Biotinylation and Purification of Surface-exposed *Helicobacter pylori* Proteins

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[Abstract] Interactions between pathogenic bacteria and host cells are often mediated by proteins found on the surfaces of the bacteria. The Gram-negative bacterium *Helicobacter pylori* is predicted to produce at least 50 surface-exposed outer membrane proteins, but there has been relatively little progress in experimentally analyzing the cell-surface proteome of this organism. Herein, we describe in detail a protocol that allows biotinylation and purification of surface-exposed *H. pylori* proteins. A comparative analysis of surface-exposed proteins identified by this biotinylation-based approach and by several other independent methods is described in a recent publication (Voss *et al.*, 2014).

Materials and Reagents

1. Tryptone (BD, catalog number: 211705)
2. Proteose peptone #3 (BD, catalog number: 211693)
3. Yeast Extract (BD, catalog number: 212720)
4. NaCl (Acros Organics, catalog number: 207790250)
5. Dextrose (Thermo Fisher Scientific, catalog number: D14-212)
6. Fetal bovine serum (Atlanta Biologicals, catalog number: S11150)
7. Tris Base (Thermo Fisher Scientific, catalog number: BP152)
8. Na_2HPO_4 (Sigma-Aldrich, catalog number: S5136)
9. KCl (Sigma-Aldrich, catalog number: P-9333)
10. 10x PBS (Corning, catalog number: 46-013-CM)
11. $MgCl_2•6H_2O$ (Thermo Fisher Scientific, catalog number: M35)
12. $CaCl_2$ (Sigma-Aldrich, catalog number: C-4901)
13. EDTA-$Na_2·2H_2O$ (Sigma-Aldrich, catalog number: E4884)
14. D-biotin (Sigma-Aldrich, catalog number: B4501)
15. S-NHS-LC-Biotin (Thermo Fisher Scientific, catalog number: 21335)
16. Zwittergent 3-14 (Fluka, catalog number: 40772)

17. Protease inhibitor cocktail (Roche Diagnostics, catalog number: 04693159001)
18. Monomeric avidin magnetic beads, Blocking buffer, Regeneration buffer (Bioclone, catalog number: MMI-101)
19. 100% (w/v) TCA (Sigma-Aldrich, catalog number: T0699)
20. Acetone (Thermo Fisher Scientific, catalog number: A16F)
21. Brucella broth (see Recipes)
22. Biotinylation buffer (see Recipes)
23. TNKCM (see Recipes)
24. Lysis buffer (see Recipes)
25. TKE (see Recipes)
26. TKEZ (see Recipes)
27. Sample dilution buffer (see Recipes)
28. Blocking buffer (see Recipes)
29. Regeneration buffer (see Recipes)
30. Wash buffer (see Recipes)
31. Elution buffer (see Recipes)

Equipment

1. CO_2 shaking incubator (ATR Biotech, model: AJ125B)
2. Bench top centrifuge (Thermo Fisher Scientific, catalog number: 75004381)
3. Sonicator (Thermo Fisher Scientific, catalog number: FB505)
4. Bench top centrifuge (Eppendorf, catalog number: 5424)
5. Ultracentrifuge (Beckman Coulter, catalog number: A94469)
6. Barnstead/Thermolyne Labquake Shaker Rotisserie (Labquake, catalog number: C400110)
7. DynaMag™- Spin Magnet (Life Technologies, catalog number: 12320D)

Procedure

A. On-cell biotinylation

1. Grow 25 ml of *H. pylori* liquid culture in Brucella broth containing 10% fetal bovine serum until late-log phase (OD_{600} = 0.7) at 37 °C.
2. Pellet all bacteria from the 25 ml culture at 3,500 *x g* for 20 min at 4 °C, and discard media.
3. Resuspend bacterial pellet in 10 ml Biotinylation buffer.
4. Pellet bacteria at 3,500 *x g* for 10 min at 4 °C, and discard buffer.

5. Resuspend bacterial pellet in 10 ml Biotinylation buffer.
6. Add S-NHS-LC-biotin to a final concentration of 200 µM.
7. Incubate on ice for 30 min.
8. Quench reaction by adding 20 ml TNKCM, incubate at room temperature for 10 min.
9. Pellet bacteria at 3,500 *x g* for 20 min at 4 °C, and discard buffer.
10. Resuspend bacterial pellet in 10 ml TNKCM, pellet bacteria at 3,500 *x g* for 10 min at 4 °C, and discard buffer. Repeat two more times for a total of three washes.

B. Bacterial lysis and subcellular fractionation

11. Resuspend bacterial pellet in 3 ml lysis buffer + protease inhibitor cocktail.
12. Sonicate (4 times, 10 sec each, at 25% maximum amplitude) on ice to lyse bacteria, pellet intact bacteria at 7,000 *x g* for 10 min, collect lysis supernatant.
13. Pellet membranes by spinning lysate at 40,000 *x g* for 30 min at 4 °C.
14. Discard supernatant.
15. Rinse membrane pellet with TKE three times.
16. Collect membrane pellet by scraping with a clean pipette tip and resuspend in 1 ml TKEZ + protease inhibitor cocktail.
17. Solubilize for 1 h at 4 °C with head-over-head mixing.
18. Spin at 100,000 *x g* for 1 h at 4 °C, collect supernatant.

C. Isolation and enrichment of biotinylated proteins

19. Dilute the supernatant 10x with sample dilution buffer (resulting in a volume of 10 ml).
20. Aliquot 50 µl of avidin magnetic bead suspension into a microfuge tube.
21. Place tube on magnet, remove storage buffer.
22. Resuspend beads in 500 µl PBS, place on magnet and discard buffer.
23. Resuspend beads in 200 µl blocking buffer.
24. Incubate beads for 10 min with head-over-head mixing.
25. Place on magnet, discard buffer, resuspend beads in 400 µl regeneration buffer.
26. Place on magnet, discard buffer, resuspend beads in 500 µl wash buffer.
27. Place on magnet, discard buffer.
28. Add 1 ml aliquot of sample described in step C19 to equilibrated beads and incubate at room temperature with head over head mixing for 10 min. Then place the tube on magnet, discard supernatant, and apply another 1 ml aliquot. Repeat this procedure until the entire 10 ml of sample is applied to the beads.
29. After entire 10 ml sample from step C19 has been incubated with the magnetic beads, resuspend beads in 1 ml wash buffer, and incubate for 5 min with head-over-head mixing.

Then place the tube on magnet and discard supernatant. Repeat two more times for a total of three washes.

30. Resuspend beads in 1 ml wash buffer and transfer the suspended beads to a clean tube.
31. To elute biotinylated proteins: place the bead suspension on magnet, discard supernatant, resuspend beads in 1 ml elution buffer, and incubate at room temperature for 10 min with head-over-head mixing.
32. Place the tube on magnet and save the supernatant. Add another 1 ml elution buffer to the beads and incubate at room temperature for 10 min with head over head mixing. Then place on the magnet and again save the supernatant. Repeat this procedure once more, resulting in a total elution volume of 3 ml.
33. Precipitate proteins by adding 1 ml TCA to the 3 ml elution (25% final concentration TCA).
34. Vortex to mix, incubate at 4 °C for at least 30 min. (Overnight storage at 4 °C is possible at this stage).
35. Spin at 21,000 *x g* for 30 min at 4 °C, discard supernatant.
36. Add 1 ml ice cold acetone, spin at 21,000 *x g* at 4 °C for 10 min, discard supernatant, repeat once for a total of two acetone washes.
37. Air dry pellet at room temperature, store at -20 °C until needed.

Notes

1. Samples can be initially analyzed by SDS-PAGE and silver staining, or by Western blotting if suitable antisera reactive with *H. pylori* outer membrane proteins are available. A comprehensive identification of the proteins present in preparations of purified biotinylated proteins can be accomplished by mass spectrometry. Results obtained using this method for biotinylation and purification of surface-exposed *H. pylori* proteins, along with a comparative analysis of results obtained using other methods, are described in detail in Voss *et al.* (2014). The use of multiple methods for identifying surface-exposed proteins helps to minimize false-positive results.
2. In parallel with the purification of biotinylated proteins from biotinylated bacteria, we recommend that non-biotinylated bacteria should be processed as a control as described previously in Voss *et al.* (2014). This comparative approach minimizes false-positive results that can arise due to non-specific protein binding to beads.

Recipes

1. Brucella broth

10 g/L tryptone
10 g/L proteose peptone #3
5 g/L NaCl
2 g/L yeast extract
1 g/L dextrose

2. Biotinylation buffer
   1x PBS
   1 mM $CaCl_2$
   0.5 mM $MgCl_2$
   1.6 mM D-biotin
   pH 7.4
3. TNKCM (for quenching of S-NHS-LC-biotin and washing bacteria)
   50 mM Tris (pH 7.4)
   100 mM NaCl
   27 mM KCl
   1 mM $CaCl_2$
   0.5 mM $MgCl_2$
4. Lysis buffer
   50 mM Tris (pH 7.4)
   1 mM $MgCl_2$
5. TKE (for rinsing membrane pellet)
   50 mM Tris (pH 7.4)
   150 mM KCl
   10 mM EDTA
6. TKEZ (for preferentially solubilizing outer membrane proteins)
   50 mM Tris (pH 7.4)
   150 mM KCl
   10 mM EDTA
   2% Zwittergent 3-14
7. Sample dilution buffer
   100 mM Na_2HPO_4 (pH 7.4)
   150 mM NaCl
8. Blocking buffer
   100 mM Na_2HPO_4 (pH 7)
   150 mM NaCl
   2 mM D-biotin
9. Regeneration buffer

100 mM glycine (pH 2.8)

10. Wash buffer (for washing magnetic beads)
    100 mM Na_2HPO_4 (pH 7.4)
    150 mM NaCl
    0.2% Zwittergent 3-14
11. Elution buffer
    100 mM Na_2HPO_4 (pH 7.4)
    150 mM NaCl
    0.2% Zwittergent 3-14
    5 mM D-biotin

Acknowledgments

The biotinylation protocol described here and in Voss *et al*. (2014) is modified from a protocol described in Sabarth *et al.* (2002). Supported by the Department of Veterans Affairs, Merit Review grant 2I01 BX000627.

References

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2. Voss, B. J., Gaddy, J. A., McDonald, W. H. and Cover, T. L. (2014). Analysis of surface-exposed outer membrane proteins in *Helicobacter pylori*. *J Bacteriol* 196(13): 2455-2471.

