# Enzymatic characterization of a lysin encoded by bacteriophage EL

Diana A. Tafoya,[1,†] Zacariah L. Hildenbrand,[1,†] Nadia Herrera,[1,†] Sudheer K. Molugu,[1] Vadim V. Mesyanzhinov,[2] Konstantin A. Miroshnikov[2] and Ricardo A. Bernal[1,*]

[1]Department of Chemistry; University of Texas at El Paso; El Paso, TX USA; [2]S.M. Shemyakin & Yu.A. Ovchinnikov Institute of Bioorganic Chemistry; Russian Academy of Sciences; Moscow, Russia

[†]These authors contributed equally to this work.

The bacteriophage EL is a virus that specifically attacks the human pathogen *Pseudomonas aeruginosa*. This phage carries a large genome that encodes for its own chaperonin which presumably facilitates the proper folding of phage proteins independently of the host chaperonin system. EL also encodes a lysin enzyme, a critical component of the lytic cycle that is responsible for digesting the peptidoglycan layer of the host cell wall. Previously, this lysin was believed to be a substrate of the chaperonin encoded by phage EL. In order to characterize the activity of the EL lysin, and to determine whether lysin activity is contingent on chaperonin-mediated folding, a series of peptidoglycan hydrolysis activity assays were performed. Results indicate that the EL-encoded lysin has similar enzymatic activity to that of the *Gallus gallus lysozyme* and that the EL lysin folds into a functional enzyme in the absence of phage chaperonin and should not be considered a substrate.

## Introduction

Bacteriophage EL is a large phage from the Myoviridae family that carries a large genome composed of 211, 215 base pairs, including 64 structural proteins. Phage EL was found to infect the gram-negative bacterium *Pseudomonas aeruginosa*.[1] EL is considered to be a novel phage in that it encodes its own chaperonin; a large, multimeric assembly that facilitates the ATP-dependent protein folding of nascent or misfolded polypeptides into their native state. Phage EL also encodes for a lytic enzyme, gp188, believed to be responsible for digesting the peptidoglycan layer of the host cell wall, thereby releasing the newly formed virions of the bacteriophage into the environment.[2] The exact role of gp188 in the lytic cycle of bacteriophage EL however, is not fully understood. It therefore remains to be determined at a future date, whether gp188 functions as a lysozyme (attack from the outside of cell for infection) or as an endolysin (peptidoglycan degradation from cytoplasmic side of cell for phage release).[3]

Previously gp188 was found to be associated with the phage chaperonin, and therefore thought to be reliant on the phage chaperonin to facilitate its folding prior to reaching an enzymatically active state.[4] Gp188 lytic activity was measured in the presence of viral chaperonin to investigate the nature of this association and to determine if there is a dependence on this association for activity. Additionally, we present the enzymatic characterization of gp188 as a lysin enzyme with peptidoglycan hydrolyzing activity.

## Results and Discussion

The EL gp188 lysin was sequenced and analyzed for sequence homologs.[1] The gene product was found to be 292 amino acids long with a molecular weight of 32 kDa, as confirmed by SDS-PAGE (**Fig. S1**). A protein Basic Local Alignment Search Tool (BLAST) search resulted in the discovery that the putative EL lysin shares sequence homology with several EF hand domain-containing proteins; in particular that of an EF hand domain-containing protein from the marine bacterium *Hahella chejunesis* (41% sequence identity, **Fig. 1**) that is characterized as having lytic activity against algal cell walls.[5] Though little is known about the EF hand domain-containing protein from *H. chejunesis*, there is a similar protein from *Escherichia coli* (Slt35) with a metal binding helix-loop-helix similar to the classic calcium-binding EF hand domain. The *E. coli* Slt35 protein has been characterized as a lytic transglycosylase.[6] The only other prokaryotic proteins that have been found to have this EF-hand metal binding domain are galactose-binding proteins.[7-9] Additionally, the BLAST search determined that the gp188 gene product (putative lysin) has a lysozyme-like domain with the potential to catalyze the

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EF      85   LTVDGIWGNGTLSGLDKAFEVYKERYRTP-----------------TYDIAWSGKVSPA  126
             L VDG+ G  T+S + K F+  K       P                   IAW G+  S
lysin   85   LVVDGVAGAATVSAI-KRFQREKAGMNKPDGRIDPGGRTLGALLGGKRLRIAWGGRTSST  143

EF     127   FTAKVKDWCGVHVPNHRAPHWLMACMAFETGQTFSPSIKNAAGSEAYGLIQFMSPAANDL  186
             F AKV   +        +P +LMACMAFETG+TFSPSI NAAGS A GLIQFM   A L
lysin  144   FNAKV---IKIAAAMDMSPDYLMACMAFETGETFSPSITNAAGSGAIGLIQFMPSTAKAL  200

EF     187   NVPLSVIRSMDQLTQLDLVFKYFEMWMKRGKRYTQLEDFYLTIFHPASVGKKADEVLFLQ  246
               +    ++ M  + QL  V KYF +        R   LED YL + +PA+VG   + LF +
lysin  201   GTSVQALKGMSAVQQLKYVEKYFSPYQ---GRLQNLEDVYLAVLYPAAVGMNPSQALFRR  257

EF     247   GSKAYLQNKGFDVDKDGKITLGEISSTLYTTYYKGL  282
             G+  Y QN GFD +KDG IT  EIS +    Y KGL
lysin  258   GTLTYTQNSGFDKNKDGIITPAEISLKVRAAYEKGL  293
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**Figure 1.** Bacteriophage EL lysin sequence alignment relative to the HCH_03670 EF hand domain-containing protein. Residues highlighted in yellow illustrate identical residues and residues highlighted in aqua indicate residues with similar properties.
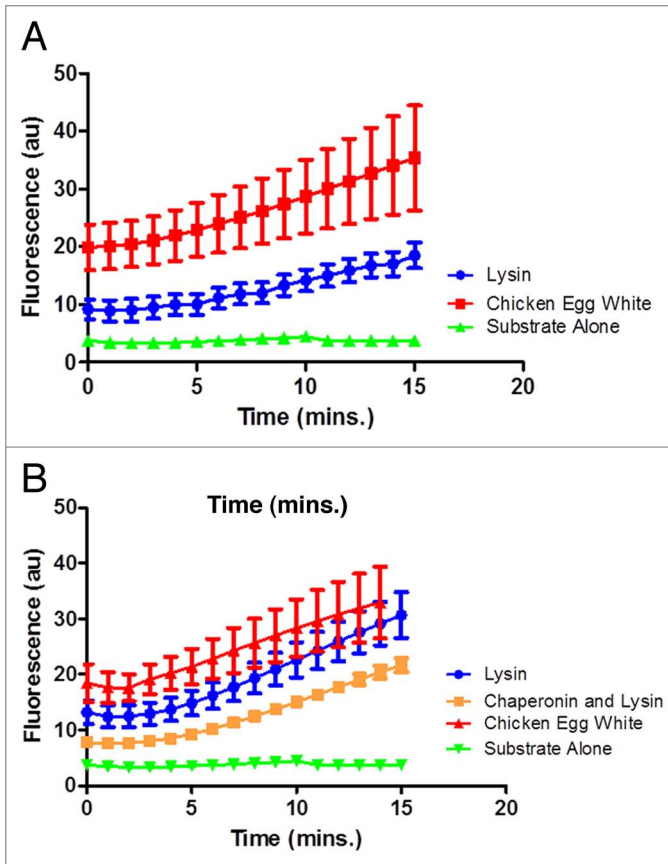


**Figure 2.** (**A**) The kinetic curves of a fluorescence-based EnzChek assay used to determine the activity of the lysin. The chicken egg white lysozyme (red) showed a higher fluorescence value when compared with the lysin (blue) by 20 fluorescence arbitrary units. As time passes, a steady increase in fluorescence is seen for both the chicken egg white and the lysin. The substrate alone (green) demonstrated a constant low fluorescence of 4 fluorescence arbitrary units. (**B**) The kinetic curves of a fluorescence-based assay used to determine the effect of chaperonin phi-EL on the activity of the lysin. As in the previous figure, the chicken egg white (red) showed a higher fluorescence than both the lysin (blue) and the lysin and chaperonin (orange) by 5 and 10 fluorescence arbitrary units respectively. Additionally, the lysin alone (blue) showed a higher fluorescence by 5 arbitrary units than the lysin combined with the phi-EL chaperonin (orange). The substrate (green) shows no increase in fluorescence as time progresses, and stays around a fluorescence arbitrary unit of 4.

hydrolysis of both peptidoglycan and chitodextrin. To explore the enzymatic potential of the EL lysin, we employed a series of lysis assays in conjunction with the chicken egg white lysozyme as a control. The lysozyme activity assay involves the use of purified peptidoglycan labeled with fluorescein that remains quenched until liberated by lysozyme (Invitrogen *EnzChek*) in addition to a turbidity assay that tests live cell lysis by the EL lysin. The EL lysin and chicken egg white lysozyme exhibited similar lytic activity as a function of a florescence (or turbidity) signal that was not observed with substrate alone (**Fig. 2A**). The observed lytic activity of the EL lysin was not enhanced by the addition of the phage chaperonin to the assay (**Fig. 2B**). Our results indicate that the enzymatic activity and the kinetics in the presence of chaperonin are not improved, supporting the notion that the EL lysin folds independently of the phage chaperonin. This observation was also corroborated by analytical size-exclusion chromatography results in which the lysin did not associate with the EL chaperonin (**Fig. S1**). These results are contrary to those presented by Kurochkina et al. 2012 where the EL lysin was assumed to be the substrate after it was found to co-purify with the phage chaperonin.[4] It was hypothesized from co-purification that the lysin is a substrate of the phage chaperonin and requires chaperonin-mediated folding of its structure to achieve enzymatic activity. Our analyses of the kinetics of lysin activity suggest that the EL lysin folds into a fully active conformation without the need for the phage encoded chaperonin. In the absence of phage chaperonin, maximum velocity ($V_{max}$), Michaelis-Menten Constant ($K_m$) and the turnover numbers ($K_{cat}$) of the lysin are similar to that of chicken egg white lysozyme (**Fig. 3**).

In conclusion, the lysin encoded by bacteriophage EL is an active enzyme capable of causing bacterial lysis and functions independently of the EL phage chaperonin, contrary to previous reports.[4] It is uncertain whether the lysin interacts with the host chaperonin system but it is clear that the bacteriophage EL encodes its own chaperonin to assist a phage protein other than the lysin that cannot be folded by the host chaperonin.

The nature of the observed lytic activity has tremendous biomedical implications and presents the possibility of using the EL lysin in the topical treatment of an assortment of bacterial infections.[10-12]

## Materials and Methods

### Expression and purification.

The lysin gene cloned into the pET28a+ vector was obtained as a gift from Vadim Mesyanzhinov (see Acknowledgments). The plasmid was transformed into BL21 *E. coli* cells by heat shock. Cells were grown in 2xTY medium with 30 µg/mL kanamycin and expression was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The EL lysin protein was purified using a His Trap affinity column (GE) and a Superose-6 size-exclusion column (GE). The EL chaperonin was also obtained as a gift from the same lab. The plasmid was transformed into BL21-DE3 *E. coli* cells by heat shock. Cells were grown in 2xTY medium with 30 µg/mL kanamycin and expression was induced with 1 mM IPTG. The protein was purified by 40% ammonium sulfate precipitation followed by size exclusion chromatography. The purified protein was then assayed for homogeneity by SDS-PAGE and protein concentration was quantified via BCA assay (Pierce) (**Fig. 2**).

### Fluorescence activity assay

Lysin activity was determined using the EnzChek Lysozyme Assay Kit (Invitrogen). This assay kit uses purified peptidoglycan labeled with fluorescein that remains quenched until liberated by a lytic enzyme. Ten micrograms of EL lysin was combined with varying amounts of substrate from the kit ranging from 0 to 10 mg. Fluorescence was read using a Fluoroscan Ascent Microplate Fluorometer (Thermo Scientific) using emission of 485–530 nm at one minute intervals for an hour at a temperature of 37°C. To assess lysin activity in the presence of chaperonin, EL chaperonin was added to the lysin reaction mixture at a ratio of 1:10 (1 µg chaperonin: 10 µg lysin) along with 5 mM of ATP and 5 mM of $MgCl_2$ solution. The sample was incubated at 25°C for 30 min. After incubation, the sample was assayed as above. Chicken egg white lysozyme was used as a positive control.

### Turbidity-based activity assay

A freeze-dried culture of *Micrococcus luteus* obtained from ATCC (ATCC-4698) was cultured in tryptic soy broth to an $OD_{600}$ of 0.8. The cells were harvested by centrifugation and were resuspended in 50mM TRIS pH 7.5, and 10 mM $MgCl_2$, to a final $OD_{600}$ of 2.0. Resuspended cells were placed in a 96-well plate in serial dilutions each in the presence of 0.2 µM of purified lysin. 0.2 µM of chicken egg white lysozyme and cells lacking enzyme were used as positive and negative controls, respectively. The kinetic turbidity assay was analyzed spectrophotometrically at $OD_{600}$. Measurements were taken in one-minute intervals over the course of 35 min.

Lineweaver-Burk plots were generated by converting the optical density for each of the wells into a quantification of the number of cells using the equation: *y = 0.000004x + 0.0241*. This equation is an approximation that serves to correlate optical density ($OD_{600}$) with the number of cells present in a solution, and was derived from *M. luteus* cells taken from a homogenous solution of known optical density that were stained with Coomassie blue and counted using a hemocytometer under a light microscope. This procedure was repeated using different solutions of varying optical densities to reduce measurement error.

### Analytical size-exclusion chromatography

Substrate binding interactions were detected as described previously.[13] Briefly, equimolar amounts of EL chaperonin and lysin were simultaneously passed through a Superose6 size-exclusion column (GE) operating with a 10 mM TRIS pH 7.5, 150 mM NaCl, and 0.02% $NaN_3$ elution buffer. The eluted fractions were then assayed by SDS-PAGE.

### Electron microscopy

Samples for electron microscopy were placed onto carbon coated copper grids by applying 3 µl of protein onto the grid for
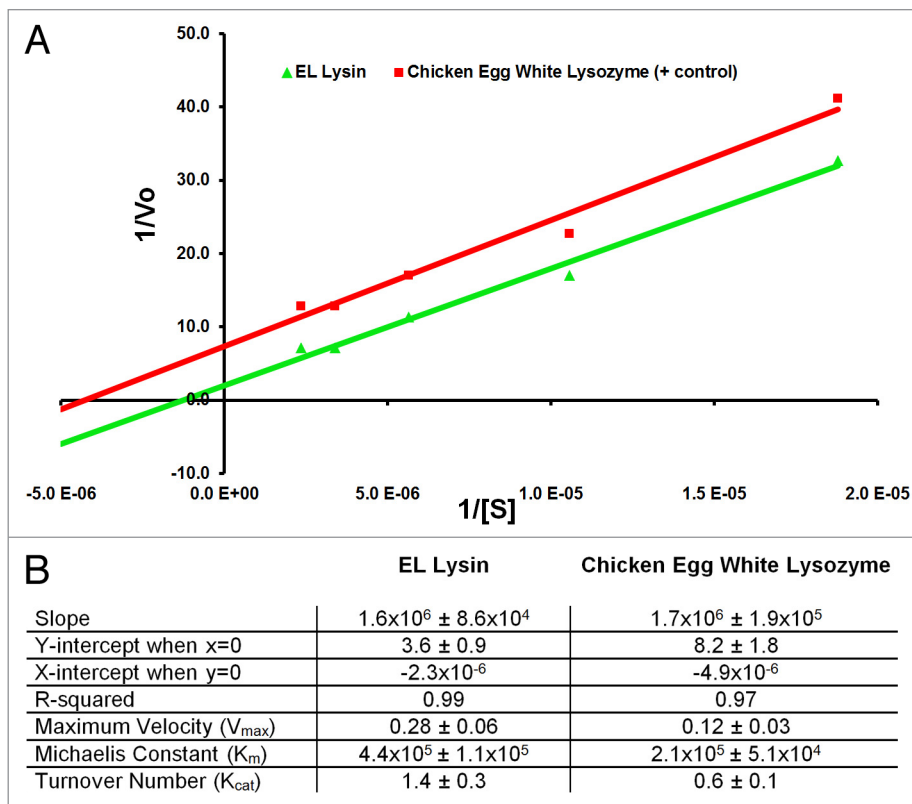


**A**



**B**

|  | EL Lysin | Chicken Egg White Lysozyme |
|---|---|---|
| Slope | $1.6 \times 10^6 \pm 8.6 \times 10^4$ | $1.7 \times 10^6 \pm 1.9 \times 10^5$ |
| Y-intercept when x=0 | $3.6 \pm 0.9$ | $8.2 \pm 1.8$ |
| X-intercept when y=0 | $-2.3 \times 10^{-6}$ | $-4.9 \times 10^{-6}$ |
| R-squared | 0.99 | 0.97 |
| Maximum Velocity ($V_{max}$) | $0.28 \pm 0.06$ | $0.12 \pm 0.03$ |
| Michaelis Constant ($K_m$) | $4.4 \times 10^5 \pm 1.1 \times 10^5$ | $2.1 \times 10^5 \pm 5.1 \times 10^4$ |
| Turnover Number ($K_{cat}$) | $1.4 \pm 0.3$ | $0.6 \pm 0.1$ |

**Figure 3.** (**A**) The Lineweaver-Burk plots derived from the results of absorbance-based assays done in triplicates and (**B**) a table with the kinetic data derived from the plots. These plots were used to derive the maximum velocity ($V_{max}$), Michaelis-Menten Constant ($K_m$) and the turnover number ($K_{cat}$) for the EL lysin and the chicken egg white lysozyme (+) control.

1 min. The protein was then blotted off with Whatman #1 filter paper. Three microliters of 2% uranyl acetate were immediately placed on the grid to act as a negative stain. The stain was then blotted off with filter paper and replaced with 3 μl of 2% methylamine tungstate. The second stain was blotted off and the grid was allowed to dry before imaging in the electron microscope.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

## Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/bacteriophage/article/25449

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