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The Rapid Isolation of Vacuoles from Leaves of Crassulacean Acid Metabolism Plants[1]

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ABSTRACT

A technique is presented for the isolation of vacuoles from *Sedum telephium* L. leaves. Leaf material is digested enzymically to produce protoplasts rapidly which are partially lysed by gentle osmotic shock and the inclusion of 5 millimolar ethyleneglycol-bis (β-aminoethyl ether)N,N'-tetraacetic acid in the wash medium. Vacuoles are isolated from the partially lysed protoplasts by brief centrifugation on a three-step Ficoll-400 gradient consisting of 5, 10, and 15% (w/v) Ficoll-400. A majority of the vacuoles accumulate at the 5 to 10% Ficoll interface, whereas a smaller proportion sediments at the 10 to 15% Ficoll-400 interface. The total time required for vacuole isolation is 2 to 2.5 hours, beginning from leaf harvest.

The yield of vacuoles is approximately 44%. The major vacuole layer is < 7% contaminated by marker enzymes from the cytoplasm and other organelles but shows no contamination by chloroplasts. Isolated vacuoles were stable for >15 hours when left in Ficoll; however, dispersion into media of various osmotic concentrations resulted in decreased stability. Addition of mercaptobenzothiazole, $CaCl_2$, $MgCl_2$, bovine serum albumin, ethylenediaminetetraacetic acid, polyethylene glycol 600, and KH_2PO_4 to the vacuole isolation media did not increase the stability of the isolated vacuoles.

This technique with only slight modifications has been used to isolate leaf cell vacuoles from the following Crassulacean acid metabolism plants: pineapple, *Kalanchoë fedtschenkoi*, and *Echeveria elegans*. Spinach leaves also were used successfully.

Recently the roles of the higher plant cell vacuole have been the subject of renewed research interest due primarily to the development of large-scale vacuole isolation techniques. These new techniques yield sufficient quantities of purified organelles for *in vitro* analysis. Beginning with the isolation work of Wagner and Siegelman (19) and Leigh and Branton (10), vacuoles now have been isolated and analyzed from a variety of plant tissues. On the basis of these studies, the number of metabolites compartmentalized within the vacuoles has been expanded to include protein (15), sucrose (11, 15), glucose, fructose, amino acids (20), cyanogenic glucosides (17), proteinase inhibitors (22), numerous hydrolytic enzymes (2, 15), organic acids (3, 6, 21), glycosinolates (4), and nicotine (18). The idea is clearly emerging from such studies that the higher plant vacuole is a versatile and dynamic organelle.

Most of the techniques used to isolate vacuoles from leaf tissue require long digestion times (4–12 h) to produce protoplasts (2, 3, 19), or a lengthy centrifugation period (2–4 h) to release vacuoles from protoplasts (12, 17). The time needed for these vacuole isolation steps makes detailed investigations of *in vivo* diurnal changes in metabolites levels impossible.

The postulated role of the vacuole in compartmentation in CAM plants is particularly interesting since the vacuole has been implicated as the nocturnal storage site of malic acid and, hence, involved in the assimilation of CO_2 in CAM plants. CO_2 is assimilated to form malic acid at night which is thought to be stored temporarily in the vacuoles. During the day, malate is removed from the vacuole and provides the substrate for light-dependent CO_2 fixation (16). This hypothesis was not tested directly until recently. Buser and Matile (3) provided evidence indicating that malic acid is present in the vacuole of *Bryophyllum daigremontiana* leaf cells; however, the method used to isolate vacuoles required about 8 h, which did not allow the diurnal flux of malate through the vacuole to be analyzed. Using the vacuole isolation technique given in this manuscript, we found both malic and isocitric acids in *Sedum* vacuoles and demonstrated a diurnal flux of malic acid and a constant level of isocitric acid (6, 7).

Because of the difficulty of adapting existing isolation techniques to the study of the participatory role of the vacuole in daily carbon assimilation in CAM plants, it was necessary to develop a rapid procedure for efficiently isolating CAM vacuoles. Here, we present a technique for rapidly isolating large quantities of intact vacuoles from mature leaves of CAM plants as well as from leaves of other plants. We assess contamination, yields, and stability. This technique has been successfully used to monitor the diurnal changes in vacuolar organic acid levels in leaf cells of *Sedum telephium*, showing that the vacuole in CAM plants is directly involved in photosynthetic CO_2 assimilation (6).

MATERIALS AND METHODS

Plant Material. *S. telephium* L. was cultivated in a sand and peat (1:1, v/v) potting soil and grown in the greenhouse under a natural photoperiod and a day to night temperature regime of 30 to 20 C. The plants were fertilized bimonthly with half-strength Hoagland solution and watered each day. One to 2 months prior to their use in experiments, the plants were transferred to a growth chamber and grown under a 30 C,15-h day (200–500 $\mu E\ m^{-2}\ s^{-1}$) and a 15 C, 9-h night. Under these conditions the plants attained a maximum diurnal acid fluctuation within 2 to 3 weeks. Other plant material in this study was harvested from greenhouse-grown plants and used immediately.

Enzyme and Chemical Assays. Cyt *c* oxidase (EC 1.9.3.1) was assayed according to Hodges and Leonard (5) with the inclusion of 0.3% (w/v) Triton X-100 and 0.1% (w/v) BSA. Catalase (EC 1.11.1.6) was assayed as described in reference 1 except that samples were sonicated briefly immediately prior to the assays.

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PEP[4] carboxylase (EC 1.1.1.46) activity was determined spectrophotometrically essentially as described by Mukerji and Ting (14). Chl was determined in 96% (v/v) ethanol by the method of Wintermans and Demots (23).

Malic and isocitric acids were determined spectrophotometrically by monitoring the reduction of NADP. The reactions were carried out in a 1-ml reaction volume containing 50 μmol Hepes-NaOH (pH 7.5) (22 C), 1 μmol $MnCl_2$, 0.5 μmol NADP and either 0.075 EU chicken liver malic enzyme (EC 1.1.1.40) (Sigma) or 0.125 EU porcine heart isocitric dehydrognase (EC 1.1.1.42) (Calbiochem). Isocitric acid in each sample was converted to its unlactonized form (9) immediately preceding each measurement.

Staining. Vacuoles and protoplasts were stained with neutral red to facilitate microscopic counting. The addition of 1 to 2 drops of 1% (w/v) neutral red in 0.6 M sucrose was sufficient to stain 5 ml of either protoplasts or vacuoles. The light microscope was used throughout this work to examine leaf tissues, protoplasts, and vacuoles.

Counting. Vacuoles and protoplasts were counted in 10 to 20-μl aliquots on a Fuchs Rosenthal ultraplane haemocytometer without a cover slip.

Protoplast Isolation. Leaves were prepared by washing in distilled H_2O, removing the midribs and margins, and peeling off both the upper and lower epidermis. The leaf material was sliced into 1- × 5-mm pieces with a razor blade and very gently vacuum-infiltrated with the enzyme incubation medium in a ratio of 1:8 (g tissue to ml medium). The incubation medium contained 0.7 M mannitol, 50 mM Mes-NaOH (pH 5.5, 22 C), 5 mM $MgCl_2$, 2.0% (w/v) Cellulysin (Calbiochem), 1% (w/v) Pectinase (Sigma), and 0.1% (w/v) BSA. Tissue digestion was carried out at 30 C and about 45 strokes/min in a reciprocal shaking water bath for 30 to 45 min. Protoplast release was routinely monitored with a light microscrope.

Digested leaf tissue was filtered through a 295-μm nylon net and the net was washed with 25 ml of a resuspension buffer containing 0.5 M mannitol, 25 mM Tris-HCl, and 5 mM EGTA (pH 8.0, 22 C). The protoplasts were collected in a large culture tube on ice and allowed to settle for 10 to 15 min. Decreasing this settling period resulted in purer protoplasts but reduced the yield. The supernatant solution was drawn off and the soft protoplast pellet was resuspended in 40 ml of resuspension buffer and allowed to settle for 10 to 15 min. The resulting pellet finally was suspended in 3 ml of fresh resuspension buffer and kept on ice. This procedure yielded a suspension of partially lysed protoplasts, most of which had plasma membranes which were beginning to rupture and pull away from the vacuole.

Vacuole Isolation. Vacuoles were isolated from the partially lysed protoplast preparation by centrifugation on a three-step discontinuous Ficoll-400 gradient. One ml of the protoplast suspension was gently layered onto the top of a Ficoll-400 gradient containing 10 ml each of 5, 10, and 15% (w/v) Ficoll-400. Each Ficoll solution was made up in 0.5 M mannitol and 25 mM Tris-HCl (pH 8.0, 22 C). The gradients were spun at 26,000 rpm (100,000g) for 30 min in a SW-27 rotor in a Beckman model L-2 ultracentrifuge. The vacuoles were removed from the gradient with a 16 gauge cannula attached to a 1-ml syringe.

RESULTS

Protoplast Isolation. A typical preparation of protoplasts obtained from *S. telephium* leaves within 1 h is shown in Figure 1. After a survey of CAM plants, *S. telephium* was chosen because large quantities of protoplasts could be isolated rapidly (<1 h) and easily from its leaves. This was important for reducing the overall time required for vacuole isolation.

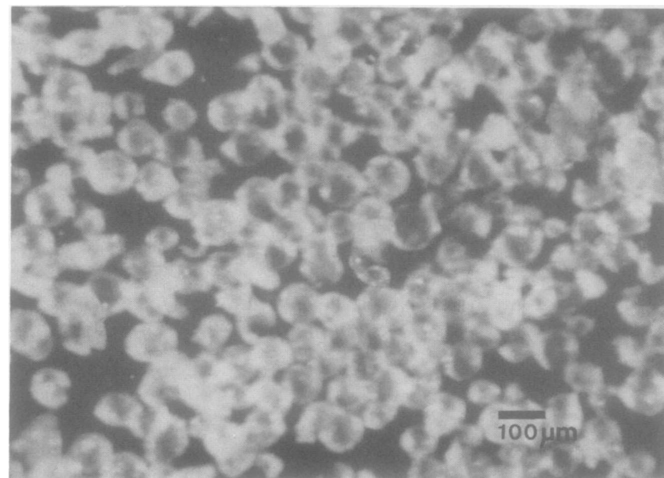

FIG. 1. A washed preparation of partially lysed protoplasts isolated after a 30-min digestion of *S. telephium* leaf tissue.

Table I. *Yields of Protoplasts and Vacuoles from S. telephium Leaves*

These leaves contained minimum levels of malic acid and were harvested in the afternoon. Three g of peeled tissue were used. The yield of protoplasts based on leaf chlorophyll was 50% and was the mean of two determinations. Values given are mean ± SD.

Total Protoplasts Recovered	Total Vacuoles Recovered from Protoplasts[a]	Yield of Vacuoles Based on Protoplasts	Yield of Vacuoles Based on Recovery of Malic Acid
		%	
$4.1 \pm 0.9 \times 10^5$ ($n = 6$)	$1.8 \pm 0.5 \times 10^5$ ($n = 8$)	44 ± 11 ($n = 8$)	53.7

[a] Vacuoles recovered from both the 5 to 10% and 10 to 15% interfaces were counted. The number of vacuoles at the 5 to 10% interface represented approximately 70 to 80% of this total.

Three g leaf material routinely yielded 3 to 5×10^5 protoplasts which accounted for 40 to 50% of the leaf Chl (Table I).

During washing, protoplasts were given a slight osmotic shock by reducing the osmotic concentration from 0.7 to 0.5 M mannitol. This decrease in the osmotic potential of the wash medium in the presence of EGTA partially lyses the protoplasts and both increases yield and decreases surface contamination visible in the light microscope of subsequently isolated vacuoles. The effect of the chelator EGTA could not be replaced by either EDTA or 2-mercaptobenzothiazole from 1 to 10 mM, nor was the subsequent isolation of vacuoles improved by the addition of 0.1 to 1% (w/v) BSA in the resuspension buffer.

Vacuole Isolation. After 30 min centrifugation, four fractions could be distinguished. The first fraction, which remained on top of the gradient, contained resuspension buffer and soluble contents of broken protoplasts and vacuoles. The second fraction consisted of a large band of vacuoles at the 5 to 10% (w/v) Ficoll interface. A third fraction located at the 10 to 15% (w/v) Ficoll interface contained primarily vacuoles with a few protoplasts and some debris adhering to the outside of the tonoplasts. The last fraction was the pellet consisting of a few intact cells and a majority of cytoplasmic organelles including chloroplasts, mitochondria, and peroxisomes.

An example of vacuoles from the major vacuole band at the 5 to 10% Ficoll interface is shown in Figure 2. To increase visibility,

[4] Abbreviations: PEP: phosphoenolpyruvic acid; EU: enzyme unit; EGTA: ethyleneglycol-bis (β-aminoethyl ether)N, N'-tetraacetic acid.

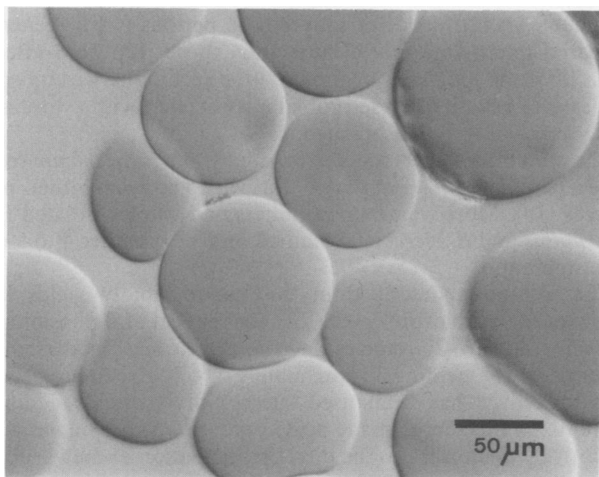

FIG. 2. Isolated vacuoles recovered from the 5 to 10% (w/v) Ficoll interface. Vacuoles were stained with neutral red and photographed with Normarski Interference optics.

the vacuoles have been stained red by adding neutral red to the protoplast resuspension buffer prior to centrifugation.

A minimum time of 30 min centrifugation was necessary to yield quantities of vacuoles sufficient for biochemical analysis. All of the analyses described here were done on vacuoles isolated after 30 min centrifugation. Increasing centrifugation time up to 4 h, however, decreased visible surface contamination of the vacuoles and caused the majority of the vacuoles to accumulate at the 10 to 15% Ficoll interface. Inasmuch as the emphasis of this isolation procedure was on minimizing isolation time, the effects of longer centrifugation times on parameters such as contamination or yield were not investigated.

Vacuole Yield. Vacuole yields may be estimated by two methods. First, the percentage of vacuoles recovered based on the number of protoplasts loaded onto the gradient gives an approximate yield of 44% (Table I). However, there is a problem associated with counting the vacuoles accurately because, for unknown reasons, there is a large range of sizes. The smaller vacuoles may be produced from larger vacuoles during centrifugation, which may introduce large errors in counting (3); hence, in these studies only vacuoles ≥15 µm in diameter were counted. Vacuoles also may lyse during handling, which would cause a low count. The second method involves the comparison of malic acid in protoplasts loaded onto the gradient with the amount recovered in the vacuole bands. This estimation does not require intact vacuoles and gave total yields of nearly 54% (Table I).

Assessment of Vacuole Contamination. Ficoll gradients were fractionated, and each 1-ml fraction was assayed for the following substances or enzymes as markers: Chl (chloroplasts), Cyt *c* oxidase (mitochondria), catalase (peroxisomes), PEP carboxylase (cytoplasm), and malic and isocitric acids (vacuoles) (Table II). Malic and isocitric acids were recovered primarily in the vacuole bands, confirming their intracellular localization in these organelles (6, 7). Most of the remaining malic and isocitric acids were on top of the gradient, presumably due to ruptured protoplasts and vacuoles as well as cytoplasmic pools. All of these acids were accounted for on the gradients.

Virtually all the Chl loaded onto each gradient was recovered in the pellet. Microscopically, only a few chloroplasts could be seen in the vacuole bands and these were exclusive to the 10 to 15% Ficoll band. No protoplasts were recovered in the major vacuole layer, but some were found in the minor vacuole layer. The results indicate there was some contamination (<7%) of the major vacuole band by marker enzymes of mitochondria, peroxisomes, and soluble enzymes of the cytoplasm in contrast to about half of the malic acid being concentrated in this band (Table II).

Table II. *Assessment of Contamination of Isolated Vacuoles by Cytoplasmic Organelles*

Contamination is based on the per cent of activity of marker enzymes or substances recovered in the vacuole bands which were loaded onto the gradients in protoplasts. Values given are the % of total activity or amount loaded on Ficoll gradients.

	Enzyme Activity or Amount in	
	Major Vacuole Band	Minor Vacuole Band
	%	
Catalase	6.3	1.28
Chl	nd[a]	nd[a]
Cyt *c* oxidase	2.5	1.8
Isocitric acid	49.0	9.5
Malic acid	47.3	6.4
PEP carboxylase	6.5	8.8

[a] nd, not detectable.

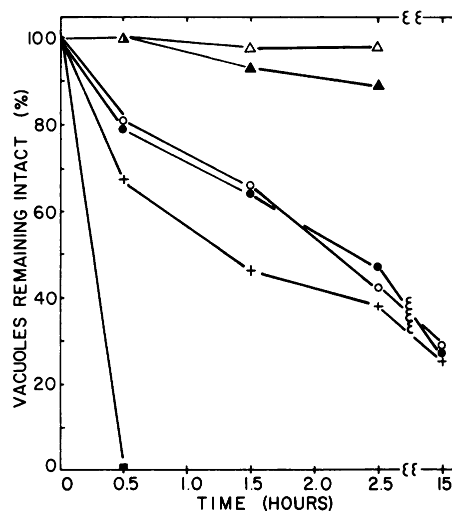

FIG. 3. The effect of osmotic concentration on vacuole stability. Isolated vacuoles were either left on the gradient at the 5 to 10% (▲——▲) or 10 to 15% (△——△) Ficoll interfaces, removed the gradient and left undiluted (●——●), or diluted 1:4 with the following concentrations of mannitol or sorbitol: 0.3 M mannitol, (■——■); 0.7 M mannitol (○——○); 5% Ficoll plus 0.5 M mannitol, 0.5 and 0.6 M mannitol, or 0.8 and 1.0 M sorbitol (+——+). All solutions contain 0.25 M Tris-HCl, (pH 8.0, 22 C) and, except for the upper curve, all vacuoles were from the 5 to 10% Ficoll interface. The points represent averages of data from three to seven experiments.

Stability. Isolated vacuoles were more stable when stored on ice as compared to room temperature; nonetheless, vacuole integrity decreased with time, regardless of temperature. In an effort to increase their stability, isolated vacuoles were suspended in various concentrations of several osmotica, and the number of intact vacuoles as a function of time was monitored. Vacuoles in their most concentrated form, left on the Ficoll gradient, remained 90 to 95% intact after 2.5 h (Fig. 3). Nearly 60% of these concentrated vacuoles retained their integrity even after 15 h on ice. Removal of vacuoles from the Ficoll gradient and dilution in any of the concentrations of osmotica tested resulted in their gradual rupture, and only about 50% remained intact after 2.5 h (Fig. 3).

The influence of various substances on vacuole stability also was examined. Addition of mercaptobenzothiazole (1 to 10 mM), low concentrations of $CaCl_2$ (1 mM), and BSA (0.1 to 1%, w/v) had little effect on increasing vacuole longevity. High concentra-

tions of $CaCl_2$ (10 mM) and all tried concentrations of $MgCl_2$, PEG 6000 (PEG-Carbowax), EDTA, and 25 mM K_2HPO_4 (pH 8.0) were found to rupture the vacuoles. BSA in higher concentrations (5%, w/v) stabilized the vacuoles slightly.

Isolation of Vacuoles from Leaves of Other Plants. Vacuoles were successfully isolated from leaves of the CAM plants *Echeveria elegans* Rose and *Kalanchoë fedtschenkoi,* both from the family Crassulaceae, by the present method. It was necessary to adjust the concentration of mannitol in the incubation medium to 0.4 M to obtain good yields of protoplasts from *K. fedtschenkoi.* The technique was also applied to leaves of *Ananas comosus* (L.). Merr, pineapple, and *Spinacia oleracea,* a C_3 plant, both of which required a longer (2 h) enzyme digestion to produce protoplasts. Hence, with slight modifications of this technique, it can be adapted for use with leaves of other plants.

DISCUSSION

A technique has been described for the isolation of intact vacuoles from *S. telephium* leaf material in sufficient quantities for biochemical analysis. This method was developed in response to a need for a vacuole isolation procedure rapid enough to allow an investigation of the diurnal metabolite changes in this cellular compartment in CAM plants.

Most existing vacuole isolation techniques developed for leaf material have two major drawbacks: (*a*) the excessively long time required for protoplast production (4–12 h) and (*b*) a large osmotic shock necessary to release vacuoles from protoplasts (2, 3, 17, 19). Development of the techniques in this manuscript was aimed at circumventing these two problems. Two factors have decreased the time necessary to isolate *S. telephium* leaf vacuoles. First, the enzyme digestion time required for protoplast release was reduced by peeling and vacuum-infiltrating the leaves. Thus, quantities of protoplasts sufficient to isolate vacuoles could be obtained in 30 to 45 min.

A second factor is the employment of only a small osmotic change which aids in protoplast rupture. It has been suggested, from work on the osmotic lysis of yeast spheroplasts to yield vacuoles, that the permeability of the tonoplast to low mol wt compounds may be affected by such treatment (13). Since the isolation technique was developed primarily to monitor changes in low mol wt metabolite levels (organic acids) in vacuoles, it was reasoned that the exposure of vacuoles to large osmotic changes should be avoided.

The facility with which vacuoles could be isolated from CAM plants depended on the acid level in the leaves. In general, *S. telephium* vacuoles are more difficult to isolate early in the morning, when leaf acid levels are highest, than late in the afternoon. To ensure good yields of usable protoplasts from leaves with high acid contents, the pH of the enzyme digestion medium had to be readjusted to pH 5.5 during the incubation. Vacuoles isolated from leaves containing large amounts of acid were also less stable than vacuoles from leaves with low acid levels.

Enzymic estimation of contamination of cytoplasmic organelles suggests that there was virtually no contamination of the vacuoles by chloroplasts; however, some contamination (<7%) by mitochondria, peroxisomes, and cytoplasm was evident. A portion of this contamination probably is due to intact protoplasts reaching the Ficoll interfaces during centrifugation, rupturing, and releasing vacuoles. This would account for the presence of the soluble cytoplasmic enzyme PEP carboxylase in vacuole fractions. This activity is not due to contamination of the vacuole fractions by the cellulytic enzymes used to prepare the protoplasts as no PEP carboxylase activity has been detected in either cellulysin or pectinase. Contamination of vacuoles by mitochondria and peroxisomes, but not by chloroplasts, may reflect differences in sedimentation coefficients of these organelles and the brief centrifugation period.

Vacuoles centrifuged for longer time periods (>1 h) generally appeared microscopically cleaner than those recovered after 30 min. Since the emphasis of the isolation technique was on speed and ease of isolation, these problems associated with cytoplasmic contamination were not pursued.

The stability of the vacuoles isolated from *S. telephium* leaves is comparable to that reported for vacuoles from other plant sources (10). Moreover, large numbers of vacuoles isolated from *A. comosus* and *S. oleracea* remained intact even after 4 to 5 days of refrigeration.

The technique described in this paper rapidly yields large numbers of vacuoles sufficient for biochemical and physiological analysis. The SW-27 rotor can accommodate six Ficoll gradients simultaneously yielding approximately 5×10^5 isolated vacuoles. This procedure has been used successfully to analyze diurnal fluctuations in vacuole malic acid content in *S. telephium,* which definitively demonstrates the direct participation of the vacuole in carbon assimilation in CAM plants (6, 7).

Recently we also have analyzed vacuoles from leaves of *E. elegans* which contain up to 15% of their dry weight as phorbic acid (8). It was found that phorbic acid is almost exclusively localized in the vacuoles of *E. elegans* leaves.

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