable results were obtained with *C.albicans* (Kapteyn *et al.*, 1995b). Presumably, Quantazyme was hindered by β-1,6 branch points in the β-1,3-glucan cell wall polymers, thereby leaving some undigested β-1,3-glucan attached to the β-1,6-glucan moiety of the proteins. Since an endo-β-1,6-glucanase could remove the β-1,3-glucan epitope, it was concluded that β-1,3-glucan was attached to protein through the β-1,6-glucan moiety (Figure 1). The presence of a protein-bound β-1,3-/β-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 β-1,3-glucanase-extractable wall proteins, including Cwp1p and α-agglutinin, could also be freed by endo-β-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 β-1,3-/β-1,6-glucan heteropolymer represents a general mechanism for anchoring cell wall proteins. In accordance with this, several *kre* mutants, which have defects in β-1,6-glucan synthesis, were found to secrete more α-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 *kre1 cwh41* double disruptants showed a 75% reduction in the cell wall β-1,6-glucan level and secretion of Cwp1p (Jiang *et al.*, 1996). The identification of a β-1,6-glucan-β-1,3-glucan heteropolymer bound to protein is also consistent with the data obtained by Fleet and Manners (1976, 1977). These authors found an alkali-soluble free polymer consisting of 90% β-1,3-glucan in one domain, and approximately 10% β-1,6-glucan and some mannose in a second region. Since alkali-treatment removes all cell wall proteins, it is tempting to speculate that the β-1,3-/β-1,6-glucan polymer described by Fleet and Manners (1976, 1977) is *in vivo* attached to protein and identical to the protein-bound β-glucan complex identified in this study.

To date, little information has been obtained about the exact carbohydrate composition and structure of the protein-bound β-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 β-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, α-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 β-1,6-glucosylated cell wall proteins (Kapteyn *et al.*, 1995a). Several other studies pointed to the involvement of GPI-anchors in the attachment of β-1,6-glucan to the wall proteins. First, a secretory reporter protein extended with the GPI-anchor addition sequence of the cell wall protein α-agglutinin became immobilized in the cell wall of *S.cerevisiae* and contained β-1,6-glucan, whereas the nonextended and secreted form was not glucosylated (Van Berkel *et al.*, 1994). Second, in contrast to intact α-agglutinin, a C-terminal truncated form, which was not GPI-anchored and was secreted into the medium, did not contain β-1,6-glucan (Lu *et al.*, 1995). Third, *S.cerevisiae cwh6* , a calcofluor white hypersensitive cell wall mutant, that showed a strongly reduced retention of α-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 β-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 α-agglutinin (Figure 5, lane 7), appeared to be non-β-glucosylated. Another line of evidence for the GPI-anchor as attachment site for β-1,6-glucan came from a preliminary compositional analysis of the aqueous HF-released β-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 β-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 cAMP-binding cell surface protein of *S.cerevisiae* (Müller *et al.*, 1992). Taken together, the data might implicate that the β-1,3/β-1,6-glucan heteropolymer is linked through its β-1,6-glucan moiety to a GPI anchor-derived structure (Figure 6). Further studies, however, are required to elucidate the exact linkage between the β-1,3/β-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.

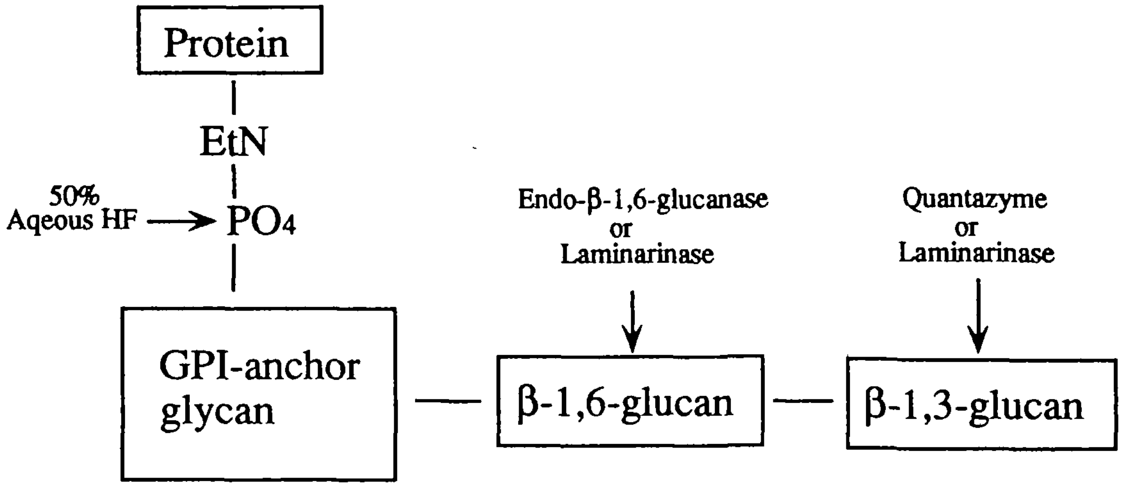

Fig. 6. Postulated attachment of a β-1,3-/ β-1,6-glucan heteropolymer (Fleet and Manners, 1976, 1977) to the GPI-anchor derived glycan moiety of yeast cell wall proteins. EtN, Ethanolamine.

Materials and methods

Materials

Phenylmethylsulfonyl fluoride, bovine serum albumin (BSA), 3-isobutyl-1-methylxanthine (IBMX), pronase E, mollusc laminarinase, bovine intestinal mucosa phosphodiesterase I (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. Quantazyme *ylg* was from Quantum Biotechnologies 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. TRAN^{35}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 (*mnn9, 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 ($Man_{10-14}GlcNAc_2$). Cells were grown at 28°C in YPD medium (1% (w/v) yeast extract (Life Technologies, Inc.), 1% (w/v) Bactopeptone (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 (a β-1,3-glucanase preparation containing small amounts of β-1,6-glucanase and α-mannanase) (0.5 U 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 Quantazyme, a pure recombinant β-1,3-glucanase (600 U 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-1,6-glucanase II (10 U 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; 100 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 [^{35}S]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, Quantazyme-, 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 Quantazyme-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 $CaCl_2$. 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 digestions of Quantazyme-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% (w/v) milk powder in phosphate-buffered saline (PBS) and incubated with affinity-purified polyclonal antibodies directed against β-1,6-glucan or β-1,3-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 (Shimoi *et al.*, 1995). In this case, a serum dilution of 1:2500 in PBS, containing 3% (v/v) BSA was 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 antiserum (Schreuder *et al.*, 1993). The β-1,6-glucan specific antibodies were raised in rabbits by using a conjugate of pustulan oligosaccharides (β-1,6-glucan) and BSA as antigen (Montijn *et al.*, 1994). This antiserum 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 preoxidized in 0.25 M $NaIO_4$ at 20°C for 60 min. The β-1,3-glucan antiserum was purified on a laminarin-Epoxy-Sepharose 6B column. Binding of the antisera was determined with goat-anti-rabbit IgG-peroxidase using ECL detection reagents. Immunoprecipitated [^{35}S]-labeled α-agglutinin was analysed on SDS–PAGE (3–15%) gels which were processed for immunoblotting with the β-1,6-glucan antiserum (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

Acknowledgements

The authors thank Stephan Brekelmans for his help with HPAEC-PAD. J.C.K. acknowledges the funding of this project by Glaxo Research Group. P.N.L. was supported by National Institute of General Medical Sciences (NIGMS).

References

Bishop,C.T., Blank,F. and Gardner,P.E. (1960) The cell wall polysaccharides of *Candida albicans*: glucan mannan and chitin *Can. J. Chem.*, **38,** 869–881.

De La Cruz,J., Pintor-Toro,J.A , Benitez,T. and Llobell,A. (1995) Purification and characterization of an endo-β-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. *J. Bacteriol.*, **177,** 1864–1871.

De Nobel,J.G., Dijkers,C., Hooijenberg,E. and Klis,F.M. (1989) Increased cell wall porosity in *Saccharomyces cerevisiae* after treatment with dithiothreitol or EDTA. *J. Gen Microbiol*, **135,** 2077–2084.

De Nobel,J.G. and Lipke,P.N. (1994) Is there a role for GPIs in yeast cell wall assembly? *Trends Cell Biol.*, **4,** 41–45.

Ferguson,M.A. (1992) Chemical and enzymatic analysis of glycosylphosphatidylinositol anchors. In Turner,A.J. and Hooper,N. (eds), *Lipid Modification of Proteins. A Practical Approach.* IRL Press, Oxford, pp. 191–230.

Fleet,G.H. (1991) Cell walls. In Rose,A.H. and Harrison, .S. (eds), *The Yeasts, Vol. 4.* Academic Press, London, pp. 199–277.

Fleet,G.H. and Manners,D.J (1976) Isolation and composition of an alkali-soluble glucan from the cell walls of *Saccharomyces cerevisiae. J. Gen. Microbiol.*, **94,** 180–192.

Fleet,G.H. and Manners,D.J. (1977) The enzymatic degradation of an alkali-soluble glucan from the cell wall of *Saccharomyces cerevisiae. J. Gen. Microbiol.*, **98,** 315–327.

Frevert,J. and Ballou,C.E. (1985) *Saccharomyces cerevisiae* structural cell wall mannoprotein. *Biochemistry,* **24,** 753–759.

Hasegawa,S. and Yanagishima,N. (1984) Alpha mating type-specific suppression and a mating type specific enhancement of sexual agglutinability in *Saccharomyces cerevisiae. Arch. Microbiol.*, **138,** 310–314.

Jiang,B., Sheraton,J., Ram,A.F.J., Dijkgraaf,G.J.P., Klis,F.M. and Bussey,H. (1996) *CWH41* encodes a novel endoplasmic reticulum membrane *N*-glycoprotein involved in β-1.6-glucan assembly. *J. Bacteriol.*, **178,** 1162–1171.

Kapteyn,J.C., Montijn,R.C., Dijkgraaf,G.J.P. and Klis,F.M. (1994) Identification of β-1.6-glucosylated cell wall proteins in yeast and hyphal forms of *Candida albicans. Eur. J. Cell Biol.*, **65,** 402–407.

Kapteyn,J.C., Dijkgraaf,G.J P., Montijn,R.C and Klis,F.M. (1995a). Glucosylation of cell wall proteins in regenerating spheroplasts of *Candida albicans. FEMS Microbiol. Lett.*, **128,** 271–277.

Kapteyn,J.C., Montijn,R.C., Dijkgraaf,G.J.P., Van Den Ende,H. and Klis,F.M. (1995b) Covalent association of β-1,3-glucan with β-1,6-glucosylated mannoproteins in cell walls of *Candida albicans. J. Bacteriol.*, **177,** 3788–3792.

Klis,F.M. (1994) Review: cell wall assembly in yeast. *Yeast,* **10,** 851–869.

Kollár,R , Petrakova,E., Ashwell,G., Robbins,P.W. and Cabib,E. (1995) Architecture of the yeast cell wall: the linkage between chitin and β(1–3)-glucan. *J. Biol. Chem.*, **270,** 1170–1178.

Kondo,K. and Inouye,M. (1991) *TIP1*, a cold shock-inducible gene of *Saccharomyces cerevisiae. J. Biol. Chem.*, **266,** 17537–17544.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature,* **277,** 680–685.

Lipke,P.N., Wojciechowicz,D. and Kurjan,J (1989) *Aga1* is the structural gene for the *Saccharomyces cerevisiae* α-agglutinin, a cell surface glycoprotein involved in cell–cell interactions during mating. *Mol Cell Biol.*, **9,** 3155–3165.

Lu,C.-F , Kurjan,J. and Lipke,P.N. (1994) A pathway for cell wall anchorage of *Saccharomyces cerevisiae* α-agglutinin. *Mol. Cell Biol*, **14,** 4825–4833.

Lu,C.-F., Montijn,R.C., Brown,J.L., Klis,F.M., Kurjan,J., Bussey,H. and Lipke,P.N. (1995) Glycosyl phosphatidylinositol-dependent cross-linking of α-agglutinin and β-1,6-glucan in the *Saccharomyces cerevisiae* cell wall. *J. Cell Biol.*, **128,** 333–340.

McConville,M.J. and Ferguson,M.A.J. (1993) The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. *Biochem. J.*, **294,** 305–324.

Montijn,R.C., Van Rinsum,J., Van Schagen,F.A. and Klis,F.M. (1994) Glucomannoproteins in the cell wall of *Saccharomyces cerevisiae* contain a novel type of carbohydrate side chain. *J. Biol Chem.*, **269,** 19338–19342.

Mort,A.J. and Lamport,D.T.A. (1977) Anhydrous hydrogen fluoride deglycosylates glycoproteins. *Anal. Biochem.*, **82,** 289–309.

Müller,G., Schubert,K., Fiedler,F. and Bandlow,W. (1992) The cAMP-binding ectoprotein from *Saccharomyces cerevisiae* is membrane-anchored by glycosyl-phosphatidyl-inositol. *J. Biol. Chem.*, **267,** 25337–25346.

Roy,A., Lu,C.-F., Marykwas,D.L., Lipke,P.N. and Kurjan,J. (1991) The *AGA1* product is involved in cell surface attachment of the *Saccharomyces cerevisiae* cell adhesion glycoprotein **a**-agglutinin. *Mol. Cell. Biol.*, **11,** 4196–4206.

Schreuder,M.P., Brekelmans,S., Van Den Ende,H. and Klis,F.M. (1993) Targeting of a heterologous protein to the cell wall of *Saccharomyces cerevisiae. Yeast,* **9,** 399–409.

Shimoi,H., Iimura,Y. and Obata,T. (1995) Molecular cloning of *CWP1*: a gene encoding a *Saccharomyces cerevisiae* cell wall protein solubilized with *Rarobacter faecitabidus* protease I. *J. Biochem.*, **118,** 302–311.

Tanner,W. and Lehle,L. (1987) Protein glycosylation in yeast. *Biochim. Biophys. Acta,* **906,** 81–99.

Tkacz,J.S. (1984) *In vivo* synthesis of β-1,6-glucan in *Saccharomyces cerevisiae.* In Nombela,C. (ed), *Microbial Cell Wall Synthesis and Autolysis* Elsevier Science Publishers BV, Amsterdam, pp. 287–295.

Terrance,K. (1983) Ph.D. dissertation, CUNY, New York.

Teunissen,A.W.R.H., Holub,E., Van Der Hucht,J., Van Den Berg,J.A. and Steensma,H.Y. (1993) Sequence of the open reading frame of the *FLO1* gene from *Saccharomyces cerevisiae. Yeast,* **9,** 423–427.

Tsai,P.-K., Frevert,J. and Ballou,C.E (1984) Carbohydrate structure of *Saccharomyces cerevisiae mnn9. J. Biol Chem.*, **259,** 3805–3811.

Valentin,E., Herrero,E., Pastor,F.I.J. and Sentandreu,R. (1984) Solubilization and analysis of mannoprotein molecules from the cell wall of *Saccharomyces cerevisiae. J. Gen. Microbiol.*, **130,** 1419–1428.

Van Berkel,M.A.A., Caro,L.H.P., Montijn,R.C. and Klis,F.M. (1994) Glucosylation of chimeric proteins in the cell wall of *Saccharomyces cerevisiae. FEBS Lett.*, **349,** 135–138.

Van Der Vaart,J.M., Caro,L.H.P., Chapman,J.W., Klis,F.M. and Verrips,C.T. (1995) Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae. J. Bacteriol.*, **177,** 3104–3110.

Van Rinsum,J., Klis,F.M. and Van Den Ende,H (1991) Cell wall glucomannoproteins of *Saccharomyces cerevisiae mnn9. Yeast,* **7,** 717–726.

Vossen,J.H., Ram,A.F.J. and Klis,F.M. (1995) Identification of *SPT14/CWH6* as the yeast homologue of hPIG-A, a gene involved in the biosynthesis of GPI anchors. *Biochim. Biophys. Acta,* **1243,** 549–551.

Wojchiechowicz,D., Lu,C.-F., Kurjan,J. and Lipke,P.N. (1993) Cell surface anchorage and ligand-binding domains of the *Saccharomyces cerevisiae* cell adhesion protein α-agglutinin, a member of the immunoglobulin superfamily. *Mol. Cell Biol.*, **13,** 22554–2563.

Yu,R.J., Bishop,C.T., Cooper,F.P., Hasenclever,H.F. and Blank,F (1967) Structural studies of mannans from *Candida albicans* (serotypes A & B), *Candida parapsilosis*, *Candida stellatoidea* & *Candida tropicalis*. *Can. J. Chem.*, **45**, 2207–2211.

Zlotnik,H., Fernandez,M.P., Bowers,B. and Cabib,E. (1984) *Saccharomyces cerevisiae* mannoproteins form an external cell wall layer that determines wall porosity. *J. Bacteriol.*, **159**, 1018–1026.

Received on November 30, 1995; accepted on January 9, 1996