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Isolation of Protoplasts and Vacuoles from Storage Tissue of Red Beet[1]

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RAINER SCHMIDT AND RONALD J. POOLE
McGill University, Department of Biology, Montreal, P.Q. Canada H3A 1B1

ABSTRACT

A fast and efficient method is described for the isolation of protoplasts and vacuoles from storage tissue of *Beta vulgaris* L. The viability of the isolated protoplasts is indicated by the development within a few hours of plasma strands with active cyclosis as well as by transport activity.

The effect of aging of the tissue on the yield and on the properties of the protoplasts is examined. Adding 1 millimolar dithiothreitol to the water during the aging of the tissue prevents a change of the cell wall structure which otherwise reduces the protoplast yield within 2 days to zero. When protoplasts are isolated from tissue after different times of aging, they show an increase in transport activity which parallels that in the intact tissue.

Plant protoplasts are being used increasingly as experimental material in biochemical investigations and membrane physiology. Appropriate procedures for the isolation of protoplasts from various tissues are described and summarized in review articles (4, 5).

Our ultimate goal is the preparation of plasma membrane vesicles from protoplasts of beet root storage tissue, for the study of transport and related phenomena. For this an efficient protoplast isolation procedure is required which does not cause irreversible damage to the protoplasts, *i.e.* by a long exposure time of the tissue to the cell wall degrading enzymes. Until now, no method is reported which fulfills these requirements. Recent papers mention the problem (6, 8) that the reported methods can be applied only to relatively soft tissues *e.g.* petals, leaves, and fruits. Published methods for the isolation of protoplasts from storage tissue such as carrot or radish (1, 9) are small scale preparations and have very long incubation times. For the preparation of vacuoles from beet root storage tissue, only one mechanical isolation procedure is reported (12). This method requires a large quantity of starting material. Since various methods are reported to release vacuoles from protoplasts (3, 7, 14, 15), we wished also to investigate this as a more efficient method of vacuole preparation. This paper describes a method for fast and efficient isolation of protoplasts and for the preparation of vacuoles.

MATERIALS AND METHODS

Preparation of Protoplasts. Roots of red beet, *Beta vulgaris* L., stored in moist Vermiculite at 7 C, were cut into small disks (0.8 mm thick, diameter 5 mm) and washed in aerated distilled H_2O for 5 h. Four g tissue were then incubated for 3 h at 25 C/ml of the digestion medium which consisted of 2% (w/v) Cellulysin, 2% (w/v) pectinase, 2% (v/v) glusulase, 1% (w/v) hemicellulase, and 1% (w/v) BSA in 33 mM Mes-Tris (pH 5.5), containing 125 mM $CaCl_2$, 125 mM KCl, 50 μg/ml chloramphenicol, and 0.2% (v/v) Aprotinin. After the incubation, the disks were separated from the incubation medium by gentle aspiration of the supernatant. The tissue slices were then suspended in ice-cold suspension medium of 50 mM Tris-HCl (pH 7.5), containing 500 mM mannitol, 100 mM $CaCl_2$, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 0.2% (v/v) Aprotinin, and 0.1% (w/v) PVP-40. The protoplasts were then freed from the soft tissue by squeezing the slices gently with a spatula. To separate the protoplasts from the remaining tissue the suspension was rinsed over Miracloth.

Purification of Protoplasts. If further purification was required, the protoplast suspension was gently layered over a Histopaque cushion and centrifuged for 10 min at 250*g* in a swinging bucket rotor. Histopaque has a density of 1.077 ± 0.001 g/cm^3 and consists of 5.7% (w/v) Ficoll type F-400 and 9% (w/v) sodium diatrizoate. The remaining cell wall debris formed a pellet. The purified protoplasts formed a narrow band at the interphase and were removed with a Pasteur pipette. A similar method was described by Larkin (10) using Lymphoprep instead of Histopaque.

Release of Vacuoles. Vacuoles were prepared essentially according to the method of Dürr *et al.* (3) with some modifications. The protoplasts were washed twice with cold 5 mM Mes-Tris (pH 6), containing 1.2 M sorbitol. At a concentration of 1×10^6/ml the protoplasts were kept for 10 min on ice in the same buffer before adding 50 μg/ml DEAE-dextran. The protoplasts were allowed to absorb the polybase for 1 min at 0–4 C. Then 100 μg/ml dextran sulfate was added to compensate a possible excess of DEAE-dextran which would be harmful for the vacuoles. To stabilize the vacuoles the pH was raised and ions were supplemented by adding 100 μl 500 mM Tris-HCl (pH 7.5), containing 1 M KCl and 50 mM $MgCl_2$, per 900 μl vacuole suspension. After this treatment the vacuole suspension contained less than 3% protoplasts.

Betacyanin Assay. The number of protoplasts and vacuoles/ml suspension as well as the number of cells/g tissue were determined by measuring the concentration of betacyanin at 545 nm. Fifty μl of a protoplast or vacuole suspension were added to 950 μl 30% (w/v) $HClO_4$. The debris was pelleted by a 2-min centrifugation in an Eppendorf centrifuge before betacyanin assay at 545 nm. The betacyanin assay was calibrated by counting protoplasts in a hemacytometer. There was a close linear relationship between *A* and titer in the range of 7×10^3 to 1.6×10^5 protoplasts/ml. To determine the number of cells/g tissue, 1 g of sliced disks were placed in 1 ml of 30% (w/v) $HClO_4$ until all the pigment was released from the disks. The supernatant was then assayed for betacyanin at 545 nm. One g of sliced tissue contained as much betacyanin as 4×10^6 protoplasts. Thus, 1 g of sliced tissue contains about 4×10^6 living cells.

Uptake Experiments. Protoplasts at a concentration of 2×10^5/

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ml were incubated in 50 mM Tris-HCl (pH 7.5), containing 1 M sorbitol and 1 mM $MgCl_2$ at 25 C. At zero time, 0.5 μCi of ^{86}Rb was added, with KCl to give a final concentration of 300 μM. At time intervals, 200-μl aliquots were taken from the reaction mixture and pipetted into 5 ml of the unlabeled incubation buffer containing additionally 100 mM KCl. The suspension was rinsed and sucked gently over a 0.45 μm Millipore cellulose nitrate filter. Shortly before the last of the solution passed the filter another 5 ml of the same solution were rinsed over it. Immediately after the rest of the solution passed through, the filter was removed and assayed for radioactivity in a liquid scintillation counter using a dioxane cocktail containing 0.5% (w/v) diphenyloxazole and 1% (w/v) naphthalene. It is important to remove the filter immediately after the rest of the solution has passed through, otherwise the protoplast burst on the filtration device before being transferred in to the scintillation vial. Uptake in the whole tissue was measured by incubation of 0.5 g of the root disks for 15 min at 25 C in the same incubation medium as described for the protoplasts but without the osmotic stabilizer sorbitol. Then the incubation medium was removed and the disks were washed twice for 5 min in 5 ml 50 mM Tris-HCl (pH 7.5). After removing the last washing solution, 1 ml of 30% (w/v) $HClO_4$ was added. After 1 h, samples of the supernatant were taken to measure the radioactivity as described before.

Aging. Slice tissue disks were aged for up to 5 days by incubating 10 g for 5 h in 1 liter of aerated distilled H_2O. Thereafter the disks were transferred into 200 ml aerated distilled H_2O containing 1 mM DTT and 25 μg/ml chloramphenicol. Protoplasts were aged at 25 C in the suspension medium containing 25 μg/ml chloramphenicol.

Chemicals. Hemicellulase, pectinase, Aprotinin, BSA, and Histopaque were obtained from Sigma. Cellulysin, dextran sulfate, and Miracloth were from Calbiochem. Glusulase was purchased from Endo Laboratories and DEAE-dextran from Pharmacia. The radioisotopes were from New England Nuclear. The other general inorganic and organic chemicals were of analytical grade and purchased from Fisher Scientific Company of BDH Chemicals Ltd.

RESULTS

Protoplast Yield. The main target of the present investigation was to develop a rapid method for the isolation of viable and metabolically active protoplasts from beet root storage tissue. The total yield of protoplasts depends on the time of washing the root disks in distilled H_2O and on the presence or absence of DTT (Fig. 1). A protoplast yield of 25% is achieved and maintained throughout the aging in the presence of DTT. Without the addition of the reducing agent, the yield drops to almost zero after 48 h due to resistance of the cell wall to degradation.

Viability. The viability of the protoplasts is demonstrated by uptake and by morphology. Protoplasts isolated from root disks after 3 days aging in distilled H_2O containing 1 mM DTT show a linear ^{86}Rb uptake up to 50 min (Fig. 2). Figure 3A shows the appearance of a typical protoplast after its isolation. If protoplasts are incubated in the suspension medium, after 3–4 h they change their morphology by forming plasma strands with cyclosis. Figure 3B represents a typical protoplast after 24 h aging.

Vacuoles. When protoplasts were treated for lysis with DEAE-dextran as described under "Materials and Methods," the polybase was rapidly absorbed and subsequently induced lysis. Figure 4A shows protoplasts shortly after lysis. The plasma membrane is still attached to the vacuole. Electrostatic interaction seems to be the reason for this since an increase in the ion concentration separates the vacuoles from the membranes (Fig. 4B).

Aging and Transport. In Figure 1 we demonstrated the aging of the tissue without reducing the protoplast yield, achieved by the addition of DTT. Figure 1 demonstrates the necessity of this procedure in order to obtain protoplasts with good transport activity. When protoplasts are isolated from tissue after different times of aging they show an increase in transport activity which parallels that in the intact tissue, (Fig. 5). Although the magnitude of transport varied slightly, the data in Figure 5 are representative of several experiments. The reduced uptake rate of the protoplasts compared with the tissue seems to be caused by the osmotic pressure of the protoplast uptake medium. The ^{86}Rb uptake in tissue is reduced from 2.51 μmol g^{-1} h^{-1} to 0.32 μmol g^{-1} h^{-1} in the presence of 1 M sorbitol. The ^{86}Rb uptake rate of the tissue in the presence of 1 M sorbitol is almost identical to the rate measured for protoplasts which is 0.3 μmol $(4 \times 10^6$ protoplasts$)^{-1}$ h^{-1}. One g of tissue contains about 4×10^6 cells.

DISCUSSION

The method described for the fast and efficient preparation of protoplasts from beet root storage tissue is the result of various experiments trying different enzymes, enzyme concentrations, and osmotic stabilizers. Concerning the different enzymes, increasing their concentration above that recommended here does not result in a faster maceration. However, a decrease in concentration results in a requirement for a longer exposure time. The enzyme mixture, Glusulase, often used for the preparation of yeast protoplasts, seems to have a key role among the enzymes. Without

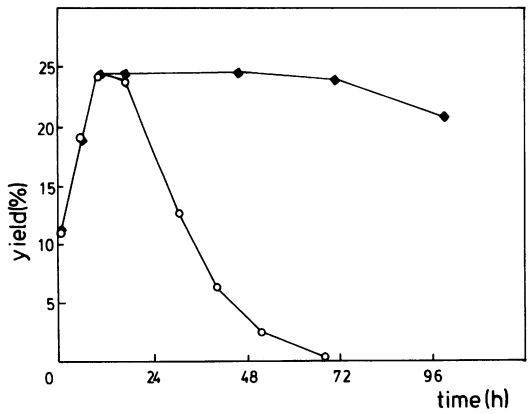

FIG. 1. Yield of protoplasts in % of the total amount of cells in the tissue (based on betacyanin) after different times of aging of tissue in distilled H_2O, in the presence (◆——◆) and absence (○——○) of 1 mM DTT.

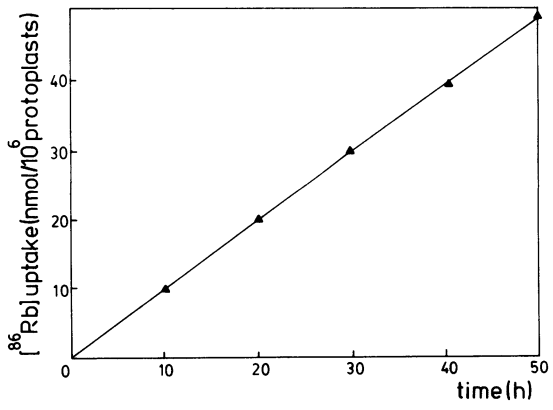

FIG. 2. ^{86}Rb uptake by protoplasts isolated by tissue after three days washing and incubated in solution containing 300 μM K. Uptake rates here and in Figure 5 are calculated on the assumption that there is no discrimination between Rb and K. Although this may not be strictly valid, it is adequate for the purpose of these experiments.

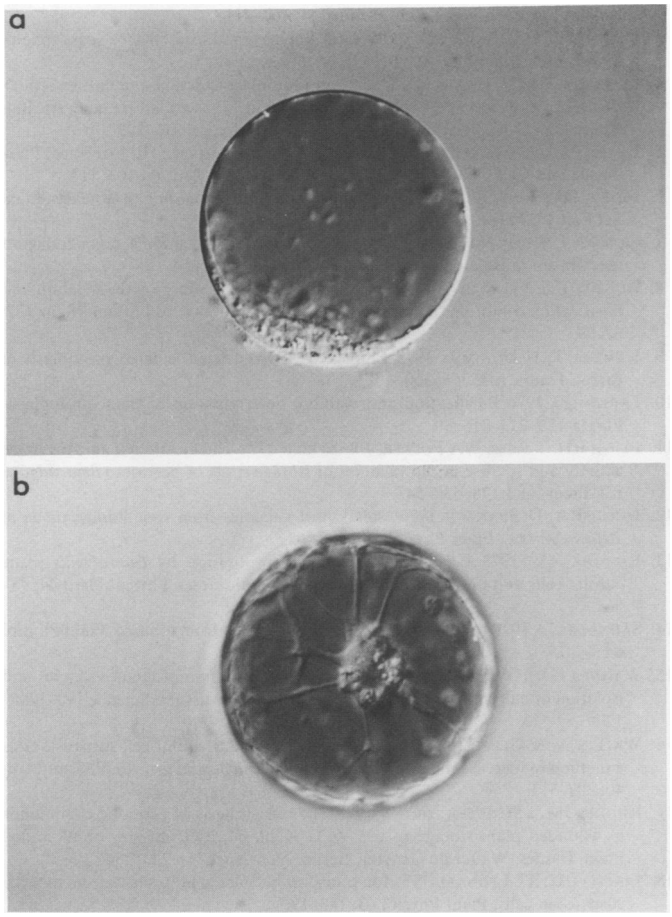

FIG. 3. Typical protoplast under Nomarski optics, 528 × magnification, (A) after isolation (B) after 24-h incubation in the suspension medium.

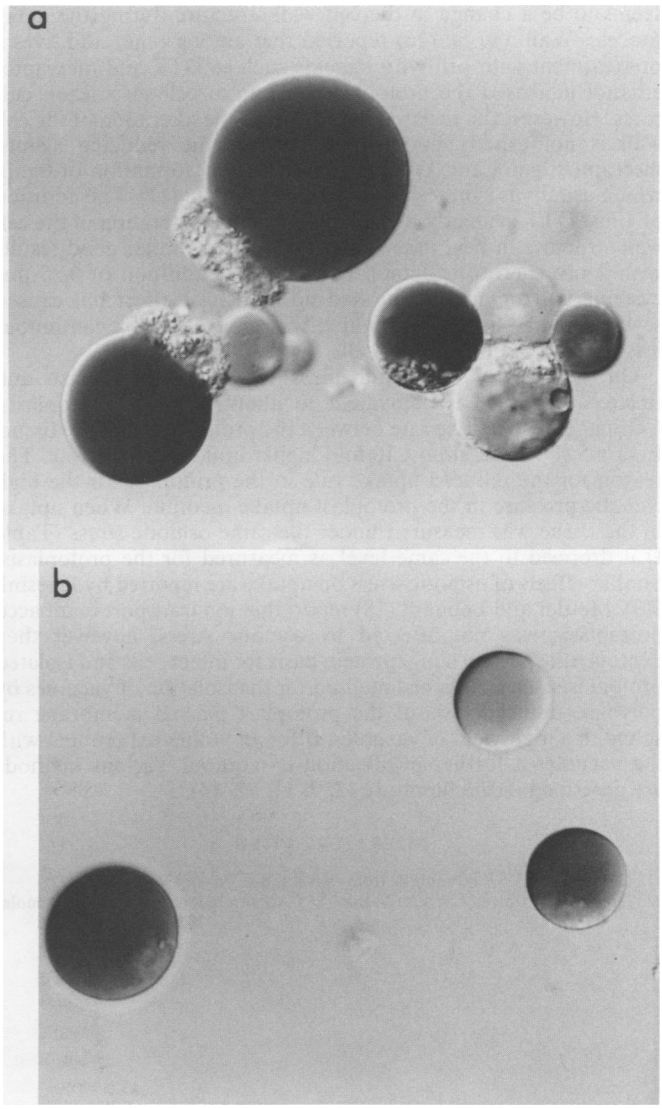

FIG. 4. Isolated vacuoles under Nomarski optics, 330 × magnification, after lysis of the plasma membrane (A) with plasma membrane still attached (B) after increasing the salt concentration.

Glusulase, the protoplast yield is almost zero and a slight reduction of its described concentration increases the necessary exposure time considerably.

It is necessary to adjust the time of washing the disks in aerated H_2O for different batches of beets to obtain the same protoplast yield. For example our first batch of beets required 5–6 h washing, whereas the second batch only needed 2–3 h.

The osmotic pressure in the digestion medium is maintained by ions. Attempts to use mannitol resulted in a large reduction of the yield. $CaCl_2$ cannot be replaced by $MgCl_2$. The presence of $MgCl_2$ seems not to affect the digestion of the cell wall but rather the viability of the cells and protoplasts, possibly by activating harmful enzymes. Additives such as BSA, Aprotinin, PVP, and chloramphenicol have no effect on the release of protoplasts. They are only protective reagents to reduce possible damage to the protoplasts, especially to their plasma membrane.

Our isolation method seems not to affect the viability of the protoplasts. We were able to demonstrate this by testing their viability in the suspension medium and by measuring ^{86}Rb transport (Fig. 5). Figure 3A compared with Figure 3B demonstrates impressively their viability and metabolically active state. We could keep protoplasts up to 8 days in the suspension medium without noticable loss or deterioration in their morphological appearance. Since the protoplasts derive from a storage tissue, they seem not to depend on an additional supplement of the medium with nutrients. Doll *et al.* (2) report, *e.g.*, a sucrose concentration up to 200 mM in beet vacuoles.

To isolate protoplasts with increased transport activity we had to overcome one severe problem. Maximum transport activity is

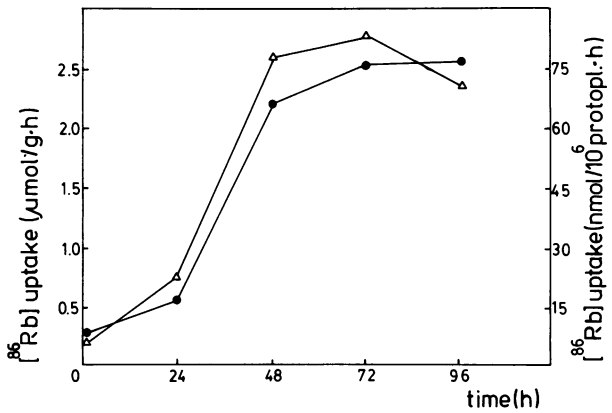

FIG. 5. ^{86}Rb uptake by the tissue (●——●) and isolated protoplasts (△——△) after different times of aging the tissue in distilled H_2O in the presence of 1 mM DTT.

achieved after aging the tissue for at least 3 days (Fig. 5). However, this caused a reduction in protoplast yield to almost zero (Fig. 1) when the tissue was aged in distilled H_2O. The reason for this

seems to be a change in the cell wall structure during the aging process. Wallin *et al.* (16) reported that among other additives a pretreatment with sulfhydryl agents such as DTT and mercaptoethanol increased the protoplast yield from cell suspension cultures. However, the reaction which causes the alteration of the cell wall is not exactly understood. Perhaps the reducing agents, mercaptoethanol and DTT, prevent the new formation of lignin which usually is synthesized in wounded tissue (17). The addition of 1 mM DTT evidently prevents the negative alteration of the cell wall structure in beet tissue (Fig. 1). We had similar good results with 2 mM mercaptoethanol. However, the addition of 0.25 mM cysteine and/or methionine had no beneficial effect but caused severe damage to the cells which was observed by a continuous loss of betacyanin from the cells.

On the basis of betacyanin measurements in protoplasts and tissue, a 1-g slice is equivalent to about 4×10^6 protoplasts. Comparing the uptake rate between the protoplasts and the tissue, Figure 5 shows an almost 10 fold higher uptake in the tissue. The reason for the reduced uptake rate in the protoplasts is the high osmotic pressure in the protoplast uptake medium. When uptake in the tissue was measured under the same osmotic stress (Table I) it dropped to the same level as measured for the protoplasts. Similar effects of osmotic stress on uptake are reported by Ruesink (13). Mettler and Leonard (18) report that ion transport in tobacco protoplasts was not affected by osmotic stress, however they express transport on a mg/protein basis for intact cells and isolated protoplasts. The described method for the isolation of vacuoles by polybase induced lysis of the protoplast plasma membrane resulted in a high yield of vacuoles. If for any other experiment with the vacuoles a further purification is required, various methods are described in the literature (2, 7, 11, 12, 14).

LITERATURE CITED

1. CAILLOUX M 1978 Protoplasts from radish tuber. Physiol Vég 15: 723–728
2. DOLL S, F RODIER, J WILLENBRINK 1979 Accumulation of sucrose in vacuoles isolated from red beet tissue. Planta 144: 407–411
3. DÜRR M, T BOLLER, A WIEMKEN 1975 Polybase induced lysis of yeast spheroplasts. Arch Microbiol 105: 319–327
4. ERIKSSON T 1977 Technical advances in protoplast isolation and cultivation. *In* W Barz, E Reinhard, MH Zenk, eds, Plant Tissue Culture and its Biotechnological Application. Springer-Verlag, Berlin, pp 313–323
5. EVANS PK, EC COCKING 1977 Isolated plant protoplasts. *In* HE Street, ed, Plant Tissue and Cell Culture. Ed 2 Vol II, Blackwell, Oxford, pp 103–117
6. FISHER DJ 1979 Studies of plant membrane components using protoplasts. Plant Sci Lett 15: 127–133
7. GUY M, L REINHOLD, D MICHAELI 1970 Direct evidence for a sugar transport mechanism in isolated vacuoles. Plant Physiol 64: 61–64
8. HUGHES BG, FG WHITE, MA SMITH 1978 Effect of plant growth, isolation and purification conditions on barley protoplast yield. Biochem Physiol Pflanz 172: 67–77
9. KAMEYA T, H UCHIMYA 1972 Embryoids derived from isolated protoplasts of carrot. Planta 103: 356–360
10. LARKIN PJ 1976 Purification and viability determination of plant protoplasts. Planta 128: 213–216
11. LEIGH RH, T REES, WA FULLER, J BANFIELD 1979 The location of acid invertase activity and sucrose in the vacuoles of storage roots of beetroot (*Beta vulgaris* L.). Biochem J 178: 539–547
12. LEIGH RA, D BRANTON 1976 Isolation of vacuoles from root storage tissue of *Beta vulgaris* L. Plant Physiol 58: 656–662
13. RUESINK AW 1978 Leucine uptake and incorporation by *Convolvulus* tissue culture cells and protoplasts under severe osmotic stress. Physiol Plant 44: 48–56
14. SAUNDERS JA 1979 Investigations of vacuoles isolated from tobacco. Plant Physiol 64: 74–78
15. WAGNER GJ, HW SIEGELMAN 1975 Large scale isolation of intact vacuoles and isolation of chloroplasts from protoplasts of mature tissues. Science 190: 1298–1299
16. WALLIN A, K GLIMELIUS, T ERIKSSON 1977 Pretreatment of cell suspensions as a method to increase the protoplast yield of *Haplopappus gracilis*. Physiol Plant 40: 307–311
17. RHODES JM, LSC WOOLTORTON 1978 The biosynthesis of phenolic compounds in wounded plant storage tissues. *In* G Kahl, ed, Biochemistry of Wounded Plant Tissues. Walter de Gruyter, Berlin, New York, pp 243–286
18. METTLER IJ, RT LEONARD 1979 Ion transport in isolated protoplasts from tobacco suspension cells. Plant Physiol 63: 183–190