

“Shaving” Live Bacterial Cells with Proteases for Proteomic Analysis of Surface Proteins

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Abstract

Surface proteins are essential molecules for the interplay between cells and the environment. They participate in many biological processes including transport, adhesion, cell–cell recognition, signaling, and other cell interactions. In pathogenic microorganisms, these molecules may act as virulence or cytotoxicity factors. Analyzing the set of surface proteins is critical to understand these processes and to identify possible targets that can be the starting point for other studies or discoveries (e.g., vaccines or diagnostics). Here I describe a proteomic procedure to identify in a fast and reliable way a set of surface-exposed proteins in bacteria, the methodology of which can be adapted to other biological systems (unicellular fungi, parasites). The protocol presented here involves “shaving” the cells cultured in broth with proteases followed by liquid chromatography–tandem mass spectrometry (LC/MS/MS) and analysis of the generated peptides. This method overcomes some important limitations of the first-generation, gel based proteomics techniques, and the “shaving” approach allows one to identify which domains from identified proteins are more accessible to proteases. These identified proteins have the highest potential to be recognized by antibodies, and thus permits the identification of potential epitopes or antigens.

Key words Surface proteins, “Shaving”, Proteomics, Proteases, Antigens, Epitopes

1 Introduction

Since the mid-1990s, the availability of genome sequences has made possible the advent of “omics” technologies (i.e., massive analysis platforms that allow the identification of hundreds or thousands of biomolecules from one sample). To understand what occurs at or through biological surfaces (cell membranes, cell walls, teguments, etc.), proteomic-based approaches provide invaluable tools capable of providing a snapshot of proteins participating in the interaction between cells and their environment. Once identified, this information helps unravel cell functions and mechanisms that may involve transport, adhesion, cell–cell recognition, signaling, and others [1]. When studying pathogenic microorganisms, the analysis of surface proteins is key to the

identification of potential targets for drugs or for vaccine or diagnostic candidate discovery, as these molecules have the highest chances to raise an effective immune response [2].

In the first decade of the proteomics era, the study of surface proteins was mainly approached using gel-based protein separation followed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis, which has been called “first generation proteomics.” This allowed the description of many surface/membrane proteomes. Researchers recognized several important limitations associated with this approach. There was a general underrepresentation of surface/membrane proteins in 2-dimension (2-D) gels, mainly due to the fact that (1) these proteins are generally synthesized in relatively low copy numbers, compared to other cellular compartments (especially when compared to cytoplasmic proteins), and (2) many of them are insoluble, particularly those having transmembrane spanning domains [3]. In addition, the protein identification from this workflow misses information about possible discrepancies/concordances between experimental and predicted topology, which is very important for projects requiring epitope or antigen descriptions for drug or vaccine discovery.

Second-generation proteomic-based approaches do not require gels for the identification of hundreds or thousands of proteins/peptides in a liquid sample, using principally LC/MS/MS platforms. In 2003, a simple and smart strategy was reported that facilitated the identification of membrane proteins and the topological characterization of domains on both sides of a biological membrane [4]. Inspired by this idea, Rodríguez-Ortega and coworkers set up a procedure to identify, in a fast and reliable way, surface proteins of pathogenic bacteria, initially for vaccine discovery purposes. This was applied to the gram-positive bacterium *Streptococcus pyogenes* [5]. The initial protocol consists of “shaving” the surface of live cells cultured in a broth with two different proteases: trypsin (the enzyme used in >99% of proteomic protocols), which specifically cleaves the C-terminal of arginine and lysine amino acid residues, and Proteinase K, a broad-spectrum, relatively nonspecific serine endopeptidase. The latter enzyme allowed the identification of pilin proteins, which are resistant to trypsin digestion. The mixture of peptides generated (called the “surfaceome” or “surfome”) is then subject to cleaning and concentration with chromatographic cartridges to remove salts and sucrose present in the digestion buffer, followed by LC/MS/MS analysis. An important factor is the control of cell lysis, as this helps avoid an excess of predicted cytoplasmic proteins in the “surfome.” In the initial procedures, this step was checked by colony forming unit (CFU) counting, although flow cytometry can also be used to assess the cell viability [6].

The procedure described here allows the identification of the most protease accessible protein regions, which was not possible with first-generation proteomics approaches. These “hot zones” can be used to determine potential epitopes for antibody recognition or to design potentially exposed polypeptides for vaccine or even diagnostics projects [7, 8]. The protocol is generally applicable to gram-positive bacteria; however, it can be applied to bacteria that are more labile, like the gram negatives, through the modification of several steps (digestion buffer, protease digestion time, etc.). The protocol has been successfully applied, as well, to other biological systems like yeast or parasites (for an extensive review, *see* [9]). Additional experimental variations may be used to improve the procedure for certain species: redigestion of “surfome” fractions to reduce the presence of protease missed cleavages; the digestion of large peptides that otherwise would be undetected by the MS instruments [10, 11]; and the use of immobilized proteases, for very labile species [12, 13].

2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Sodium azide does not need to be added to the reagents.

2.1 Shaving Protocol Components

1. Culture broth (*see* **Note 1**).
2. Sterile material for microbiological culture (disposable pipettes, flasks, etc.).
3. Falcon-type tubes (50 mL volume).
4. Wash buffer: prepare phosphate-buffer saline solution (PBS), pH 7.4. For 1 L solution, add 8.181 g NaCl, 0.2 g KCl, 2.68 g Na₂HPO₄, and 0.245 g NaH₂PO₄ to a 1 L graduated cylinder or a glass beaker. Dissolve salts in 800 mL of distilled water, adjust the pH to 7.4 with HCl, and then bring to a final volume of 1 L. Sterilize it by filtering or autoclaving.
5. Trypsin digestion buffer: PBS/30% sucrose, pH 7.4. Prepare it by dissolving 30 g sucrose in 80 mL PBS, pH 7.4, and then bring to a final volume of 100 mL. Sterilize it by filtering (*see* **Note 2**).
6. 1.5 mL low-binding tubes (Eppendorf® Protein LoBind microcentrifuge tubes).
7. Sequencing grade modified trypsin (Promega, Madison, WI, USA) (*see* **Note 3**). Store trypsin at −20 °C.

8. 0.22- μm pore-size filters (Millex[®]-GV PVDF 0.22- μm filter units, 13 mm diameter (EMD Millipore, Billerica, MA, USA) (*see* **Note 4**).
9. Top-down agitation rotor.

2.2 Cleaning of the “Surfome”

1. Oasis[®] HLB cartridges, 1 cc (Waters Corporation, Milford, MA, USA).
2. Prepare 10% formic acid by the addition of 10 mL of 100% formic acid to 100 mL distilled water. Store it in a glass flask.
3. Equilibration solution for Oasis[®] HLB cartridges: prepare 80% acetonitrile (HPLC grade) by mixing 80 mL acetonitrile with 20 mL distilled water.
4. Wash solution for Oasis[®] HLB cartridges: prepare 2% acetonitrile (HPLC grade)/0.1% formic acid by mixing 2 mL acetonitrile with 97 mL distilled water, and adding 1 mL of 10% formic acid.
5. Elution solutions for Oasis[®] HLB cartridges: prepare 10%, 20% and 50% acetonitrile (HPLC grade)/0.1% formic acid by mixing 10, 20 or 50 mL acetonitrile with 89, 79 or 49 mL distilled water, respectively, followed by the addition of 1 mL of 10% formic acid.
6. Speed-vacuum concentrator system.
7. Vacuum manifold and vacuum pump, or 5 mL automatic pipette.

3 Methods

Be cautious when working with pathogenic microorganisms. Take appropriate precautions and wear personal protective equipment (e.g., lab coat, latex gloves, and goggles). Be sure to fulfill the biological safety standards in terms of protection levels according to an organism's risk. The protocol described here is adapted from the original one described for the first time for *Streptococcus pyogenes* [5].

3.1 “Shaving” Protocol for Gram-Positive Bacteria

1. Grow the bacterial culture to the desired OD₆₀₀, normally corresponding to mid-exponential phase (*see* **Notes 5** and **6**).
2. Pellet the bacteria normally by centrifugation at 3500 $\times g$, 10 min, 4 °C.
3. Resuspend the pelleted bacteria in PBS (*see* **Note 7**). Repellet the bacteria as in **step 2**.
4. Repeat **step 3** two more times.
5. Resuspend the bacterial pellet in PBS/30% sucrose, pH 7.4 in a 1.5 mL low-binding tube at a ratio of 800 μL buffer per each 100 mL of initial bacterial culture.

6. Add 5 µg/mL of sequencing grade, modified trypsin.
7. Incubate the suspension for 30 min at 37 °C with top-down agitation (*see* **Notes 8** and **9**).
8. Pellet the “shaved” bacteria by centrifugation at $3500 \times g$, 10 min, 4 °C and recover the supernatant containing the peptide fraction (“surfome”) in a clean, sterile low-binding tube.
9. Filter the supernatant (“surfome”) with a 0.22-µm pore-size filter (*see* **Note 10**).
10. Optionally, if the trypsin digestion has not worked well (e.g., too many large peptides with many trypsin missed cleavage sites), redigest the “surfome” with 2 µg trypsin overnight at 37 °C with top-down agitation (*see* **Note 11**).

3.2 Cleaning the “Surfome” with Oasis[®] HLB Extraction Cartridges

Cleaning can be done with a vacuum manifold system or by pushing the liquids through cartridge resin with a 5 mL tip pipette, using a 5 mL pipette for generating pressure (*see* **Note 12**). According to the manufacturer’s instructions, the use of the cartridges involves the following steps: equilibration, sample loading, washing, and elution.

1. Equilibrate Oasis[®] HLB extraction cartridges with 0.6 mL of 80% acetonitrile.
2. Add 0.6 mL of 0.1% formic acid.
3. Load the sample (in our hands, loading 150 µL of the “surfome” of any streptococcal species mixed with 450 µL PBS works well) (*see* **Note 13**).
4. Wash the sample twice with 0.6 mL of 2% acetonitrile/0.1% formic acid.
5. Elution in three steps with 0.6 mL of each of the following solutions (*see* **Note 14**):
 - (a) 10% acetonitrile/0.1% formic acid
 - (b) 20% acetonitrile/0.1% formic acid
 - (c) 50% acetonitrile/0.1% formic acid
6. Dry in a speed-vacuum system (*see* **Note 15**).
7. Resuspend the pellet in 100 µL 2% acetonitrile/0.1% formic acid (you can divide it among three tubes, or resuspend the first tube, then transfer the volume to the second one and resuspend, and then transfer it to the third one and resuspend). Keep the sample in a low-binding tube.
8. At this stage, the sample is ready for MS/MS analysis (*see* **Note 16**). Otherwise, it can be stored at −20 °C for some months (*see* **Notes 17** and **18**).

4 Notes

1. The protocol described here is adapted from [5], which can be generally applied to most gram-positive bacteria. For microorganisms of the *Streptococcus* genus, it works well using a complex broth like Todd-Hewitt. However, Olaya-Abril et al. [6] used a chemically defined medium supplemented with ethanolamine to reduce cell lysis. The occurrence of cell lysis is a potentially major drawback of this proteomic procedure. If the lysis is too extensive, nonspecific peptides may mask the desired targets of this protocol, i.e., surface-attached proteins will be hidden and/or underestimated within the vast amount of peptides in the sample. Choosing the right culture medium is very important to assure the success of this protocol, and the medium may vary for each organism. Cell lysis can be assessed by counting CFUs [5] or by flow cytometry [6], which requires more complex equipment, but is more precise.
2. As indicated in **Note 1**, a critical aspect of this protocol is the control of cell lysis. This can be achieved, in part, through the use of isotonic buffers when handling microorganisms, especially at the protease digestion step. For most gram-positive bacteria, digestion is performed using an isotonic buffer in PBS with a high concentration of sugars (sucrose, raffinose). The pH is adjusted to 7.4 to be near the optimal value of the trypsin. However, if other proteases are used, changes may be made to the buffer composition and/or the pH. This concept can be illustrated by experiments with proteinase K, a nonspecific protease. This enzyme has a high turnover number, so if its activity is not controlled, it may cleave all substrates very rapidly. One way to control the activity is either to lower the pH to reduce its activity or to avoid/reduce buffer Ca^{2+} , which normally acts as an activator. Rodríguez-Ortega et al. [5] determined empirically that a buffer consisting of PBS/30% sucrose, pH 6.0, without added Ca^{2+} works well for a 20–30 min digestion.
3. As previously described in **Note 2**, other proteases can be used according to the purpose and/or protein target(s). If the targets are recalcitrant, as seen with trypsin-resistant proteins or proteins with potentially hidden specific cleavage sites, nonspecific proteases like proteinase K can be used. It has also been described for the use of immobilized enzymes (e.g., trypsin on agarose beads), which theoretically will reduce cell disruption because of reduced protease penetration into the cell wall. Immobilized enzymes also reduce the numbers of generated peptides, as they have lower turnover numbers [12, 13].

4. Filters of 4 mm diameter may be used, but they clog more easily and must be changed during the filtration operation. This may lead to loss of some sample amounts.
5. Ideally at the mid-exponential phase, bacteria are in the most active division phase with minimal cell death. Many gram-positive bacteria express a lot of surface proteins during this phase. The optimal moment of the growth phase in which cells are harvested will depend on the organism and/or the research purpose. The protocol described here is well adapted for *Streptococcus* sp. At the mid-exponential phase ($OD_{600} = 0.25\text{--}0.30$), the cell concentration is approximately 10^8 cells/mL. In 100 mL cultures there is enough material for recovering a high amount of peptides. Nevertheless, it has been proven with bacteria from 25 mL culture that there is still sufficient material for protease digestion and peptide recovery.
6. Follow specific safety recommendations when handling pathogenic microorganisms.
7. Avoid resuspending the pelleted bacteria by pipetting excessively, as this could break the cells. It is better to vortex gently.
8. The digestion time with the protease may influence cell viability. If it is too long, it can favor cell lysis. Therefore, it should be set up empirically. For most gram-positive organisms, 30 min gives a good yield in terms of peptide recovery, without compromising significantly cell viability. A study on the effect of trypsin digestion time on peptide yield and cell lysis is available in [6].
9. A top-down agitation rotor may be used to improve protease-cell contact during digestion, especially when using narrow tubes. By rotating at low rpm, the bacterial suspension will be in continuous movement within the tube and thus avoid settling of cells at the bottom of the tube, which would prevent contact with the protease. The rotor may be placed in a chamber at 37 °C.
10. As the digestion volume is normally less than 1 mL, a 1-mL syringe is recommended to filter the “surfome” fraction. Push the syringe piston slowly to avoid that the filter is dislodged from the syringe if it is clogged. This is quite probable if using 4-mm diameter filters. Clogging may be due to an incomplete removal of cells and subsequent cell contamination of the supernatant. To avoid this, the “surfome” fraction can be centrifuged.
11. If the LC/MS/MS analysis contains too many large peptides with consensus trypsin sites that were not cleaved, the trypsin added to the solution may have not worked adequately [10]. Under these circumstances, a redigestion of the primary “surfome” can be performed. For this, 2 µg of trypsin are

added and the “surfome” is incubated for a minimum of 2 h (or overnight). This improves significantly the digestion yield.

12. The easiest way to make the liquids pass through the cartridge resin is by creating a negative pressure from the bottom side of the cartridge, using a vacuum pump coupled to a manifold system, on which the cartridges are placed. This operation takes only a few seconds for liquid passing through the resin. If this system is not available, it can be done using a 5 mL automatic pipette. The tip of the pipette is placed on the upper side of the cartridge, and the piston is slowly pushed to make the liquid pass through the resin.
13. As previously indicated in the notes, the amount of sample to load in the resin must be determined empirically. To the author’s knowledge, for all the streptococcal species 150 μ L of the “surfome” contains sufficient peptide material to be detected by LC/MS/MS. But, as expected, factors like the efficiency of the protease digestion, the amount of bacterial cells, and other parameters, may require modifications to this volume. Although the cartridges have a high retention capacity, the sample flow-through can be passed again through the resin.
14. According to the manufacturer’s instructions, one elution step with a high concentration of organic solvent is enough to elute the retained molecules. However, the author has observed that a second elution with the same solvent concentration still elutes peptides. This may be due to the small volume of the resin and the low interaction time between the resin and the solvent. A clear improvement is achieved by carrying out three elution steps, as described. The three obtained volumes may be kept separately or mixed together.
15. Be patient. It may take a few hours to completely evaporate the volume, especially for the elution fraction at 10% acetonitrile.
16. For all the streptococcal species with which the author has experience, there is enough peptide material in the resuspended “surfome” after cleaning with the cartridges to be detected by LC/MS/MS. Moreover, the sample can be diluted without losing detection capacity.
17. Use low-binding tubes. It has been observed that, when using normal, non low-binding tubes, the number of different peptides identified by LC/MS/MS dramatically decreases after some months.
18. To be sure that the cleaning process with the Oasis[®] HLB cartridge has worked well, keep both the sample flow-through and the wash fractions, and vacuum-concentrate them, together with the elution fractions. In there are no peptides in the eluted fraction(s), the presence of peptides can be quickly checked in the sample flow-through and the wash fraction by MALDI-TOF MS.

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