Isolation of intact vacuoles from Arabidopsis rosette leaf–derived protoplasts

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Vacuoles are very prominent compartments within plant cells, and understanding of their function relies on knowledge of their content. Here, we present a simple vacuole purification protocol that was successfully used for large-scale isolation of vacuoles, free of significant contamination from other endomembrane compartments. This method is based on osmotic and thermal disruption of mesophyl-derived Arabidopsis protoplasts, followed by a density gradient fractionation of the cellular content. The whole procedure, including protoplast isolation, takes approximately 6 h.

INTRODUCTION

Large-scale isolation and characterization of cellular organelles offers excellent opportunities in plant systems biology[1]. Detailed qualitative and quantitative knowledge of the composition of a particular organelle allows for investigation of subcellular pathways through the identification of novel protein components and the analysis of cellular sinks. This also facilitates detailed comparative studies between species or between mutant and wild-type plants. In contrast to some organelles, such as mitochondria and chloroplasts, that are relatively easy to obtain in a pure form[2,3], plant vacuoles are extremely fragile, and their isolation using conventional tissue homogenization and fractionation schemes can be remarkably challenging and often results in a subcellular fraction without the same properties as intact mature vacuoles[4]. The first successful technique to overcome these difficulties was based on protoplast isolation and subsequent gentle release of vacuoles by osmotic shock[5,6]. Later large-scale isolation protocols employed gradient fractionation of osmotically released organelles[7,8], and the method was further optimized by application of both osmotic and thermal shocks to the isolated protoplasts[9,10]. As an alternative to the protoplast methods, which are applicable only to relatively soft tissues, vacuoles can be isolated by slicing the plant material and releasing contents into a medium with a perfectly adjusted osmoticum[11]. In subsequent studies, numerous vacuolar constituents have been determined, including sugars and amino acids[12], hydrolases[13] and ions[14].

The increasing impact of proteomic studies on plant biology[15,16] has triggered renewed interest in organelle purification and led to two independent proteomic studies focused on intact vacuoles derived from different plant tissues[17,18]. In contrast to a relatively intricate protocol describing intact vacuole purification from an Arabidopsis cell suspension[18], we present a simple, reliable method for vacuole isolation from Arabidopsis leaf mesophyl protoplasts in great detail[17]. This protocol is based on one-step Ficoll gradient fractionation of osmotically and thermally lysed protoplasts under simple chemical conditions. This method has been used successfully in our laboratory for biochemical characterization of vacuolar contents using both proteomics and immunological approaches. The focus of this protocol on vegetative tissues has allowed for comparative studies of a mutant plant line that was affected in terms of vacuolar protein processing and degradation[19]. Therefore, this protocol provides an exceptional opportunity for comparative studies between multiple plant lines without generation of stable cell suspension cultures.

Procedural comments

This protocol is designed to be performed within 1 d. It consists of two steps, which include the isolation of the protoplast followed by a gradient fractionation. The buffers should be made fresh, and the plant tissues cannot be frozen. It is recommended that the experimenter wear gloves during the whole procedure.

MATERIALS

REAGENTS

- $CaCl_2 \cdot 2H_2O$ (Fisher, cat. no. C614)
- Cellulase Onozuka R-10 (1.0 U mg^{-1}) (Serva, Heidelberg, Germany, cat. no. 16419) ! CAUTION Harmful; avoid inhalation and contact with skin.
- EDTA (J.T. Baker, cat. no. 893) (see REAGENT SETUP)
- Ficoll (Sigma, cat. no. F4375)
- Macerozyme R-10 (0.55 U mg^{-1}) (Serva, Heidelberg, Germany, cat. no. 28032) ! CAUTION Harmful; avoid inhalation and contact with skin.
- Mannitol (Fisher, cat. no. M120) (see REAGENT SETUP)
- β-Mercaptoethanol (Sigma, cat. no. M3148) ! CAUTION Toxic; avoid inhalation, ingestion or contact with skin, eyes or mucous membranes. Dangerous for the environment.
- 2-(N-morpholino)ethanesulfonic acid (VWR, cat. no. EM6110) (see REAGENT SETUP)
- $Na_2HPO_4 \cdot 7H_2O$ (Sigma, cat. no. S9390)
- $NaH_2PO_4 \cdot H_2O$ (Sigma, cat. no. S9638)
- Neutral Red dye (Sigma, cat. no. N7005) (see REAGENT SETUP)
- Acetic acid
- Solid NaOH
- Milli-Q water
- KOH (1 M)
- Lysis buffer (see REAGENT SETUP)
- Chloroform
- Wash buffer (see REAGENT SETUP)
- Protoplast enzyme solution (see REAGENT SETUP)
- Vacuole buffer (see REAGENT SETUP)

EQUIPMENT

- Allegra 6 benchtop centrifuge (Beckman Coulter, cat. no. 366802)

- Axioskop 40 Pol upright microscope (Zeiss)
- 50-ml BD Falcon centrifuge tubes, polypropylene, conical bottom, sterile (BD Bioscience; VWR, cat. no. 21008-178)
- Benchtop open air Excella shaker model E1 (New Brunswick scientific)
- Cellector Tissue Sieve Thermo EC (150 mesh) (VWR, cat. no. 62399-872)
- GH-3.8 rotor, swinging bucket, 3,750 r.p.m., 3,200*g* (Beckman Coulter, cat. no. 360581)
- 250 ml Kimax flasks with side arm (Fisher, cat. no. 10-181D)
- 150 ml Pyrex brand fleaker beaker (Fisher, cat. no. 02599)
- Pyrex 5 ml reusable wide-tip serological pipettes, TD, color-coded, colored markings (Corning Inc., cat. no. 7078B-5)
- SW 40 Ti rotor, swinging bucket, 6 × 14 ml, 40,000 r.p.m., 285,000*g* (Beckman Coulter, cat. no. 331302)
- Ultra-clear centrifugation tubes, 14 ml, 14 × 95 mm (Beckman Coulter, cat. no. 344060)

REAGENT SETUP

0.5 M EDTA Dissolve 74.45 g EDTA in 300 ml Milli-Q water. Adjust pH to 8 with solid NaOH. Add Milli-Q water until the total volume is 400 ml.

30% (wt/vol) Ficoll Dissolve 4.5 g Ficoll in Milli-Q water to a total volume of 15 ml. Heat the solution carefully for several minutes in a 65 °C water bath and vortex until completely dissolved. The Ficoll solution can be prepared 1 d ahead.

▲ **CRITICAL** The 30% Ficoll solution should not be exposed to excessive heat. Ficoll is difficult to dissolve, but heat the solution for a short time without boiling it.

1 M mannitol Dissolve 72.87 g mannitol in 400 ml Milli-Q water.

50 mM MES, pH 5.6 Dissolve 1.07 g MES in 80 ml Milli-Q water. Adjust pH to 5.6 with 1 M KOH. Add Milli-Q water until the total volume is 100 ml.

0.1% Neutral Red solution Mix 0.1 g Neutral Red, 200 µl 1% acetic acid and 50 µl chloroform. Add Milli-Q water until the total volume is 100 ml.

0.2 M sodium phosphate, pH 7.5 Mix 16 ml solution A (27.6 g $NaH_2PO_4 \cdot H_2O$ per liter; 0.2 M) and 84 ml solution B (53.65 g $Na_2HPO_4 \cdot 7H_2O$ per liter; 0.2 M).

0.2 M sodium phosphate, pH 8.0 Mix 5.3 ml solution A (27.6 g $NaH_2PO_4 \cdot H_2O$ per liter; 0.2 M) and 94.7 ml solution B (53.65 g $Na_2HPO_4 \cdot 7H_2O$ per liter; 0.2 M).

Lysis buffer Mix 3 ml 1 M mannitol, 5 ml 30% Ficoll, 300 µl EDTA pH 8.0, 375 µl 0.2 M sodium phosphate pH 8.0 and 75 µl Neutral Red[20]. Add Milli-Q water until the total volume is 15 ml. Keep at 37 °C.

Vacuole buffer Mix 4.5 ml 1 M mannitol, 250 µl 0.2 M sodium phosphate pH 7.5 and 40 µl 0.5 M EDTA pH 8.0. Add Milli-Q water until the total volume is 10 ml. Keep on ice.

4% Ficoll solution Mix 4.5 ml vacuole buffer and 3 ml lysis buffer. Keep at room temperature (~25 °C).

Wash buffer Mix 60 ml 1 M mannitol and 30 ml 50 mM MES pH 5.6. Add Milli-Q water until the total volume is 150 ml.

Protoplast enzyme solution Dissolve 0.3 g cellulase, 0.3 g macerozyme, 0.12 g $CaCl_2 \cdot 2H_2O$ in 30 ml wash buffer. Keep at 37 °C with occasional vortexing until the enzymes are completely dissolved. Add 10.5 µl β-mercaptoethanol.

! **CAUTION** β-Mercaptoethanol is toxic and dangerous for the environment. Handle using appropriate safety equipment and measures. Cellulase and macerozyme are harmful.

PROCEDURE

Plant material

1| Remove rosette leaves from 35-day-old Arabidopsis plants. The plants should be grown under short-day conditions (8 h light) at 18 °C in small pots, one plant per pot.

? **TROUBLESHOOTING**

Protoplast isolation

2| Collect no more than 2 g rosette leaf tissue.

3| Cut the leaves into 2 mm strips using a razor blade.

4| Place the processed leaves and 30 ml protoplast enzyme solution into a 250-ml Kimax flask with side arm and apply vacuum for 10 min.

! **CAUTION** β-Mercaptoethanol is toxic and dangerous for the environment. Cellulase and macerozyme are harmful.

5| Cover the flask openings with Parafilm and incubate the tissues with the protoplast enzyme solution for 4 h on a benchtop open air Excella shaker model E1 in the dark. Shake at 70 r.p.m.

6| Wash the protoplasts by straining through a Cellector Tissue Sieve Thermo EC into a 150-ml Pyrex brand fleaker beaker (150 mesh). Rinse the Cellector Tissue Sieve Thermo EC gently with 5 ml wash buffer.

7| Transfer the protoplasts carefully into a 50-ml BD Falcon centrifuge tube.

8| Rinse the fleaker with an additional 5 ml wash buffer.

9| Spin down the protoplasts in an Allegra 6 benchtop centrifuge at 80*g* at 20 °C for 20 min.

10| Remove and discard the supernatant using a Falcon disposable polystyrene serological pipette.

11| Gently re-suspend the pellet in approximately 30 ml wash buffer.

12| Spin (as described in Step 9) and wash once more with wash buffer to remove protoplasting enzymes.

13| Check the isolated protoplasts under the light microscope. You may wish to keep some unlysed protoplasts for comparative/enrichment analysis. Proceed with the remainder as described below.

Vacuole isolation

14| Add 10.5 ml pre-warmed lysis buffer to the protoplasts using a Falcon disposable polystyrene serological pipette.

15| Re-suspend well, yet gently, by pipetting 5–8 times up and down using a 5-ml Pyrex reusable wide-tip serological pipette.

16| Wait for 2 min.

17| Inspect the protoplast disruption and the vacuole release under the light microscope (**Fig. 1**).

18| Transfer 5 ml of the solution into an ultra-clear centrifugation tube (two tubes per sample) using a 5 ml Pyrex reusable wide-tip serological pipette.

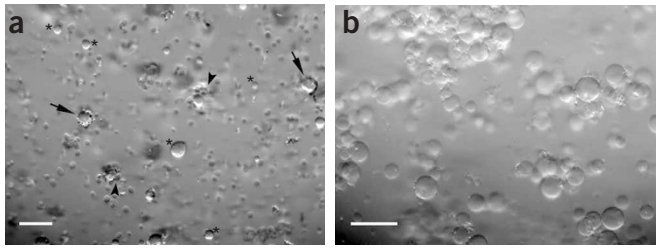

Figure 1 | Micrographs of lysed protoplasts and purified vacuoles. (**a**) Bright-field micrograph of osmotically/thermally lysed protoplast, Step 17. Arrows: lysed protoplasts; arrowheads: released plastids; asterisks: released vacuoles. (**b**) Bright-field micrograph of purified vacuoles, Step 23. Scale bar = 50 μm.

19| Overlay with 3 ml 4% Ficoll solution using a 10 ml Falcon disposable polystyrene serological pipette.
▲ **CRITICAL STEP** The layering of the gradient has to be done very carefully but quickly; try to avoid mixing the consecutive layers.

20| Layer 1 ml ice-cold vacuole buffer on the top of the gradient.
▲ **CRITICAL STEP** The layering of the gradient has to be done very carefully but quickly; try to avoid mixing the consecutive layers.

21| Spin for 50 min at 71,000*g* at 10 °C in an Optima L-90K ultra-centrifuge using slow acceleration and slow deceleration settings.

22| Vacuoles should be visible as a pink layer on the interface between 0 and 4% Ficoll. Remove them carefully using a pipette.
? TROUBLESHOOTING

23| Check the vacuoles for purity under the light microscope (**Fig. 1**).
? TROUBLESHOOTING

● **TIMING**
Vacuole isolation can be performed in approximately 6 h.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reasons	Solution
1	No vacuoles detected at the interface	The amount of tissue was exceeded	Use 2 g of leaves
22	No vacuoles detected at the interface	The Ficoll stock solution was overheated	Avoid excessive heat
23	A few vacuoles detected at the interface	The plant material has aged and is senescing	Use fresh, 35-day-old rosette leaves

ANTICIPATED RESULTS

The typical amount of protein found in the final vacuolar sample is 50 μg. The purity of the vacuole fraction extracted according to this protocol has been studied and discussed extensively[17,19,21]. The presence of different markers of the endomembrane system and other organelles in the vacuolar fraction has been examined using immunoblot analysis. The vacuolar markers AtALEU, γ-TIP and SYP22 were enriched in the vacuolar fractions, but AtVPS45 (TGN), SYP21 (PVC), AtSEC12 (ER) and Hsp93 (chloroplasts) were not present at a detectable level in the vacuolar fractions, indicating that the fractions were not contaminated with these organelles.

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1. Girke, T., Ozkan, M., Carter, D. & Raikhel, N.V. Towards a modeling infrastructure for studying plant cells. *Plant Physiol.* **132**, 410–414 (2003).
2. Millar, A.H., Sweetlove, L.J., Giege, P. & Leaver, C.J. Analysis of the Arabidopsis mitochondrial proteome. *Plant Physiol.* **127**, 1711–1727 (2001).
3. Fitzpatrick, L.M. & Keegstra, K. A method for isolating a high yield of Arabidopsis chloroplasts capable of efficient import of precursor proteins. *Plant J.* **27**, 59–65 (2001).
4. Matile, P. & Wiemken, A. Vacuoles and spherosomes. *Methods Enzymol.* **31**, 572–578 (1974).
5. Cocking, E.C. A method for the isolation of plant protoplasts and vacuoles. *Nature* **187**, 962–963 (1960).
6. Strobel, G.A. & Hess, W.M. Evidence for the presence of the toxin-binding protein on the plasma membrane of sugarcane cells. *Proc. Natl. Acad. Sci. USA* **71**, 1413–1417 (1974).
7. Wagner, G.J. & Siegelman, H.W. Large-scale isolation of intact vacuoles and isolation of chloroplasts from protoplasts of mature plant-tissues. *Science* **190**, 1298–1299 (1975).

8. Boller, T. & Kende, H. Hydrolytic enzymes in the central vacuole of plant-cells. *Plant Physiol.* **63**, 1123–1132 (1979).
9. Sonnewald, U., Brauer, M., von Schaewen, A., Stitt, M. & Willmitzer, L. Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or apoplast: a powerful tool for studying sucrose metabolism and sink/source interactions. *Plant J.* **1**, 95–106 (1991).
10. Gomez, L. & Chrispeels, M.J. Tonoplast and soluble vacuolar proteins are targeted by different mechanisms. *Plant Cell* **5**, 1113–1124 (1993).
11. Leigh, R.A. & Branton, D. Isolation of vacuoles from root storage tissue of *Beta vulgaris* L. *Plant Physiol.* **58**, 656–662 (1976).
12. Wagner, G.J. Content and vacuole/extravacuole distribution of neutral sugars, free amino acids, and anthocyanin in protoplasts. *Plant Physiol.* **64**, 88–93 (1979).
13. Nishimura, M. & Beevers, H. Hydrolases in vacuoles from castor bean endosperm. *Plant Physiol.* **62**, 44–48 (1978).
14. Granstedt, R.C. & Huffaker, R.C. Identification of the leaf vacuole as a major nitrate storage pool. *Plant Physiol.* **70**, 410–413 (1982).
15. Canovas, F.M. *et al.* Plant proteome analysis. *Proteomics* **4**, 285–298 (2004).
16. Rossignol, M. *et al.* Plant proteome analysis: a 2004–2006 update. *Proteomics* **6**, 5529–5548 (2006).
17. Carter, C. *et al.* The vegetative vacuole proteome of *Arabidopsis thaliana* reveals predicted and unexpected proteins. *Plant Cell* **16**, 3285–3303 (2004).
18. Shimaoka, T. *et al.* Isolation of intact vacuoles and proteomic analysis of tonoplast from suspension-cultured cells of *Arabidopsis thaliana*. *Plant Cell Physiol.* **45**, 672–683 (2004).
19. Rojo, E., Zouhar, J., Carter, C., Kovaleva, V. & Raikhel, N.V. A unique mechanism for protein processing and degradation in *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **100**, 7389–7394 (2003).
20. Dubrovsky, J.G. *et al.* Neutral red as a probe for confocal laser scanning microscopy studies of plant roots. *Ann. Bot. (Lond)* **97**, 1127–1138 (2006).
21. Ahmed, S.U. *et al.* The plant vacuolar sorting receptor AtELP is involved in transport of NH(2)-terminal propeptide-containing vacuolar proteins in *Arabidopsis thaliana*. *J. Cell. Biol.* **149**, 1335–1344 (2000).