SOP: PP024.9
Modified: 7/8/2019 MCL

Purification of PhoS1 (38kDa protein) from the CFP

Materials and Reagents:

1. Culture filtrate proteins (CFP) from *M. tuberculosis* or 50% cut supernatant (see SOP PP035)
2. Ammonium sulfate
3. Endotoxin-free water
4. Dialysis buffer (10mM ammonium bicarbonate, 1mM DTT)
5. Concanavalin A-Sepharose 4B resin (Sigma, C9017)
6. ConA Binding Buffer A: 50mM KH_2PO_4, 500mM NaCl, 1mM each of $MgCl_2$, $CaCl_2$, $MnCl_2$ and DTT (note 1)
7. ConA Elution Buffer B: Binding buffer with 0.225 M Methyl D-mannose (note 2)
8. 5 ml HiTrap phenyl sepharose column
9. Phen Seph Buffer A: 1M ammonium sulfate, 50mM Sodium Phosphate, 1mM DTT, pH 7.4 (note 3)
10. Phen Seph Buffer B: 50mM Sodium Phosphate, 1mM DTT, pH 7.4 (note 4)
11. 12 x 75 mm disposable culture tubes
12. Polypropylene oak ridge tubes
13. Dialysis tubing, 3500 MWCO
14. Dialysis tank
15. Amicon ultra-15, 30K MWCO
16. Amicon ultra-15, 10K MWCO
17. Biorad Econopump chromatography system
18. Sorvall centrifuge

Protocol:

1._____ If starting from CFP, thaw at 4°C overnight. If starting with 50% supernatant, skip to step 8.

2._____ Add a stir bar, place on a stir plate and begin stirring. Slowly add ammonium sulfate to 40% saturation (note 5).

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

4._____ Remove stir bar and centrifuge the CFP/ammonium sulfate solution at 10,000 x g, 4°C for 30 minutes.

5._____ Decant to supernatant into a clean bottle and save the pellet at 4°C (note 6).

6._____ To the supernatant, slowly add ammonium sulfate to 50% saturation (note 5), being sure to calculate based on the starting concentration of 40%.

7._____ Repeat incubation and centrifugation. Decant supernatant into a clean bottle and save the pellet at 4°C (note 7).

8._____ To the supernatant, slowly add ammonium sulfate to 70% saturation (note 5), calculated based on the starting concentration of 50%.

9._____ Repeat incubation and centrifugation.

10._____ Decant supernatant. The pellet is the 70% ammonium sulfate cut to be used for 38kDa starting material.

11._____ Resuspend the pellet in 25-30 mL 10 mM ammonium bicarbonate and transfer to dialysis tubing.

12._____ Dialyze in 7 L dialysis buffer at 4°C for 4-16 hours.

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

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

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

16._____ Quantitate protein by BCA (see SOP: SP003), and lyophilize.

17._____ Into an open column, pour a volume of Con-A resin which will give a ratio of 1 ml packed resin: 2 mg protein (note 8).

18._____ Pack and equilibrate the column with 3-5 column volumes (CV) ConA Binding Buffer at a flow rate of 2 ml/min using the Biorad Econopump (note 9).

19._____ Resuspend the dried sample in ConA Binding Buffer at a concentration of 1 mg/ml.

20._____ Load the sample onto the column at 1 ml/min and pass the flow through back over the column. Collect as "ConA Flow Thru".

21._____ Run the following gradient at 1.5 ml/min:
3 CV Binding Buffer A Wash
4 CV A→B Gradient
1 CV 100% Elution Buffer B Hold
Collect the Wash as "ConA Wash", then collect 40 fractions during the gradient.

22._____ Run 8 µl of each fraction on a gel.

23._____ Once it is determined which fractions contain 38kDa, run 8 µl of each on a western blot probed against Rv1411c (a common contaminant that is difficult to remove), being sure to include a positive control (CFP or recombinant Rv1411c).

24._____ Pool all of the fractions that are predominantly 38kDa with little-to-no Rv1411c contamination.

25._____ Concentrate the pool using an amicon ultra-15 30K MWCL and wash three times with 10 mM ammonium bicarbonate.

26._____ Quantitate by BCA, visualize the pool by SDS-PAGE, and lyophilize.

27._____ Suspend lyophilized ConA pool in Phenyl Sepharose Buffer A at a concentration of 1 mg/ml and stir or rock at 4°C overnight (note 10).

28._____ Pump 25 ml (5 CV) of endotoxin-free water through the HiTrap column at 1 ml/min to elute the storage buffer.

29._____ Pump 25 ml of Buffer A through the column at 1 ml/min to equilibrate the column.

30._____ Centrifuge the resuspended sample at 3000 rpm, 4°C, for 10 minutes to remove any precipitate.

31._____ Pump the sample (supernatant) onto the column at a flow rate of 1 ml/min, the pass the flow through back over the column. Collect as "Phen Seph Flow Thru".

32._____ Run the following gradient at 1 ml/min:
2 CV Buffer A Wash (10 min)
20 CV A→B Gradient (100 min)
10 CV Buffer B Clean-Up (50 min)

Collect the wash as “Phen Seph Wash”, then collect 40 x 2.5 min fractions during the gradient. Collect the final clean up as “Phen Seph Clean-Up” (note 11).

33._____ Run 10 µl of each fraction on a gel and check for Rv1411c contamination by western blot.

34._____ Pool all fractions containing clean 38kDa.

35._____ Concentrate using amicon ultra-15 10K MWCO and wash three times with 10 mM ammonium bicarbonate.

36._____ Run BCA, gel, and blot using IT-23 (+) and Rv1411c (-) antibodies, for QC.

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

Notes:

1. Con A Binding Buffer A:
   To 80 ml of endotoxin-free water stirring on a stirplate, add the following:

KH_2PO_4	0.69 g
NaCl	2.92 g
$MgCl_2 \cdot 6H_2O$	20.3 mg
$CaCl_2 \cdot 2H_2O$	14.7 mg
$MnCl_2 \cdot 4H_2O$	19.8 mg
DTT	15.4 mg

   After all reagents have gone into solution, add NaOH dropwise until the pH is 5.7. Transfer to a graduated cylinder and bring to final volume of 100 ml with endotoxin-free water.
2. Con A Elution Buffer B: Dissolve all the above reagents in 70 ml of endotoxin-free water plus D-mannose 9.01 g. Titrate to pH = 5.7 and bring to final volume of 100 ml.
   Con A Elution Buffer C: Dissolve all the above reagents in 70 ml of endotoxin-free water plus Methyl α-D-mannopyranoside 9.71 g. Titrate to pH = 5.7 and bring to final volume of 100 ml.
3. Buffer A:
   To 70 ml of endotoxin-free water stirring on a stir plate, add the following reagents:

ammonium sulfate	13.22 g
NaH_2PO_4	0.114 g
Na_2HPO_4	1.0865 g
DTT	15.3 mg

   When all reagents have gone into solution, titrate to pH = 7.4. Bring final volume to 100 ml with endotoxin-free water.
4. Buffer B:
   To 70 ml of endotoxin-free water which is stirring on a stir plate, add the following reagents:

NaH_2PO_4	0.114 g
Na_2HPO_4	1.0865 g
DTT	15.3 mg

   When all reagents have gone into solution, titrate to pH=7.4. Bring final volume to 100 ml with endotoxin-free water.
5. Determine the appropriate amount of ammonium sulfate using the calculator at http://www.encorbio.com/protocols/AM-SO4.htm.
6. The pellet is the 40% ammonium sulfate cut and is used as starting material for Ag85 (see SOP PP020 and PP021) and Mpt32 (SOP PP022). This material should be resuspended and dialyzed along with the other ammonium sulfate cuts, then freeze-dried and stored at -80°C for future use.
7. The pellet is the 50% ammonium sulfate cut and is used as starting material for GroES (see SOP PP035). This material should be resuspended and dialyzed along with the other ammonium sulfate cuts, then freeze-dried and stored at -80°C for future use.
8. Measure approximately 1.4 ml resin slurry for every 1 ml of desired packed resin. For large preparations of CFP, it may be necessary to run more than one column, due to the amount of time necessary to run large columns.
9. The column can be packed and equilibrated the day before use, as long as there is sodium azide present in the buffer. All buffers containing sodium azide must be disposed of as hazardous waste.

10. Even if the protein appears to go into solution it will often crash out of solution on the phenyl sepharose column, therefore overnight incubation is essential.
11. To clean the column for storage, pump 5 column volumes of water, then 5 column volumes of 20% ethanol. Cap both ends of the column and store at 4°C.