SOP: PP021.6
Modified: 2/23/2017 by MCL

Preparation of Purified Ag85 Individual Components (a, b, c)

Materials and Reagents:

1. Culture filtrate proteins (CFP) from *M. tuberculosis*, 300-600 mg
2. Ammonium bicarbonate
3. Dithiothreitol (DTT)
4. Sodium azide
5. MilliQ Water
6. Ammonium sulfate
7. Buffer A: 10 mM Potassium phosphate (pH 6.8), 1 mM EDTA, 1 mM DTT
8. Buffer B: 10 mM Tris-Base (pH 8.9), 1 mM EDTA, 1 mM DTT
9. Buffer C: 10 mM Tris-Base (pH 8.9), 1 mM EDTA, 1 mM DTT, (filter through 0.45μm filters before adding ethylene glycol) 50% ethylene glycol (v/v)
10. 100X Sodium Azide Stock Solution (2% NaN_3 in water)
11. 20% ethanol
12. Dialysis buffer (10 mM Ammonium bicarbonate, 1 mM DTT)
13. Trypsin modified sequencing grade
14. 15 % SDS-PAGE gels
15. 13x100 mm polypropylene culture tubes
16. 10 cc syringe
17. Transfer pipettes
18. 10 ml plastic disposable pipettes
19. Deplasticized tubes (see SOP: SP021)
20. 7 L Dialysis tank
21. Dialysis tubing (3,500 Da MWCO)
22. Filter bell funnel with Pall membrane filter (catalog number P/N 66548)
23. Lyophilizer flask
24. Waters HPLC system (high flow)
25. Lyophilizer
26. Waters fraction collector
27. 20 ml HiPrep Phenyl Sepharose HPLC column (GE Healthcare, Catalog #29018184)
28. Waters injection needle
29. High speed centrifuge
30. 250 ml, Centrifuge bottles
31. F16/250 rotor
32. ESI ion trap mass spectrometer
33. 120 ml Superdex-75 HPLC size exclusion column (GE Healthcare, Catalog # 17-1068-01)
34. Size Exclusion Buffer: PBS (pH7.4), 1mM DTT, 0.1% n-octylthioglucoside
35. 0.2μm filter
36. Amicon Ultra-15, 30000 MWCO (Fisher, Catalog # UFC903024)

Protocol:

1._____ Thaw the CFP at 4°C overnight.

2._____ Add a stir bar, place on a stir place and begin stirring. Slowly add ammonium sulfate to 40% saturation (note 2).

3._____ Stir at room temperature until ammonium sulfate is completely dissolved, then transfer sample to 4°C and stir for 4-16 hr.

4._____ Remove stir bar and centrifuge the CFP/ammonium sulfate solution at 27,000 x g, 4°C for 1 hour.

5._____ Prepare 7 L of dialysis buffer.

6._____ Decant the supernatant into a clean container (note 3). The pellet is the 40% ammonium sulfate cut.

7._____ Suspend the protein pellet in approximately 25-30 ml of dialysis buffer and pipet it into the dialysis tubing. Close the dialysis tubing and place the tube into the dialysis tank.

8._____ Dialyze at 4°C for 4-16 hours.

9._____ Change the dialysis buffer (7 L) and dialyze at 4°C for 4-16 hours.

10._____ Change the dialysis buffer to 7 L of 10 mM ammonium bicarbonate (without DTT) and dialyze at 4°C for 4-16 hours.

11._____ Collect the protein solution from the dialysis tubing and rinse the dialysis tubing with a minimal volume of fresh 10 mM ammonium bicarbonate. Place the protein solution along with the rinse in a sample tube.

12._____ Determine the protein concentration using the BCA assay (see SOP SP003).

13._____ Lyophilize the protein (see SOP SP004) (note 4).

14._____ Make phenyl sepharose buffers A, B, and C. Filter all of the buffers using the pall filter bell and 0.45 μm filters (make sure the filter bell has been cleaned and there is a new filter for each buffer).

15._____ Connect the 60 ml HiPrep Phenyl Sepharose HPLC column to the HPLC system (notes 5 and 6).

16._____ Wash the Phenyl Sepharose column with 5 column volumes (300 ml) of filtered water, at a flow rate of 2.0 ml/min, to remove the ethanol.

17._____ Prime line C with buffer C, prime line B with buffer B, prime line A with buffer A, line D in HPLC-grade water (note 7).

18._____ Equilibrate the Phenyl Sepharose column with 2 column volumes (120 ml) of buffer A.

19._____ Suspend the lyophilized protein in buffer A so that the final protein concentration is between 1.5 and 2.0 mg/ml.

20._____ Filter the protein suspension through a 0.2 μm filter.

21._____ Start the Empower HPLC program, select the Phenyl Sepharose method and set up the chromatography run (note 8).

22._____ Set up fraction collector for 120 minute wait, followed by 105 x 3 minute fractions.

23._____ Place 60 μl of 100X Sodium Azide Stock Solution in each fraction tube (optional: see note 9).

24._____ Draw 10 ml of the filtered protein solution into a 10 ml syringe. Attach the Waters injection needle. Tap the syringe to move any air bubbles to the top and expel all air from syringe and needle.

25._____ Move the HPLC injection lever to “load”, insert the needle into the injection lever and expel the liquid by pushing on the plunger. After all the liquid has been dispensed, remove the needle from the injection lever, move the lever to “inject”.

26._____ If more injections are required, wait 6 minutes, then repeat injection (steps 24-25). Repeat as many times as necessary to inject all material, **being sure to collect and save the flow through from the injection and wash** (note 10).

27._____ After injecting approximately half of the sample, wash the column for 1 column volume (60ml) before loading the remaining sample.

28._____ Before the final injection, click on the “inject” icon on the Empower software so that the program is initiated with the final injection.

29._____ Upon completion of the run, remove the tube holder from the fraction collector and remove 8 µl from each fraction and place in a 0.65 ml eppendorf tube for analysis by SDS-PAGE.

30._____ Speak with PI or lab manager to determine whether to continue on with step 30 or begin SOP: PP053 ‘Ag85a,b,c MALDI-TOF/TOF MS Protocol’ and then continue at step 37 of this protocol.

31._____ Place the fractions from the fraction collector tray into a test tube rack and store at -20°C.

32._____ Add 2 µl of 5X loading buffer to the aliquots and run on SDS-PAGE and stain with coomassie, simply blue stain, or other mass spec compatible stain (see SOP: SP007, SP013 for coomassie staining, or manufacturer instructions for other stains).

33._____ Cut the Ag85 spots from every other fraction and place in deplasticized tubes.

34._____ Follow the SOP for modified in-gel digestion (see SOP SP021).

35._____ Prep the samples for analysis by ES-MS-MS.

36._____ Once the MS and MS/MS data are collected, analyze this data using the Sequest software to determine which fractions contain the individual components (A, B, and C) of the Ag85 complex.

37._____ Pool individual components according to the MS/MS or MALDI data and SDS-PAGE results.

38._____ Use centricon-70 or amicon ultra-15 to concentrate pools. Wash three times with 10 mM ammonium bicarbonate to remove any residual buffers Determine protein amount by BCA.

39._____ Run 2 µg of the pooled components on SDS-PAGE to check purity (note 12).

40._____ Lyophilize the protein.

41._____ Set up the Superdex-75 size exclusion column on the waters HPLC.

42._____ Wash the column in at least 1CV (120 ml) water.

43._____ Equilibrate the column in at least 1CV size exclusion buffer.

44._____ Resuspend the dry sample in no more than 7 ml size exclusion buffer.

45._____ Filter the protein suspension through a 0.2 µm filter.

46._____ Start up the Empower program and select the S-75 method set (note 13).

47._____ Inject sample and start fraction collector as in step 24-25 (note 14).

48._____ Run 10 µl of each fraction on a gel.

49._____ Pool all fractions containing clean Ag85.

50._____ Concentrate using amicon ultra-15 30,000 MWCO centrifugal device and wash three times with 10mM ambic (note 14).

51._____ Run BCA, and run 1 and 2 µg on gel and western blot (SOP: SP011) (develop using CS-90 primary antibody) for QC.

52._____ Make aliquots (default quantity = 0.25 mg), lyophilize, and store at -80°C.

Notes:

1. Preparation of Phenyl Sepharose Buffer A:
   Prepare 1 M Stock Solution of KH_2PO_4 (250 mL)
   KH_2PO_4 34.02 g
   Check pH to confirm that it is within the working range listed on the product label (4.1-4.5)
   Dilute to 10 mM working stock when ready to use.
   Prepare 1 M Stock Solution of K_2HPO_4 (250 mL)
   K2HPO4 43.55 g
   Check pH to confirm that it is within the working range listed on the product label (8.7-9.3)
   Dilute to 10 mM working stock when ready to use.
   Buffer A (1L):

10 mM KH_2PO_4	600 mL
10 mM K_2HPO_4	400 mL
0.5 M EDTA	2 mL
DTT	154 mg (added when ready to use)
pH 6.8	

   Adjust pH by adding more monobasic (lowers pH) or dibasic (raises pH) potassium phosphate. Or if more drastic changes are needed, a small amount of phosphoric acid can be added to lower pH. Because the total volume can change significantly using this method, it is suggested that you calculate the amount of EDTA needed after the final volume is achieved.
2. Determine the appropriate amount of ammonium sulfate using the calculator at http://www.encorbio.com/protocols/AM-SO4.htm.
3. The supernant can be immediately used for further ammonium sulfate cuts, or saved at -20°C (see SOP SP035 and SP024).
4. Both Ag85 and Mpt32 are purified from the 40% cut and the purification steps can be performed in either order. You can either proceed with the protocol as written here, or run the sample over a ConA column for Mpt32 purification and collect the flow through for running on the phenyl sepharose column (see SOPs PP019 and PP022). Performing the protocol in this order can help reduce the amount of protein applied the phenyl sepharose column, which allows for better protein binding.
5. Before using the HPLC and Empower HPLC program, read the HPLC SOP:SP025 or have lab personnel trained in the use of the HPLC assist you in setting up the liquid chromatography of the Ag85.
6. A 20 ml Phenyl Sepharose column is also available for smaller samples. If using a different size column, adjust the times in the program listed in note 8 to accommodate the necessary volumes.
7. This order is best so that the main line is in buffer A for the start of the column.
8. The run parameters are as follows:
   Flow rate = 2 ml/min
   Fractions = 105 x 3 min fractions, starting at 120 min
   Column capacity = 600 mg protein
   Column Volume (CV) = 60 ml

5 CV	Injection/Buffer A Wash	150 min
2 CV	A→B Gradient	60 min
2 CV	100% B	60 min
1 CV	100% B→50% B/50% C Gradient	30 min
1 CV	50% B/50% C	30 min
1 CV	50% B/50% C→100% C Gradient	30 min
2 CV	100% C	60 min
0.5 CV	C→A Gradient	15 min
2 CV	100% A	60 min
		495 min = 8hr 15min

9. Sodium azide is used as a preservative to keep anything from growing in the samples if they are stored for long periods of time, however all buffers and samples containing sodium azide must be discarded as hazardous waste. Therefore, adding sodium azide directly to the sample tubes reduces the amount of waste produced compared to adding it to the HPLC buffers themselves. If the samples will be processed immediately, sodium azide can be omitted entirely.
10. If the 40% cut was not already passed over a ConA column as described in note 4, the phenyl sepharose flow through should be saved for Mpt32 purification (see SOPs PP019 and PP022). Be sure to process the flow through in a timely manner, and that DTT is maintained in the dialysis to preserve protein integrity of the Mpt32. In addition, Ag85B will often elute in the Buffer A wash, so it is important to analyze the flow through and wash for the presence of Ag85. If necessary, the wash can be exchanged into a higher buffer concentration (50mM potassium phosphate) a passed back over the column to increase recovery.
11. If sodium azide was added to the HPLC fractions, the initial amicon/centricon eluate and the first buffer exchange eluate must be disposed of as hazardous waste.
12. The Ag85 components should be approximately 90-95% pure as determined by Mass Spec, SDS-PAGE and silver staining.
    - If there is contamination from other proteins, continue on with the remaining steps of the SOP.
    - If more separation of the components is required based on mass spectrometry results (for example, if A didn't separate from C), repeat liquid chromatography with the Phenyl Sepharose column following the previously described procedure.
    - If the protein is determined to be pure, proceed to step 52.
13. The program for the Sephadex-75 column is as follows:

    Flow Rate = 1.5 ml/min
    Fraction program = 20 minute wait
    30 x 2 min fractions
    30 minute wash
14. Unlike the phenyl sepharose column, only one injection is done for the size column.
15. The large membrane size will help to remove any remaining low molecular weight contaminants.

References:

Belisle J.T., V.D. Vissa, T. Sievert, K. Takayama, P.J. Brennan, and G.S. Besra. 1977. Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. Science **276**: 1420-1422