

Analysis of D3-,4-,5-Phosphorylated Phosphoinositides Using HPLC

Teun Munnik

Abstract

Detection of polyphosphoinositides (PPIs) is difficult due to their low chemical abundance. This problem is further complicated by the fact that PPIs are present as various, distinct isomers, which are difficult, if not impossible, to separate by conventional thin layer chromatography (TLC) systems. PPIs in plants include PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,5)P₂, and PtdIns(4,5)P₂. Here, a protocol is described analyzing plant PPIs using ³²P-orthophosphorus pre-labeled material. After extraction, lipids are deacylated and the resulting glycerophosphoinositol polyphosphates (GroPInsPs) separated by HPLC using a strong anion-exchange column and a shallow salt gradient. Alternatively, PPIs are first separated by TLC, the lipids reisolated, deacylated, and the GroPInsPs then separated by HPLC.

Key words Polyphosphoinositides, Glycerophosphoinositolphosphates, GroPIns, PPI isomers, Deacylation, Mono-methylamine, PtdInsP, PtdInsP₂

1 Introduction

Polyphosphoinositides (PPIs) represent only a minor fraction of the phospholipids of eukaryotic membranes, yet they are extremely important for signal transduction and membrane trafficking. Their concentration is so low and their ionization and chemical properties unfavorable, that they normally do not appear in any of the lipidomic type of analyses that are currently routine (1–4).

The most abundant plant PPI is phosphatidylinositol phosphate (PtdInsP), being present in three different isomers, with the inositol headgroup phosphorylated either at the 3-, 4-, or 5-position (i.e., PtdIns3P, PtdIns4P, PtdIns5P). PtdIns4P is the most abundant, representing ~80 % of the PtdInsP pool, with PtdIns3P and PtdIns5P combined ranging between 5 and 15 % (5–7). Plants exhibit only two phosphatidylinositol biphosphate (PtdInsP₂) isomers, i.e., PtdIns(4,5)P₂ and PtdIns(3,5)P₂ (8). Compared to mammalian systems, plant PtdInsP₂ levels are extremely low, 30–100-fold

lower than PtdInsP, which in animals is present in approx. equal amounts (4, 9). Most (>95 %) of the PtdInsP₂ pool consists of PtdIns(4,5)P₂ with tiny amounts of PtdIns(3,5)P₂ being present and made in response to osmotic stress (8). Earlier, the isomer PtdIns(3,4)P₂ had been reported (10) but this lipid was probably mistaken for PtdIns(3,5)P₂, which had not yet been described at that time. In animals cells, PtdIns(3,4)P₂ is a breakdown product of PtdIns(3,4,5)P₃ which is also lacking from plants.

The low amounts of PPIs make them relatively difficult to study. One of the ways to get around this is to use ³²P_i labeling and exploit the sensitive detection possible with this isotope. ³²P_i-label is rapidly taken up by plant cells and quickly incorporated into ATP and CTP pools to label phospholipids. Because PPIs turn over more rapidly than structural phospholipids, they are preferentially ³²P-labeled (5, 6, 11–13). Isomers are, however, difficult (4,5 - 3,5) if not impossible (3-,4-,5P) to separate by thin layer chromatography (6, 8). Hence, deacylation by *mono*-methylamine and HPLC analysis of the resulting glycerolphosphoinositol phosphates (GroPInsPs) using a strong anion exchanger is required of which a detailed procedure is described here, below.

2 Materials

2.1 Plant Material, ³²P-Labeling, Lipid Extraction, and TLC

1. See detailed procedures described in Chapter 1, Subheadings 2.1–2.5.
2. Spray gun with distilled water.
3. Hair dryer.
4. Strong plastic kitchen foil.

2.2 Deacylation

1. Geiger counter.
2. 2 ml Eppendorf Safe-lock tubes.
3. Mono-methylamine reagent: For 100 ml, take 42.8 ml 25 % (w/v) mono-methylamine (Sigma), 45.7 ml MeOH, and 11.5 ml *n*-ButOH (14). Store at 4 °C and keep it cold! (See Note 1).
4. Deacylation wash solution: H₂O/MeOH/*n*-ButOH (42.8:45.7:11.5).
5. Thermoblock, 53 °C.
6. N₂ (g).
7. Vacuum centrifuge with cold trap.

2.3 Fatty Acid Removal

1. Geiger counter.
2. *n*-ButOH/petroleum ether 40–60°/ethyl formate (20:4:1).
3. 2 ml “Safe-lock” reaction tubes (Eppendorf).
4. 0.5 ml-reaction tubes.

2.4 Desalting

1. Cation exchange resin, Bio-Rad AG 50W \times 8 200–400, H⁺ form.
2. Empty PD-10 columns.
3. Geiger counter.

2.5 HPLC

1. HPLC.
2. Partisil 10 SAX (Whatman) column with guard; Alternatively, a Zorbax SAX column (DuPont) can be used.
3. Buffer A = water.
4. Buffer B = 1.0 M ammonium phosphate (pH 3.35, phosphoric acid) (*see Note 2*).
5. Liquid scintillation vials (5 ml).
6. Liquid scintillation fluid (Packard).
7. Liquid scintillation counter.

3 Methods
3.1 ³²P-Labeling of Plant Material and Lipid Extraction

A detailed procedure for lipid labeling and extraction is described in Chapter 1 (Subheadings 3.1–3.5), illustrating methods for suspension-cultured plant cells, seedlings, roots, and leaf disks.

3.2 Deacylation of Phospholipids

To remove the fatty acids of labeled phospholipids, a mono-methylamine deacylation procedure is used, which is based on the method of Clarke and Dawson (15) with modifications as described by Munnik et al. (5, 16) and Meijer et al. (7).

1. Add 400 μ L mono-methylamine reagent (careful, DO NOT inhale, keep it cold!) to the dried lipid extract.
2. Incubate for 30 min (NOT longer!) at 53 °C in a fume hood.
3. Evaporate the mono-methylamine vapor using streaming air or N₂ (g) as the vapor would damage the vacuum pump. Be careful to not to inhale the fumes!
4. After 30–60 min, when the mono-methylamine vapor is gone, samples can be dried further using a vacuum centrifuge at room temperature.
5. Dissolve samples in 500 μ L distilled H₂O.

To remove the fatty acids:

6. Add 600 μ L [*n*-ButOH/petroleum ether 40–60°/ethyl formate] (20:4:1; by vol.).
7. Vortex; remove organic phase (= upper phase).
8. Wash lower water phase with 375 μ L [*n*-ButOH/petroleum ether 40–60°/ethyl formate] (20:4:1; by vol.).
9. Transfer the water phase to a clean 0.5 ml-reaction tube.
10. Dry samples in a vacuum centrifuge at 60 °C. (This may take a few hours).

3.3 Deacylation of TLC Purified Phospholipids

In instances when phospholipids were first separated by TLC, HPLC analysis can still be performed. For TLC analysis, see Chapter 1.

1. Dry the TLC plate in a fume hood for 30–60 min, so that all organic-solvent is gone.
2. Wrap the TLC plate in strong kitchen foil.
3. Visualize the ^{32}P -labeled lipids by exposing an autoradiography film (Kodak X-OMAT) to the TLC plate for 30 min to 2 h (*see Note 3*).
4. Develop the autoradiography film and dry it with a hairdryer.
5. Fix the film in front of the TLC plate (silica-side) using clips.
6. Hold the TLC plate in the light such that the spots of the film shine through (*see Note 4*).
7. Indicate the spots on the back side (glass side) of the TLC plate using a water-resistant marker.
8. When done, remove the film, clips and foil.
9. Use a spray gun with water to wet the silica of the TLC plate. Spray in short intervals (*see Note 5*).
10. Upon spraying, the TLC plate becomes transparent so that the marker on the glass-side starts to shine through.
11. Use a small/thin spatula to scrape-off the PPI spots from the plates and collect the silica into “Safe-Lock” reaction tubes (2 ml) (*see Note 6*).
12. Dry samples for 15 min in a vacuum centrifuge.
13. Add 400 μL mono-methylamine reagent (careful, DO NOT inhale, keep it cold! *See Note 1*).
14. Incubate for 30 min (NOT longer!) at 53 °C in a fume hood.
15. Remove silica by centrifugation ($13,000\times g$) for 2 min and collect the supernatant in a new reaction tube. Wash the silica again with “mono-methylamine *wash* solution” ($\text{H}_2\text{O}/\text{MeOH}/n\text{-ButOH}$ (43:46:12; by vol.)).
16. If necessary, repeat step 15, and check whether there is still radioactivity present in the silica remnant.
17. Dry total supernatant under streaming air or N_2 (g) for 60 min, then transfer to a vacuum centrifuge at room temperature. Mono-methylamine fumes are bad for the vacuum pump, too! Therefore, only proceed after all mono-methylamine vapor has been eliminated.
18. Dissolve samples in 500 μL distilled H_2O .
19. To remove the fatty acids, add 600 μL [$n\text{-ButOH}$ /petroleum ether 40–60 °/ethyl formate] (20:4:1; by vol.).
20. Continue as in Subheading 3.2, step 7.

3.4 Desalting Protocol for HPLC Analysis of Deacylated Phospholipids

1. Dissolve deacylated sample into 100 μL H_2O .
2. Desalt sample with cation exchange column (Bio-Rad AG 50W $\times 8$ 200–400, H^+ form):
 - (a) Pipet 500 μL of the cation exchange “slurry” into an empty PD-10 column.
 - (b) Elute the solvent.
 - (c) Add 500 μL H_2O ; elute.
 - (d) Wash with another 1,000 μL of H_2O (i.e., Total = minimum of 3 column volumes).
 - (e) Load 100 μL sample (*see* **Note 4**) onto the column.
 - (f) Elute ^{32}P -GroPInsPs from column by adding $x\mu\text{L}$ H_2O (see below):
 - (g) Determine x by adding 100 μL aliquots and following the radioactivity with a Geiger counter on the collected fractions and on the column itself. ~ 500 μL should be sufficient.
 - (h) Elute the rest of the samples with the same volumes.
3. Dry by vacuum centrifugation (60°C).
4. Store at -20°C in H_2O in a volume appropriate for loading onto the HPLC (100–500 μL).

3.5 HPLC Analysis of Deacylated $^3\text{H}/^{32}\text{P}$ -Labeled PPIs (Glycerophos phoinositides)

Deacylated lipids are routinely separated by anion-exchange HPLC using a Partisil 10 SAX column and a discontinuous gradient of water (buffer A) and 1.0 M ammonium phosphate, pH 3.35 (phosphoric acid; Buffer B), at a flow rate of 1.0 ml/min.

1. Equilibrate the HPLC column with H_2O prior to sample loading (6 column volumes).
2. Filtrate the deacylated sample over a 0.45 μm filter to remove particles.
3. Load the sample onto the HPLC column using a glass syringe with a blunt-ended needle. Sample loops of 100, 200, or 500 μL can be used.
4. Elute the sample with the following nonlinear gradient of Buffer B: 0–10 min, 0 % B; 10–80 min, 5 % B; 80–140 min, 35 % B; 140–145, 100 % B; 145–160 min 0 % B.
5. Collect 1 ml fractions.
6. Add 4 ml scintillation fluid to each fraction and determine the radioactivity by liquid scintillation counting (*see* **Note 7**).
7. Individual peaks can be identified following periodate treatment by HPLC analysis (17) and/or the use of ^3H -standards, including Ins, GroPIns, Ins1P, Ins3P, Ins4P, GroPIns3P, GroPIns4P, Ins(1,4) P_2 , Ins(1,5) P_2 , GroPIns(3,4) P_2 , GroPIns(4,5) P_2 , Ins(1,3,4) P_3 , and Ins(1,4,5) P_3 .

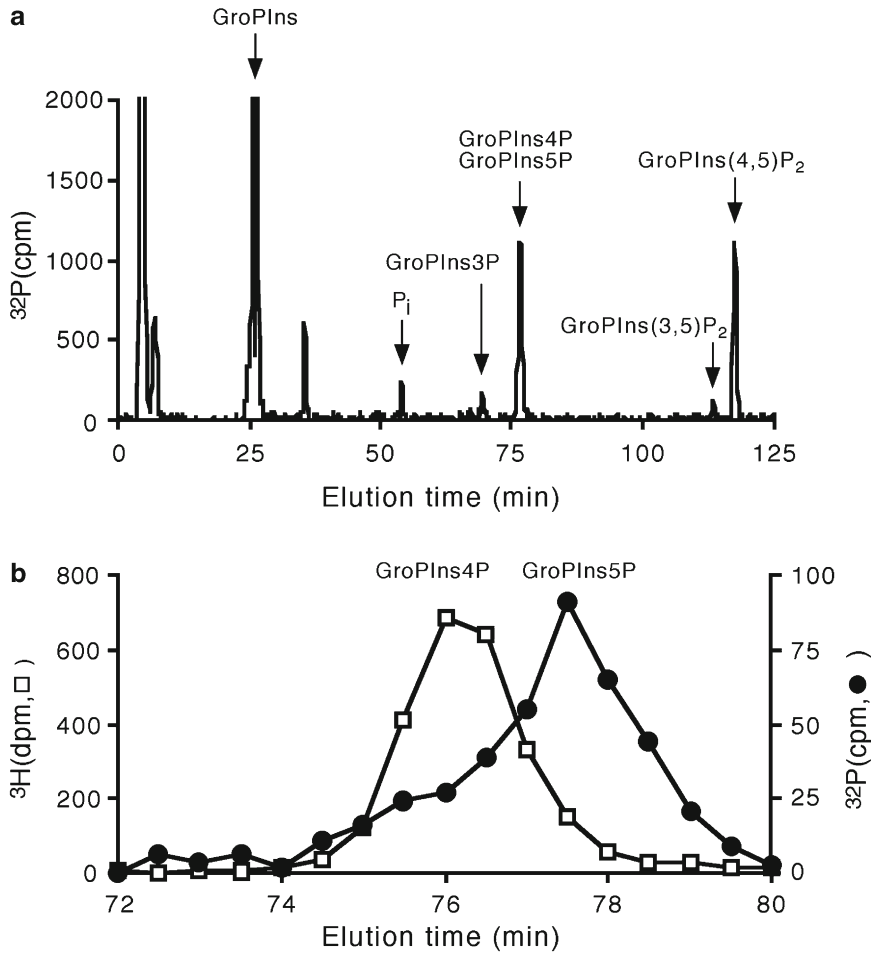


Fig. 1 Anion-exchange HPLC of deacylated phospholipids. **(a)** *Chlamydomonas* cells were radiolabeled with ^{32}P - P_i for 1 h and then treated for 2.5 min with 150 mM NaCl. Lipids were extracted, deacylated, and the water-soluble products separated by anion-exchange HPLC. **(b)** Separation of GroPIns4P and GroPIns5P. ^3H -GroPIns4P and ^{32}P -GroPIns5P standards were prepared as indicated and separated by HPLC using a more shallow salt gradient and fractions collected at 30 s intervals. Radioactivity was determined by scintillation counting. Adapted with permission from (7)

8. Because the peaks of GroPIns4P and GroPIns5P differ in their retention times by less than 20 s (Fig. 1a), a modified gradient can be utilized, collecting fractions of 0.5 ml: 0–45 min, 0–1.5 % Buffer B; 45–46 min, 1.5–2.4 % Buffer B; 46–80 min, 2.4–4.5 % Buffer B; 80–81 min, 4.5–6.0 % Buffer B; 81–141 min, 6.0–35.0 % Buffer B; 141–142 min, 35–100 % Buffer B; 142–147 min, 100 % Buffer B; 147–150 min 100–0 % Buffer B; 150–180 min 0 % Buffer B wash. The latter gradient produced a 1.5 min peak-to-peak separation of GroPIns4P and GroPIns5P (see Fig. 1b).

4 Notes

1. Mono-methylamine is dangerous. The fumes are *toxic* and *explosive*! Store at 4–8 °C and keep it cold!
2. Alternatively, 1.25 M NaH₂PO₄, pH 3.7 (with H₃PO₄) can be used as Buffer B. It is the P_i that is important. Obviously, one has to adjust the gradient to separate all GroPInsPs.
3. Radioactivity of the PPI spots should be high enough, otherwise you will not be able to get a proper signal after deacylation and HPLC separation (since compounds elute from the column in more than one fraction).
4. Stay behind an 1-cm Perspex screen for radiation safety at all times and wear gloves and safety glasses.
5. Spray in short bursts so that the silica has time to take-up the water (i.e., do not soak it immediately).
6. First try on the side to see how well the silica comes off the glass plate. Too wet will make it too fluffy, if the silica is too dry than it “breaks.” When it is right, it scrapes like butter on a knife, sticking together.
7. By adding nonradioactive ADP and ATP and monitoring with a UV detector, specific fractions can be taken rather than the whole run. This saves scintillation fluid and counting.

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