

Purification of 38 kDa Antigen of *Mycobacterium Tuberculosis* by Two Dimensional Preparative Electrophoresis

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[Abstract] Serodiagnosis of tuberculosis using purified native antigens is one of the approach for the early detection of TB. This protocol gives the details for growing *Mycobacterium tuberculosis* in liquid culture and one proven way to get a good yield of the total secreted antigens in the culture supernatant. In addition the procedure for purification of the 38 kDa antigen from the total culture filtrate antigens is also explained. 38 kDa is one of the specific antigen for *Mycobacterium tuberculosis* complex and in this particular study it was used as one of the antigens for serodiagnosis of different category of *tuberculosis* patients.

Materials and Reagents

1. *Mycobacterium tuberculosis* (*M tuberculosis*) H37Rv
2. Glycerol
3. Potassium dihydrogen-orthophosphate (anhydrous)
4. Magnesium sulphate (7H₂O)
5. Citric acid
6. Ferric ammonium sulphate
7. Sodium glutamate
8. Casein hydrolysate
9. Milli Q water
10. Sodium azide
11. BCA assay kit (Pierce Antibodies, catalog number: 23252)
12. Protease inhibitor (Roche Diagnostics, catalog number: 05-056-489-001)
13. Phenyl methyl sulfonyl fluoride (PMSF)
14. Urea
15. Digitonin (Sigma-Aldrich, catalog number: D141-100MG)
16. CHAPS
17. Carrier ampholytes
18. 6x SDS sample buffer
19. Glass beads

20. Protein dialysis bag (Thermo Fisher Scientific, catalog number: 88244)
21. McCartney bottle
22. Millipore filter (0.22 micron) (EMD Millipore, catalog number: SLAP02550)
23. Sauton's liquid medium (see Recipes)

Equipment

1. Centrifuges
2. Magnetic stirrer
3. Preparatory Preparative isoelectric focusing (IEF) cell (Bio-Rad Laboratories, Rotofor, catalog number: 170-2906)
4. Model 491 Prep Cell (Bio-Rad Laboratories, catalog number: 170-2926)
5. Bacterial incubator
6. Protein concentrator 3 kDa cut-off-stirred cell (Life Technologies, Ambion®, catalog number: 5123)
7. Circulating water bath
8. 3 mm single glass beads

Procedure

- A. Preparation of culture filtrate antigens from *Mycobacterium tuberculosis*
 1. *M. tuberculosis* H37Rv colonies were transferred from the Lowenstein Jensen (LJ) slopes to 2 ml of Sauton's liquid medium. LJ media is widely used in *M. tuberculosis* culture and contains glycerol, malachite green, coagulated egg and poured in McCartney bottles to solidify. Bacterial clumps are treated with 2 ml of LB media and 3 mm single glass beads, briefly vortex and spin the suspension briefly at low speed or leave it undisturbed for a few minutes for the clumps to settle and transfer the supernatant containing the bacteria for culture.
 2. The bacterial cell suspension was then transferred to 10 ml of Sauton's liquid medium in a McCartney bottle for incubation at 37 °C for 2 weeks.
 3. The bacilli were then transferred to 200 ml of Sauton's liquid medium and grown as shaker culture for 4 weeks. Inoculum from log phase culture is usually added to the culture media in 1/100th dilution for propagation of large volume mycobacterial culture.
 4. The log phase cells were again transferred to a 4 L culture flask containing 1.5 L of Sauton's medium and grown as stationary culture for four weeks at 37 °C.
 5. The mycobacterial cells continue to multiply during this period and form a surface pellicle.

6. The bacilli in the culture were harvested by centrifugation at 5,000 rpm (3,000 x g) for 30 min.
7. The culture supernatant was sterile filtered using Millipore filter (0.22 micron) to ensure complete removal of the bacilli and the supernatant containing the culture filtrate antigen was concentrated using 3 kDa cut-off membrane in stirred cell.
8. The protein content in the culture filtrate antigen was estimated using BCA assay kit.
9. The antigen was then distributed into smaller volumes along with sodium azide (0.002%) and protease inhibitor, PMSF at a final concentration of 2 mM per ml each.
10. The Culture Filtrate Antigen (CFA) was then stored at -70 °C for later use. This was used as the starting material to purify 38 kDa and 30 kDa antigens.

B. Purification of 38 kDa antigen of *Mycobacterium tuberculosis* by two dimensional preparative electrophoresis.

1. Purification of the 38 kDa antigen

The 38 kDa lipoprotein of *M. tuberculosis* H37Rv CFA was purified by two dimensional electrophoresis (2-D). The first dimensional separation was based on the pI value of the protein using Preparative isoelectric focusing technique, followed by Preparatory SDS-PAGE wherein the proteins were separated based on their molecular weight.

Two dimensional preparative electrophoresis (2-D)

a. Preparative isoelectric focusing

The preparatory IEF cell was used for the first dimensional separation of proteins based on the fact that all proteins have a pH dependent net charge. During electrofocusing, the proteins are concentrated in discrete liquid fractions at their respective isoelectric points in an established pH gradient where the net charge on the protein is zero.

i. Sample preparation

Whole *M. tuberculosis* H37Rv CFA (100 mg) was dialysed overnight against Milli Q water (4 L with 3- 4 changes of water advised at 3 -4 h interval) and then treated with 4 M urea (13.2 g), 0.2% Digitonin (137.5 mg of 80% stock), 0.2% CHAPS (110 mg), 10% glycerol (5.5 ml) and 1% Carrier ampholytes pH range 4-6 (0.5 ml). The final volume was made up to 55 ml with Milli Q water.

ii. Experimental conditions

The sample was loaded onto the preparatory IEF cell and focusing was carried out at 12 W constant power for 4-5 h at RT with cooling provided by a circulating cold water bath. According to the manufacturer's instructions the Rotofor cell must be run with cooling or excessive heating may occur, damaging the unit. A refrigerated circulating water bath is recommended to keep the coolant temperature at 4 °C. The initial

conditions were 500 V and 20 mA (30 min). At equilibration (4-5 h), the conditions were 1,200 V and 10 mA. Twenty fractions of 2.5 ml each were collected, their pH measured, and their polypeptide composition was analyzed by SDS-PAGE.

b. Preparatory SDS-PAGE

The Model 491 Prep Cell was used for the continuous elution electrophoresis. During the run, samples were electrophoresed through a cylindrical SDS-PAGE. After optimization, 10% gel was used, wherein, the RF (Relative Front) value of 38 kDa protein was between 0.55-0.6.

i. Sample preparation

Rotofor fractions containing 38 kDa antigen were pooled, dialyzed extensively against distilled water at 4 °C. The proteins concentrated (savant speed vac concentrator) up to 2.5 ml were dissolved in 0.5 ml of 6x SDS sample buffer (composition of 6x SDS sample buffer: 0.35 M Tris HCl (pH 6.8), 10% SDS, 30% glycerol, 9.3% DTT, do not make up the volume) and applied to the model 491 Prepcell for purification.

ii. Experimental conditions

Total volume of sample applied: 3 ml

Percentage of resolving gel: 10%

Height of resolving gel: 6 cm

Percentage of stack gel: 4%

Height of stack gel: 1.5 cm

Run buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS

Elution buffer: PBS (0.01 M, pH 7-7.2)

Run condition: 12 W for 8 h

- c. After elution of Bromophenol blue tracking dye, the fractions were collected at the rate of 1.5 ml/min. The polypeptide composition of every tenth fraction was analyzed by SDS-PAGE. When the region with protein of interest was identified, every fraction within that region was analyzed to determine the level of contamination. Fractions containing the purified 38 kDa antigen were pooled and concentrated.

Recipes

1. Sauton's liquid medium

Glycerol 30 ml

Potassium dihydrogen -orthophosphate (anhydrous) 0.5 g

Magnesium sulphate (7H₂O) 0.5 g

Citric acid 2.0 g

Ferric ammonium sulphate 0.05 g

Sodium glutamate 4.0 g
Casein hydrolysate 500 mg
Milli Q water 950 ml

The components of the medium were mixed and dissolved completely using a magnetic stirrer. The medium was adjusted to pH 7.2 with 40% Potassium hydroxide (KOH) (Approximately 5 ml of 40% KOH is needed). The medium was then made up to 1 L with Milli Q water and sterilized at 15 lb psi for 20 min.

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