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Protocol for Assessing Lysozyme-Like Activity in *Galleria mellonella* Hemolymph



Forked from [Protocol for Assessing Phenoloxidase Activity in *Galleria mellonella* Hemolymph](#)

DOI

<https://dx.doi.org/10.17504/protocols.io.dm6gpm4y5gzp/v1>

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Galleria protocols



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We are still developing and optimizing this protocol

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Abstract

Bacterial or fungal infection in *Galleria mellonella* larvae produces an immune response in the host, affecting the antimicrobial enzymatic activity as the body tries to fight back the foreign elements causing the infection.

This is a protocol to evaluate lysozyme-like enzymatic activity in the cell-free hemolymph of *Galleria mellonella* larvae previously infected with a bacterial or fungal strain.

I designed this experiment to be run in parallel and/or use the same hemolymph samples obtained with the "Protocol for Assessing Phenoloxidase Activity in *Galleria mellonella* Hemolymph"

The anticoagulant buffer is taken from the described in DOI: 10.1159/000168009

Guidelines

Total Protein Quantification (for Normalization):

Use a Bradford or BCA Protein Assay Kit (e.g., from Bio-Rad or Thermo Fisher).

Standards: Prepare a standard curve using Bovine Serum Albumin (BSA).

Follow the kit's microplate protocol to determine the total protein concentration in the CFH samples. This will allow you to express lysozyme-like activity as "Activity/mg protein," providing a more accurate assessment of enzyme activity, especially if sample collection varies.

Materials

Anticoagulant Solution:

93 mM NaCl, 100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM Na₂EDTA, and 0.1 mM phenylthiourea, pH 4.6. Filter-sterilized through a 0.45 nm pore filter

Micrococcus luteus lyophilized cells:

Prepare a suspension of 0.2 mg/ml in 0.1 M sodium phosphate buffer (pH 6.2-6.5).

Sterile PBS

Hen Egg White Lysozyme

1.5 ml Eppendorf tubes

Crushed ice tray

Liquid nitrogen for snap-freezing

Refrigerated ultracentrifuge

Safety warnings

- ❗ Ensure all equipment and reagents are sterile to prevent contamination.
It is crucial to keep all samples on ice until they are processed to maintain enzyme activity.

Ethics statement

Ensure to follow all safety and ethical guidelines when handling live larvae and biological materials.

Before start

Prepare anticoagulant buffer 93 mM NaCl, 100 mM glucose, 30 mM trisodium citrate, 26 mM citric acid, 10 mM Na₂EDTA, and 0.1 mM phenylthiourea, pH 4.6



The day before

- 1 Grow 3 ml of an overnight bacteria culture at 37 °C in an incubator with vigorous shaking (150-200 rpm)

Bacteria preparation

10m

- 2 Take the overnight bacterial culture and transfer 2 ml into an Eppendorf tube
- 3 Centrifuge at no more than 1000 rcf, for 10 min at 4 °C.
- 4 Discard the supernatant and resuspend the bacterial pellet in 1 ml of sterile PBS
- 5 Measure the absorbance at 600 nm and dilute the suspension so it contains about 10^6 cells/ml

10m



Larvae injection

6h

- 6 Select *Galleria mellonella* larvae in their 7th instar (usually, above 400 mg in weight) and distribute in Petri dishes as groups of 5 for each condition.
- 7 Prepare a Hamilton syringe with 10 µl of bacterial suspension or sterile PBS in case of control.
- 8 Grab a larva by its sides with the thumb and the index fingers and flip it upside down to expose its abdomen
- 9 Localize the hind/left proleg of the larva and carefully insert the needle of the syringe about 5 mm into the body of the larva
- 10 Inject the contents of the syringe slowly to prevent any leaking, and remove the syringe.
- 11 Return the larva to their respective dish and repeat for all the larvae and conditions.



- 12 Transfer the Petri dishes to an incubator and incubate the larvae for 0-6 hours at 37 °C

6h

Material Preparation

2m

- 13 Dispense 50 µl of anticoagulant buffer into 1.5 ml Eppendorf tubes, and register their respective mass (1 tube per larva).
- 14 Transfer the tubes to an ice tray and wait a couple of minutes so the contents of the tubes are ice-cold.

2m



Hemolymph Collection

10m

- 15 Optional: Anesthetize the larvae on ice for 5-10 minutes to minimize movement during hemolymph collection
- 16 Surface sterilize the larvae by dipping them in 70% ethanol for a few seconds, then rinse with sterile PBS or water.
- 17 Surface sterilize the blades of a pair of micro scissors by dipping them in ethanol and allowing them to air dry.
- 18 With the scissors, make a small cut in a proleg of the anesthetized larva.
- 19 Gently squeeze the larva to collect the dripping hemolymph (aim for 50-100 µl per larva) into a pre-chilled tube containing 50 µl of ice-cold anticoagulant buffer.
- 20 Place the tube back on ice immediately after collection.
- 21 Repeat the collection steps for all the larvae.

10m



Preparation of Cell-Free Hemolymph (CFH)

10m



- 22 Register the weight and add ice cold anticoagulant buffer to ensure the final ratio of hemolymph to buffer is 1:1
- 23 Centrifuge the tubes at 4°C, 4000 x g for 10 minutes to separate cellular debris from the hemolymph. 10m 
- 24 Carefully transfer the clear supernatant (cell-free hemolymph) to a new pre-chilled tube. 
- 25 Aliquot the CFH to avoid freeze-thaw cycles.
- 26 Snap-freeze aliquots in liquid nitrogen and store at -80°C until ready for the PO activity assay. 

Lysozyme-like Activity Assay

35m

- 27 Prepare a series of HEWL dilutions in 0.1 M sodium phosphate buffer to create a standard curve (0, 0.1, 0.5, 1, 5, 10, 20 µg/ml).
- 28 In one or several wells of a microplate, add 90 µl of the *M. luteus* suspension.
- 29 Pre-incubate the mixture at room temperature for 5 minutes. 5m
- 30 Dilute the CFH sample in in 0.1 M sodium phosphate buffer at a ratio of 1:10.
- 31 Add 10 µl of the diluted CFH solution (or HEWL standard) to each well to start the enzymatic reaction. 
- 32 Immediately measure the absorbance at 450 nm using a spectrophotometer or microplate reader.
- 33 Record the absorbance every 30 seconds for a total duration of 30 minutes. 30m



Data analysis

- 34 Calculate the slope of the linear increase in absorbance ($\Delta A_{450}/\text{min}$). This rate is proportional to the lysozyme-like activity.
- 35 Normalize the PO activity to the total protein concentration in the CFH.

Protocol references

Bidla, Gawa, Thomas Hauling, Mitchell S. Dushay, and Ulrich Theopold. "Activation of insect phenoloxidase after injury: endogenous versus foreign elicitors." *Journal of Innate Immunity* 1, no. 4 (2009): 301-308.