

Activity Assays for Bacteriophage Endolysin PlyPy

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[Abstract] Bacterial viruses (bacteriophages) escape and kill their host by degrading the bacterial peptidoglycan layer through the mechanism of enzymes called endolysins: peptidoglycan degrading enzymes. The method included here is useful for the initial characterization of any endolysin, regardless of the specific catalytic domain (as long as the activity results in a reduction in the optical density), in order to determine its optimal enzymatic (lytic) activity. This protocol is specific for the *Streptococcus pyogenes* phage endolysin PlyPy, but can be adapted for any peptidoglycan degrading enzyme.

Materials and Reagents

1. *Streptococcus pyogenes* (*S. pyogenes*) strain MGAS 315
2. Purified phage lysin PlyPy (or other lysins of interest)
3. Brain heart infusion (BHI) broth (BD, catalog number: 237500)
4. Agar (BD, catalog number: 214010)
5. Tris (Thermo Fisher Scientific, catalog number: BP152-1)
6. HCl (Thermo Fisher Scientific, catalog number: A144S-500)
7. CaCl₂·2H₂O (Thermo Fisher Scientific, catalog number: C70-500)
8. MgCl₂·7H₂O (AMRESCO, catalog number: 0288-100G)
9. ZnCl₂ (Fluka, catalog number: 96468-50G)
10. DTT (Sigma-Aldrich, catalog number: D0632-10G)
11. Sodium acetate (Thermo Fisher Scientific, catalog number: S210-500)
12. NaCl (Thermo Fisher Scientific, catalog number: S271-3)
13. PlyPy activity buffer (see Recipes)

Equipment

1. 37 °C incubator
2. Spectrophotometer (Bio-Rad Laboratories, SmartSpec™, model: 3000)
3. Table centrifuge (Eppendorf, model: 5810 R)

4. pH meter (Thermo Fisher Scientific, Accumet™, model: 15)
5. 15 ml tubes (Falcon®, catalog number: 352097)
6. 50 ml tubes (Falcon®, catalog number: 352098)
7. Cuvettes (Sarstedt AG, catalog number: 67.742)
8. 96-well flat bottom plates (Falcon®, catalog number: 35-1172)
9. 96-well plate reader (Molecular Devices, SpectraMax Plus Reader)

Procedure

Note: Streak S. pyogenes strain MGAS 315 on a BHI agar plate (or other plates supporting the growth of your bacteria, including blood agar and THY agar plates) and allow to incubate overnight at 37 °C until single colonies are visible.

1. Inoculate one colony of MGAS 315 in 5 ml BHI in a 15 ml tube. Incubate overnight at 37 °C.
2. Preheat BHI by adding 45 ml BHI to a 50 ml tube, and incubate overnight at 37 °C.
3. Transfer the 5 ml overnight culture of MGAS 315 into the preheated BHI (1:10 dilution), and incubate until OD₆₀₀ ~ 0.3. The time for the culture to reach this OD₆₀₀ varies depending on what strain is used, but will in general take 3-4 h for *S. pyogenes*.
Note: Cells can be taken at other OD₆₀₀ values, but bacteria are generally more susceptible to the effect of endogenously added lysins when in mid log phase as compared to stationary phase cells. Therefore it is recommended to do a growth curve of your bacteria to determine the mid log phase before conducting any further experiments.
4. Harvest cells by centrifugation (4,000 x g; 15 min; 4 °C).
5. Discard supernatant, and wash cells three times in 20 mM Tris-HCl (pH 6.8) (4,000 x g; 15 min; 4 °C).

Note: Other buffers might prove more efficient for other peptidoglycan degrading enzymes, but buffers in the pH-range 6-8 are usually a good starting point since bacteria generally tolerate this pH well, and most lysins are highly active within this pH range.

6. Resuspend the washed cells in 20 mM Tris-HCl (pH 6.8) to an OD₆₀₀~1.4.
Note: For best experimental resolution, a final OD₆₀₀ of 0.5-1.0 is recommended. Washed cells will be diluted 1:1 in upcoming steps, yielding a final OD₆₀₀ = 0.7.
7. Add 100 µl washed cells to the wells of a sterile 96-well plate.
Note: For consistency and statistical reliability, we recommend all samples to be run in at least triplicates.
8. Add 100 µl buffer [20 mM Tris-HCl (pH 6.8)] containing divalent cations at varying concentrations.
CaCl₂: 0-100 mM

MgCl₂: 0-100 mM

ZnCl₂: 0-5 mM

Note: Since many endolysins rely on the presence of divalent cations for their activity, it is critical to optimize this condition before moving on to other optimizations. Several other divalent cations might affect the activity of lysins, including but not limited to Mn²⁺, Fe²⁺, Co²⁺, Cu²⁺ and Ni²⁺.

9. Add 1 µg of your peptidoglycan hydrolyzing protein (PlyPy).

Note: The amount of lysin needed to be added is dependent on the activity of the lysin and needs to be determined empirically. A few µg lysin is a good starting point.

10. Insert the 96-well plate into the SpectraMax Plus Reader, and incubate the plate at 37 °C for 60 min. Shake the plate and measure the OD₆₀₀ every 30 sec.

11. Plot the data in an Excel sheet and analyze the results.

12. Repeat steps 9-12, including the necessary divalent cations at an empirically determined concentration in the buffer, but now optimize the conditions for other factors, including but not limited to:

NaCl: 0-1,000 mM

DTT: 0-20 mM

Temperature: 20-50 °C

13. To determine the optimal pH, exchange the 20 mM Tris-HCl (pH 6.8) used in steps 6, 7 and 9 to buffers of your choice, including but not limited to:

20 mM sodium acetate (pH 5.0)

20 mM sodium acetate (pH 5.5)

20 mM sodium acetate (pH 6.0)

20 mM Tris-HCl (pH 6.0)

20 mM Tris-HCl (pH 6.5)

20 mM Tris-HCl (pH 7.0)

20 mM Tris-HCl (pH 7.5)

20 mM Tris-HCl (pH 8.0)

20 mM Tris-HCl (pH 8.8)

14. To finally determine the activity of your lysin:

- a. Wash and resuspend your cells in activity buffer (optimized buffer based on your empirical values) to an OD₆₀₀~1.4.

- b. Add 100 µl cells and 100 µl activity buffer to each well in a 96-well plate.

Note: Take into account that the maximum volume in the wells will be 200 µl.

Therefore, less than 100 µl activity buffer will be added to wells that will also contain lysin (diluted in activity buffer). The exact volume depends on your initial concentration of the lysin.

- c. Add your lysin in serial dilutions, ranging from 0.1-51.2 $\mu\text{g/ml}$.
Note: It is important to run all samples in triplicates to account for any outliers.
- d. Incubate for 15 min at 37 °C in a SpectraPlus Max Reader, shaking and measuring OD₆₀₀ of the samples every 30 sec.
- e. Plot the data in an Excel sheet and analyze the results.
- f. The dilution closest corresponding to a 50% reduction of the starting OD corresponds to 1 Unit (Loeffler *et al.*, 2001). Most characterized lysins have an activity of 0.5-1 U/ μg .

Representative data

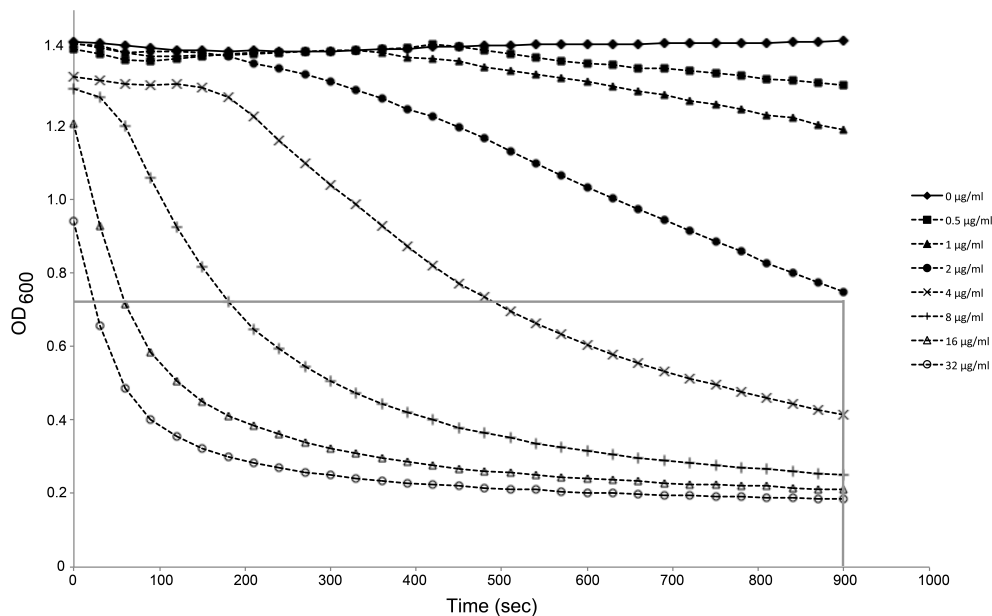


Figure 1. Activity determination of PlyPy. Washed *S. pyogenes* cells were incubated in activity buffer at 37 °C for 15 min with varying concentrations of PlyPy. The optical density (600) was measured every 30 sec in a SpectraPlus Max Reader. The mean value of triplicates is shown. For simplicity, a gray line has been added, representing the OD_{50%} at 900 sec (15 min), thus corresponding to one Unit. In this representative figure, 2 $\mu\text{g/ml}$ corresponds closely to one Unit; e.g. the activity of the lysin is 0.5 U/ μg . Note the fast initial drop in optical density in samples with high concentration of lysin, stressing the necessity of reading your sample as soon as possible after adding the lysin.

Recipes

1. PlyPy activity buffer
 - 20 mM Tris-HCl (pH 6.8)
 - 2 mM CaCl₂

100 mM NaCl

Sterile filter the solution (0.22 µm) before usage

Acknowledgements

This protocol was published in a more abbreviated form in the full-length article by Lood *et al.* (2014). This study was supported in part by USPHS grant AI11822 and by a grant from Contrafect Inc. to V.A.F. and by a grant from the Tegger Foundation to R.L.

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