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Report of false positives when using zymography to assess peptidoglycan hydrolytic activity of an endopeptidase with multiple LysM domains.

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Abstract

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Highlights

-False positives peptidoglycan hydrolytic activities were revealed by zymography

-False positives zymography results do not reflect loss of peptidoglycan binding strength

-Zymography artifact is linked to the net protein charge

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Keywords: *Bacillus subtilis*; LysM; zymography; peptidoglycan; NlpC/P60 endopeptidase.

1 Introduction

Bacteria are shielded from environmental stresses by a cell wall, which contains a peptidoglycan (PG) layer. PG is a three-dimensional mesh-like structure composed of repeating units of MurNAc and GlcNAc. Long polymers containing MurNAc-GlcNAc repeats are linked to each other by peptide stems, enabling the formation of a complex 3D network [1]. Although the structure of PG is very rigid, it has also to be highly dynamic during bacterial cell division processes, notably, to facilitate the separation of the two daughter cells and neosynthesis of PG [2–4]. During these phases, PG remodeling is mediated by autolysins that are a group of PG-cleaving enzymes with different substrate specificities. These enzymes either cleave the carbohydrate backbone, the peptide stem, or the bond between MurNAc and the peptide stem or the peptide stem at specific positions. The complex and insoluble nature of PG renders the development of activity assays to characterize autolysins very challenging [5]. Zymography, a widely used technique allowing in-gel visualization of substrate hydrolysis, helps to overcome this challenge [6]. The principle of zymography is based on the migration of a protein of interest into a native or non-denaturing electrophoresis gel in which the substrate is incorporated. The substrate hydrolysis can be then detected where the protein end-migrates [6]. It has been extensively used to study matrix metalloproteases in eukaryotes [7] but also to study bacterial PG hydrolytic enzymes.

To study autolysin activities by zymography, bacterial cell wall or bulk PG can be incorporated in the polyacrylamide gels. After gel electrophoresis, in native or denaturing conditions of the purified protein of interest or even crude protein extracts, the PG is stained and any presence of a white zone of clearing corresponding to the end migration of the protein would indicate hydrolysis of PG. When separation is performed under denaturing conditions, the gel is extensively washed to remove sodium dodecyl

sulfate (SDS) and the protein is refolded slowly by placing the gel into a renaturation buffer prior to staining.

We studied the PG binding properties of the CwlS protein from *Bacillus subtilis* [8]. This enzyme is a NlpC/P60 D,L-endopeptidase capable of cleaving the PG peptide stem between the 2nd and 3rd amino acids. CwlS possesses a repeat of four LysM domains that are known binding modules of chitin and MurNAc-GlcNAc polymers [9]. The CwlS encoding gene is essential for daughter cell separation in *B. subtilis* [10]. In the course of characterizing CwlS and CwlS mutants by biochemical approaches, we noticed that the apparent hydrolytic activity was not correlated to the catalytic properties observed on the zymograms. In this study, we, therefore, report these observations with the aim of drawing attention to the potential misinterpretation of results of this widely used technique.

2 Materials and methods

2.1 Cloning, Protein expression and Purification

CwlS wild-type (CwlS_FL), 3LysM-Cata, 2LysM-Cata, 1LysM-Cata, Cata, FL_Mut and 4LysM constructs were essentially cloned and purified as previously described [8]. The CwlS-C326A mutant was generated with the CCTGCCGGTTTTGATGCCAGCGGCTTCATT forward primer and AATGAAGCCGCTGGCATCAAAACCGGCAGG reverse primer using the QuikChange Lightning Site-Directed Mutagenesis Kits (Agilent Technologies) according to the manufacturer's instructions and using the CwlS-FL gene as a template. This mutant was expressed and purified as similar as wild-type CwlS.

2.2 Zymography

To purify *B. subtilis* cell wall, *B. subtilis* cells (ATCC 6056) were grown in 100 ml LB media overnight at 30 °C. The cells were centrifuged at 10,500 *g* for 15 min, and washed twice with 50 mM Phosphate pH 7.5. The cell lysate was then centrifuged at 30,000 *g* for 20 min, resuspended in 0.5% SDS, and incubated at 60 °C for 30 min. The cells were centrifuged at 30,000 *g* for 20 min and washed with water. This step was repeated five times. Extracted *B. subtilis* cell wall was digested with 100 µg ml-1 trypsin in 1 M Tris-HCl pH 7 overnight at 37 °C. The cell wall was then centrifuged at 30,000 g for 20 min and washed with 1 M Tris-HCl pH 7. This step was repeated once with 1 M Tris-HCl

pH 7 and 1 M NaCl, once with 1 M Tris-HCl pH 7, and six times with water. Zymography gels were prepared by adding 0.4% (w/w) cell wall extract to 15% SDS-PAGE. 20 µg of protein in loading buffer were loaded in each lane. 10 µg BSA and 0.5 µg lysozyme were added as negative and positive control respectively. After SDS-PAGE, the gels were incubated in 50 mM Tris-HCl pH 8 buffer for 48 hours, then stained with 0.1% (w/v) methylene blue diluted in 0.01% KOH.

2.3 Assessment of the endopeptidase activity using turbidity activity assay

The endopeptidase activity assay was performed as previously described [8].

2.4 Overall protein net charge determination

The overall net charges of the protein variants at pH 7 were determined using this server: https://pepcalc.com/protein-calculator.php. The protein charges were calculated according to the following formula: $Z=\sum_i N_i \frac{10^{pKa_i}}{10^{pH}+10^{pKa_i}}-\sum_j N_j \frac{10^{pH}}{10^{pH}+10^{pKa_j}}$ where N_i indicates the number, and pKa_i the pKa values, of the N-terminus and the side chains of positively charged amino acids: Arginine, Lysine, and Histidine. The *j*-index relates to the C-terminus and the negatively charged residues: Aspartic Acid, Glutamic Acid as well as Cysteine and, Tyrosine amino acids.

3 Results and discussion

The CwlS study was undertaken to reveal the role of the N-terminal region of the protein containing multiple LysM domains (Figure 1A). The LysM domain is a known PG binding domain [9], so we initially performed a study to assess if the number of LysM repeats was conferring substrate-binding strength. We showed that the CwlS affinity towards PG and chitin polymers is indeed influenced by the numbers of LysM repeats which cooperated to bind these long polymers [8]. The dissociation constants (K_d) determined for chitin and MurNAc-GlcNAc polymers using the Microscale Thermophoresis method demonstrated that the K_d increased alongside LysM deletion (Figure 1A) [8]. A positive correlation between carbohydrate affinity and the number of LysM domains was also confirmed in studies using NlpC/P60 from *Thermus thermophilus* [11] and the AtlA autolysin from *Enterococcus faecalis* [12].

In this study, we assessed if the deletion of LysM modules could influence the PG hydrolysis efficacy of CwlS. We performed zymography experiments using non-denaturing gels containing bulk PG from *B. subtilis*. Full-length wild-type CwlS protein (CwlS-FL) seemed capable of cleaving PG as a white zone of clearing corresponding to the end-migration position of the protein was observed after methylene blue staining of zymography gels (Figure 1B). We then assessed the PG-cleaving abilities of single to four LysM deletion in CwlS mutants and noticed that the more impaired the CwlS mutants were in their PG binding capacities the lower their abilities were to form white zones of clearing on zymogram (Figure 1A and 1B). It appeared that CwlS-FL and 3LysM-Cata proteins that have similar dissociation constants for PG, cleaved PG the most efficiently as indicated by most distinct zones of clearing in zymograms compare with 2LysM-Cata, 1LysM-Cata and Cata mutants that were strongly impaired in PG binding. These mutants presented less pronounced zones of clearing. The Cata mutant that did not possess any PG binding module produced barely detectable zone of clearing. This observation could have been considered in perfect agreement with our previously published results showing that these mutants were impaired in their capacity to hydrolyze *Bacillus subtilis* culture which could be explained by their loss of ability to anchor to the cell wall [8].

Counter-intuitively, a protein mutant (4LysM) that did not contain any catalytic domain but only the four LysM domains also generated zones of clearing on zymograms as pronounced as the wild-type protein, CwlS-FL (Figure 2A). Puzzled by the apparent PG hydrolytic activity of a CwlS variant that does not actually contain a catalytic domain, we further generated a catalytically dead mutant (CwlS-C326A) by site-directed mutagenesis of a catalytic cysteine that is present in the active site of the protein; this cysteine residue is strictly conserved in NlpC/P60 endopeptidases and is essential for PG hydrolysis [13–15]. Surprisingly, the CwlS-C326A mutant also generated a white zone of clearing in zymograms with equal intensity as the wild-type protein (Figure 2A).

To rule out that neither the 4LysM nor the CwlS-C326A mutant possessed PG hydrolytic properties, we assessed their activities on exponentially growing planktonic *B. subtilis* cultures. We added the purified proteins to *B. subtilis* cultures and monitored the cell density over 140 min. If the proteins are active, they should lyse the cells, and this is reflected by a drop in turbidity readings. CwlS-FL and the positive control, lysozyme, hydrolyzed *B. subtilis* as indicated by a drop in optical density of ~ 0.3 units (Figure 2B) in about 2 hours. In contrast, an increase in optical cell density was

observed for cells treated with the 4LysM and CwlS-C326A mutants, similar to the negative controls, BSA or protein buffer. The inability of the 4LysM and CwlS-C326A mutants to hydrolyze *B. subtilis* cultures (Figure 2B) strongly indicated that their corresponding zones of clearing on the zymograms do not reflect the enzymatic activity. One could consider in such a case, that zymography was in fact reflecting PG binding event and not PG hydrolysis.

Interestingly, the ChiZ protein from *Mycobacterium tuberculosis* that is part of the divisome and possesses a single LysM domain in its C-terminus also gave positive results on a zymogram albeit not being confirmed by other methods to be a PG hydrolase [16]. The authors elegantly proposed that the high net positive charge of ChiZ is in fact impairing toluidine blue coloration. Toluidine blue is indeed a positively charged compound that stains negatively charged PG, thus positively charged protein would repulse toluidine blue. The authors further demonstrated that the highly positively charged CrgA protein with no PG-related function also displayed apparent PG hydrolytic activity on zymogram [16].

We therefore calculated the net charge of all our CwlS variants (Figure 1A). With the exception of the Cata mutant with a net charge of 5, the other proteins have all very high net positive charges, from 12 to 33 as compared with ChiZ that has a net charge of 10. The intensity of the zones of clearing of the different CwlS version is indeed correlated with net positive charges. The Cata mutant with a net charge of +5 generates indeed a rather weak zone of clearing compare with the CwlS-FL that has a net charge of +33. To completely rule out the possibility that the positive zymogram result observed is not due to the loss of peptidoglycan binding strength but is rather related to the net positive charge of the protein, we included an additional mutant (FL-Mut) in our zymography assays. The FL-Mut (Figure 1A) presents twelve mutations, with three residues in each of the four LysM domains mutated into Ala. The mutagenesis was driven by docking studies which revealed amino acids with side chains that could potentially interact with carbohydrates as described in [8]. This mutant is highly impaired in its capacity to bind PG but very importantly has a similar net positive charge as the wild-type protein (Figure 1A). As assessed on zymogram (Figure 2C), this FL-Mut also resulted in a zone of clearance that was as pronounced as the CwlS-FL protein and the CwlS-C326A catalytic mutant that both possess similar net positive charges. Furthermore, the FL_Mut apparent activity is more pronounced than the CwlS variant that possessed only a catalytic domain and has a much lower net positive charge

(Figure 2C). Altogether our data provides compelling evidence that the apparent activity of all the CwlS variants presented in this study, as assessed by zymography, is not correlated to PG binding strength but more probably to the net positive charge of the protein.

Kohler et *al*., reported similar zymogram false positive when studying the autolysin AtlA from *Neisseria gonorrhoeae* [17]. The authors noticed that a catalytic dead mutant had apparent PG hydrolysis activity on zymogram although this mutant was, in fact, inactive in PG degradation assay or cell lysis assays [17]. The authors proposed that the protein was able to bind PG, therefore avoiding the PG coloration by toluidine blue. We calculated the net charge of AtlA, and noticed that it also has a high positive value of 10. Altogether, the studies of ChiZ, AtlA and CwlS, all possessing high net positive charges are therefore in favor of the hypothesis of impairment of coloration due to charges repulsion [16].

We hereby demonstrated that zymography could trigger false-positives when probing for PG hydrolysis activities of a multiple containing LysM protein. This communication is nonetheless not intended to discredit the use of zymography, which has been extensively and successfully used in autolysin hydrolysis assays for decades, but to create awareness that the data should be interpreted with caution. Zymography has in most cases been proven to be trustworthy as hydrolytic activities have successfully been confirmed by additional techniques. To avoid such artifact, Escobar et *al.*, proposed to compare the results of gels revealed with and without a refolding step [16]. Alternatively or in complement, we would advise to determine the net charge of the protein of interest, and if possible, to assess the activities of a catalytic dead version alongside a wild-type version of the protein when performing zymography assessments, and most certainly to confirm the PG hydrolase activity by another method [5].

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Declarations of interest

All authors declare no conflict of interest.

Authors contribution

JW and MB designed research. JW performed experiments. JW and MB analyzed data. MB supervised research. MB and JW wrote the manuscript.

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Figures legends

Figure 1: Determination of the peptidoglycan hydrolytic activity of CwlS mutant by zymography

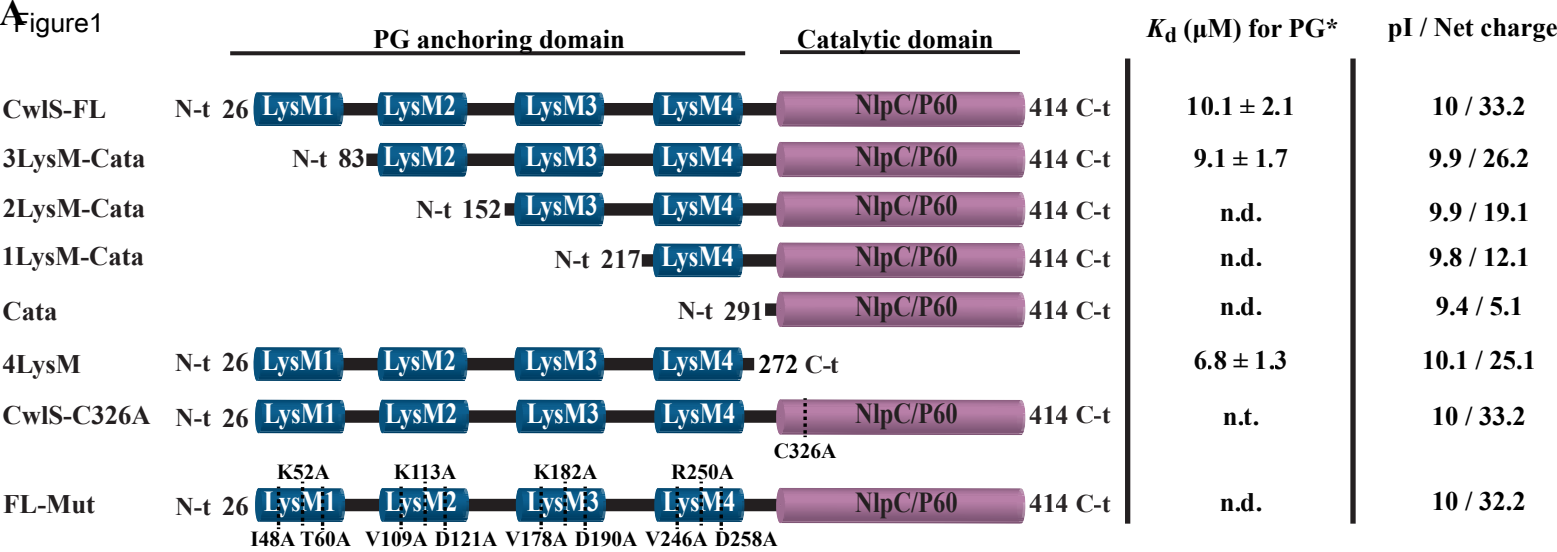
A-Schematic representation of the CwlS variants assessed in this study with the PG-anchoring and catalytic domains indicated. The CwlS PG-anchoring domain corresponds to the LysM modules present in the protein. N-t and C-t stand for N-terminus and C-terminus respectively. *Indicates the dissociation constants (K_d) previously determined by microscale thermophoresis using *Staphylococcus aureus* PG tetramers [8], ± represents the standard deviation (with 95% confidence interval). N.d. indicates that binding was not detectable and n.t. indicates that binding was not tested. The isoelectric point (pI) and the overall net charges of the CwlS variants (without its peptide signal of secretion) at a pH of 7 are shown. The pI and the overall net charge at pH 7 of the different protein variants were determined using this server: https://pepcalc.com/protein-calculator.php. **B**-Determination of the apparent PG hydrolytic activity of the different CwlS variants with one, two, three, or all LysM domains deleted. M indicates the protein molecular weight marker. Bovine serum albumin (BSA) and lysozyme were used as negative and positive controls, respectively. The dotted lines signify that the two gels are independent but were run simultaneously in the exact same conditions. Empty lanes in the gels were cropped.

Figure 2: Assessment of the PG hydrolytic properties of CwlS mutants by zymography and upon incubation with planktonic *B. subtilis* culture

A-Zymography assessment of the apparent PG hydrolytic activity of wild-type CwlS and mutated versions devoid of a catalytic domain (4LysM) or containing a mutation in the catalytic domain (C326A) rendering it inactive. **B-***B. subtilis* cell lysis capacities of the different CwlS variants. A drop of OD at 600 nm indicates cell lysis. The data is representative of three independent experiments. **C-** Comparison of the apparent PG hydrolytic activity of the FL_Mut variant versus CwlS_FL, CwlS_C326A and Cata mutants.

Figure1

A


PG anchoring domain
Catalytic domain
K_d (μM) for PG*
pI / Net charge
CwlS-FL
N-t 26
LysM1
LysM2
LysM3
LysM4
NlpC/P60
414 C-t
10.1 ± 2.1
10 / 33.2
3LysM-Cata
N-t 83
9.1 ± 1.7
9.9 / 26.2
2LysM-Cata
N-t 152
n.d.
9.9 / 19.1
1LysM-Cata
N-t 217
n.d.
9.8 / 12.1
Cata
N-t 291
n.d.
9.4 / 5.1
4LysM
272 C-t
6.8 ± 1.3
10.1 / 25.1
CwlS-C326A
C326A
n.t.
10 / 33.2
FL-Mut
K52A
K113A
K182A
R250A
I48A T60A
V109A D121A
V178A D190A
V246A D258A
n.d.
10 / 32.2

B

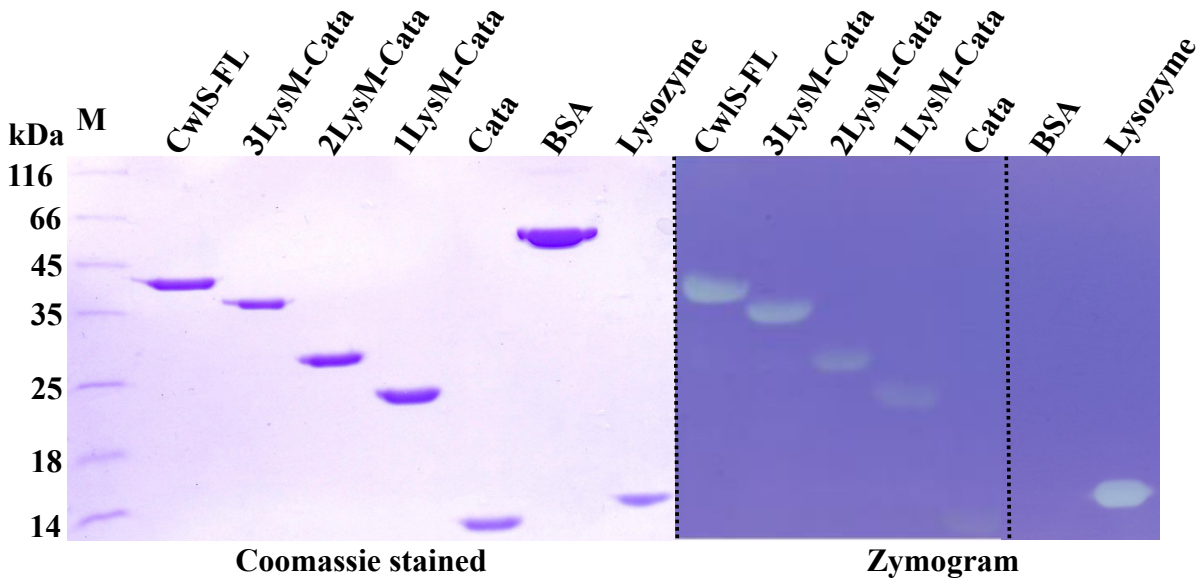
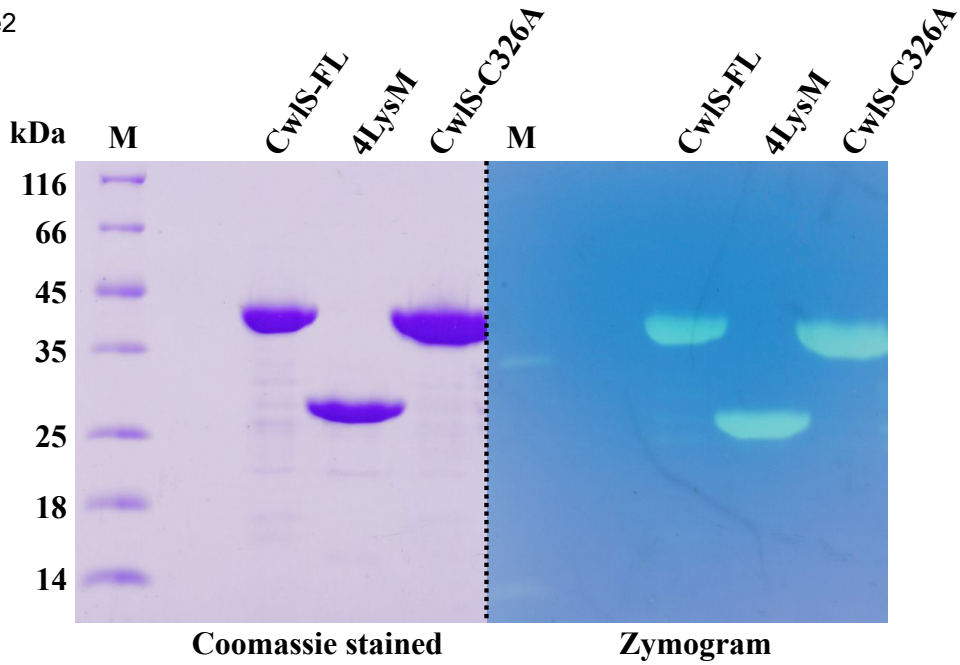
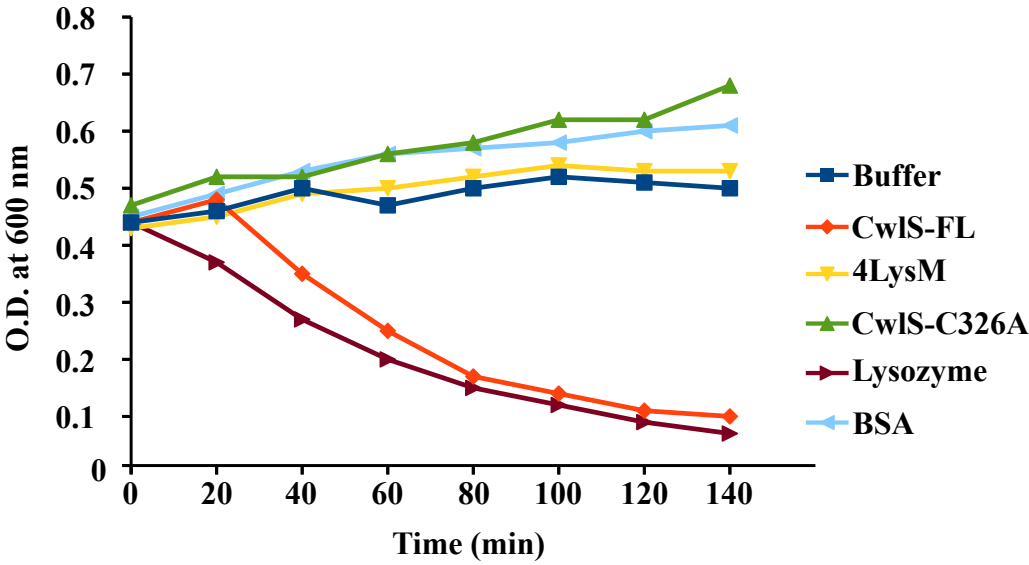

kDa
M
CwlS-FL
3LysM-Cata
2LysM-Cata
1LysM-Cata
Cata
BSA
Lysozyme
116
66
45
35
25
18
14
Coomassie stained
Zymogram

Figure2

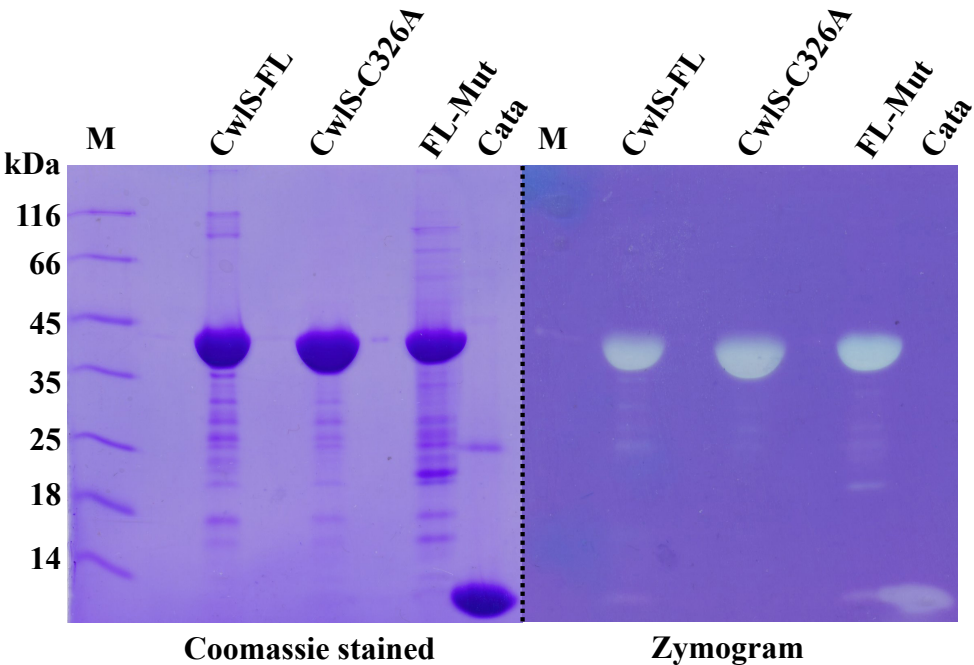
A


kDa
M
CwlS-FL
4LysM
CwlS-C326A
M
CwlS-FL
4LysM
CwlS-C326A
116
66
45
35
25
18
14
Coomassie stained
Zymogram

B


O.D. at 600 nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
Time (min)
Buffer
CwlS-FL
4LysM
CwlS-C326A
Lysozyme
BSA

C


kDa
M
CwlS-FL
CwlS-C326A
FL-Mut
Cata
M
CwlS-FL
CwlS-C326A
FL-Mut
Cata
116
66
45
35
25
18
14
Coomassie stained
Zymogram