SOP: PP020.4
Modified: 02/23/2017 by MCL

Preparation of Purified Ag85 Complex

Materials and Reagents:

1. Culture filtrate proteins (CFP) from *M. tuberculosis* (~300mg)
2. Ammonium bicarbonate
3. MilliQ Water
4. Ammonium sulfate
5. Buffer A: 50 mM Potassium phosphate (pH 6.8), 1 mM EDTA, 1 mM DTT (1L) (note 1)
6. Buffer B: 10 mM Tris-Base (pH 8.9), 1 mM EDTA, 1 mM DTT (1L)
7. Buffer C: 10 mM Tris-Base (pH 8.9), 1 mM EDTA, 1 mM DTT, 50% ethylene glycol (v/v) (500ml)
   *** Buffers A, B, and C must be made fresh every 14 days- DTT must be refreshed every day***
8. 70% ethanol
9. Dialysis buffer (10 mM Ammonium bicarbonate, 1 mM DTT)
10. 15% SDS-PAGE gels
11. 13x100 mm polypropylene culture tubes
12. 10 cc syringe
13. Transfer pipets
14. 150 mL plastic container
15. 10 ml plastic disposable pipets
16. Dialysis tank
17. Dialysis tubing (3,500 Da MWCO)
18. Filter bell funnel with Pall membrane filter (catalog number P/N 66548)
19. Lyophilizer flask
20. Waters HPLC system (high flow)
21. Lyophilizer
22. Waters fraction collector
23. 60 ml Phenyl Sepharose HPLC column
24. Waters injection needle
25. Amicon ultrafiltration system with a 10,000 MWCO membrane (catalog number PLGC07610)
26. High speed centrifuge
27. Centrifuge bottles, 250 ml
28. F16/250 rotor
29. 120 ml Sephadex-75 HPLC size exclusion column
30. Size Exclusion Buffer: PBS (pH7.4), 1mM DTT, 0.1% n-octylthioglucoside
31. Amicon ultra-15 30,000 MWCO centrifugal device
32. 0.2 µm acrodisc syringe filter

Protocol:

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

2._____ Add a stir bar, place on a stir plate 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 buffers using the pall filter bell and 0.45µm filters (make sure the filter bell has been cleaned and there is a new filter for each buffer).

15._____ Connect the 60 ml 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 HPLC grade 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 (note 7).

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

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

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

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

22._____ 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.

23._____ 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 and move the lever to “inject”.

24._____ If more injections are required, wait 6 minutes, then repeat injection (steps 22-23). 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 9).

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

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

27._____ Use centricon-70 or Amicon to concentrate pools. Wash three times with 10 mM ammonium bicarbonate to remove any residual buffers (note 10).

28._____ Determine the protein concentration by BCA assay (SOP: SP003).

29._____ Run a gel of the pooled Ag85 complex to check purity (note 11).

30._____ Lyophilize the protein.

31._____ Set up the Sephadex-75 size exclusion column on the waters HPLC.

32._____ Wash the column in 120 ml water.

33._____ Equilibrate the column in 120 ml size exclusion buffer.

34._____ Resuspend the dry sample in approximately 7 ml size exclusion buffer.

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

36._____ Start up the Empower program and select the S-75 method set (note 12).

37._____ Inject sample and start fraction collector as in step 22-26 (note 13).

38._____ Run 8 µl of each fraction on a gel and silver stain (SOP: SP007 and SP012).

39._____ Pool all fractions containing relatively clean Ag85.

40._____ Concentrate using amicon ultra-15 30,000 MWCO centrifugal device and wash three times with 10 mM ammonium bicarbonate.

41._____ Run BCA, gel, and western blot (SOP: SP011) using CS-90 antibody, for QC.

42._____ Make aliquots (default quantity = 0.5 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 50 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 50 mM working stock when ready to use.
   - Buffer A (1L):

50 mM KH_2PO_4	600 mL
50 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 supernatant 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 column flow thru 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.
6. A 20 ml Phenyl Sepharose column is also available for smaller samples. If using this 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 = collect flow thru, wash, and gradient (120-315 min) as separate pools
    Column capacity = 600 mg protein
    Column Volume (CV) = 60 ml

4 CV	Injection/Buffer A Wash	120 min
0.5 CV	A→B Gradient	15 min
1.5 CV	100% B	45 min
0.5 CV	B→C Gradient	15 min
3 CV	100% C	90 min
0.5 CV	C→A Gradient	15 min
2 CV	100% A	60 min
		360 min = 6 hr

9. 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.
10. See SOP: PP006 for details on how to set up the amicon (steps 1-12). Disregard the use of the 10 L amicon reservoir. Check the amicon every 30 minutes. When the sample is concentrated down, turn off the nitrogen and vent the system. Open the lid and add the ammonium bicarbonate to wash. After the last wash, remove the sample and rinse the membrane.
11. The Ag85 complex should be approximately 90-95% pure as determined by SDS-PAGE and silver staining. If this level of purity has been achieved, then move on to the QC (step 43). If more purification is required, continue on with the remaining steps of the SOP.
12. 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

13. Unlike the phenyl sepharose column, only one injection is used for the size column.

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