Density Gradient Centrifugation for Enrichment and Identification of GFP-tagged Chitosomal Microvesicles of Filamentous Fungi

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[Abstract] Density gradient centrifugation has been utilized to characterize the subcellular distribution of physiologically relevant enzymes in yeasts and filamentous fungi (Leal-Morales *et al.*, 1988; Martínez *et al.*, 1989; Kamada *et al.*, 1991). This approach is now potentiated by protein tagging and live imaging techniques, which make possible to relate a single protein with, for example, a discrete population of intracellular vesicles and their *in vivo* dynamics (Verdín *et al.*, 2009; Fajardo-Somera *et al.*, 2013; Sánchez-León *et al.*, 2015). Here, we describe the density gradient centrifugation and fractionation analysis of cell-free homogenates of a *Neurospora crassa* (*N. crassa*) strain that expresses CHS-6 chitin synthase fused to the green fluorescent protein (Riquelme *et al.*, 2007).

Materials and Reagents

1. Tygon R-3603 tubing, I.D. 1/16 inch (Saint-Gobain, catalog number: AAC00002)
2. Grade 1 Qualitative Filter Papers (GE Healthcare, catalog number: 1001-090)
3. *Neurospora crassa* conidia
4. Sucrose (Sigma-Aldrich, catalog number: S0389)
5. Sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$) (Thermo Fisher Scientific, Fisher Scientific, catalog number: S369-500)
6. Sodium phosphate dibasic anhydrous (Na_2HPO_4) (Sigma-Aldrich, catalog number: S-7907)
7. Protease Inhibitor Cocktail, Complete ULTRA tablets EDTA-free (Roche Diagnostics, catalog number: 04693132001)
   Note: Currently, it is "Sigma-Aldrich, catalog number: 04693132001".
8. Glass beads (500 µm) (Bio Spec Product, catalog number: 11079105)
9. 10% (w/v) sucrose in 33 mM (final) phosphate buffer (pH 8.2) (steam sterilize and store at 4 °C until use)

10. 65% (w/v) sucrose in 33 mM (final) phosphate buffer (pH 8.2) (steam sterilize and store at 4 °C until use)
11. D-Biotin (Faga Labs, catalog number: CAS-58-85-5)
12. Vogel's complete medium (see Recipes)
13. 50x salt solution (see Recipes)
14. Trace element solution (see Recipes)
15. 100 mM phosphate buffer (pH 8.2) (see Recipes)
16. Laemmli buffer (see Recipes)

Equipment

1. Braun MSK 50 ml shaking bottles (LABEQUIP LTD, catalog number: 8541302)
2. VWR® Standard Hot Plate Stirrers (VWR International, catalog number: 12365-382)
3. Quick-seal centrifuge tube (Beckman Coulter, catalog number: 344326)
4. Shaker incubator (Lab-LineX, model: Orbit Environ Shaker)
5. Vacuum filtration system
6. Braun MSK cell homogenizer (LABEQUIP LTD, catalog number: 953030)
7. Bright field microscope (Olympus, model: Vanox-S)
8. Beckman L8-70M Ultracentrifuge (pre-cooled at 4 °C) (Beckman Coulter)
9. Type 70Ti rotor (pre-cooled at 4 °C) (Beckman Coulter)
10. GM-40 Linear Gradient maker (C. B. S. Scientific)
11. Peristaltic (Multi-staltic) pump (Buchler Lab, catalog number: N/A)
12. Tube sealer (Beckman Coulter, catalog number: 342420)
13. Density gradient fractionator (ISCO, 185)
    Note: This model is not available anymore. Brandel BR-186 Gradient Fractionator with Syringe Pump can be used instead.
14. CIGNET™ Fraction collector (ISCO)
    Note: This item is also discontinued, but Bio-Rad 2110 Fraction Collector can be used instead.
15. 6505 W UV-Vis Spectrophotometer (Bibby-scientific, Jenway)
16. Refractometer (ZEISS, catalog number: 12230)

Procedure

A. Homogenate (lysate) preparation

1. Inoculate 400 ml Vogel's Complete Medium (Vogel, 1956) with *Neurospora crassa* conidia (1 x 10^6 conidia/ml) in a 1 L flask. Incubate for 14-20 h at 30 °C, 200 rpm.
2. Harvest mycelium by vacuum filtration into Whatman filter paper number 1. Wash twice with 200 ml cold sterile distilled H_2O to eliminate remnant culture media and then

twice with 50 ml cold 33 mM phosphate buffer (pH 8.2) to equilibrate mycelium for the next step.

3. Mix 10 g wet mycelium and 10 ml 5% (w/v) sucrose in 33 mM phosphate buffer (pH 8.2), supplemented with a protease inhibitor cocktail (1 tablet/50 ml) into a Braun bottle and add 20 g of glass beads (500 µm; 2 g/g mycelium). Keep all solutions, tubes and bottles at 4 °C to prevent protein degradation.
4. Homogenize in a Braun homogenizer 4 x 30 sec in presence of a CO_2 stream directed to the Braun bottle so that a temperature increase during homogenization is prevented. The CO_2 line must be firmly attached to the metallic arm of the Braun homogenizer so that the CO_2 stream can be directed to the bottle. After homogenization, verify the broken cells by observing the sample in a bright field microscope.
5. Transfer homogenate into a 50 ml tube (on ice) and centrifuge at 1,000 *x g* (R_{av}), 10 min, 4 °C.
6. Save the supernatant at 4 °C using a glass pipette to avoid the lipid layer formed on top. This will be the homogenate or lysate, which would contain mitochondria, microvesicles, ribosomes, and soluble proteins. Discard pellet containing whole cells, cell walls, and nuclei.

B. Density gradient centrifugation

1. Construct a 10-65% sucrose linear gradient (10% and 65% sucrose stock solutions must be prepared in final 33 mM phosphate buffer, pH 8.2) in a quick-seal centrifuge tube using a linear gradient maker and a peristaltic pump set up to obtain a flow rate of 3.5 ml/min (Figure 1).
2. Gently layer the *N. crassa* homogenate (supernatant saved in step A6) on top of the 10-65% sucrose gradient by using a Pasteur pipette via single drops close to the surface of the sucrose gradient. Grease the centrifuge inlet to seal and cap the centrifuge tube. Centrifuge at 184,000 *x g* (R_{av}), for 4 h and 4 °C in a pre-cooled Beckman rotor 70Ti.
3. Fractionate the gradient from the top with an ISCO fractionation system using 70% (w/v) sucrose as chase at a flow rate of 6 ml/min. Make sure no air bubbles form in the tube system that conducts the chase sucrose to the gradient or else they will break it. Collect 2 ml fractions (3 fractions/min) into ice-cold tubes and keep at 4 °C.
4. Characterize each fraction by measuring absorbance at 280 nm, refraction index (to infer the density) and, ideally, an enzyme activity associated to intracellular particles or vesicles (Figure 2A). Refractometers output the refractive index, which can be converted to density (g/ml) using this table: http://homepages.gac.edu/~cellab/chpts/chpt3/table3-2.html.
5. Mix 50 to 100 µl of each fraction with Laemmli buffer (2x), boil for 5 min and store at -20 °C until analysis by Western blot (Figure 2B).

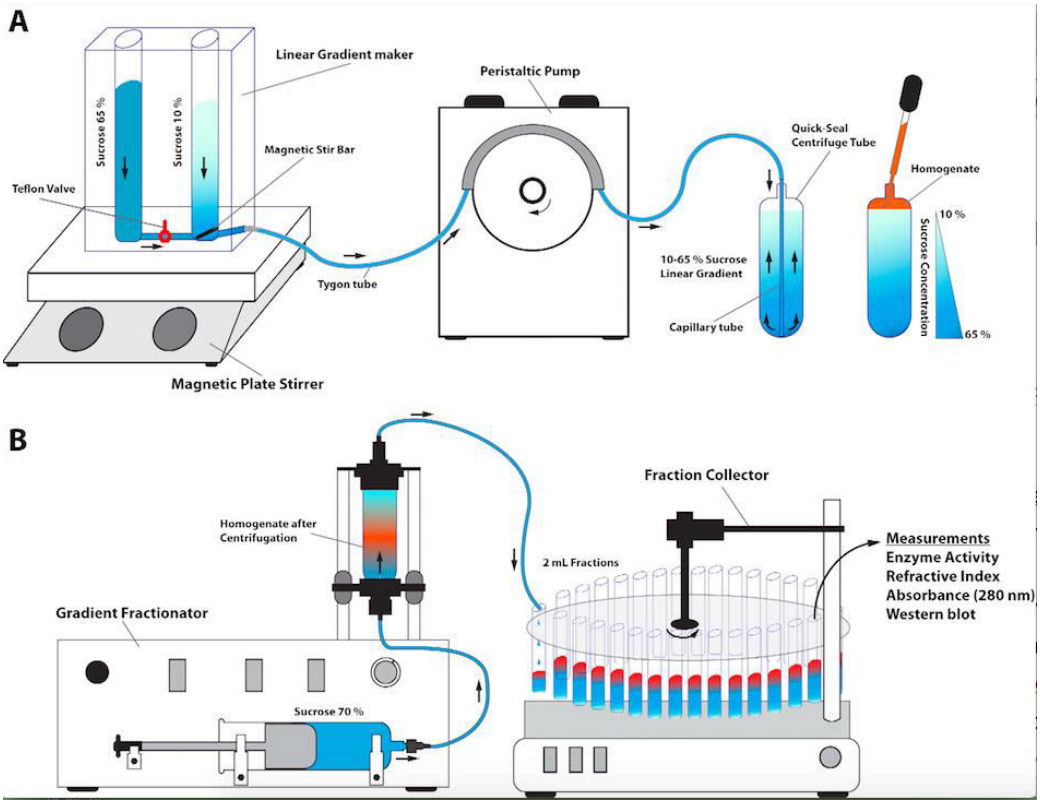

Figure 1. Preparation and fractionation of sucrose density gradients. A. To prepare the density gradients, sucrose stock solutions are independently poured into each column of the gradient maker (keep locked the Teflon valve that connects one column to the other). A magnetic stir bar is placed in the 10% sucrose solution column, from which a silicon tube (purged of any bubble) exits towards the peristaltic pump and, via a capillary tube attached to the tip of the silicon one, is introduced into the quick-seal centrifuge tube down to the bottom. After turning the peristaltic pump on, the Teflon valve is opened to allow the sucrose solutions mixing. Then, increasing concentration sucrose flows to the quick-seal tube. Lower concentration sucrose arrives first to the tube, which is pushed up by higher concentration sucrose (the capillary tip is always touching the quick-seal tube bottom). Immediately before the transfer of total volume of the sucrose gradient to the quick-seal tube, turn off the peristaltic pump and take out the capillary tube very gently to avoid breaking the gradient. The remaining volume of the quick-seal tube is filled up with the mycelial lysate. B. After centrifugation and isopycnic separation of mycelial homogenates, the gradient is carefully fractionated using a 70% sucrose solution as chase. This solution is injected into the quick-seal tube from the bottom so that the gradient is integrally pushed up toward the exit tubing and the fraction collector. Before fractionation, it is critical to purge any bubble from the syringe that contains the chase solution and the tubing that conducts it to the bottom of the quick-seal tube; otherwise, the gradient could be broken. Collected fractions are subsequently characterized (enzyme activity, refractive index,

absorbance, *etc.*) and stored at 4 °C.

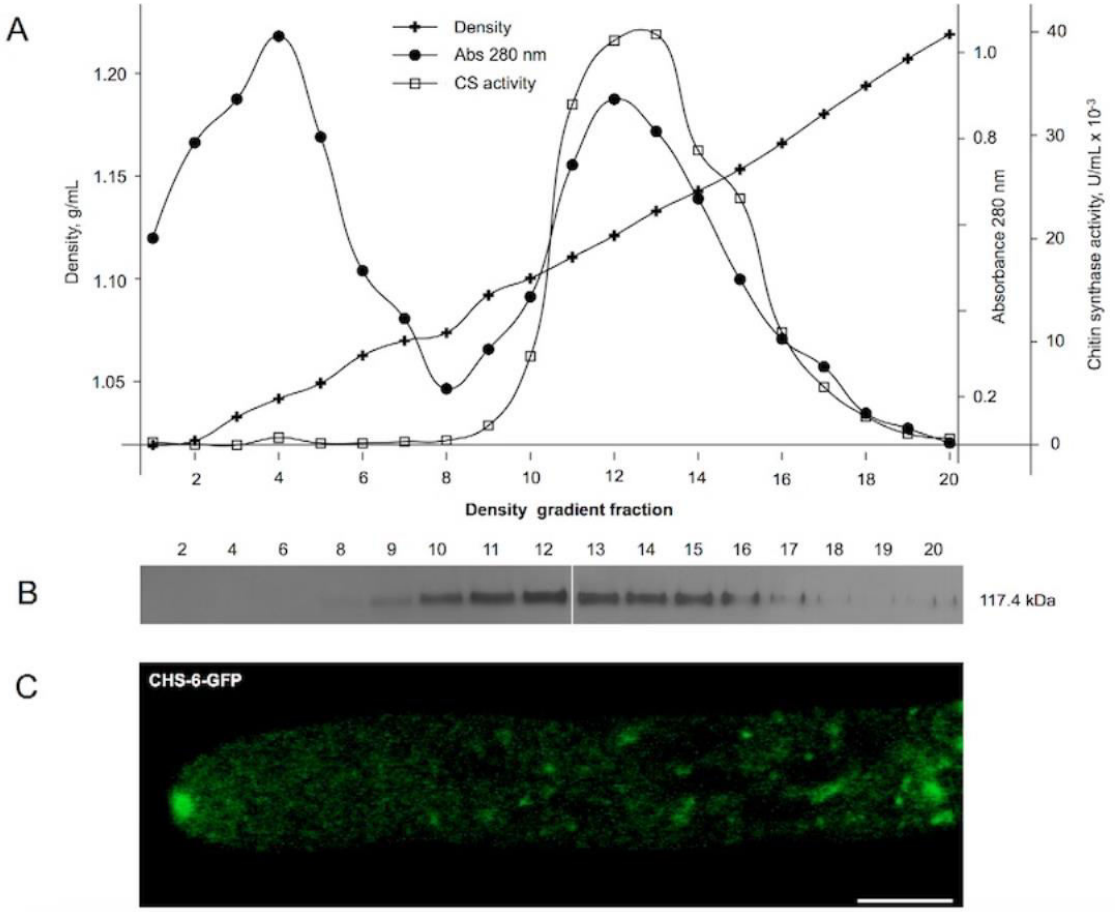

Figure 2. Density gradient centrifugation of homogenates of *N. crassa* expressing CHS-6 chitin synthase co-translationally fused to GFP (Riquelme *et al.*, 2007). A. Density (inferred from the refractive index), total protein (Abs 280 nm) and chitin synthase activity were estimated for each fraction of the density gradient collected. Chitin synthase activity sedimented at 1.13 g/ml, the buoyant density of chitosomes. B. Western blot analysis demonstrated the presence of CHS-6-GFP around fraction 13 (1.13 g/ml). C. Live imaging of a *N. crassa* hyphae by confocal microscopy showed that CHS-6-GFP localizes at the tip mainly at the core of the Spitzenkörper (an apical body that directs the growth of the hypha), where microvesicles (chitosomes) accumulate, as confirmed by transmission electron microscopy (Riquelme *et al.*, 2002). Scale bar, 5 μm

Recipes

1. Vogel's complete medium
   Dilute 50x salt solution 50 fold with distilled water
   Add:
   Sucrose 15 g/L

0.5% yeast extract

0.5% N-Z case (casamino acids)

Steam sterilize and store at room temperature

2. 50x salt solution

$Na_3C_6H_5O_7 \cdot 5.5\ H_2O$	Sodium citrate	420 mM
KH_2PO_4	Potassium phosphate monobasic anhydrous	1.8 M
NH_4NO_3	Ammonium nitrate anhydrous	1.2 M
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate	40.5 mM
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate	34 mM
Trace element solution*		500 µl
Biotin solution (0.1 mg/ml, in ethanol)		250 µl

Bring up to 100 ml with ddH_2O

Add 2 ml of chloroform as preservative

Alternatively, steam sterilize and store at 4 °C

3. Trace element solution

Note: Concentrations in brackets are final concentrations.

Citric Acid $\cdot H_2O$	5.0 g (238 mM)
$ZnSO_4 \cdot 7H_2O$	5.0 g (174 mM)
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	1.0 g (25.5 mM)
$CuSO_4 \cdot H_2O$	0.25 g (14 mM)
$MnSO_4 \cdot H_2O$	0.05 g (2.3 mM)
H_3BO_3 anhydrous	0.05 g (8 mM)
$Na_2MoO_4 \cdot 2H_2O$	0.05 g (2.3 mM)

Bring up to 100 ml with ddH_2O

Add 1 ml of chloroform for storage at room temperature

4. 100 mM phosphate buffer (pH 8.2)

$NaH_2PO_4 \cdot H_2O$	1.23 g
Na_2HPO_4	12.92 g
H_2O	up to 1 L

5. Laemmli buffer

0.5 M Tris (pH 6.8)	1.25 ml
10% SDS	2.0 ml
Glycerol	2.5 ml
ddH_2O	9.5 ml
Bromophenol blue	1 mg

Stored at -20 °C
Add 25 µl β-mercaptoethanol to 475 µl Laemmli buffer before use
Dilute samples 1:2 in Laemmli buffer and boil for 4 min

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