BBA 75008

PREPARATION AND COMPOSITION OF THE PROTOPLAST MEMBRANE OF *CANDIDA UTILIS*

C. GARCÍA MENDOZA AND J. R. VILLANUEVA
Instituto de Biología Celular, C.S.I.C., Madrid (Spain)

(Received May 5th, 1966)
(Revised manuscript received September 15th, 1966)

SUMMARY

1. Protoplasts of the yeast *Candida utilis* can be prepared by controlled streptzyme treatment. When such protoplasts are resuspended in a dilute solution, rapid disruption occurs, and the protoplasmic constituents are released. Lysis is dependent on the concentrations of different cations in the surrounding medium.

2. The membrane fraction can be isolated as a dark-yellow pellet by centrifugation in the presence of 0.01 M Mg^{2+}. Phase-contrast microscopy shows the presence of membrane-like bodies. Electron microscopy reveals no organized structure on the membrane surfaces.

3. Isolated cell membranes have been found to consist largely of protein and lipids. Traces of carbohydrate and ribonucleic acid were also found.

INTRODUCTION

With the advent of improved biochemical and electron miscroscopic techniques, membranous elements have been detected in a large variety of cells. Studies carried out during the last ten years have shown that the cell walls of several kinds of yeasts are composed of large amounts of glucan and mannan, but contain little protein and lipids. On the other hand, the osmotic barrier, the membrane, in yeast cells is usually assumed to be lipoprotein in nature, as it is in bacteria, though few analyses have been performed. Recently BOULTON AND EDDY[1] and BOULTON[2] have reported that a membrane fraction isolated from *Saccharomyces cerevisiae* protoplasts contained lipids and protein as the major components.

GARCÍA MENDOZA AND VILLANUEVA[3] and GASCÓN, OCHOA AND VILLANUEVA[4] have prepared protoplasts of *Candida utilis* by treatment with lytic enzymes (streptzymes) from Streptomyces GM or Micromonospora AS. The protoplasts are sensitive to osmotic shock; when disruption occurs by dilution of the medium, the delicate membrane can sometimes be maintained and isolated as a "ghost" from the lysate[5].

The present paper describes some investigations on the chemical composition of the protoplast membrane of this organism.

MATERIAL AND METHODS

Organisms

The organism used in these studies was *C. utilis* CE CT 1061 (obtained from the Colección Española de Cultivos Tipo). The medium used for growth of the organism contained per l: glucose, 20 g and yeast extract, 3 g. The culture was always harvested in the early exponential phase of growth, since preliminary experiments revealed the greater susceptibility of young cells to the lytic enzyme action.

Formation of protoplasts

With slight modifications, the method employed for the preparation of *C. utilis* protoplasts is essentially that previously described[3,4]. The suspension of yeast (5 mg dry wt. per ml) was incubated at 37° with shaking in the presence of the enzyme preparation and mannitol as stabilizing agent (final concentration 0.8 M). The process of conversion into protoplasts required 1 to 2 h, and could be followed by microscopic observation. The protoplasts were centrifuged at 3000 × *g* for 15 min and washed in 0.8 M mannitol to remove the lytic enzyme preparation.

Preparation of protoplast membranes

Protoplast membranes were prepared by dilution of the protoplast preparation. As preliminary experiments showed that lysis was dependent on the salt concentration of the medium, the following method was used. An iced solution containing 10^{-4} M Tris buffer (pH 7.2) and 10^{-4} M Mg^{2+} was used, and when disruption was complete a solution of the same buffer containing $MgCl_2$ was added to give a final concentration of 10^{-2} M Mg^{2+}; this cation has been shown to prevent lysis of the yeast protoplast and further disintegration of the membranes, as described elsewhere. The membrane fraction was obtained by centrifugation at 15 000 × *g* for 15 min. The dark-yellow pellet, very loosely packed, was purified by washing several times with the same buffer.

The membrane preparations were freeze-dried and stored in a vacuum desiccator over P_2O_5 prior to carrying out chemical analysis.

Microscopy

At all stages of preparation and lysis of the protoplasts the percentage of intact cells, protoplasts and ghosts, as well as total cell numbers, were monitored by direct phase-contrast microscopic observations.

Samples of the membrane preparations obtained were also examined by electron microscopy. The preparations were shadowed according to classical techniques with an alloy of gold and palladium in order to observe the characteristic surface of the membranes.

Chemical methods

The membranes were separated into lipid and non-lipid fractions by ether extraction after a preliminary treatment with methanol to disrupt any lipoprotein complexes[6]. The extracts were combined, centrifuged to remove traces of suspended material and evaporated. Both lipid and defatted materials were dried *in vacuo* over P_2O_5.

Total nitrogen was estimated by the method of UMBREIT, BURRIS AND STAUF-

FER[7], followed by addition of Nessler's reagent. Total phosphorus was determined by the Fiske and SubbaRow method (see ref. 7). Routine protein determinations were performed with the procedure of LOWRY *et al.*[8]. However, this method gave rather low results with membranes, and for some studies of the composition of membranes, Kjeldahl determinations were used.

Hexoses were determined by the anthrone method of TREVELYAN AND HARRISON[9]. Nucleic acid was fractionated by a modification of the SCHNEIDER[10] procedure. Total nucleic acid was estimated spectrophotometrically. DNA was determined with the diphenylamine reagent and RNA was determined by the orcinol reaction[12].

Samples of membrane preparations were hydrolyzed under different conditions (either 6 M or 4 M HCl, 15 h, for amino acids; 2 M HCl, 4 h, for amino sugars and 1 M HCl, 15 h, for sugars; all at 105°) and after removing the acid, one-dimensional chromatograms were run in several solvents. Amino acids were detected by spraying with ninhydrin, or by means of specific reagents.

Sugars were detected with aniline phthalate[13] and hexosamines were estimated by the method of Morgan and Elson as modified by RONDLE AND MORGAN[14].

RESULTS

Effect of salts and pH on protoplast disruption

In our early experiments we observed that lysis of yeast protoplasts was dependent on the concentration of salts in the surrounding medium. The effects of several cations on lysis showed that monovalent cations (Li^+, Na^+, K^+, NH_4^+) prevented lysis at concentrations of 10^{-1} M, while the same concentration of mannitol yielded 100% lysis (mannitol as stabilizing agent is used at concentration of 0.8 M). Bivalent cations were found to prevent complete lysis even at concentrations of 10^{-2} M, and the order of efficiency was $Mg^{2+}>Ca^{2+}>Mn^{2+}>Fe^{2+}$, which correlates with their affinity for phosphate groups.

The effect of pH between 5 and 8 was studied. No observable changes were found when the pH was near 7. However, at pH 5 and pH 8 some inhibitory effect on the protoplast disruption was apparent.

Effects of different enzymes on protoplast and membrane preparations

Treatment of protoplasts of *C. utilis* with commercial proteolytic enzymes such as trypsin, papain, carboxypeptidase, protease and proteinase, during a 3-h incubation, did not affect the integrity of the protoplasts. Likewise, there was no detectable change in the appearance of the isolated membranes after incubation with these enzyme preparations.

On the other hand, when protoplasts were incubated in the presence of crystalline ribonuclease, rapid lysis occurred with bursting. This effect took place within the first 15 min of incubation. No visible effect of ribonuclease on the membrane preparations was observed.

One lipase tested (from wheat germ) produced lysis of the protoplasts; others (hog pancreas, steapsin and 448 lipase) did not. However, when acting on membrane preparations, all lipases produced some degradative effect as seen under the phase-contrast microscope.

The enzymes chitinase and β-1,3-glucanase did not affect the integrity of the

protoplasts or of the membrane preparations. All the enzymes were tested at a final concentration of 200 μg/ml with the exception of trypsin which was used at 500–1000 μg/ml.

Microscopic studies

The strepzyme attacks yeast cells at the equatorial zone, resulting in the release of protoplasts[15]. Microscopic observation of the suspension during the first stage of the incubation revealed a large number of protoplasts and of empty cell walls. On prolong-

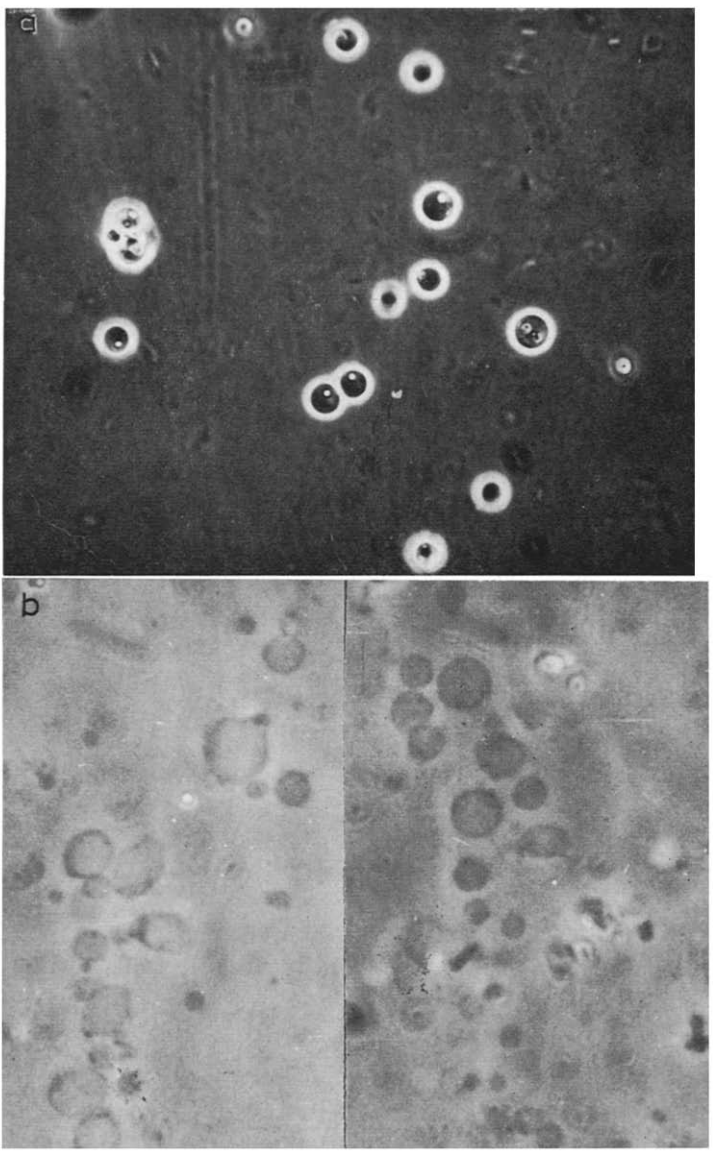

Fig. 1. Phase-contrast micrograph of: a. protoplasts of *C. utilis* (× 6000); b. membranes of *C. utilis* (× 8000).

ed incubation the remaining cell walls were completely dissolved leaving a suspension of 100% protoplasts. These are convenient for subsequent lysis and cell membrane recovery.

Phase-contrast micrographs of protoplasts and cell membrane preparations used in the present work are illustrated in Fig. 1. Typical structures suggesting membranes free from particulate debris are shown in Fig. 1b. The appearance of these structures was slightly different in the presence and in the absence of Mg^{2+}, those exposed to the action of the Mg^{2+} showing greater contrast although in both cases the

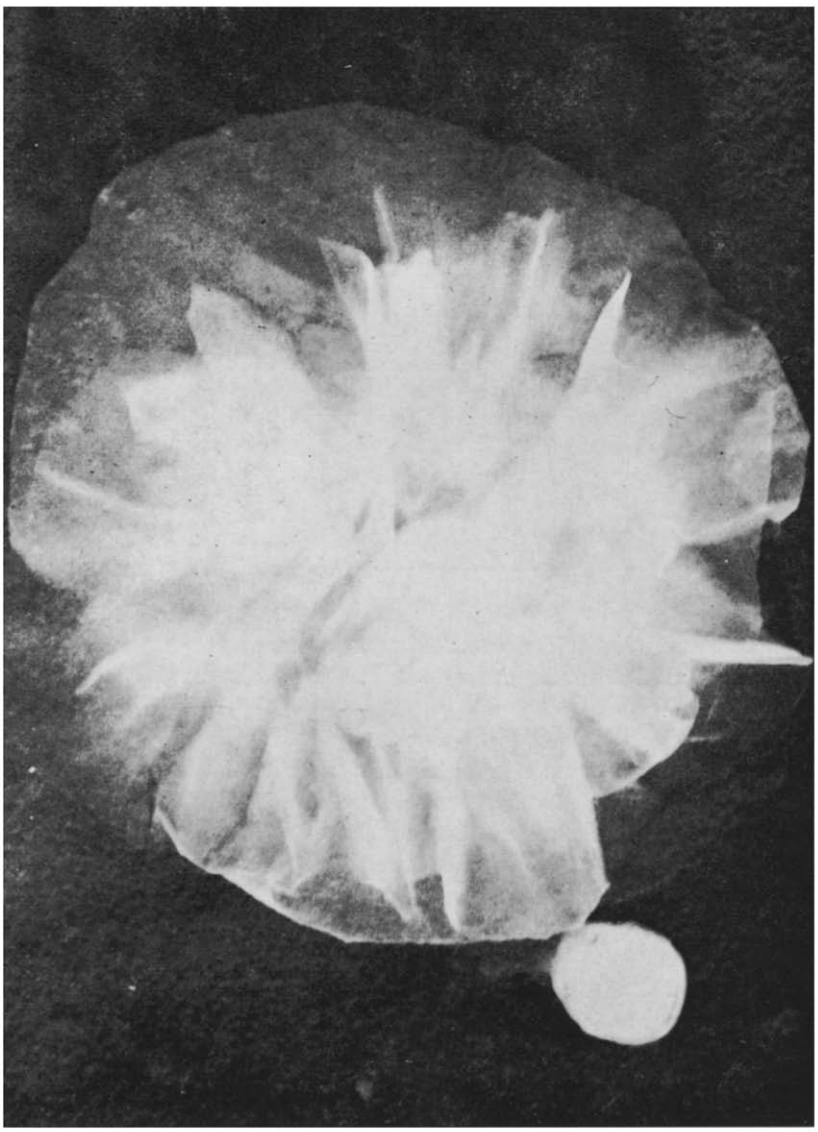

Fig. 2. Electron micrograph of a membrane of *C. utilis* (× 60 000).

preparation exhibited very little contrast on examination under the phase-contrast microscope.

Attempts to obtain membranes of uniform size by using synchronized cultures were unsuccessful, mainly owing to the necessity of employing very young cultures.

Using frozen-etched baker yeast cells MOOR AND MÜHLETHALER[16] have recently reported that the plasma membrane possesses invaginated grooves. Between these structures, hexagonal patterns of roughly circular electron-opaque regions are clearly visible. Our preparations have not revealed any organized structure on the surface of the *C. utilis* membranes (Fig. 2). Small electron-dense granules, possibly ribosomes, have at times been observed adhering to membranes.

Chemical composition of the cytoplasmic membranes

Three separate batches of membranes have been analysed (Table I). The agreement of values suggests that under the conditions of preparation a product of reproducible composition was obtained. The protoplast membrane of *C. utilis* comprises

TABLE I

CHEMICAL COMPOSITION OF CELL MEMBRANES OF *C. utilis*

All figures expressed as percentage of dry weight of cell membrane.

Batch No.	*Protein*	*Lipids*	*Carbohydrate*	*RNA*	*DNA*
I	37.7	41.1	5.0	1.2	0
II	39.3	39.8	4.9	1.1	0
III	38.6	40.4	5.3	1.0	0
Mean	38.5	40.4	5.2	1.1	0

about 10% of the cell dry weight. The isolated membranes were composed almost entirely of protein and lipids. A small amount of carbohydrate and traces of nucleic acid were also present.

Chromatograms of hydrolysates of membranes showed that most of the commonly occurring amino acids are present in the protein, and the relative amounts were fairly constant from batch to batch. Arginine was detected only in traces, but tyrosine (which was found in a very small quantity in the wall of *C. utilis*[17]) appeared as a significant component. The total carbohydrate content was found to be 5%, hexoses (glucose, mannose and galactose) being the only components. No pentoses nor hexosamines were detected. The total nucleic acid content of 1% corresponds to RNA, since no DNA was detected.

The absorption spectra of supernatants from 0.1 M HCl hydrolysates (1 h at 100°) of defatted membranes of *C. utilis* showed maxima at 270 mμ. From the ratio of absorption at 280 mμ to that at 260 mμ (nearly 1.0) it can be concluded that no more than traces of nucleic acid were present. These results are in agreement with those obtained by chemical analysis.

Further analysis of the lipid components has not been carried out.

DISCUSSION

The preparations obtained in this study are similar to the cell membrane, cytoplasmic membrane or "ghost" preparations of other investigators from a microscopic and chemical point of view. Chemically they resemble the preparations obtained from the Gram-positive bacteria[18] in that the major fractions are protein and lipids. However, these investigators found a higher percentage of carbohydrate in their membrane preparations. BOULTON AND EDDY[1] and BOULTON[2] found that a membrane fraction isolated from baker's yeast contained protein and lipids as the main components, although it possessed small amounts of carbohydrates, RNA and DNA.

The cell membrane of *C. utilis* consists largely of protein and lipids, each accounting for 40% of the dry weight of the membrane preparations. The lipid content is higher than that recorded for bacterial protoplast membranes, which contain 15–30% lipid[18–21].

The total amount of carbohydrate was significantly lower than that reported for other membrane preparations. Lack of hexosamines (glucosamine) can be interpreted as meaning that no contamination with cell-wall material occurred, since this amino sugar is present in the wall of *C. utilis*[17].

The presence of traces of RNA in these membrane preparations was not unexpected. Small amounts of nucleic acids have always been associated with isolated membranes of bacterial protoplasts. It is difficult to decide whether the traces of residual nucleic acid found were contaminants or a true membrane component, since the amount of RNA has been shown to be dependent on the Mg^{2+} concentration of the surrounding medium.

The results indicate that, under the conditions described, very little or no contamination, either with cell-wall or cytoplasmic material, occurred in the *C. utilis* membrane preparations.

REFERENCES

1 A. A. BOULTON AND A. A. EDDY, *Biochem. J.*, 82 (1962) 16P.
2 A. A. BOULTON, *Exptl. Cell Res.*, 37 (1965) 343.
3 C. GARCÍA MENDOZA AND J. R. VILLANUEVA, *Nature*, 195 (1962) 1326.
4 S. GASCÓN, A. G. OCHOA AND J. R. VILLANUEVA, *Can. J. Microbiol.*, 11 (1965) 573.
5 C. GARCÍA MENDOZA AND J. R. VILLANUEVA, *Can. J. Microbiol.*, 9 (1963) 900.
6 R. REICHERT, *Helv. Chim. Acta*, 27 (1944) 961.
7 W. W. UMBREIT, R. H. BURRIS AND J. F. STAUFFER, *Manometric Techniques and Tissue Metabolism*, Burgess, Minneapolis, 1949, p. 273.
8 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
9 W. E. TREVELYAN AND J. S. HARRISON, *Biochem. J.*, 50 (1952) 298.
10 W. C. SCHNEIDER, *J. Biol. Chem.*, 161 (1956) 293.
11 K. BURTON, *Biochem. J.*, 62 (1956) 315.
12 W. MEJBAUM, *Z. Physiol. Chem.*, 258 (1939) 117.
13 S. M. PARTRIDGE, *Nature*, 164 (1949) 433.
14 C. M. J. RONDLE AND W. T. J. MORGAN, *Biochem. J.*, 61 (1955) 586.
15 C. GARCÍA MENDOZA AND J. R. VILLANUEVA, *Nature*, 202 (1964) 1241.
16 H. MOOR AND K. MÜHLETHALER, *J. Cell. Biol.*, 17 (1963) 609.
17 M. NOVAES AND J. R. VILLANUEVA, *Biochim. Biophys. Acta*, 78 (1963) 797.
18 A. R. GILBY, A. V. FEW AND K. MCQUILLEN, *Biochim. Biophys. Acta*, 29 (1958) 21.
19 C. WEIBULL AND L. BERGSTROM, *Biochim. Biophys. Acta*, 30 (1958) 340.
20 M. D. YUDKIN, *Biochem. J.*, 82 (1962) 40P.
21 G. D. SHOCKMAN, J. J. KOLB, B. BAKAY, M. J. CANOVER AND G. TOENNIES, *J. Bacteriol.*, 85 (1963) 168.