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Protocol status: Working
We use this protocol and it's working

Purification of Methylglucose Lipopolysaccharides (MGLP)

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ABSTRACT

Methylglucose lipopolysaccharides (mGLPs) are poly-methylated polysaccharides (PMPSs) that are acylated with short-chain fatty acids, uniquely synthesized by bacteria belonging to the Actinomycetales order. mGLPs are composed of ~20 glucose (Glc) units, most of them O-methylated (6-*O*-Me-Glc, Glc, and a 3-*O*-Me-Glc in a ratio of 11:8:1). Acyl functions in mGLPs are heterogeneous and contain acetyl, isobutyryl, succinyl, and octanoyl groups. mGLPs also known as 6-O-methylglucose-containing-lipopolysaccharides are believed to be essential in *Mycobacterium tuberculosis* and participate in the regulation of lipid metabolism, protecting fatty acids from degradation. Additionally, this cytosolic bacterial component is a potent stimulator of $\gamma_9\delta_2$ T cells.

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MATERIALS

1. ~100 g γ-irradiated H37Rv M. tuberculosis cells
2. 10:10:3 (chloroform:methanol:water)
3. 35 mL Teflon Oak Ridge tubes
4. Lyophilizer
5. 500 mL bottle
6. Chloroform, HPLC grade
7. Methanol, HPLC grade
8. MilliQ water
9. Magnetic stir bar
10. Magnetic stir plate
11. Glass tubes, 13 x 100 mm + Teflon caps
12. N2 bath
13. Light Sep-Pak C18 reverse phase columns (Waters, WAT023501)
14. Glass syringe, 10 mL
15. Glass vials, 2 mLs + Teflon caps
16. Glass pipets (5, 10 mL)
17. Rubber pipet bulb
18. Amicon® Ultra-15 Centrifugal Filter Unit, 10 kDa MWCO
19. Cytiva Microsep Advance Centrifugal Devices with Omega Membrane 3kDa
20. Pasteur pipettes with bulbs
21. Acetonitrile, HPLC grade
22. Water bath sonicator

PROTOCOL MATERIALS

α-Cyano-4-hydroxycinnamic acid **Bruker Catalog #8201344** Step 29

SAFETY WARNINGS

All the procedures described in the protocol should be performed in a biosafety level 2 laboratory with the proper personal protective equipment. The bacterial cells described in the initial steps have to be previously tested for viability before their use in the BSL2 laboratory setting. All steps involving organic solvents must be performed in a chemical fume hood.

Total lipid extraction

1 Obtain approximately 100 g Sample *Mycobacterium tuberculosis (Mtb)* cells and freeze dry by lyophilization.

Note

Before starting this protocol, *Mtb* cell inactivation must be tested and confirmed.

2 Determine the dry weight of the cells and transfer them to a 500 mL glass bottle.

3 Delipidate cells by adding 10mL of 10:10:3 [vol/vol/vol] chloroform: methanol: water per 1 gr of the *Mtb* cells. Add a stir bar and stir Overnight in a chemical fume hood. Make sure the bottle is tightly closed.

4 Transfer overnight extract into 35 mL Teflon Oak Ridge tube(s) and centrifuge at 20m
27000 x g, 15°C, 00:20:00
Note: The number of Teflon tubes would depend on the volume obtained in step 3.

5 Decant organic supernatant and preserve it in a labeled bottle.

6 Transfer the cells from tubes back to 500 mL bottle and repeat delipidation (steps 2 -5) two more times, reducing incubation time to 2 hours for each repeat. Be sure to vortex the cell pellet for one minute before repeating.

Note

The Overnight step can be done for either the 1st or 2nd round of extraction.

7 Collect all extracts into the same bottle (started on step 5).

8 Measure total volume and aliquot 0.5 mL into a previously weighed 2 mL glass vial. Dry under a Nitrogen bath and calculate mg/mL of total volume based on the net weight obtained.

9 Take 50 mg of your 10:10:3 total lipid and completely dry down via Nitrogen bath. Resuspend in MilliQ water at a concentration of 5 mg/mL (10 mL total). Use a water bath sonicator to thoroughly resuspend – may take several minutes. If necessary, can also add more water to further dilute and ensure complete resuspension.

Note

Do not let water resuspension sit out to avoid contamination build-up. If necessary, can store overnight in the fridge. Thorough resuspension is important to ensure no clogging during the 10 kDa filtration.

Removal of high molecular weight molecules

5m

10 Prepare your Amicon® Ultra-15 Centrifugal Filter Unit 10 kDa MWCO by adding 5 mL of MiliQ water and centrifuging at 3500 x g, 20°C, 00:05:00. Dispose the flow-through. 5m

Note

This step will remove any impurities from the amicon filter before using it with the sample. It is important to not let the filter over spin and dry out the filter membrane.

11 Add your total lipid extract from step 9 to the Amicon® Ultra-15 Centrifugal Filter Unit 10 kDa MWCO and 30m centrifuge at 3500 x g, 20°C, 00:30:00, **keeping the flow-through**. Check volume level: if there is only a small amount of flow-through, increase speed to 3750 – 4000 x g.

12 Repeat centrifugation until most of the volume is passed and the retentate volume is ~ 1 mL.

13 Rinse 10 kDa filter membrane using Pasteur pipette by adding 2 – 3 mL of MiliQ water, pipette up and down several times. Centrifuge for 10 minutes and **collect the flow-through**.

Note

Ensure the total volume of flow-through does not exceed 15 mL. If it does, transfer flow-through to a new tube before adding more water

Concentration of mGLP (~3.5-4 kDa)

14 Transfer pooled flow-through (from steps 11-13) to a Cytiva Microsep Advance Centrifugal Devices with Omega Membrane 3kDa (maximum capacity 4 mL).

15 Centrifuge at 3500 x g, 20°C for 10 minutes and dispose of flow-through. Add more of the 10 kDa flow-through until max capacity (4 mL) is reached again in the Cytiva Microsep.

Continue this process until all the 10 kDa flow-through has been passed through the 3 kDa filter and the retentate volume is reduced to ~ 1 mL.

16 Transfer retentate from previous step to 13 x 100 tube and thoroughly rinse 3 kDa filter 2X with 1 mL of water, pipetting up and down to collect any residual product before transferring to 13 x 100 tube. Record the total volume obtained.

17 Determine the total amount of crude material in 3 kDa retentate by aliquoting 0.5 mL into a pre-weighted 2 mL vial. Calculate mg/mL of total 3KDa crude material.

18 Aliquot 0.5 mg of 3 kDa retentate into a 13 x 100 tube and completely dry down on a N2 bath. Resuspend in 500 uL of water and dilute 1:10 by adding 4.5 mL of additional water.

Note

Use a conservative approach of no more than 0.5mg of crude mGLP – Over-saturation of the Sep-Pak cartridge can result in lower yield.

19 Prepare your Sep-Pak C18 cartridge, adding in order the following solutions, using a 10 mL glass syringe:
a. 4 mL 95% acetonitrile
b. 5 mL 100% methanol
c. 2 mL 80% methanol in chloroform

20 Equilibrate your cartridge by adding 3 mL of water; leave enough water in the column so that it is saturated and a droplet is visible at the bottom. Let sit for 5 minutes.

21 Load your diluted crude sample into the column and slowly elute.

22 Wash with 4 mL of water (no need to collect the flow-through).

23 Perform a series of elutions with the following, with a wash step in between each elution, using 4 mL of water :
a. 4 mL 30% methanol in water
b. 4 mL 60% methanol in water
c. 4 mL 90% methanol in water

Collect each elution and washes steps in a labeled and pre-weighted 13 x 100 tube.

Note

Caution: For the optimal function of the Sep-Pak C18 cartridge, the flow-rate when eluting liquid through the filters needs to go very slowly (i.e., one drop per 2 seconds).

24 Repeat Sep-Pak chromatography for as much material is needed. Around 6 to 8 cycles of Sep-Pak preparations would be needed for 50 mg of the 10:10:3 extract (obtained in step 9).

25 In the N_2 bath, dry the 60%, and 90% fractions obtained in the Sep-Pak runs, separately (mGLP is enriched mostly in the 60% fraction and in a less extent in the 90% fraction).

26 Determine the dried weight of the 60% and 90% fraction and resuspend in 0.5 mL of water. Record the concentration obtained for each fraction.

27 Apply 10µg -20µg of these fractions into an analytical TLC plate and run the plate in 56:38:10 Chloroform: Methanol: Water. After this, stain the TLC plate with α-Naphtol.

Figure 1. Example of TLC result using 10 ug of the 60% fraction obtained during mGLP purification.

28 After combining the 60% and 90% fractions, the yield of purified mGLP is about 4% of the 10:10:3 obtained material from step 9 (starting with 50 mg of the 10:10:3 extract, the yield of is approximately 4 mg).

29 Perform the MALDI-TOF for QC.

Note

For MALDI, use HCCA α-Cyano-4-hydroxycinnamic acid **Bruker Catalog #8201344** matrix, using the sandwich method in the negative mode.

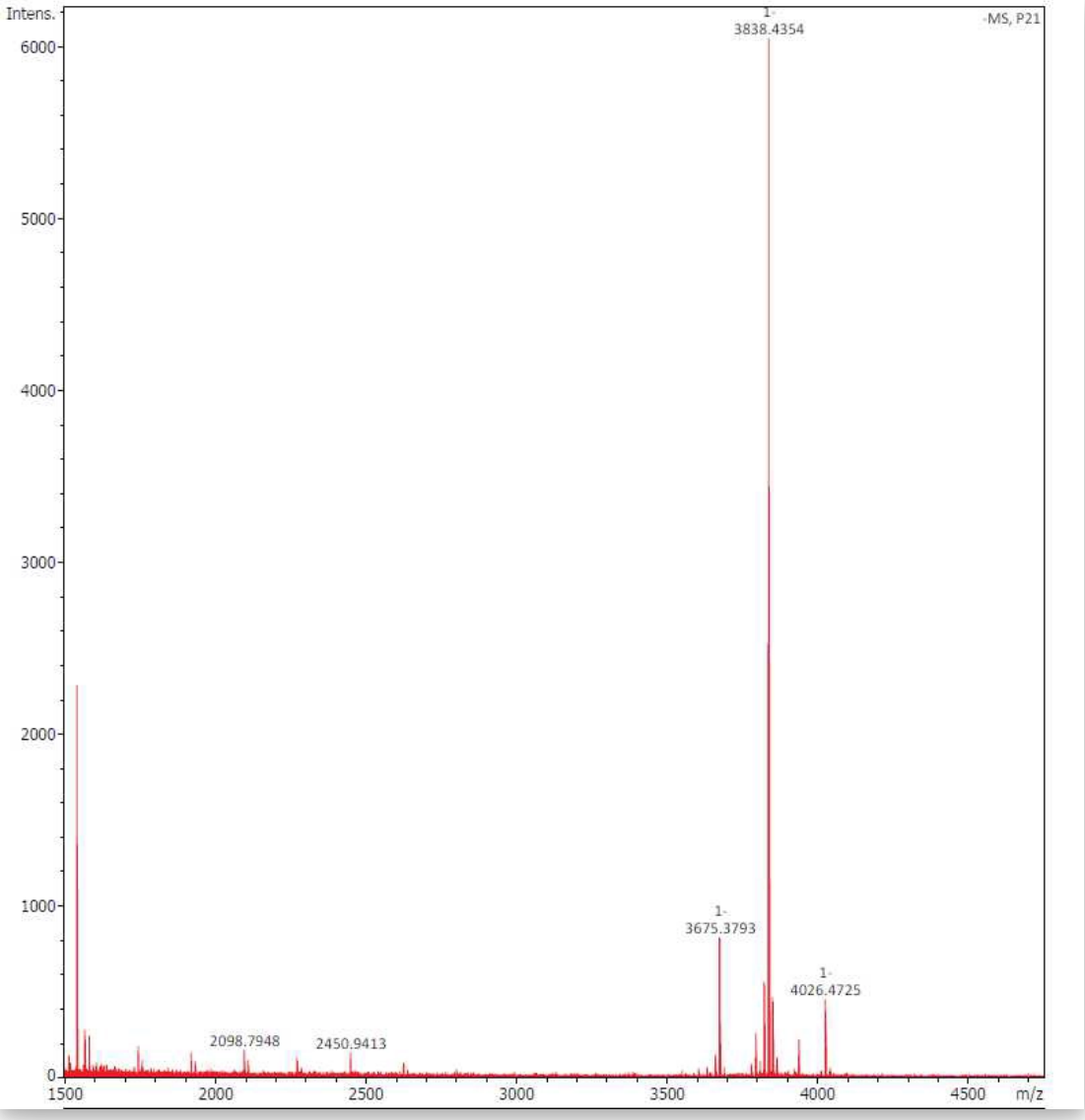

Figure 2. Example of MALDI-TOF results from purified mGLP. m/z values between 3500 to 4100, in this images predominant peak is at 3838.4354 m/z, with secondary peaks at 3675.3793 and 4026.4725 m/z.