


Laboratory Exercise

The Disruptive Effect of Lysozyme on the Bacterial Cell Wall Explored by an *in-Silico* Structural Outlook

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Abstract

The bacterial cell wall, a structural unit of peptidoglycan polymer comprised of glycan strands consisting of a repeating disaccharide motif [N-acetylglucosamine (NAG) and N-acetylmuramylpentapeptide (NAM pentapeptide)], encases bacteria and provides structural integrity and protection. Lysozymes are enzymes that break down the bacterial cell wall and disrupt the bacterial life cycle by cleaving the linkage between the NAG and NAM carbohydrates. Lab exercises focused on the effects of lysozyme on the bacterial cell wall are frequently incorporated in biochemistry classes designed for undergraduate students in diverse fields as biology, microbiology, chemistry, agronomy, medicine, and veterinary medicine. Such exercises typically do not include structural data. We describe here a sequence of computer tasks designed to illustrate and reinforce both

physiological and structural concepts involved in lysozyme effects on the bacterial cell-wall structure. This lab class usually lasts 3.5 hours. First, the instructor presents introductory concepts of the bacterial cell wall and the effect of lysozyme on its structure. Then, students are taught to use computer modeling to visualize the three-dimensional structure of a lysozyme in complex with bacterial cell-wall fragments. Finally, the lysozyme inhibitory effect on a bacterial culture is optionally proposed as a simple microbiological assay. The computer lab exercises described here give students a realistic understanding of the disruptive effect of lysozymes on the bacterial cell wall, a crucial component in bacterial survival. © 2017 by The International Union of Biochemistry and Molecular Biology, 46(1):83–90, 2018.

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Introduction

The most abundant and diverse prokaryote microorganisms in our planet are bacteria, which are surrounded by a sophisticated and complex structure called bacterial cell wall. This structure is important because it not only defines the bacterial cell shape, but it also provides protection in

hostile and very often unpredictable environments, both in biological and nonbiological systems [1].

The main component of bacterial cell wall is peptidoglycan, which is composed of alternating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), linked by β -(1,4) bonds [2].


Gram-positive bacteria have a single lipid membrane surrounded by a cell wall composed of a thick layer of peptidoglycan, while in Gram-negative bacteria the cell wall consists of a thin layer of peptidoglycan placed between the cytoplasmic and outer lipid membranes (Fig. 1A). There has been an indefatigable search throughout the years for different strategies to disrupt the bacterial cell wall to fight against invasion of pathogenic bacteria.

Lysozyme is an enzyme that cleaves peptidoglycan in bacterial cell walls by catalyzing the hydrolysis of β -(1,4) linkages between the NAM and NAG saccharides (Fig. 1B, upper) [3].

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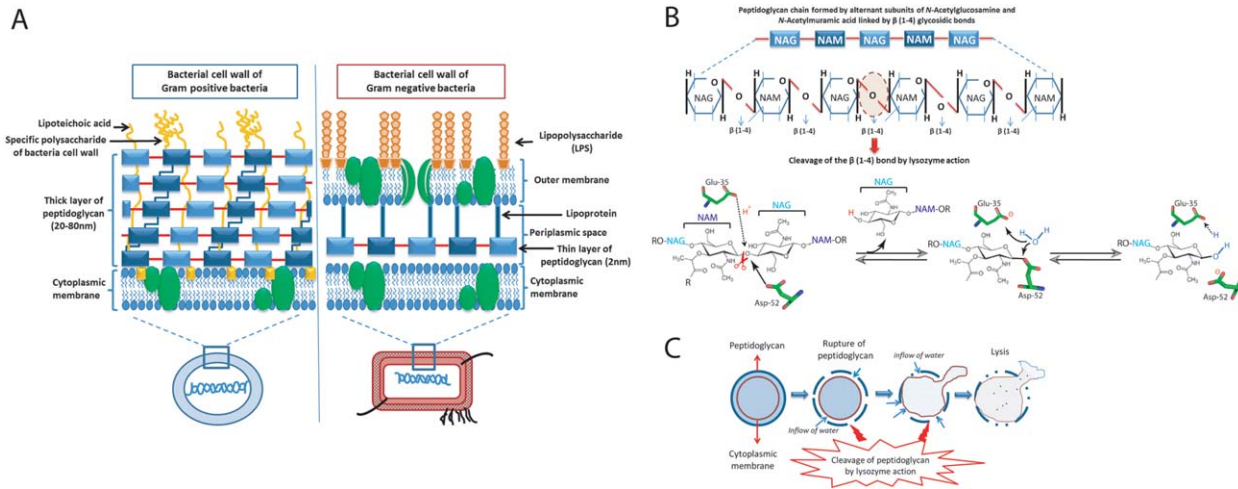


FIG 1

(A) Structural comparison between the bacterial cell wall of characteristic Gram-positive and Gram-negative microorganisms. (B) Upper; Specific cleavage sites on the peptidoglycan chain for lysozyme, which catalyzes the hydrolysis of the β -1,4 bond between N-acetylmuramic acid and N-acetylglucosamine. Bottom; Mechanism of action of a lysozyme. In the enzyme-substrate complex, the Glu-35 residue donates a proton (red) to the glycosidic bond, generating an oxonium that is stabilized by Asp-52 via a covalent bond. The enzyme-substrate intermediate complex reacts with a water molecule (light blue), where Glu-35 accepts a proton from the product of hydrolysis and leaves the enzyme unchanged. References: R= cell wall oligosaccharide chain, R'= cell wall peptide side chain. (C) Progressive effect of lysozyme on the peptidoglycan of bacteria and lytic activity. Schemes adapted from W. Carrillo (2013) [4]. [Color figure can be viewed at wileyonlinelibrary.com]

In this enzymatic reaction, the residue Glu-35 acts as an acid, donating a proton to the glycosidic bond, generating an oxonium that is then stabilized by a covalent bond with Asp-52. The addition of a water molecule completes the hydrolysis and regenerates the protonated form of Glu-35 (Fig. 1B, bottom).

Lysozyme has been relevant for the progress of protein biochemistry [5], and its disruptive effect on the bacterial cell wall has helped to develop and improve new antibiotics [6]. In addition, since the determination of the crystal structure of hen egg-white lysozyme (HEWL) reported by Blake et al. [7], several studies that shed light onto its mechanism of action have been carried out.

In a liquid medium, the exposure of exogenous lysozyme to bacteria allows for a straightforward observation of the damage on the peptidoglycan integrity. The progressive rupture of bacterial cell wall by lysozyme gives rise to cells called protoplasts or spheroplasts, on which there is a continuous inflow of water from the external medium leading to an increase in pressure that finally results in cellular lysis (Fig. 1C).

The study of the structure-function relationships in proteins is essential in teaching protein biochemistry, as it provides a more realistic overview of biochemical processes. However, this methodology is not frequently implemented in biochemistry lab classes. This article describes the disruptive effect of lysozyme on the bacterial cell wall in a programmed undergraduate laboratory class of 3.5 hours, usually addressed to teachers and students with no previous knowledge on structural biology. Specifically, the

purpose of the computational class proposed here is to introduce structural concepts to explain the lysozyme effects on the cell wall integrity in bacteria. Optionally, we suggest the inclusion of a simple descriptive test to observe the lysozyme lytic activity *in vivo*.

Materials and Methods

Crystallographic Structure Analysis of Lysozyme

The coordinates of the hen egg-white lysozyme crystal structure bound to the bacterial cell-wall trisaccharide NAM-NAG-NAM were obtained from the Protein Data Bank (PDB, <http://www.rcsb.org/pdb/>) under the accession code 9LYZ. Molecular structure visualization and analysis was performed with PyMOL [8]. The Educational-Use-Only PyMOL version can be freely downloaded from the website (<https://www.pymol.org/>), and easily installed on a basic computer running *Windows* or *Linux*. Additional help and instructions to perform tutorials and exercises outside the scope of this work can be found at the PyMOL website as well as the PyMOL Wiki (<http://www.pymolwiki.org>).

Bacterial Strain and Culture Conditions (Optional)

Gram-positive bacteria *Micrococcus luteus* ATCC 9341 (Oliva MDLM, UNRC) were grown in Luria-Bertani (LB) medium on a rotator shaker (200 rpm) at 30°C. For maintenance of strains and testing on plates, the medium was solidified using 1.5% agar-agar. However, the techniques used in these lab experiments are not specific to this Gram-positive bacterium; other model bacteria may be readily

used depending on the availability, and the methodology adapted accordingly.

Antibacterial Activity of Lysozyme (Optional)

The lysozyme inhibition effect was performed by the agar disc diffusion method. A bacterial culture at mid-log phase (100 μ l, $A_{600} \approx 0.5$ – 0.6) was spread on top of Petri plates with 20 ml LB culture medium containing 1.5% agar. After drying the plates, sterile filter paper discs (7 mm diameter) were soaked in 1 mg/ml, 2.5 mg/ml, 10 mg/ml, and 50 mg/ml of lysozyme solutions (Sigma-Aldrich L6876), and then placed on the plates. Sterile water was placed on the filter paper disc as a negative control. The plates were incubated at 30°C for 24 h, and later on the results were assessed by measuring the inhibition zones around the disc paper. (Note: the plates can be stored at 4°C, if the class is not thought to be performed on the next day).

Hazard Avoidance

The bacterial strain used in these laboratory experiments belongs to nonpathogenic Biosafety Level 1 microorganisms. However, the students should be instructed on how to work properly when handling bacterial cell cultures and extracts. Additional biosafety guidelines may be found at the American Society of Microbiology website (<http://www.asm.org/index.php/education-2/22-education/8308>).

Results and Discussion

The computational class described here is designed to be performed in a session of 3.5 hours in a computer classroom. The computers should have internet access and the free academic version of the program PyMOL installed, as detailed in the Materials and Methods section. Before starting, the instructor should explain the theoretical background based on the concepts described in the introduction section.

“*In-Silico*” Visualization of the 3D Structure of Lysozyme in Complex with Bacterial Cell-Wall Fragments

Visualizing 3D protein models, typically using computer programs, is one of the most important methods employed to understand the biological function of proteins.

The aim of this class is to interactively examine a 3D structure of lysozyme available at the Protein Data Bank (PDB) and to determine the interactions between the enzyme and bacterial cell-wall fragments (hereafter called lyso-CWF), to analyze the disruptive effect of this protein on bacterial cell wall integrity from a structural point of view.

Fetching the 3D Lyso-CWF Structure File from the PDB Website

Students should be divided into working groups of 2–3 members. First, each group should go to the PDB webpage (<http://www.rcsb.org/pdb/>) and download the file containing the 3D structure of lysozyme in complex with bacterial cell wall fragments. Since 1971, the PDB archive has freely served as a weekly updated repository of information about the 3D structures of proteins, nucleic acids, and complex assemblies to the global community [9, 10]. All structures in this database, which are deposited by researchers from all over the world, are carefully validated and fully documented. Each deposited 3D structure is assigned with a four-alphanumeric code, called the “PDB accession code.”

In this study, the students will proceed with the survey by typing the PDB code 9LYZ in the section highlighted with a red box in Fig. 2A. This code identifies the X-ray crystallographic structure of hen egg-white lysozyme in complex with the bacterial cell wall trisaccharide fragment NAM-NAG-NAM (lyso-CWF) [11]. Next, the students should save the file containing the atomic coordinates, the so-called PDB file. To perform this, they should proceed to the section “Download Files” (Fig. 2B), and select the “PDB

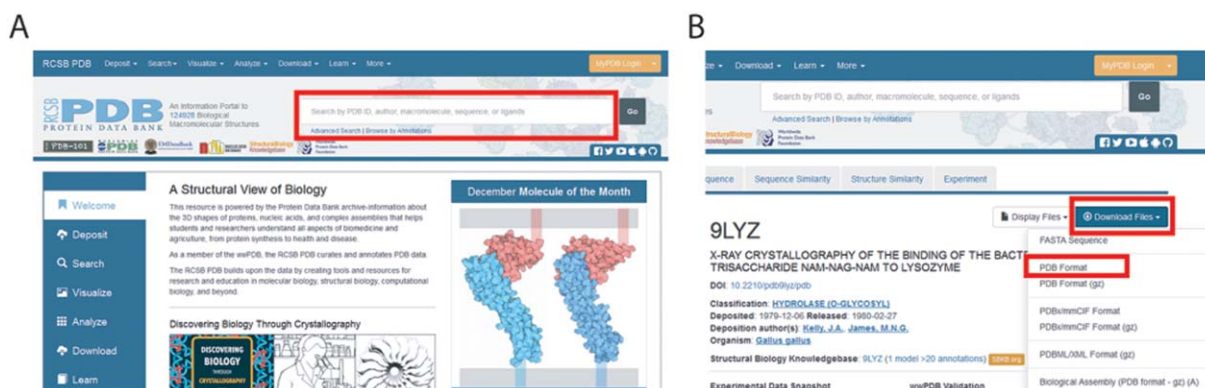


FIG 2

Home web page of the Research Collaboratory for Structural Bioinformatics - Protein Data Bank (RCSB PDB). (A) Screenshot of the search window highlighted with a red box. (B) Screenshot of the atomic coordinates file downloading menu highlighted with a red box. [Color figure can be viewed at wileyonlinelibrary.com]

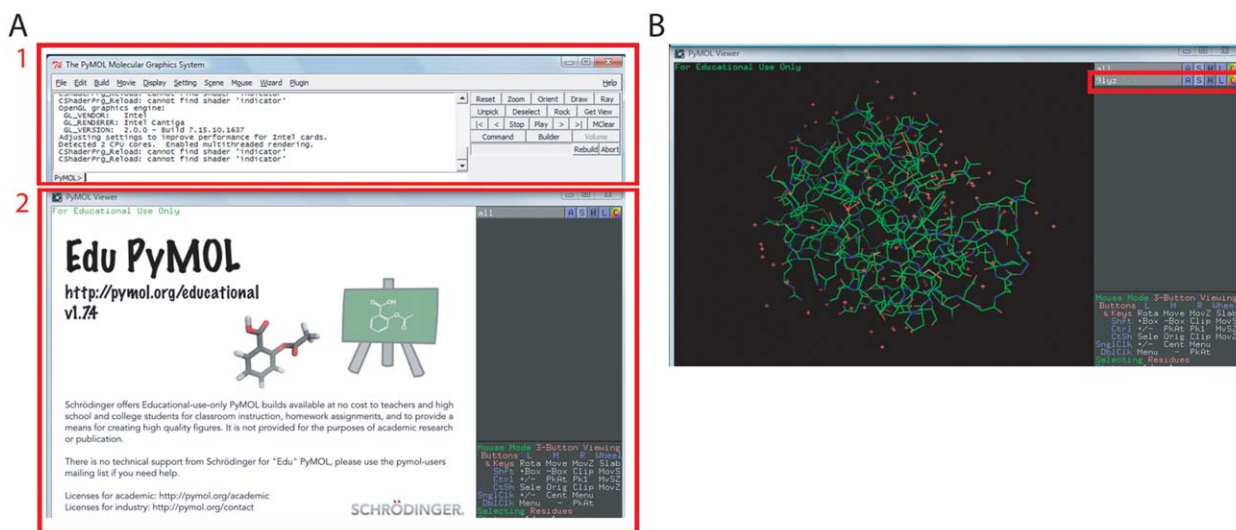


FIG 4 *PyMOL program graphical overview. (A) PyMOL appearance directly after launch. Two windows can be observed, a control window (1-upper), and a graphics window (2-bottom). (B) The 9lyz.pdb file visualization after loading it into PyMOL. The protein structure is colored in green (carbon), blue (nitrogen), red (oxygen), and yellow (sulfur). Water molecules are shown as red asterisks. The PDB identifier “9lyz” is highlighted with a red box in the upper right panel of the graphics window. [Color figure can be viewed at wileyonlinelibrary.com]*

molecules considerably smaller than the protein and are usually called ligands (i.e. metal ions, water molecules, substrates, inhibitors, etc.).

Here, it is important to notice that sometimes these molecules can be found in the protein structure because the researchers intentionally added them during the structure resolution process to (i) stabilize the protein during its expression and purification or (ii) examine where they are placed in the protein structure (i.e. substrates, inhibitors, cofactors, among others) and how they are stabilized by the respective protein-ligand atomic interactions. Thus, ligands can frequently provide us with essential atomic insight into the mechanism and function of the protein under study.

In this particular PDB file, three different ligand molecules are present in the following protein residues: AMU, β -N-acetylmuramic acid; NAG, N-acetylglucosamine, and HOH, water (Fig. 3B) (please notice that both AMU (aka NAM) and NAG are the sugar components in the peptidoglycan structure as represented in Fig. 1B).

Getting Started: lyso-CWF 3D Structural Analysis with PyMOL

Once the students are acquainted with the PDB file information, the file 9lyz.pdb should be opened with the program PyMOL (a molecular visualization system) to interactively observe and analyze the lysozyme 3D structure in the presence of cell wall fragments.

PyMOL is an open-source molecular graphics program widely utilized in structural biology research laboratories for looking at 3D molecular structures, rendering quality

figures for publications, and making dynamic videos of molecules, among other functions.

There is a vast, helpful, and well-documented literature about how to use the PyMOL interface (webpages, tutorials, publications, etc), which the teacher may recommend, specifically the tutorial “*Practical PyMOL for Beginners*” from the PyMOL Wiki webpage (https://pymolwiki.org/index.php/Practical_Pymol_for_Beginners) before this class. Our purpose in using PyMOL in this course is to exclusively describe from a structural point of view how lysozyme recognizes the bacterial cell wall fragments to induce their disruption.

Loading the lyso-CWF 3D coordinates. First of all, the PyMOL program must be opened, and an interface will come into view (Fig. 4A), where two windows can be seen (1-upper, a control window where several commands can be executed; and 2-lower, a graphical window where the protein structure and objects are displayed).

To start, the students should look for the 9lyz.pdb file in their computers by clicking on file > open in the control window. Once the 3D structure of lysozyme in the presence of cell wall fragments is open, a display as shown in Fig. 4B should appear on the screen where the PDB identifier “9lyz” is listed at the upper right panel of the graphics window. Next, the students should proceed on the structure examination as indicated below.

Moving, rotating and zooming on the 3D structure in the graphics window. Using the mouse, different points of view of the structure can be easily reached.

- Rotate (pressing the left mouse button and dragging)
- Zoom (pressing the right mouse button and dragging)

Move (pressing the middle mouse button and dragging)

Notice that atoms are placed on the vertices, colored in green (carbon), blue (nitrogen), red (oxygen), and yellow (sulfur), while the bonds among them are represented as line segments. Water molecules are shown as red asterisks. On this step, let students familiarize themselves with the use of the mouse.

Identifying the protein backbone and residue side chains. To examine the protein folding, the protein backbone can be displayed as cartoon representation for clarity. Students should go to the upper right panel of the graphics window 9lyz>S(show)>cartoon (Supporting Information Fig. S1). On this representation, the secondary structure elements along the protein are shown, contrasting with the different residue side chain conformations. All these conformational arrangements give rise to the 3D protein scaffold.

Looking for the ligands (cell wall fragments). The pocket of atomic contacts between the protein residues and specific ligands gives key information about the enzymatic mechanism. In this step, the cell wall components AMU and NAG bound to the protein will be recognized, and the respective residues involved in their stabilization will be identified.

First, the primary structure of the protein (the sequence of amino acids) must be displayed.

To achieve this, the students must go to display>sequence in the control window (Supporting Information Fig. S2). This will show the sequence of amino acids of the protein and the bound ligands (note that each letter represents an amino acid in the protein primary structure using the so-called FASTA code). At the end of the sequence (pulling the gray rectangle toward the right by holding the left mouse button on it), the bound ligands observed in the protein structure are shown. In this case, several water molecules (represented by the “O” letter), and the sugars β -N-acetylmuramic and N-acetylglucosamine (represented by “AMU” and “NAG,” respectively) are noted, as specified at the end of section “Opening the 3D Lyso-CWF Structure File”. Be aware that the saccharide chain AMU-NAG-AMU observed in this structure is a fragment of the bacterial cell wall illustrated in Fig. 1 (please note that AMU = NAM).

By clicking on every ligand code in the sequence bar with the left mouse button, this particular ligand can be selected (and unselected) to define it within the protein structure. Following this instruction, the students must identify the AMU-NAG-AMU ligands in the structure. To make the ligand visualization easier, students must go to the upper right panel of the graphics window sele>S(show)>sticks (Supporting Information Fig. S3).

Subsequently, the students should recognize the interactions between lysozyme and the bacterial cell wall components by identifying the protein catalytic residues Glu-35 and Asp-52. On the sequence of amino acid bar, the students should look for the residues desired and select them. Once selected, go to the upper right panel of the graphics

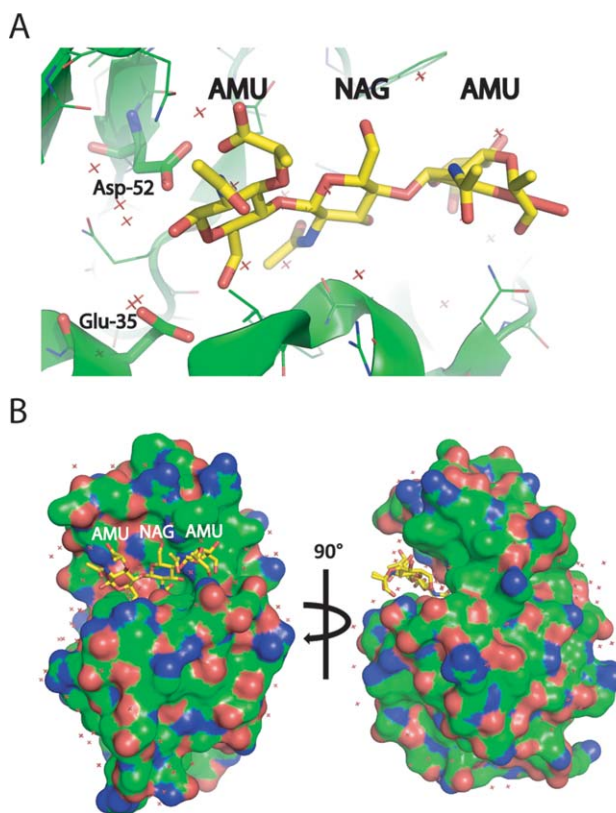


FIG 5

Interaction of lysozyme with the fragment saccharide chain AMU-NAG-AMU of the bacterial cell wall. (A) View of catalytic residues Glu-35 and Asp-52 interacting with the AMU ligand within the active site shown in capped sticks, with carbons in green, oxygens in red, and nitrogens in blue. The ligands AMU-NAG-AMU are shown as sticks with carbons in yellow. (B) 3D surface representation of the trisaccharide ligand buried into the lysozyme active site. Protein and ligand atom colors are depicted according to (A). Two different orientations of the macromolecule related with a 90° turn are depicted for easy recognition of the ligands and their binding pocket. Water molecules are shown as red crosses. [Color figure can be viewed at wileyonlinelibrary.com]

window sele>S(show)>sticks, as specified in the Supporting Information Fig. S3.

It must be noted that different protein orientations and movements can be performed as specified in the section “Moving, rotating and zooming on the 3D structure in the graphics window”, and residue names and numbers can be listed in the upper windows by clicking on them in the protein structure.

The students should be able to note the close interactions between lysozyme catalytic residues and the AMU and NAG ligands, which make hydrolysis of the bacterial cell wall possible (Fig. 5A). In this section, there are two points to keep in mind: i) in this protein structure, only the interactions with AMU are visible as the hydrolysis already



FIG 6

Lytic activity of lysozyme on *Micrococcus luteus* ATCC 9341. Filter papers were soaked in solutions containing different concentrations of lysozyme: (1) 1 mg/ml; (2) 2.5 mg/ml; (3) 10 mg/ml; (4) 50 mg/ml, and (5) sterile water as negative control. Different levels of inhibition are appreciated according to the lysozyme concentration. [Color figure can be viewed at wileyonlinelibrary.com]

occurred, and the NAG adjacent to the AMU was released (please see the mechanism reaction in Fig. 1B, bottom); ii) the bacterial cell wall is comprised of long chains of AMU (NAM) and NAG saccharides, and not only the three molecules shown in this protein structure.

Finally, as an overview of the protein-ligand interaction, the protein structure will be shown as a surface representation. This can be done on the upper right panel of the graphics window by clicking on 9lyz>S(show)>surface (Supporting Information Fig. S4). By this protein structure illustration, it is possible to note the protein shape (folding) and how the ligands are bound to it. Notice here how the AMU (NAM) and NAG ligands are perfectly trapped within the protein active site (Fig. 5B). Due to this ideal contact between protein and glycan strands of repeating disaccharide motifs NAM-NAG, lysozyme interacts with the bacterial cell wall facilitating its rupture. As a result, bacterial cells are no longer viable as observed in the antibacterial activity assays described below in section “Simple Microbiological Assay for the Detection of Lysozyme Antibacterial Activity (Optional)”.

Tip: It is important to highlight here that several capabilities of PyMOL can be implemented to go further with the exploration on the protein-ligand interactions, such as recognition of other close residues, distance and angle measurements, recognition of van der Waals contacts, salt-bridge and hydrogen-bond interactions, etc. As mentioned before, teachers may motivate students to find more instructions on the PyMOL website as well as on the PyMOL Wiki (<http://www.pymolwiki.org>).

Simple Microbiological Assay for the Detection of Lysozyme Antibacterial Activity (Optional)

To complement the *in-silico* structural analysis performed above, we propose an antibacterial lysozyme activity experiment as a supplementary assay, which may be developed by the teacher before the class. This assay described in the Material and Methods section is easy to perform, not demanding sophisticated laboratory facilities and expensive materials, which might also be adaptable to be performed by the students. The main aim of this optional appendix of the class is that the students experimentally detect the inhibition of a bacterial culture caused by the lysozyme lytic activity (Fig. 6).

Concluding Remarks and Perspectives

Educational biochemistry laboratory practices are frequently performed by protein-reagent *in-vivo* and *in-vitro* assays, but protein structural analyses are rarely incorporated. Paradoxically, a deep understanding of 3D protein structures allows us to have a more detailed knowledge on the molecular function of proteins, their modes of action, and consequently the biochemical processes that they carry out. The lab class proposed here aims to explore structural data to allow for a more effective understanding of the lysozyme effect on the bacterial cell-wall structure. By the end of the lab session, the students will be able to understand how a physiological effect produced by a protein can be explained from its 3D structure, giving them a glance closer to reality.

As the structural analysis briefly detailed in this article can be implemented for any protein whose 3D structure has been elucidated, we encourage teachers to further merge *in-vivo/in-vitro* assays with structural data to promote students critical thinking on biochemistry issues.

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Contributions

The project was designed by E.D.P., L.H.O., and W.G. Microbiological assays were performed by E.D.P. Structural



analysis was developed by L.H.O. The manuscript was written by all authors.

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