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Chapter 12.

Measure of peptidoglycan hydrolase activity

Running head: peptidoglycan remodelling enzymes

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Summary: Most of the gene clusters encoding multiprotein complexes of the bacterial cell envelope, such as conjugation and secretion systems, Type IV pili and flagella, bear a gene encoding an enzyme with peptidoglycan hydrolase activity. These enzymes are usually glycoside hydrolases that cleave the glycan chains of the peptidoglycan. Their activities are spatially controlled to avoid cell lysis and to create localized rearrangement of the cell wall. This is assured by interaction with structural subunits of the apparatus. Here, we describe protocols to test the peptidoglycan hydrolase activity of these proteins *in vitro* and in solution.

Key words: cell wall, localized degradation, peptidoglycan, lytic transglycosylase, remazol blue.

1. Introduction

The peptidoglycan is a mesh-like structure that provides the shape and protection against external pressure to bacterial cells. It is composed of glycan chains resulting from the polymerization of *N*-acetylmuramic acid (MurNAc)-*N*-acetylglucosamine (GlcNAc) disaccharides. These chains are linked by peptide stems that differ from one species to the other. With pores of ~ 2 nm, the cell wall constitutes a physical barrier for the passage of macromolecules and for the assembly of cell envelope spanning complexes (***1-3***). Most trans-envelope multi-protein machineries have therefore evolved dedicated enzymes that locally degrade the cell wall to provide sufficient space for their assembly and insertion without compromising the bacterial shape and survival (***3,4***). These enzymes usually cleave the β-1,4 bond between the *N*-acetylmuramic acid and the *N*-acetylglucosamine of the glycan chains and form non-reducing 1,6-anhydromuropeptides characteritics of lytic transglycosylases

(LTGs) (***4-7***). Genes encoding these enzymes are found associated in Type III secretion, Type IV secretion or flagellum gene clusters (***3,4,6***). The best-studied specialized LTGs are FlgJ and SltF that are associated with flagellar assembly (***8-10***), and EtgA and VirB1 that are necessary for the biogenesis of the Type III (T3SS) and Type IV (T4SS) secretion systems, respectively (***5,11-16***). However, these enzymes could be deleterious for the bacterial cell and therefore their activity needs to be restricted to the site of assembly in order to avoid breaches in the cell wall. Studies have provided evidence that the LTGs are recruited via specific interactions to subunits of the machine (***17-19***), and in a few cases, that these interactions stimulate the activity of the LTG (***19,20***).

Methods have been developed and used to test whether putative LTGs have peptidoglycan hydrolase activities. An indirect approach is to clone the gene encoding the putative LTG to a signal sequence in order to address the protein to the periplasm of *Escherichia coli* and follow the cell growth after induction as overproduction of the LTG causes cell lysis (***21,22***). More direct protocols have been developed using purified LTGs, including zymogram (***23,24***). However, this technique, which consists to subject the purified LTGs to SDS-PAGE in a gel supplemented with purified peptidoglycan, has limits such as the refolding of the protein after migration. Additional approaches, performed in solution, do not need the denaturation and refolding steps. These turbidometric assays, detailed below, are methods to follow the activity of the purified LTG on peptidoglycan or peptidoglycan labelled with the Remazol Brilliant Blue (RBB) dye (***25,26***). The peptidoglycan assay relies on the decrease of absorbance of the peptidoglycan solution (***25***), whereas the RBB assay relies on the release on the dye captured into the peptidoglycan net (***26***) in the presence of the LTG. In addition, more precise approaches, such as the analysis of peptidoglycan degradation products released after incubation of the peptidoglycan with the purified protein by reverse-phase high-

performance liquid chromatography coupled to mass spectrometry (***27,28***), allow to define the site of cleavage of the enzyme.

2. Material

2.1. Peptidoglycan purification

1. 8% SDS solution: Resuspend 8 g of sodium dodecyl-sulfate resuspended in 100 mL sterile distilled water.
2. 20 mM Tris-HCl, pH8.0, 100 mM NaCl: Dissolve 2.43 g of Tris(hydroxymethyl) aminomethane and 5.84 g of NaCl in 1 L of sterile distilled water. Adjust the pH at 8.0 with 1 M HCl.
3. 20 mM Tris-HCl, pH7.2, 50 mM NaCl: Dissolve 2.43 g of Tris(hydroxymethyl) aminomethane and 2.92 g of NaCl in 1 L of sterile distilled water. Adjust the pH at 7.2 with 1 M HCl.
4. 0.5 M NaCl: Dissolve 29.22 g of NaCl in 1 L of sterile distilled water.
5. α-amylase stock solution (100×): 20 mg/mL α-amylase in 20 mM Tris-HCl, pH7.2. Store at -20 °C.
6. Pronase stock solution (100×): 20 mg/mL pronase in 20 mM Tris-HCl, pH7.2. Incubate the pronase stock solution for 1 hour at 56°C. Store at -20 °C.
7. French press, Emulsiflex apparatus or any apparatus to disrupt bacterial cells.
8. Vortex.
9. Water bath at 96°C.
10. Incubator at 37°C.
11. Ultracentrifuge (Beckman with TLA100.3 and TLA100.4 rotors, or equivalent).

2.2. Turbidometric analyses of peptidoglycan degradation

1. 60 mM MES, pH6.0, 180 mM NaCl buffer: Dissolve 11.71 g of 2-(*N*-morpholino)ethanesulfonic acid and 10.52 g of NaCl in 1 L of sterile distilled water.
2. Purified protein to be tested.
3. Lysozyme stock solution: 10 mg/mL egg-white lysozyme in sterile distilled water.
4. Incubator at 37°C.
5. Spectrophotometer.

2.3. Peptidoglycan labelling with Remazol Brilliant Blue.

1. 400 mM NaOH: dissolve 16 g of NaOH in 1 L of sterile distilled water.
2. Remazol Brilliant Blue stock solution (10×): Dissolve 1.566 g of Remazol Brilliant Blue R (Sigma-Aldrich) in 10 mL of sterile distilled water.
3. 1 M HCl: Dilute 10 mL of HCl 37% solution (10 M) with 90 mL of distilled water.
4. PBS buffer: Dissolve 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, 0.2 g of KCl and 8 g of NaCl in 1 L of sterile distilled water. Adjust pH to 7.4 with 1 M HCl.
5. Incubator at 37°C.
6. Vortex.
7. Ultracentrifuge (Beckman with TLA100.3 rotor, or equivalent).

2.4. RBB-labelled peptidoglycan degradation assay

1. PBS buffer: Dissolve 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4, 0.2 g of KCl and 8 g of NaCl in 1 L of sterile distilled water. Adjust pH to 7.4 with 1 M HCl.
2. Purified protein to be tested.
3. Ethanol 96° or absolute.
4. Lysozyme stock solution: 10 mg/mL egg-white lysozyme in sterile distilled water.

5. Incubator at 37°C.

6. Ultracentrifuge (Beckman with TLA100.3 rotor, or equivalent).

7. Spectrophotometer.

3. Methods

3.1. Peptidoglycan purification

The peptidoglycan purification protocol is adapted from (***29,30***).

1. Grow the cells in 400 mL of the appropriate medium until the culture reaches an A_{600} ~ 1-1.2.
2. Harvest cells by centrifugation at 10,000 × *g* for 20 min at 4°C. Resuspend cells in 20 mL of 20 mM Tris-HCl, pH8.0, 100 mM NaCl. Break the cells by three passages at the French press or using an Emulsiflex apparatus.
3. Pellet cell envelopes by centrifugation at 400,000 × *g* (90,000 rpm in a Beckman TLA-100.4 rotor) for 45 min at 4°C. Resuspend cells in 10 mL of 0.5 M NaCl.
4. Add 10 mL of 8% SDS and incubate for 1 hour at 96°C.
5. Leave the solution at room temperature overnight.
6. Pellet the peptidoglycan by ultracentrifugation at 400,000 × *g* at 25°C for 45 min (*see* **Note 1**).
7. Resuspend the peptidoglycan fraction in 10 mL of 0.5 M NaCl and add 10 mL of 8% SDS. Incubate for 30 min at 96°C.
8. Pellet the peptidoglycan by ultracentrifugation at 400,000 × *g* at 25°C for 30 min and resuspend the peptidoglycan in 10 mL of water.
9. Repeat step 8 two times.

10. Resuspend peptidoglycan in 10 mL of 20 mM Tris-HCl, pH7.2, 50 mM NaCl supplemented with 200 µg/mL of α-amylase and 200 µg/mL of pronase. Incubate overnight at 37°C.
11. Add 10 mL of 8% SDS and incubate for 1 hour at 96°C.
12. Pellet the peptidoglycan by ultracentrifugation at 400,000 × *g* at 25°C for 30 min and resuspend the peptidoglycan in 10 mL of water.
13. Repeat step 12 two times.
14. Resuspend the peptidoglycan pellet in 1 mL of water. Store at 4°C.

3.2. Turbidometric analyses of peptidoglycan degradation

1. Dilute 125 µL of the purified peptidoglycan suspension obtained at step 14 in section 3.1. with 875 µL of 60 mM MES, pH6.0, 180 mM NaCl and incubate at 37°C for 30 min. Use three tubes for each reaction to measure peptidoglycan hydolysis in triplicate.
2. Measure the A_{600} for each tube (*see* **Note 2**).
3. Add 2-5 nmoles of the protein to be tested to each tube and incubate at 37°C (*see* **Note 3**).
4. Measure the A_{600} every 10 minutes and plot the difference of absorbance (absorbance at time *t* subtracted from the initial absorbance) against time (*see* **Note 4**).

A typical example of the turbidometric peptidoglycan assay is shown in Fig. 1.

3.3. Peptidoglycan labelling with Remazol Brilliant Blue.

The peptidoglycan labelling protocol is adapted from (***26***).

1. Mix 250 µL of the purified peptidoglycan fraction obtained at step 14 in section 3.1. with 250 µL of 400 mM NaOH and incubate for 30 min at 37°C.

2. Add the Remazol Brilliant Blue dye to the mixture at the final concentration of 25 mM. Vortex and incubate the mixture overnight at 37°C.

3. Add 500 µL of 1 M HCl and mix by vortexing.

3. Pellet the peptidoglycan by ultracentrifugation at 400,000 × *g* at 25°C for 30 min and resuspend the peptidoglycan in 2 mL of water.

4. Repeat step 3 two times.

5. Resuspend the peptidoglycan pellet in 250 µL of PBS buffer. Store at 4°C.

3.4. RBB-labelled peptidoglycan degradation assay

1. Dilute 10 µL of RBB-labelled peptidoglycan obtained at step 5 in section 3.3. with 90 µL of PBS buffer and incubate at 37°C for 30 min. Use nine tubes for each reaction to measure peptidoglycan hydrolysis in triplicate, at three different times.

2. Add 0.2-0.5 nmoles of the protein to be tested to the mixture and incubate at 37°C (*see* **Note 3**). This step corresponds to time zero.

3. Add 100 µL of ethanol in three tubes 30 min after time zero, to quench the reaction.

4. Pellet the peptidoglycan by ultracentrifugation at 400,000 × *g* at 25°C for 30 min.

5. Measure the A_{595} of the supernatant.

6. Repeat steps 3 and 4 1 hour and 4 hours after time zero.

A typical example of the dye release assay is shown in Fig. 2.

4. Notes

1. Do not incubate at 4°C to avoid SDS precipitation.

2. Typically, an A_{600} ~ 0.4-0.7 is measured from peptidoglycan purified from *Escherichia coli*.

3. Control assays include incubation of the peptidoglycan suspension with (i) buffer and (ii) purified lysozyme. Ideally, additional controls include incubation of the peptidoglycan with (i) the protein to be tested but bearing amino-acid substitutions in the catalytic site (if known or predicted) and (ii) the wild-type protein in presence of 100 µM of bulgecin A, an inhibitor of lytic transglycosylases (***31***).

4. The initial rate of the hydrolysis reaction (in AU/min/mol) can be calculated from the slope of the initial linear curve.

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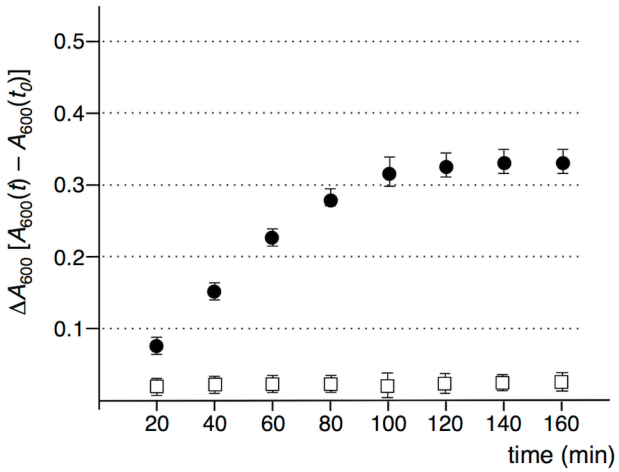
Figure Legends

Figure 1. LTG activity measured by the peptidoglycan hydrolysis assay. A representative example of peptidoglycan degradation is shown. Purified peptidoglycan was incubated with buffer (open square) or purified LTG (closed circles) and the absorbance at 600 nm (A_{600}) was measured every 20 minutes. The difference of absorbance at time *t* minus the absorbance at time zero (ΔA_{600}) was plotted against time (in min).

Figure 2. LTG activity measured by the RBB release assay. A representative example of peptidoglycan degradation is shown. (**A**) RBB-labelled peptidoglycan was incubated with buffer (open bars) or purified LTG (+ LTG, blue bars) and the absorbance at 595 nm (A_{595}) of the supernatant was measured after 0.5, 1 and 4 hours of incubation. (**B**) Photographs of supernatant fractions of RBB-labelled peptidoglycan incubated with buffer (left tube) or purified LTG (+ LTG, right tube) after 4 hours of incubation.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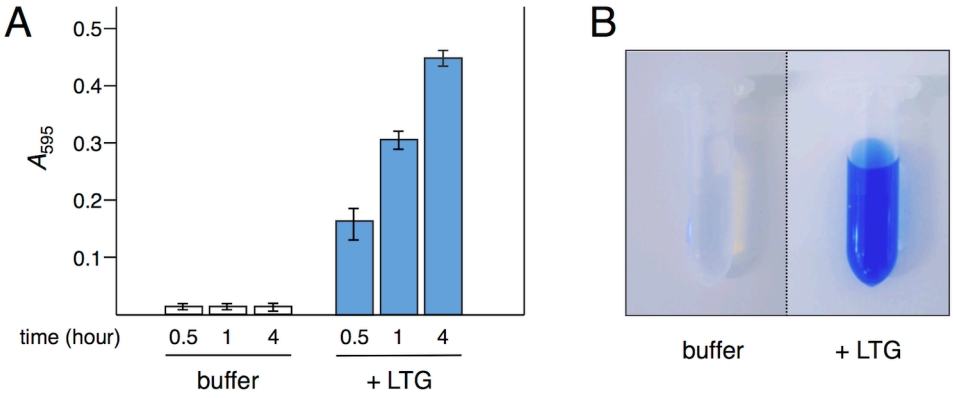
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