Chapter 19

Purification of a Vesicle–Vacuole (V) Fraction from *Aspergillus*

Anindya Chanda, Ludmila V. Roze, and John E. Linz

Abstract

Recent studies conducted in our laboratory demonstrate that *Aspergillus parasiticus* synthesizes and stores aflatoxin in transport vesicles and endosomes. Proteomics data suggest that enzymes involved in the synthesis of other secondary metabolites as well as enzymes involved in response to heat, osmotic, and oxidative stress also localize to these subcellular organelles. In order to better understand how cells integrate the regulation and function of secondary metabolite biosynthesis and stress response, it is important to understand the composition and function of the membrane-bound organelles that house this biosynthetic machinery. Isolation of vesicles, endosomes, and vacuoles (V fraction) is, therefore, an essential method to study secondary metabolism in *A. parasiticus* at the cellular level. Here, we describe a "one-step density gradient" method for purification of a highly heterogeneous cell fraction consisting of transport vesicles, endosomes, and vacuoles from protoplasts prepared from *A. parasiticus* cells harvested during aflatoxin synthesis.

Key words: Protoplast, Vesicle, Endosome, Vacuole, One-step density gradient, Aflatoxin, Secondary metabolism, *Aspergillus parasiticus*

1. Introduction

Vesicles and vacuoles are known to contain enzymes associated with secondary metabolism in plants and fungi. Examples include enzymes involved in the biosynthesis of alkaloids (1, 2) and flavonoids (1, 3) in plants and the non-ribosomal peptide cyclosporin (4), the β-lactam antibiotic penicillin (5), and the polyketide aflatoxin (6–8) in filamentous fungi. Our recent studies indicate that these organelles play a functional role in the biosynthesis of secondary metabolites apart from their proposed role in sequestering the enzymes to protect host cells from self-toxicity

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(1). In order to elucidate their function in secondary metabolism, we developed a method to isolate a pure fraction containing transport vesicles, endosomes, and vacuoles.

Protocols to isolate vesicles and vacuoles from fungi (4, 5, 9–14) and plants (15–19) generally involve multistep flotation, density gradients, and/or differential centrifugation procedures. However our studies with *Aspergillus parasiticus*, a fungus that synthesizes the secondary metabolite and carcinogen aflatoxin, suggest that the high degree of size and density heterogeneity of these subcellular organelles in *A. parasiticus* makes it impractical to directly apply previously published procedures.

We describe here a "one-step density gradient" method for purification of a cell fraction that contains heterogeneous transport vesicles, endosomes, and vacuoles from *A. parasiticus* (20). The method includes the following steps: (1) preparation of protoplasts from mycelia; (2) release of vesicles, endosomes, and vacuoles from purified protoplasts; and (3) fractionation of the subcellular organelles using the "one-step density gradient." We successfully used this method to perform proteomic analysis (manuscript in review), to conduct feeding studies (21), and to functionally link these subcellular organelles with aflatoxin synthesis (21).

2. Materials

2.1. Buffers

Protoplast buffer. 1.2 M $MgSO_4$ and 10 mM KH_2PO_4. Adjust solution to pH 5.8 with 1 N NaOH with continuous stirring, filter sterilize, and store at 4°C (see Note 1).

Protoplast solution. Prepare this solution just prior to protoplast isolation. Add 50 mg each of Driselase and Lysing Enzymes (from Sigma) per ml of protoplast buffer. Filter sterilize the solution before use. Determine the volume of the solution used to prepare protoplasts based on the wet weight (explained under methods) of the mycelia; use 1 ml of protoplast solution per 40 mg (wet weight) of mycelia (see Note 2).

Protoplast separation buffer. 0.6 M sorbitol, 100 mM Tris–HCl (pH 7.0; use 1 N NaOH). Autoclave and store at 4°C.

Protoplast storage buffer ("Protobuffer"). 1.2 M sorbitol, 10 mM Tris–HCl (pH 7.5; use 1 N NaOH). Autoclave and store at 4°C.

Protoplast lysis solution. 0.6 M sorbitol, 10 mM Tris–HCl, 0.025% (vol/vol) Triton-X 100 (pH 7.5). Prepare solution just prior to use. Dilute protobuffer 1:1 with 10 mM Tris–HCl (pH 7.5). Then add 0.025% (vov/vol) Triton-X 100. A 500 ml stock protoplast lysis solution can also be prepared and stored at 4°C.

Sucrose cushion. 3 M sucrose, 1.2 M sorbitol, 10 mM Tris–Cl, pH 7.5. Prepare solution in a 50 ml disposable conical centrifuge tube. Add 27 g sucrose, fill the tube to the 50 ml mark with protobuffer, and dissolve using a vortex mixer. The sucrose may take up to 2 h to dissolve. Prepare the solution just as you initiate the procedure and it will be ready when needed. Store solution at 4°C.

2.2. Equipment

1. Refrigerated benchtop clinical centrifuge equipped with a swinging bucket rotor (Sorvall Legend RT, Kendro Laboratory Products, Osterode, Germany).
2. Clinical centrifuge with swinging bucket rotor (Model 59A, Fisher Scientific, Pittsburg, PA).

2.3. Other Accessories

1. Miracloth (Calbiochem, San Diego, CA).
2. Cheese cloth.
3. Nylon mesh (Pore size 30 μm, SpectrumLabs, Rancho Dominguez, CA).
4. 50 ml conical disposable centrifuge tubes (Corning, NY).
5. Transfer pipettes (5 ml capacity).
6. Ice bucket with ice.
7. Funnel.
8. Spatula.
9. Paper towels.
10. 0.45 μm filters (Millex, Millipore, Billerica, MA) and syringes 20 ml, 60 ml (Becton Dickinson, Franklin Lakes, NJ) for filter sterilization.
11. Forceps.
12. Balance to measure wet weight of mycelia.
13. Shaking water bath incubator.

3. Methods

Carry out all procedures at room temperature (RT) unless otherwise specified. The flowchart of the method is described in Fig. 1.

3.1. Protoplast Preparation

1. Inoculate 100 ml YES growth medium in a 250 ml Erlenmeyer flask at 10^5 conidiospores per ml and incubate with shaking at 150 rpm, in the dark, with five glass beads, at 30°C, for 36 h.
2. Harvest mycelia by filtration through a miracloth-lined funnel. Do not squeeze mycelia during filtration (see Fig. 2a).

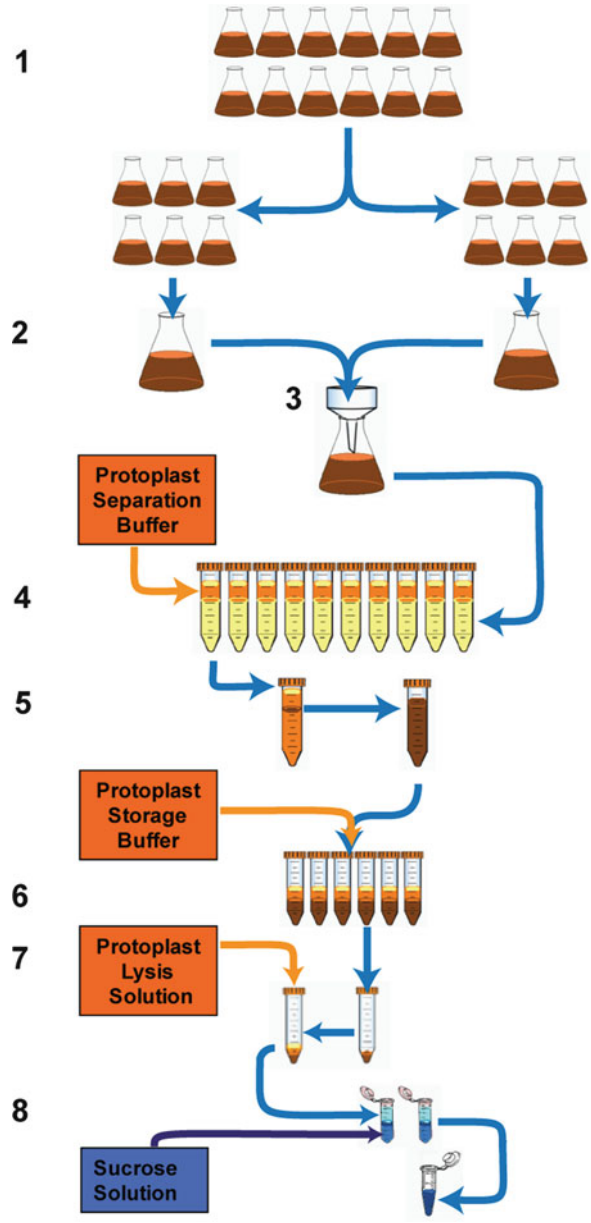

Fig. 1. Flowchart illustrating the entire procedure of the isolation of a pure V-fraction for our routine experiments. (*1*) Grow *Aspergillus parasiticus* culture for 36 h, 30°C. (*2*) Harvest mycelia into two flasks and carry out protoplasting for 5–7 h, 30°C. (*3*) Filter protoplasts into a single flask, RT. (*4*) Aliquot protoplast suspension (25 ml) into 50 ml conical centrifuge tubes and overlay 15 ml of Protoplast Separation Buffer onto each aliquot. Centrifuge for 15 min at 1,500 × *g*, RT. (*5*) Just above the interface, collect 3 ml of protoplast suspension (visible as a "fuzzy band") from each tube and combine collected protoplasts in one tube. (*6*) Aliquot protoplast suspension (5 ml) into 50 ml tubes and add 5 ml of Protoplast Storage Buffer into each tube, mixing gently; centrifuge for 10 min at 1,000 × g, RT. During centrifugation prepare sucrose cushion: Add 0.9 ml of High-Density Sucrose into each of the two 2 ml microcentrifuge tubes; keep at RT. (*7*) Remove supernatant saving approximately 0.1 ml and collect the pellets into a 50 ml tube on ice. Adjust volume to 1 ml with Protoplast Storage Buffer. Add 1 ml of Protoplast Lysis Solution, mix thoroughly by pipetting up and down, and perform lysis for 10 min on ice. (*8*) Carefully layer 1 ml of lysed protoplasts on 0.9 ml of sucrose cushion (two tubes in total) and centrifuge at 3,000 × *g* for 30 min, RT. Take 100–150 μl of a thin layer of vesicles/vacuoles located just above the visible band.

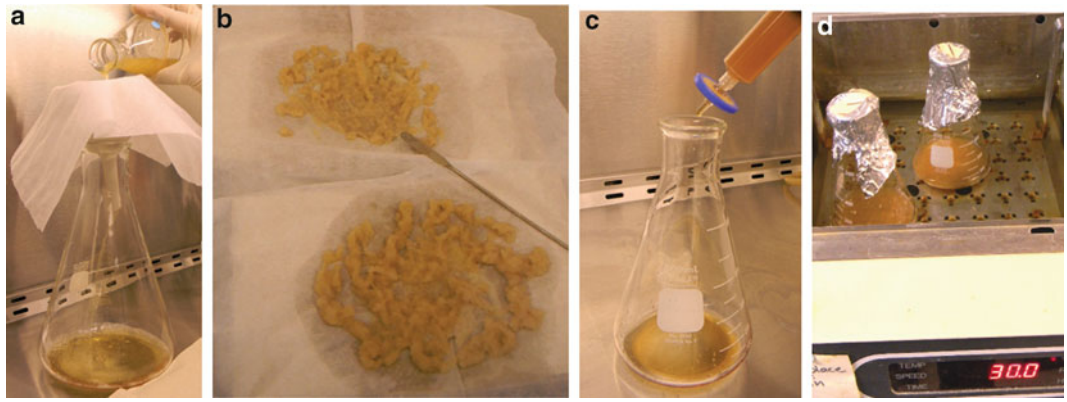

Fig. 2. Protoplasting. (**a**) Mycelia are filtered from the growth medium using a miracloth placed on a funnel. (**b**) The remaining media from the mycelia are soaked out by spreading out the mycelia on the miracloth that is placed on a bed of paper towels. (**c**) While the mycelia dry on the paper-towel bed, the enzyme solution is prepared and filter-sterilized into a sterile conical flask. (**d**) The dried mycelia are then transferred into the flasks containing the enzyme solution and placed in the incubator that was preset to 30°C.

3. Place the miracloth carrying mycelia on a bed of paper towels approximately 6 cm in thickness and spread the mycelia evenly with spatula to remove excess medium from the mycelia (see Fig. 2b).
4. As the mycelia are drying, prepare the protoplast solution (see Note 2) and set the shaking water bath incubator to 30°C.
5. Using the forceps and spatula, tear the mycelia into small pieces. Transfer 4 g (wet weight) into 100 ml protoplast solution in a 250 ml conical flask.
6. Incubate conical flasks containing the mycelia and protoplast solution in the shaking water bath incubator (30°C) for 7 h with shaking at 100 rpm (see Note 3).

3.2. Protoplast Isolation

7. After incubation, filter the protoplast solution using a layer of cheese cloth and a second layer of nylon mesh to remove larger pieces of mycelia from the solution (see Fig. 3a).
8. Transfer 25 ml protoplast solution into a 50 ml disposable conical centrifuge tube and carefully layer protoplast separation buffer on top of this solution up to 40 ml using a transfer pipette (see Fig. 3b). Be careful not to mix protoplast solution and protoplast separation buffer.
9. Centrifuge the tubes at 1,500×*g* for 15 min at 4°C using a refrigerated clinical centrifuge. Protoplasts will form a band just above the interface of the two layers (see Fig. 3c). Collect the protoplasts with a transfer pipette, being careful not to disturb the layers. The protoplasts can normally be collected in a total volume of 5 ml.

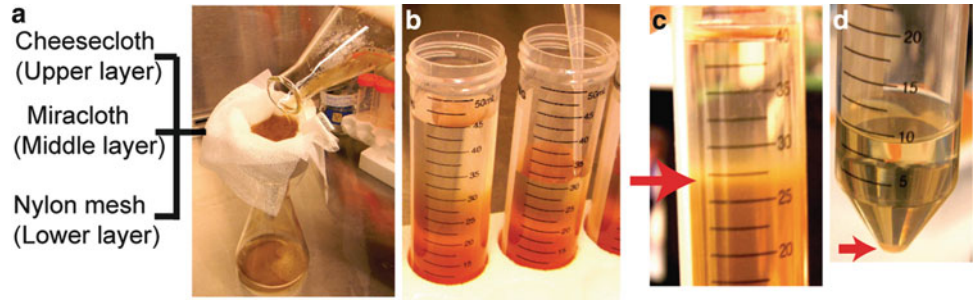

Fig. 3. Protoplast isolation. (**a**) Protoplasting broth is filtered through a tri-layer consisting of cheese-cloth (*upper layer*), miracloth (*middle layer*), and nylon mesh (*lower layer*). (**b**) The filtrate is then aliquoted in 25 ml volumes into fresh 50 ml conical tubes and protoplast separation buffer is layered on top of the filtrate up to 40 ml mark. (**c**) Protoplasts accumulate at the interface of layers and form a "fuzzy" band (denoted by a *red arrow*) after centrifugation of the tubes (step 8). (**d**) The band is collected and recentrifuged with protoplast storing buffer (steps 9–11). After centrifugation (step 11), the protoplasts collect as a white precipitate (denoted by a *red arrow*) at the bottom of the conical tube.

10. Transfer protoplasts collected to a fresh 50 ml conical disposable centrifuge tube.
11. Dilute protoplasts with protoplast storage buffer to a total volume of 10 ml.
12. Centrifuge this solution at 1,000 × *g* for 10 min at RT in a clinical centrifuge. Protoplasts will pellet at the bottom of the tube as a white precipitate (see Fig. 3d).
13. Carefully remove the supernatant from the protoplast pellet with a transfer pipette (see Note 4).
14. Gently resuspend all protoplasts in 1 ml total volume of protoplast storage buffer by carefully pipetting up and down with a transfer pipette. Vigorous mixing will lyse the protoplasts.

3.3. Protoplast Lysis and the Release of Vesicles, Endosomes, and Vacuoles

15. Transfer 1 ml of protoplasts obtained in step 14 to a 50 ml disposable conical centrifuge tube placed on ice. Add 1 ml of protoplast lysis solution.
16. Protoplast lysis will be observed within 1 min after addition of lysis buffer. At 2–3-min intervals, pipette the solution up and down vigorously to enhance release of vesicles, endosomes, and vacuoles. Allow lysis to continue for 10 min.
17. As lysis proceeds, pipette 1 ml of sucrose cushion into two 2 ml microcentrifuge tubes.
18. After completion of lysis, carefully layer 1 ml of lysis solution onto the sucrose cushion in each microcentrifuge tube.

3.4. Isolation of the Vesicle–Vacuole (V) Fraction

19. Centrifuge the microcentrifuge tubes in a benchtop clinical centrifuge equipped with a swinging bucket rotor at RT for 30 min at 3,000 × *g*.

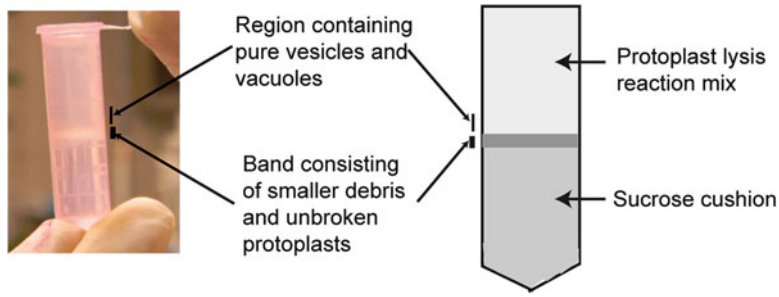

Fig. 4. Isolation of pure vesicle–vacuole fraction (V-fraction). The figure on the *left* shows an Eppendorf tube after centrifugation as mentioned in step 18. The schematic on the *right* shows the region from where the vesicle–vacuole fraction should be isolated.

20. After centrifugation, a visible band that contains cell debris and unbroken protoplasts will form at the interface between the layers (see Fig. 4). Just above this band is the pure vesicle–vacuole (V) fraction. Using a 200 µl pipette, carefully remove approximately 200 µl containing (V) fraction while avoiding the visible band at the interface. This procedure results in (V) fraction with >95% purity. If additional purity is required, (V) fraction combined from two tubes (400 µl) can be re-banded on a fresh sucrose cushion (see Note 5). To perform biochemical assays, (V) fraction can be stored for up to 60 min on ice. To perform proteomic analysis, (V) fraction can be stored for up to 30 days at −80°C.

4. Notes

1. Soon after addition of NaOH dropwise to the solution, $Mg(OH)_2$ flakes begin to appear. Continuous stirring helps to dissolve the flakes resulting in an increase in pH. Therefore, add NaOH slowly and make sure that flakes dissolve completely before adding additional NaOH.
2. For routine (V) fraction isolation, we use 1 g of Driselase and 1 g of Lysing Enzyme to digest mycelia obtained from six flasks incubated for 36 h after inoculation. Under these conditions we routinely obtain approximately 4 g (wet weight) of mycelia per flask.
3. After 3 h, monitor appearance of protoplasts every hour by placing a drop of the protoplast solution on a slide with a transfer pipette and observing the sample under a light microscope.

4. Always use a transfer pipette to gently remove the supernatant from the top of the tube. Avoid pouring off the supernatant in order to prevent loss of protoplasts.
5. To reband vesicles, endosomes, and vacuoles in (V) fraction, add 0.6 M sorbitol, 10 mM Tris–Cl, pH 7.5 (protoplast lysis solution minus Triton X-100) to a final volume of 1 ml. Layer this solution on a fresh 1 ml sucrose cushion in a 2 ml microcentrifuge tube and repeat steps 19 and 20.

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