Autolytic Release of Mannoproteins from Cell Walls of *Saccharomyces cerevisiae*

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Autolysis of purified *Saccharomyces cerevisiae* cell walls resulted in the release of several components thought to be mannoprotein in nature, since they were retained by Concanavalin A-Sepharose. The most abundant was a 29 kDal molecule which was also a major species among mannoproteins solubilized by the β -glucanase complex Zymolyase. There was a concomitant release of glucose and mannose oligosaccharides and of β -glucanase activity. Glucanase and protease inhibitory treatments considerably lowered the release of mannoproteins. The use of different protease inhibitors during autolytic incubations interfered with at least two proteases (one of them being a cysteine protease) apparently involved in the solubilization and partial degradation of mannoproteins from the walls.

INTRODUCTION

Autolysis of yeast cell walls during *in vitro* incubations is due to hydrolytic enzyme activities present in the organelle (Fleet & Phaff, 1974; Phaff, 1977; Reichelt & Fleet, 1981; Notario, 1982; Hien & Fleet, 1983*a*; Fleet, 1984). In *Saccharomyces cerevisiae*, two exo-(1 $\rightarrow$ 3)- β -glucanases and four endo-(1 $\rightarrow$ 3)- β -glucanases have been detected (del Rey *et al.*, 1979; Sánchez *et al.*, 1982; Hien & Fleet, 1983*a*), which may act *in vivo* in wall structuring during vegetative growth and sporulation (del Rey *et al.*, 1979; Hien & Fleet, 1983*b*). These glucanases might also be responsible for the degradation of walls during *in vitro* autolysis. The glucanase molecules themselves are released into the surrounding medium during this process (Hien & Fleet, 1983*a*). However, other enzyme activities such as periplasmic chitinase (Elango *et al.*, 1982) or mannanase (Maddox & Hough, 1971) may also play a role in yeast wall autolysis, as well as hypothetical envelope-associated proteases.

Recently, we have studied the mannoprotein species solubilized by purified Zymolyase (a β -glucanase complex of bacterial origin; Kitamura *et al.*, 1974) from *S. cerevisiae* walls (Pastor *et al.*, 1984). Essentially, material of high molecular mass and a 29 kDal mannoprotein were released; these components appeared to interact preferentially with the glucan network of the wall (Shibata *et al.*, 1983; Pastor *et al.*, 1984). Elorza *et al.* (1985) have extended this study to the mannoproteins released by Zymolyase from *Candida albicans* walls and found that they have a pattern similar to that of *S. cerevisiae* mannoproteins.

In this paper, we compare the mannoprotein molecules solubilized by Zymolyase with those released by autolysis in *S. cerevisiae* and we investigate the main enzyme activities responsible for their release.

Abbreviations: Endo H, endo- β -N-acetylglucosaminidase H; PHMB, *p*-hydroxymercuribenzoate; PMSF, phenylmethylsulphonyl fluoride; TLCK, *N* α -*p*-tosyl-L-lysine chloromethyl ketone.

METHODS

Strain, growth conditions and cell labelling. *Saccharomyces cerevisiae* X-2180-1A (from the Yeast Genetic Stock Center, University of California, USA) was used. Cells were grown at 28 °C in yeast nitrogen base medium without amino acids (Difco), supplemented with 2% (w/v) glucose. For mannoprotein labelling, [U - ^{14}C]protein hydrolysate [0.2 μ Ci ml $^{-1}$ (7.4 kBq ml $^{-1}$); sp. act. 56 mCi (2072 MBq) per mg-atom carbon] was usually added to the cultures at early exponential phase (about 0.2 mg cell dry wt ml $^{-1}$) and incubation continued for 4 h (up to 0.5–0.6 mg cell dry wt ml $^{-1}$). In some experiments the radioactive precursor was added at other growth stages.

Wall autolysis. To isolate and purify walls, cells were washed twice with distilled water, then resuspended in 1 mM-phenylmethylsulphonyl fluoride (PMSF) and broken by agitation with glass beads in a Braun homogenizer (five periods of 1 min). After sedimenting the walls (1200 g, 10 min), they were washed once with 1 mM-PMSF, five times with distilled water and then twice with autolysis buffer (50 mM-sodium acetate pH 5.5). All the washings were done at 0 °C.

In the standard autolysis procedure, purified walls were resuspended in autolysis buffer (100 μ l of the latter for walls obtained from 5 mg cell dry wt), plus 5 mM-sodium azide, and incubated at 37 °C in a New Brunswick rotatory shaker at 75 r.p.m. Incubation was continued for 48 h, then walls were sedimented and the released material was recovered from the supernatant by precipitation with 3 vols ethanol (16 h at 4 °C) containing PMSF at 1 mM (final concentration).

Wall treatment with Zymolyase. Zymolyase 5000 was purified by gel filtration and used for solubilization of wall mannoproteins in the presence of 5 mM-PMSF, as described by Pastor *et al.* (1984).

Mannoprotein analysis. Mannoproteins solubilized either by autolysis or by Zymolyase treatment from radioactively-labelled walls were analysed by SDS-PAGE and fluorography as described previously (Valentin *et al.*, 1984; Pastor *et al.*, 1984). Concanavalin A-Sepharose 4B binding and digestion by endo- β -N-acetylglucosaminidase H (Endo H) of the released mannoproteins were as described by Pastor *et al.* (1984).

Chromatography of amino acids. Radioactive samples were concentrated to dryness by heating, then resuspended in 6 M-HCl and treated at 100 °C for 24 h. The solution was neutralized using Dowex AG1 $\times$ 8 (carbonate form), and the supernatant was lyophilized and redissolved in distilled water. The samples were then passed through a Dowex 50W $\times$ 2 column (1 $\times$ 5 cm) and, after extensive washing of the latter, amino acids were eluted with 0.5 M-ammonia. The eluate was lyophilized, redissolved in 100 μ l distilled water and then applied to Whatman no. 1 paper. Ascending chromatography (up to 20 cm) of duplicate samples was carried out using water-saturated phenol as eluant. One of the chromatograms was cut into 1 cm pieces and the radioactivity determined in each of these, while the other chromatogram was sprayed with a solution of 0.2% (w/v) ninhydrin in methanol/acetic acid/water (50:1:49, by vol.).

Chromatography of sugars. Autolysis supernatants were hydrolysed in 3 M-HCl (105 °C, 4 h). Hydrolysates were neutralized using Dowex AG1 $\times$ 8 (carbonate form), concentrated by lyophilization and subjected to descending chromatography for 40 h on Whatman no. 1 paper in ethyl acetate/pyridine/water (8:2:1, by vol.), together with sugar standards.

Enzyme activities. β -1,3-Glucanase and mannanase activities were determined using laminarin and yeast mannan as respective substrates (Somogyi, 1952), followed by analysis for reducing sugar. Protease activity using Azocoll as substrate was measured as described by Saheki & Holzer (1974).

Analytical determinations. Total sugar was determined by the phenol/sulphuric acid method using D-glucose as standard (Dubois *et al.*, 1956). Reducing sugar was determined according to Nelson (1944), also with D-glucose as standard.

Chemicals. Dowex AG1 $\times$ 8, Dowex 50W $\times$ 2, dithiothreitol, laminarin, glucono- δ -lactone and all the protease inhibitors were from Sigma. Zymolyase 5000 and Endo H were from Miles Laboratories. SDS and other electrophoresis reagents were purchased from Bio-Rad. Azocoll was from Hoechst-Behring. [U - ^{14}C]Protein hydrolysate was from Amersham.

RESULTS

Optimal conditions for autolysis of purified walls

To quantify the release of mannoproteins during autolysis of *S. cerevisiae* walls, the latter were obtained from cells grown in the presence of a mixture of radioactively-labelled amino acids. Under these conditions, most of the radioactivity incorporated into walls corresponds to the mannoprotein fraction (Valentin *et al.*, 1984).

It has been reported that autolysis of yeast cell walls due to endogenous glucanases occurs at a pH optimum of between 5.0 and 5.5 (Fleet & Phaff, 1974; Notario, 1982; Hien & Fleet, 1983a). Thus, all the experiments in this study were done with *S. cerevisiae* walls resuspended in 50 mM-acetate buffer pH 5.5. Incubations were done at 37 °C since we found that at this temperature, as

Fig. 1

Fig. 2

Fig. 1. Mannoproteins from radioactively-labelled walls of *S. cerevisiae* X-2180-1A solubilized by autolysis (lane *a*) and then treated with Endo H (lane *b*), or by digestion of the walls with purified Zymolyase (lane *c*). Released mannoproteins were ethanol-precipitated and analysed by SDS-PAGE and fluorography. Their positions are indicated on the right-hand side.

Fig. 2. Fluorography of mannoproteins from *S. cerevisiae* walls released by autolysis (lane *a*) and then retained by Concanavalin A-Sepharose 4B (lane *b*). The position of the 29 kDa protein is indicated on the right-hand side.

with *C. albicans* (Elorza *et al.*, 1985), the amount of radioactivity released was double that at 30 °C. Pretreatment of the walls with reducing agents such as dithiothreitol (either before or during incubation in the autolysis buffer) did not improve the level of released radioactivity (data not shown).

To determine if the growth stage of the culture modified the release of wall mannoproteins, radioactive samples taken at the exponential, early and late stationary phases were analysed. Values for radioactivity released from the walls, normalized by the amount of cells (dry weight) in the samples, were independent of the growth stage; in subsequent experiments samples were taken during the late exponential phase.

Individual mannoproteins solubilized during autolysis

Using the optimal conditions for autolysis, about 20% of the radioactivity incorporated into walls from [¹⁴C]protein hydrolysate was solubilized after 48 h. Prolonged incubation did not increase the amount of solubilized radioactivity. Only about 40% of this radioactivity was ethanol-precipitable and the efficiency of precipitation could not be increased by using acetone or by adding carriers such as yeast mannan or bovine serum albumin.

Material released and precipitated with ethanol was analysed by SDS-PAGE [Fig. 1, lane (*a*)]. The most abundant of the released molecules was a 29 kDa mannoprotein with a sugar moiety *N*-glycosidically-linked to the peptide, since it was sensitive to Endo H digestion, giving a deglycosylated molecule of 26 kDa [Fig. 1, lane (*b*)]. This 29 kDa molecule is also preferentially

Fig. 3. (a) Release of sugar residues during autolysis of purified walls of *S. cerevisiae*. ●, Total sugar; ○, reducing sugar. (b) Enzyme activities during autolysis of purified walls of *S. cerevisiae*. ○, β -Glucanase in the supernatant; ●, β -glucanase in the wall pellet; □, mannanase in the supernatant; ■, mannanase in the wall pellet. One unit of enzyme activity is defined as the amount of enzyme that forms 1 nmol product min⁻¹, under the conditions of the assay.

Table 1. Effect of glucanase and protease inhibitors on the solubilization of mannoproteins during autolysis of *S. cerevisiae* cell walls

Walls were continuously labelled with [¹⁴C]protein hydrolysate as described in Methods. Inhibitors were incorporated separately into the autolysis buffer for the whole 48 h incubation period. Results are the mean of at least three independent experiments. Radioactivity solubilized in control samples corresponds to 7060 c.p.m. for walls obtained from 5 mg (dry wt) of cells.

Treatment	Radioactivity solubilized from walls (%)	Solubilization relative to control (%)	Solubilized radio-activity ethanol-precipitable (%)	Radioactivity solubilized and ethanol-precipitated (%)
Control	20.0	100.0	41.5	8.3
Glucono- δ -lactone (300 mM)	13.7	68.5	38.8	5.3
Boiling (5 min)	3.7	18.5	74.0	2.7
ZnSO ₄ (100 mM)	3.5	17.5	38.4	1.3
HgI ₂ (10 mM)	5.6	28.0	77.0	4.3
PHMB (5 mM)	7.3	36.5	83.8	6.1
Iodoacetamide (200 mM)	9.0	45.0	63.4	5.7
Antipain (500 μ M)	7.4	37.0	67.3	5.0
Chymostatin (500 μ M)	8.2	41.0	76.4	6.3
PMSF (5 mM)	10.3	51.5	43.0	4.4
<i>o</i> -Phenanthroline (5 mM)	12.8	64.0	34.4	4.4
EDTA (5 mM)	16.0	80.0	85.5	13.7
EGTA (5 mM)	20.7	103.5	31.1	6.5
TLCK (500 μ M)	26.7	133.5	35.3	9.4
Leupeptin (500 μ M)	23.4	117.0	41.7	9.8
Elastatinal (500 μ M)	21.0	105.0	54.4	11.4
8-Hydroxyquinoline (5 mM)	20.5	102.5	46.2	9.5
Pepstatin A (100 μ M)	18.6	93.0	28.4	5.3

released by Zymolyase digestion of cell walls [Fig. 1, lane (c); Pastor *et al.* (1984)]. None of the other proteins released by autolysis were Endo H-sensitive. However, most of them were retained by Concanavalin A-Sepharose 4B (Fig. 2), an indication of their glycoprotein nature. Since resistance to Endo H implies lack of *N*-glycosidically-linked mannose chains, the glycoprotein nature of these molecules must be due to short mannose chains *O*-glycosidically-linked to the peptide moieties.

β -Glucanase activities in Zymolyase act on the glucan network of the yeast wall releasing heterogeneous mannoprotein material of high molecular mass (larger than 120 kDa), in addition to the 29 kDa species (Pastor *et al.*, 1984). About 70% of these mannoproteins larger than 120 kDa remained in the walls after the 48 h autolysis period, as determined by digesting

the pellets of autolysed walls with Zymolyase (data not shown). However, a prominent band of 100 kDal appeared in the supernatant [Fig. 1, lane (a)]. This is about the same size as the *N*-deglycosylated form of the large heterogeneous material solubilized by Zymolyase (Pastor *et al.*, 1984).

Enzyme activities responsible for wall autolysis

During the autolytic process a significant amount of carbohydrate was released from the walls (Fig. 3a). Comparison of the levels of total sugar and reducing sugar present in the solution indicates that short saccharide chains are released into the medium. The nature of the liberated residues was determined by paper chromatography after extensive acid hydrolysis. About 60% of the sugar was glucose, the remainder being mostly mannose, with only a very small amount of *N*-acetylglucosamine. This is a glucose to mannose ratio similar to that existing in intact walls (Phaff, 1971; unpublished results of the authors). Partially in accordance with the data of Hien & Fleet (1983a), the level of glucanase activity in the wall decreased slightly during autolysis, with a corresponding appearance of activity in the supernatant only after 48 h (Fig. 3b). Some previously cryptic mannanase activity was also measurable in the wall pellet after extensive autolysis, but the level of this enzyme(s) in the supernatant was almost undetectable (Fig. 3b). Nevertheless, we cannot discount the possibility of this minor mannanase activity being an artifact due to small amounts of glucan contaminating the mannan substrate. The results shown in Fig. 3 were fully reproducible and are of a representative experiment.

Since only about 40% of the radioactivity previously incorporated into mannoproteins and released into the medium during autolysis was ethanol-precipitable, there was a possibility that the non-precipitable radioactivity was due to small peptides resulting from the action of some protease activity existing in the wall and acting during the autolysis process. To test this, the material that was non-precipitable by ethanol was hydrolysed in strong acid and then chromatographed. Radioactivity appeared in two regions, one of lower mobility mainly corresponding to neutral or basic amino acids and another (containing most of the radioactivity) of higher mobility corresponding to hydrophobic amino acids. Although these data pointed to the existence of wall proteases acting during the autolytic process, we were unable to detect any protease activity either in the wall pellet or in the released material when endogenous wall mannoproteins solubilized by Azocoll or SDS were used as substrates.

Effect of enzyme inhibitors on wall autolysis

To confirm the role of endogenous glucanases and proteases in wall autolysis and mannoprotein release, the effect of several inhibitors of these activities was tested (Table 1). Glucono- δ -lactone [known to inhibit *exo*- β -glucanases and, to a lesser extent, *endo*- β -glucanases (Abd-El-Al & Phaff, 1968; Farkaš *et al.*, 1973)] partially inhibited the autolytic solubilization of radioactivity incorporated into mannoproteins, confirming the role of glucan hydrolysis in the process. Boiling the walls (for 5 min in autolysis buffer) prior to the 48 h incubation, a treatment that inactivates glucanases (Notario, 1982; Hien & Fleet, 1983a) and possibly proteases, also drastically inhibited the release of radioactivity from walls.

Hg²⁺, Zn²⁺, *p*-hydroxymercuribenzoate (PHMB), iodoacetamide, antipain and chymostatin were the most effective protease inhibitors tested, while a partial effect was also observed with PMSF, *o*-phenanthroline and EDTA. In most cases, a higher inhibitory effect on solubilization of radioactivity correlated with an increased amount of ethanol-precipitated radioactivity; that is, although less radioactivity was released from the walls in the presence of such inhibitors, this preferentially appeared as long peptide chains. As a whole, the amount of radioactivity solubilized and ethanol-precipitated was only slightly diminished by the first group of protease inhibitors (Table 1). Treatment with glucono- δ -lactone plus any of the protease inhibitors effective in reducing the autolytic release of radioactivity resulted in a solubilization level similar to that of the glucanase inhibitor alone (not shown in detail). On the other hand, when PHMB and PMSF were present together, the radioactivity released was less than that released by either of them alone.

The effect of glucanase and protease inhibitors on the autolytic solubilization of individual mannoproteins was studied by SDS-PAGE. Treatments inhibiting glucanase activities

Fig. 4. Fluorography of mannoproteins released by wall autolysis in the presence of glucanase or protease inhibitory treatments. Lanes: (a), control; (b), 5 min boiling of the walls; (c), 300 mM-glucono- δ -lactone; (d), 10 mM- Hg^{2+} ; (e), 5 mM-PHMB; (f), 5 mM-PMSF; (g), 5 mM-8-hydroxyquinoline. The position of the 29 kDa protein is indicated on the right-hand side.

negatively affected the release of all the mannoprotein species [Fig. 4, lanes (b) and (c)], while protease inhibitors such as Hg^{2+} or PHMB facilitated the release of several molecular species which did not appear in their absence [Fig. 4, lanes (d) and (e)]. In this case, the 29 kDa mannoprotein was one of the more abundant, together with the 100 kDa species. The 29 kDa mannoprotein was also preferentially solubilized in the presence of PMSF [Fig. 4, lane (f)] or *o*-phenanthroline (not shown). With inhibitors that had no effect on the autolytic solubilization of radioactivity, the released mannoproteins were similar to those of the control [Fig. 4, lane (g), for 8-hydroxyquinoline].

DISCUSSION

Hydrolysis of the *S. cerevisiae* glucan network by glucanases present in the Zymolyase complex solubilizes several mannoprotein species, mainly heterogeneous material of high molecular mass and a 29 kDa mannoprotein (Pastor *et al.*, 1984). We have proposed that these molecules interact closely with glucan and, in fact, the large material seems to be covalently-linked to glucan (Shibata *et al.*, 1983; Pastor *et al.*, 1984). Other authors have also shown the role of exogenous β -glucanases in the release of mannan from yeast cell walls (Bacon *et al.*, 1969; Fleet & Manners, 1977).

The 29 kDa mannoprotein is also released during the autolytic process, together with other molecules not present in the Zymolyase supernatant. We cannot discount the possibility that at least part of the latter may result from an artifactual alteration of intrinsic wall mannoproteins. The existence of a mannanase activity in the *S. cerevisiae* cell wall has been reported (Maddox & Hough, 1971). Also, some protease(s) may be present in the wall (see below) and introduce artifacts in the mannoprotein band patterns detected by SDS-PAGE. The material larger than

120 kDal (the major component released by Zymolyase digestion) only appears in minor amounts in the supernatant of autolysed walls. However, a 100 kDal band, similar in size to the *N*-deglycosylated product of the former (Pastor *et al.*, 1984) and no longer Endo H-sensitive was observed in the autolysis supernatant. This 100 kDal molecule was still retained by Concanavalin A-Sepharose 4B, due probably to the presence of *O*-glycosidically-linked oligomannose chains (unpublished results of the authors).

Mannoprotein solubilization under the conditions reported in this paper is concomitant with the release of glucose (as short saccharide chains) to the incubation medium. This confirms the essential role of endogenous glucanases in wall autodegradation [for review, see Fleet (1984)]. Thus, autolytic solubilization of mannoproteins may parallel that caused by Zymolyase and the species released may correspond to those in close interaction with the glucan skeleton. Partial destruction of the latter may alter the whole wall structure, consequently releasing some of the mannoprotein constituents of the wall matrix (Sentandreu *et al.*, 1984). Autolysis of *C. albicans* cell walls also releases a protein material (Notario, 1982) that is separated by SDS-PAGE into four (blastospores) or two (mycelial cells) bands depending on the cell type (Elorza *et al.*, 1985).

The role of glucan hydrolysis in mannoprotein solubilization was confirmed by the lower amount of mannoprotein released in the presence of glucanase inhibitory treatments (glucono- δ -lactone, boiling of the walls). Although glucono- δ -lactone is a good inhibitor of *exo*- β -glucanases, it only partially inhibits *endo*- β -glucanases (Abd-El-Al & Phaff, 1968; Farkaš *et al.*, 1973) and this may explain the residual level of mannoprotein release in its presence. Metal ions such as Hg^{2+} and Zn^{2+} also inhibit yeast glucanases (Notario *et al.*, 1976; Villa *et al.*, 1978; Notario, 1982). We have shown their interfering effect on the solubilization of wall mannoproteins, but their possible role in the inhibition of some protease must also be considered, at least in the case of Hg^{2+} .

The protease contribution to the autolytic release of radioactivity is shown by the action of several protease inhibitors. Two classes of effects were observed: (i) some of the inhibitors (mercurials, iodoacetamide, antipain, chymostatin) significantly lowered the release of radioactivity while increasing the precipitability by ethanol of the released material, and (ii) other inhibitors (PMSF, *o*-phenanthroline) affected autolysis to a lesser extent and the precipitability of the solubilized material was the same as in their absence. From these results, it appears that at least two proteases (or protease groups) may be acting during autolysis. One of them would probably be a cysteine protease sensitive to agents interacting with essential thiol groups of the molecule. This protease would hydrolyse previously released peptides to short amino acid chains that were non-precipitable by ethanol. The other protease is less easy to characterize from the nature of the second group of inhibitors, and the existence of more than a single enzyme seems plausible. These proteases would be effective in the release of peptides from the wall structure, without extensively degrading them. Our inability to detect any protease activity in autolysed walls or in the released supernatant might be caused by the lack of accessibility of the substrates used in the assay to the protease molecules of the walls, and/or to the instability of the released enzyme molecules.

Hydrogen and thiol bonds may be important in the interactions between mannoproteins in the wall (Valentin *et al.*, 1984), and covalent linkages between glucan and the large size mannoprotein material seem to exist (Shibata *et al.*, 1983; Pastor *et al.*, 1984). The treatment of yeast cell walls with exogenous proteases causes the release of mannan (Russell *et al.*, 1973). The possibility therefore exists that limited endoproteolysis of some mannoprotein molecules would detach peptide chains from the wall network. In any case, wall proteases would only be effective in solubilizing mannoprotein material after partial autolytic degradation of glucan, as shown by the effect of glucono- δ -lactone and protease inhibitors when present together in the incubation mixture.

To date, only two proteases (aminopeptidases) are thought to be localized outside the yeast cytoplasmic membrane (Frey & Röhm, 1979; Trumbly & Bradley, 1983). The relationship between these proteases and those whose existence is suggested from our results remains to be elucidated.

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REFERENCES

- ABD-EL-AL, A. T. H. & PHAFF, H. J. (1968). Exo- β -glucanases in yeast. *Biochemical Journal* **109**, 347–360.
- BACON, J. S. D., FARMER, V. C., JONES, D. & TAYLOR, I. F. (1969). The glucan components of the cell wall of baker's yeast (*Saccharomyces cerevisiae*) considered in relation to its ultrastructure. *Biochemical Journal* **114**, 557–576.
- DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**, 350–356.
- ELANGO, N., CORREA, J. U. & CABIB, E. (1982). Secretory character of yeast chitinase. *Journal of Biological Chemistry* **257**, 1398–1400.
- ELORZA, M. V., MURGUI, A. & SENTANDREU, R. (1985). Dimorphism in *Candida albicans*: contribution of mannoproteins to the architecture of yeast and mycelial cell walls. *Journal of General Microbiology* **131**, 2209–2216.
- FARKAŠ, V., BIELY, P. & BAUER, S. (1973). Extracellular β -glucanases of the yeast *Saccharomyces cerevisiae*. *Biochimica et biophysica acta* **21**, 246–255.
- FLEET, G. H. (1984). The occurrence and function of endogenous wall-degrading enzymes in yeast. In *Microbial Cell Wall Synthesis and Autolysis*, pp. 227–238. Edited by C. Nombela. Amsterdam: Elsevier.
- FLEET, G. H. & MANNERS, D. J. (1977). The enzymic degradation of an alkali-soluble glucan from the cell walls of *Saccharomyces cerevisiae*. *Journal of General Microbiology* **98**, 315–327.
- FLEET, G. H. & PHAFF, H. J. (1974). Glucanases in *Schizosaccharomyces*. Isolation and properties of the cell wall associated (1 $\rightarrow$ 3)- β -glucanases. *Journal of Biological Chemistry* **249**, 1717–1728.
- FREY, J. & RÖHM, K. H. (1979). External and internal forms of yeast aminopeptidase II. *European Journal of Biochemistry* **97**, 169–173.
- HIEN, N. H. & FLEET, G. H. (1983a). Separation and characterization of six (1 $\rightarrow$ 3)- β -glucanases from *Saccharomyces cerevisiae*. *Journal of Bacteriology* **156**, 1204–1213.
- HIEN, N. H. & FLEET, G. H. (1983b). Variation of (1 $\rightarrow$ 3)- β -glucanases in *Saccharomyces cerevisiae* during vegetative growth, conjugation and sporulation. *Journal of Bacteriology* **156**, 1214–1220.
- KITAMURA, K., KANEKO, T. & YAMAMOTO, Y. (1974). Lysis of viable yeast cells by enzymes of *Arthrobacter luteus*. II. Purification and properties of an enzyme, Zymolyase, which lyses viable yeast cells. *Journal of General and Applied Microbiology* **20**, 323–344.
- MADDUX, I. S. & HOUGH, J. S. (1971). Yeast glucanase and mannanase. *Journal of the Institute of Brewing* **77**, 44–47.
- NELSON, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *Journal of Biological Chemistry* **153**, 375–380.
- NOTARIO, V. (1982). β -Glucanases from *Candida albicans*: purification, characterization and the nature of their attachment to cell wall components. *Journal of General Microbiology* **128**, 747–759.
- NOTARIO, V., VILLA, T. G. & VILLANUEVA, J. R. (1976). Purification of an exo- β -D-glucanase from cell-free extracts of *Candida utilis*. *Biochemical Journal* **159**, 555–562.
- PASTOR, F. I. J., VALENTIN, E., HERRERO, E. & SENTANDREU, R. (1984). Structure of the *Saccharomyces cerevisiae* cell wall: mannoproteins released by zymolyase and their contribution to wall architecture. *Biochimica et biophysica acta* **802**, 292–300.
- PHAFF, H. J. (1971). Structure and biosynthesis of the yeast cell envelope. In *The Yeasts, Vol. 2, Physiology and Biochemistry of Yeasts*, pp. 135–210. Edited by A. H. Rose & J. S. Harrison. London: Academic Press.
- PHAFF, H. J. (1977). Enzymatic yeast cell wall degradation. *Advances in Chemistry* **160**, 244–282.
- REICHEL, B. Y. & FLEET, G. H. (1981). Isolation, properties, function and regulation of endo-(1 $\rightarrow$ 3)- β -glucanases in *Schizosaccharomyces pombe*. *Journal of Bacteriology* **147**, 1085–1094.
- REY, F. J. DEL, SANTOS, T., GARCIA-ACHA, I. & NOMBELA, A. C. (1979). Synthesis of 1,3- β -glucanases in *Saccharomyces cerevisiae* during the mitotic cycle, mating and sporulation. *Journal of Bacteriology* **139**, 924–931.
- RUSSELL, I., GARRISON, I. F. & STEWART, G. G. (1973). Studies on the formation of sphaeroplasts from stationary phase cells of *Saccharomyces cerevisiae*. *Journal of the Institute of Brewing* **79**, 48–54.
- SAHEKI, T. & HOLZER, H. (1974). Comparisons of the tryptophan synthase inactivating enzymes with proteinases from yeast. *European Journal of Biochemistry* **42**, 621–626.
- SANCHEZ, A., VILLANUEVA, J. R. & VILLA, T. G. (1982). *Saccharomyces cerevisiae* secretes two exo- β -glucanases. *FEBS Letters* **138**, 209–212.
- SENTANDREU, R., HERRERO, E., MARTINEZ, J. P. & LARRIBA, G. (1984). Biogenesis of the yeast cell wall. *Subcellular Biochemistry* **10**, 193–235.
- SHIBATA, N., MIZUGAMI, K., TAKANO, K. & SUZUKI, S. (1983). Isolation of mannan-protein complexes from viable cells of *Saccharomyces cerevisiae* X2180-1A wild type and *Saccharomyces cerevisiae* X2180-1A-5 mutant strains by the action of Zymolyase-60,000. *Journal of Bacteriology* **156**, 552–558.
- SOMOGYI, M. (1952). Notes on sugar determination. *Journal of Biological Chemistry* **195**, 19–23.
- TRUMBLY, R. & BRADLEY, G. (1983). Isolation and characterization of aminopeptidase mutants of *Saccharomyces cerevisiae*. *Journal of Bacteriology* **156**, 36–48.
- VALENTIN, E., HERRERO, E., PASTOR, F. I. J. & SENTANDREU, R. (1984). Solubilization and analysis of mannoprotein molecules from the cell wall of *Saccharomyces cerevisiae*. *Journal of General Microbiology* **130**, 1419–1428.
- VILLA, T. G., LACHANCE, M. A. & PHAFF, H. J. (1978). β -Glucanases of the yeast *Kluyveromyces fragilis*: partial purification and characterization. *Experimental Mycology* **2**, 12–25.