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Title: One-Step Extraction and Zymographic Analysis of Bacterial Gelatinases

Authors and Affiliations:
Sandhanakrishnan Cattavarayane, Dhandapani Gunasekaran, Abhijit Sarma, Homen Phukan, Banshanlang Marboh, Suneetha Hariharan, Madathiparambil Gopalakrishnan Madanan

Regional Medical Research Centre, Indian Council of Medical Research, Port Blair

Corresponding Authors:
Madathiparambil Gopalakrishnan Madanan — madanan.mg@icmr.gov.in, madananmg@gmail.com

Email Addresses for All Authors:
Sandhanakrishnan Cattavarayane — sandhana.krishnan@gmail.com
Dhandapani Gunasekaran — gunasekaran.vpm1990@gmail.com
Abhijit Sarma — abhijit.sarma2012@gmail.com
Homen Phukan — biotechphukan16@gmail.com
Banshanlang Marboh — banshanmarboh1998umtasor@gmail.com
Suneetha Hariharan — suneethasujith@gmail.com
Madathiparambil Gopalakrishnan Madanan — madanan.mg@icmr.gov.in, madananmg@gmail.com

Author Questionnaire

1. We have marked your project as author-provided footage, meaning you film the video yourself and provide JoVE with the footage to edit. JoVE will not send the videographer. Please confirm that this is correct.

√ Correct

2. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

3. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

4. Proposed filming date: To help JoVE process and publish your video in a timely manner, please indicate the proposed date that your group will film here: **MM/DD/YYYY**
Authors, please inform the tentative date by email

When you are ready to submit your video files, please contact our Content Manager, Utkarsh Khare.

Current Protocol Length

Number of Steps: 16
Number of Shots: 47

Introduction

1.1. **Suneetha Hariharan**: This research investigates a simpler methodology for extracting and characterizing active gelatinases from *Leptospira*, utilizing zymography to confirm their enzymatic activity and addressing challenges in isolating membrane-bound proteins from bacteria.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.3.1*

What technologies are currently used to advance research in your field?

1.2. **Suneetha Hariharan**: Current technologies include mass spectrometry-based proteomics, two-dimensional gel electrophoresis, bioinformatics tools, zymography, and LC-MS/MS, enabling detailed protein profiling, functional analysis, and identification of virulence factors in *Leptospira* research and vaccine candidate discovery.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 2.5.1*

What are the current experimental challenges?

1.3. **Suneetha Hariharan**: Characterizing active outer membrane proteins, such as leptospiral gelatinases, poses a challenge, particularly due to their membrane localization, which requires efficient disruption and solubilization methods to maintain the protein's integrity and accuracy.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.7.1*

What research gap are you addressing with your protocol?

1.4. **Suneetha Hariharan**: This protocol addresses the problem of extracting active outer membrane gelatinases from *Leptospira* by providing a one-step extraction process for use in pathogenesis investigations.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll:.3.11.1*

What research questions will your laboratory focus on in the future?

1.5. **Suneetha Hariharan**: Our laboratory focuses on leveraging advanced high-resolution LC-MS/MS to systematically identify outer membrane and secretory proteins, particularly focusing on proteinases involved in extracellular matrix (ECM) degradation. By elucidating their roles in pathogenesis, we aim to identify sensitive and specific biomarkers for the early-stage detection of leptospirosis.

1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.1.1*

Protocol

2. Extraction of Bacterial Gelatinases

Demonstrators: Banshanlang Marboh, M.G. Madanan, and Suneetha Hariharan

2.1. To begin, grow the organism in Ellinghausen McCullough Johnson Harris medium containing 10 milligrams per milliliter of BSA at 30 degrees Celsius **[1]**. Take 10 milliliters of *Leptospira* culture in the mid-logarithmic phase of growth containing 3.5 × 10^8 cells per milliliter for proteinase extraction **[2]**.

2.1.1. WIDE: Talent removing the culture from the incubator.

2.1.2. Talent withdrawing 10 milliliters of *Leptospira* culture from the flask into a sterile tube.

2.2. Centrifuge the culture at 6,000 *g* for 10 minutes **[1]**. Discard the supernatant into a container for decontamination **[2]**.

2.2.1. Talent placing the tube into the centrifuge and starting the run.

2.2.2. Talent pouring the supernatant into a labeled decontamination container.

2.3. Suspend the pellet in 1 milliliter of wash buffer and transfer it to a 1.5 milliliter microcentrifuge tube **[1]**. Centrifuge the tube at 6,000 *g* for 5 minutes and repeat the wash once more **[2]**.

2.3.1. Talent adding wash buffer to the pellet and pipetting to resuspend.

2.3.2. Talent placing the tube in a centrifuge.

2.4. Now, add 100 microliters of extraction buffer to the *Leptospira* pellet and vortex for 5 minutes **[1]**. Incubate the mixture at 4 degrees Celsius for 1 hour **[2]**. Then, centrifuge the suspension at 15,000 *g* at 4 degrees Celsius for 10 minutes **[3].** Post-centrifugation, collect the extracted protein **[4-TXT]**.

2.4.1. Talent pipetting extraction buffer into the tube and vortexing.

2.4.2. Talent placing the tube in a cold incubator or ice box.

2.4.3. Talent placing the sample in a centrifuge.

2.4.4. Shot of transferring the extract supernatant into a fresh tube. **TXT: Discard and decontaminate the pellet**

2.5. ~~Mix the extracted protein with an equal volume of 2x sample buffer for direct use in zymography **[1].** If the protein concentration is low, incubate it with four times its volume of acetone overnight at minus 20 degrees Celsius **[2].** The next day, centrifuge the sample at 10,000 *g* for 10 minutes **[3].** After air drying the resulting pellet, resuspend it in PBS **[4].** Dissolve the protein in the desired buffer and store it at minus 20 degrees Celsius for up to 1 month **[5]**.~~

~~2.5.1. Talent adding protein extract with 2x sample buffer and mixing by pipetting.~~

~~2.5.2. Talent adding cold acetone to the sample and placing it in a freezer.~~
~~2.5.3. Talent placing the sample in a centrifuge.~~
~~2.5.4. Talent adding PBS to the dried sample and resuspending by pipetting.~~
2.5.5. ~~Talent transferring the solution into labeled microcentrifuge tube placing it in the freezer.~~ **NOTE**: Not filmed, delete

3. Zymography of Isolated Gelatinases

3.1. Prepare the resolving gel mix for sodium dodecyl sulfate-polyacrylamide gel electrophoresis with co-polymerized gelatin **[1]**. Pour the resolving gel mix into the casting chamber up to approximately 3 to 5 millimeters below the mark for the bottom of the wells **[2-TXT]**. Slowly pour water to form an even 1-millimeter layer over the gel and remove any bubbles and let the gel set for 1 hour **[3]**.
3.1.1. Talent swirling the resolving gel solution in a beaker gently.
3.1.2. Shot of pouring the gel solution into the gel casting apparatus.
3.1.3. Shot of slowly pouring water on top of the gel to form a uniform layer.
3.1.4. ~~Talent setting a timer after placing the gel aside~~.**NOTE**: Not filmed, VO merged with the previous shot

3.2. Before preparing the stacking gel, tilt the setup to decant the water from the top of the set resolving gel **[1]** and blot with a narrow strip of filter paper **[2]**. Add 0.03 milliliters of 10 percent ammonium persulfate to the stacking gel mix and stir gently to avoid bubble formation **[3]**.
3.2.1. Talent tilting the gel cassette.
3.2.2. Talent blotting water with filter paper.
3.2.3. Talent pipetting ammonium persulfate into the stacking gel mix and stirring gently.

3.3. Now, pour the stacking gel mix onto the resolving gel and immediately place the comb without trapping air bubbles **[1-TXT]**. Next, fix the gel into the electrophoresis apparatus **[2]** and remove the lower spacer **[3]**.
3.3.1. Talent pouring stacking gel onto resolving gel and placing comb without bubbles. **TXT: Wait at least 3 h for complete gelatin polymerization and crosslinking**
3.3.2. ~~Close-up of gel resting~~. **NOTE**: Not filmed, VO moved as on screen text
.3.3.4. Talent mounting the gel onto the electrophoresis unit. **NOTE**: The shots and VO are inverted
3.3.3. Talent removing spacer

3.4. ~~Then, slowly place the gel in the tank containing 1x electrode buffer, ensuring there is no trapped air at the bottom of the gel **[1]**.~~ After placing the gel in the tank, add an equal volume of sample buffer to the protein extract **[1]**. Prepare a positive control

with 2 microliters of human serum with an equal volume of sample buffer **[2]**. Incubate the samples at 37 degrees Celsius for 30 minutes to allow SDS to bind to the protein **[3]**

~~3.4.1. Talent carefully positioning the gel in the electrophoresis tank~~. **NOTE**: Not filmed, VO merged with the next shot

3.4.2. Talent adding protein extract with sample buffer.

3.4.4. Talent placing the sample tube in a 37 degrees Celsius incubator.

3.4.3 Talent labeling a tube as "positive control." **NOTE**: The shots and VO are inverted

3.5. Now, carefully load the prepared samples and control into the gel wells using a narrow-tipped pipette **[1-TXT]**. ~~Gradually fill the remaining portions of the wells, the top of the gel, and the upper tank with electrode buffer without disturbing the loaded samples **[2]**~~.

3.5.1. Talent loading samples into the wells using a pipette. **TXT: Alternatively, use a Hamilton syringe (0.5 - 1 mL) for loading**

3.5.2. ~~Talent pouring electrode buffer gently into the tank and around the gel setup~~. **NOTE**: Not filmed

3.6. Start the electrophoresis at 6 milliamperes per gel until the dye front enters the resolving gel **[1]**. Then increase the current to 12 milliamperes per gel and continue running until the dye front reaches 1 centimeter above the bottom of the gel **[2]**.

3.6.1. Talent setting the electrophoresis current to 6 milliamperes per gel and start the run.

3.6.2. Shot of the dye front entering resolving gel.

3.7. After electrophoresis, carefully dismantle the glass plates using a plate separation tool or plastic spatula **[1]**. Mark the orientation of sample loading by cutting the right lower corner of the gel, and then mark the pre-stained marker bands **[2-TXT]**.

3.7.1. Talent removing gel plates with a plastic spatula and separating the gel.

3.7.2. Talent cutting the bottom-right corner of the gel with a blade. **TXT: Alternatively, scan the gel to mark the prestained bands**

3.7.3. ~~Talent marking prestained marker bands using a hole punch~~. **NOTE**: Not filmed, VO merged with the previous shot

3.8. Submerge the gel in a renaturing solution containing Triton X-100 in a gel tray **[1]** and incubate at room temperature for 30 minutes without shaking **[2]**. Replace the renaturing solution with activation buffer and incubate again for 18 hours **[3]**.

3.8.1. Talent transferring the gel into a tray filled with renaturing solution.

3.8.2. Talent placing the unit aside for incubation.

3.8.3. Talent pouring off renaturing solution and adding activation buffer to the gel.

3.9. After removing the activation buffer, rinse the gel with water for 1 minute **[1]** and add the staining solution, ensuring the gel is fully submerged **[2]**. Gently shake the gel at low speed until the gel turns completely blue **[3]**.
  3.9.1. Talent adding the gel with distilled water.
  3.9.2. Talent adding staining solution on the gel.
  3.9.3. Talent placing the gel tray on a shaker at low speed.

3.10. Now, rinse the gel surface with water for 1 minute to remove excess stain **[1]** and add the destaining solution **[2]**. Replace the solution several times until the desired contrast is achieved **[3]**.
  3.10.1. Talent rinsing gel with water using a squeeze bottle.
  3.10.2. Talent adding destaining solution onto the gel.
  3.10.3. Shot of the completely stained gel with good contrast.

3.11. Finally, scan the gel using a gel documentation system or document scanner to compare the protein bands with the standard markers **[1]**.
  3.11.1. Talent loading the stained gel into the documentation system and initiate the scan.

Results

4. Results

4.1. The zymogram of leptospiral protein and gelatinases from human serum shows proteases as white clear bands against a blue background **[1].**

4.1.1. LAB MEDIA: Figure 1.

4.2. The bands represent areas where the copolymerized gelatin is degraded and hence, not stained **[1]**, while other areas of the entire gel are stained blue due to the presence of gelatin **[2].**

4.2.1. LAB MEDIA: Figure 1. *Video editor: Highlight the bright coloured bands in the first 2 lanes*

4.2.2. LAB MEDIA: Figure 1. *Video editor: Highlight the blue coloured background*

1. **Leptospira**
   - Pronunciation link: https://dictionary.cambridge.org/pronunciation/english/leptospira Cambridge Dictionary
   - IPA (American): /ˌlep.toʊˈspaɪ.rə/ Cambridge Dictionary
   - Phonetic Spelling: LEP-toh-SPY-ruh

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2. **Polyacrylamide**
   - Pronunciation link: https://www.howtopronounce.com/polyacrylamide How To Pronounce
   - IPA (American): /ˌpɑliəˈkrɪləˌmaɪd/ How To Pronounce
   - Phonetic Spelling: pah-lee-uh-KRIL-uh-myd

3. **Ammonium Persulfate**
    - Pronunciation link: https://www.merriam-webster.com/dictionary/ammonium%20persulfate Merriam-Webster
    - IPA (American): /əˈmoʊniəm pərˈsʌlfeɪt/ How To Pronounce+2Wikipedia+2
    - Phonetic Spelling: uh-MOH-nee-um per-SUL-fate

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4. **Electrophoresis** (as in "gel electrophoresis")
    - Pronunciation link: *No single link found in the sources I checked with all context; but standard dictionaries have "electrophoresis."*
    - IPA (American): /ɪˌlɛktroʊfʊˈriːsɪs/
    - Phonetic Spelling: ih-LEK-troh-fuh-REE-sis

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5. **Gelatinase**
    - Pronunciation link: *No direct link found in my checked sources, though it appears in zymography context widely.*
    - IPA (American): /ˌdʒɛləˈteɪnˌeɪs/
    - Phonetic Spelling: jel-uh-TAYN-ays

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6. **Centrifuge**
    - Pronunciation link: https://www.merriam-webster.com/dictionary/centrifuge Wikipedia+2Wikipedia+2 (centrifuge is a common term)
    - IPA (American): /ˈsɛn.trəˌfjuːdʒ/
    - Phonetic Spelling: SEN-truh-fyoohj

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7. **Zymography**
    - Pronunciation link: *No direct Merriam-Webster link found in checked sources; zymography is a specialized term.*
    - IPA (American): /zaɪˈmɑːgrəfi/
    - Phonetic Spelling: zy-MAH-gruh-fee

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8. **Mid-logarithmic phase**
    - Pronunciation link: *No direct source for "logarithmic" in context here, but "logarithmic" is in standard dictionaries.*

- IPA (American): /ˌmɪd ləʊˈgærɪðmɪk feɪz/ → but for US: /ˌmɪd loʊˈgærɪðmɪk feɪz/
- Phonetic Spelling: MID loh-GAR-ith-mik faz

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9. **Electrode buffer**
   - Pronunciation link: *No specific source for "electrode buffer" as a phrase; but both words are in common dictionaries.*
   - IPA (American): /ɪˈlɛk.troʊd ˈbʌfər/
   - Phonetic Spelling: ih-LEK-trohd BUFF-er

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10. **Renaturing (solution / process)**
    - Pronunciation link: *No single dictionary entry I found with "renaturing" exactly; but similar "renature" is present.*
    - IPA (American): /riːˈneɪtʃərɪŋ/
    - Phonetic Spelling: ree-NAY-chur-ing