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Preparation of peptidoglycan-depleted soluble microbial extract

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SUBJECT AREAS

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Introduction

Unknown microbial ligand(s) other than peptidoglycan (PG) in the soluble microbial extract (SME) can induce Dual oxidase (DUOX) activity and generate reactive oxygen species in the *Drosophila* gut epithelia. We describe a simple method for the preparation of the PG-depleted SME (SME-ΔPG) by treatment with recombinant peptidoglycan recognition protein (PGRP)-LB, a PG-degrading enzyme. (SME-ΔPG) has been successfully used for dissecting the PG-independent immune signaling pathways in *Drosophila* gut. The (SME-ΔPG) will be a potentially useful material to dissect PG-independent host response to microbes in diverse biological systems.

Reagents

E. coli BL21 (DE3) cells for recombinant protein expression (Novagen, cat. no. 69450)

Erwinia carotovora carotovora 15 (obtained from Bruno Lemaitre Laboratory, EPFL, Switzerland)

Plasmid containing the full-length *PGRP-LB* gene

LB (Luria-Bertani Broth) MILLER (Novagen, cat. no. 71753)

Sodium phosphate monobasic (SIGMA Aldrich, cat. no. S0751)

Sodium phosphate dibasic (SIGMA Aldrich, cat. no. S0876)

NaCl (Fluka Analytical, cat. no. 71379)

Imidazole (ACROS Organics, cat. no. 122020020)

Dithiothreitol (DTT) (Fisher Scientific, cat. no. BP172-25)

β-Mercaptoethanol (β-ME) (Fluka Biochemika, cat. no. 63690)

Ampicillin sodium salt (SIGMA, cat. no. A9518)

Isopropyl-D-thiogalactopyranoside (IPTG) (Gold BioTechnology Inc, I2481C100)

Ni-NTA Agarose resin (Quiagen, cat. no. 30250)

TEV-protease (Invitrogen)

PBS

Methanol

Reagent setup

Ampicillin-LB broth: 2.5 % LB Broth MILLER. Autoclave for 30 min. Store at room temperature. Add

ampicillin to a final concentration of 100 µg/ml before use.

Ampicillin-LB agar plates: 2.5 % LB Broth MILLER 1.5 % Bactor-Agar. Autoclave for 30 min. Allow LB agar to cool to 50 °C and then add ampicillin to a final concentration of 100 µg/ml. Dispense into sterile Petri dishes and allow to set at room temperature. Store the plates at 4 °C.

Cell lysis buffer: 20 mM sodium phosphate pH 7.6, 0.1 M NaCl and 2 mM β-ME.

40 mM imidazole wash buffer: Add 3 M imidazole pH 8.0 into the cell lysis buffer to a final concentration of 40 mM.

200 mM imidazole wash buffer: Add 3 M imidazole pH 8.0 into the cell lysis buffer to a final concentration of 200 mM.

Dialysis buffer: 20 mM sodium phosphate, pH 7.6, and 1 mM DTT.

1M NaCl wash buffer: Add 5 M NaCl to a final concentration of 1M into the dialysis buffer.

Equipment

Econo-Column chromatography column (Bio-Rad, cat. no. 737-4250)

Spectra/Por molecular porous membrane tubing (Spectrum Laboratories Inc)

HiTrap Q (GE Healthcare Bio-Sciences, cat. no. 17-1154-01)

HiTrap SP (GE Healthcare Bio-Sciences, cat. no. 17-1152-01)

VivaSpin 20 centrifugal concentrator with 10 K MWCO (Sartorius stedim biotech, cat. no. VS2001)

Incubator shaker with temperature control

UV-vis spectrophotometer (Varian Inc)

High speed refrigerated centrafuge (Hanil scientific industrial)

Probe sonicator (Sonic & Materials Inc)

AKTA HPLC system (GE Healthcare Bio-Sciences)

Grind kit for Precellys 24 (tube containing 0.5 mm glass beads, Bertin Technologies).

Precellys 24 Homogenizer (Bertin Technologies).

Lyophilizer

Procedure

A. Preparation of recombinant PGRP-LB.

1. Transform *E. coli* BL21 (DE3) cells by heat shock according to the manufacturers' protocol with the pProEx HTa vector containing the full-length *PGRP-LB* gene. Plate the transformed cells onto ampicillin-LB agar plates. Grow at 37 °C overnight.
2. Pick a single colony and inoculate a starter culture of 2 ml ampicillin-LB medium. Place the culture in an incubator overnight at 37 °C and 200 r.p.m.
3. Next morning, inoculate 50 ml ampicillin-LB medium in a shaker flask. Place the culture in an incubator at 37 °C and 200 r.p.m. for 2 h.
4. Inoculate 1 liter ampicillin-LB medium in a 2-liter flask with the culture from Step 3.
5. Grow the cultures in an incubator shaker at 37 °C and 200 r.p.m. until the optical density at 600 nm reaches 1.0.
6. Place the flasks in an ice water bath and rapidly cool the cultures to 18 °C.
7. Induce protein expression by adding IPTG to a final concentration of 1 mM. Continue to grow the cultures at 18 °C and 200 r.p.m. for 4 h.
8. Transfer the cells to centrifuge tubes and harvest the cells by centrifuging for 15 min at 5,000g and 4 °C.
9. Discard the supernatant and collect the cell pellet.
10. Resuspend the pellet in 50 ml of cell lysis buffer at 4 °C with glass rod until the suspension is homogeneous.
11. Place the suspension on ice and lyse the cells using sonication in a series of 10-s pulses to avoid heating until the suspension loses viscosity.
12. Centrifuge for 50 min at 15,000g and 4 °C to pellet cellular debris.
13. Collect the supernatant. Then, filtrate the supernatant through a 0.45 μm pore membrane.
14. Prepare the 5 ml of Ni-NTA agarose resin according to the manufacturers' instructions and equilibrate the resin with the cell lysis buffer. All purification steps

should be carried out at 4 °C with ice-cold buffers.

15. Load the supernatant collected from the Step 13 onto a column containing the equilibrated Ni-NTA agarose resin.
16. Wash the column with 50 ml cell lysis buffer.
17. Wash the column with 50 ml 40 mM imidazole wash buffer.
18. Elute the protein with 15 ml 200 mM imidazole wash buffer.
19. Transfer the elution fraction from the Step 18 into a dialysis membrane bag (molecular weigh cut-off of 3.5 KDa).
20. Immerse the bag in dialysis buffer (more than 50 times the volume of the elution fraction) and agitate the bag with a magnetic stir bar at 4 °C for 4 h.
21. Transfer the content of the bag to a clean beaker.
22. Add TEV-protease according to the manufacturers' instructions and incubate or overnight at 4 °C.
23. Check the cleavage by running SDS-PAGE and comparing the apparent molecular weight of PGRP-LB before and after the TEV-protease treatment.
24. Equilibrate a HiTrapQ column connected to the AKTA HPLC system with the dialysis buffer.
25. Load the sample from the Step 23 onto the HiTrapQ column. Collect and save the flow-through containing the unbound material.
26. Apply a linear gradient from the dialysis buffer to NaCl wash buffer over 15 column volumes.
27. Fractions containing PGRP-LB are identified using SDS-PAGE. PGRP-LB is found in the flow-through and low-salt fractions.
28. Equilibrate a HiTrapSP column connected to the AKTA HPLC system with the dialysis buffer.

29. Collect the fractions from the Step 27 and load the sample onto the HiTrapSP column. Collect and save the flow-through containing the unbound material.
30. Apply a linear gradient from the dialysis buffer to the NaCl wash buffer over 15 column volumes.
31. Fractions containing PGRP-LB are identified using SDS-PAGE. PGRP-LB is found in the flow-through and low-salt fractions.
32. Concentrate PGRP-LB from the Step 31 to 6 mg/ml using an VivaSpin 20 centrifugal concentrator following the manufacturers' instructions.
33. Freeze in liquid nitrogen in 100 µl aliquots and store at -80 °C.

B. Preparation of SME.

1. Inoculate a culture of 100 ml LB medium with a bacterial strain, *Erwinia carotovora carotovora 15*.
2. Grow at 30 °C for 16 h with vigorous shaking (~300 rpm).
3. Harvest the bacteria by centrifugation at 3,000 x g for 15 min at 4 °C.
4. Wash the pellet 2 times with 1 ml PBS.
5. Prepare ~200 mg (wet weight) of bacterial pellet.
6. Resuspend the bacterial pellet in 2 ml PBS.
7. Transfer to the centrifuge tube containing 0.5 mm glass beads (Grind kit for Precellys 24, Bertin Technologies)
8. Cell lysis by using Precellys 24 homogenizer at 6,500 rpm for 15 sec by repeating 3 times (Bertin Technology).
9. Lyophilze the the lysate.
10. Resuspend the dry pellet with 2ml of 100% methanol.
11. Centrifuge at 13,000 rpm for 5 min.
12. Lyophilize the supernatant.

13. Dissolve the dry pellet with 1 ml H_2O.

C. Preparation of SME-▲PG.

1. Add 560 ng of recombinant PGRP-LB to SME (50 mg).
2. Incubate the mixture for 1 h at 25 °C.
3. The depletion of PG in SME-ΔPG can be confirmed by examining the absence of *Diptericin* gene (PG-dependent gene) induction in SME-ΔPG treated *Drosophila* mbn2 cells.

Timing

Expression in *E. coli* (steps 1-9) TIMING 3 d

Cell lysis (steps 10-13) TIMING 4 h

Nickel affinity purification (steps 14-18) TIMING 3-4 h

Removal of imidazole (steps 19-21) TIMING 5 h

Cleavage of affinity tag (steps 22-23) TIMING 12 h

Anion exchanger chromatography (steps 24-27) TIMING 4 h

Cation exchanger chromatography (steps 28-31) TIMING 4 h

Concentration and storage of PGRP-LB (steps 32-33) TIMING 2 h

Preparation of SME TIMING 22h.

Preparation of SME-ΔPG TIMING 1h

Critical Steps

It is important to place the flasks in an ice water bath and rapidly cool the cultures to 18 °C (Step 6 of the procedure A).

Anticipated Results

1. We typically obtain 2 mg of highly purified PGRP-LB from 1 liter culture.
2. We routinely obtain 50 mg (dry weight) of SME from 100 ml culture.
3. The SME-ΔPG is virtually free of PG as evidenced by that SME-ΔPG could not induce *Diptericin* (PG-dependent target gene) expression in the mbn2 cells (**Fig. 1**), but still could induce reactive oxygen species in the same cells (**Fig. 2**).

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Figures

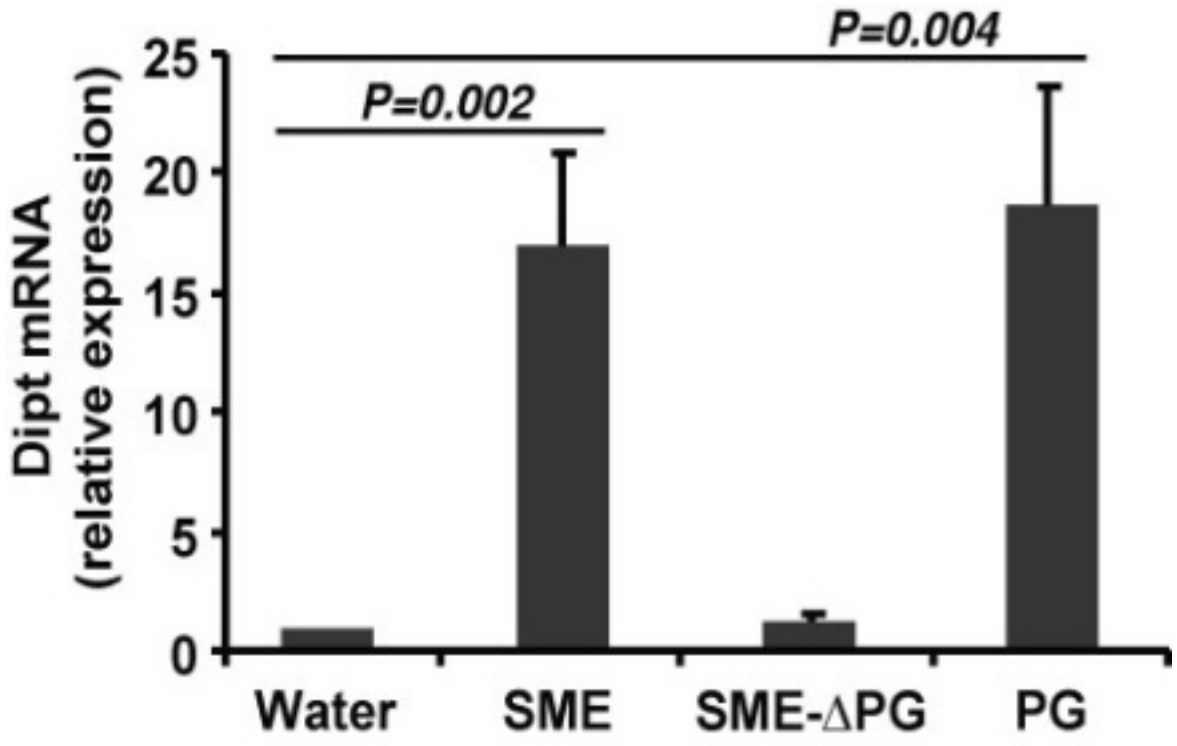

Figure 1

PG-dependent _Diptericin_ gene induction in mbn2 cells. _Drosophila_ mbn2 cells were treated with SME (1mg/ml), SME-ΔPG (1mg/ml) or monomeric PG (0.4 nM). Diptericin (9 h treatment) expression was measured by real-time PCR. Target gene expression in water-treated control mbn2 cells was taken arbitrarily as 1, and the results are shown as relative expression. Data represent at least three independent experiments (mean and s.d.).

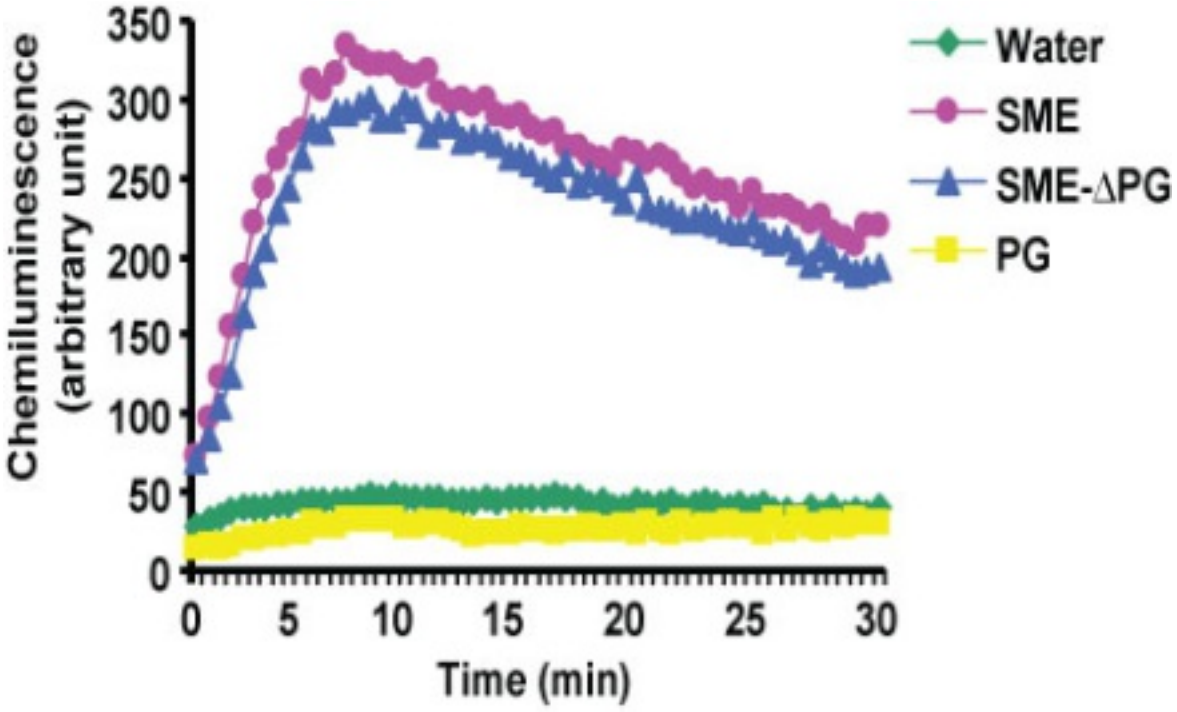

Figure 2

PG-independent reactive oxygen species generation in mbn2 cells. _Drosophila_ mbn2 cells were treated with water, SME (10 μg/ml), SME-ΔPG (10 μg/ml) or monomeric PG (0.4 nM). ROS generation was then measured using a luminal-based method. The results shown here are representative of at least three independent experiments all of which showed similar results.

Coordination of multiple dual oxidase–regulatory pathways in responses to commensal and infectious microbes in drosophila gut

by Eun-Mi Ha, Kyung-Ah Lee, You Yeong Seo, +4
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