

Lectin Binding Analysis of *Streptococcus mutans* Glycoproteins

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[Abstract] Bacterial glycoproteins are of increasing interest due to their abundance in nature and importance in health and infectious diseases. However, only a very small fraction of bacterial glycoproteins have been characterized and its post-translational modification machinery identified. While analysis of glycoproteins can be achieved through various techniques, this is often limited by the specific characteristics of individual proteins such as type and level of glycosylation. Lectins are sugar-binding proteins that recognize specific glycoconjugates in a manner similar to antigen-antibody interactions. Here, we describe a simple method for the detection of glycoproteins using lectin-based Western blot analysis, which can be applied to different organisms and coupled with various other strategies for complementary analysis.

Materials and Reagents

1. Bacterial whole cell lysates
2. Brain heart infusion medium (BHI) (BD Biosciences, catalog number: 237500)
3. Biotinylated lectin [Vector Laboratories, for Wheat Germ Agglutinin (WGA), catalog number: B-1025]
4. HRP-conjugated streptavidin (Cell Signaling Technology, catalog number: 3999)
5. Tween 20 (Sigma-Aldrich, catalog number: P2287)
6. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A9418)
7. Enhanced chemiluminiscent detection kit (GE Healthcare, catalog number: RPN2108)
8. Autoradiography films (Carestream Health, catalog number: 864-6770)
9. NaCl (J.T.Baker®, catalog number: 3628-01)
10. KCl (J.T.Baker®, catalog number: 3045-01)
11. Na₂HPO₄ (J.T.Baker®, catalog number: 3827-01)
12. KH₂PO₄ (J.T.Baker®, catalog number: 3246-01)
13. Tris base (J.T.Baker®, catalog number: 4109-02)
14. Sodium dodecyl sulfate (SDS) (Sigma-Aldrich, catalog number: L3771)
15. Glycine (Thermo Fisher Scientific, catalog number: BP381-1)
16. 30% Acrylamide/Bis solution (Bio-Rad Laboratories, catalog number: 161-0158)

17. Tetramethylethylenediamine (TEMED) (Life Technologies, catalog number: 15524-010)
18. Ammonium persulfate (Bio-Rad Laboratories, catalog number: BP179-25)
19. Glycerol (Alfa Aesar, catalog number: A16205)
20. Methanol (BDH Chemicals, catalog number: BDH1135-4LP)
21. Bromophenol blue (Sigma-Aldrich, catalog number: B5525)
22. 1x phosphate buffer solution (PBS) (see Recipes)
23. Resolving gel (see Recipes)
24. Stacking gel (see Recipes)
25. 2x sample buffer (see Recipes)
26. Running buffer (see Recipes)
27. Transfer buffer (see Recipes)
28. Blocking solution (see Recipes)
29. Primary solution (see Recipes)
30. Secondary solution (see Recipes)
31. Washing solution (see Recipes)

Equipment

1. 0.1 mm glass beads (Research Products International)
2. Polyvinylidene fluoride (PVDF) membranes (EMD Millipore, catalog number: P2563-10EA)
3. 3 mm Whatman paper (GE Healthcare)
4. CO₂ Incubator
5. Sterile culture tubes (15 ml)
6. Screw cap tubes (1.7 ml)
7. Bead beater (BioSpec Products)
8. Protein electrophoresis running apparatus
9. Protein transfer apparatus
10. Power supply
11. Rocker
12. Film developer
13. pH meter

Procedure

1. Preparation of whole cell protein lysates

- a. Grow bacterial cells [*Streptococcus mutans* (*S. mutans*) OMZ175] in 10 ml BHI broth at 37 °C in a humidified 5% CO₂ atmosphere.
 - b. After 18 h, stationary phase cultures (OD₆₀₀~0.9) are washed twice (2x) by centrifuging at 4,000 rpm for 10 min, discarding supernatants and resuspending pelleted cells with 1x PBS in original culture volume. After the second wash, cells are concentrated 5x by adding five times less the original culture volume of 1x PBS. The concentrated suspension is then transferred to a 1.7 ml screw cap tube containing approximately 500 µl of glass beads.
 - c. Whole cell protein lysates are obtained using a bead-beater at maximum setting (3,450 oscillations per min) and the suspensions are homogenized for three cycles of 30 sec with 2 min of incubation on ice between cycles.
 - d. Alternative mechanical methods to obtain whole cell protein lysates such as sonication or French-press, with or without freeze-thawing cycles, are also acceptable.
2. Protein lysates are separated on a 10% SDS-PAGE and subsequently transferred to a PVDF membrane.
 - a. For gel preparation, the cassette is assembled with glass and silica plates with 0.75-1.0 mm spacers and placed on a caster.
 - b. Resolving gel is prepared, poured and let to polymerize for approximately 30 min.
 - c. Stacking gel is prepared, poured and a comb with desired number of wells inserted. Allow gel to polymerize (~ 30 min).
 - d. Remove cassette from caster and place on running apparatus.
 - e. Prepare samples by mixing equal volume of sample and 2x sample buffer and boil for 10 min. Incubate on ice for 1 min, quick spin tube to settle any sediment and load sample on gel.
 - f. Run SDS-PAGE at 100 V for 2 h.
 - g. Transfer “sandwich” is assembled (Figure 1) in the presence of transfer buffer. Gel is removed from plates and placed in assembling “sandwich”.

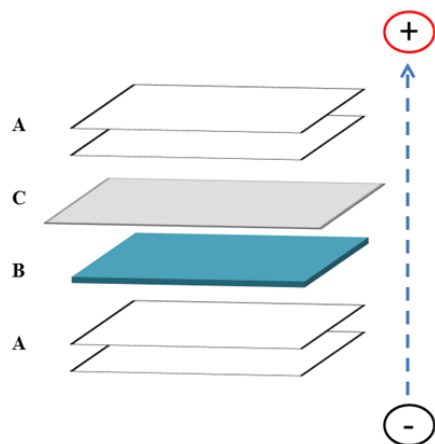


Figure 1. Schematic of transfer “sandwich” assembling for Western blotting. In the presence of transfer buffer, place two (2) pieces of Whatman paper A soaked in transfer buffer followed by the gel with separated proteins B, the PVDF membrane C soaked in methanol then transfer buffer, and two (2) more pieces of soaked Whatman papers. Arrow shows the direction of protein transfer.

- h. An oversized piece of PVDF membrane, to cover the entire gel, is soaked in methanol first followed by transfer buffer and then placed on top of the gel.
 - i. The rest of the transfer “sandwich” is completed and proteins are transferred to the membrane for 1 h at 100 V in the presence of transfer buffer and an ice unit block to absorb heat produced during transfer.
 - j. Sandwich is disassembled, membrane is removed and any extra piece of membrane that is bigger than the gel is cut out.
3. Block membrane with 5% BSA solution for 18-20 h at 4 °C with slow rocking. Wash membrane 3x by adding 20 ml of washing solution and slow rocking for 5 min to remove excess BSA. Then, incubate the membrane with the primary solution (biotinylated lectin in 0.5% BSA) at room temperature for 1 h with slow rocking.
 4. Wash membrane 3x followed by the addition of the secondary solution (HRP-streptavidin in 0.5% BSA) and incubate at room temperature for 1 h with slow rocking.
 5. After incubation with secondary solution, wash the membrane 4x to visualize biotinylated lectins bound to glycoproteins using enhanced chemiluminescent detection kit, mix equal volumes of detection solution 1 and detection solution 2 (for a final volume of 0.125 ml per cm² of membrane) and incubate for 1 min at room temperature.
 6. Drain excess detection solution and place membrane in an autoradiography cassette for film exposure. Due to the variability of carbohydrate content on glycoproteins, different exposure of the membrane to the film should be performed.

Representative data

A representative image of Cnm analysis for different strains of *S. mutans* with anti-rCnmA and biotinylated-WGA is shown below.

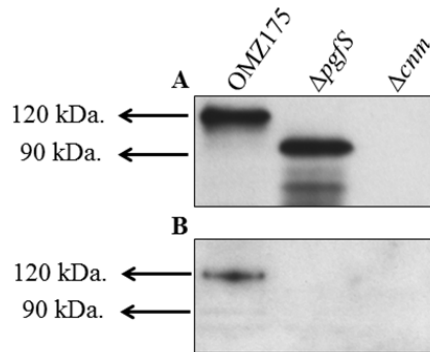


Figure 2. Example of lectin binding analysis of *S. mutans*. Cnm is a surface protein of invasive *S. mutans* that undergoes PgfS-dependent glycosylation (Aviles-Reyes *et al.*, 2014). A. Size difference in Cnm due to PgfS modification can be observed using anti-rCnmA. B. The presence of glycoconjugates attached to Cnm in OMZ175 was determined using biotinylated wheat germ agglutinin.

Notes

1. Due to the variability of the type and the level of glycosylation for each protein, protocol optimization may be required for successful identification of glycoproteins by lectin analysis.
2. Control glycoproteins with known reactivity to specific lectins should be included in each experiment.

Recipes

1. 1x PBS
 - 137 mM NaCl
 - 2.7 mM KCl
 - 10 mM Na₂HPO₄
 - 2 mM KH₂PO₄
2. Resolving gel (10 % Tris glycine SDS-PAGE; 10 ml)
 - 4.0 ml of H₂O
 - 3.3 ml of 30% acrylamide

- 2.5 ml of 1.5 M Tris (pH 8.8)
- 0.1 ml of 10% SDS
- 0.1 ml of 10% APS
- 8 μ l of TEMED
3. Stacking gel (3 ml)
 - 2.1 ml of H₂O
 - 0.5 ml of 30% acrylamide
 - 0.38 ml of 0.5 M Tris (pH 6.8)
 - 0.03 ml of 10% SDS
 - 0.03 ml of 10% APS
 - 6 μ l of TEMED
4. 2x sample buffer (200 ml)
 - 50 ml of 0.5 M Tris (pH 6.8)
 - 8 g of SDS
 - 40 ml of glycerol
 - H₂O to 200 ml
 - Add bromophenol blue until dark
5. Running buffer
 - 25 mM Tris
 - 192 mM glycine
 - 0.1% SDS
 - pH 8.3
6. Transfer buffer (1 L)
 - 200 ml of methanol
 - 3.0 g of Tris
 - 14.4 g of glycine
 - H₂O to 1 L
7. Blocking solution
 - 5% BSA (w/v) in 1x PBS
8. Primary solution
 - 0.5% BSA (w/v) in 1x PBS
 - 0.1% Tween 20
 - 20 μ g/ml biotinylated lectin
9. Secondary solution
 - 0.5% BSA (w/v) in 1x PBS
 - 0.1% Tween 20
10. Washing solution

1x PBS + 0.1% Tween 20

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References

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