Laboratory Exercise

An All-Inclusive and Straightway Laboratory Activity to Solve the Three-Dimensional Crystal Structure of a Protein[S]

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Abstract

X-ray crystallography provides structural information of molecules at the atomic level, being a central technique at the forefront of science and technology. However, crystallography teaching is not usually implemented in biochemistry lab classes due to its complex execution by nonexpert users. Here, we report the basic step-by-step workflow performed by crystallographers in order to solve the three-dimensional structure of a protein. All these activities were executed in a course for Latin-American graduate students with no previous knowledge on X-ray crystallography entitled "Crystallography in Structural Biology: why do we need a protein crystal, and how do we get it?." We would like to share our experience with the educational research community, with the main purpose being to enrich teaching in biochemistry and structural molecular biology by performing a series of interesting laboratory and computer experiments.

Keywords: *Laboratory exercises; molecular visualization; protein structure function and folding; computer-based learning; X-ray crystallography*

Introduction

Three-dimensional (3D) high-resolution protein structures provide a deep knowledge on protein molecular functions and their modes of action in biochemical processes. X-ray crystallography is still today the most powerful technique to visualize protein structures at the atomic level. As such, approximately 90% of the biological macromolecule structures deposited in the Protein Data Bank [1] have been determined by X-ray crystallography, exposing its impact within structural biology.

Standard classes for upper-division undergraduate and graduate education in Biochemistry generally include visualization of protein structures, but usually lack activities on protein structure determination by X-ray crystallography, mainly due to the complexity to execute this technique by nonexpert teachers.

Several articles can be found in the educational literature on protein crystallization [2–6], X-ray diffraction [7, 8], and structure resolution [9, 10]. Usually, these reports deal only with the individual steps, leaving incomplete the workflow performed by protein crystallographers. Only a few reports encompass all these activities in a single paper [11, 12]; however, from our point of view, they require previous X-ray crystallographic expertise.

In 2017 and 2018, we organized a course for graduated students of Chemistry, Biochemistry, Biology, Biotechnology, and related sciences entitled "Crystallography in Structural Biology: why do we need a protein crystal, and how do we get it?" funded by the CELFI program (Latin-American

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Center for Interdisciplinary Formation, www.celfi.gob.ar). This educational program, sponsored by the Argentinian Ministry of Science (MINCyT), aims at the training of Argentinian and Latin-American graduate students on specific topics that require the interaction of diverse areas of knowledge. A total of approximately 30 students from nine different Latin-American countries were selected to attend the two editions of the course, which were held at the University of Buenos Aires, Argentina. Due to the exceptional outcome achieved, we report here the main activities performed during the course (adapted to cover the most relevant steps in only four classes of experiments and tutorials) with the aim of sharing our experience with the educational research community.

The comprehensive workflow herein described is intended to implement the protein structure resolution pipeline using X-ray crystallography in an easier way for educational purposes, using the hen egg-white lysozyme protein as an example.

Materials and Methods

Commercial Lysozyme

Lysozyme from chicken egg can be purchased from Sigma-Aldrich® (Saint Louis, MO, code L6876) as a lyophilized powder with a purity ≥90%.

Materials for Crystal Growing and Manipulation

All necessary materials can be purchased from Hampton Research (Aliso Viejo, CA, www.hamptonresearch.com), with the following product codes: Sitting drop Cryschem plates, HR3-158; Sealing tape, HR4-506; Magnetic crystal wand, HR4-729; Magnetic crystal caps, HR4-733; and Mounted cryo loops of different sizes, HR4-918.

Crystallization Trials

Crystallization assays are to be performed by means of the sitting drop vapor diffusion method. Briefly, lysozyme is dissolved in water in different concentrations (30—50—70 mg/ml), and mixed in a 1:1 volume ratio with a precipitant solution consisting of 0.1 M sodium acetate—acetic acid (NaAc/HAc) buffer, pH 4.0 + variable sodium chloride (NaCl) concentrations (1.1—1.2—1.3—1.4 M) or 0.1 M Tris(hydroxymethyl)aminomethane—hydrochloric acid (Tris/HCl) buffer, pH 7.5 + variable sodium nitrate ($NaNO_3$) concentrations (0.1—0.2—0.3—0.4 M) (see Supporting Information for the preparation of these crystallization solutions). The crystallization plates should be prepared in duplicate and incubated overnight at room temperature and at 4 °C in the absence of vibrations and direct sunlight.

Data Collection and Processing

Diffraction data from a single crystal were collected *in-house* at 100 K on a Bruker D8 QUEST microfocus copper anode X-ray diffractometer (Karlsruhe, Germany) equipped with a PHOTON 100 CMOS detector. For the data collection, the generator was set up at 50 kV and 1 mA. Data were collected with an oscillation of 0.5° (rotation in Φ angle) and with an X-ray exposure time of 30 seconds per image. X-ray diffraction data were then processed to a maximum resolution of 1.8 Å in the tetragonal $P4_32_12$ space group with XDS [13]. A total of 5% of the reflections were separated at that stage for cross-validation purposes. Detailed information on data collection parameters and processing statistics are shown in Supporting Information Table S1.

Structure Resolution, Model Building, and Refinement

Lysozyme is proposed to be solved by the molecular replacement method with the program PHASER [14] using the reported atomic coordinates of lysozyme (PDB code: 1HEL), with the last 21 C-terminal residues truncated. The obtained model should be refined using default parameters with the program REFMAC5 [15]. The incomplete region is manually traced in COOT [16].

Software Downloads and Installations

All programs required for this activity are freely available on the package provided by the CCP4 Software for Macromolecular X-ray Crystallography [17] and easily installed on a computer running *Windows*. Additional instructions are provided in the CCP4 website (http://www.ccp4.ac.uk/). The COOT version for *Windows* (WINCOOT) can be also downloaded from the developer webpage (http://www.ysbl.york.ac.uk/~lohkamp/coot/wincoot.html).

Graphical Representation of the Model

Molecular structures were represented using educational-use-only PyMOL [18] (https://pymol.org/edu/?q=educational/). Electron density maps were calculated with PHENIX [19] and represented using PyMOL 1.8.x.

Results and Discussion

Course Overview Description

The original full courses were taught in 2 weeks. In the first week, the following concepts were introduced: structure and function of biomolecules, protein expression and purification, crystallization, the crystal nature, symmetry elements, X-ray properties, and X-ray diffraction. The second week encompassed topics related to structural resolution methods, refinement, modeling, validation, and structural analysis. In this second week, a hands-on lab experience including protein crystallization, crystal manipulation, X-ray diffraction data collection and processing, and a tutorial on protein structure resolution were carried out. All these latter activities are entirely offered in this article including the processing files.

The condensed activities presented in this article are intended to be executed in four classes (about 3–4 hours each), which can be sequential or interrupted (Table I).

TABLE I *Outline of the proposed activities*

Class	*Activity*
1	Theoretical background
2	Crystallization trials
3	X-ray diffraction data collection (demonstration)
4	Structure resolution and visualization

Class 1: Theoretical Background

The *xyz* coordinates of the atoms present in a molecule (or macromolecule in the case of proteins) can be obtained by means of single-crystal X-ray diffraction [20]. Conventional crystals are ordered and periodic arrangements of molecules. The diffraction phenomenon occurs when crystals are irradiated with X-rays, which causes the cooperative dispersion of the incident beam into precise directions by the electron clouds in the well-ordered 3D array of molecules (Fig. 1*A*). This is possible due to the similar order of magnitude (around 10^{-8} cm or 1 Å) between the wavelength of the X-rays and the interatomic distances in the molecules present in the crystal lattice. As a result of this interaction, a set of reflections or diffraction "spots" are recorded from a series of images (also called diffraction patterns or diffraction frames) (Fig. 1*B*), where the crystal is rotated.

Each spot is an X-ray electromagnetic wave reflected by the crystal consisting of an amplitude ($|F_{hkl}|$) and a phase (φ_{hkl}), the so-called structure factor, which contains information of all atoms in the crystal. Based on the positions and intensities of these spots in the diffraction patterns, which are classified by the Miller indices or the "hkl" triads, the atomic coordinates of the crystallized molecule can be determined.

The amplitudes can be calculated from the square root of the scattered X-ray intensities, but the phases are lost during data collection and cannot be recorded. In other words, X-ray detectors are not able to recognize at what time the different electromagnetic waves arrive. This is called "the phase problem" in crystallography. Fortunately, a series of different methods have been developed to solve this difficulty. Some of them involve the incorporation of heavy atoms in the crystals and even the measurement of the diffraction intensities using different X-ray wavelengths (the so-called "experimental phasing" methods), while others take advantage of homologous structures already solved for obtaining an initial set of phases ("molecular replacement"). Once initial phases are calculated, electron density maps of the crystal are generated, which allow for the tracing of the molecule, or in other words, the placement of the atoms that build up the protein. The process ends with iterative molecular modeling and crystallographic refinement steps.

Extensive bibliography can be found on the basics of X-ray crystallography and therefore it is not the objective of this article to go in-depth on this topic. However, didactic and user-friendly books [20–22] and webpages [23–25] can be consulted if desired.

We would like to recall here that, although a basic overview on the particular concepts mentioned above is recommended before starting the activities, it is not necessarily needed to go further on it, as this practice is thought to be executed by teachers and students with no previous knowledge on X-ray crystallography.

Class 2: Crystallization Trials

The first experiment to be performed consists in the overnight crystallization of the protein of interest, hen egg-white lysozyme, by means of the sitting drop vapor diffusion method [22]. Lysozyme is stable, relatively cheap, and (most importantly) easy to crystallize in comparison to the great majority of proteins. It is recommended to work with no more than 3–4 students per group. With the aim of helping the teacher and students with the execution of this section, a note for instructors (as supplement to the information presented in the

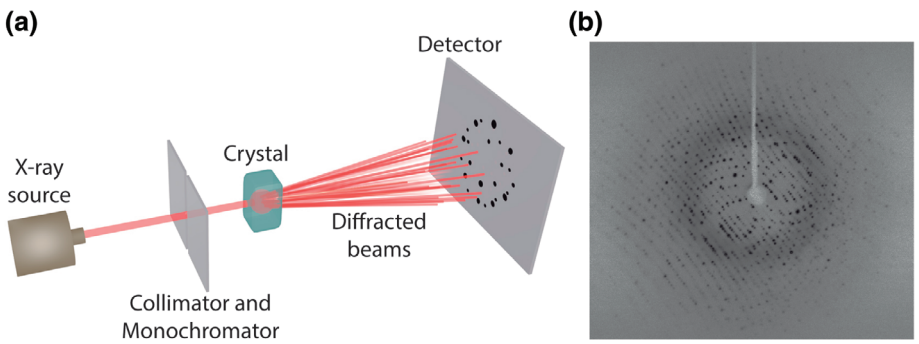

FIG 1 *Experimental setup used to collect X-ray diffraction data from crystals. (a) Collimated monochromatic X-rays hit a crystal mounted on a goniometer. As a result, the electron clouds of the crystal scatter the beams, which are recorded as dark spots on a detector giving rise to a diffraction pattern. The positions and intensities of these spots are used for obtaining a 3D picture of the electron density within the crystal. (b) Actual diffraction pattern recorded from a protein crystal. [Color figure can be viewed at wileyonlinelibrary.com]*

Materials and Methods section), as well as a student's handout, are provided in Supporting Information.

The crystallization setup consists of a mixture of protein and crystallization solution placed on a concave pedestal in equilibrium with a higher volume of crystallization solution in a bottom reservoir (Fig. 2*A*). A total of 24 different crystallization conditions are tested using Hampton Research Cryschem plates (Aliso Viejo, CA) (Fig. 2*B*), varying 1) the concentration of the protein, 2) the concentration of the precipitants (NaCl or $NaNO_3$), and 3) the pH of the mixtures (using NaAc/HAc or Tris/HCl as buffers). The system is isolated by means of an adhesive tape cover and incubated at room temperature or at 4 °C. Then, water vapor starts diffusing from the pedestal droplet into the reservoir to equal the water chemical potential. This leads to the concentration of both the protein and the chemicals present in the crystallization solution in the tiny droplet, which can eventually lead to the formation of lysozyme crystals.

After overnight equilibration, the student will observe each of the drops carefully under a microscope and discuss the results observed. Usually, 20–40× magnification is enough, and the presence of a polarizer and analyzer is highly recommended. Some of the expected results are presented in Fig. 2*C*–2*E*. The students should evaluate the influence of 1) the concentration of lysozyme, 2) the concentration of NaCl or $NaNO_3$, and 3) the incubation temperature in the number, size, shape, and quality of the crystals formed.

Class 3: X-Ray Diffraction Data Collection (Demonstration)

On this session, the students learn how the crystals are picked up from the crystallization droplet, how they are mounted on a classical diffractometer, and how the X-ray diffraction patterns are recorded. As these activities demand manipulation of liquid nitrogen as well as the availability of an X-ray diffractometer equipment, they are shown by a series of pictures and briefly explained below.

First, a nylon cryo loop attached to a magnetic wand (Fig. 3*A*) is introduced into the crystallization droplet and one crystal is "fished" from below and taken out with a vertical movement (this experiment is done under the microscope). If the action is successful, the crystal should stay attached to the loop as in Fig. 3*B*. Mounted crystals in the magnetic pins are then transferred to a drop containing crystallization solution added with a so-called cryoprotectant (i.e. glycerol, PEG 400), which avoids the formation of crystalline ice. Later, crystals are plunged into liquid nitrogen (77 K) for a few seconds (Fig. 3*C*), and then introduced in plastic vials with the help of a metal forceps (Fig. 3*D*) and immediately transferred to an X-ray source or stored in

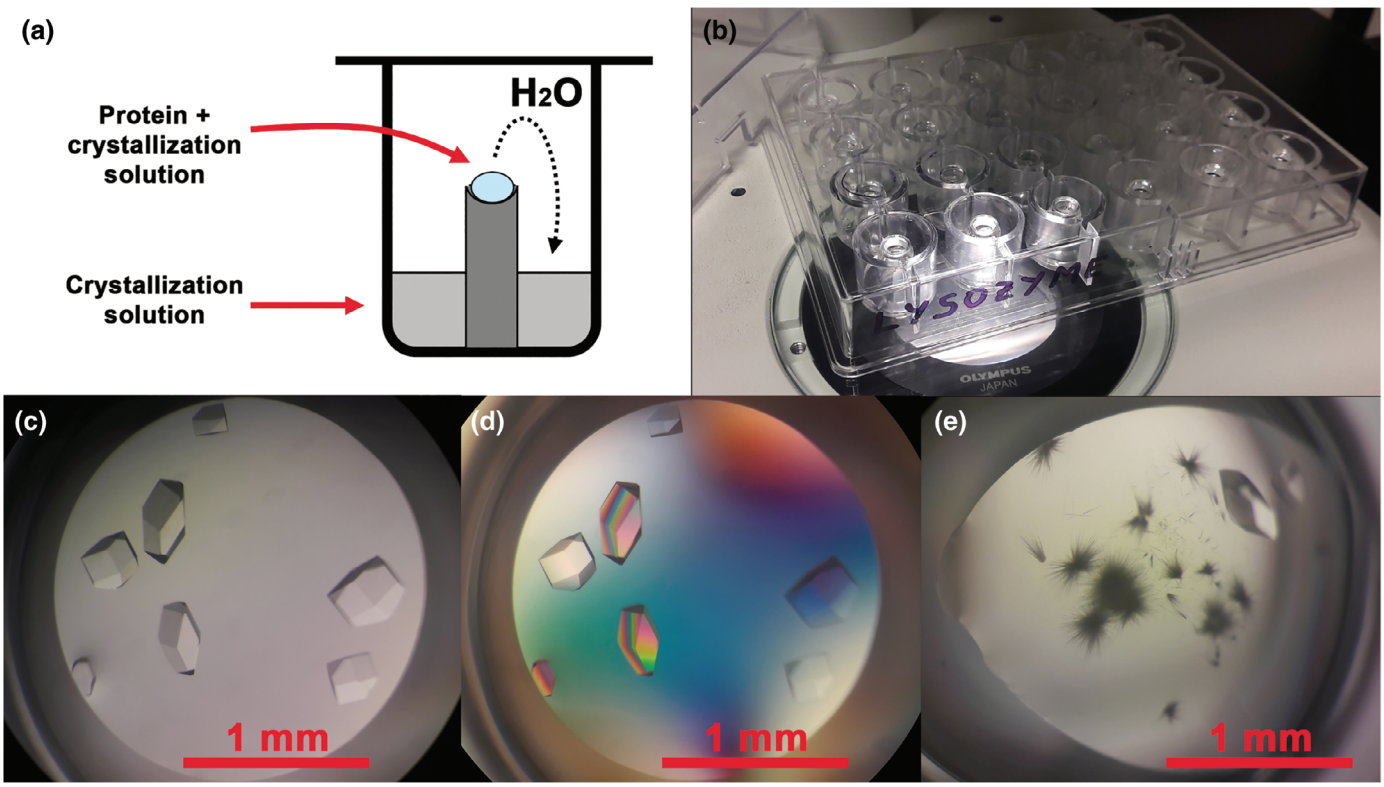

FIG 2 *Sitting drop vapor diffusion crystallization. (a) Overall sketch of the method. (b) Hampton Research Cryschem plate. (c) Lysozyme crystals visualized under the microscope. (d) Same as (c) but with the use of a polarizer and analyzer. (e) Presence of dendrites (dark needle-shaped aggregates) together with crystals. [Color figure can be viewed at wileyonlinelibrary.com]*

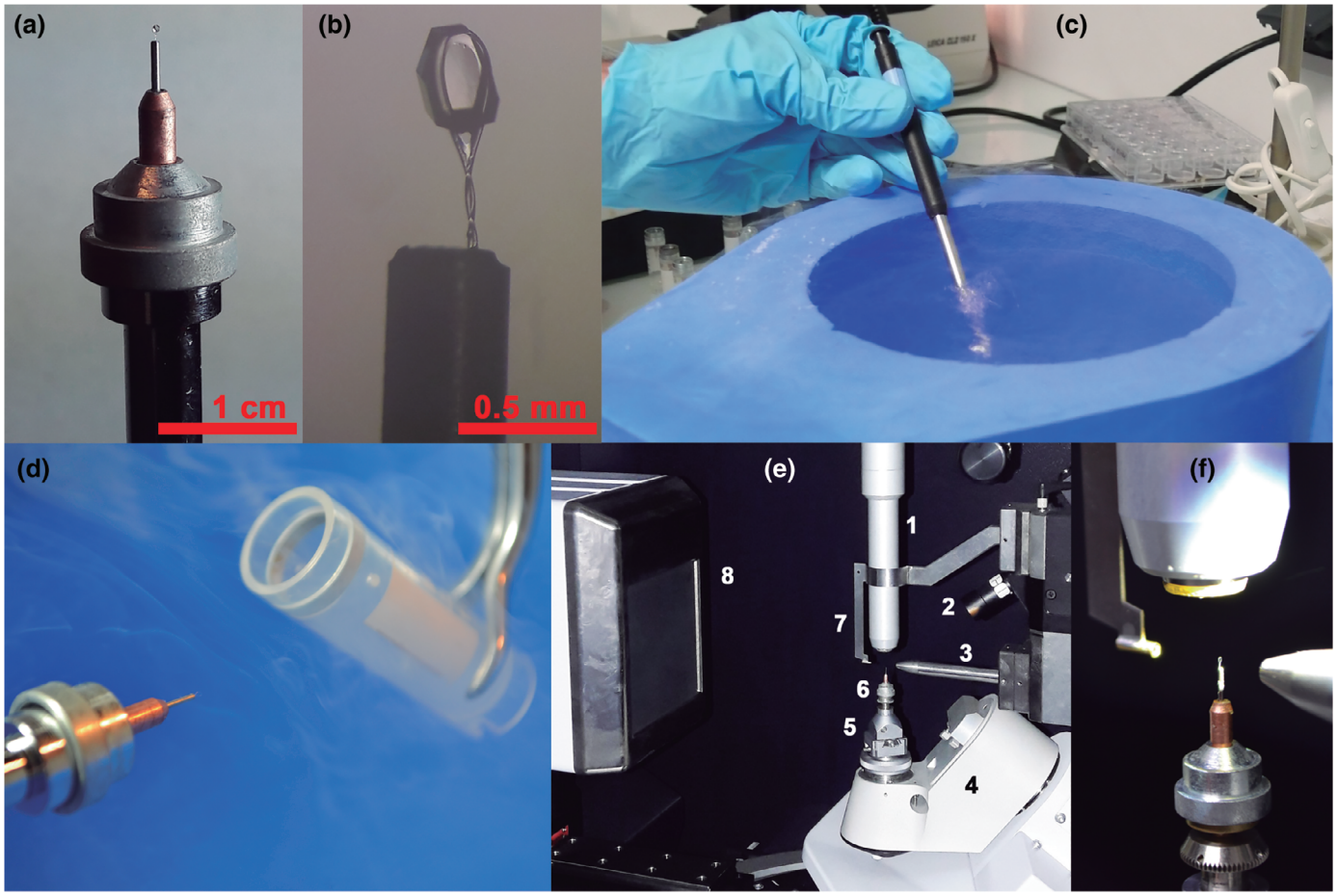

FIG 3

Mounting of the samples for X-ray diffraction data collection. (a) Lysozyme crystal (top) trapped in a Hampton Research cryo-loop. A magnetic wand (bottom) holds the crystal cap system in place. (b) Visualization of a mounted crystal in the microscope. (c) Cooling of a crystal sample in liquid nitrogen. (d) Placement of a magnetic pin holding a crystal into a plastic vial. (e) Experimental setup for X-ray diffraction using a Bruker D8 QUEST microfocus diffractometer. (1) Low temperature crystal cooling device. (2) Video camera. (3) X-ray collimator. (4) Goniometer. (5) Goniometer head. (6) Mounted lysozyme crystal. (7) Beam stop. (8) X-ray detector. (f) Detail of the sample placement in the X-ray diffractometer. [Color figure can be viewed at wileyonlinelibrary.com]

liquid nitrogen containers called "dewars." It is important to mention that this procedure can be performed at room temperature avoiding the liquid nitrogen manipulation only for teaching proposes using the tools for crystal manipulation specified in the Materials and Methods section.

For the diffraction experiment, the magnetic pin containing the vitrified cooled crystal is directly mounted on the goniometer head of the diffractometer under a cold nitrogen stream (100 K). For our course, we used a Bruker D8 QUEST microfocus diffractometer (Fig. 3*E*–3*F*).

The first step in diffraction data collection comprises the so-called test images, in which the crystal is exposed for a short time to the X-ray radiation with the aim of evaluating its diffraction quality. This process can be repeated after rotating the crystal 90° relative to the X-ray beam, to check both for the correct centering of the sample and the presence of a homogeneous diffraction signal. At this point, the shape and arrangement of the crystal spots are evaluated (i.e. geometry and symmetry) (Fig. 4). Additional test frames can also be taken with the modification of the exposure time and the crystal-to-detector distance, to evaluate the effect of these parameters in the diffraction image. The last step consists in the collection of a complete dataset. For this purpose, we have collected a total of 360 frames, each of them with an exposure of 30 seconds and an oscillation of 0.5°, which covered a total of 180° rotation of the crystal.

Class 4: Structure Resolution and Visualization

The common required steps to process the measured diffraction data in order to obtain the 3D structure of a protein are the following: 1) Indexing; 2) Integration, 3) Scaling and reduction, 4) Phasing, 5) Model building, and 6) Refinement [20]. Briefly, indexing identifies the geometrical arrangement of the spots, defining the symmetry of the crystal; integration gives an intensity value I and a background value $\sigma(I)$ for each spot; scaling and reduction combine the

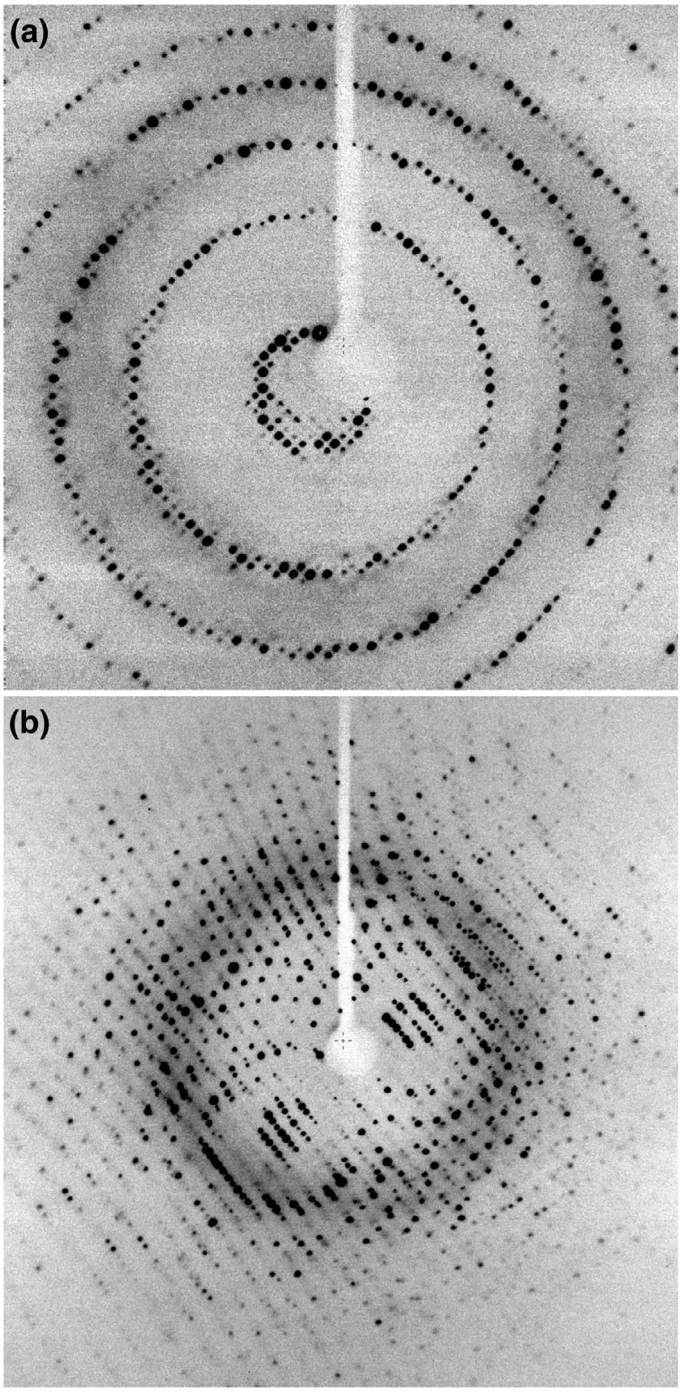

FIG 4

Lysozyme X-ray diffraction patterns. The vertical white shadow is generated by the beam stop. Panels (a) and (b) correspond to the diffraction signal observed for different orientations of the same crystal, with the latter at a smaller crystal-to-detector distance, which results in closer spot separations. Detailed information on data collection parameters are shown in Supporting Information Table S1.

integrated values into a set of structure factor amplitudes $|F_{hkl}|$ fixing several empirical errors produced during the data collection; phasing finds initial structure factor phases φ_{hkl} that cannot be measured; model building places the atomic model into the electron density map; and refinement adjusts and fixes the atomic model into the electron density map, calculating an improved set of phases. It is important to state that steps (v) and (vi) are iterative until a final model is reached.

The computer lab activity reported here (Supporting Information) is a step-by-step guide to perform steps (iv), (v) and (vi) to solve the 3D crystal structure of lysozyme, using the X-ray diffraction data collected.

As mentioned previously, the phasing procedure aims at solving the phase problem existing in crystal diffraction. Fortunately, there are several ways to recover the lost phases using experimental or computational methodologies, depending on the protein molecule under investigation. One of them is the molecular replacement method (MR) [26], in which a homologous protein structure (called the search model) is used as a "phase-donor" in order to merge these phases with the measured intensities of the target protein crystal, giving rise to an electron density map using a Fourier transform. This method involves sequential rotation and translation movements of the structure model protein into the unit cell of the target protein crystal until a good match with the experimental data is obtained.

In the aforementioned structure resolution tutorial, the phasing step will be performed by MR using the already known lysozyme crystal structure (PDB code 1HEL) [27] as a search model, but lacking (on purpose) the last 21 C-terminal residues. As a result, after the truncated model protein is placed, the calculated the electron density map will clearly expose where these C-terminal missing residues should be traced and refined (Fig. 5*A*). Later, based on the available sequence of amino acids from the protein, the students should model the missing residues into the 3D electron density map, and refine them until a good match between the location of the atoms and the electron density map is achieved, preserving the expected stereochemistry of the individual amino acid residues (Fig. 5*B*).

This computer lab activity is designed to be performed individually or in group work during a session of 3–4 hours in a computer classroom. The computers should have the CCP4 suite [17] installed, following the instructions specified in Materials and Methods section.

Each computer should be provided with a folder containing the four files needed for the execution of the tutorial (Supporting Information): an *mtz* file containing the structure factor amplitudes $|F_{hkl}|$ where the steps (i), (ii), and (iii) had already been executed, a *pdb* file containing the atomic coordinates for the C-terminally truncated lysozyme structure used as search model, a *fasta* file containing the full-length protein sequence, and an additional *docx* file containing the location of the secondary structure elements in the protein sequence as well as the numbering and missing residues for ease of the modeling step. Once the full-length lysozyme structure is solved, visualization analysis of

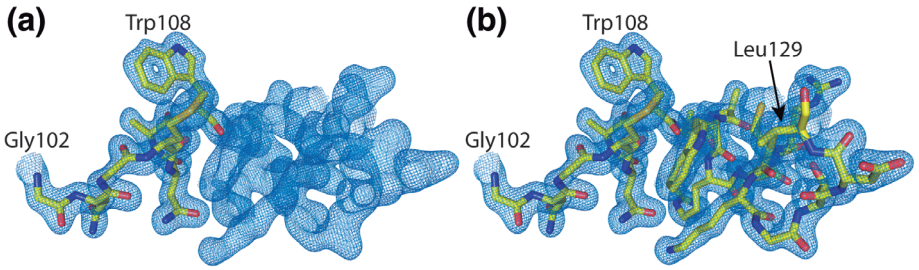

FIG 5

Model building and tracing of the C-terminus of lysozyme into the electron density map obtained after MR at 1.8 Å resolution. (a) Fragment of the truncated lysozyme structure (residues 1–108) placed in the electron density map revealing the missing region to be modeled. Only residues from Gly102 to Trp108 are shown for clarity. (b) Same as (a) but with the missing residues already traced and modeled. The last C-terminal residue (Leu129) is highlighted. The protein is represented in sticks with carbon atoms in yellow, oxygen atoms in red, nitrogen atoms in blue, and sulfur atoms in wheat. The 2Fo–Fc Fourier map used to build the model is shown as a blue mesh contoured at the 1σ level. [Color figure can be viewed at wileyonlinelibrary.com]

the 3D final model may be also performed following our previously reported teaching activity [28].

Course Bottlenecks and their Handling

Although all students succeeded in growing excellent quality lysozyme crystals, there were some specific points where difficulties arose. For most of the students, it was their first time in direct contact with macromolecular crystallography software. As such, many of them expressed frustration at not being able to follow the instructions flawlessly and required the assistance of the supervisors in the lysozyme structure resolution tutorial. Their first encounter with the different programs took a considerable amount of time for them just to get familiar with the commands, mouse controls, and output files and formats. Additionally, the main scientific problem came afterward, that is, modeling and refinement of the lysozyme crystal structure. A good way to handle these pitfalls, which are definitely a consequence of the limited amount of time for all proposed activities, is having all programs installed in the student's computers when the course ends and allowing remote assistance from the supervisors if the students are not able to finish the tutorial in the scheduled times. The great advantage of macromolecular crystallography in this point is that the required data processing software is free of charge, with several user-friendly tutorials [29], and able to run in a regular personal desktop computer.

Student Assessment and Feedback

Students were evaluated individually by means of a written examination on the last day and a laboratory report to be sent not more than two weeks after the course ended (one report per group). For the former, a multiple-choice quiz with 33 questions covering all aspects discussed in the course was designed. There were four options per question with only one correct answer in each case, and 90 min time for its completion. Students were allowed to have free access to their own class notes, printed Powerpoint slides and crystallography books. The great majority of them reached at least 28 correct answers. For the laboratory report (see Supporting Information), the students were asked to present crystal pictures and discuss how the different variables affected the outcome of the experiments.

To finish, the professor performances as well as the quality and general expectations of the course were evaluated by the students through an online anonymous feedback questionnaire. Absolutely, all students expressed highly positive comments in the items "Global quality of the course" and "Expectations covered," whereas most of them asked for a longer time dedicated to the computational section in the item "Changes and suggestions for future versions of the course." Students also highlighted the importance of this regional initiative supported by the CELFI program for the development of structural biology in Latin-America.

Final Remarks

X-ray crystallography is a state-of-the-art technique broadly used by the scientific community for solving the 3D atomic coordinates of small molecules and biological macromolecules. Although this technique is believed to be executed by individuals with expertise, we are confident that the activities here detailed are suitable for Biochemistry and Biology students with no previous knowledge in the field. Thus, these lab classes aim to familiarize the students with X-ray crystallography, performing the basic steps that a crystallographer usually does to solve the 3D structure of proteins. In addition, most college and university departments have the lab equipment required and the materials are inexpensive, therefore, it may be applied both for the upper-division and the graduate level.

By the end of the lab sessions, the students should be able to understand why crystals are needed, how they are obtained, how crystals should be analyzed by means of X-ray diffraction, and which kind of information can be obtained from the diffraction data.

Acknowledgments

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Conflict of Interest

The authors declare no competing financial interest.

Hazards and Safety Precautions

The experiments described are designed to be executed with low-risk chemicals (lysozyme, NaCl, $NaNO_3$, Tris, and diluted acetic acid and hydrochloric acid). However, students should be instructed on how to work properly when handling chemical reagents. During the crystallization procedure, we recommend the implementation of basic safety guidelines such as the use of gloves, glasses, and laboratory coats.

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