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EVALUATION OF THE SILICA MICROBEAD METHOD FOR ISOLATION OF RED BEET PROTOPLAST PLASMA MEMBRANE SHEETS

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The silica microbead procedure was utilized for the isolation of plasma membrane sheets from protoplasts of a higher plant, the red beet (*Beta vulgaris* L.). Membrane yields, as determined by recovery of an exogenous membrane marker were approx. 75%. The plasma membrane fraction contained the enzyme marker, pH 6.5, vanadate-sensitive, K^+-stimulated, Mg^{2+}-ATPase and small amounts of mitochondria, endoplasmic reticulum, and possibly tonoplast. The silica microbead procedure was also used for the isolation of intact vacuoles from microbead-coated protoplasts.

Introduction

To investigate physiological functions of the plasma membrane in higher plants, it is important to be able to isolate plasma membrane in a form representative of the native membrane, free of contaminating organelles. The most common procedure for obtaining purified plant plasma membrane consists of homogenization of intact tissue [1–4], or more recently of protoplasts [5–8], followed by differential and gradient centrifugation. This approach results in the isolation of vesicles which are enriched with respect to putative plasma membrane markers such as the pH 6.5 K^+-stimulated Mg^{2+}-ATPase [1–7] or glucan synthetase II [2,4,6]. It is presently not known whether such vesicles are representative of the entire plasma membrane or if they constitute selected plasma membrane domains.

A rapid technique for obtaining purified plasma membrane sheets from *Dictyostelium discoidium* [9] and *Saccharomyces cerevisiae* [10] has recently been reported. The procedure is based upon coating cells with a layer of cationic silica microbeads. The microbeads are of high density (2.5 g/cm^3) and adhere tenaciously to the plasma membrane. The microbead-coated protoplasts are then lysed and centrifuged through a sucrose density gradient. The coated plasma membrane sheets rapidly sediment to form a pellet. Cytoplasmic debris and organelles remain distributed throughout the gradient. Plasma membrane can be obtained in approx. 1 h.

This paper describes the application of this

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Abbreviations: Mes, 4-morpholineethanesulfonic acid; PNPP, *p*-nitrophenyl phosphate.

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technique for the isolation of a plasma membrane-enriched fraction from protoplasts of red beet (*Beta vulgaris* L.) root storage tissue. A modification of the procedure, which permits the isolation of intact vacuoles, is also described.

Materials and Methods

Microbead synthesis and preparation. Silica microbeads ranging from 10 to 50 nm in diameter were obtained from the Nalco Chemical Co., Oak Brook, IL. Microbeads were converted to a positively charged form as previously described [19].

Immediately before each experiment, microbeads (30%, w/w) were diluted by suspending 0.25 ml in 6 ml of coating buffer which contained 800 mM sorbitol/20 mM KCl/10 mM $MgCl_2$/25 mM sodium acetate (pH 5.0). The diluted microbead solution was then centrifuged at $1500 \times g$ for 10 min to remove aggregates and the supernatant used.

Protoplast isolation. Red beets (*B. vulgaris* L.) were obtained commercially and stored at 7 °C in moist vermiculite until use. Protoplasts were isolated by the procedure of Schmidt and Poole [11] using either the digestion medium described, or a slightly modified one. The general procedure was as follows: tissue slices were incubated overnight at 7 °C in 0.1% (v/v) β-mercaptoethanol. The slices were then suspended in a digestion medium which consisted of 2% (w/v) cellulysin (Calbiochem-Behring), 10% (v/v) pectinase (Sigma), 2% (v/v) β-glucuronidase-sulfatase (Glusulase, Endo Labs, Garden City, NY, or β-glucuronidase, Sigma Type H3), 1% (w/v) hemicellulase and 1% (w/v) bovine serum albumin in 33 mM Tris-Mes buffer (pH 5.5), containing 125 mM $CaCl_2$, 125 mM KCl, 50 μg/ml chloramphenicol (Sigma) and 0.25% (v/v) aprotinin (Sigma). The modified digestion medium contained lower levels of $CaCl_2$ and KCl (20 mM) and in addition, had 20 mM $MgCl_2$ and 600 mM sorbitol.

Protoplasts were released by gentle squeezing of the soft tissue and separated from undigested tissue by passage through cheesecloth. Protoplasts were purified by three 5 min centrifugations at $250 \times g$, resuspending in coating buffer. Prior to each experiment, the protoplast concentration was adjusted to $(3–4) \cdot 10^6$/ml.

Membrane isolation. In a 50 ml centrifuge tube, 2 ml of the diluted microbead solution were further diluted with 1 ml of coating buffer. Using a wide-bore pipette, 3 ml of the protoplast suspension were then added. Immediately, the solution was gently vortexed and 6 ml of an overcoating buffer consisting of 10 mg/ml polyacrylic acid, M_r 90000 (Aldrich Chemical Co.), in 800 mM sorbitol/20 mM KCl/10 mM $MgCl_2$/25 mM sodium acetate buffer (pH 6.0) was added.

The mixture was then centrifuged at $140 \times g$ for 2 min and the supernatant, which contained excess microbeads and overcoating agent, was discarded. The protoplasts were resuspended in a wash buffer consisting of 900 mM sorbitol/5 mM Na_2EDTA/5 mM dithioerythritol/4 mM Tris-HCl (pH 7.5), and centrifuged again at $140 \times g$ for 2 min.

The pellet containing microbead-coated protoplasts was then suspended in 3–10 ml of 5 mM Na_2EDTA/4 Tris-HCl (pH 7.5) (lysis buffer) plus 5 mM dithioerythritol and vortexed thoroughly. Complete lysis occurred within 15 min.

Microbead-coated membranes were initially purified by two differential centrifugations at 2000 $\times g$ for 5 min. The pellet was then suspended in 3–6 ml of lysis buffer and layered on top of a discontinuous gradient containing 2 ml each of 50% (w/w), 40% (w/w), and 30% (w/w) sucrose. This gradient was centrifuged for 15 min at 6000 $\times g$ in a Beckman SW 28.1 rotor. The pellet was then suspended in 0.5–2.0 ml of lysis buffer and assayed for markers and protein.

Vacuole release. The membrane isolation procedure described above was modified to obtain the release of vacuoles from microbead-coated protoplasts: 1 ml protoplasts $(1–3.5 \cdot 10^6$/ml) was washed twice by centrifugation at $140 \times g$ for 3 min in a modified coating buffer comprising 800 mM sorbitol/25 mM sodium acetate (pH 5.5). After the second centrifugation, the pelleted protoplasts were suspended in 5.93 ml of modified coating buffer, 0.27 ml of microbeads was added and the preparation was centrifuged at $50 \times g$ for 2 min. The microbead-coated protoplasts were resuspended in an overcoating solution consisting of 800 mM sorbitol, 10 mM Na_2EDTA and 10 mg/ml polyacrylic acid in 25 mM sodium acetate (pH 7.0).

After overcoating, the protoplasts were centrifuged at 50 × *g* for 3 min. The pellet was gently resuspended in 6 ml of vacuole release buffer (900 mM sorbitol/20 mM Tris-HCl (pH 9.0)) and incubated at 4°C for approx. 30 min. During this time, vacuoles were released from the microbead-coated protoplasts.

Following vacuole release, 3 ml of a solution containing 1.8 M sorbitol/30 mM Na_2EDTA/74 mM Tris-HCl (to which sufficient 1 M HCl was added to bring the final 9 ml suspension to pH 7.4) was added to the 6 ml vacuole suspension. Thus, the final composition of the vacuole suspension prior to the purification was 1.2 M sorbitol/10 mM Na_2EDTA/38 mM Tris-HCl (pH 7.5).

To separate intact vacuoles from lysed protoplasts, the vacuole suspension was layered on a discontinuous Histopaque gradient of 0%, 5%, 10% 15%, (v/v) Histopaque (Sigma) in 1.2 M sorbitol/10 mM Na_2EDTA/2.5 mM Tris-HCl (pH 7.5) and centrifuged at 400 × *g* for 10 min.

The vacuoles were removed from the 5%/10% and 10%/15% interfaces and diluted with lysis buffer and the plasma membrane-enriched pellet was resuspended in 25 mM Tris-SO_4 (pH 7.5). The suspension of vacuoles was centrifuged at 200 × *g* to pellet any contaminating microbead-coated membranes. The 200 × *g* supernatant was centrifuged at 60000 × *g*. The 60000 × *g* pellet (tonoplast fraction) was resuspended in 2.5 mM Tris-SO_4 (pH 7.5).

Protoplast surface labelling with concanavalin A. ^{125}I-labelled concanavalin A was prepared by the procedure of Cuatrecasas [12]. To label protoplasts, 100 μl ^{125}I-labelled concanavalin A ($1.8 \cdot 10^7$ cpm/ml) was added to 6.0 ml of purified protoplasts. Labelled protoplasts were separated from unbound ^{125}I-labelled concanavalin A by centrifugations at 250 × *g*, resuspending after each centrifugation in 6 ml of coating buffer. The concentration of the labelled protoplasts was $1.5 \cdot 10^6$ per ml. Of the total $2.6 \cdot 10^6$ cpm added to the protoplast suspension, $8.0 \cdot 10^4$ cpm became bound, representing a binding efficiency of 3.1%.

Marker and protein assays. Cytochrome *c* oxidase, NADH-dependent cytochrome *c* reductase and NADPH-dependent cytochrome *c* reductase were assayed as described by Hodges and Leonard [1].

ATPase and PNPPase activities were determined in an assay medium containing 36.7 mM Tris-Mes (pH 6.5 or 8.5), 3 mM Tris-ATP or PNPP, 3 mM $MgSO_4$ and 55.5 mM KCl. The assay was started with the addition of 100 μl of the sample (10–100 μg protein) to 200 μl of the assay medium. The reaction was allowed to proceed at 38°C for 20–50 min, depending on the amount of substrate hydrolyzed. Inorganic phosphate release was determined according to the method of Berenblum and Chain [13]. Corrections were made for nonenzymatic substrate hydrolysis and for inorganic phosphate present in the fraction.

DNA was assayed fluorimetrically by the method of Thomas and Farquhar [14]. Protein was measured after trichloroacetic acid precipitation using a modification [15] of the assay of Lowry et al. [16]. Silica was assayed by the method of Iler [17].

Results

To prepare red beet plasma membrane sheets, protoplasts were coated with microbeads and overcoated with polyacrylate. The anionic polymer was added to neutralize exposed positively charged regions on the microbead-coated surface and to crosslink to the microbeads to form a continuous pellicle [9,10,18]. The neutralization prevents binding of negatively charged organelles and proteins. Microbead-coated protoplasts were osmotically lysed and purified by differential and sucrose gradient centrifugation. Although the overall sequence of steps is similar to that used for the isolation of plasma membrane from yeast [10] and *Dictyostelium* [9], the large size and fragility of the plant protoplasts necessitated mixing reagents gently, while proceeding rapidly during the coating and overcoating steps. This significantly reduced protoplast rupture, which can occur either spontaneously or as the result of centrifugation.

Several polyanionic overcoating agents were tested. The most effective was polyacrylic acid, M_r 90000. Polyacrylic acids of lower molecular weight resulted in the release of microbeads from the membrane. When dextran sulfate was tested, significant amounts of soluble acid phosphatase bound to the microbeads upon protoplast lysis, contaminating the plasma membrane preparation.

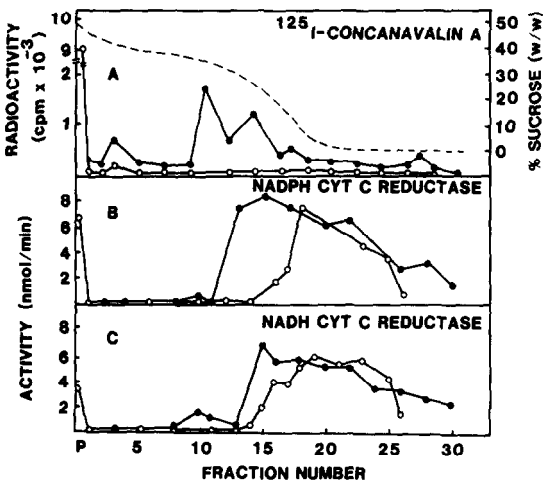

Fig. 1. Distribution of ^{125}I-labelled concanavalin A and marker enzymes after sucrose density gradient centrifugation of microbead-coated (○——○) and uncoated (●——●) red beet protoplast lysates. (A) Distribution of radioactivity; (B) NADPH-dependent cytochrome *c* reductase activity; (C) NADH-dependent cytochrome *c* reductase activity. The dashed line indicates density. Protoplasts were labelled and coated as described under Materials and Methods. After removal of excess microbeads and polyacrylic acid, protoplasts were resuspended in 10 ml lysis buffer and incubated for 8 h at 4°C. The 8 h incubation was found to be necessary to release organelles entrapped within plasma membrane sheets when initial lysates are not washed prior to sucrose gradient centrifugation. 6 ml lysate was layered onto a discontinuous sucrose gradient containing 2 ml of 50, 40, 36, and 30% sucrose, and 5 mM dithiothreitol in 50 mM Tris buffer (pH 7.5). The uncoated protoplast lysate was prepared similarly, except the addition of microbeads and blocking reagent was omitted. The gradients were centrifuged at 6000× *g* in a SW 28.1 (Beckman) rotor for 20 min. After centrifugation, 0.5 ml fractions were collected and assayed for radioactivity, marker enzyme activity and protein. The membrane pellet was resuspended in 0.75 ml lysis buffer and assayed as above.

Marker studies

To demonstrate plasma membrane position and recovery in the sucrose gradient, protoplasts were labelled with ^{125}I-labelled concanavalin A prior to the coating step. The presence of contaminating organelles was monitored by the assay of marker enzymes. As a control experiment, the lysate from non-microbead-coated ^{125}I-labelled concanavalin A labelled protoplasts was subjected to the same purification procedure. The sucrose gradients were fractionated and each fraction was analyzed for

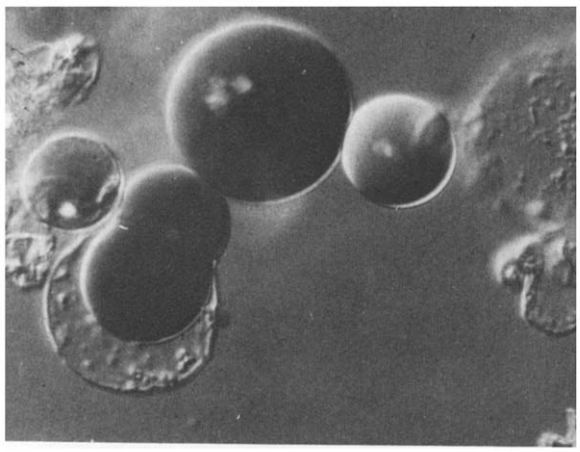

Fig. 2. Phase-contrast micrograph showing vacuole release, vacuoles and a microbread-coated plasma membrane sheet (×340) magnification.

label, marker enzyme activity, protein, and betacyanin.

The results of a typical experiment where lysates were layered directly onto sucrose gradients omitting the differential centrifugation step are shown in Fig. 1. The primary difference between the microbead-coated and uncoated protoplast sucrose gradient was the presence of a pellet in the microbead-coated gradient. Large membrane sheets of the type shown in Fig. 2 were observed upon microscopic examination. The distribution of the ^{125}I-labelled concanavalin A marker is shown in Fig. 1A. Approx. 75% of the radioactivity present on the microbead-coated gradient was recovered in the membrane pellet. Label in the non-coated cells was located at several places throughout the gradient, indicating that cell lysis produced plasma membrane vesicles or sheets with different buoyant densities. In some experiments, protoplasts were surface-labelled with diazotized ^{125}I-labelled sulfanilic acid (New England Nuclear). The distribution of radioactivity with this label was similar to that of ^{125}I-labelled concanavalin A.

With respect to marker enzyme activity, Fig. 1B and C show that less than 10% of the NADH- and NADPH-cytochrome *c* reductase activities penetrated into the sucrose gradient. The internal vacuolar marker, betacyanin, also remained in the upper layer.

Marker enzyme recovery in the membrane pel-

TABLE IA

RECOVERY OF MITOCHONDRIAL AND ENDOPLASMIC RETICULUM MARKER ACTIVITIES

	Protein (mg/ml)	Vol. (ml)	Specific activity [a] (μmol/min per mg protein)			Recovery (%)			
			NADPH-cyt *c* reductase	NADH-cyt *c* reductase	Cyt *c* oxidase	Protein	NADPH-cyt *c* reductase	NADH-cyt *c* reductase	Cyt *c* oxidase
Protoplast lysate	0.47	7 [b]	0.25	0.38	0.022	100	100	100	100
Plasma membrane pellet Lysate layered directly on gradient	1.4	1	0.016	0.027	0.006	43	2.6	3.1	12.4
Plasma membrane pellet Lysate layered on gradient after two low-speed centrifugations [c]	0.92	0.75	0.018	0.028	0.001	28	1.5	0.75	0.46

TABLE IB

RECOVERY OF CONCANAVALIN A AND SILICA PLASMA MEMBRANE MARKERS

	Protein (mg/ml)	Vol. (ml)	^{125}I-labelled concanavalin A			Silica		
			cpm/mg protein	% Recovery	Enrichment	μg silica/mg protein	% Recovery	Enrichment
Protoplast lysate	0.69	4 [b]	5356	100	–	0.9	100	–
Plasma membrane pellet Lysate layered directly on gradient	1.4	0.5	15089	71	2.8	2.7	82	3.0
Plasma membrane pellet Lysate layered on gradient after two low speed centrifugations [c]	0.80	0.5	14755	40	2.8	4.4	76	4.9

[a] Specific activities varied between beets obtained from different sources.
[b] Amount layered on gradient.
[c] 2000 × *g* for 10 min.

let is summarized in Table I. When the lysate was layered directly onto the sucrose gradient, approx. 3% of the NADH- and NADPH-cytochrome *c* reductase activities co-sedimented with the pellicle-coated plasma membrane. Of interest is that an equivalent amount of each of these activities was associated with the plasma membrane surface marker in the non-microbead-coated sample (Fig. 1B and C). This result suggested that the endoplasmic reticulum and mitochondria might be entrapped within the plasma membrane ghosts or that they are intimately associated with the plasma membrane by way of the cytoskeleton. Mitochondrial contamination, as judged by the presence of cytochrome *c* oxidase, was particularly high. It was found that this contamination could be reduced by washing the plasma membrane ghosts three times with lysis buffer, centrifuging for 5 min at 2000 × *g*. Such washings lowered the mitochondrial contamination from approx. 12% to less than 1% in the final plasma membrane preparation (Table I). Levels of NADPH- and NADH-cytochrome *c* reductase also decreased.

The possibility of nuclear contamination in the plasma membrane pellet was examined in a separate experiment by comparing the amount of

DNA found in the membrane pellet with the amount present in the total lysate. DNA contamination was less than 1%, suggesting that intact nuclei did not co-purify with the plasma membrane.

Based on the ^{125}I-labelled concanavalin A to protein ratios in the lysates and pellets (Table IB), washing and sucrose gradient centrifugation resulted in a plasma membrane enrichment of approx. 3-fold. Since the combination of washing and exposure to sucrose resulted in the dissociation of almost 50% of the radioactive concanavalin A from the membrane, this figure may underestimate the true magnitude of membrane purification. Silica to protein ratios were also measured (Table IB). About 90% of the silica present in the lysate was recovered in the membrane pellet. This represents an approx. 5-fold purification of silica with respect to protein.

ATPase recovery and vacuole release

Vanadate-sensitive ATPase activity at pH 6.5 in the presence of Mg^{2+} and KCl is considered to be a red beet plasma membrane marker [3,19]. Such activity was present in the plasma membrane fraction prepared by the microbead procedure (Table II).

Vanadate-insensitive ATPase activity at high pH (Table II) may indicate the presence of tonoplast membranes [20]. The regular microbead procedure (Table II, line a) gives an ATPase activity at pH 8.5 about 80% of that at pH 6.5. However, this can be reduced to about 30% (Table II, line b) after a treatment designed to release intact vacuoles before pelleting the plasma membrane. This suggests that the regular procedure does not remove tonoplast contamination even though it appears to remove other organelles quite successfully (see above). This is understandable if one notes that in these mature highly vacuolated cells, the tonoplast appears appressed to the plasma membrane over a considerable part of its area [21,22]. A similar reduction in pH 8.5 ATPase was also obtained by sonication of the microbead-plasma membrane preparation in the presence of 0.25 M KI and recentrifugation (data not shown). Table II also demonstrates that tonoplast membranes prepared by the vacuole-release procedure show the expected higher ATPase activity at pH 8.5 than at pH 6.5.

TABLE II

ATPase ACTIVITY FROM MEMBRANE FRACTIONS

Membranes were assayed in the presence of 3 mM Mg^{2+} and 55 mM K^+. Vanadate, when added was at a concentration of 100 μM. a, b and c represent three separate membrane isolations.

Fraction	Vanadate	ATPase activity (μmol/mg protein per h)	
		pH 6.5	pH 8.5
(a) Plasma membrane	−	1.14	0.86
	+	0.67	0.73
(b) Plasma membrane after vacuole release	−	0.54	0.19
	+	0.10	0.21
(c) Tonoplast fraction	−	0.32	1.01
	+	0.14	1.07

Nonspecific phosphatase activity in the plasma membrane fractions was assayed using the substrate *p*-nitrophenylphosphate. Its level was typically about 50% of that of ATPase at pH 6.5. Briskin and Poole [3] demonstrated that nonspecific phosphatase activity can be removed from conventionally prepared plasma membrane fractions of red beet by treatment with 0.25 M KI. This treatment did not appear to remove phosphatase from microbead membrane preparations.

Discussion

The microbead method provides a rapid means for obtaining enriched plasma membrane in the form of sheets from protoplasts of higher plants. It is unique with respect to other methods for obtaining plasma membrane from plant protoplasts [5–8] since it avoids harsh disruptive procedures such as homogenization. Since the outer plasma membrane surface is coated with a microbead-polyanion pellicle, the ultimate utility of this method may be to provide a starting material for selectively probing the cytoplasmic surface of the plant plasma membrane to examine cytoskeletal interactions and to conduct transbilayer protein mapping as demonstrated with the plasma membrane of *Dictyostelium* [9]. Scanning electron microscopy indicated that the entire cell was coated by the pellicle. In addition, once cells are coated with the microbeads, which takes seconds, lateral movement of

membrane proteins appears to be stopped (Chaney, L.K. and Jacobson, B.S., personal communication). In which case, transient physiological states of the plasma membrane, such as in the patching of lectins can be captured and the changes that these states might exert on plasma membrane-cytoskeletal associations analyzed.

The current method should be viewed as a supplement rather than a replacement for existing plasma membrane isolation methods. In various experiments, total cell protein recoveries ranged between 15 to 25%. Since it is generally assumed that the plant plasma membrane, like mammalian membranes, contains a lower fraction of total cellular protein, it is possible that other contaminating material may be present. On the other hand, in this highly vacuolated tissue where the amount of cytoplasm is very small [21,22], the plasma membrane of the red beet may contain a larger proportion of the cell protein than is found in the plasma membrane of other cell types. Purifications of plant plasma membrane of approx. 20-fold with regard to a particular marker have been obtained [7], however, in most plant plasma membrane purification studies, numerical indices of plasma membrane purification have not been reported. It is likely that techniques based upon membrane disruption result in the formation of inside and outside vesicles as well as open sheets. The purification of a marker therefore does not necessarily mean the purification of a representative sample of the plasma membrane. This is a less likely problem if one can isolate the plasma membrane in high yields. The microbead procedure usually provided 75% yields (Table I). The use of the microbead method coupled with traditional plasma membrane isolation procedures could provide a more accurate estimate of the true index of plant plasma membrane purity as well as provide a starting material for studies of membrane composition, enzyme activity or membrane protein purification.

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