Preparation of Pneumococcal Proteins for Western Blot Analysis

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[Abstract] This protocol was developed in a study aimed to determine the cellular localization of the lysin of pneumococcal phage SV1 (Frias *et al.,* 2013). We obtained proteins from the surface of *Streptococcus pneumoniae* by elution with choline or those secreted to the medium. The analysis by Western blot of these fractions allowed us to demonstrate that the phage lysin localizes to the cell wall, associating with choline residues in the teichoic acids. Hence, protein extracts can be used to determine the localization of uncharacterized proteins and can also be useful for other biochemical analyses such as protein identification. This protocol can be easily adapted to different pneumococcal strains and growth conditions and it is well suited to isolate other proteins of interest.

Materials and Reagents

1. Pneumococcal cells
2. Mitomycin C (MitC) (0.1 µg/ml) (Sigma-Aldrich, catalog number: M0503)
3. 1x PBS (10x PBS pH 7.2) (Life Technologies, Gibco®/Invitrogen®, catalog number: 70013-016)
4. 50 mM Tris pH 7.5
5. 2% (w/v) Choline chloride in 1x PBS (Sigma-Aldrich, catalog number: C7527)
6. NaCl (AppliChem, catalog number: A46615000)
7. Tris-HCl (Bio-Rad Laboratories, catalog number: 161-0799)
8. Glycerol (AppliChem, catalog number: A2364, 5000)
9. SDS (Bio-Rad Laboratories, catalog number: 161-0416)
10. β-mercaptoethanol (Sigma-Aldrich, catalog number: M3148)
11. Bromophenol blue (Bio-Rad Laboratories, catalog number: 161-0404)
12. Casamino acids
13. L-Tryptophan
14. L-Cysteine·HCl
15. Glutamine
16. Adenosine

17. Uridine
18. Nicotinic acid
19. Pyridoxine
20. Ca-pantothenate
21. Thiamine-HCl
22. Riboflavin
23. Biotin
24. Asparagine
25. Casein-based semisynthetic medium C+Y (Lacks and Hotchkiss, 1960) (see Recipes)
26. Loading buffer 5x (see Recipes)
27. C+Y with 2% choline chloride (see Recipes)

Equipment

1. Water bath at 37 °C to grow bacterial cultures
2. Cell density meter (Biochrom WPA CO8000 Cell Density Meter) (Biochrom, catalog number: 80-3000-45)
3. Centrifuges
4. 0.2 µm low-binding-protein membrane (DISMIC-03CP) (Advantec, catalog number: 03CP020AS)
5. 0.22 µm membrane filter (Frilabo, catalog number: 1520012)
6. Amicon Ultra-15 centrifugal filter unit, cut-off 10 kDa (Merck Millipore, catalog number: UFC901024)
7. Western blot equipment

Procedure

A. Extraction of choline-binding proteins by choline wash

1. Grow lysogenic cells without holin activity, hence incapable of lysis, in C+Y at 37 °C until OD_{600} nm of approximately 0.9 is reached (overnight culture). We used a lysogenic strain since this protocol was developed to determine the localization of the phage lysin. Moreover, since holin function activates phage-mediated lysis, we eliminated holin activity in this strain to avoid possible phage lysin escape (Frias *et al.,* 2013).
2. Dilute 1:100 in 7 ml of fresh C+Y and continue incubation at 37 °C to an OD_{600} nm of 0.2–0.25, which takes approximately 2 h. Then, induce the phage by treating with Mitomycin C (MitC) at a final concentration of 0.1 µg/ml or left untreated as control.

3. Take samples (7 ml) at different time points, for instance in 20 min-intervals, after MitC treatment. In the case of untreated cultures, collect the samples at the same time points after the culture reached OD_{600} nm 0.2-0.25.
4. Harvest the cells by centrifugation (3,200 *x g* for 10 min at 4 °C).
5. Wash the cells once with 0.5 culture volumes (3.5 ml) of cold 1x PBS.
6. To obtain the total cell pellet fraction, suspended the cells in 200 µl of 50 mM Tris pH 7.5 and store at -20 °C.
7. For choline wash, gently suspend the PBS washed cells (in step 5) in 200 µl of 2% choline chloride (w/v) prepared in 1x PBS and incubate 30 min at 4 °C without agitation to elute the choline binding proteins (avoiding cell lysis).
8. As control for the specificity of the choline wash in removing only choline binding proteins, incubate cells in the same conditions with 1x PBS or 2% (w/v) NaCl prepared in 1x PBS.
9. Collect bacteria by centrifugation (3,200 *x g* for 15 min at 4 °C). To obtain the cell pellet fraction after choline extraction, wash the pellet once with 0.5 volumes of cold 1x PBS, suspended in 200 µl of 50 mM Tris pH 7.5 and store at -20 °C.
10. Filter the supernatant, which corresponds to the choline wash fraction, through a 0.2 µm low-binding-protein membrane to ensure the removal of all bacteria. Store at -20 °C.

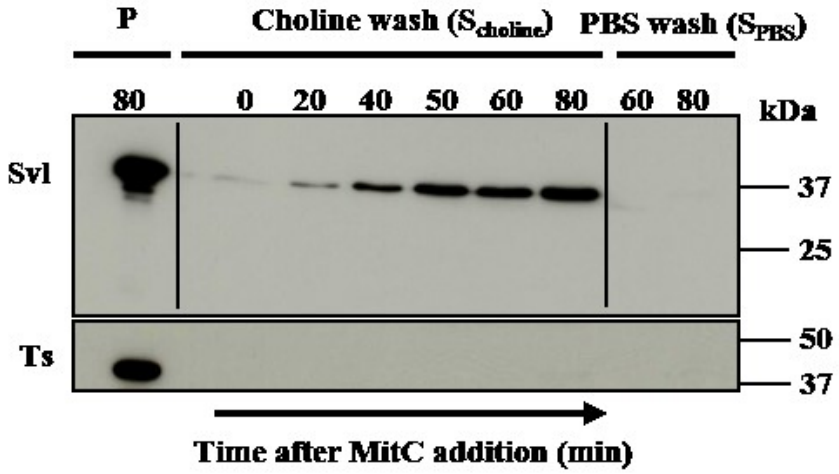

Figure 1. Choline extracts increasing amounts of phage lysin (a choline-binding protein) from the cell surface. Equal aliquots were taken at the indicated times from MitC-treated lysogenic cultures (without holin activity). Cells were harvested by centrifugation and directly suspended in Tris buffer (cell pellet fraction, P) or choline washed (choline wash fraction, $S_{choline}$). As control, cells collected at 60 and 80 min were washed with PBS (S_{PBS}). All fractions were tested by Western blotting for the phage lysin Svl (37 kDa) presence with the appropriate antibody. P and S fractions were also tested

for the cytoplasmic elongation factor Ts (43 kDa) to control for cell lysis (Frias *et al.*, 2013).

11. Separate proteins on SDS-PAGE: Boil 5–15 µl of the pellet fractions and 45 µl of the supernatant fractions for 5 min with 1x loading buffer and load onto the gel. You will need the antibody for your protein to visualize the protein on Western blot and it is important to control for possible cell lysis using an antibody for a known cytoplasmic protein (Figure 1). When comparing the amount of the protein of interest between samples, do not forget to normalize by a loading protein control. As an alternative to loading equal sample volumes followed by normalization, determine the protein concentration of each sample and load the same amount of total protein in each lane.

B. Preparation of culture medium fractions in the presence of 2% choline

1. Grow lysogenic cells without holin activity in C+Y at 37 °C until OD_{600} nm of approximately 0.9 is reached (overnight culture).
2. Dilute 1:100 in 7 ml of fresh C+Y and continue incubation at 37 °C to an OD_{600} nm of 0.2-0.25.
3. Collect cells by centrifugation (3,200 *x g* for 10 min at 4 °C) and discard the supernatant.
4. Suspend lysogens in 7 ml of C+Y with 2% choline chloride, treat the cells with MitC (0.1 µg/ml) to induce the phage and continue the incubation (alternatively, bacteria can be grown in the absence of choline).
5. Take samples (7 ml) at different time points after MitC treatment.
6. Harvest the cells by centrifugation (3,200 *x g* for 10 min at 4 °C). To obtain the cell pellet fraction, wash the cells once with 0.5 volumes of cold 1x PBS, suspend in 200 µl of 50 mM Tris pH 7.5 and store at -20 °C.
7. Collect the supernatant, which corresponds to the culture medium fraction, and filter through a 0.22 µm membrane filter. Besides the secreted proteins, this fraction also includes the choline binding proteins which are eluted from the pneumococcal surface in the presence of 2% choline chloride in the growth medium. Note that the choline binding proteins can be extracted using procedure described in Section I.
8. Concentrate the supernatant 35-fold (final volume of 200 µl) by centrifugation (3,200 *x g* at 4 °C for approximately 15 min) on an Amicon Ultra-15 centrifugal filter unit (cut-off 10 kDa). It is recommended that the molecular weight cut off of the membrane is at least 3 times smaller than the molecular weight of the protein being retained. Since we wanted to study the 37 kDa phage lysin, we selected a 10 kDa cut off.
9. Note that this experiment also allows to test for the membrane permeabilizing effect of a specific compound since in this case one expects the release of cytoplasmic proteins into the culture medium. If this is the goal of the experiment, after challenging the (MitC-

treated) cultures with the compound of interest in step 4, the samples are processed as described in the following steps.

10. Separate proteins on SDS-PAGE: Boil 5–15 µl of the pellet fractions and 45 µl of the supernatant fractions for 5 min with 1x loading buffer and load onto the gels. You will need the antibody for your protein to visualize the protein on Western blot and it is important to control for eventual cell lysis using an antibody for a known cytoplasmic protein.

Recipes

1. C+Y (pH 8), 463 ml
   400 ml of PreC (A)
   13 ml of Supplement (B)
   10 ml of 1 mg/ml (w/v) glutamine in water
   10 ml of Adams III (C)
   5 ml of 2% (w/v) pyruvate in water
   15 ml of 1 M potassium phosphate (KPO_4) buffer pH 8
   10 ml of 5% (w/v) yeast extract in water.
   (A) PreC, 2,000 ml
   2.42 g of sodium acetate anhydrous
   10 g of casamino acids
   0.01 g of L-Tryptophan
   0.1 g of L-Cysteine·HCl
   Add dH_2O to 2,000 ml
   Adjust to pH 7.4-7.6
   Autoclave
   Store at room temperature
   (B) Supplement, 213 ml
   30 ml of 3 in 1 Salts (D)
   60 ml of 20% (w/v) glucose in water
   3 ml of 50% (w/v) sucrose in water
   60 ml of 2 mg/ml (w/v) adenosine in water
   60 ml of 2 mg/ml (w/v) uridine in water
   Filter sterilize (0.22 µm)
   Store at 4 °C
   (C) Adams III, 400 ml
   0.8 g of asparagine

0.08 g of choline
0.64 ml of 1% (w/v) $CaCl_2$ in water
64 ml of Adams I (E)
16 ml of Adams II (F)
Add dH_2O to 400 ml
Filter sterilize (0.22 µm)
Store in the dark at 4 °C
(D) 3 in 1 salts, 100 ml
10 g of $MgCl_2 \cdot 6H_2O$
0.05 g of $CaCl_2$ anhydrous
0.02 ml of 0.1 M $MnSO_4 \cdot 4H_2O$
Add dH_2O to 100 ml
Autoclave
Store at room temperature
(E) Adams I, 200 ml
0.03 g of nicotinic acid
0.035 g of pyridoxine
0.12 g of Ca-pantothenate
0.032 g of thiamine-HCl
0.014 g of riboflavin
0.06 ml of 0.5 mg/ml (w/v) biotin in water
Add dH_2O to 200 ml
Filter sterilize (0.22 µm)
Store in the dark at 4 °C
(F) Adams II, 100 ml
0.05 g of $FeSO_4 \cdot 7H_2O$
0.05 g of $CuSO_4 \cdot 5H_2O$
0.05 g of $ZnSO_4 \cdot 7H_2O$
0.02 g of $MnCl_2 \cdot 4H_2O$
1 ml of HCl concentrated
Add dH_2O to 100 ml
Autoclave
Store at room temperature

2. 5x loading buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 10% β-mercaptoethanol), 8 ml
   1 ml of 0.5 M Tris-HCl (pH 6.8)
   1.6 ml of Glycerol

1.6 ml of 10% SDS

0.8 ml of β-mercaptoethanol

0.4 ml of 0.5% (w/v) bromophenol blue in water

Add dH_2O to 8 ml

Store at -20 °C

3. C+Y with 2% choline chloride, 100 ml

   2 g of choline chloride

   Add C+Y to 100 ml

   Filter sterilize (0.22 µm)

   Store at 4 °C

References

1. Frias, M. J., Melo-Cristino, J. and Ramirez, M. (2013). Export of the *pneumococcal* phage SV1 lysin requires choline-containing teichoic acids and is holin-independent. *Mol Microbiol* 87(2): 430-445.
2. Lacks, S. and Hotchkiss, R. D. (1960). A study of the genetic material determining an enzyme in *Pneumococcus*. *Biochim Biophys Acta* 39: 508-518.