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Sample Preparation for Proteomic Analysis of Barnacle Basal Materials

DR. ERIC O. MCGHEE

Laboratory for Molecular Interfaces Branch
Center for Biomolecular Science and Engineering Division

DR. DAGMAR LEARY

Laboratory for Biomaterials and Systems Branch
Center for Biomolecular Science and Engineering Division

DR. WILLIAM J. HERVEY
DR. CHRISTOPHER SPILLMANN

Laboratory for Bio/Nano Science and Technology Branch
Center for Biomolecular Science and Engineering Division

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Briefly, this method utilizes sample buffer extraction of barnacle proteins, denaturation, separation via gel electrophoresis based on molecular weight and size. Once vertical separation is achieved, gel image is obtained and protein bands are cut out at the corresponding molecular weights, cutting pattern is recorded, and in-gel digestion using trypsin is performed. This protocol requires a minimum two working days to be completed.

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EXECUTIVE SUMMARY

This standard operating procedure (SOP) describes how to extract and process a complex protein mixture for LC-MS proteomics analysis. Gel electrophoresis for protein separation and in gel digestion is used to make the protein mixtures compatible with LC-MS/MS processing. Successful application of this SOP to samples collected from the basal region of barnacles is described herein but the SOP can be applied to other complex protein mixtures. The protocol was successfully used to improve understanding of barnacle adhesion to aid in developing antifouling materials and protecting the marine infrastructure.

Briefly, this method utilizes sample buffer extraction of barnacle proteins, denaturation, separation via gel electrophoresis based on molecular weight and size. Once vertical separation is achieved, gel image is obtained and protein bands are cut out at the corresponding molecular weights, cutting pattern is recorded, and in-gel digestion using trypsin is performed. This protocol requires a minimum two working days to be completed.

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SAMPLE PREPARATION FOR PROTEOMIC ANALYSIS OF BARNACLE BASAL MATERIALS

1. INTRODUCTION

Marine infrastructure exposed to the environment is subjected to harsh conditions where chemical and physical forces can degrade capabilities. In addition to environmental factors, fouling of surfaces by plants or animals can also play an important role in the degradation of marine infrastructure and performance. Biofoulers span protists (*e.g.* algae), microbes and microbial films, sea grasses, a range of higher order organisms. For example, on large Naval ships there is significant increased operational and maintenance cost incurred by the elevated drag forces and increased fuel expenditure from hull biofouling [1]. Therefore, an ongoing challenge is the development of next-generation marine coatings that target problematic organisms and otherwise minimize their impact on the environment.

A prominent macroscopic biofouler is the acorn barnacle [2], an organism known to colonize on a wide variety of natural and synthetic surfaces. As hermaphroditic arthropods, barnacles undergo metamorphosis from their final larval stage as a cyprid to a sessile adult where they produce a tenacious cement to attach permanently to a substrate. Barnacles have been a source of interest dating back to Darwin [3] and, more recently, for the purposes of understanding the mechanism and composition of interfacial cement layer, which appears to be a secreted material [4]. Currently, there is not a conclusive explanation of the mechanism underlying barnacle adhesion, which appears to be distinct from adhesive chemistries identified in other marine organisms [5-7]. However, several hypotheses have been proposed: hydrophobic forces [8-12]; repetitive sequence motifs similar to those found in silk proteins [13] promoting nanofibril formation [14-18]; protein polymerization as a specialized form of wound healing [4]; and protein post-translational modifications [19]. These hypotheses highlight how much remains to be understood about the mechanism of cement production in adult acorn barnacles.

Understanding barnacle adhesion is complicated by the active processes occurring at the leading edge of the expanding base or obscured underneath it. Observation of the leading edge of growth in juvenile barnacles via confocal microscopy has highlighted the biological complexity of this region [14, 15, 20, 21].

Early academic efforts identified several proteins in barnacle cement [8, 22, 23]. More recently, a number of unique proteins in the adhesive have been identified [12, 13, 24-30]. Combined, these efforts have culminated in the identification of ~90 proteins in the adhesive proteome of the acorn barnacle *Amphibalanus amphitrite* [13, 26, 27] and help promote this species a model organism for antifouling research [2].

Development of a standard method for the collection, extraction, and downstream proteomic analysis of barnacle cement is critical to continue to understand the composition of this key material and aid to standardize the types of experiments and analysis for inter-species comparisons. Here, a method to separate previously collected barnacle adhesive is described as a standardized approach to subsequent proteomic analysis. Note, this method is not exclusive to barnacle cement preparations, but is generalized to apply to the separation and initial preparation of most protein rich samples for downstream analysis.

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2. PROTEIN SEPARATION OF BARNACLE BASE MATERIALS

2.1 Method

Note: wear gloves at all times when collecting and handling samples to avoid introduction of unwanted contaminants

2.1.1 Equipment

- Gel electrophoresis vertical gel separation system, or similar stacked gel electrophoresis electrode separation system (Currently in the lab: XCell SureLock Mini-Cell Electrophoresis System)

Figure 1- example of gel electrophoresis system

- Power supply compatible with gel electrophoresis system

Currently in the lab: Thermo Electron Co., EC250-90

- Gel imaging equipment

Currently in the lab: BioRad, Gel Doc™ EZ Imager

- Light box (optional) for identifying bands and cutting gels
- Gel knife
- Razor blade
- ~1 L reservoir basin (Tupperware or pipette tip box useful) for washing and staining the gel
- Speed vac
- 1L glass bottle
- Low attachment (LA) pipette tips and tubes

example: Eppendorf™ LoBind

2.1.2 Materials

- 20x MES (2-morpholin-4-ylethanesulfonic acid; catalog no.: NP0002 NuPAGE™ MES SDS Running Buffer (20X) volume 500 mL) or MOPS (3-(N-morpholino)propanesulfonic acid; catalog no.: NP0001NuPAGE™ MOPS SDS Running Buffer (20X) volume:500 mL) running buffer

There are two choices of running buffers for SDS-PAGE gels: MES and MOPS. MES has a lower pKa than MOPS enabling the gel to run faster, thus resulting in better separation of proteins at lower molecular weights. MOPS buffer provides better separation at higher molecular weights.

- 18Ω Milli Q water in clean glass bottle
- Sample buffer containing lithium dodecyl sulfate 4x buffer solution

Catalog no. NP0007 NuPAGE™ LDS Sample Buffer (4X) volume 10 mL

Alternatively buffer kits can be used: NP0060 NuPAGE™ MES SDS Buffer Kit (for Bis-Tris Gels), contains: NuPAGE MES SDS Running Buffer (20X, 500 mL, Cat. No. NP0002), NuPAGE Sample Reducing Agent (10X, 250 µL, Cat. No. NP0004), NuPAGE Antioxidant (Cat. No. NP0005), NuPAGE LDS Sample Buffer (4X, 10 mL, Cat. No. NP0007) or NP0050 NuPAGE™ MOPS SDS Buffer Kit (for Bis-Tris Gels), contains: NuPAGE MOPS SDS Running Buffer (20X, 500 mL, Cat. No. NP0001), NuPAGE Sample Reducing Agent (10X, 250 µL, Cat. No. NP0004), NuPAGE Antioxidant (Cat. No. NP0005), NuPAGE LDS Sample Buffer (4X, 10 mL, Cat. No. NP0007)

- Molecular weight marker

Example: Thermo Scientific™ PageRuler™ Prestained Protein Ladder, 10 to 180 kDa, Catalog number: 26616

- Coomassie blue stain solution

For proteomics, we prefer the Bio-Safe™ Coomassie (161-0786) — Bio-Safe Coomassie Brilliant Blue G-250 stain is fast, simple, sensitive, and convenient. Because it has very low amounts of methanol, and does not fix the gels, digestion is much easier to complete.

- SDS-PAGE gel pack

Here, a 10% NuPAGE bis-tris gel pack was used to perform the separation (catalog no.: NP0301BOX NuPAGE™ 10%, Bis-Tris, 1.0 mm, Mini Protein Gel, 10-well), but use of NuPAGE gels is not a necessity; however gels have to be compatible with the gel running apparatus!

- LC-MS-grade water (Fisher optima or similar)
- LC-MS-grade Acetonitrile (Fisher optima or similar)
- 3% Formic acid (prepared by mixing Formic Acid, LC-MS Grade ampule with LC-MS grade water to achieve 3% v/v concentration)
- 1M Ammonium Bicarbonate in LC-MS water
- Trypsin (Promega catalog no.: V5111)
- Dithiothreitol (DTT)
- Iodoacetamide (IAA)

List of materials used in our laboratory and their specification can be found here: https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-gel-electrophoresis/protein-gels/nupage-bis-tris-gels.html

2.1.3 General procedure

The sample used in this procedure should be a protein containing solution mixed into an adequate buffer. Here, LDS is used as the buffer. The collection of and preparation of material specific to barnacle base materials is included in a separate report, but briefly, this material should be collected directly into buffer, homogenized as gently as best as possible, and collected at the highest concentration possible degree for maximum protein content during PAGE separation. This sample is referred to as the *sample solution*.

1. Clean and rinse electrophoresis assembly 3x in Milli Q water and air dry
2. Assemble electrophoresis system with fresh gel kit

To prepare the gel cassette, remove the comb from the top of the gel and white strip from the bottom of the gel prior to adding to assembly.

3. If only single gel will be run, use a blank in the place of the second gel to seal the cartridge.
4. Mix 45 mL of chosen 20x running buffer with 855 mL Milli Q water and gently mix, avoiding bubbles
5. Pour buffer solution into PAGE device - over pour and allow overflow into overflow chamber
6. Wash each well by pipetting running buffer into it. Ensure wells are not collapsed.
7. Add 5 uL of ladder solution to first column, this is the ladder column
8. Add 5 uL of sample buffer with LDS 4x to next column, this column is a blank column
9. Add 15 uL of each sample solution to sample wells
   a. Best to separate columns with 5uL LDS 4x to avoid samples mixing across lanes

10. Apply electrodes to assembly and run power supply on constant voltage

Running voltage: voltage can be tuned to achieve desired separation speeds but should be kept low enough to not generate high currents (>2A) in chamber. This user finds a voltage near 110V to work well, balancing separation speed and current.

11. Check every 30 minutes until sample line reaches depth needed; stop before sample solvent front runs off the gel
12. Take out gel pack and carefully crack open using gel knife
13. Remove gel from the plastic sheet

Removing gel from NuPAGE container: gently removing the gel can be a challenge but cracked gels can be avoided by first cutting off the foot of the gel (the L-shaped region near the bottom), then gently rocking the slightly submerged gel parallel to the water surface until the gel slides off the plastic into the water. This may take a bit of coercing, but should allow gentle removal. If assistance is needed, gently prying one corner when submerged in water should provide a crease for water to enter.

14. Place in 1L reservoir basin filled with Milli Q water and gently shake 3x 10 minutes, replacing water each time
15. Dump water and add staining solution to reservoir

Staining solution: coomassie blue is used here, but can be changed to suit user needs. If the staining solution is changed, the destaining protocol must be adjusted accordingly. The amount required is simply an amount that would cover the gel, allowing it to move around in the container when on the shake table. Typically, 50 mL is used, but this is container dependent.

16. Stain at least 3 hours. Longer is acceptable but destaining will take longer.
17. Properly dispose of staining solution and gently rinse gel and container with Milli Q water to remove residual staining solution. Be careful not crack gel
18. Fill reservoir with a large amount of Milli Q water and shake gently for a few hours or overnight if needed, change water often if faster destaining is desired
19. Dispose of the water and check for bands in sample lanes and ladder lane
20. Continue replacing water until it becomes clear and bands are visible, using the ladder column as a reference when sample bands are difficult to identify
21. Replace water and place in fridge until ready to continue

2.1.4 Imaging gel

1. Select proper light sheet for gel: white light for coomassie blue
2. Carefully lay gel onto lighting portion of sheet. Sheet can be wet to help gel slide
3. Insert light sheet into machine
4. Prepare imaging protocol in software, paying attention to exposure time and light power for best results
5. Save and print to pdf
6. Remove light sheet and gather gel carefully into new container of water
7. Print copy for use during cutting

2.1.5 Cutting and destaining gel

1. Slide gel onto glass sheet and place onto light box

Light box is optional but helps when identifying lightly stained bands. If light box is used, take extra care to prevent gel overheating and to prevent unwanted protein modification.

2. On printed sheet, mark the MW of the ladder bands.
3. Identify region to cut out of gel on printed sheet, then cut that section out using a clean razor blade, and ensure the cutting pattern is recorded properly by labeling tubes

Attempt to minimize blank gel by sectioning bands neatly around stained regions

4. Place cut out band into 1.5mL Eppendorf tube
5. Repeat cutting and place into new tube, each time identifying region on printed sheet

6. In each respective tube, use a pipette tip to chop gel sections into smaller chunks
7. Make a 50 mL solution of 250mM ammonium bicarbonate in 1:1 Acetonitrile:LCMS water
   a. This is the destaining solution
8. Add 1 mL destaining solution and incubate at least 1hr
9. Remove and collect the solution as hazardous waste, being careful not to remove gel
10. Repeat 3x or until destained; if longer periods of destaining are needed it is advised to place the tubes to the fridge to prevent unwanted protein modifications and loss.
11. Extract and waste liquid being careful not to remove gel
12. Use a 200 uL pipette tip to chop the gel into 1mm nominal pieces
13. Add 1 mL 100% LCMS Acetonitrile to dehydrate gel
14. Remove and waste liquid and allow to air dry 1-2 mins inside tube
15. Store in 4C until ready for next steps

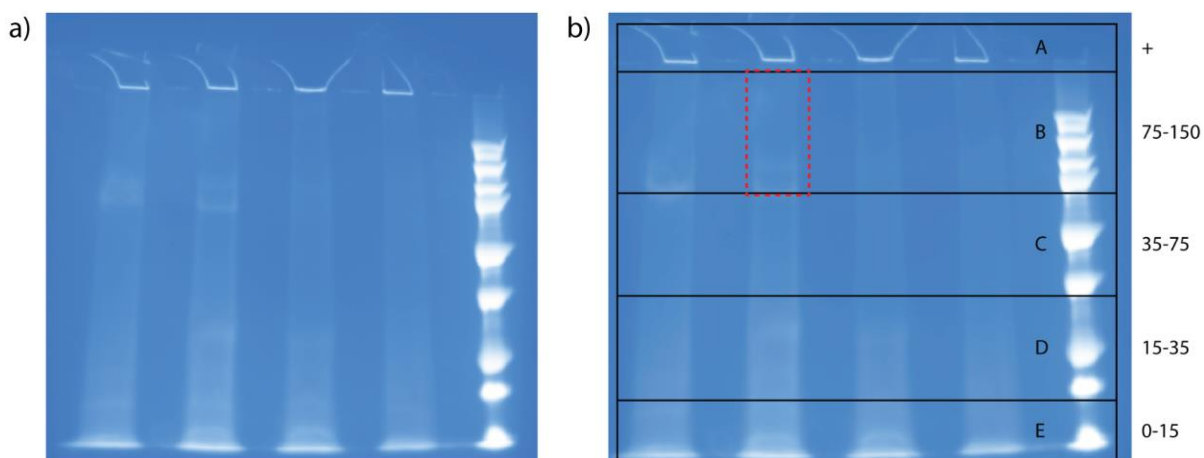

Fig. 2 — Result of electrophoretic separation of protein mixture through a 10% NuPAGE gel using MOPS buffer. a) Image of bands after separation; the first four columns correspond to true sample while the final column on the right corresponds to the reference ladder sample. b) Samples are identified by molecular weight region from reference ladder and cut out. Black boxes correspond to identified regions for follow-on in-gel digestion. Red dashed box shows cutting boundary for an example region.

2.1.6 Digesting peptides from gel

Note: volumes described below are not universal. Volumes should be targeted at use of minimal quantities so to concentrate extraction as much as possible. This volume target may change depending on the quantity of gel in the tube - i.e., more gel, more volume. Importantly, ensure complete flooded coverage of protein-containing gel across all steps.

1. Make a 1M solution of DTT in 100 mM ammonium bicarbonate (AmBicarb) LCMS water and make a dilution to 10 mM DTT with 100 mM AmBicarb
2. Add 200 uL 10 mM DTT solution to gel containing tubes
3. Incubate 1 hr
4. Remove and waste liquid
5. Add 1mL 100% LCMS Acetonitrile to collapse gel
6. Remove and waste liquid and allow to air dry 1-2 mins inside tube
7. Make 1M solution of IAA in 100 mM AmBicarb LCMS water and dilute to 25 mM (or 2.5x DTT solution concentration)
8. Add 200 uL 25 mM IAA solution to gel containing tubes

9. Incubate 1 hr in dark
10. Remove and dispose of the liquid
11. Add 1mL 100% LCMS Acetonitrile to dehydrate the gel
12. Remove and dispose of the liquid and allow to air dry 1-2 mins inside tube
13. Make Trypsin solution by adding 1 mL 100 mM AmBicarb LCMS water to trypsin bottle
14. Add ~200 uL trypsin solution to gel tubes, enough to cover gel pieces.
15. Incubate 8 hrs

Trypsin incubation time: the time necessary for trypsinization of proteins can vary and may need to be customized to user needs. Over-trypsinization is a real possibility and timeframes longer than 8 hours should be avoided. Over-trypsinization can result in poor LCMS readings as trypsin fractions could drown relevant data.

16. Make 1:1 Acetonitrile:LCMS water solution containing 3% formic acid
    a. This is the extraction solution
17. Add 150 uL extraction solution to gel containing trypsin solution and vortex mix gently and tap tube on solid surface to force solution down off walls
18. Extract liquid and place into new tube. Pipet carefully to avoid small gel pieces from being transferred.
    a. This is the LC-MS sample
19. Add 150 uL extraction solution to gel and vortex mix gently and tap down
20. Extract liquid and place into LCMS sample tube
21. Repeat 3x
22. Add 100% Acetonitrile
23. Vortex mix and tap down
24. Extract liquid and place into tube containing LCMS sample
25. Speed-vac these samples dry

Speed-vac timeframe: evaporation timeframes depend on total volume of sample. Extended operation of evaporator once sample has been evaporated sufficiently should be avoided.

26. Store dry peptide samples in -20C until they can be analyzed by LC-MS.

3. CONCLUSIONS

Use of the described method allows a user to vertically separate a protein mixture and allow better protein identification LC-MS based proteomics.

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