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Structural and Immunological Studies on the Protoplast Membrane of the Yeast *Candida utilis*

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Cell membranes of the yeast *Candida utilis* isolated by lysis of protoplasts have been shown to be lipoprotein in nature. Electron microscopy shows that Mg^{++} is responsible for maintaining the integrity of the membrane. A close serological relationship was found between membranes and cell walls isolated from the yeast. This relationship was exhibited not only by membranes obtained by strepzyme treatment but also by those obtained from the action of helicase enzyme. No such relationship existed between membranes and whole cells. Related data have been obtained by treatment of yeasts with different digestive enzymes. All of the results suggest that the protoplast membrane possesses traces of structural cell wall material. This material is detectable by serological tests, but not by electron microscopy.

The present work was carried out to elucidate the architecture of the yeast cell and in particular the structure of the cell membrane of *Candida utilis*. It has been assumed, without much direct evidence, that the protoplast membrane of yeast possesses a lipoprotein structure. Recently, Boulton (1) and Garcia Mendoza and Villanueva (7) reported that membranes obtained by enzymatic methods have protein and lipids as major components, with small amounts of carbohydrates and nucleic acids. By mechanical treatment, Matile et al. (9) obtained a preparation of membranes of *Saccharomyces cerevisiae* containing protein, lipids, and an appreciable amount of polysaccharide composed of mannose.

In this report, special attention has been paid to the relationship between the structure and immunology of membrane preparations which we previously studied from a morphological and chemical point of view (7). Electron microscopy of these preparations has not revealed the presence of traces of residual cell wall material, which may be present in the preparations of membranes obtained by enzymatic methods; thus, the immunological behavior of these membranes was compared with that of isolated cell walls and whole cells.

MATERIALS AND METHODS

Organism, growth conditions, and harvesting. C. utilis CECT 1061 was grown in a medium containing glucose and yeast extract, as previously described (7). The cultures were harvested in the exponential phase of growth.

Preparation of protoplasts and protoplast membranes. Yeast protoplasts were usually prepared by means of strepzyme treatment [enzyme preparation from *Micromonospora* AS (8)]. When another enzymatic source was required, commercial helicase was used (enzymes from the gut juice of the snail *Helix pomatia* prepared by L'Industrie Biologique Francaise, Gennevilliers, France) according to the method first described by Eddy and Williamson (3) and slightly modified by Davies and Elvin (4).

Protoplast membranes were always prepared by lysis of the protoplast preparations in the presence of 0.01 M Mg^{++} (7).

Preparation of cell walls. Yeast cells were disrupted in a B. Braun mechanical disintegrator (Georgii Kobold, Elektro-Motoren Apparatebau, Stuttgart, Germany), and the cell walls were purified by washing and differential centrifugation (5). In some experiments, an additional washing of the walls with the detergent Duponol-C (sodium lauryl sulfate USP, Fisher Scientific Co., New York, N.Y.) was carried out. Other times, when small fragments of cell walls were required, the Ribi Cell Fractionator was used.

Chemical analyses. Preliminary results on the chemical composition of the membrane have been described elsewhere (7). Chemical analyses of cell walls of *Candida utilis* were previously performed by Novaes and Villanueva (14).

Electron microscopy. Specimens were fixed with 2% $KMnO_4$ at room temperature for 2 hr. The fixed samples were dehydrated through 25, 50, 75, and 100% acetone. During dehydration, the material was stained overnight in 2% uranyl acetate dissolved in 75% acetone. The specimens were embedded in Durcupan ACM (Fluka, Buchs SG, Switzerland), cut with an LKB Ultratome Microscope 4804 Nife, using glass-knives, and picked up on Formvar-coated grids.

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Sections were examined in an EM 9 Zeiss electron microscope.

Serological tests. Antisera were prepared by prolonged immunization of four mature rabbits with suspensions of highly purified membranes, obtained from the strepzyme-treated cells. The animals were injected weekly, for a period of 4 weeks, by the subcutaneous route, with a suspension of material containing 6, 10, 10, and 10 mg (dry weight), respectively, in Freund adjuvant (Difco). Subsequently, intravenous injections were administered twice a week for a 3-week period. These injections consisted of a suspension of increasing amounts of membranes (2, 3, 5, 7, 9, and 11 mg) in physiological saline; the dose per injection was 1 ml. Rabbits were bled by cardiac puncture 9 days after the last injection. The blood was allowed to clot. The serum was removed, centrifuged to separate blood cells, and frozen until needed. Merthiolate was added to a final concentration of 1:10,000.

Agglutination tests were performed by mixing dilutions of antiserum with suspensions of membranes in saline. The tubes were incubated at 37 C for 1 hr and left overnight in a refrigerator. Agglutination was measured by the settling pattern, and the titer was reported as the highest dilution showing activity. In some cases, it was necessary to test the antibodies against the antigens by the hemagglutination method (10).

Enzymatic digestion. Some agglutination experiments were carried out in which the membranes, cell walls, or whole cells used as antigenic material had been pretreated with different digestive enzymes, such as ribonuclease and wheat germ lipase (both from Koch Light Laboratories, Colnbrook, England), Pronase (from Calbiochem, Los Angeles, Calif.), strepzyme and helicase (both described previously), and a crude preparation of glucanases from *Penicillium brefeldianum* QM 1.872, known for its high activity. The last preparation contained mainly β1–3 and β1–6 glucanases and β-glucosidase, and traces of β1–4 glucanase and mannanase (no proteolytic activity); it was prepared according to the method of Reese et al. (16). Some of these enzymes had been shown earlier (7) to possess a degradative effect against the antigens cited above.

RESULTS

Fine structure of membranes. In an attempt to elucidate the structure of the protoplast membrane of yeasts, a careful microscopic observation of these materials was carried out. Electron micrographs of pellets of membranes of *C. utilis* obtained by the method previously described (7) provided a clear identification of plasma membrane components, in addition to giving some indication of the extent of contamination with cell wall or cytoplasmic constituents. A typical shadowed membrane preparation has been shown elsewhere (7).

Figures 1 and 2 are electron micrographs of sectional specimens of the membrane preparation. As can be seen, the yeast membrane does not show a very well-defined unit membrane structure, although sometimes a triple-layered structure could be distinguished (Fig. 3).

The thin sections of the membranes obtained in the presence of a high percentage of Mg^{++} show more clearly delimited structures than those obtained with low Mg^{++} concentrations. Occasionally, the presence of some remaining membrane-associated structures which may be formed by disintegrating membranous material was also observed (Fig. 3, arrows).

Figure 4 shows a thin section of a protoplast of *C. utilis* before lysis.

A parallel study in the electron microscope of membranes prepared either by strepzyme or helicase treatment of cells provided additional information. Preparations obtained by means of *Micromonospora* enzymes were always cleaner—and more free from cell wall debris—than were those obtained by the action of helicase.

Immunological results. The serological properties of protoplast membranes of *C. utilis*, prepared by strepzyme treatment, were studied by agglutination tests. Intact homologous antigen (membranes) reacted with the four sera that had been obtained. Membranes solubilized by ultrasonic treatment (Table 1) behaved similarly.

The agglutination reactions observed with cell walls of *C. utilis*, either intact or fragmented, are shown in Table 2. To eliminate all possible traces of membranes in the preparations of cell walls, the detergent Duponol and different enzymes were tested. The walls pretreated with Duponol, ribonuclease, and lipase gave strong agglutination with antiserum-prepared membranes. Another sample of cell walls was treated with Pronase to digest the possible fragments of membranous protein adhering to the walls, and the extent of hydrolysis was checked by the ninhydrin reaction. (Pronase is considered capable of catalyzing the hydrolysis of all peptide bonds.) The results of these experiments (Table 2) indicate a close serological relationship between the membrane and the cell walls. Agglutination could not be detected when cell walls of a different microorganism (*Streptomyces griseolus*) were used, thus showing that this reaction is specific for the antigen.

If these treatments of the cell walls eliminated all traces of membranous material, it appears that the serum must possess antibodies against small fragments of the cell wall that still adhered to the membranes; that is, the membrane preparation used as antigen contained some contaminant cell wall material.

To verify this hypothesis, heated cells of *C. utilis* and heated cells semidigested by a short treat-

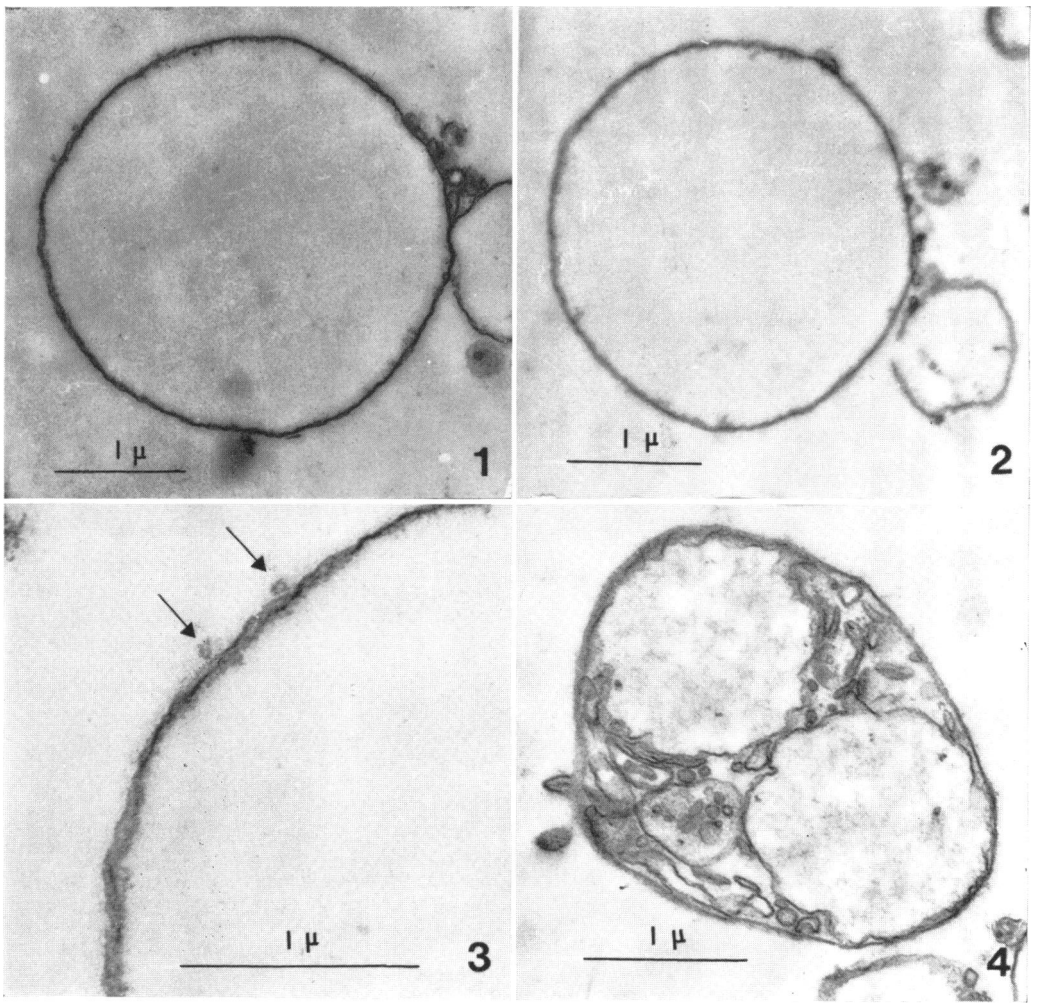

FIG. 1. *Thin section of Candida utilis membranes in the presence of 10^{-2} M Mg^{++}. × 10,400.*
FIG. 2. *Thin section of Candida utilis membranes in the presence of 10^{-4} M Mg^{++}. × 19,000.*
FIG. 3. *Higher magnification of Fig. 1, showing a region of Candida utilis membrane. × 28,800. Small vesicular bodies can be seen (arrows).*
FIG. 4. *Thin section of a Candida utilis protoplast. × 22,800.*

ment with strepzyme [a lytic preparation which first attacks the outer layer of the cell wall, until a preferential digestion at the equatorial region is observed to liberate the protoplast (17)] were incubated in the presence of antiserum. The first antigen did not show agglutinating activity, but the second did (Table 3). This last result was verified by hemagglutination.

From these results, it can also be assumed that the inner and not the outer layer of the cell wall is responsible for the agglutination reactions.

Finally, other enzymatic treatments were carried out with membranes obtained either by strepzyme or by helicase treatments. Both preparations were pretreated with strepzyme, helicase, or the mixture of glucanases prior to carrying out the agglutination. The results of these experiments (Table 4) show that membranes obtained by both of the lytic enzymes and pretreated with the glucanase preparation lose some of their agglutinating ability. This loss does not occur, at least to the same extent, after pretreatment with the lytic enzymes.

This result is in agreement with the concept that the inner layer of the cell wall is responsible for the agglutination in the presence of antiserum, not only of isolated cell walls and semidigested cells but also of preparations of membranes.

TABLE 1. *Agglutinin titers of the four sera against homologous antigen*[a]

Serum no.	Dilution of serum				
	1:8	1:16	1:32	1:64	1:128
1	++	±	±	−	−
2	++	++	±	±	−
3	++	++	±	±	−
4	++	++	±	±	−

[a] The antigens (membranes) were used suspended in saline, at a concentration of 10 mg (dry weight)/ml, either intact or solubilized by ultrasonic treatment. The low agglutinin titers obtained are due to the high concentration of antigen used (the same that was used in the last immunizing injection, in order to have defined results).

TABLE 2. *Results of cross-agglutination of serum prepared against cell membranes with cell walls*[a]

Antigen	Treatment	Agglutination
Cell walls of *Candida utilis* (obtained in the B. Braum Disintegrator)	None	++++
	Duponol	++++
	Ribonuclease	++++
	Lipase	++++
	Pronase	++++
Cell walls of *C. utilis* (obtained in the Ribi Cell Fractionator)	None	++
	Duponol	++
	Ribonuclease	++
	Lipase	++
	Pronase	++
	Ribonuclease	++
	Duponol + ribonuclease + lipase	++
	Duponol + Pronase	++
	Ribonuclease + Pronase	++
	Lipase + Pronase	++
	Ribonuclease + lipase + Pronase	++
Cell walls of *Streptomyces griseolus*	None	−

[a] Symbols: ++++, rapid agglutination; ++, slow agglutination. Duponol, ribonuclease, lipase, and Pronase were used at a concentration of 1%, 200 μg/ml, 200 μg/ml, and 300 μg/ml, respectively. The incubation time with the enzymes was 3 hr, except in the case of Pronase when it was 8 hr. The serum was used at a dilution of 1:8. Cell walls were used at a concentration of 10 mg (dry weight)/ml.

TABLE 3. *Results of cross agglutination of serum prepared against cell membranes with whole cells*[a]

Antigen	Agglutination
Candida utilis cells heated for 10 min at 100 C	−
C. utilis cells semidigested with strepzyme (after heating)	++

[a] Heated cells were used at a concentration of 10 mg (dry weight)/ml. Strepzyme was used for 1 hr under the same conditions as described to prepare protoplasts. The dilution of serum was 1:8.

TABLE 4. *Changes in the results of agglutination after treatment of the membranes with glucanases*[a]

Antigen	Treatment	Agglutination
Membranes (from strepzyme-treated cells)	None	++
	Strepzyme	++
	Helicase	+
	Glucanase preparation	±
Membranes (from helicase-treated cells)	None	±
	Strepzyme	+
	Helicase	+
	Glucanase preparation	±

[a] Membranes were used at a concentration of 10 mg (dry weight)/ml. Strepzyme and helicase were used under the same conditions described for the preparation of protoplasts, for 3 hr. The preparation of glucanases was used for 6 hr at a concentration of 5 mg of protein/ml. The dilution of serum was 1:8.

Additional information could be obtained from this last experiment. Membranes prepared from helicase-treated cells behaved in a rather similar way to those prepared by means of strepzyme, indicating that both preparations contain some contaminating cell wall material.

DISCUSSION

Protoplasts of yeasts can be obtained by helicase or strepzyme treatment. When such protoplasts are resuspended in a dilute solution, rapid disruption occurs and the membranes can be isolated from the lysate by differential centrifugation in the presence of Mg^{++} (1, 7). Lipids and proteins are the major components of these membranes.

The cell walls of yeasts are composed of large amounts of glucan and mannan and also contain

protein and lipids (from 6 to 12% and 1 to 13.5%, respectively). However, regardless of possible differences, many of the wall fractions are probably composed of walls and fragments of cytoplasmic membranes, at least this appeared to be the case when the organisms were disrupted in a mechanic disintegrator (Mickle). In bacteria, this has been demonstrated with the electron microscope by Boy de la Tour, Bolle, and Kellenberger (2).

We do not think that the small vesicles near the membrane are remaining fragments of the cell wall, but it is possible they come from the unit membrane structure. Mühlethaler, Moor, and Szarkowski (11) have postulated a model for the structure of the unit membrane, according to data obtained with freeze-etched preparations. Since the coiled protein molecules of the protein layer of the unit membrane are easily denatured, owing to the chemical fixatives, we can assume that the vesicular material of the membrane preparations is denatured protein from the unit membrane structure.

Sectioned *C. utilis* membranes have shown in some cases the appearance of triple-layered structures (Fig. 3), but recent electron microscopic studies have not yet revealed a complex structure of such membranes when they are sectioned in situ (Fig. 4). A possible explanation of this phenomenon lies in the fact that the fine structure of different biological materials is greatly influenced by environmental conditions.

The fact that membranes obtained in the presence of a higher percentage of Mg^{++} show more clearly delimited structures leads to the assumption that Mg^{++} can play an important role in the maintenance of membrane structure after lysis of the protoplasts.

Our studies show that a close serological relationship exists between the protoplast membrane and the cell wall of the yeast *C. utilis*. The same serological relationship was found with membranes and strepzyme-semidigested cells, but no such relationship existed when heated whole cells were tested. Similar results were obtained by using membranes from helicase-treated cells.

The principal conclusion to be drawn is that, although electron microscopy has not revealed the presence of any traces of cell wall material on the surface of the membranes, in serological behavior, cell wall and protoplast membrane preparations are very similar. This may indicate that the outer layer of the membranes we obtained possesses substances serologically related to those of the cell wall. This is in agreement with the results reported by Ottolenghi (15) in studies with yeast protoplasts from a morphological point of view.

When yeast cells are treated with strepzyme (6, 17), the protoplasts emerge through a hole made in the equatorial region of the cells, leaving apparently empty cell walls which later disappear. There are no visible traces of cell wall material on the protoplast membrane; however, on the basis of all the data available, it can be concluded that the protoplast leaves the cell, keeping a small amount of cell wall on the membrane. A further treatment of membranes with enzymes is not sufficient to eliminate this material, but it can be degraded by the glucanase preparation.

The present results suggest also that the inner layer of the cell wall, deprived of all possible traces of membranous material, is serologically different from the outer wall; it may also be chemically different, as has been demonstrated already in *Saccharomyces* by Mundkur (12) and Northcote (13).

Thus, the chemical and serological data indicate that some small fragments of cell wall material, probably a complex of glucan plus protein, remain adhering to the surface of yeast membranes after treatment with yeast cell wall digestive enzymes (either helicase or strepzyme), possibly because these enzymes are not capable, at least under the conditions described above, of degrading all these fragments.

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