Cell Wall Proteins Liberated by Zymolyase from Several Ascomycetous and Imperfect Yeasts

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(Received 12 March 1987; revised 26 May 1987)

Eight yeast species (the ascomycetes *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii*, *Hansenula wingei*, *Saccharomycopsis lipolytica*, *Schizosaccharomyces pombe* and *Pichia scolyti*, and the imperfect yeasts *Candida albicans* and *Rhodotorula glutinis*) have been compared with respect to the proteins solubilized by glucanase treatment of amino-acid-labelled, purified walls. Except for *R. glutinis*, a significant quantity of radioactively labelled protein material was liberated by this treatment. Among the major protein components solubilized was a material larger than 100 kDa (heterogeneous in size in some species) and a molecule ranging in size from 31·5 to 34 kDa depending on the yeast species. The large material was of glycoprotein nature, with the sugar portion *N*-glycosidically linked to the protein moiety; in some species, this material did not bind concanavalin A (ConA), indicating the presence of terminal sugar residues different from mannose, glucose or glucosamine. The form of 31·5–34 kDa was present in all the species studied except *R. glutinis*; it was a mannoprotein, except in *Schiz. pombe*, which possessed a 31·5 kDa form not sensitive to tunicamycin or endoglycosidase H and not recognized by ConA. Antigenic cross-reactivity was observed between the protein moieties of the 33 kDa form of *Sacch. cerevisiae* and the species of equivalent size in *C. albicans* and *H. wingei*. Similar partial proteolysis patterns were obtained for the 33 kDa form of *Sacch. cerevisiae* and the 34 kDa forms of *C. albicans* and *P. scolyti*.

INTRODUCTION

The yeast cell wall consists of three main kinds of polymers (Bacon, 1981; Wessels & Sietsma, 1981): glucan (mainly of the β -glucan type), which forms the structural network of the wall; chitin, a minor structural polymer present on the bud scars and near the plasma membrane in smaller amounts (Horisberger & Vonlanthen, 1977); and glycoprotein molecules usually formed by a mannose polysaccharide (sometimes termed mannan) covalently linked to protein moieties. These mannoproteins appear as an amorphous material filling the network formed by the structural polymers.

The chemical structure of wall mannan in *Saccharomyces cerevisiae* and some other yeast species is well known (Ballou, 1982). In *Sacch. cerevisiae*, it is composed of a (1 $\rightarrow$ 6)- α -linked backbone with short lateral chains formed by (1 $\rightarrow$ 2)- α - and (1 $\rightarrow$ 3)- α -linked mannose residues. This also appears to be the overall structure of mannan in *Kluyveromyces lactis* (Douglas & Ballou, 1980) and *Zygosaccharomyces rouxii* (Hamada *et al.*, 1984). In *Hansenula wingei* (1 $\rightarrow$ 2)- α - and (1 $\rightarrow$ 3)- α linkages are interspersed in the (1 $\rightarrow$ 6)- α backbone (Yen & Ballou, 1974).

Much less is known about the protein moiety of wall mannoproteins, probably because classical methods for extraction involved destruction of the peptide moiety. Recently, the (1 $\rightarrow$ 3)- β -glucanase complex Zymolyase has been employed to digest the glucan skeleton of the cell wall

Abbreviations: ConA, concanavalin A; Endo H, endo- β -*N*-acetylglucosaminidase H.

of *Sacch. cerevisiae* and liberate glucan-associated mannoproteins for further analysis (Pastor *et al.*, 1984; Zlotnik *et al.*, 1984; Frevert & Ballou, 1985; Valentin *et al.*, 1986). Two main mannoprotein components are liberated from *Sacch. cerevisiae* walls: (i) a material heterogeneous in size (larger than 120 kDa) whose *N*-linked peptide moiety runs as 90–100 kDa in acrylamide gels; and (ii) a 33 kDa mannoprotein possessing a single *N*-linked chain of mannose residues. A similar pattern is obtained from *K. lactis* cell walls (Herrero & Boyd, 1986). This same approach has been employed with *Candida albicans* (Elorza *et al.*, 1985; Sundstrom & Kenny, 1985), where the large material may be resolved into several defined bands in polyacrylamide gels.

In this paper, we have extended the range of yeast species for which the proteins solubilized by Zymolyase from purified cell walls have been analysed. Six ascomycetous yeasts were studied: *Sacch. cerevisiae*, *Z. rouxii*, *H. wingei*, *Saccharomycopsis lipolytica*, *Schizosaccharomyces pombe* and *Pichia scolyti* (Kreger-van Rij & Kurtzman, 1984). Among the imperfect yeasts, *C. albicans* [with counterparts among ascomycetous forms (Meyer *et al.*, 1984)] and *Rhodotorula glutinis* [related to perfect basidiomycetous forms (Yamada & Kondo, 1973; Bastide *et al.*, 1979)] were also considered.

METHODS

Strains. The following strains were employed: *Sacch. cerevisiae* X-2180-1A (from the Yeast Genetic Stock Center, University of California, USA), *C. albicans* ATCC 26555, *Z. rouxii* NCYC 583, *H. wingei* CECT 1465, *Saccharomycopsis lipolytica* CECT 1240, *Schiz. pombe* CECT 1375, *P. scolyti* ATCC 24212, and *R. glutinis* NCYC 162. Cultures were maintained in solid Winge medium (3 g yeast extract, 20 g glucose and 15 g agar per litre) and periodically restreaked in fresh medium.

Growth and labelling conditions. The eight yeast species studied were able to grow at 28 °C in Yeast Nitrogen Base (YNB) liquid medium (Difco) supplemented only with 2% (w/v) glucose. The osmotolerant yeast *Z. rouxii* grew better in 20% glucose and this sugar concentration was employed in subsequent experiments with this species. During exponential growth, optical density (at 600 nm) doubling times were: *Sacch. cerevisiae*, 135 min; *C. albicans*, 90 min; *Z. rouxii*, 187 min; *H. wingei*, 103 min; *Saccharomycopsis lipolytica*, 107 min; *Schiz. pombe*, 216 min; *P. scolyti*, 183 min; and *R. glutinis*, 150 min. These values are the means of four separate measurements. All the species grew as yeast cells, except *P. scolyti* and *R. glutinis*, which formed rudimentary pseudohyphae; in the case of *P. scolyti*, the pseudohyphae were branched.

Cells were suspended at 5 µg dry wt ml⁻¹ in the appropriate liquid medium and incubated overnight at 28 °C for growth up to 250 µg dry cells ml⁻¹ before beginning the experiments. For radioactive labelling of peptides, 11.1 kBq [U-¹⁴C]protein hydrolysate ml⁻¹ (Amersham, 2.07 GBq per milliatom) was added to cultures in YNB medium plus 2% glucose (20% for *Z. rouxii*); incubation was continued at 28 °C for a period equivalent to one and a half doubling times of OD₆₀₀. For sugar labelling, cells grown in YNB medium plus 2% glucose (20% for *Z. rouxii*) and 2% (w/v) casein hydrolysate were washed twice with distilled water and resuspended at identical cell concentration in YNB medium with the same composition except that glucose was at 0.05% (0.2% for *Z. rouxii*) and [U-¹⁴C]glucose (Amersham) was present at 7.4 kBq ml⁻¹ (8.88 GBq ml⁻¹); incubation was continued at 28 °C for one doubling time of OD₆₀₀.

Wall purification, protein solubilization and analysis and sugar fractionation. Walls were purified after mechanical breakage and successive washings with distilled water, as described by Sanz *et al.* (1985). Proteins were solubilized from purified walls by incubating these with Zymolyase 20T (Miles Laboratories) (50 µg enzyme complex for the quantity of wall material obtained from 1 mg dry cells) in 10 mM-Tris/HCl, pH 7.0, at 30 °C for 210 min. Endo-β-*N*-acetyl glucosaminidase H (Endo H) treatment and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of the liberated material, as well as fluorography of the radioactive gels, were done as described by Pastor *et al.* (1984). Purified walls, labelled in the sugar moiety, were fractionated as described by Murgui *et al.* (1985).

Partial proteolysis of individual peptides. Individual bands were cut from SDS-PAGE gels and treated with V8 protease (Boehringer) from *Staphylococcus aureus* inside a second gel (Cleveland, 1983). Electrophoretic conditions were adjusted so that digestion occurred over a 4 h period through the stacking gel.

Immunoblotting procedure. Proteins were blotted from polyacrylamide gels to nitrocellulose paper and the 33 kDa mannoprotein was immunodetected with rabbit-raised antiserum against its deglycosylated form (diluted 1:2000). The procedure described by Towbin *et al.* (1979) was essentially employed, except that 3% (w/v) bovine serum albumin acted as blocking agent and 0.05% Tween 20 was included in the washing solutions. Peroxidase-labelled goat anti-rabbit IgG (diluted 1:3000) were used as indicator antibodies, with 4-chloro-1-naphthol as chromogenic reagent.

Concanavalin A-peroxidase (ConA-peroxidase) staining of mannoproteins. Proteins separated in polyacrylamide

gels were blotted to nitrocellulose membranes and mannoproteins were detected by indirect (ConA-mediated) peroxidase staining, using the protocol described by Millette & Scott (1984).

RESULTS AND DISCUSSION

Effect of Zymolyase on radioactively labelled, isolated walls

A mixture of radioactive amino acids was employed to label cell protein in cultures corresponding to the eight yeast species. Zymolyase treatment of isolated walls released between 2 and 4% of total cell radioactivity in all cases except *R. glutinis*, where less radioactivity was solubilized by the glucanase complex (Table 1).

The above values were correlated with the proportion of radioactive glucose incorporated into several polysaccharide fractions of isolated walls (Table 1). Thus, most of the radioactivity in cell walls of *R. glutinis* appeared as an alkali-insoluble material insensitive to Zymolyase; this is in accordance with observations showing that cell walls of *R. glutinis* contain high levels of chitin (Berthe *et al.*, 1981) and explains the low amount of protein material liberated by the treatment with (1→3)- β -glucanases. Compared with the other yeast species, *Z. rouxii* and *Schiz. pombe* contained a smaller amount of alkali-insoluble material susceptible to Zymolyase degradation; this fraction corresponds to alkali-insoluble glucan mostly with (1→3)- β -linkages (Manners *et al.*, 1973). In fact, it has been shown that the composition of *Schiz. pombe* cell walls differs considerably from that of other yeasts such as *Sacch. cerevisiae*, since they lack chitin and have reduced levels of (1→3)- β -glucan, which is substituted by (1→6)- β - and (1→3)- α -glucans (Bush *et al.*, 1974; Manners *et al.*, 1974).

The presence of mannan in the wall (alkali-soluble Fehling-precipitable material) is characteristic of the yeast morphology among fungi (Bartnicki-Garcia, 1968). Thus, the two species forming pseudohyphae in the culture conditions employed (*P. scolyti* and *R. glutinis*) contained somewhat lower amounts of this fraction than the species with yeast morphology (Table 1). It must be considered, however, that mannan structure may vary significantly among yeast species and this may affect precipitability by Fehling solution (Spencer & Gorin, 1973). The Fehling-precipitable material from cell walls of *Schiz. pombe* corresponds to galactomannan instead of mannan (Bush *et al.*, 1974). On the other hand, the proportion of alkali-soluble material non-precipitable by Fehling solution (in *Sacch. cerevisiae* this corresponds to a minor β -glucan fraction) was highly variable among the species tested (Table 1).

Table 1. *Solubilization of wall mannoproteins by Zymolyase and polysaccharide fractionation of isolated cell walls*

Exponentially growing cultures (30 ml) at 250 μ g dry cells ml⁻¹ were labelled with either [U-¹⁴C]protein hydrolysate or [U-¹⁴C]glucose, as described in Methods. Amino-acid-labelled walls were treated with Zymolyase and glucose-labelled ones were fractionated with regard to their sugar components.

Organism	Percentage cell radioactivity solubilized by Zymolyase*	Percentage wall radioactivity in:				
		Alkali-soluble material		Alkali-insoluble material		
		Fehling-precipitable	Fehling-non-precipitable	Acid soluble	Acid-insoluble Zymolyase-digestible	Acid-insoluble Zymolyase-indigestible
<i>Sacch. cerevisiae</i>	2.73 (5958)	31.3	9.6	1.5	41.9	15.7
<i>C. albicans</i>	3.19 (6554)	20.4	15.8	1.9	46.5	15.4
<i>Z. rouxii</i>	2.16 (4796)	31.4	8.8	1.0	28.7	30.1
<i>H. wingei</i>	3.73 (6187)	30.9	14.6	1.7	40.6	12.2
<i>Saccharomycopsis lipolytica</i>	2.19 (4299)	23.4	6.0	0.5	44.7	25.4
<i>Schiz. pombe</i>	2.15 (6505)	34.9	17.3	4.2	17.9	25.7
<i>P. scolyti</i>	3.92 (6834)	11.9	22.6	1.0	47.5	17.0
<i>R. glutinis</i>	0.76 (6771)	3.0	6.4	6.3	16.3	68.0

* Total radioactivity ($10^{-3} \times$ c.p.m.) in cell protein in parenthesis.

Fig. 1. Proteins from radioactively labelled walls of the indicated yeast species solubilized by Zymolyase (lanes *a*) and then treated with Endo H (lanes *b*). Released proteins were ethanol precipitated and analysed by SDS-PAGE and fluorography. The gel was overexposed for the samples corresponding to *R. glutinis*, since these contained one-fourth of the radioactivity present in the other samples. Numbers at the right-hand side correspond to the molecular masses (in kDa) of standard proteins migrating at the indicated positions in parallel runs. The positions of specific proteins described in the text are also indicated (their molecular masses were calculated from calibration curves based on the migration of the standard proteins, in two independent experiments).

Individual proteins solubilized by Zymolyase digestion of the walls

The material released by Zymolyase treatment of amino-acid-labelled walls was analysed by SDS-PAGE and fluorography of the gels (Fig. 1). In all the yeasts studied, except *Schiz. pombe* and *R. glutinis*, a material with low mobility in the gels (in the region corresponding to 100 kDa or more) was one of the major protein components liberated. Although in Fig. 1 this material appears diffuse, that in *C. albicans* and *H. wingei* could be resolved in more defined bands when shorter exposure times were employed for the fluorography (data not shown). Some amount of large diffuse material also appeared in *Schiz. pombe* but it was hardly observed in *R. glutinis*.

Another major protein component liberated by Zymolyase digestion of the walls was a band of 33 kDa in *Sacch. cerevisiae* (Valentin *et al.*, 1986; and Fig. 1), 33.5 kDa in *Saccharomycopsis lipolytica* and

Fig. 2. Proteins solubilized by Zymolyase from radioactively labelled walls obtained from untreated (lanes a) or tunicamycin-treated cultures, which received $10 \mu\text{g ml}^{-1}$ of the drug (lanes b). The following amounts of radioactivity were loaded: 12000 c.p.m. for *Schiz. pombe*, 15000 c.p.m. for *Z. rouxii*, and 18000 c.p.m. for the other yeasts. The migratory positions of standard proteins of known molecular mass (in kDa) are indicated on the left. Specific protein molecules described in the text are also indicated.

Z. rouxii, and 34 kDa in *C. albicans*, *H. wingei* and *P. scolyti* (Fig. 1). In *Schiz. pombe* and *R. glutinis* major bands of 31.5 and 37.7 kDa respectively also appeared in this region of the gels.

The Zymolyase-solubilized material from the eight yeasts was treated with Endo H to study the glycoprotein nature of the liberated molecules (Fig. 1). The 33 kDa species of *Sacch. cerevisiae* was converted into a 30.5 kDa form, and the 34 and 33.5 kDa species of *C. albicans*, *Z. rouxii*, *H. wingei*, *Saccharomycopsis lipolytica* and *P. scolyti* were all converted into 31.5 kDa forms. The 31.5 kDa form of *Schiz. pombe* was not affected by Endo H, while the 37.5 kDa form of *R. glutinis* disappeared on Endo H treatment with the concomitant appearance of a 35 kDa band.

With respect to the larger material, treatment with Endo H (Fig. 1) resulted in the disappearance of most of it in *P. scolyti*, whereas no effect was observed in *C. albicans*, *Z. rouxii*, *H. wingei*, *Saccharomycopsis lipolytica* and *Schiz. pombe*. Partial digestion of this low-mobility band from *Sacch. cerevisiae* resulted in the appearance of a band of 90–100 kDa, as already reported (Pastor *et al.*, 1984).

To confirm the results obtained with Endo H, the incorporation of Zymolyase-solubilizable protein material in the walls of cells treated with tunicamycin (a drug that inhibits *N*-glycosylation) was studied (Fig. 2). Total incorporation of radioactivity into this fraction in treated cells was inhibited in *Z. rouxii*, *H. wingei*, *Saccharomycopsis lipolytica*, *Schiz. pombe* and *P. scolyti* by 55, 38, 58, 52 and 31% respectively, relative to control cells. Other studies have shown levels of inhibition of the same order in *Sacch. cerevisiae* (Pastor *et al.*, 1984) and *C. albicans* (M. V. Elorza, A. Murgui and R. Sentandreu, unpublished). No study was done on *R. glutinis* due to the low amount of Zymolyase-solubilizable proteins in this species. In the five yeast species for which tunicamycin treatment was carried out, incorporation of the large material was especially affected, even in those cases where this material was not sensitive to Endo H. Taken together, the results suggest that in these yeasts, as well as in *Sacch. cerevisiae* and *C. albicans*, the large material consists of glycoprotein molecules in which the sugar chains are *N*-glycosidically linked to the peptide moiety. The lack of sensitivity to Endo H may be due to the protective role of the large sugar chains against digestion by the glycosidase. Unfortunately, the above results do not allow us to know the size of the protein moiety on the large glycoprotein material, since non-glycosylated molecules are apparently not incorporated efficiently into the walls.

Tunicamycin-treated cells incorporated into the walls 31.5 kDa non-glycosylated derivatives of the 34 or 33.5 kDa forms of *Z. rouxii*, *H. wingei*, *Saccharomycopsis lipolytica* and *P. scolyti* (Fig.

Fig. 3. ConA-peroxidase staining of the material solubilized by Zymolyase from isolated walls (obtained from 6 mg dry cells) of *Sacch. cerevisiae* (lane a), *C. albicans* (lane b), *Z. rouxii* (lane c), *H. wingei* (lane d), *Saccharomycopsis lipolytica* (lane e), *Schiz. pombe* (lane f), *P. scolyti* (lane g) and *R. glutinis* (lane h). Numbers (in kDa) correspond to molecular species described in the text.

2). A species of 31.5 kDa is also incorporated into the walls of tunicamycin-treated cells of *C. albicans* (M. V. Elorza, A. Murgui and R. Sentandreu, unpublished). Confirming the results with Endo H, the 31.5 kDa species of *Schiz. pombe* was not sensitive to tunicamycin.

ConA-peroxidase staining of the Zymolyase-solubilized wall material

The presence of terminal residues of mannose in the Zymolyase-liberated wall glycoproteins was studied by ConA-peroxidase staining (Fig. 3). This is an extremely sensitive method that detects mannoproteins after blotting from polyacrylamide gels to nitrocellulose paper (Hawkes, 1982). With respect to the larger material, only that from *Sacch. cerevisiae*, *C. albicans*, *H. wingei* and *P. scolyti* bound ConA, as shown by a positive peroxidase reaction. Therefore, in *Z. rouxii*, *Saccharomycopsis lipolytica* and *Schiz. pombe* the large glycoprotein material must contain terminal residues in the saccharide chains different from mannose, glucose or glucosamine. In *Z. rouxii*, however, a material that remained in the stacking gel during electrophoresis became stained (data not shown).

The 33 kDa glycoprotein of *Sacch. cerevisiae*, the 33.5 kDa ones of *Z. rouxii* and *Saccharomycopsis lipolytica*, and the 34 kDa one of *C. albicans*, *H. wingei* and *P. scolyti* were all able to bind the lectin (Fig. 3), which demonstrates their mannoprotein nature. In *Schiz. pombe*, neither the large heterogeneous material nor the 31.5 kDa species became stained. In this respect, it must be considered that in this yeast mannan is substituted by galactomannan (Bush *et al.*, 1974) and the possibility of saccharide chains containing terminal residues of galactose exists. Since the 31.5 kDa form of *Schiz. pombe* was not sensitive to Endo H or tunicamycin (Figs 1 and 2), the more plausible explanation for the results regarding this molecule is that it lacks sugar residues. However, the presence of a protein-saccharide linkage different from the *N*-glycosidic one cannot be ruled out.

Relationship between the 33 kDa species of Sacch. cerevisiae and molecular species of similar size in other yeasts

The antigenic relationship between the 33 kDa species of *Sacch. cerevisiae* and the species of similar size present in the other yeasts was analysed. Polyclonal antibodies raised against purified deglycosylated 30.5 kDa molecules of *Sacch. cerevisiae* (Sanz *et al.*, 1987) were used to

Fig. 4. Immunoblot of Zymolyase-solubilized proteins from walls of *Sacch. cerevisiae* (lane a), *C. albicans* (lane b), *Z. rouxii* (lane c), *H. wingei* (lane d), *Saccharomycopsis lipolytica* (lane e), *Schiz. pombe* (lane f), *P. scolyti* (lane g) and *R. glutinis* (lane h). Polyclonal antibodies against the peptide moiety of the *Sacch. cerevisiae* 33 kDa molecule were employed. Only the relevant region of the blotted paper is shown.

Fig. 5. Partial proteolytic digests of the 33 kDa mannoprotein of *Sacch. cerevisiae* and the 34 kDa mannoproteins of *C. albicans* and *P. scolyti*, using V8 protease from *Staphylococcus aureus* (10 ng enzyme in lanes a, 30 ng in lanes b and 100 ng in lanes c). For each individual digest, the original labelled band was obtained by Zymolyase digestion of walls corresponding to 10, 13 and 7 mg dry cells of *Sacch. cerevisiae*, *C. albicans* and *P. scolyti* respectively. Arrows indicate the respective undigested forms. Numbers at the left-hand side correspond to the migratory positions of standard proteins of known molecular mass (in kDa).

compare the Zymolyase-solubilized material from the eight yeast species by Western blotting (Fig. 4). The antibodies gave significant reaction with the 34 kDa species of *C. albicans* and *H. wingei*. For the other yeasts, faint bands of cross-reactive material were observed; these were caused by non-specific reactions since they also appeared when preimmunized antiserum was employed (data not shown). Recently, it has been shown that the antibodies against the 33 kDa molecules of *Sacch. cerevisiae* also react against a 33 kDa molecular species of *K. lactis* (Herrero & Boyd, 1986).

As another approach to studying the structural relationship among these 33–34 kDa major species of the yeast cell walls, the molecules from *Sacch. cerevisiae*, *C. albicans* and *P. scolyti* were partially digested with staphylococcal V8 protease (Fig. 5). Similar proteolytic patterns were obtained for the three yeasts, indicating an overall similarity among the structures of the 33 kDa molecules of *Sacch. cerevisiae* and the 34 kDa molecules of *C. albicans* and *P. scolyti*. It should be remarked that the similarity between the proteolytic patterns of *Sacch. cerevisiae* and *P. scolyti* does not correspond with antigenic cross-reactivity.

Therefore, the presence of a major mannoprotein in the range 31.5–34 kDa with common structural properties seems to be characteristic of ascomycetous yeasts and related imperfect forms. The closest relationship (indicated by antigenic cross-reactivity) is observed among budding species with true yeast morphology. Nevertheless, the presence of these molecules is not exclusive to cells with yeast morphology, since the 34 kDa species is also present in the walls of mycelium-forming cells of *C. albicans* (Elorza *et al.*, 1985).

There are other immunological studies of protein similarity among yeast species. Lachance & Phaff (1979) found that exo- β -glucanases were poorly conserved in species of *Kluyveromyces*. On the other hand, a strong cross-reactivity between plasma membrane ATPases of *Schiz. pombe* and *Candida tropicalis* has been observed (Blasco *et al.*, 1983). The 31.5–34 kDa molecular species studied here seem to be rather well conserved among a wide range of yeast species and their possible role as integral proteins in the cell wall is being studied.

This work was supported by grant 3432-83 from the Comisión Asesora de Investigación Científica y Técnica. P.S. was the recipient of a predoctoral grant from the Spanish Ministry of Education and Science.

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