SOP: PP052

Isolation of Native PGL-I from *M. leprae*

Materials and Reagents:

1. Supernatant containing *M. leprae* whole cells from step 10 in SOP: PP050
2. Lyophilizer
3. Erlenmeyer flask, 500 ml, with glass stopcock
4. Chloroform, ACS grade or HPLC grade
5. Methanol, ACS grade or HPLC grade
6. Magnetic stir bar
7. Parafilm
8. Magnetic stir plate
9. Whatman filter cone, (cat. # 1202-270, grade 2V, 270mm)
10. Whatman filter paper, 17 CHR
11. 500 ml round-bottomed flask
12. Rotovap
13. Glass tube, 50 ml, with PTFE cap
14. Glass pipettes, 1 ml, 5 ml, 10 ml
15. Glass Pasteur pipettes
16. Glass chromatography column, with Teflon stopcock, L= 37.5 cm, I.D.= 2 cm, V= 117.8 ml, or similar
17. Ring stand
18. Glass wool
19. Graduated Cylinders, 100 ml, 500 ml
20. Silica gel, 60 Å, 70-230 mesh
21. Beaker, 400 ml
22. Air hose
23. Glass bottles (x3), with PTFE caps, 500 ml
24. Glass stir rod
25. Glass bottles (x20), with PTFE caps, 125 ml
26. Round-bottomed flasks (x20), 100 or 500 ml
27. Glass tubes (x20), with PTFE caps, 10 ml
28. Analytical TLC sheets, silica gel 60 (EMD 5554-7)
29. TLC tank
30. 10 µl microdispenser tubes
31. α-naphthol
32. Water, distilled
33. Nitrogen evaporator
34. Preparative TLC plates, silica gel 60, 1000 µm thick, with fluorescent indicator (Whatman 4861-840)

Protocol:

1._____ Lyophilize the supernatant obtained from step 6 in SOP: PP050. Record dry weight. Start with ~10 g of dry weight (note 1).

2._____ Transfer material to 500 ml Erlenmeyer flask with glass stopcock using chloroform-methanol (2:1). Suspend material in 10 ml of chloroform-methanol (2:1) per 1 g of lyophilized material. Add metal stir bar, place glass stopcock on flask, stir for 1-4 days at room temp inside fume hood (notes 2 & 3).

3._____ Cut filter paper to fit inside Buchner funnel and filter the extracted material. Collect the eluent in appropriate sized, pre-weighed, round-bottomed flask.

4._____ Evaporate the solvent using the Rotovap and obtain dry weight of crude lipid (notes 4 & 5).

5._____ Suspend crude lipid in chloroform to yield a concentration of 50-200 mg/ml. Transfer about 2 g of suspended crude lipid to a 50 ml tube with PTFE membrane cap.

6._____ Set up glass column with reservoir in fume hood with the ring stand and pack with glass wool. Elute ~40 ml 100% chloroform through the column, collect eluent and discard (note 6).

7._____ Weigh the appropriate amount of silica gel 60 and transfer to a 400 ml beaker. Add 60-150 ml of chloroform slowly to the silica gel in order to create slurry. Pour directly into the column, place the beaker under the nozzle of the column to collect the eluent (chloroform), then open stopcock. Gentle tapping on the side of the column or low air pressure may be used to 'pack' the silica gel more tightly.

8._____ Allow the solvent line to fall to the top of the silica gel, and then close the stopcock. Do **NOT** allow the solvent line to fall below the top of the silica gel column. This can be a stopping point. If leaving the column overnight, add ~50 ml or more of chloroform to ensure the chloroform level does not fall below the silica gel, and place glass stopcock on top.

9._____ Pour the collected eluent back into the column, place the beaker under the nozzle to collect the eluent, and then open the stopcock. Allow the solvent line to fall even with the top of the silica gel and then close the stopcock; do not allow the solvent line to fall below the silica gel. Gentle tapping and low air pressure may be used to compact the silica gel. Sprinkle sand on top of silica to help keep integrity of column.

10._____ Discard the eluent as hazardous waste. Measure 40 ml chloroform and pour into column, open the stopcock. Allow the solvent to fall to about 2 inches from the top of the silica gel and close stopcock. Collect eluent and discard.

11._____ Make each solvent as follows in 500 ml bottles with PTFE membrane caps:

1% methanol-99% chloroform = 3 ml methanol + 297 ml chloroform.
2**% methanol-98% chloroform** = 8 ml methanol + 392 ml chloroform.
3% methanol-97% chloroform = 12 ml methanol + 388 ml chloroform.

Measure the chloroform first, then measure and add the methanol.

12._____ Open the stopcock to drain the solvent from the column until the solvent line is even with the top of the silica gel, then close the stopcock. Discard the eluent.

13._____ Gently add the 2 g of suspended crude lipid to the column using a glass Pasteur pipette. Place beaker under the column nozzle and open the stopcock to allow the crude lipid to absorb into the column until the solvent line is even with the silica gel, then close the stopcock. It may be necessary to stir the top inch of the silica gel with a glass stir rod and apply low air pressure to facilitate the solubility of the crude lipid. Collect the eluent and discard.

14._____ Begin collecting fractions 1-20 as follows. For each fraction, add one column volume (CV = 1ml solvent per g silica gel) of the corresponding solvent to the column carefully with a Pasteur pipette and then elute the solvent until level with the top of the silica gel. Low air pressure may be used to speed up the flow rate of the column. Collect each eluent in their respective 125 ml bottle with PTFE cap labeled 1-20: (notes 7 & 8)

Fraction 1: 100% chloroform
Fraction 2: 100% chloroform
Fraction 3: 100% chloroform
Fraction 4: 100% chloroform
Fraction 5: 1% methanol-99% chloroform
Fraction 6: 1% methanol-99% chloroform
Fraction 7: 1% methanol-99% chloroform
Fraction 8: 1% methanol-99% chloroform
Fraction 9: 2% methanol-98% chloroform
Fraction 10: 2% methanol-98% chloroform
Fraction 11: 2% methanol-98% chloroform
Fraction 12: 2% methanol-98% chloroform
Fraction 13: 2% methanol-98% chloroform
Fraction 14: 2% methanol-98% chloroform
Fraction 15: 3% methanol-97% chloroform

Fraction 16: 3% methanol-97% chloroform
Fraction 17: 3% methanol-97% chloroform
Fraction 18: 3% methanol-97% chloroform
Fraction 19: 3% methanol-97% chloroform
Fraction 20: 3% methanol-97% chloroform

For fraction 20, add the solvent to the column and elute and collect the entire volume of the column. This is the only time to allow the solvent to elute completely through the silica gel, i.e. allow the solvent line to fall below the silica gel.

15._____ Transfer each fraction to a pre-weighed round-bottomed flask (100-500 ml), being sure to keep the fractions labeling.

16._____ Evaporate the solvent for each fraction using the Rotovap. Record dry weight of each fraction, as this serves as a rough estimate of lipid recovered at this point.

17._____ Resuspend each lipid fraction in chloroform-methanol (2:1) to yield a concentration of 10 mg/ml and transfer to a 10 ml tube with PTFE cap, while maintaining respective labeling (1-20). The lipid may be resuspended at a higher concentration if necessary to allow the volume to fit in a 10 ml tube.

18._____ Perform analytical TLC of each fraction, while including proper controls. The solvent system should be chloroform:methanol (90:5) and the TLC should be developed using α-naphthol (note 9 & 10)

19._____ Pool the fractions containing bands indicative of PGL-I and that contain little contamination of other lipids in an appropriate sized pre-weighed round-bottomed flask and evaporate the solvent using the Rotovap. Record dry weight.

20._____ Resuspend PGL-I in 2-7 ml chloroform-methanol (2:1) and transfer to a pre-weighed 10 ml tube with PTFE cap. Evaporate solvent using the Nitrogen bath and record final weight of PGL-I.

21._____ Pool the fractions containing bands indicative of PGL-I and that contain some contamination of other lipids for further PGL-I purification via Preparative TLC. Repeat step #20.

QC:

22._____ Run purified samples on TLC and scan.

23._____ Perform an ELISA, doing an antigen titration using purified PGL-I sample starting with 1 μg and continuing with a 2-fold dilution. Use CS-48 undiluted as primary antibody and A5153 α–mouse 1:2500 in 3% BSA/PBS as secondary antibody. Do NOT use any tween in buffers!!!!

24._____ Aliquot as needed.

Notes:

1. See SOP SP004 for use of the lyophilizer.
2. When performing MALDI-MS use HPLC grade solvents, otherwise ACS grade solvents may be used.
3. All use of organic solvents and filtering should take place in a chemical fume hood.
4. All solvents should be properly discarded as hazardous waste.
5. See SOP SP037 for operation of the rotovap.
6. Recommendation for column size is 20 g of silica gel per gram of crude lipid. ~1 g of silica gel = 1 ml of solvent. The length of the stacking column should not exceed 16 cm in length, so changing to a larger diameter makes it more time efficient, a thinner column will not affect the yield, but will be more time consuming. Volume (ml) = $\pi r^2 h$, r= radius h= height of column.
7. The first four fractions are chloroform and serve to 'wash' the column as well as facilitate the absorption of crude lipid onto the column. Once the solvent line is level with the top of the silica gel in the column, add the solvent corresponding to the particular fraction and then elute the solvent while collecting the eluent until the solvent line is level with the top of the silica gel once again. Each fraction collected will contain some void volume from the previous solvent added; therefore, fraction 20 serves to purge the column of all the void volume.

8. Using air pressure while eluting the column will change the flow rate, however, does not seem to affect the point at which PGL-I is eluted from the column, at 2%-3% methanol, 98%-97% chloroform, respectively.
9. See SOP SP033 for running TLC.
10. Transfer of materials using microdispenser tubes may be done without a microdispenser pipette.