

Zymogram Assay for the Detection of Peptidoglycan Hydrolases in *Streptococcus mutans*

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[Abstract] Peptidoglycan hydrolases or autolysins are enzymes capable of cleaving covalent bonds in bacterial peptidoglycan cell wall layer. They can participate in the cell division process, in the release of turnover products from peptidoglycan during cell growth, and in cell autolysis induced under particular conditions. The protocol for zymogram presented below should enable the identification of such enzymes through their separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis containing bacterial cells as substrate.

Materials and Reagents

1. Bacterial strain (*S. mutans* UA159 wild-type strain or other *S. mutans* strains)
2. Todd-Hewitt broth (BD Biosciences)
3. Yeast-Extract (BioShop)
4. Tris Base
5. NaCl
6. Sodium dodecyl sulfate (SDS)
7. Triton X-100
8. KOH
9. MgCl₂
10. Glycine
11. Glycerol
12. Bromophenol blue
13. Methylene blue
14. 40% Acrylamide/Bis solution (37.5:1 acrylamide: bisacrylamide) (BioShop)
15. Ammonium persulfate (Sigma-Aldrich)
16. TEMED (BioBasic, Inc.)
17. Ethanol
18. Isopropanol
19. dH₂O
20. Filter paper
21. Precision Plus Protein Prestained Standards (Bio-Rad Laboratories)

22. THYE broth (see Recipes)
23. 20 mM Tris, 100 mM NaCl (pH 7.4) (see Recipes)
24. 1.5 M Tris (pH 8.8) (see Recipes)
25. 0.5 M Tris (pH 6.8) (see Recipes)
26. SDS-PAGE loading buffer (see Recipes)
27. Tris-Glycine SDS running buffer (see Recipes)
28. Zymogram renaturing buffer (see Recipes)
29. Staining solution (see Recipes)
30. 10% separating gel solution (see Recipes)
31. 4% stacking gel solution (see Recipes)

Equipment

1. 15-ml canonical tubes
2. Flasks
3. 1.5 ml microcentrifuge tubes
4. Refrigerated centrifuge
5. Refrigerated microcentrifuge
6. CO₂ incubator
7. Spectrophotometer
8. Disposable plastic cuvettes
9. Protein mini gel cassettes
10. Heating block module
11. Power supply
12. Orbital shaker
13. 37 °C temperature chamber

Procedure

- A. Preparation of the bacterial substrates incorporated into the gel
 1. Start 5 ml culture of *S. mutans* UA159 wild-type strain (or other *S. mutans* strains) in THYE broth into a 15-ml canonical tube and incubate overnight statically at 37 °C in air with 5% CO₂.
 2. Inoculate 300 ml of fresh THYE broth with 1% of the overnight preculture into a 500-ml flask.
 3. Incubate the culture statically at 37 °C in air with 5% CO₂ until an optical density at 600 nm (OD₆₀₀) of 0.2 is reached.

4. Harvest the cells by centrifugation at 10,000 x *g* for 10 min at 4 °C.
5. Wash the cells using 5 ml of 20 mM Tris, 100 mM NaCl buffer (pH 7.4) and resuspend the cell pellet in 1.0 ml of 1.5 M Tris buffer (pH 8.8).
6. Keep the cells at -20 °C until used.

B. Preparation of bacterial whole-cell extracts

1. Start 5 ml overnight culture of *S. mutans* UA159 wild-type strain (or other *S. mutans* strains) in THYE broth into a 15-ml canonical tube and incubate statically at 37 °C in air with 5% CO₂. Whole cell extract of a mutant strain deficient in the peptidoglycan hydrolase under study can also be analyzed concomitantly as negative control to confirm the specificity of the hydrolytic band(s) observed.
2. Inoculate 10 ml of fresh THYE broth with 1% of the overnight preculture into a 15-ml canonical tube.
3. Incubate the culture statically at 37 °C in air with 5% CO₂ until the desired OD₆₀₀ is reached. If the expression profile of the targeted peptidoglycan hydrolase is not known, we recommend to harvest cells at different optical densities corresponding to early log, mid-log, early stationary, and late stationary phase of growth.
4. Harvest the cells by centrifugation at 10,000 x *g* for 10 min at 4 °C.
5. Keep the cell pellet at -20 °C until used.
6. Resuspend the cell pellet in 20 µl of SDS-PAGE loading buffer freshly prepared.
7. Heat the samples at 95 °C for 10 min. Keep the samples on ice until loading.

C. Preparation of the zymogram gel

1. Clean glass plates, spacers, and combs with ethanol and completely dry before use. Assemble the gel cassette following the manufacturer's instructions.
2. Prepare 10% separating gel solution (see Recipe 9).
3. Transfer the separating gel solution (approx. 3.8 ml per small gel) to the casting chamber between the glass plates and fill up to about 0.7 cm below the bottom of the comb when the comb is in place.
4. Add a small layer of isopropanol to the top of the gel prior to polymerization to straighten the level of the gel. Once the gel has polymerized, remove the isopropanol layer by several washes with dH₂O, and dry with filter paper.
5. Prepare 4% stacking gel solution (see Recipe 10).
6. Pour the stacking gel solution (approx. 2.5 ml per small gel) on top of the separating gel until the space is full, and then insert the appropriate comb. Once the gel has polymerized, carefully remove comb.
7. Remove the gel cassette from the casting stand and place it in the electrode assembly as recommended by the manufacturer.

8. Pour Tris-Glycine SDS running buffer into the opening of the casting frame between the gel cassettes. Add enough buffer to fill the wells of the gel. Fill also the region outside of the frame.
9. Load the samples (from Section B, step B-7) into each well as well as 5 μ l of the Precision Plus Protein Prestained Standards.
10. Connect the electrophoresis tank to the power supply. Run the gel at a constant voltage between 125-200 volts until the dye front is near the bottom of the gel.

D. Peptidoglycan hydrolase detection

1. Remove the gel from the electrophoresis chamber, allow the gel to peel away and gently drop into a container. Wash the gel twice in 100 ml of dH₂O for 30 min at room temperature under constant agitation.
2. Incubate the gel in 100 ml of zymogram renaturing buffer for 30 min at room temperature under constant agitation. This step is necessary to renature the peptidoglycan hydrolases.

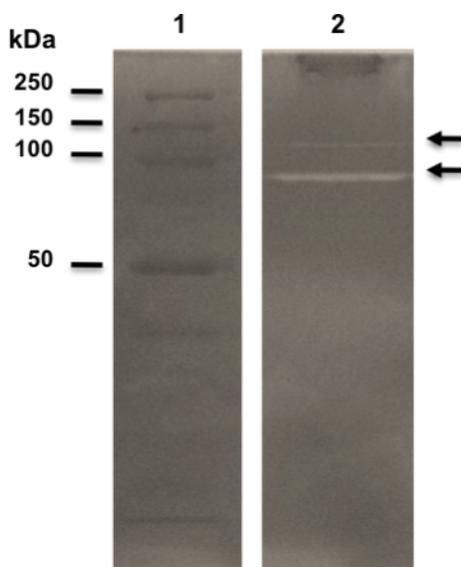


Figure 1. Zymogram activity gel after methylene blue staining. Heat-killed cells of *S. mutans* were used as substrate and were incorporated into a 10% SDS-PAGE gel. (1) Molecular size marker (Precision Plus Protein Prestained Standards). (2) Whole-cell extract of *S. mutans* UA159 wild-type strain. The two hydrolytic bands (arrows) observed correspond to the unprocessed (upper) and processed (lower) forms of the AtlA autolysin.

3. Replace the zymogram renaturing buffer with fresh zymogram renaturing buffer and incubate the gel at 37 °C in a temperature chamber under constant agitation until clear hydrolytic band(s) appear, usually between 16 h and 48 h. The proteolytic activity appears as clear bands against a white background.

4. Optional staining step: Decant the buffer and add 100 ml of staining solution, and incubate the gel at room temperature under constant agitation between 15 min and 2 h. Regions without staining are indicative of lysis (Figure 1). The proteolytic activity appears as clear bands against a blue background.

Recipes

1. THYE broth
 Dissolve 15 g of Todd-Hewitt and 1.5 g of Yeast Extract in 400 ml of dH₂O
 Once dissolved, bring up to a final volume of 500 ml with dH₂O
 Autoclave for 20 min at 120 °C
 Store at room temperature
2. 20 mM Tris, 100 mM NaCl buffer (pH 7.4)
 Dissolve 2.42 g of Tris Base, and 5.84 g NaCl in 800 ml of dH₂O
 Once dissolved, adjust the pH to 7.4, and then bring up to a final volume of 1 L with dH₂O
 Store at 4 °C
3. 1.5 M Tris pH 8.8 buffer
 Dissolve 181.71 g of Tris Base in 800 ml of dH₂O
 Once dissolved, adjust the pH to 8.8, and then bring up to a final volume of 1 L with dH₂O
 Store at 4 °C
4. 0.5 M Tris pH 6.8 buffer
 Dissolve 60.57 g of Tris Base in 600 ml of dH₂O
 Once dissolved, adjust the pH to 6.8, and then bring up to a final volume of 1 L with dH₂O
 Store at 4 °C
5. SDS-PAGE loading buffer (0.25 M Tris pH 6.8, 2% SDS, 10% glycerol, bromophenol blue)
 Dissolve 0.3 g of Tris Base, 0.2 g SDS, 1.0 ml glycerol, and traces of bromophenol blue in 7 ml of dH₂O
 Once dissolved, bring up to a final volume of 10 ml with dH₂O
6. Tris-Glycine SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS)
 Dissolve 3.03 g of Tris Base, 14.4 g glycine, and 1 g SDS in 800 ml of dH₂O
 Once dissolved, bring up to a final volume of 1 L with dH₂O
 Store at 4 °C
7. Zymogram renaturing buffer (20 mM Tris, 50 mM NaCl, 20 mM MgCl₂, 0.5% Triton X-100, pH 7.4)
 Dissolve 2.42 g of Tris Base, 2.92 g NaCl, and 4.06 g MgCl₂ in 800 ml of dH₂O
 Adjust the pH to 7.4
 Add 5 ml of Triton X-100, and bring up to a final volume of 1 L with dH₂O

- Store at 4 °C
8. Staining solution (0.1% methylene blue, 0.01% KOH)
Dissolve 0.1 g of methylene blue and 0.01 g KOH in 100 ml of dH₂O
Store at room temperature
 9. 10% separating gel solution
Mix the following reagents in a clean flask (total volume for 4 small gels):
7.4 ml dH₂O
3.7 ml 40% acrylamide/bis
4 ml of bacterial substrate (from Section A, step A-6) boiled for 10 min just prior use
100 µl 10% SDS
50 µl 10% ammonium persulfate
5 µl TEMED
 10. 4% stacking gel solution
Mix the following reagents in a clean flask (total volume for 4 small gels):
6 ml dH₂O
2.5 ml 0.5 M Tris (pH 6.8)
1.0 ml 40% acrylamide/bis
100 µl 10% SDS
100 µl 10% ammonium persulfate
25 µl TEMED

References

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