Demannosylated LAM

SOP: PP059.1
Modified: 10/12/2022 Megan Stookey

Materials and Reagents:
1. QC verified purified Lipoarabinomannan (for demannosylation)
2. *M. smegmatis* purified Lipoarabinomannan
3. H37Rv purified Lipoarabinomannan
4. Sodium Acetate
5. Zinc sulfate
6. Digest Buffer: 0.02M Sodium Acetate, 5mM Zinc, pH 4.5
7. α-Mannosidase from Canavalia ensiformis (Jack bean) M7257-Sigma
8. Proteinase K stock solution (10mg/ml in water)
9. 37°C water bath
10. 13 X 100 mm glass tubes w/ lids
11. 100 kDa amicon ultra centrifugal filter
12. Micro BCA kit
13. Water, endotoxin-free
14. Stir plate
15. SDS-PAGE gel supplies
16. Alditol Acetate supplies
17. Concanavalin A lectin peroxidase
18. PBS
19. HRP substrate
20. Endozyme II recombinant Factor C Endotoxin Assay Kit – 890030 bioMerieux

Protocol:
1. _____ Take 1mg of alpha-mannosidase (Jack Bean) per every 3mg of LAM and centrifuge the Jack bean α-mannosidase solution at 16,000xg for 20 min (α-mannosidase concentration will vary per lot purchased)

2. _____ Discard supernatant from the centrifuged α-mannosidase (Jack Bean), then resuspend the pellet at a concentration of 1 mg/1 ml in Digest Buffer: 0.02M Sodium Acetate + 5mM Zinc pH:4.5 Buffer.

3. _____ Resuspend dried LAM at a concentration of 5 mg/ml in Digest Buffer.

4. _____ Add 0.5 mg α-mannosidase per 3 mg of LAM and incubate for 8 hours in 37°C water bath.

5. _____ After 8 hours, add the remaining 0.5 mg of α-mannosidase per 3 mg of LAM into the tube and incubate for an additional 16 hours in 37°C water bath.

6. _____ Take the sample and pass it over a 100 kDa amicon ultra centrifugal filter a total of 3 times making sure to keep the eluate as this is where the LAM should be located (note 1)

7. _____ For each 1 ml of sample, add 10 µl of Proteinase K stock solution for a final concentration of 0.1 mg proteinase K/ml. Incubate at 37°C overnight

8. _____ Dialyze the digest for 24 hours in running DI-water using the 3,500 MWCO Slide-A-Lyzer cassette (note 2)

9. _____ Move cassette to endotoxin free water for 24 hour dialysis.

10. _____ Remove from dialysis and transfer to a new 13 X 100 mm glass tube

11. _____ Run Micro BCA assay to estimate protein concentration. Run 10µg, 5µg and 2.5µg, calculated from original LAM concentration. Total protein amount must be less than 10% of the total LAM product (i.e. < 1 mg protein/10 mg LAM)

12. _____ Run a gel for quantitation: Using a smegLAM standard with known concentration, create a standard curve on the gel ranging from 1µg - 5µg, and run the demannosylated sample in various amounts (usually 0.5µl – 2 µl). Silver stain the gel (note 3) and use the Image J software (note 4) to create a linear regression to calculate the amount of demannosylated LAM based on densitometry.

13. _____ Run 2 µg of sample, 2µg H37Rv LAM, and 5µg HSPX on western blots (note 5). Develop using Con A (note 6) and CS-35 (anti-LAM antibody). The demannosylated LAM should have a size shift of compared to H37Rv LAM. HSPX is used to show lack of glycosylation

14. _____ Run a gel to further indicate demannosylation: Run 5µg of sample, 5µg H37Rv LAM, and 5µg HSPX. Silver stain the gel.

15. _____ Run Endozyme assay per manufacturers protocol and calculate endotoxin amount relative to the sample concentration. Endotoxin amount must be less than 10 ng/1 mg of LAM

16. _____ Freeze dry by lyophilization

Notes:

1. Follow manufacturer instructions to equilibrate the 100kDa filter and then when adding in sample make sure to wash the retentate multiple times to make sure all the LAM flows out into the eluate
2. Soak cassette in DI water for 20 minutes prior to sample injection
3. See SOP SP007 for running SDS-PAGE gels, and SOP SP012 for Silver Staining (use periodic acid step)
4. See SOP SP079 for Quantitation of LAM by Image J
5. See SOP SP011 for Western Blot
6. Follow manufacturer protocol for Con A western blot, but reduce the concentration of ConA lectin peroxidase to 5µg/10ml
7. If additional confirmation of demannosylation is needed, the sample can be run on GCMS for linkage analysis. To prepare the sample for analysis, a NaOH permethylation (Ciucanu, et.al.) needs to be done on 100 µg of the demannosylated LAM as well as Smeg and regular H37Rv LAM, followed by preparation of alditol acetate derivatives (SOP SP022).
   Materials:

   1. NaOH pellet (must be kept in decanter)
   2. Dry anhydrous DMSO
   3. Pasteur pipette
   4. Autosampler vials
   5. Mortar and pestle
   6. ACS grade Chloroform
   7. Methyl Iodide

GCMS: Linkages, Retention Times, and Ions

Linkage	Retention Time	Ions
t-Ara	8.53	118,129,161
2-Ara	10.12	129, 130, 161, 190
5-Ara	10.77	118, 129, 189
t-Man	11.54	102, 118, 129, 145, 161, 162, 205
3,5-Ara	12.16	118
2-Man	13.09	129, 161, 190
6-Man	13.69	102, 118, 129, 162, 189
2,6-Man	15.38	129, 190

- Demannosylation can be confirmed if no 2-Man peak can be found in the sample

References:

Ciucanu, I and Kerek, F et al. (1984) A simple and rapid method for the permethylation of carbohydrates study

Snaith, S. M., & Levvy, G. A. (1968). Purification and properties of alpha-D-mannosidase from jack-bean meal. *The Biochemical journal*, *110*(4), 663–670.