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Isolation of Cell Membranes from *Saccharomyces cerevisiae* for Evaluation of Their Protein Composition

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Abstract—We describe a simple method for the isolation of membrane fractions from *Saccharomyces cerevisiae* yeasts containing a complex of plasma membranes and cell walls. The method is based on cell disruption on an INBI flow disintegrator. This device spares subcellular structures, which simplifies the isolation of cell membranes. The membrane fraction obtained by this method was suitable for studies of the protein composition of these structures by means of two-dimensional gel electrophoresis.

The plasma membrane of *Saccharomyces cerevisiae* yeasts includes more than 170 proteins with known biological functions and amino acid sequences [1]. The functions of these proteins are mainly associated with transport processes. Several proteins are involved in biogenesis of the yeast cell wall contacting the plasma membrane. The cell wall contains approximately 90 proteins whose amino acid sequence was determined previously [1]. However, molecular functions of various protein components of yeast cell membranes (including the plasma membrane and cell wall) remain unknown.

Studies of the protein composition of biological structures (e.g., yeast plasma membrane) constitute a topical problem. Their results will allow the evaluation of metabolic characteristics at the level of the protein (proteome). Protein analysis by two-dimensional electrophoresis (a sensitive method of high-resolution) is an approach to solving this problem [2].

In the case of yeast plasma membranes, implementation of this approach requires a simple and reproducible method for isolating the membrane fraction. Two groups of methods of isolation of these structures have been developed, which differ in their technique of cell disruption: enzymatic and mechanical. At present, all methods that have gained wide recognition include a stage of spheroplast isolation (using glucohydrolases), which makes them laborious and expensive [3]. These methods are associated with (1) prolonged exposure of the cells to enzymes (lyticase, DNase, and RNase) at high temperatures, (2) the use of gradient centrifugation and Dounce precision homogenizer, (3) treatment with ultrasound and concanavalin A, and (4) ultracentrifugation [3].

However, the most effective methods for the isolation of *S. cerevisiae* membranes (ghosts) are based on mechanical disintegration of the cells. The resulting plasma membranes are of higher quality compared to those isolated via the spheroplast stage [3]. The common drawback of devices disintegrating cells with glass beads is that mechanical treatment cannot be regulated. This results in severe fragmentation of subcellular structures, which complicates the procedure for the isolation of cell walls.

This work was designed to simplify the method for the isolation of membranes. This goal may be achieved by disrupting the cells in an INBI flow disintegrator [4], which preserves the integrity of subcellular structures [5].

MATERIALS AND METHODS

Isolation of cells. Experiments were performed with *S. cerevisiae* strain Y503 [6], obtained from the State Research Institute of Genetics (Moscow). Cells were grown in a medium containing 0.67% YNB Difco (base substrate; BD, USA) and 0.3% D(+)-glucose monohydrate (sole carbon source; Merch, Germany). The pH was brought to 5.5 with KOH. Cells were grown in 750-ml flasks with 100 ml of the medium on a stir plate at 28°C for 12 h. The cell suspension was washed three times with distilled water at 0°C and pelleted at 3400 *g* for 5 min. Washed cells (3.8 g wet weight) were suspended in 30 ml of medium M containing 0.6 M mannitol, 10 mM HEPES (pH 7.2), and 1 mM ethylenediaminetetraacetic acid sodium salt (EDTA-Na).

Disintegration of cells. The cell suspension was cooled to 0°C and passed through a flow disintegrator [4] in medium M. The disk of the device was spun at 6000 rpm. The flow rate was 0.3 ml/min. The jacket of the stationary disk was cooled with water. The receiving device was placed in an ice bath. The pressure at the disks and the flow rate of the suspension were adjusted in such a way so as to ensure 50% cell disintegration in the effluent (as assessed by microscopy).

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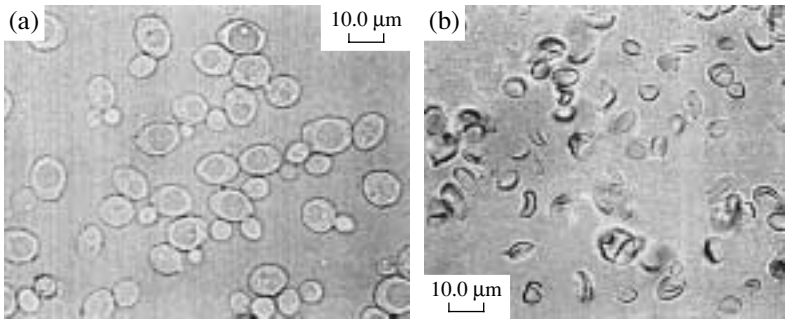

Fig. 1. (a) *S. cerevisiae* Y503 cells in the stationary growth phase and (b) cell membranes obtained in a flow disintegrator and isolated by low-speed differential centrifugation (×1000).

Isolation of membrane fractions. The suspension of disrupted cells was diluted with medium M (suspension volume 30 ml) and centrifuged on a K23 centrifuge (Janetzki, Germany) at 5000 *g* for 10 min. The pellet containing membranes and non-disrupted cells was washed two times with medium M (30 ml) under the same conditions and suspended in 70 ml low-tonicity LT medium (1 mM EDTA-Na and 10 mM Tris, pH 7.2) to remove subcellular structures. The suspension was centrifuged two times at 300 *g* for 5 min to separate cells. Membranes were pelleted at 3400 *g* for 10 min. The pellet was suspended in a small volume of LT medium. Protein concentration in the suspension was 6 mg/ml. Protein content was measured by the method of Bradford with modifications [7, 8]. The purity of the preparation was determined using an Axiolab light microscope (Carl Zeiss, Germany).

Two-dimensional electrophoresis. The membrane suspension (40 μl) was diluted with buffer C (80 μl), containing 2% SDS, 5% dithiothreitol, and 20% glycerol, and maintained in a water bath at 90°C for 5 min. Urea (53 mg) and bromophenol blue (5 μl, 0.05%) were added. Portions (40 and 80 μl) of the solubilized fraction were separated by isoelectric focusing [9, 10] with ampholytes (pH 5–8; LKB, Sweden). The separation was performed in tubes (length, 150 mm; inner diameter, 2.5 ± 0.1 mm). The gel-forming solution contained 4% acrylamide, 0.24% bisacrylamide, 2% ampholytes, 48% urea (extra pure), 2% Nonidet P-40, 0.006% TEMED, and 0.01% ammonium persulfate. The upper (cathode) and lower (anode) solutions included 20 mM NaOH and 10 mM H_3PO_4, respectively. Isoelectric focusing started at 100 V. The voltage was increased three times at 1-h intervals (by 100 V), resulting in the value of 500 V after 4 h. The procedure lasted for 20 h.

Electrophoretic separation of proteins in the second direction was performed on polyacrylamide gel (PAG) plates ($150 \times 150 \times 1.5$ mm). The solution for the separating gel contained 11.7% acrylamide, 0.3% bisacrylamide, 0.375 M Tris–HCl (pH 8.8), 0.02% TEMED, and 0.02% ammonium persulfate. The solution for the concentrating gel included 5% acrylamide, 0.12% bisacrylamide, 0.125 M Tris–HCl (pH 6.8), 0.1% SDS, and 0.03% ammonium persulfate. The electrode solution contained 25 mM Tris, 0.192 M glycine, and 0.1% SDS. Electrophoresis was performed at 38 mA (per plate) for 2.83 h. Proteins were developed by the method of Wray [11].

Electrophoretograms were calibrated (molecular weight and pH) using protein sets (Pharmacia Fine Chemicals), which included rabbit muscle phosphorylase b (97.2 kDa, pI 6.77), BSA (65.9 kDa, pI 5.71), chicken ovalbumin (42.8 kDa, pI 5.19), bovine erythrocyte carbonic anhydrase (29 kDa, pI 7.93), soybean trypsin inhibitor (19.8 kDa, pI 4.85), and bovine α-lactalbumin (13.6 kDa, pI 4.76). Theoretical values of the molecular weight and p*I* were calculated from theamino acid sequences (shown in brackets).

RESULTS AND DISCUSSION

The method based on the shock effect of glass microbeads is used for the mechanical disintegration of *S. cerevisiae* cells [12, 13]. In a variety of disintegrators used for this purpose (Vibrogen Cell Mill, Dyno Mill Glass Beads Grinder, and Braun MSK Homogenizer), disrupted cells are exposed to repeated disintegration, resulting in the formation of small vesicles. However, this method fails to ensure the complete separation of cell wall fragments from plasma membranes, even though it requires high-speed centrifugation and two cycles of fractionation in a sucrose concentration gradient (6 h each) [12]. In this work, the cells were disrupted using a flow disintegrator [4] in a single cycle of disruption between disks, followed by the immediate removal from the workspace of the devoce. Subcellular structures remained undamaged since the distance between disks was determined by the size of cells [5]. This approach allowed us to obtain non-disintegrated membranes that could be rapidly separated from the fraction of soluble proteins, subcellular structures, and intact cells by low-speed differential centrifugation (Fig. 1).

Some proteins in the cell wall are covalently bound to the carbohydrate polymer that forms this structure. They are not solubilized with the detergent in buffer C

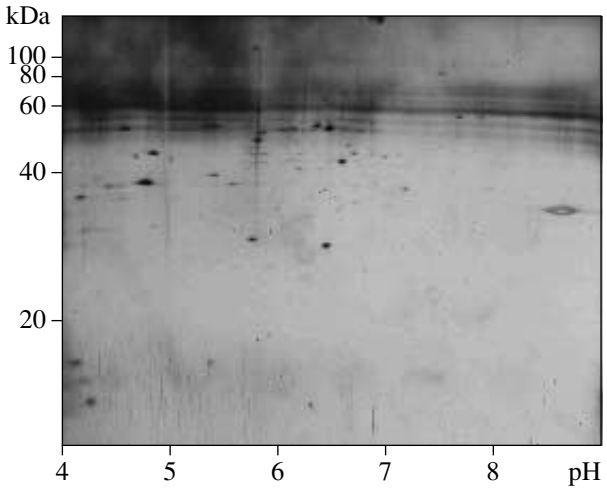

Fig. 2. Two-dimensional SDS-PAGE (11.7% PAG) of proteins extracted from membranes (plasma membrane and cell wall) of *S. cerevisiae* Y503 cells. The sample contained 160 μg of protein per gel. Proteins were developed with silver by the method of Wray [11]. The gel fragment is shown without the lower part (not containing proteins with molecular weights below 15 kDa).

(see Materials and Methods) and cannot permeate the gel during isoelectric focusing and electrophoresis. For the majority (68%) of cell wall proteins, the p*I* values are in the range 3.7–4.5; these proteins therefore appear in the acidic area of the electrophoretograms, where only 5% of plasma membrane proteins are found. These features simplify the identification of membrane proteins. Separation of a membrane sample containing 160 μg protein per gel is shown in Fig. 2. Thirty protein spots were revealed. The sample with a twofold lower protein content (not shown) was subjected to separation for the identification of major proteins. Table 1 illustrates 15 proteins present in both electrophoretograms and shows theoretical values of their characteristics estimated from the primary structure [1].

Proteins were identified by the method of Wilkins *et al.* [14]. It was assumed that the estimated molecular weight and p*I* of a protein could differ from the theoretical values by 20 kDa and 0.25, respectively. When the estimated value corresponded to several proteins, we assumed that the sample included protein typical of cell growth conditions (aerobiosis, YNB medium, stationary phase, and low glucose concentration). The first and second digits in the columns listing the protein characteristics (molecular weight and p*I*) represent the results obtained at protein concentrations of 80 and 160 μg per gel, respectively. Two-dimensional PAGE yielded highly reproducible results. The position of proteins in the gels was determined with an accuracy of 6.9 (molecular weight) and 0.14% (p*I*).

These data show that the use of a flow disintegrator allows the isolation of a fraction of *S. cerevisiae* cell walls suitable for studying the protein composition of plasma membranes and yeast cell walls by two-dimensional gel electrophoresis. This approach is less laborious than the isolation of membranes (ghosts) from spheroplasts [15, 16]. The method proposed may be used for identifying proteins in yeast plasma membrane and cell wall (by comparing the protein composition of wild strains and transformants lacking the gene encoding the protein in question).

Two-dimensional electrophoresis of proteins from *S. cerevisiae* membranes

Estimated protein characteristics		Prospective gene, encoded protein, and theoretical characteristics of the protein			
molecular weight, kDa	pI	gene	protein	molecular weight, Da	pI
100–105	5.9–5.9	CHS1	Chitin synthase	129918	6.17
53–57	6.6–6.5	HXT4	High-affinity hexose transporter	62043	6.37
52–56	4.9–4.7	FET3	Ferroxidase	72360	4.45
53–56	5.7–5.5	HOL1	Cation transporter	65348	5.56
52–56	6.3–6.3	PHO84	Phosphate transporter	64381	6.41
52–56	6.7–6.6	TNA1	Nicotinic acid permease	60134	6.80
51–54	5.7–5.9	JEN1	Lactate and pyruvate transporter	69376	5.77
51–54	6.2–6.2	PHO89	Na/phosphate symporter	62653	6.41
47–52	5.9–5.9	LEM3	Transcription regulator	47437	5.77
43–46	5.2–4.9	FET4	Low-affinity Fe^{2+} transporter	62791	5.26
44–47	6.9–6.8	MEP2	Ammonium ion transporter	53400	6.64
44–46	7.3–7.1	AGP3	General amino acid transporter	61051	7.52
41–45	6.8–6.7	MEP3	Low-affinity ammonium ion transporter	53689	6.49
34–39	5.2–4.8	ZRT2	Zn^{2+} transporter	46363	4.93
32–33	8.8–9.0	FTR1	High-affinity F^{2+} transporter expressed in aerobiosis	45722	8.53

REFERENCES

1. The Saccharomyces Genome Database (http://www.yeastgenome.org).
2. Gygi, S.P., Corthals, G.L., Zhang, Y., Rochon, Y., and Aebersold, R., *Proc. Natl. Acad. Sci. USA*, 2000, vol. 97, no. 17, pp. 9390–9395.
3. Zinser, E. and Daum, G., *Yeast*, 1995, vol. 11, no. 6, pp. 493–536.
4. Shol'ts, K.F., Rabinovich, Ya.M., Zvyagil'skaya, R.A., Mosolova, I.M., Pinus, E.A., and Kotel'nikova, A.V., USSR Inventor's Certificate no. 464329, *Byull. Izobret.*, 1975, no. 11, p. 14.
5. Shol'ts, K.F., Rabinovich, Ya.M., Zvyagil'skaya, R.A., Mosolova, I.M., Pinus, E.A., and Kotel'nikova, A.V., *Dezintegratsiya mikroorganizmov*, (Disintegration of Microorganisms), Pushchino-na-Oke: NTsBI Akad. Nauk SSSR, 1972, pp. 192–196.
6. Abramov, Sh.A., Kotenko, S.Ts., Dalgatova, B.I., Mamaev, A.T., and Peisakhova, D.S., USSR Inventor's Certificate no. 1284998, *Byull. Izobret.*, 1987, no. 3, p. 111.
7. Bradford, M.M., *Anal. Biochem.*, 1976, vol. 72, no. 1/2, pp. 248–254.
8. Aliverdieva, D.A. and Shol'ts, K.F., *Prikl. Biokhim. Mikrobiol.*, 1984, vol. 20, no. 6, pp. 823–830.
9. O'Farrell, P.H., *J. Biol. Chem.*, 1975, vol. 250, no. 10, pp. 4007–4021.
10. Malygin, A.G., *Usp. Biol. Khim.*, 1993, vol. 33, pp. 173–213.
11. Wray, W., *Anal. Biochem.*, 1981, vol. 118, no. 1, pp. 197–203.
12. Serrano, R., *Methods Enzymol.*, 1988, vol. 157, pp. 533–544.
13. Goffeau, A. and Dufour, J.-P., *Methods Enzymol.*, 1988, vol. 157, pp. 528–533.
14. Wilkins, M.R., Gasteiger, E., Wheeler, C.H., Lindskog, I., Sanchez, J.C., Bairoch, A., Appel, R.D., Dunn, M.J., and Hochstrasser, D.F., *Electrophoresis* (Weinheim, Fed. Repub. Ger.), 1998, vol. 19, no. 18, pp. 3199–3206 (cited by http://us.expasy.org/tools/multiident).
15. Bussey, H., Saville, D., Chevallier, M.R., and Rank, G.H., *Biochim. Biophys. Acta*, 1979, vol. 553, no. 2, pp. 185–196.
16. Tschopp, J. and Schekman, R., *J. Bacteriol.*, 1983, vol. 156, no. 1, pp. 222–229.