SOP: PP026.4
Updated 2/26/17

Purification of $PIM_{1,2}$

Materials and Reagents

1. Methanol, chloroform, HPLC-grade
2. Acetone, ACS grade
3. Graduated cylinder, glass 100 ml
4. Chemical fume hood.
5. Magnetic stir plate
6. Whatman filter funnels, 125 mm diameter
7. Round-bottom flasks, 1 L, 250 ml
8. Rotary evaporator
9. Metal spatula
10. Sorvall centrifuge bottles, compatible with organic solvents
11. Pasteur pipets
12. PIM standard
13. TLC equipment (note 1)
14. Glass tubes, 13 x 100 mm + Teflon caps
15. Glass vials, 2 ml + Teflon caps
16. Preparative TLC plates (Merck 1.05715.0001)
17. Analytical TLC sheets (Merck 1.05548.0001)
18. TLC tanks, large
19. Glass pipets, 10 ml
20. Rubber pipet bulb
21. Vortex
22. Reagents and equipment for alditol-acetate derivation (note 2)

Protocol

1._____ Obtain 10:10:3 total lipid (TL) from cells used as starting material for preparation of lipoaribinanmannan.

2._____ Use rotary evaporation to reduce volume of TL down to 60-80 mL.

3._____ Obtain acetone pre-equilibrated at –20°C.

4._____ Working in the hood, transfer the cold acetone to a 1 L bottle, then slowly pipet the concentrated 10:10:3 TL into the acetone. (note 3)

5._____ Allow the trituration to proceed at –20°C overnight.

6._____ Transfer entire volume of trituration to sterile 250 ml Sorvall centrifuge bottles compatible with organic solvents. (note 4)

7._____ Centrifuge at 20,000 x g at 4°C for 30 minutes.

8._____ Decant supernatant into a beaker for evaporation.

9._____ Allow the pellets to briefly dry in the fume hood.

10._____ The acetone-insoluble material can be resuspended in $CHCl_3/CH_3OH$ (2:1) in the centrifuge bottles or tubes and transferred to an appropriate round-bottom flask to reduce volume via rotovap. (note 5)

11._____ Apply the extract to preparative TLC plates in the hood and run in solvent system 65/25/4 $CHCl_3/CH_3OH/H_2O$. (note 6)

12._____ Transfer PIM-specific silica to Teflon tubes for extraction. (note 7)

13._____ Add at least 10 ml 2:1 $CHCl_3/CH_3OH$ to each tube and, ensuring that caps are tight, briefly vortex.

14._____ Centrifuge at 3,000 rpm at 4°C for 10 minutes.

15._____ Transfer the organic supernatant to round bottom flasks, filtering through small filter cones fitted into a glass funnel. (note 8)

16._____ Repeat steps 13 to 15 twice for a total of 2 extractions.

17._____ Dry PIM extract for this set of plates via rotary evaporation.

18._____ Resuspend in small volume of 2:1 and transfer to glass tube. (note 9)

19._____ Evaluate by analytical TLC using solvent system 65/25/4 $CHCl_3/CH_3OH/H_2O$. (note 10)

20._____ Run at least 20 preparative HPTLC plates before combining appropriate crude $PIM_{1,2}$ extracts for final polishing.

21._____ Evaluate the total amount of crude $PIM_{1,2}$ extract by drying in a tared 2 ml glass vial.

22._____ Resuspend PIM and apply to final prep plates, developing as before. (note 11)

23._____ Evaluate by analytical TLC in triplicate. (note 12)

24._____ Once $PIM_{1,2}$ has been confirmed with TLC, make alditol acetate derivatives of the sample in duplicate, using 50 and 100 µg material. Include $PIM_{1,2}$ control and neutral sugar standards. (note 2)

25._____ Perform gas chromatography on derivatives to confirm presence of mannose and inositol.

26._____ Perform MALDI-TOF analysis. (note 13)

Notes

1. See Thin Layer Chromatography, SOP SP033, for a complete list of equipment and reagents.

2. See Preparation of Alditol-Acetate Derivatives, SP022.

3. A fluffy white precipitate will form. The purpose of the trituration is to selectively precipitate PIM1,2 and other glycolipids with similar solubility. TDM, SL-I, and very polar lipids tend to be acetone-soluble, hence this is essentially an enrichment technique. Ratio of TL extract to acetone should be 1:10.

4. If the total volume is less than 500 ml, it may be more efficient to centrifuge iterively in several Teflon Oakridge tubes.

5. Minimize the time that 2:1 sits in the non-Teflon bottles. Use vortexing and scraping with spatula to assist in resuspension.

6. Start with 0.5 ml per plate. If separation of $PIM_{1,2}$ from other bands determined by pattern under short-wave UV is good, subsequent plates can be loaded with incrementally more total lipid. The quantity of total lipid applied to each plate, and total overall, can be determined by taring a 2.0 ml glass vial and drying down a small aliquot. This will help calculate the efficiency of extraction relative to previous lots. One plate in the first set should have 25 µg $PIM_{1,2}$ loaded near the edge to confirm which band is actually $PIM_{1,2}$.

7. Stain edges of TLC plates with α-naphthol to discern the total lipid bands and the PIM-specific band. Outline PIM-specific area on prep plate with pencil, visualizing under short-wave UV light. Alternatively, hold the plates up to visible light to see a “light” band aligning with the PIM control. Working in the hood, scrape away

silica below PIM area with a glass slide and discard. Carefully scrape PIM area and distribute to 50 ml Teflon tubes.

8. Select a round-bottom flask sufficient such that the total volume of three rounds of extraction will not exceed ½ the volume capacity of the flask. This will make drying the lipid via rotary evaporation more efficient.

9. Use 3-4 ml 2:1 to resuspend crude PIM in the flask, transfer to 13x100 mm tube, then add 1-2 ml 2:1 rinse of flask. Dry down in nitrogen bath, then resuspend in 200-500 µl 2:1.

10. Run 10 µl on analytical HPTLC (10x10 cm) along with $PIM_{1,2}$ standard, developing with 65/25/4 chloroform/methanol/water, and stain with $CuSO_4$ only. This is the most sensitive stain and will show in this system any impurities, usually a single spurious band below $PIM_{1,2}$. This should be done for each set of plates prior to combining for the final clean-up. Some will be sufficiently clear of bands as to not require further polishing; up to 60% of crude PIM cleaned up in this way can be lost.

11. Use no more than 5 mg per plate. Follow the same extraction protocol described in steps 11-18.

12. Run 10-20 µg on 3 10x10 mm HPTLC plates using 65/25/4 solvent system. Develop with α-naphthol, copper sulfate, and Dittmer-Lester. The first two should be charred with the heating gun to activate staining, but that sprayed with Dittmer-Lester will come up on its own after 10 min or so. Do not char this one, but do scan immediately, as the color will fade. A second band between the baseline and PIM will indicate phosphatidyl-inositol contamination, visible on the CuSO4-visualized and Dittmer-Lester TLC sheets.

13. Apply 1-2 µg using DHB matrix, and include PIM control from previous lot. One should see a peak cluster at 1693 m/z in negative mode, with peaks separated by 14 amu.

References

Brennan, P. J. and C. E. Ballou. Journal of Biological Chemistry. 1967. 242:3046.
Khoo, K.-H., *et. al.* Glycobiology. 1995. 5:117.

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