β -Glucosylated proteins in the cell wall of the black yeast *Exophiala (Wangiella) dermatitidis*

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Wild-type cells of the pathogenic black yeast *Exophiala (Wangiella) dermatitidis* grown in a low-pH ascorbate medium became less melanized and less resistant to Zymolyase. This was accompanied by increased staining with fluorescently labelled concanavalin A. The sugar composition of wild-type and mutant cell walls was, except for the presence of galactose, similar to that of *Saccharomyces cerevisiae*. Digestion of mutant cell walls with laminarinase released galactomannoproteins. In addition, the released cell wall proteins contained glucose and reacted with affinity-purified 1,6- β -glucan antiserum, indicating that they are linked to 1,6- β -glucan. It is proposed that 1,6- β -glucosylated cell wall proteins generally occur among ascomycetes.

Keywords: cell wall proteins, 1,6- β -glucan, galactomannoproteins, black yeast, *Exophiala dermatitidis*

INTRODUCTION

Exophiala (Wangiella) dermatitidis, a black yeast with affinity to the ascomycete family Herpotrichiellaceae, is an uncommon agent of (sub)cutaneous mycoses and deep mycoses. Traumatic mycoses result from contaminated materials from the environment (Crosby *et al.*, 1989). The infection route of systemic mycoses is presently unknown. Two prevalent types have been reported: (i) subclinical colonization of the lungs of patients with cystic fibrosis (Haase *et al.*, 1991; Kusenbach *et al.*, 1992), and (ii) neurotropic dissemination (Hohl *et al.*, 1983; Matsumoto *et al.*, 1984), mostly in otherwise healthy patients. The latter clinical type has hitherto been found in Asia only (Hiruma *et al.*, 1993; Matsumoto *et al.*, 1993). Systemic dissemination in European patients with impaired natural immunity is transient, without causing any major disorder (Blaschke-Hellmessen *et al.*, 1994; Kabel *et al.*, 1994). The two types of systemic mycoses are caused by strains which are indistinguishable from each other, and therefore the possibility of racial host factors has been surmised (Uijthof *et al.*, 1994).

The cell wall has been proposed to play a significant role

in the pathogenicity of black yeasts. The presence of melanin and carotene in the cell wall protects the organism against environmental stress, such as dehydration and UV irradiation (Geis & Szaniszló, 1984), but also against the oxygenic burst of mammalian phagocytes. In addition, melanin plays a role in resistance against cell wall lytic enzymes, for example chitinase and 1,3- β - and 1,6- β -glucanase (Taylor *et al.*, 1987; Polak, 1989; Dixon *et al.*, 1991). Dixon *et al.* (1987, 1992) found that melanized strains are more virulent than non-melanized mutants. However, factors other than melanin must also be involved, since non-melanized mutants remain pathogenic to some extent (Dixon *et al.*, 1987, 1989; Polak & Dixon, 1993).

In *Saccharomyces cerevisiae*, about 50 % of the cell wall dry weight is accounted for by mannoproteins, and the other 50 % is composed of β -glucans and a small amount of chitin (Fleet, 1991; Klis, 1994). The β -glucans can be subdivided into 1,3- β -glucan (80 %) and 1,6- β -glucan (20 %) (Fleet, 1991). The 1,3- β -glucan gives the cell wall its strength, forming a network in which proteins are anchored. Mannoproteins are of two types: the SDS-extractable mannoproteins, which are loosely associated with the 1,3- β -glucan network (Zueco *et al.*, 1986; Hård *et al.*, 1989; Herscovics & Orlean, 1993) and the SDS-resistant glucanase-extractable mannoproteins, which can only be released from the wall by digestion with 1,3- β -glucanases (Pastor *et al.*, 1984), and mostly carry N- and/or O-glycan side-chains. The O-linked glycans are

Abbreviations: ConA, concanavalin A; GN, glucosamine/N-acetylglucosamine; Endo H, endoglycosidase H; FITC, fluorescein isothiocyanate; SG, Sabouraud glucose.

short linear chains consisting of between one and five mannose residues linked to serine or threonine (Herscovics & Orlean, 1993). The N-linked glycans consist of a chitobiose unit linked to asparagine, elongated with up to 150–200 mannose residues (Ballou, 1990). A third type of carbohydrate side-chain containing 1,6- β -linked glucose residues and 1,6- α -linked mannose residues has recently been described (van Rinsum *et al.*, 1991; Montijn *et al.*, 1994). These 1,6- β -glucan-containing side-chains were insensitive to endo-glycosidase H treatment or β -elimination, indicating that they were distinct from N- and O-glycan side-chains. Recently, it was demonstrated that 1,6- β -glucan chains are cross-linked to 1,3- β -linked glucose residues (Kapteyn *et al.*, 1995, 1996). In *S. cerevisiae*, the cell wall proteins α -agglutinin, Cwp1p and Tip1p have been shown to be 1,6- β -glucosylated. These proteins are all rich in serine/threonine and contain a glycosyl-phosphatidylinositol (GPI) attachment signal (Lu *et al.*, 1995; Van der Vaart *et al.*, 1995). It is believed that the 1,6- β -glucan side chain is linked to the protein through the GPI-anchor (Kapteyn *et al.*, 1994; Lu *et al.*, 1994; Kapteyn *et al.*, 1996). Lu *et al.* (1995) have shown that 1,6- β -glucan becomes attached to α -agglutinin upon incorporation into the cell wall. Attachment of 1,6- β -glucan to proteins is a prerequisite for their incorporation in the cell wall (Van Berkel *et al.*, 1994), which in turn is essential for cell viability (Roemer *et al.*, 1993).

In *E. dermatitidis*, α -mannan, β -glucan and chitin are present in the cell wall (Geis, 1981; Szaniszló *et al.*, 1983). α -Mannan is present in the outer layer, while β -glucan is generally distributed over the cell wall. β -Glucan appears at least to a large extent to consist of 1,3- β -glucan, because melanin-deficient mutants (Taylor *et al.*, 1987) and cells albinized with tricyclazole (Dixon *et al.*, 1991) proved to be sensitive to Zymolyase, a 1,3- β -glucanase. In this paper we show that wild-type cells of *E. dermatitidis* grown in low-pH ascorbate medium became less melanized and less resistant to Zymolyase. In addition, we show that cell walls of *E. dermatitidis* are similar to those of *S. cerevisiae*, both in sugar composition and with respect to the presence of 1,6- β -glucosylated cell wall proteins.

METHODS

Yeast strains and growth conditions. *E. dermatitidis* (CBS 207.35) was obtained from the Centraalbureau voor Schimmelcultures, Baarn, the Netherlands. Cells were grown in liquid 1/2 Sabouraud glucose (SG) medium containing 0.5% (w/v) Bactopeptone (Difco), 1% (w/v) glucose (Merck), 0.001% (w/v), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.001% (w/v) KH_2PO_4 overnight at 30 °C (exponential cells) on a rotary shaker at 150 r.p.m. in order to culture the fungus in its yeast form, or in YPD liquid medium containing 1% (w/v) yeast extract (Oxoid), 1% (w/v) special peptone (Oxoid), 2% (w/v) D-glucose (Merk) for 4 d at 30 °C (stationary phase) on a rotary shaker at 150 r.p.m. A mutant strain of *E. dermatitidis* (ATCC 44504), which is defective in the *mel3* gene, was obtained from Dr G. Haase (Institut für Medizinische Mikrobiologie, Klinikum RWTH Aachen, Aachen, Germany). Cells were grown overnight at 30 °C in liquid SG medium containing 1%

(w/v) Bactopeptone, 1% (w/v) glucose and were shaken at 150 r.p.m.

Inhibition of melanin synthesis on solid medium or in liquid medium. Cells were grown on low-pH ascorbate medium plates (pH 3.0), containing 1.17% (w/v) Bacto yeast carbon base (YCB; Difco), 0.33% (w/v) ethylamine.HCl (Fluka) and 0.8% (w/v) agar. Filter-sterilized YCB solution and sterile solutions of ascorbic acid (Merck) (final concentration 0.1%, w/v) were added to autoclaved agar after cooling to 50 °C (Butler & Lachance, 1987).

Liquid medium consisted of 1.17% (w/v) YCB and 0.33% (w/v) ethylamine.HCl. A filter-sterilized solution of ascorbic acid was added to a final concentration of 0.1% (w/v). The cultures were incubated overnight at 30 °C, 150 r.p.m.

Isolation of cell walls. Cells were isolated by centrifugation for 20 min at 6000 r.p.m. at 4 °C, and subsequently suspended in 100 μg sodium azide ml^{-1} (Baker Chemicals) and 5 μg cycloheximide ml^{-1} (Fluka). Cells were broken by glass beads and cell walls were isolated from the glass beads and washed three times with 1 M NaCl, 1 mM PMSF (Boehringer Mannheim), and subsequently washed with 1 mM PMSF (Montijn *et al.*, 1994).

Isolation of cell wall proteins by extraction with SDS and laminarinase. Cell wall proteins were isolated as previously described (Montijn *et al.*, 1994). Cell walls were boiled in 2% (w/v) SDS to isolate SDS-extractable proteins. The supernatant was isolated by centrifugation, and SDS extraction was repeated once for total removal of SDS-extractable proteins (Schreuder *et al.*, 1993). The SDS extract was desalted by gel filtration on a Bio-Gel P-6 polyacrylamide gel column. SDS-extracted cell walls were washed three times with 100 mM sodium acetate (BDH), pH 5.5, 1 mM PMSF, to remove the SDS. To isolate the glucanase-extractable proteins, the washed cell walls were resuspended in the same buffer (0.5 g fresh wt ml^{-1}) and mollusc laminarinase (Sigma) was added at 0.25 U (g cell wall) $^{-1}$. After incubation for 2 h at 35 °C, a further 0.25 U enzyme (g cell wall) $^{-1}$ was added, followed by an additional incubation for 2 h at 35 °C. The undigested material was pelleted at 14000 r.p.m. for 5 min and the glucanase-extractable proteins were collected from the supernatant.

Mannoproteins were purified by concanavalin A (ConA)–Sephacrose affinity chromatography as described by Van Rinsum *et al.* (1991).

Endo H digestion. To release high mannose N-chains from cell wall glycoproteins, extracts were digested with Endo H (endoglycosidase H recombinant from *E. coli*, Boehringer Mannheim). Forty-microlitre samples of the various extracts containing 3% (v/v) β -mercaptoethanol and 0.4% (w/v) SDS were boiled for 5 min to denature the proteins; 150 μl 50 mM sodium acetate, pH 5.5, containing several protease inhibitors (2 mM EDTA, 1 mM PMSF, 0.42% pepstatin and 0.3% leupeptin), and 16 μl Endo H were added. The mixture was incubated overnight at 37 °C. The proteins were precipitated with 90% (v/v) ice-cold acetone at –20 °C for 2 h, pelleted at 14000 r.p.m. in a cooled Eppendorf centrifuge at 4 °C, and dissolved in 30 μl sample buffer. The digested glycoproteins were subjected to SDS-PAGE followed by either silver staining or Western analysis.

Exo- α - or exo- β -galactosidase treatment of cell wall proteins. Laminarinase extracts were treated with exo- α - or exo- β -galactosidase (Bahl & Agrawal, 1969). Acetone-precipitated and freeze-dried proteins were resuspended in 0.05 M citrate buffer (Merck), pH 3.9, and 2 μl α - (20 mU) or β - (4 mU) galactosidase (from *Aspergillus niger*, Sigma) were added.

These samples were incubated at 37 °C for 1 h. The proteins were precipitated by adding 90 % (v/v) acetone for 2 h at -20 °C, followed by centrifugation in a cooled Eppendorf at 4 °C at 14000 r.p.m. for 5 min; the supernatants were stored separately.

Carbohydrate analysis. To determine carbohydrate composition the fractions were hydrolysed with 2 M trifluoroacetic acid for 4 h at 100 °C. Since this results in deacetylation of N-acetylglucosamine, no discrimination between N-acetylglucosamine and glucosamine could be made. The glucosamine detected was referred to as GN. The hydrolysate was lyophilized. The monosaccharide compositions of both fractions were examined by high-pH anion-exchange chromatography with pulsed amperometric detection (Dionex Bio LC Carbohydrate system) as previously described (Montijn *et al.*, 1994).

SDS-PAGE and Western blot analysis of cell wall proteins. SDS-PAGE of the cell wall proteins was performed as described previously (Montijn *et al.*, 1994). Proteins were either stained by the silver staining method as described by De Nobel *et al.* (1989) or transferred electrophoretically to Immobilon PVDF (Millipore) membranes for Western analysis. The membrane was blocked with 5 % (w/v) non-fat milk powder (Elk, DMV Campina) in PBS [0.8 % (w/v) NaCl (Merck), 0.02 % (w/v) KCl (Merck), 0.144 % (w/v) Na₂HPO₄ (Merck), 0.024 % (w/v) KH₂PO₄ (Merck)] and incubated with a dilution (1:5000) of affinity-purified 1,6- β -glucan antibodies (Lu *et al.*, 1995) in PBS/3 % (w/v) BSA. Binding of the antibodies was detected by goat anti-rabbit IgG-peroxidase conjugates (Pierce) and developed using a chemiluminescence detection kit (ECL kit, Western blotting detection reagents, Amersham) as described by Kapteyn *et al.* (1995). For competitive Western analysis the proteins were transferred to the blot, blocked with PBS/3 % BSA, washed with PBS and incubated with a dilution of the specific antibodies together with 100 μ M of either pustulan (1,6- β -glucan; Hoechst) or laminarin (1,3- β -glucan; Fluka). For the oxidation of 1,6 linkages, mild periodate treatment was performed as described previously (Montijn *et al.*, 1994).

Analytical methods. Protein was assayed with the BCA protein assay reagent (Pierce) with bovine serum albumin as a reference protein. Carbohydrate was measured with phenol/sulphuric acid (Dubois *et al.*, 1956) with mannose as reference.

Fluorescent labelling of cells. For the fluorescent labelling of whole cells, concanavalin A-fluorescein isothiocyanate (ConA-FITC, Sigma) was used. Cells (100 μ l) were washed twice with PBS and centrifuged for 30 s at 14000 r.p.m. Cells were resuspended in 50 % (v/v) ethanol and washed twice with PBS. After the washing step with ethanol, cells were centrifuged at 3000 r.p.m. for 5 min. After 2 h incubation in the dark in 10 % (v/v) ConA-FITC/PBS, cells were washed twice with PBS. Cells were resuspended in PBS and stored in the dark until they were used for microscopy. For microscopic examination an Olympus Automatic Photomicrographic System, model PM-10 ADS, was used and photographs were taken with Kodak Ektachrome 1600 ASA film.

Zymolyase resistance. Zymolyase resistance was expressed as the time needed to decrease the optical density of a cell suspension in the presence of Zymolyase to 50 % of the original value. Cells (10⁷ cells) were suspended in 1 ml 0.1 M potassium phosphate (KH₂PO₄/K₂HPO₄; Merck), pH 7.5, containing 0.3 mg Zymolyase 20T ml⁻¹ at 23 °C. The stock solution of Zymolyase (from *Arthrobacter luteus*, Seikagaku) was dissolved in 50 % (v/v) glycerol. OD₅₃₀ was monitored.

RESULTS

Inhibition of melanin synthesis by growth on low-pH medium

Melanin synthesis can be inhibited in the black yeast *Phaeococcomyces* sp. by growth on a low-pH ascorbate medium (Butler & Lachance, 1986). To test whether melanin synthesis could also be inhibited in *E. dermatitidis*, wild-type cells were cultured on different media. When wild-type cells were grown on SG medium, they had a typical dark appearance. When grown on a low-pH ascorbate medium, the colonies became significantly less melanized. Similar results were obtained with liquid low-pH ascorbate medium. However, the degree of melanization was still higher than in a non-melanized *mel3* mutant.

Zymolyase resistance

To investigate the possibility of isolating cell wall proteins, wild-type cells and *mel3* mutant cells were treated with Zymolyase, a 1,3- β -glucanase preparation. Cells were grown on SG and low-pH ascorbate medium. Results varied significantly depending on the degree of melanization. Wild-type cells grown in SG were resistant to Zymolyase treatment. However, wild-type cells became sensitive to Zymolyase when grown in low-pH ascorbate medium. After 28 min, 50 % of the cells had lysed as a consequence of cell wall degradation. As expected, mutant cells grown in either medium lysed even more rapidly in the presence of Zymolyase: 50 % of the *mel3* cells grown in SG medium had lysed after 11 min, while 50 % of the *mel3* cells grown in low-pH ascorbate medium had already lysed after 8 min. The fact that both wild-type and mutant cells became more sensitive to Zymolyase when grown in low-pH ascorbate medium indicates that in this medium the cell wall is weakened, thereby facilitating cell lysis by Zymolyase. This is also consistent with microscopic observations of the cells. Cells cultured in low-pH ascorbate medium were more spherical and larger than yeast cells grown in SG medium, indicating that the cell wall was indeed weakened (compare Fig. 1c and d).

ConA-FITC labelling of cell walls

Wild-type cells of *E. dermatitidis* grown in SG medium were labelled with ConA-FITC and examined by light microscopy. An exponential-phase culture containing mostly yeast cells and some hyphal cells displayed variable labelling intensity. Hyphal elements were fluorescent, while almost no fluorescent yeast cells were detected (Fig. 1a). Stationary-phase yeast cells, however, were moderately labelled (Fig. 1b). Wild-type cells from the exponential phase which had been cultured in low-pH ascorbate medium were moderately labelled (data not shown). Non-melanized mutant yeast cells grown in either normal SG medium or ascorbate medium showed a high intensity of labelling (Fig. 1c and d). Taken together, these data show that the efficiency of

Fig. 1. ConA-FITC labelling. Cells were isolated and labelled with ConA-FITC. Cells on the left side of the figure were photographed with normal light; those on the right side were photographed with fluorescent light. (a) Wild-type cells grown in SG medium isolated in the exponential phase; (b) wild-type cells grown in SG medium isolated in the stationary phase; (c) mutant cells grown in SG medium isolated in the exponential phase; (d) mutant cells grown in low-pH ascorbate medium isolated in the exponential phase.

ConA-FITC labelling of yeast cells is dependent on the growth phase and the degree of melanization.

Composition of the cell wall

Walls of wild-type and mutant cells consisted of *N*-acetylglucosamine (or glucosamine, see Methods), galactose, glucose and mannose (Table 1). Walls from mutant cells were isolated and treated with hot SDS and laminarinase for the isolation and characterization of cell wall proteins. Only 10% of the total cell wall proteins of the mutant cells could be extracted by hot SDS. Since walls from mutant cells were susceptible to glucanase digestion, proteins could be released by laminarinase (a 1,3- β -glucanase preparation). Carbohydrate analysis demonstrated that the SDS-extractable proteins and laminarinase-extractable proteins contained glucose (Table 1). In *S. cerevisiae*, glucosylated proteins are exclusively found in glucanase extracts (Van Rinsum *et al.*, 1991; Montijn *et al.*, 1994). The high amount of mannose in both protein fractions is probably derived from *O*- and *N*-mannosyl chains as found in *S.*

cerevisiae. Indeed, proteins in these fractions were sensitive to Endo H, indicating that *N*-chains are present (Figs 2 and 3). Galactose was also found in both fractions, but no decrease in the amount of galactose could be determined after treatment with α - and β -exo-galactosidase (not shown). Carbohydrate analysis, however (see Methods), revealed an unexpected decrease of the glucose content of these fractions. This result could be explained by contaminating activities in the enzyme preparations used: both α - and β -galactosidase have minor α - and β -glucosidase activities (Bahl & Agrawal, 1969). This implies that galactose is not present in terminal positions of the carbohydrate chains.

Identification of cell wall proteins

Mutant cells were grown in SG medium. Cell wall proteins were released from isolated cell walls by hot SDS and laminarinase. Laminarinase extracts were purified by ConA-Sepharose affinity chromatography to remove laminarinase (Van Rinsum *et al.*, 1991). Proteins (10 μ g per lane) were separated by SDS-PAGE on 2.2 to

Table 1. Sugar composition of isolated walls and wall proteins from *E. dermatitidis*

Starting material*	Sugar composition (%)			
	GN	Gal	Glc	Man
Walls from:				
WT (exponential phase)	1.1	4.4	55.5	39
WT (stationary phase)	1.5	4.4	68.1	26
<i>mel3</i> (exponential phase)	0.5	5.5	67	27
Proteins† from:				
<i>mel3</i> (SDS extract)	0.6	8.8	6.6	84
<i>mel3</i> (glucanase extract)	0.3	34.6	2.1	63

* WT, wild-type cells; *mel3*, non-melanized mutant cells.

† Proteins were purified by ConA-Sepharose affinity chromatography.

Fig. 2. Protein profiles of various cell wall fractions of the non-melanized mutant of *E. dermatitidis*. Lanes 1 and 2, SDS-extractable proteins; lanes 3 and 4, laminarinase-extractable proteins. The extracts in lanes 2 and 4 were digested with Endo H. Proteins were separated by SDS-PAGE (2.2–20%) and visualized by silver staining. Molecular masses are indicated in kDa.

20% gradient gels and visualized by silver staining (Fig. 2). The SDS extract of the mutant contained a major protein with a mean molecular mass of 335 kDa and less abundant proteins with lower molecular masses (lane 1). No major Endo-H-sensitive SDS-extractable proteins could be detected in this experiment (lane 2), but some minor bands disappeared after Endo H digestion. However, Endo H-sensitive proteins could be detected when this fraction was analysed by Western analysis using purified 1,6- β -glucan antibodies (Fig. 3).

Laminarinase extracts of the mutant grown in SG medium were also analysed by SDS-PAGE. Although

Fig. 3. Western analysis of cell wall proteins using affinity purified 1,6- β -glucan antibodies at a serum dilution of 1:5000. Binding of the antibodies was detected with a goat anti-rabbit IgG-peroxidase conjugate. Lanes 1 and 2, SDS-extractable proteins; lanes 3 and 4, laminarinase-extractable proteins. The extracts in lanes 2 and 4 were digested with Endo H. Molecular masses are indicated in kDa.

sufficient laminarinase-extracted proteins of cells grown in SG were loaded on the gel, not many proteins could be visualized by silver staining (Fig. 3, lane 3). Faint low-molecular-mass bands were detected. After Endo H treatment a high molecular mass smear became visible (lane 4). A low-molecular-mass band of 71 kDa was also detected. These proteins probably originated from hypermannosylated proteins of highly variable molecular mass that only gave rise to visible bands after Endo H treatment. This phenomenon has previously been demonstrated for laminarinase extracts of *Candida albicans* (Kapteyn *et al.*, 1994).

Identification of 1,6- β -glucan side chains on cell wall proteins

Recently, it was shown by SDS-PAGE and Western analysis that laminarinase-extractable cell wall proteins of *S. cerevisiae* and *C. albicans* contain 1,6- β -glucan side-chains (Montijn *et al.*, 1994; Kapteyn *et al.*, 1994). Protein extracts of *E. dermatitidis* mutant cell walls were subjected to Western analysis using affinity-purified anti-1,6- β -glucan serum to test whether 1,6- β -glucosylated proteins could also be detected in *E. dermatitidis*. In the SDS extract of mutant cells a smear with a molecular mass > 390 kDa was recognized by the antibodies (Fig. 3, lane 1). The major protein of 335 kDa visualized by silver staining (Fig. 2, lanes 1 and 2) was not recognized by the antibodies, indicating that only a part of the SDS-extractable protein carries 1,6- β -glucan. After Endo H digestion, the antibodies revealed a polydisperse smear with a lower molecular mass (lane 2). This demonstrated that N-chains were released. As the 1,6- β -glucan signal did not decrease in strength, this

indicated that the 1,6- β -glucan chains were resistant to Endo H digestion. Laminarinase digests of the mutant showed a heavily labelled smear ranging in mass from 80 to > 500 kDa (Fig. 3, lane 3). After Endo H treatment, the mobility of these glucosylated proteins was reduced to a smear with a molecular mass of 40 to 450 kDa (lane 4).

To confirm that the epitope on the proteins contained 1,6- β -glucose residues, the following control experiments were carried out. In a competitive Western analysis, 100 μ M pustulan (a 1,6- β -glucan) inhibited the binding of the antibodies (Montijn *et al.*, 1994), whereas laminarin (a 1,3- β -glucan) did not significantly affect the binding, even at a concentration of 200 μ M (data not shown). Furthermore, it was shown that the epitope was sensitive to oxidation by periodate, consistent with the notion that the epitope was composed of 1,6-linked carbohydrates (data not shown).

DISCUSSION

The cell wall of *E. dermatitidis* is composed of carbohydrates, glycoproteins and melanin. Carbohydrate analysis demonstrated that the wall consisted of glucose, (N-acetyl)glucosamine, mannose and galactose. To explore the possibility of extracting cell wall proteins by 1,3- β -glucanase digestion, cells were subjected to a Zymolyase-resistance assay. Melanized wild-type yeast cells resisted the lytic effect of Zymolyase, a 1,3- β -glucanase preparation. However, when wild-type cells were grown on low-pH ascorbate medium, melanin synthesis was partly inhibited and the 'albinized' cells became less resistant to Zymolyase. This is in agreement with Butler & Lachance (1986) who demonstrated that albinized cells of the black yeast *Phaeococcomyces* sp. became sensitive to Zymolyase. Non-melanized *mel3* mutants were even more sensitive to Zymolyase digestion, confirming that the sensitivity for Zymolyase is dependent on the degree of melanization. However, it was also shown that mutant cells grown in low-pH ascorbate medium were more sensitive to Zymolyase than mutant cells grown in SG medium. This indicates that the composition of the cell wall is dependent on the culture medium as was shown previously for *S. cerevisiae* (McMurrough & Rose, 1967).

In *S. cerevisiae* it has been shown that mannan in the wall is, at least partially, exposed on the outside, since the wall reacts strongly with ConA (Tkacz *et al.*, 1971; Ballou, 1982; Frevert & Ballou, 1985; Kukuruzinska *et al.*, 1987; Pringle *et al.*, 1989). In *E. dermatitidis*, melanin seems to mask the mannoproteins of the cell wall, since wild-type exponential-phase yeast cells could not be labelled by ConA-FITC (Fig. 1a), whereas some labelling was obtained with albinized wild-type yeast cells (not shown). Non-melanized mutant cells labelled very well. In contrast to wild-type yeast cells, wild-type hyphal elements showed a high intensity of fluorescence. Stationary-phase yeast cells of the wild-type displayed a moderate labelling with ConA-FITC. Two explanations are possible for these observations: (i) the manno-

proteins in hyphal cells or in the stationary phase of yeast cells are not masked by melanin, or (ii) an extracellular polysaccharide capsule, which is composed of α -glucan and can also be recognized by ConA, is formed in these cells (Kwon-Chung *et al.*, 1992; De Hoog *et al.*, 1995).

Since wild-type cells of *E. dermatitidis* were resistant to Zymolyase, *mel3* mutants were used for the isolation and characterization of cell wall proteins. In glucanase extracts and in SDS extracts, proteins containing mannose, galactose, (N-acetyl)glucosamine, and glucose were found. Western analysis revealed that at least part of the glucose present in both extracts was 1,6- β -linked (Fig. 3). This is in contrast to *S. cerevisiae*, where SDS-soluble cell wall proteins are not 1,6- β -glucosylated (Lu *et al.*, 1995; Montijn *et al.*, 1994). Addition of 1,6- β -glucan to the cell wall proteins is correlated with their incorporation (van Berkel *et al.*, 1994). Kapteyn *et al.* (1996) have shown that cell wall proteins were cross-linked to 1,3- β -glucan through their 1,6- β -glucan moiety. Possibly, the presence of 1,6- β -glucosylated proteins in the SDS extract of *Exophiala* is caused by an accumulation of cell wall precursors which are not yet linked to 1,3- β -glucan. If pulse-chase experiments could support this hypothesis, the 1,6- β -glucosylated SDS-extractable proteins would provide an excellent tool for cross-linking studies.

The presence of 1,6- β -glucosylated proteins is not unique to *E. dermatitidis*. 1,6- β -Glucosylated proteins have been found in other ascomycetes such as the 'classical' yeast *S. cerevisiae* (Montijn *et al.*, 1994), the dimorphic yeast *Candida albicans* (Kapteyn *et al.*, 1994, 1995, 1996), the yeast *Hansenula polymorpha* (H. Caro, personal communication), and in the 'true' filamentous fungus *Fusarium oxysporum* (Schoffemeer *et al.*, 1996). This indicates that this type of linkage is a general phenomenon among ascomycetes.

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