Formal Typed Report

Egg-White Lysozyme Crystallization by Hanging Drop Vapor Diffusion

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Abstract

Hen Egg White (HEW) lysozyme is a highly prevalent and easily acquired enzyme with antimicrobial uses in a variety of industries. Variables surrounding its crystallization are therefore important in understanding the enzyme's properties, structure, and function. 18 HEW lysozyme crystallizations were performed by hanging drop diffusion using a sodium acetate buffer pH 4.5, and sodium bromide precipitant. Crystal composition was confirmed by crush and dye absorption tests. Higher buffer concentration (0.1 M) yielded the best crystals at both 55 and 90 mg/mL lysozyme concentrations. Lower precipitant concentrations (0.30 and 0.45 M) were also superior conditions, while higher concentration (0.60 M) yielded primarily salt crystals.

Introduction

Lysozymes are antimicrobial enzymes involved in immune activity both inside and outside of the animal kingdom.[1] they function by destroying bacterial cell walls by hydrolyzing β (1→4) glycosidic linkages from N-acetylmuramic acid to N-acetylglucosamine in cell wall peptidoglycans.[2] Because of its ubiquitousness and natural antimicrobial activity, lysozyme has been used in pharmaceuticals and medicine as a general antibiotic, and in the food industry as a preservative.[3] Though the protein is present in a variety of species, hen egg white (HEW) lysozyme (RCSB PDB ID code: 1LYS[4]) represents the most easily accessible lysozyme, due to the relatively inexpensive cost of acquiring and processing chicken eggs. Lysozyme makes up approximately 3.5% of functionally important proteins in egg white.[3]

HEW lysozyme (figure 1) consists of 6 alpha helices and 5 beta sheets.[4] Its dimensions are 30×30×45 Å. The protein is roughly ellipsoidal, with a substrate binding cleft that spans one face of the molecule. The protein has a pI of 11.0.[2]

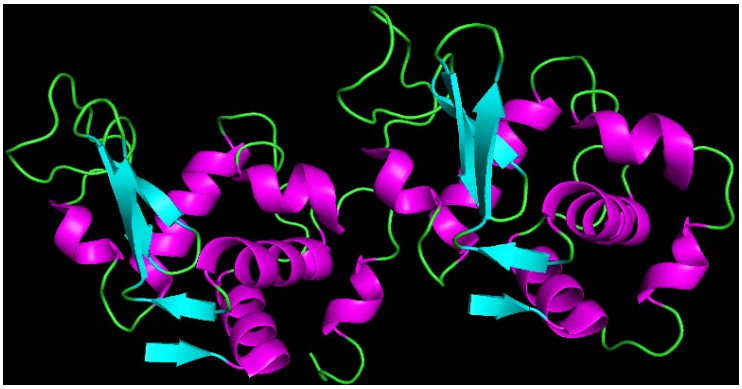

Figure 1: 3D Structure of 2 hen egg white lysozymes (RCSB PDB ID: 1LYS).

Crystallography is often the most difficult step in the process of three-dimensional protein analysis because general factors important in crystallization are not fully understood.[5] In order to examine the properties of this important protein, it is necessary to determine the conditions that yield the most suitable crystals for X-ray diffraction crystallography first. Such crystallography requires crystallization of a protein into an ordered three-dimensional array of molecules. As a prelude to such analysis, trial crystallizations are performed in order to identify ideal conditions for a protein. During this process, multiple attempted crystallizations are performed under a variety of precipitant, buffer, and protein concentrations.

Crystallization requires creation of a supersaturated solution of the macromolecule that simultaneously allows it to retain its natural state. This is done by adding mild precipitating

agents, such as neutral salts, to the solution, or by manipulating other solvent parameters, like pH, temperature, or ionic strength. Upon supersaturation, protein is slowly forced out of solution and forms crystals.[5]

Microdialysis crystallization is one such method of achieving supersaturation of macromolecules. This technique usually relies on adjusting ionic strength to achieve supersaturation. Samples are placed into a Microdialysis button at a specific ionic strength. A dialysis membrane, that excludes large molecules is then secured over the button, preventing proteins from leaving the Microdialysis chamber. The button/membrane apparatus is then placed into solution and small compounds can diffuse out of the chamber until the chamber solution reaches supersaturation.[6]

Vapor diffusion is cited as a simple and cost effective method of crystallization, and is the most widely used method of crystallization for these reasons.[6] In these cases water diffuses out of the protein solution into the reservoir solution below, increasing relative supersaturation. The two most common methods of vapor diffusion are sitting and hanging drop diffusion. In sitting drop diffusion, a drop is placed on a concave micro-bridge over a reservoir solution. Water diffuses from the drop into the reservoir. Hanging drop vapor-diffusion, as performed in our experiment, functions when a drop of protein solution suspended over a reservoir achieves supersaturation when water moves from the drop to the reservoir solution.

In the process of conducting a crystallization screen, salt crystals are often encountered, so it must be demonstrated that a macromolecule, not salt, crystal has been formed. This is accomplished via a battery of qualitative tests that rely on the inherent chemical differences between salt and protein crystals.[7] Salt crystals are often described as 'crunchier' than crystallized proteins, which shatter very easily.[6] Whereas salt crystals will form distinct cracks when pressure is applied and will break cleanly, protein crystals are much more fragile, shattering under a miniscule amount of pressure. This is because while salts are composed entirely of strong ionic bonds between component molecules, Na^+ and Br^-, for example, protein crystals are stabilized by intermolecular bonds, which are much weaker compared to the total surface area of the protein.[7] These intermolecular forces also allow for more space between atoms, so protein crystals will readily absorb small molecule dyes, while salt crystals will not. Proteins are also expected to have lower birefringence than salts, so crystal optical properties are also assessed after crystallization.[7]

Methodology

HEW lysozyme was crystalized at 20 °C by hanging drop diffusion. Equal 2.5 μL volumes of protein and well solution containing sodium acetate buffer pH 4.5, NaBr precipitant, and lysozyme solution were combined to create hanging drops in a 3×6 well apparatus. Wells in this apparatus were filled with 1 mL of well solution. Well rows A, B, and C contained NaBr precipitant molarities of 0.30 M, 0.45 M, and 0.60 M, respectively. Columns 1-3 contained fixed lysozyme concentration of 55 mg/mL, while columns 4-6 contained lysozyme at 90 mg/mL. Columns 1 and 4 contained 0.0250 M sodium acetate buffer, columns 2 and 5 contained 0.0625 M buffer, and columns 3 and 6 contained 0.100 M buffer. Wells were stored at 20 °C in the dark, allowed to crystallize for 5 weeks and checked weekly to monitor the progress of crystal formation. After 5 weeks, the three largest crystals obtained, those from wells A3, A6, and B3, were characterized by examination under a polarized microscope, methylene blue dye

absorption, and finally a crush test. This battery of tests was designed to ascertain whether protein or salt crystals were formed, as well as their optical and molecular properties.

Results and Discussion

Optimal crystallization

The best crystallization was achieved in well A3 (see figure 2), which quickly yielded a single rectangularly elongated hexagonal crystal. Such a crystal is ideal because its large size and uniformity is most easily analyzed by x-ray crystallography. Other wells yielded multiple discrete crystals within single droplets. Wells in row A all yielded crystals with varying degrees of success, whereas roughly half of wells in row B yielded crystals. In contrast, wells in row C yielded very small crystals, if any, and these were all engulfed in fuzzy salt crystals by the end of their development period (as shown in figure 3). Similarly, many wells in rows A and B became engulfed in different fuzzy crystals (depicted in figure 4), though these were distributed evenly throughout the drop, rather than appearing in clumps as was the case in row C wells. It is suspected that these are due to convection heating of the solution by the lamp used to illuminate the sample during observation, and subsequent cooling when the well apparatus was returned to room-temperature storage. This hypothesis is consistent with the results of X.H. Tang et al., who studied the effect of variations in cooling rate on lysozyme crystals under similar solution conditions. The study found that faster solution cooling yielded crystals engulfed in similar-looking smaller, evenly distributed crystals.[8] While it is hypothesized that these are crystallized salts, wells that did not yield optimal crystals were not subjected to protein crystal tests, as the sample would not be suitable for x-ray crystallography. Therefore, this hypothesis remains to be confirmed.

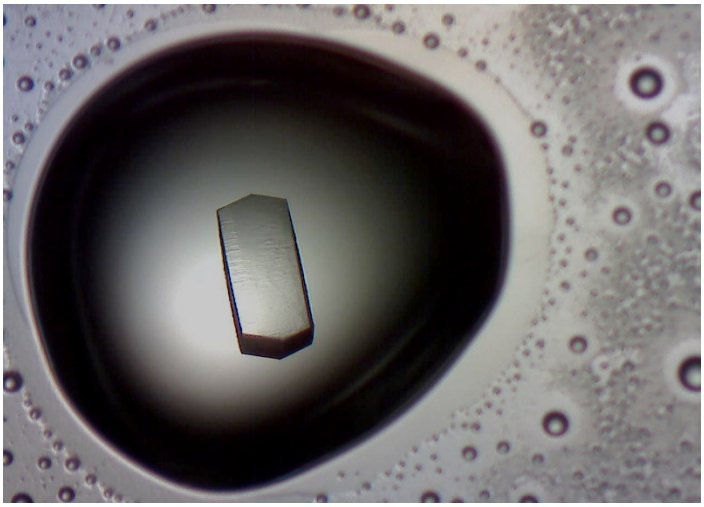

Figure 2: Hexagonal crystal from well A3 (unstained)

Figure 3: Crystallization result of well C2 (unstained).

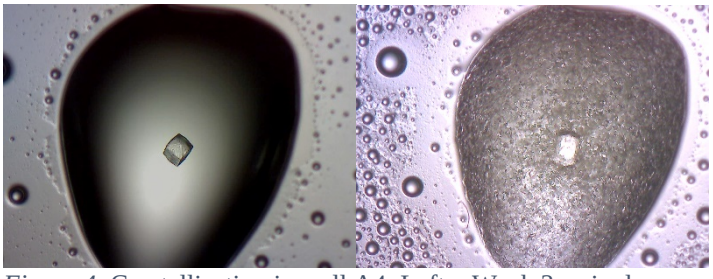

Figure 4: Crystallization in well A4: Left – Week 3 – single crystal forming. Right – week 4 – miniscule crystals of unknown composition cover the single lysozyme crystal.

Crystal Morphology

Crystals demonstrated a rectangularly elongated hexagonal geometry upon crystallization. This geometry is consistent with prior literature on lysozyme crystallization.[9] Specifically, the phenomenon of rectangular elongation is consistent with the pH of 4.5 used in our experiment, as prior research has demonstrated that lysozyme crystal growth is more rectangular-cuboidal at pH 4.2, and more simple-cuboidal at pH 5.4.[10] Size of crystals varied, with smaller crystals having a top-facing surface area of roughly 0.47 mm^2, and the largest crystal having a top-facing surface area of roughly 0.53 mm^2.

Protein crystal tests

Crush Test

Upon subjection to a crush test, crystals A3, A6, and B3 readily broke into a very fine particulate (see figures 5-7). This indicates that these crystals were formed from protein, rather than salt crystallization, as salt crystals would have provided more resistance to pressure and would have broken cleanly.

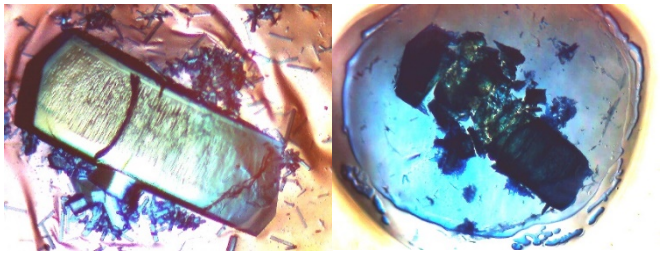

Figure 5: Right – Intact crystal from well A3 (methylene blue stain). Left – Crushed crystal from well A3 (methylene blue stain)

Absorption of Methylene Blue Dye

Presence of solvent channels within a crystal allows for the absorption of small molecule dyes, whereas salt crystals lack the solvent channels necessary to absorb dyes.[6] In order to demonstrate the presence of such solvent channels, methylene blue was introduced into the crystal droplets, and was absorbed within 5 minutes (see figures 5-7). This demonstrates that the crystals consist of crystallized lysozyme, not salt.

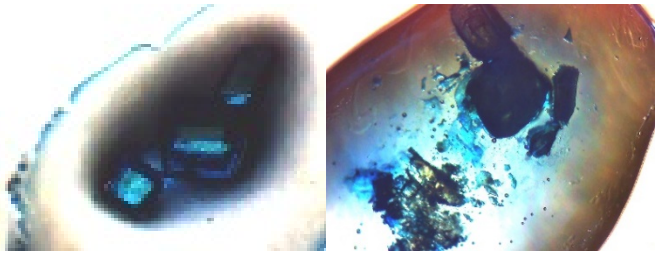

Figure 6: Left – Crystal from well A6 (methylene blue stain). Right – Crushed crystal from well A6 (methylene blue stain).

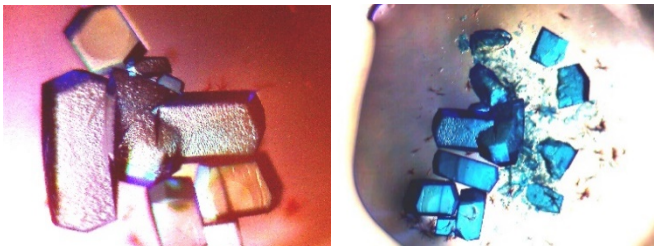

Figure 7: Left – Crystal from well B3 (methylene blue stain). Right – Crushed crystal from well B3 (methylene blue stain).

Birefringence

Crystals were examined under a polarized light microscope and were found to be strongly birefringent, illustrated in figure 8.

Conclusion

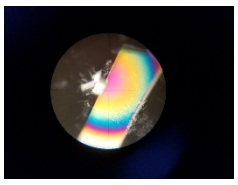

Figure 8: Strong birefringence of crystal A3

Best crystallizations occurred at high buffer concentrations (0.10 M), and low precipitant concentrations (0.30 and 0.45 M) for both 55 and 90 mg/mL protein concentrations, but precipitant concentration is the more important factor, evidenced by the stark difference in quality between crystallizations from rows A and B compared to those from row C. Overall, these results are consistent with prior literature published on HEW lysozyme crystallization both with respect to pH-specific geometry and the effect of temperature on crystal quality. It is notable that while these HEW lysozyme crystals have many physical properties expected of a protein crystal, they demonstrate higher birefringence than would be expected, as proteins are not typically birefringent.

References

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