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Thin-Layer Chromatography of Phospholipids

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Abstract

Thin-layer chromatography (TLC) is a technique that has been routinely used for the separation and identification of lipids. Here we describe an optimized protocol for the steady state labeling, separation, and quantification of yeast phospholipids using 1-D TLC.

Keywords

Thin-layer chromatography; Phospholipids; Extraction; Orthophosphate ($^{32}P_i$); Phosphatidic acid; Phosphatidylserine; Phosphatidylethanolamine; Phosphatidylcholine; Phosphatidylglycerol; Cardiolipin; Phosphatidylinositol; Zymolyase; *Saccharomyces cerevisiae*

1 Introduction

Phospholipids constitute a major component of cell membranes, where they play essential roles in vital cellular processes. They are derived from the precursor phosphatidic acid (PA) and include phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidylinositol (PI), which is further phosphorylated to form a variety of phosphoinositides.

Increasingly, diseases are found to be associated with alterations in composition and levels of phospholipids. For example, Barth syndrome results from defects in cardiolipin levels and species [1, 2]; Charcot-Marie-Tooth disease is linked to altered levels of the phosphoinositide PI3,5P_2 [3]; and Alzheimer's disease and cystic fibrosis are both associated with altered levels of PE and PC [4, 5]. The finding that altered phospholipids are implicated in many diseases necessitates quick and relatively easy methods for the detection and quantification of specific phospholipids.

Thin-layer chromatography (TLC) was first described in 1938 [6]. Since then, TLC has been used for the routine separation and identification of lipids. Its use offers several advantages in terms of cost, convenience, and simplicity. The method depends on the use of: (1) A stationary phase, most commonly silica gel, with particle size ranging from 10 to 50 μm for standard TLC, and 5 μm for high-performance TLC (HPTLC), and (2) a mobile phase, generally consisting of a combination of organic solvents. The composition varies in different protocols.

Phospholipids can be separated by 1-D or 2-D chromatography. 2-D TLC results in more sensitive separation; however, the number of samples is limited to one per plate. While 1-D

TLC is somewhat less sensitive, multiple samples can be analyzed simultaneously on a single plate and in a shorter time than in 2-D TLC.

Here, we demonstrate the use of an optimized protocol for 1-D TLC for the detection and quantification of phospholipids from *Saccharomyces cerevisiae*.

2 Materials

2.1 Growth and Labeling of Phospholipids

1. Culture media: (a) Complex medium (yeast peptone dextrose, YPD): 1 % (w/v) yeast extract, 2 % (w/v) bactopeptone, 2 % (w/v) glucose, and (2 % (w/v) agar for plates only). (b) Synthetic medium (SM): To prepare 1 L of SM, use 0.69 g of vitamin-free yeast base mix, 2.01 g of ammonium sulfate, 20 g of glucose, vitamins, inositol, and amino acids as needed. The vitamin-free yeast base mix is based on the Difco recipe and contains the following: potassium phosphate (monobasic): 0.5 g; magnesium sulfate (anhydrous): 0.25 g; sodium chloride: 0.05 g; calcium chloride: 0.05 g; boric acid: 0.25 mg; copper sulfate (cupric sulfate anhydrous) 0.02 mg; potassium iodide: 0.05 mg; ferric chloride: 0.1 mg; manganese sulfate (monohydrate): 0.2 mg; sodium molybdate (molybdic acid, sodium salt): 0.1 mg; zinc sulfate: 0.2 mg.
2. Zymolyase 20-T (*see* Note 1).
3. Zymolyase buffer (50 mM Tris–SO_4 (pH 7.5), 1.2 M glycerol, 100 mM sodium thioglycolate): Weigh 0.6 g Tris base and transfer to a beaker. Add 60 mL dH_2O. Add 22.10 g of 50 % glycerol and 1.14 g of sodium thioglycolate. Mix and adjust pH to 7.5 using 1 N H_2SO_4. Make up to 100 mL with dH_2O. Store at 4 °C (*see* Note 2).
4. [$^{32}P_i$] orthophosphate: 10 μCi/mL for each sample used for steady state labeling (*see* Note 3).

2.2 Extraction

1. Extraction solvent: Chloroform/methanol 2:1 (v/v).
2. Microcentrifuge.

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[1]Zymolyase is available in two preparations, 20-T and 100-T, with lytic activities of 20,000 units/g and 100,000 units/g, respectively. For this protocol, the less expensive 20-T preparation is sufficient.

[2]Just before use, weigh appropriate amount of zymolyase and dissolve in prewarmed zymolyase buffer using gentle pipetting up and down. Do not vortex, as the enzyme may lose activity.

[3]The half-life of ^{32}P is relatively short (14.29 days). Therefore, purchase the radioactive ^{32}P; as close as possible to the time of the experiment. Measure and record the initial activity (A_0) as soon as you receive it. Use the following formula to calculate the amount of radioactivity at the time the experiment is begun:

$$A = A_0 \times (0.5)^{t/(1/2)t}$$

where A = current activity, A_0 = initial activity, t = number of days since order was received, ½t = half-life of ^{32}P, which is 14.29 days.

3. Agitator/Stirrer.

2.3 Chromatography

1. TLC plates (Whatman LK5 Silica gel 150 Å).
2. Prewash solution: Chloroform/methanol 1:1 (v/v). Prepare 150 mL of prewash solution by mixing 75 mL of chloroform with 75 mL of methanol.
3. Boric acid solution 1.8 % (w/v in 100 % ethanol): Weigh 9.0 g boric acid and add to 500 mL ethanol (*see* Note 4).
4. TLC tank lined with filter paper: Cut an appropriate size of filter paper to completely line all sides of the tank.
5. Developing solution (mobile phase): Chloroform/ethanol/water/tri-ethylamine 30:35:7:35 (v/v/v/v). Make a total of about 160 mL. Mix together 45 mL chloroform, 52.5 mL ethanol, 10.5 mL water, and 52.5 mL tri-ethylamine. Keep in an air-tight container until ready to use.

3 Methods

3.1 Steady State Labeling of Phospholipids

1. Streak the desired yeast strain on a fresh YPD plate and grow until colonies are about 2 mm in diameter.
2. Prepare an overnight culture by picking one colony and transferring it to 5 mL of the desired medium (YPD or SM).
3. Inoculate each of three tubes containing 2 mL of the desired media with cells from the overnight culture to a starting A_{550} of 0.05 (*see* Note 5).
4. To two tubes only, add 20 μCi $^{32}P_i$; (10 μCi/mL culture). Use the third tube (with no ^{32}Pi added) to monitor the absorbance.
5. To achieve steady state labeling, incubate all three tubes in a rotary shaker at the desired temperature for 5–6 generations (*see* Note 6).
6. Harvest the cells by centrifuging at 5,500 × *g* for 5 min, and wash once with 1 mL distilled water. Remove the supernatant. *In this and all subsequent steps, discard radioactive materials properly*.
7. To facilitate the extraction of phospholipids, digest the cell wall by adding 1 mL zymolyase solution to the cell pellets (*see* Note 7). Mix and keep on stirrer for 15 min at room temperature.

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[4]Boric acid improves the resolution of the separated phospholipids by acting as an adsorbent modifier [7]. The use of 1.8 % boric acid enhances the separation of PG from PE [8].

[5]To ensure consistency, inoculate 6 mL of medium with the overnight culture, measure A_{550} and then aliquot into three tubes.

[6]This usually takes about 8 h, but the time could vary depending on the strain used. It is advisable to do a growth curve for each strain before starting labeling experiments, in order to determine the time required for growth of 5–6 generations.

[7]As the cells progress from the log phase to the stationary phase, the cell wall becomes more difficult to digest, and the amount of zymolyase required will vary accordingly. For example: use 1.5 mg/mL for cells in early log phase, 2.0 mg/mL for mid-log phase, and 2.5 mg/mL for stationary phase.

8. Harvest the spheroplasts (the cells without the cell wall) by centrifuging at 5,500 × *g* for 5 min. Discard the supernatant, which contains the zymolyase and all debris.

3.2 Extraction of the Phospholipids

1. To lyse the spheroplasts and extract the phospholipids, add 250 μL of chloroform/methanol 2:1 (v/v), resuspend and incubate on stirrer at room temperature for 45 min to 1 h.
2. To separate the organic phase containing the phospholipids from the aqueous phase containing non-lipid cellular material, add 50 μL of water, vortex, and centrifuge at 1,000 × g for 1 min.
3. Using a pipette with a fine tip, or a Hamilton syringe, carefully remove the lower (organic) phase and transfer to a microcentrifuge tube. The sample can be loaded directly onto the TLC plate or concentrated under a gentle stream of nitrogen at room temperature (*see* Note 8).

3.3 Preparation of the TLC Plates (See Fig. 1)

1. At least 2 h before starting, line the TLC tank with filter paper and equilibrate for at least 2 h with chloroform/ethanol/water/tri-ethylamine 30:35:7:35 (v/v/v/v), keeping the tank covered with a lid.
2. To remove impurities from the TLC plates, pre-develop plates with chloroform/methanol 1:1 (v/v) in a clean tank and air-dry in a fume hood.
3. Prepare a 1.8 % boric acid solution in 100 % ethanol. Pour into a glass tray that can easily accommodate the TLC plate. Fill the glass tray to a depth of about 2 cm. Dip the TLC plate in the solution for 2 min, making sure that it is uniformly impregnated with boric acid (*see* Note 9). Air-dry the plate for 15 min, then activate it at 100 °C for 15 min. Let it cool.
4. Loading lanes: (a) Using a fine point pencil, divide the pre-adsorbent zone (the lower thick area of the plate) into 2 cm fractions. (b) Scrape a width of ~0.5 cm using a flat spatula or a razor blade. (c) Using a fine tip, spot or streak 20–30 μL of the phospholipid sample onto the pre-adsorbent area at about 1.5 cm from the base. Allow sample to dry (*see* Note 10).
5. Gently lower the loaded TLC plate into the pre-equilibrated chromatography tank, making sure the samples are above the surface of the developing solvent (*see* Note 11).

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[8] The samples can be completely dried under nitrogen, resuspended in chloroform/methanol 2:1 (v/v), and stored at 4 °C in a plexiglass box until needed.

[9] Aside from boric acid, other chemical modifiers of silica gel have been reported, including EDTA, ammonium sulfate, silver nitrate, or oxalate [9–11].

[10] Applying the sample as a fine streak allows for clear distinction of bands that may migrate in close proximity.

[11] Before placing the loaded TLC plate in the tank, make sure there is enough developing solvent in the tank. Most of what is added earlier to equilibrate the tank is absorbed by the filter paper during the equilibration process. It is important that the sample spots remain above the surface of the developing solvent. Otherwise, the samples could leach into the solvent instead of ascending up the TLC plate.

6. Place the lid on the tank and allow the solvent (the mobile phase) to ascend to about 1 cm from the top of the plate (*see* Note 12).
7. Remove the plate from the tank, mark the level of the solvent front by making a small etch on both sides of the plate, and air-dry completely in a fume hood.
8. Cover the TLC plate with plastic wrap, avoiding creases, and place the plate in an imaging cassette, face up. Place the phosphorimaging film on top, lock the cassette, and expose for 15–60 min.
9. Remove the film from the cassette and scan it using a Storm/Typhoon phosphorimager system. Separation of phospholipids from wild-type and *crd1* mutant cells, which lack CL, and accumulate PG, is shown in Fig. 2.
10. Quantify intensity of the bands using available software (e.g., ImageQuant).
11. To determine the *rate of synthesis* of phospholipids, a slightly modified procedure is followed (*see* Note 13).

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[12]This takes 1.5–2 h. Keep checking the level of the solvent to ensure that samples do not reach the top.
[13]The protocol described above is used for steady state labeling, which allows the detection of phospholipids present in the cells. However, to determine the *rate of synthesis* of phospholipids, pulse-labeling should be used. The procedure is the same as above except for the following: (a) the number of cells should be higher thus, the culture volume should be 10 mL instead of 2 mL. (b) The amount of $^{32}P_i$ should be higher, use 50 μCi/mL culture instead of 10 μCi/mL culture. (c) Cells are labeled for 15 min only, after which they are harvested and phospholipids are extracted. This is done for every time point during which the rate of synthesis is being monitored.

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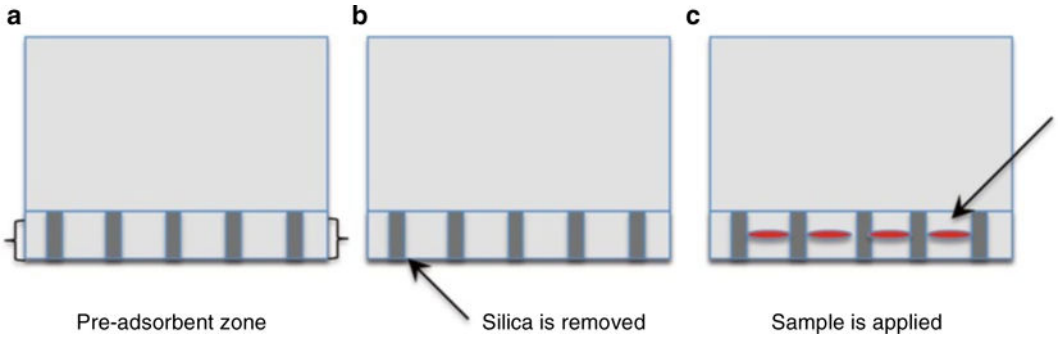

Fig. 1.

TLC plate preparation for loading several samples onto the same plate. (**a**) The pre-adsorbent zone is divided into several zones using a fine tip pencil. (**b**) The silica from a width of about 0.5 cm is gently scraped off. (**c**) The sample is applied as a fine streak at about 1.5 cm from the base of the plate

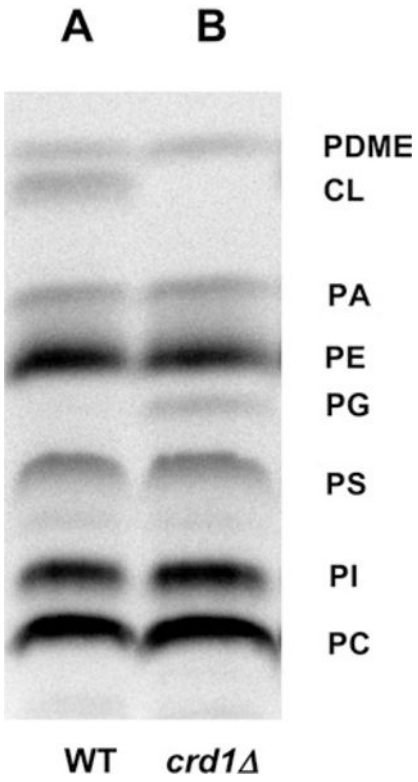

Fig. 2.

Separation of phospholipids by 1-D TLC. Cells were grown in YPD, labeled with $^{32}P_i$, washed and digested with zymolyase. Lipids were extracted from spheroplasts with chloroform/methanol 2:1 (v/v), and then analyzed by 1-D TLC. Phospholipids from wild-type cells (**A**), and from *crd1* mutant cells (**B**), which lack CL are depicted. Individual lipid species were identified by the comigration of standards. *PDME* phosphatidyldimethylethanolamine, *CL* cardiolipin, *PA* phosphatidic acid, *PE* phosphatidylethanolamine, *PG* phosphatidylglycerine, *PS* phosphatidylserine, *PI* phosphatidylinositol, *PC* phosphatidylcholine