

Metabolic Labeling of Yeast Sphingolipids with Radioactive D-erythro-[4, 5-³H] dihydrosphingosine

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[Abstract] Yeast sphingolipids can be metabolically labeled with exogenously added radioactive precursors. Here we describe a method to label ceramides and complex sphingolipids of *Saccharomyces cerevisiae* with a radioactive ceramide precursor, D-erythro-[4, 5-³H] dihydrosphingosine. This protocol is used to study the biosynthesis, transport and metabolism of sphingolipids in yeast.

Materials and Reagents

1. Yeast strain (*Saccharomyces cerevisiae*)
2. D-erythro-[4,5-³H]-dihydrosphingosine ([³H]DHS) (60 Ci/mmol) (American Radiolabeled Chemicals, catalog number: ART-46)
3. Sodium fluoride (NaF) (Sigma-Aldrich, catalog number: S1504)
4. Sodium azide (NaN₃) (Sigma-Aldrich, catalog number: S2002)
5. Glass beads (0.5 mm) (Yasui Kikai, catalog number: YGB05)
6. Chloroform (Sigma-Aldrich, catalog number: 05-3400)
7. Methanol (Sigma-Aldrich, catalog number: 19-2410)
8. 1-Butanol (Sigma-Aldrich, catalog number: 03-4560)
9. Ammonium hydroxide (Sigma-Aldrich, catalog number: 221228)
10. Acetic acid (Sigma-Aldrich, catalog number: 01-0280)
11. Thin layer chromatography silica gel 60 (TLC plate) (aluminium sheet 20 x 20 cm) (Merck KGaA, catalog number: 105553)
12. N₂ gas
13. Distilled water (H₂O)
14. Dextrose (D-glucose) (Sigma-Aldrich, catalog number: 07-0680)
15. Yeast extract (Oriental Yeast, catalog number: OYC42008000)
16. Polypeptone (Wako Chemicals USA, catalog number: 513-76611)
17. Yeast nitrogen base without amino acids and ammonium sulfate (BD Difco™, catalog

- number: 233520)
18. Adenine (Sigma-Aldrich, catalog number: A9126)
 19. Leucine (Nacalai Tesque, catalog number: 20327-62)
 20. Histidine (Nacalai Tesque, catalog number: 18116-92)
 21. Lysine (Sigma-Aldrich, catalog number: L5626)
 22. Uracil (Nacalai Tesque, catalog number: 35824-82)
 23. Ammonium sulfate (Sigma-Aldrich, catalog number: A2939)
 24. Tryptophan (Nacalai tesque, catalog number: 35607-32)
 25. Yeast extract-peptone dextrose (YPD) liquid medium (see Recipes)
 26. Synthetic minimal dextrose (SD) liquid medium (see Recipes)
 27. Water-saturated 1-butanol (see Recipes)

Equipment

1. Tritium-sensitive imaging plate (Fujifilm, catalog number: BAS-TR2040)
2. Microcentrifuge tube
3. Shaking incubator (TAITEC, model: BR-43FL · MR)
4. Swinging bucket centrifuge (TOMY, model: LC-201)
5. Microcentrifuge (TOMY, model: MX-301)
6. Water bath shaker (TAITEC, model: PERSONAL-11)
7. Bath-type ultrasonic cleaner (AS ONE, model: US-2R)
8. Pressure gas blowing concentrator (EYELA, model: MGS-2200)
9. TLC developing tank
10. Hair dryer
11. FLA-7000 imaging system (GE Healthcare Life Sciences, model: Typhoon FLA 7000)

Software

1. FLA-7000 image software (ImageQuant TL analysis)

Procedure

- A. Cell culture and metabolic labeling
 1. Inoculate yeast cells in YPD liquid medium at 25 °C with gyratory shaking at 175-200 rpm overnight.
 2. Dilute the culture with SD liquid medium to an OD₆₀₀ of 0.01-0.02 and culture with gyratory shaking at 175-200 rpm overnight.

3. When the OD₆₀₀ of the culture is 0.2-0.6, transfer the culture to a 50 ml conical tube.
4. Spin down yeast cells by a swinging bucket centrifuge at 2,000 x g for 5 min at room temperature (RT) and remove supernatant.
5. Resuspend the pellet in 20 ml SD liquid medium, spin down at 2,000 x g for 5 min at RT and remove supernatant. Repeat this step at least three times.
6. Resuspend cells in SD liquid medium to get an OD₆₀₀ of 20 and transfer 0.5 ml of cell suspension to a new 50 ml conical tube.
7. Incubate at 25 °C for 20 min with reciprocal shaking.
8. Add 4 µCi of [³H] DHS to the cell culture and incubate at 25 °C for 1-4 h with reciprocal shaking. If necessary, lipid synthesis inhibitors are added before the labeling.

B. Lipid extraction and alkaline hydrolysis

1. To stop metabolic labeling, chill the 50 ml conical tube on ice and add 250 mM NaF and 250 mM NaN₃ to a final concentration of 10 mM.
2. Transfer the labeled culture to a 1.5 ml microcentrifuge tube (tube #1).
3. Collect yeast cells by a microcentrifuge at 20,000 x g for 5 min at 4 °C, remove supernatant and resuspend yeast cells in 1 ml cold water. Repeat this step at least three times.
4. Adjust volume of cell suspension to 66 µl with cold water and vortex well.
5. Add 0.3 g of glass beads and vortex well at low temperature (vortex for 30 sec and chill on ice for 1-2 min, repeat at least three times).
6. Add 440 µl of chloroform-methanol (CM; 1/1, v/v), vortex well and centrifuge at 20,000 x g for 5 min at RT.
7. Transfer the supernatant to a new 1.5 ml microcentrifuge tube (tube #2).
8. In order to extract the radiolabeled lipids remaining in tube #1, add 200 µl of chloroform-methanol-water (CMW; 10/10/3, v/v/v) in tube #1, sonicate for approximately 5-10 min in bath-type ultrasonic cleaner until the pellet is completely suspended, centrifuge at 20,000 x g for 5 min at RT and transfer the supernatant to tube #2.
9. Dry the combined supernatants in tube #2 completely with N₂ gas using pressure gas blowing concentrator.
10. Add 80 µl of CMW, vortex well and centrifuge at 20,000 x g for 1 min at RT.
11. To deacylate glycerophospholipids by mild alkaline hydrolysis, add 16 µl of 0.6 N NaOH in methanol, vortex well and incubate at 30 °C for 3 h.
12. Centrifuge at 20,000 x g for 1 min at RT.
13. To neutralize, add 16 µl of 0.6 N acetic acid in methanol, vortex well and centrifuge at 20,000 x g for 1 min at RT.
14. Dry the reaction mixture completely with N₂ gas using pressure gas blowing concentrator.

15. To desalt, add 100 μ l of water to tube #2, vortex well and spin down at 20,000 \times g for 1 min at RT.
16. Further add 200 μ l of water-saturated 1-butanol, vortex well, centrifuge 20,000 \times g for 5 min at RT and transfer the butanol (upper) phase containing sphingolipids to a new 1.5 ml microcentrifuge tube (tube #3).
17. In order to collect the radiolabeled sphingolipids remaining in tube #2, add 200 μ l of water-saturated 1-butanol to tube #2, vortex well, centrifuge 20,000 \times g for 5 min at RT and transfer the butanol phase to tube #3. Repeat this step one more time.
18. Add 100 μ l of water to the combined butanol phase in tube #3, vortex well, centrifuge 20,000 \times g for 5 min at room temperature and transfer the butanol phase to a new 1.5 ml microcentrifuge tube (tube #4).
19. Dry the butanol phase in tube #4 completely with N_2 gas using pressure gas blowing concentrator.

C. Lipid separation and analysis

1. Add 25 μ l of CMW to tube #4, vortex well and centrifuge at 20,000 \times g for 1 min at RT.
2. Then load total sample on TLC plate.
3. Place the plate in a glass TLC developing tank and develop with chloroform-methanol-4.2 N ammonium hydroxide (9/7/2, v/v/v) solvent mixture.
4. When wetting front reaches within 1 cm of the top of TLC plate, remove the plate from the tank and dry it at RT.
5. When the plate is completely dried with a hair dryer, set it in exposure cassette and expose it to a tritium-sensitive imaging plate for few hours-few days.
6. Capture image and quantify signals with FLA-7000 image analyzer and software (Figure 1A).

D. Ceramide extraction and analysis

1. Add a few drops of water on the area containing ceramides of TLC plate, which is in 2 or 3 cm inside from the top edge of the plate.
2. Collect the silica of the area by scraping with a spatula and transfer to a 1.5 ml microcentrifuge tube (tube #5).
3. Add 400 μ l of CM, sonicate for approximately 5 min in bath-type ultrasonic cleaner until the silica is completely suspended, centrifuge at 20,000 \times g for 5 min at RT and transfer the supernatant to a new 1.5 ml microcentrifuge tube (tube #6).
4. Add 200 μ l of CM to tube #5, vortex well, centrifuge at 20,000 \times g for 5 min at RT and transfer the supernatant to tube #6.
5. Dry the combined supernatants in tube #6 completely with N_2 gas using pressure gas

blowing concentrator.

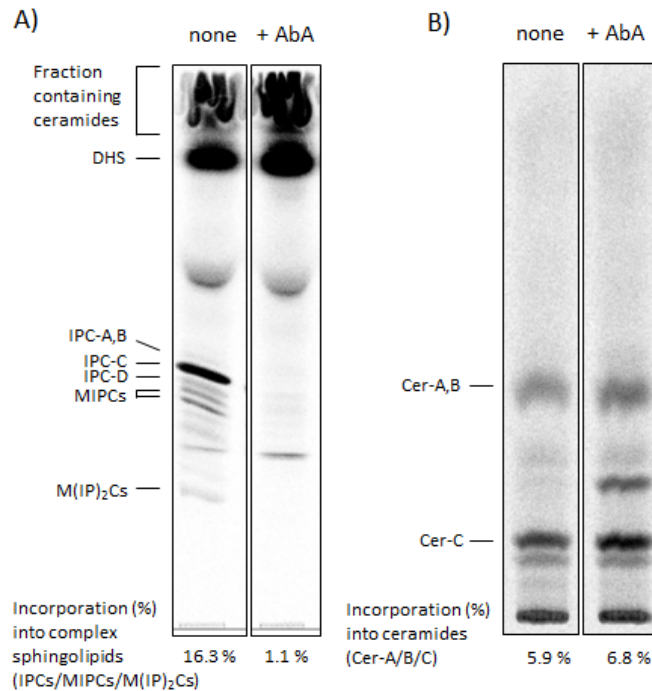


Figure 1. Captured images showing the distribution of complex sphingolipids (A) and ceramides (B) on TLC plates and quantified data. Wild-type cells were labeled with [³H]DHS at 25 °C for 3 h in the absence or presence of 2 µg/ml aureobasidin A (AbA), a specific inositol phosphorylceramide (IPC) synthase inhibitor (Funato and Riezman, 2001; Kajiwara *et al.*, 2012). The labeled lipids were extracted, subjected to mild alkaline hydrolysis and analyzed by TLC with chloroform-methanol-4.2 N ammonium hydroxide (9/7/2, v/v/v) solvent mixture (A). Fractions containing ceramides in (A) (Funato and Riezman, 2001) were collected by scraping, and the radiolabeled ceramides were extracted from the silica and analyzed by TLC with chloroform-methanol-acetic acid (190/9/1, v/v/v) solvent mixture (B). Incorporation of [³H]DHS into complex sphingolipids (IPC-A,-B,-C,-D, MIPCs and M(IP)₂Cs) and into ceramides (Cer-A, -B and -C) was quantified and determined as percentage of the total radioactivity in (A). IPC-A,-B,-C,-D and Cer-A,-B,-C are different IPC subclasses and ceramide species, respectively (Haak *et al.*, 1997). The complex sphingolipids and ceramides can be identified by using mutants defective in the biosynthesis of specific sphingolipid species (Haak *et al.*, 1997), by chemical treatment of isolated radiolabeled lipids (Puoti *et al.*, 1991; Funato and Riezman, 2001) or by using the lipid standards that are isolated from [³H] inositol labeled sphingolipids (Puoti *et al.*, 1991; Haak *et al.*, 1997). MIPC, mannosyl inositolphosphorylceramide; M(IP)₂C, mannosyl di(inositolphosphoryl)ceramide.

6. Add 100 μ l of water to tube #6, vortex well and spin down at 20,000 \times g for 1 min at RT.
7. Further add 200 μ l of water-saturated 1-butanol, vortex well, centrifuge 20,000 \times g for 5 min at RT and transfer the butanol phase containing ceramides to a new 1.5 ml microcentrifuge tube (tube #7).
8. In order to collect the radiolabeled ceramides remaining in tube #6, add 200 μ l of water-saturated 1-butanol to tube #6, vortex well, centrifuge 20,000 \times g for 5 min at RT and transfer the butanol phase to tube #7. Repeat this step one more time.
9. Dry the butanol phase in tube #7 completely with N₂ gas using pressure gas blowing concentrator.
10. Add 25 μ l of CMW to tube #7, vortex well and centrifuge at 20,000 \times g for 1 min at RT.
11. Then load total sample on TLC plate.
12. Place the plate in a glass TLC developing tank and develop with chloroform-methanol-acetic acid (190/9/1, v/v/v) solvent mixture.
13. When wetting front reaches within 1 cm of the top of TLC plate, remove the plate from the tank and dry it at RT.
14. When the plate is completely dried with a hair dryer, set it in exposure cassette and expose it to a tritium-sensitive imaging plate for few hours-few days.
15. Capture image and quantify signals with FLA-7000 image analyzer and software (Figure 1B).

Recipes

1. YPD liquid medium
 - 20 g Dextrose
 - 10 g Yeast extract
 - 20 g Polypeptone
 - 40 mg Uracil
 - 40 mg Adenine
 - 40 mg Tryptophan
 - Add H₂O to 1 L
 - Sterilize by autoclaving.
2. SD liquid medium
 - 20 g Dextrose
 - 1.7 g Yeast nitrogen base without amino acids and ammonium sulfate
 - 5.0 g Ammonium sulfate
 - 80 mg Uracil
 - 80 mg Adenine

- 80 mg Leucine
 - 80 mg Histidine
 - 80 mg Lysine
 - Dissolve in 800 ml H₂O
 - Adjust pH to 5.8-6.0 with 1 M NaOH
 - Adjust volume to 990 ml with H₂O
 - Sterilize by autoclaving
 - Add 10 ml of tryptophan solution (8 mg/ml) sterilized by filtration.
3. Water-saturated 1-butanol
- Mix equal volumes of 1-butanol and H₂O
 - Shake overnight at RT and leave
 - The resulting upper phase is water-saturated 1-butanol.

Acknowledgments

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