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False positives in using the zymogram assay for identification of peptidoglycan hydrolases

Cristian A. Escobar[b], Timothy A. Cross[a,*]

[a] Department of Chemistry and Biochemistry, Institute of Molecular Biophysics, and National High Magnetic Field Laboratory, Florida State University, Tallahassee, FL 32310 USA

[b] Institute of Molecular Biophysics, and National High Magnetic Field Laboratory, Florida State University, Tallahassee, FL 32310 USA

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ABSTRACT

Zymogram assays have been used extensively to identify novel peptidoglycan hydrolases. In this study it is reported that the zymogram is susceptible to false positive results when highly positively charged proteins are assayed. As an example, we report on the case of the ChiZ membrane protein from the *Mycobacterium tuberculosis* divisome, which previously was described as a peptidoglycan hydrolase. Even though the full length ChiZ protein was able to produce positive assay results, other direct methods for measuring peptidoglycan hydrolysis do not provide convincing evidence that ChiZ has peptidoglycan hydrolysis activity. We show that the false positive result is produced by the highly positively charged N-terminal region of ChiZ. Thus, we developed a zymogram control that can be used to identify false positives results. This control assay lacks the refolding step in the normal zymogram assay. For lysozyme the control assay shows no activity, while the N-terminal region of ChiZ shows a false positive result. Given the limitations of the zymogram assay to reliably identify peptidoglycan hydrolases, we recommend using the zymogram control assay together with other methods to evaluate possible peptidoglycan hydrolysis activity.

Introduction

The Zymogram assay is the primary assay for the identifying the functionality of peptidoglycan hydrolase enzymes. Here, we report a false positive result using this assay and note that false positives have been reported in the literature from other labs leading to incorrect identification of protein functionality. Furthermore, we have identified the cause for the false positive results that has led to a misidentification of the protein's functionality. Peptidoglycan (PG) remodeling is a key cellular process in which peptidoglycan is synthesized and hydrolyzed during bacterial proliferation. One important group of enzymes called peptidoglycan hydrolases are essential for PG remodeling. These enzymes can be classified into several groups depending on the bonds broken during PG hydrolysis [1]. Identification of new PG hydrolases has been achieved primarily through the use of zymogram assays, this method was developed in the late 80's and it is been used to date for that purpose [2]. The zymogram assay consists of running an SDS-PAGE gel of the protein to be tested with substrate for peptidoglycan hydrolysis suspended in the gel matrix; usually this substrate is a gram positive bacterium, such as *Micrococcus lysodeikticus* or pure PG. After, the gel is incubated in refolding buffer it is then stained with methylene blue that is known to bind to PG. Thus, proteins able to hydrolyze PG are revealed as clear bands in a blue background. Since this method is fast and easy, it has been applied to identify new proteins with peptidoglycan hydrolysis activity using whole cell lysates [3,4] and even to evaluate enzymatic activity under different conditions [5].

Despite the advantages of the zymogram assay, it has been observed that the results from this method do not always agree with other experimental tools used to further characterize PG hydrolases. For instance, it was concluded that the membrane anchored protein SpoIID from *Bacillus subtilis* is a PG hydrolase based mostly on the zymogram assay [6]. However, SpoIID did not show PG hydrolase activity in solution based assays [7]. The same case was observed for the protein EnvC from *E. coli* [8,9]

This work explains the cause of such false positive results by studying the case of ChiZ, a membrane protein from *M. tuberculosis*, which was identified as a PG hydrolase using primarily the zymogram assay [10]. ChiZ is one of many proteins associated with the *M. tuberculosis* divisome. It has a single transmembrane helix, a cytoplasmic N-terminus and periplasmic C-terminus. The periplasmic domain is homologous with LysM domains that bind peptidoglycan. The transmembrane domain has small residue motifs suggesting that this helix

* Corresponding author.
E-mail address: cross@magnet.fsu.edu (T.A. Cross).

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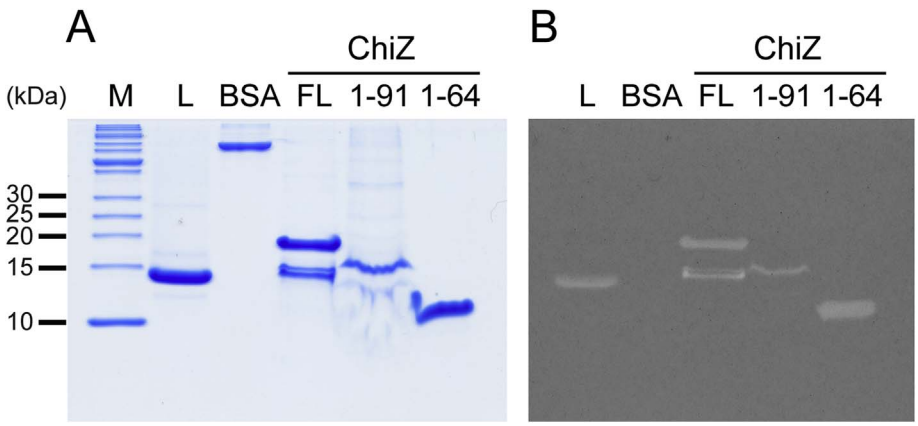

Fig. 1. Zymogram assay of ChiZ constructs. A) SDS-PAGE that shows the different ChiZ constructs used for the zymogram assay. B) Result of the zymogram assay. Lysozyme (L) and Bovine serum albumin (BSA) were used as positive and negative controls, respectively. The gel includes ChiZ without the LysM domain (1–91), the N-terminal soluble region (1–64), and the full length protein (FL).

binds to other protein transmembrane helices. It is further known that ChiZ binds to both FtsQ and FtsI [11], each of which has a single TM helix and FtsI is known to be a transpeptidase [11]. When the cytoplasmic N-terminus of ChiZ was determined to be a PG hydrolase it was speculated that Lipid II, the peptidoglycan precursor that is transported from the cytoplasm to the periplasm for PG assembly might be hydrolyzed. This hypothesis was consistent with elongated *Mtb* bacilli upon ChiZ overproduction and a modest increase in cell size of *chiZ* deletion mutants [10].

Material and methods

Zymogram assay

The zymogram assay was performed as described previously [10]. SDS-PAGE was run using a 16% gel containing 0.1% (wt/vol) of *Micrococcus lysodeikticus* cells (Sigma) as substrate. Then, the gel was washed three times by soaking it in water for 10 min. After washing, the gel was incubated in refolding buffer (25 mM tris-HCl at pH 7.0, 1% (wt/vol) Triton-X 100) for 16 h at 37 °C and then washed as before. After that the gel was stained with 0.1% (wt/vol) methylene blue (Acros) in 0.01% (wt/vol) KOH solution for 3 h. The gel was washed overnight with water to remove excess stain.

Zymogram assay control

A zymogram assay control was performed in the same way as the normal zymogram except that the refolding step for the protein was removed. Thus, after the SDS-PAGE was run the gel was washed and immediately stained with methylene blue solution.

Purification of ChiZ N-terminal constructs

Purification of ChiZ N-terminal constructs ($ChiZ_{1-64}$, $ChiZ_{1-54}$, $ChiZ_{1-47}$, and $ChiZ_{1-37}$) proceeded as follows: after protein expression in *E. coli* BL21 cells, the cells were resuspended in 20 mM tris-HCl pH 8.0 containing 500 mM NaCl and 6 M urea. The cells were lysed using a French Press. Insoluble debris was pelleted by centrifugation at ~250,000 × g for 30 min. Protein purification proceeded using nickel affinity chromatography (Ni-NTA resin, Qiagen). The column was washed with 20 mM tris-HCl pH 8.0 containing 500 mM NaCl and 20 mM imidazole. Then, the protein was eluted in the same buffer containing 400 mM imidazole. The His-tag was cleaved using TEV protease. To remove the TEV protease the protein preparation was passed through a Ni-NTA column and then washed with 20 mM imidazole.

Fluorescence cell wall hydrolysis assay

The cell wall hydrolysis assay was performed as previously reported [10]. Briefly, 50 µg of fluorescamine labeled *M. tuberculosis* peptidoglycan was mixed with ChiZ in 50 mM tris-HCl pH 7.0 and 0.25% (wt/vol) with a final volume of 100 µL. The hydrolysis reaction was performed at 37 °C for 2 h and then stopped by addition of 100 µL of 4 M LiCl. Insoluble peptidoglycan was pelleted by centrifugation and the supernatant was used for fluorescence measurements. Fluorescence emission was measured at 482 nm using an excitation wavelength of 390 nm. Negative controls were performed using samples without protein and peptidoglycan substrate. Positive control was performed using Lysozyme.

Results

ChiZ peptidoglycan hydrolysis, a false positive case

Previous studies of the ChiZ membrane protein demonstrated that the N-terminal region was responsible for hydrolyzing peptidoglycan. As can be seen in Fig. 1, the zymogram assay of the full length protein (lower band corresponds to a degradation product), first 91 residues (N-terminal region and transmembrane helix) and N-terminal region alone (first 64 residues) are all able to produce clear bands suggesting that the N-terminal region is capable of hydrolyzing peptidoglycan. Fig. 2A shows the result of cell wall hydrolysis assay using fluorescent labeled *M. tuberculosis* peptidoglycan demonstrating that ChiZ has no significant activity when compared to controls without substrate. It is clearly observed that there is an apparent increase in activity when the protein concentration is increased (Grey bars), but the same increase in fluorescence emission with protein concentration is observed when no substrate is used (Blue bars). Even though, there is a small difference between treatments (Grey bars) and only protein controls (Blue bars), this difference is not significant when compare to lysozyme positive control (Fig. 2B). This demonstrates that ChiZ is giving rise to a false positive result in the zymogram assay.

False positive results are produced by positively charged proteins

To understand how a false positive result can be produced it is necessary to understand how the zymogram assay works. This assay relies on staining PG with methylene blue, which forms a complex with peptidoglycan [12]. Thus, regions where peptidoglycan has been hydrolyzed, such as in the area of the hydrolase band, appear clear against the dark blue background. The nature of the interaction in the methylene blue-peptidoglycan complex appears to be electrostatic since methylene blue is a positively charged organic molecule (Fig. 3A) and peptidoglycan is a negatively charged biopolymer that is rich in acidic amino acids, such as glutamic acid and diaminopimelic acid in some cases. Based on this, it could be anticipated that proteins with a high net positive charge density could give a false positive result by simply repelling the methylene blue from an area of the gel where the protein