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# *Saccharomyces cerevisiae* Mannoproteins Form an External Cell Wall Layer That Determines Wall Porosity

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**A β-glucanase (Z-glucanase) from Zymolyase was freed from a protease (Z-protease) by affinity chromatography on $\alpha_2$-macroglobulin–Sepharose columns and used to solubilize proteins from isolated cell walls of *Saccharomyces cerevisiae*. The cell wall proteins were labeled with $^{125}I$ and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The bulk of the labeled material had very low mobility. Its mannoprotein nature was demonstrated by precipitation with specific antibodies and by conversion to a band with an average molecular weight of 94,000 after incubation with endo-β-*N*-acetylglucosaminidase. The intact mannoproteins were hydrolyzed by Z-protease, but were resistant to the enzyme when the carbohydrate was first removed by endo-β-*N*-acetylglucosaminidase. In intact cells, lysis of cell walls by Z-glucanase required a previous incubation with Z-protease, which led to solubilization of most of the $^{125}I$-labeled proteins. Other proteases that did not attack the cell wall mannoproteins were unable to substitute for Z-protease. The specific effect of Z-protease is consistent with the notion that mannoproteins form a surface layer of the cell wall that penetrates the wall to some depth and shields glucans from attack by Z-glucanase. Mannoproteins, however, do not appear to cover the inner face of the cell wall, because isolated cell walls, in contrast to intact cells, were completely solubilized by Z-glucanase in the absence of protease. The function of mannoproteins in determining cell wall porosity was highlighted by the finding that horseradish peroxidase ($M_r$, 40,000) causes lysis of cells that had been treated with Z-protease. Depletion of mannoproteins by Z-protease also resulted in the disappearance of a darkly stained surface layer of the cell wall, as observed by electron microscopy. Other agents that facilitate cell lysis by Z-glucanase, such as 2-mercaptoethanol, digitonin, and high concentration of salts, caused little or no solubilization of mannoprotein. We assume that they perturb and loosen the structure of the mannoprotein network, thereby increasing its porosity. The implications of our results for the construction of the yeast cell wall and the anchoring of mannoprotein to the cell are discussed.**



The fungal cell wall is an essential component of the cell. Because of its rigidity, the cell wall prevents lysis of the protoplast in a hypotonic environment, protects the cell from mechanical injury, and determines cellular shape. It also retains certain periplasmic enzymes that break down nutrients to components that can be transported through the plasma membrane. Such is the case with invertase and acid phosphatase in *Saccharomyces cerevisiae* (1). Another function of the cell wall, based on its porosity, is to limit the size and type of substances that may come in contact with the plasma membrane.

In *S. cerevisiae*, the cell wall consists mainly of glucose and mannose polymers, in approximately equal amounts (8). Mannan is linked to a protein moiety (27) and should therefore be designated as mannoprotein. Although a great deal is known about the structure (5) and biosynthesis (5, 21) of the carbohydrate portion of the mannoprotein, much less information is available about the protein moiety (27, 28). In particular, it is not known whether one or more polypeptide chains are present. Several lines of evidence, based on the use of anti-mannan antibodies (5) and of concanavalin A (ConA) (12, 30), indicate that mannose polymers are available at the external surface of the cell wall, but it is not known how deeply the mannoprotein reaches into the wall.

With regard to the functional aspects, the predominantly β(1→3)-linked glucan appears to be responsible for the structural integrity of the cell wall. Thus, isolated cell walls can be solubilized almost entirely by a purified β(1→3)glucanase (3, 15, 34; this paper). On the other hand, the mannoprotein can be extracted from the cell wall of intact cells without much change in cell shape (18). On the basis of this evidence, the mannoprotein has come to be regarded as a filling material, enmeshed in the glucan structural network; there is, however, no clear notion of its function.

Recently, it has been reported that lysis of the cell wall of intact cells of *S. cerevisiae* by a β(1→3)glucanase from *Arthrobacter* sp. (in the commercial preparation, zymolyase) or *Oerskovia* sp. requires the concomitant or previous treatment with another agent, either a protease or a thiol (14, 25, 34). The nature of these agents implies that a protein(s) is involved in protecting the structural glucan from glucanase action. We found that digitonin or high concentrations of salts could also facilitate glucanase action (see below). In the present study, we pursued these observations further in the hope of gaining a better understanding of the nature and distribution of the cell wall mannoprotein(s) and of its possible role in controlling wall porosity.

## MATERIALS AND METHODS

**Yeast and growth conditions.** The diploid strain of *S. cerevisiae* X2180 (ATCC 26109) was used throughout. Cells were grown in a minimal medium and harvested at the mid-logarithmic phase as described previously (7).

**Chemicals.** Outdated human blood plasma, as a source of

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$\alpha_2$-macroglobulin, was obtained from the Blood Bank of the National Institutes of Health Clinical Center. Cibacron Blue F-3-GA, laminarin, digitonin, ConA-Sepharose, lactoperoxidase, horseradish peroxidase, and Coomassie brilliant blue R were all products of Sigma Chemical Co. (St. Louis, Mo.). Sepharose CL6B, Sephacryl S-300, and cyanogen bromide-activated Sepharose 4B were purchased from Pharmacia Fine Chemicals, Inc. (Piscataway, N.J.). Bio-Gel CM30 and Bio-Gel P-150 were from Bio-Rad Laboratories (Richmond, Calif.). Zymolyase 60,000 was purchased from Miles Laboratories (Elkhart, Ind.). A sample of zymolyase 60,000 was also generously provided by K. Kitamura, Kirin Brewery Co., Takasaki, Japan. Endo-β-*N*-acetyl-glucosaminidase (endo-H) was obtained either from Miles Laboratories or from Health Research Incorporated. Goat anti-$\alpha_2$-macroglobulin antibodies and goat anti-rabbit immunoglobulin G (IgG) were from Cappel Laboratories (Cochranville, Pa.). Antibodies against the α(1→3)mannosyl group of yeast mannan were kindly provided by C. E. Ballou, University of California, Berkeley. Protein A (Pansorbin), pustulan, and Hide Powder Azure were bought from Calbiochem-Behring (San Diego, Calif.), and $Na^{125}I$ was from New England Nuclear Corp. (Boston, Mass.). Bakers' yeast mannan was obtained as described by Cifonelli and Smith (9).

**$\alpha_2$-Macroglobulin.** $\alpha_2$-Macroglobulin was purified by a combination of the methods of Virca et al. (33) and Kurecki et al. (16). Briefly, to human plasma (600 ml) 50% polyethylene glycol (average molecular weight, 4,000) was added at 4°C to a final concentration of 4.5%. The precipitate obtained after 30 min of constant stirring was discarded. More 50% polyethylene glycol was added to the supernatant fluid, to obtain a final concentration of 15%. After 30 min of stirring, the precipitate was removed by centrifugation and dissolved in 0.05 M Tris-chloride (pH 8) containing 0.15 M NaCl to a final volume of 70 ml. This solution was fractionated on a Cibacron blue-Sepharose CL6B column as described by Virca et al. (33). The fractions containing $\alpha_2$-macroglobulin, as determined by immunodiffusion with antibody against the protein, were pooled, applied to a zinc chelate column with a bed volume of 200 ml, and eluted by the method of Kurecki et al. (16). The fractions containing material that precipitated with goat anti $\alpha_2$-macroglobulin antibodies were concentrated by ultrafiltration to 20 ml and applied to a Sephacryl S-300 column (400-ml bed volume) previously equilibrated with 0.05 M Tris chloride (pH 8.6) containing 0.5 M NaCl. After eluting with the same buffer, the fractions that contained $\alpha_2$-macroglobulin were pooled and concentrated by ultrafiltration. When the purified material was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, a major band and several minor ones were detected.

**Protease-free glucanase *(Z-glucanase)*.** $\alpha_2$-Macroglobulin was coupled to CNBr-activated Sepharose 4B under the conditions specified by the manufacturer, with an initial proportion of 17 mg of $\alpha_2$-macroglobulin per g (dry weight) of gel. Approximately 80% was bound, as measured by depletion of 280 nm-absorbing material in solution.

Zymolyase 60,000 (67.5 mg) was dissolved in 9 ml of 0.05 M Tris (pH 7.5). Some insoluble material was eliminated by centrifugation, and then the solution was applied to a column (0.9 by 11 cm) of $\alpha_2$-macroglobulin–Sepharose, previously equilibrated with 0.05 M Tris (pH 7.5). The same buffer was used for elution, and fractions were assayed for β(1→3)glucanase and for protease. The fractions containing the former activity and devoid of the latter were pooled. Either no protease was found in the eluate, or a trace emerged at the tail end of the glucanase peak. The recovery of β(1→3)glucanase activity was about 90%. During purification, β(1→3)glucanase activity was assayed with laminarin as the substrate, essentially as described by Fleet and Phaff (10), by measuring the liberation of reducing power. One unit is defined as that amount of enzyme that gives rise to the liberation of 1 μmol of product (calculated as glucose) per min at 30°C. The purified preparation was free from mannanase and β(1→6)glucanase as judged from the lack of liberation of reducing power (10) with yeast mannan and pustulan, respectively, as substrates.

**Purification of Z-protease.** The procedure of Kitamura (13), which makes use of Bio-Gel CM-30 and Bio-Gel P-150 columns, was used for Z-protease purification. In some cases, it was necessary to apply a linear gradient of increasing phosphate concentration between 0.01 and 0.1 M to elute the enzyme from the Bio-Gel CM-30 column. The purified preparation showed one major and one very faint band upon SDS-gel electrophoresis. The protease from zymolyase (Z-protease) and other proteases were assayed with Hide Powder Azure as previously described (32). One unit of activity represents the amount of enzyme that yields one unit of absorbance at 595 nm in 1 h.

**Preparation of cell walls.** The previously reported procedure (24), involving breakage of cells with glass beads, was used, with the following modifications. Cells were not treated with Formalin before disruption. To eliminate contaminating proteins as much as possible, cell walls were washed five times with distilled water and twice with each of the following solutions: 5 M LiCl, water, 3% SDS, and water.

**Solubilization of cell walls.** Cell walls (about 200 μl as a pellet) obtained from 500 mg (wet weight) of cells were incubated overnight at 30°C with 100 μl (0.7 U) of Z-glucanase and 100 μl of 0.05 M Tris-chloride (pH 7.5). Sodium azide was added to give a final concentration of 0.02%. After incubation, a small amount of material that remained insoluble was eliminated by centrifugation.

**ConA-Sepharose chromatography.** To separate mannoproteins from glucanase after solubilization of cell walls, the digest from 200 μl of cell walls was applied to a ConA-Sepharose column (5-ml bed volume) previously equilibrated with 0.05 M Tris-chloride (pH 7.5) containing 0.5 M NaCl. If the cells or cell walls had not been labeled with $^{125}I$, a small amount of iodinated and solubilized material from a previous experiment (ca. 25,000 cpm) was added as marker. Elution was started with equilibration buffer and continued until the effluent was free of radioactivity. Glycoproteins were eluted with 0.5 M α-methylmannoside in equilibration buffer. Fractions containing radioactivity were pooled and concentrated by ultrafiltration to a final volume of 2 ml. When so desired, the α-methylmannoside present in the eluate was removed by overnight dialysis against water.

**Iodination. (i) Intact cells or isolated cell walls, chloramine T method.** Cells (50 mg [wet weight]) were suspended in 1.25 ml of 0.05 M potassium phosphate, pH 7.5 (buffer A), and 0.5 mCi of $Na^{125}I$ was added (usually in a volume of 20 μl), followed by 0.5 ml of chloramine T (2 mg/ml) in buffer A. After 7 min of incubation at room temperature, the reaction was stopped by the addition of 0.5 ml of sodium metabisulfite (4 mg/ml) in buffer A and 20 μl of 1 mM KI. After centrifugation, the cells were washed four or five times with buffer A. The same procedure was applied to cell walls (100 μl, as a pellet) prepared as described above.

**(ii) Intact cells, lactoperoxidase method.** To 50 mg of cells suspended in 1.25 ml of buffer A, 100 μl of lactoperoxidase (1 mg/ml) was added, followed by 0.5 mCi of $Na^{125}I$ (in 10 μl).

The reaction was started by adding 20 μl of 3% $H_2O_2$. Two more additions of $H_2O_2$ were made at 7-min intervals. After 21 min at room temperature, the reaction was stopped with 2 ml of tyrosine (1 mg/ml) in buffer A, followed by 20 μl of 1 mM KI. Cells were centrifuged and washed as above.

**(iii) Solubilized cell walls, chloramine T method.** To 100 μl of solubilized cell wall material purified on a ConA-Sepharose column as described above and resulting from 33 mg (wet weight) of original cells, 100 μl of buffer A and 0.5 mCi of $Na^{125}I$ (10 μl) were added, followed by 100 μl of chloramine T (10 mg/ml) in buffer A. After 2 min the reaction was stopped with 200 μl of sodium metabisulfite (8 mg/ml) and 20 μl of 1 mM KI. Excess $Na^{125}I$ was eliminated by subjecting the mixture to ConA-Sepharose chromatography as outlined above.

**(iv) Solubilized cell walls, lactoperoxidase method.** To 100 μl of solubilized cell wall material, 100 μl of buffer A and 50 μl of lactoperoxidase (1 mg/ml) were added, followed by 0.5 mCi of $Na^{125}I$ in 10 μl. The reaction was started with 20 μl of 3% $H_2O_2$; two more additions of $H_2O_2$ were made after 7 and 14 min. At 21 min, 200 μl of tyrosine (1 mg/ml) and 20 μl of 1 mM KI were added to stop the reaction. Excess $Na^{125}I$ was eliminated by chromatography on ConA-Sepharose as described above.

**Endo-H treatment.** To 20 μl of $^{125}I$-labeled, solubilized wall material containing 100,000 cpm, 20 μl of citrate-phosphate buffer, pH 5 (11), and 10 μl (0.01 U) of endo-H were added. One drop of toluene was added, and the mixture was incubated for 24 h at 37°C. A second addition of endo-H was followed by another 24-h incubation.

**Precipitation of mannoprotein with antimannan antibody.** To samples of $^{125}I$-labeled, solubilized cell wall material containing about 100,000 cpm, 15 μl of rabbit antibody against α(1→3)-linked mannosyl units was added, followed by 25 μl of 0.05 M Tris-chloride buffer (pH 7.5). The mixtures were stored at 4°C overnight, whereafter 15 μl of goat anti-rabbit IgG was added. After 3 h at room temperature, 20 μl of 10% Pansorbin (protein A) was added. The mixture was incubated for 30 min at room temperature and centrifuged. Radioactivity was measured in the pellet and supernatant fluid. The pellet was washed with 0.05 M Tris-chloride (pH 7.5) containing 0.5 M NaCl and counted again. Less than 10% of the radioactivity was lost in the washing. For SDS-gel electrophoresis, 20 μl of 4% SDS was added to the pellet. Heating for 2 min in a boiling water bath resulted in dissolution of the pellet.

**Protease treatment of $^{125}I$-labeled material.** Protease treatment was applied to solubilized cell wall material that had been originally labeled either at the stage of intact cells, as cell walls, or after solubilization of cell walls with Z-glucanase. Also, protease treatment was either preceded or followed by an incubation with endo-H, which was carried out as outlined above. After incubation with either one of the two enzymes, the reaction mixture was boiled for 2 min before the addition of the other enzyme. In general, to an amount of mannoprotein containing ca. 120,000 cpm as $^{125}I$ in approximately 20 μl, 25 μl of 0.05 M Tris-chloride (pH 7.5) and 25 μl (4 U) of Z-protease were added. Incubation was for 30 min at 30°C, and the reaction was stopped by immersion in a boiling water bath as described above.

**SDS-polyacrylamide gel electrophoresis.** Slab gel electrophoresis in the presence of SDS was performed essentially as specified by Laemmli (17). The concentration of acrylamide was 3% for the stacking gel and 9% for the separating gel. After electrophoresis, gels were stained with 0.5% Coomassie brilliant blue R and dried in a slab gel drier. Autoradiography was performed with Kodak X-Omat XAR-5 film, with an exposure time of 2 to 10 days.

**Treatment of cells with horseradish peroxidase.** To 25 mg (wet weight) of cells, 0.47 ml of 0.05 M Tris-chloride (pH 7.5) and 30 μl (4.8 U) of Z-protease were added. After 30 min of incubation at 30°C with shaking, the cells were centrifuged and washed twice with 0.5 ml of 0.05 M Tris-chloride (pH 7.5). The pellet was suspended in either 0.125 ml of 0.05 M Tris-chloride, pH 7.5 (control), or in 0.125 ml of horseradish peroxidase (1 mg/ml) in the same buffer. The suspensions were rotated for 1 h at room temperature. Samples of cells that had not been treated with Z-protease were carried through the same procedure.

## RESULTS

**Solubilization, $^{125}I$ labeling, and electrophoretic separation of cell wall proteins.** To isolate wall proteins in an undenatured state, cells were first disrupted by shaking with glass beads; the resulting walls were isolated by centrifugation and purified by extensive sequential washing with water, 5 M lithium chloride, and 3% SDS to remove all extraneous proteins. The walls were then solubilized by incubation with Z-glucanase. To facilitate the detection of the wall proteins, they were labeled with $^{125}I$. Iodination was performed at any one of three stages, i.e., either on the intact cells, on the cell walls, or on the solubilized proteins. In the last case, the labeled mannoproteins were further purified by affinity chromatography on ConA-Sepharose columns to eliminate unreacted $^{125}I$ and any iodinated protein from the glucanase preparation. Most of the radioactivity (84 ± 12%, average of five experiments) was adsorbed on the column, and 93.5 ± 5% of the adsorbed material was eluted by 0.5 M α-methylmannoside.

When the solubilized proteins were subjected to SDS-polyacrylamide gel electrophoresis and revealed by autoradiography, a very similar pattern emerged for all three conditions (Fig. 1, lanes A, C, and E). Most of the material moved only slightly or not at all from the origin. By cutting gels into slices and counting the radioactivity, we found that about 80% of the radioactivity was in the area above the 67K marker. All gels showed a strong and rather wide band at about 67K and several minor bands of lower molecular weight. Decreasing the acrylamide cross-linking did not substantially improve the pattern. In an attempt to increase the mobility of the proteins by removal of the bulky carbohydrate moiety, the preparations were treated with endo-H (29) before electrophoresis. In all cases, a marked change ensued (Fig. 1, lanes B, D, and F). Most of the material at the origin disappeared, and a new, diffuse band could be seen, corresponding approximately to the position of the 94K standard. In addition to the band at 67K, another band (Fig. 1, lane B, indicated by a) was found in the endo-H digest from intact cells, but not from the other preparations. In the same figure, b indicates the position of a minor band of lane A, which disappeared after endo-H treatment, probably giving rise to the band of similar intensity that can be seen just above the 30K marker.

**Precipitation of mannoproteins with anti-mannan antibodies.** To characterize the cell wall proteins, the iodinated preparations were treated with antibodies against the α(1→3)mannosyl linkage found in mannan (4); 70 to 80% of the labeled material in each of the three preparations was precipitated by antibody (Table 1). After endo-H treatment, the amount of iodinated material in the precipitate was decreased by about 50%. Electrophoresis of the precipitated material in SDS-polyacrylamide gels revealed similar pat-

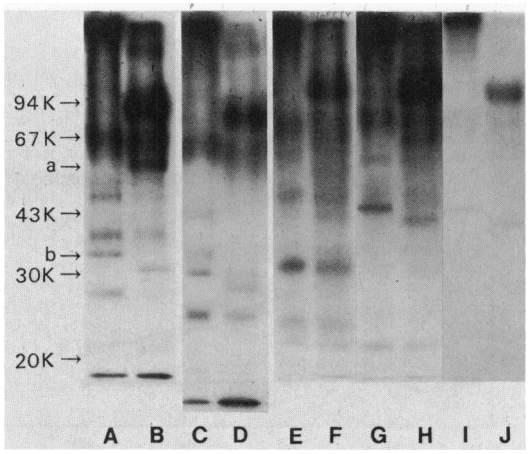


FIG. 1. SDS-polyacrylamide gel electrophoresis of iodinated cell wall proteins. Iodination was performed by the chloramine T method on intact cells (lanes A and B), on cell walls (lanes C and D), or on solubilized cell wall proteins (lanes E and F). In lanes G through J, iodination was carried out by the lactoperoxidase procedure, either on solubilized proteins (lanes G and H) or on intact cells (lanes I and J). Lanes A, C, E, G, and I represent untreated samples, and lanes B, D, F, H, and J represent samples after treatment with endo-H. Each pair corresponds to a different gel, hence the difference in mobility of the bands. The numbers on the left side indicate the positions of molecular weight standards for lanes A and B only. Gel electrophoresis standards were either from Bio-Rad or from Pharmacia and consisted of (molecular weights within parentheses) phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,500), and either lysozyme or α-lactalbumin (both 14,400). In the other gels the positions of major bands relative to the corresponding standards were the same. Gels were subjected to autoradiography as described in the text.

terns to those found in untreated samples, except that the minor bands with molecular weights below 40,000 to 50,000 were not visible (Fig. 2, lanes C and D, compared with controls in lanes A and B, respectively). In Fig. 2 only the results obtained with proteins labeled after solubilization are shown; an essentially identical pattern was seen when iodination was carried out either on intact cells or on cell walls.

**Mannoproteins on external and internal wall layers.** Chloramine T, because of its small size, would be expected to penetrate the cell wall easily and to lead to the labeling of inner as well as superficial wall proteins. This assumption is confirmed by the efficiency of the chloramine T method in labeling either intact cells, isolated cell walls, or solubilized wall proteins and by the similar electrophoretic patterns in all three cases (Fig. 1). To ascertain whether any difference in composition exists between surface and internal layers of the wall, iodinations with chloramine T and lactoperoxidase were compared. Lactoperoxidase, because of its large size ($M_r$, 93,000), cannot enter the wall and thus should lead only to surface labeling. The amount of incorporation into intact cells with the peroxidase method was only 1.3% of that obtained with chloramine T. This was not due to an intrinsic difference in the efficiency of the two procedures. When a preparation of solubilized wall proteins was subjected to iodination, the incorporation with peroxidase was 88% of that obtained with chloramine T, and the electrophoretic pattern of the material was also practically identical (Fig. 1, compare lanes G and H with E and F, respectively). The material labeled in intact cells was solubilized and subjected to gel electrophoresis. Although the intensity of the bands was weaker because of the very small amount of radioactivity available, the main bands showed the same positions as in the preparations labeled either with peroxidase in solubilized material or with chloramine T (Fig. 1, lanes I and J).

TABLE 1. Precipitation of iodinated mannoproteins with antimannan antibody

| Material subjected to iodination[a] | % of total counts in precipitate | |
|---|---|---|
| | Control | After endo-H |
| Whole cells | 78 | 25 |
| Cell walls | 73 | 40 |
| Solubilized cell walls | 69 | 46 |

[a] In every case the precipitation was carried out on solubilized material. When intact cells were labeled, cell walls were prepared and subsequently lysed with glucanase. Labeled cell walls were also solubilized with glucanase. Conditions for endo-H treatment and for antibody precipitation are described in the text.

**Effect of carbohydrate moiety of mannoproteins on Z-protease action.** Previous reports (14, 26, 34) and our own results (see below) on the need of both a glucanase and a protease component for cell wall solubilization by the commercial preparation zymolyase prompted us to investigate the action of the protease (Z-protease) on wall mannoprotein. Gel electrophoresis patterns of cell wall proteins after treatment with Z-protease did not differ substantially from those of the untreated control, except that some decrease in intensity was usually observed in the area near the top of the running gel, and some bands of high mobility appeared (date not shown). Incubation with endo-H after protease, however, caused a striking decrease in most of the bands observed after electrophoresis (Fig. 3, lane D). If the treatments were inverted, i.e., endo-H before Z-protease (Fig. 3, lane C), little change in the bands was observed, an indication that attachment of the carbohydrate moiety to the protein is required for Z-protease action. Experiments in which proteins were separated by gel chromatography on Sephacryl S-200 yielded similar results, except that some protein degra-

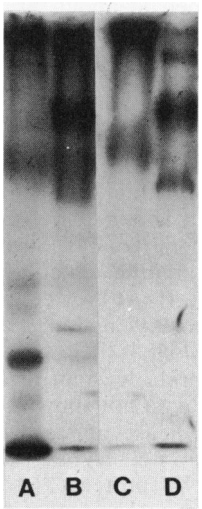


FIG. 2. SDS-gel electrophoresis of cell wall glycoproteins precipitated by anti α(1→3)mannosyl antibody. Results shown were obtained with a preparation labeled with $^{125}I$ after solubilization of cell walls and further purified by chromatography on ConA-Sepharose. Lanes A and B show controls, without addition of antibody, i.e., original preparation (A), and endo-H treated preparation (B). Lanes C and D show material precipitated with antibody from original preparation (C) and endo-H-treated preparation (D).


FIG. 3. Effect of Z-protease on cell wall mannoproteins, as observed by SDS-gel electrophoresis. Lanes: A, original preparation; B, after endo-H treatment; C, endo-H treatment followed by Z-protease treatment; D, Z-protease treatment followed by endo-H treatment. The preparation shown had been iodinated after solubilization of cell walls. Similar results were obtained when iodination was carried out on intact cells or on isolated cell walls.

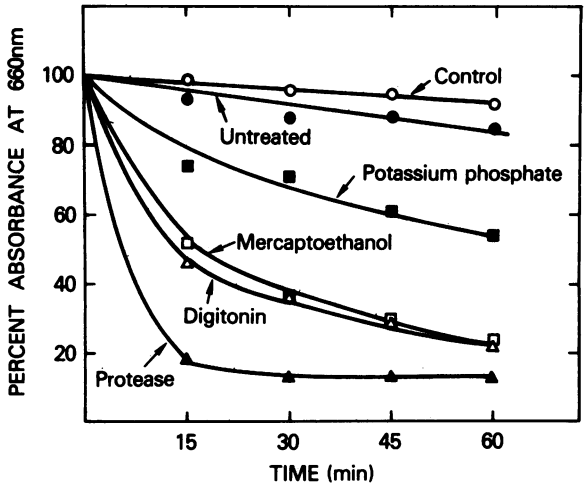


FIG. 4. Effect of different treatments of cells on subsequent lysis by Z-glucanse. Cells (25 mg [wet weight]) were incubated in a total volume of 0.5 ml with each of the following mixtures: ▲, 4.8 U of Z-protease in 50 mM Tris-chloride (pH 7.5); □, 0.42 M 2-mercaptoethanol in 25 mM 2-(*N*-morpholino)-ethanesulfonic acid (pH 6.3); △, 0.1% digitonin in 25 mM 2-(*N*-morpholino)-ethanesulfonic acid (pH 6.3); ■, 1 M potassium phosphate (pH 7.5). The control (○) contained 0.5 ml of 50 mM Tris-chloride (pH 7.5). All mixtures were incubated for 30 min at 30°C with shaking and centrifuged for 5 min at 1,800 × *g*. The cells were washed twice with 50 mM Tris-chloride (pH 7.5) and suspended in 2 ml of the same buffer. To 1 ml of each suspension, 4 ml of 50 mM Tris-chloride (pH 7.5) and 25 μl (0.034 U) of Z-glucanase were added. The mixtures were incubated at 30°C with shaking, and turbidity was monitored at 15-min intervals in a Coleman Junior spectrophotometer at 660 nm.

dation by Z-protease was observed even after preincubation with endo-H (data not shown).

**Effect of different agents on cell wall lysis by Z-glucanase.** The effect of Z-protease on mannoproteins and its peculiar specificity appeared to provide a rationale for the participation of this enzyme in cell wall lysis by zymolyase. To investigate the effect of this and other agents on the action of the glucanase contained in zymolyase on intact cells, it was necessary to separate the glucanase from the protease. The converse operation, i.e., preparation of glucanase-free protease, was carried out as described by Kitamura (13), but in our hands the yield of glucanase in this procedure was very small. A glucanase preparation devoid of proteolytic activity was, however, obtained in about 90% yield by passing a zymolyase solution through an affinity column of $\alpha_2$-macroglobulin, a plasma protein that is a general proteolytic enzyme inhibitor (6).

The protease-free glucanase had very little effect on untreated cells of *S. cerevisiae*, but, as already reported (14), rapidly lysed cells that had been previously treated with Z-protease (Fig. 4). Incubation with 2-mercaptoethanol also facilitated, albeit to a lesser degree, glucanase action, as communicated by others (26, 34). Surprisingly, digitonin, a detergent, had a facilitating effect as large as that of 2-mercaptoethanol (Fig. 4). At high concentrations (ca. 1 M), salts also had some positive action. The most effective was potassium phosphate (Fig. 4), followed in order by potassium chloride, lithium chloride, cesium or magnesium chloride, and sodium chloride (data not shown).

The effect of Z-protease appears to be quite specific. Seven other proteases, i.e., trypsin, chymotrypsin, subtilisin, *Staphylococcus* V8 protease, proteinase K, pronase, and thermolysin, used in the amount that yielded the same activity with Hide Powder Azure, did not significantly affect glucanase action on intact cells (data not shown). Increasing the concentration of subtilisin, proteinase K, and thermolysin 50, 100, and 200-fold, respectively, did not result in increased lysis. The unusual specificity of Z-protease for mannoproteins seemed to afford a possible explanation for the lack of activity of the other proteases. Accordingly, four of those enzymes, i.e., trypsin, subtilisin, proteinase K, and pronase, were tested on solubilized cell wall proteins before or after endo-H incubation. None of the enzymes caused a significant decrease in the intensity of the protein bands (Fig. 5).

Treatment of iodinated cells with Z-protease released 87% of the label into the medium (Table 2). Of the other agents that facilitate the action of Z-glucanase, only 2-mercaptoethanol had some effect. The release of iodinated material by 0.1% digitonin or 1 M potassium phosphate was negligible.

**Effect of Z-protease on cell wall structure.** The accessibility of glucan to Z-glucanase, a protein of molecular weight 58,000 (13), in protease-treated cells indicated an increased porosity of the cell wall after proteolytic action. Because the reaction product of horseradish peroxidase is easily stainable for electron microscopy, an attempt was made to use this enzyme as a probe for penetration of high-molecular-weight substances into the cell wall. Surprisingly, we found that protease-treated cells were osmotically lysed by subsequent incubation with horseradish peroxidase (Fig. 6). Lysis in this case apparently involved damage to the plasma membrane without loss of cell wall, because the cells maintained their shape. We conclude that the protease treatment caused the appearance in the wall of pores large enough to allow passage of the peroxidase ($M_r$, 40,000) and contact of the enzyme with the plasma membranes.

Cells that were treated with Z-protease and extracted with buffer solutions were examined by electron microscopy (Fig. 7). The images indicate that a dark-staining outer layer was largely removed after treatment of intact cells with Z-protease. The wall in treated cells was irregularly eroded and had lost the stainable material (Fig. 7b). The loss in overall

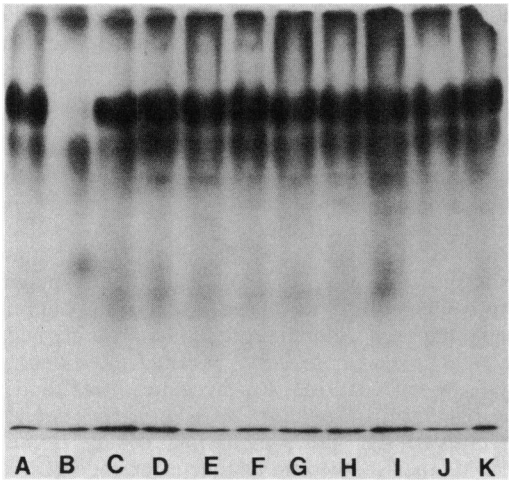


FIG. 5. SDS-gel electrophoresis of iodinated cell wall proteins after incubation with different proteases and endo-H. Lanes: A, wall proteins after endo-H digestion; B, D, F, H, and J, proteins after protease treatment followed by endo-H digestion; C, E, G, I, and K, proteins treated first with endo-H and then with protease. The proteolytic enzymes used were Z-protease (lanes B and C; 9.6 U); trypsin (lanes D and E; 2.6 μg); subtilisin (lanes F and G; 2.4 μg); proteinase K (lanes H and I; 0.74 μg); and pronase (lanes J and K; μg). The cell wall protein was obtained from iodinated cells, and the total amount of radioactivity per incubation was 120,000 cpm. When protease was added first, incubation was for 30 min in a total volume of 80 μl. The reaction was stopped by boiling, and 20 μl of citrate-phosphate buffer (pH 5) was added, followed by 0.01 U of endo-H. After 24 h at 37°C in a toluene atmosphere, another sample of endo-H was added, and the incubation was repeated. In parallel tubes, the treatments were inverted, i.e., incubation with endo-H preceded that with protease.

wall thickness by treated cells corresponds well to the thickness of the dark staining layer in the controls (i.e., about 30% of the wall thickness).

The extensive changes elicited in the cell wall by protease treatment did not result, in most cases, in the death of the cell. When protease-treated cells were plated on YEPD agar (1% yeast extract, 2% peptone, 2% glucose), an 80% survival rate, compared with a control, was observed.

**Lysis of isolated cell walls by Z-glucanase.** On the assumption that Z-protease was removing a surface layer of protein from intact cells and thus exposing glucan to glucanase action, the effect of zymolyase and of Z-glucanase was studied on isolated cell walls. In these, both outer and inner surfaces are accessible to enzymes. If the protecting layer is not present on the inner face of the wall, Z-glucanase might be able to attack the glucan in the absence of protease. This was indeed the case (Fig. 8). Z-glucanase was able to effect a nearly complete lysis of the walls, although at a lower rate than complete zymolyase. In the preparation of the cell walls used in this experiment, washings with LiCl and SDS were omitted to avoid removing any protein layer that might be present.

As mentioned above, the ability of Z-glucanase to lyse cell walls in the absence of Z-protease was used to advantage in this study to obtain solubilized preparations of cell wall proteins.

TABLE 2. Liberation of radioactivity from $^{125}$I-labeled cells by different treatments[a]

| Treatment | % of total radioactivity solubilized |
|---|---|
| None | 7 |
| Protease | 87 |
| 1 M Potassium phosphate (pH 7) | 6 |
| 0.1% Digitonin | 11 |
| 0.21 M 2-mercaptoethanol | 23 |

[a] Labeling of cells and treatment with different agents were as described in the text.

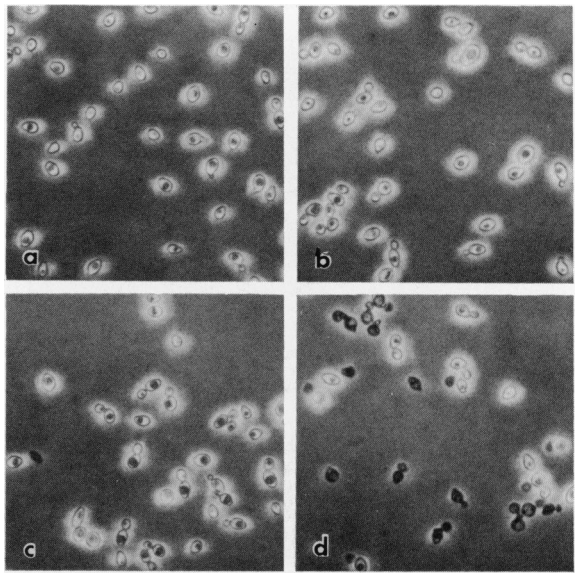


FIG. 6. Lytic effect of horseradish peroxidase on Z-protease-treated cells. a, Untreated control; b, peroxidase-treated control; c, Z-protease-treated cells with no peroxidase; d, Z-protease-treated cells plus peroxidase.

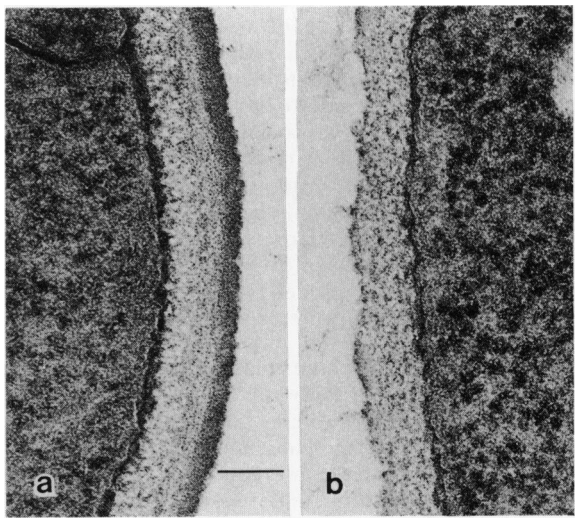


FIG. 7. Wall appearance of Z-protease-treated cells. a, Control, b, protease-treated cells. Cells were treated identically, except that protease was omitted from incubation in control cells. After incubation in protease or buffer, cells were fixed in 3% glutaraldehyde for 30 min and rinsed in 0.1 M sodium phosphate buffer (pH 6.8), followed by a prolonged rinse in 0.05 M Tris-hydrochloride (pH 7.6). The cells were postfixed in 1% $OsO_4$, enrobed in agar, and embedded in Epon 812. Sections were stained with 1% aqueous uranyl acetate and lead citrate. Bar, 0.1 μm.

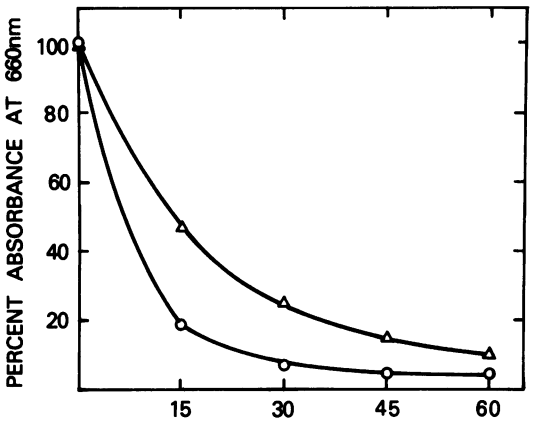


FIG. 8. Lysis of cell walls by zymolyase or Z-glucanase. The incubation mixture contained, in a total volume of 5 ml, cell walls from 100 mg (wet weight) of cells, 0.05 M Tris-chloride (pH 7.5), and either zymolyase 60,000 (○) or Z-glucanase (△). The amount of glucanase activity in each incubation mixture was 0.21 U. The cell walls used in this experiment were washed with water only, with omission of both LiCl and SDS washings.

## DISCUSSION

To understand the localization and function of yeast cell wall proteins, a methodology was needed for their isolation in a native form. In previous studies, wall mannoproteins were extracted from cells either by autoclaving (23) or by heating with alkali (9), procedures that may result in substantial damage to the protein moiety. We used only mechanical and enzymatic means to solubilize the wall proteins. Cell walls were prepared by breaking cells with glass beads and were first thoroughly washed and then solubilized with a purified glucanase. The glucanase had been completely freed from proteolytic activity by passage through an $\alpha_2$-macroglobulin–Sepharose affinity column. Because of the broad spectrum of proteases that are inhibited by $\alpha_2$-macroglobulin, this procedure may be of wide applicability for the removal of proteases from cell extracts. Because of the mild conditions used in their extraction, the solubilized wall proteins may be expected to have retained their original properties unchanged. A procedure based on the same principle was described very recently by Shibata et al. (28). These authors used crude zymolyase in which the protease was inhibited with phenylmethylsulfonyl fluoride, a method better suited than ours for large-scale preparations. Nevertheless, zymolyase was used by Shibata et al. on intact cell preparations rather than on cell walls. The resulting extract contains all periplasmic proteins and may be contaminated by cytoplasmic materials if any lysis of the protoplasts formed in the incubation takes place. Furthermore, preliminary experiments in our laboratory on gel chromatography of the solubilized mannoproteins on Sephacryl S-200 and S-300 (data not shown) indicate a much higher molecular weight for the bulk of the proteins than that reported by Shibata et al., suggesting that those authors might have failed to inhibit Z-protease completely. This criticism does not apply to the recent work of Novick and Schekman (20), who used a purified glucanase from *Oerskovia* sp. to lyse the cell wall in intact cells.

It was also necessary to develop a procedure that would enable us to follow through our studies the protein moiety of glycoproteins in which the carbohydrate portion is by far the major component (about 95% [28]). Labeling with $^{125}I$ offered several advantages, including specificity for the protein moiety and sensitivity of product detection. Furthermore, the alternative use of the lactoperoxidase or chloramine T method allowed differentiation between surface and inner cell wall proteins. One limitation of the iodination procedure is that the intensity of labeling depends on the tyrosine content of the proteins.

The bulk of the solubilized wall protein was of very high molecular weight as indicated by low relative mobility on SDS-polyacrylamide gel electrophoresis. A similar result was reported by Novick and Schekman (20), although in their gels the mobilities of iodinated proteins appear to be different from those we observed. Most of the high-molecular-weight material is apparently glycoprotein, because a significant increase in mobility resulted after depletion of carbohydrate with endo-H. Furthermore, more than 80% of the material was absorbed on ConA-Sepharose columns and subsequently eluted with α-methylmannoside. Some of the faster bands that showed no change after endo-H treatment were still found after adsorption-elution on ConA-Sepharose. They might contain serine- or threonine-linked oligosaccharides (4) that would be resistant to endo-H (29). It should also be taken into account that endo-H may be unable to release all the carbohydrate chains (31); therefore, the main, diffuse band of average molecular weight 94,000 appearing after endoglycosidase incubation probably corresponds to a mixture of proteins with various amounts of bound carbohydrate. Accordingly, the material precipitated by antibody against $\alpha(1 \rightarrow 3)$mannosyl residues from endo-H digests, although decreased in amount with respect to the untreated sample, showed the 94K band. The effect of mannan antibodies confirmed the expectation that at least the major glycoproteins were mannoproteins, although some of the minor bands did not appear upon electrophoresis of the immunoprecipitate.

When intact cells were iodinated by the lactoperoxidase method, which would be expected to label only the surface protein, incorporation was only a small fraction of that obtained with the chloramine T procedure; yet, the same major bands were observed after electrophoresis. It may be concluded that the major mannoproteins are present both at the surface and in deeper layers of the wall (Fig. 9). It seems therefore reasonable to assume that the mannoproteins are shielding the cell wall glucan (Fig. 9) from attack by glucanases, particularly Z-glucanase; the facilitation of cell wall

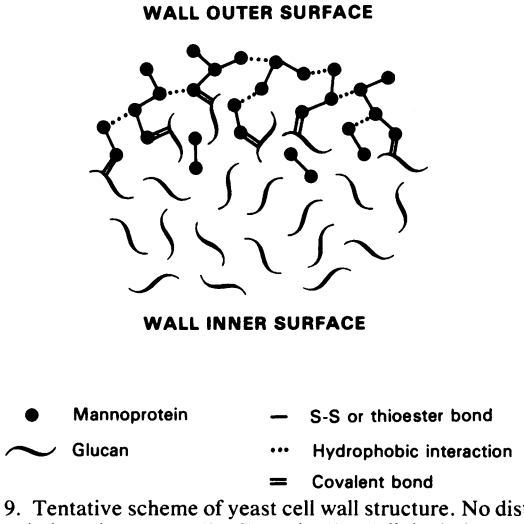


FIG. 9. Tentative scheme of yeast cell wall structure. No distinction is made here between β(1→3)- and β(1→6)-linked glucans.

lysis by previous incubation of the cells with Z-protease (Fig. 4) would result from elimination of the shield. This concept is strengthened by the peculiar specificity exhibited by Z-protease. Only the intact mannoproteins were good substrates for the enzyme, and removal of the sugar moiety by endo-H resulted in loss of susceptibility to protease (Fig. 3). Kitamura (14) postulated a similar specificity, based on inhibition of Z-protease by mannan. This behavior is consistent with the notion that the substrate attacked by Z-protease to uncover the glucan layer of the cell wall is mannoprotein in nature rather than some other putative wall protein. Further reinforcement for this view is provided by the finding that other proteinases, which were ineffective in facilitating the action of Z-glucanase, were also unable to attack the mannoproteins.

Removal of most of the mannoproteins from *S. cerevisiae* cell walls by Z-protease resulted in important changes. A loss of the outer dark layer of the wall was the major visible structural alteration. The porosity of the wall was also strikingly increased. Not only did glucan become accessible to Z-glucanase, but horseradish peroxidase was apparently able to cross the wall and interact with the plasma membrane, causing cell lysis. This result recalls the finding of Yphantis and his associates (35) that small basic proteins such as cytochrome *c* and protamine can cause lysis of yeast cells. Horseradish peroxidase also is a basic protein (isoelectric point, 7.2 [22]) and may act on the plasma membrane in the same way, although the possibility of a mechanism based on its enzymatic activity cannot be discarded. Although unable to affect normal cells because of its size, the peroxidase can reach the membrane and damage it in the protease-treated cells because of their increased porosity. This case illustrates the importance of the mannoprotein for survival of the yeast cell. Although removal of the mannoprotein does not cause immediate cell death, the cell would remain at the mercy of any basic or positively charged polyelectrolyte (35) in its surroundings. By contract, the molecular weight limit for easy penetration through the intact cell wall seems to be about 700 (25).

If the function of mannoprotein is to protect the cell from external injury, there would be no need for its presence at the inner face of the wall. This appears to be the case. The ability of Z-glucanase to lyse isolated cell walls, in which the inner surface is exposed to enzymatic action, in the absence of protease indicates that glucan is not protected by mannoprotein from the inside (Fig. 9).

If the mannoprotein is responsible for the porosity of the cell wall, how can we explain the facilitation of glucanase action by 2-mercaptoethanol, digitonin, or high concentration of salts, conditions that lead to little or no release of mannoprotein from the cell? 2-Mercaptoethanol, which also facilitates the effect of snail gut enzymes on the wall, may act by breaking disulfide or perhaps thioester (8) linkages between mannoprotein molecules and thus give rise to a more open structure that would allow the passage of large molecules. For digitonin, we postulate binding to hydrophobic regions of mannoprotein molecules with the creation of hydrophilic channels, whereas high salt concentration may alter the conformation of mannoproteins in ways that create openings in wall structure. The effects of all these agents point to a much greater flexibility of the mannoprotein network in the cell wall than one would have suspected.

Our findings about the distribution of mannoprotein raise some fundamental questions about the construction of the yeast cell wall. First, how does the mannoprotein find its way to an outer layer of the cell wall? Other mannoproteins, such as periplasmic enzymes, appear to be secreted by exocytosis by small vesicles (19). It is probable, although not yet proven, that the wall mannoprotein moves through the same pathway, but this is not sufficient to explain its external location. Second, what anchors the mannoprotein to the cell wall? Hydrolysis of glucan by glucanase in isolated cell walls results in solubilization of the mannoprotein. Therefore, even if the protein molecules are joined by disulfide groups and hydrophobic bonds, this is not sufficient to ensure their insolubility. One is thus led to the conclusion that mannan must be covalently linked to the insoluble glucan (Fig. 9). A similar view was expressed by Bacon (2). The finding that small amounts of glucose are tenaciously retained by mannoproteins obtained by lysis of cell walls by Z-glucanase (28) supports, but does not prove, this hypothesis. Additional structural work is undoubtedly required to settle this point.

In this discussion we have used, for clarity and simplicity, the word layer to designate regions of the wall rich in mannoprotein and glucan, respectively. It should be kept in mind, however, that there probably is an intermediate zone, of as yet unknown thickness, where the polymers are intermixed, as partially shown in Fig. 9.

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