
CRYSTALLIZATION OF LYSOZYME

CHEM 110L - INTRODUCTORY BIOCHEMISTRY LABORATORY

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ABSTRACT

The objective of this experiment is to learn basic principles and methods in modern protein crystallography by running crystallization trials of lysozyme and analyzing the quality of the resulting crystals. Protein crystallography allows for the determination of three-dimensional structure of molecules, which in turn helps to understand their function and mechanism of action. Importantly, analyzing the active site structure of proteins enables us to elucidate the details of enzymatic catalysis via structural analysis and computational modeling.

1 Introduction

Proteins grow from supersaturated solutions, which can be obtained by slowly increasing the concentration of precipitating agents, the concentration of the protein, or altering the pH. A popular method relies on vapor diffusion from a hanging drop. This so-called 'hanging drop' method enables a simultaneous increase in the concentration of the protein and the precipitating agent. It has been successfully employed to grow uniform crystals with well-defined edges and morphology in comparison to other techniques.¹

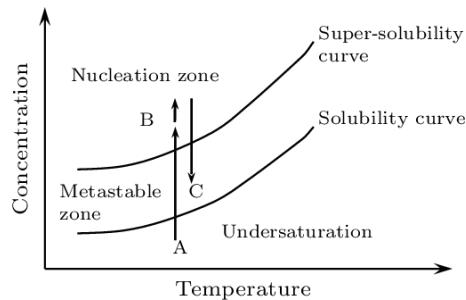


Figure 1: Crystallization phase diagram of vapor diffusion.²

Nucleation largely determines the outcome of crystallization. When the nucleation rate is high, a large amount of crystals form nearly simultaneously and grow to similar sizes. However, when nucleation is slow, the supersaturation of the solution drops slowly, allowing for the nucleation of new crystals at different points in time. Thus, a population of crystals of various sizes forms. Thus, the size, size distribution, morphology, and other properties of the crystals grown in a crystallization experiment may be controlled for by altering factors that enable slower nucleation rates.

Determining the three-dimensional structure of a molecule with diffraction methods requires quality crystals of pure protein. A high quality crystal is a single crystal of sufficient size that lacks major crystal defects (cracks) and has a small number of minor crystal defects. In general, different proteins require different conditions for crystallization,

*Not a final draft.

and predicting the conditions necessary to crystallize a completely novel protein is a difficult task. A trial-and-error approach is often more successful than using scientific reasoning to determine these conditions. Thus, we will set up 6 trials with different conditions, and analyze the resulting crystals or lack thereof.³

1.0.1 pH

Generally, the solution pH determines the net charge and charge distribution of the sample, which effects intermolecular interactions. Tight and precise intermolecular contacts must be formed to ensure stability of the crystal lattice. For proteins, the solubility will generally decrease as the pH value approaches the isoelectric point (at pH 11 for lysozymes). This holds true for lysozyme; the majority of lysozyme structures uploaded to the Protein Data Bank (PDB) were grown between pH 4.5 and 5.0, and crystals grown at higher successive pH values are of lesser quality. Thus, the pH of the protein and precipitant solutions will be within pH 4.5 to 5.0.⁴

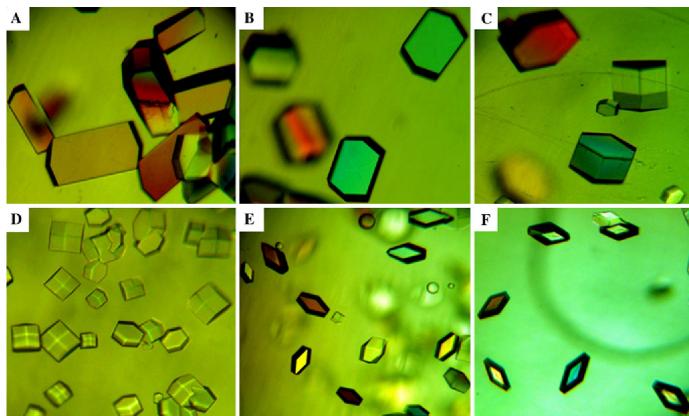


Figure 2: **Micrographs ($\times 100$ magnification)** of lysozyme crystals obtained at 15°C under different pH values: **a** 4.2, **b** 5, **c** 6, **d** 7, **e** 8, and **f** 8, 10 mg/mL.⁴

1.0.2 Buffers and ionic strength

There are a variety of buffers that have been used previously for lysozyme crystallization.^{5,6} The sodium acetate (NaAc) buffer has a buffering range of pH 3.6 - 5.6 and has been widely and successfully applied in biochemistry research.

1.0.3 Additional precipitating agents

The addition of salts in precipitating solutions is generally considered to increase the quality of crystals via the effect of non-specific stabilizing actions between the salt and the protein of interest. However, high salt concentration may result in salting in (precipitation) or denaturation of the protein, rather than crystallization. Negatively-charged chlorine ions occupy the majority of available positively charged sites on the molecular surface of lysozyme.¹¹ Only one chlorine ion binding site is available in the tetragonal lysozyme crystal;^{14,15} thus, because crystal formation relies on the removal of the other ions, crystallization is more difficult when the concentration of chlorine ions in solution is increased. Additionally, increased salt concentration decreases the solubility of the protein in sodium acetate buffer.

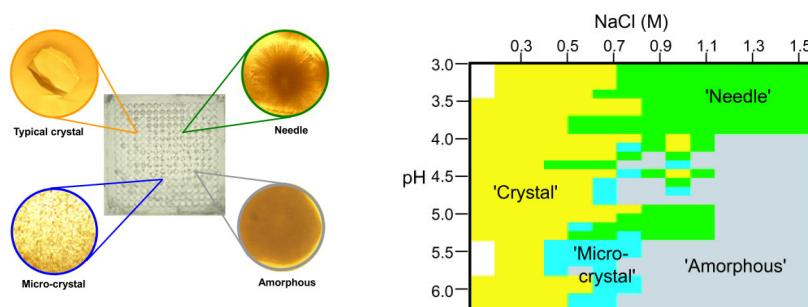


Figure 3: **Control of lysozyme crystal morphology by ionic strength and pH.**⁷

Ionic liquids (ILs) have been used to manipulate crystallization of macromolecules. ILs change the intermolecular contacts, and thus the shape of the crystals. The addition of ILs also generally seems to increase crystal size.^{8,9} This phenomenon can be attributed to the fact that the formation of the crystal nucleus is involved in the protein's transition to its secondary structure.¹⁰ To some degree, the pH and ionic strength can be varied to control for the desired morphology of the lysozyme crystals.⁷

High molecular weight linear polymers have also been used as precipitating agents.¹¹ For example, small concentrations of polyethylene glycol (PEG) have been used to enhance crystal nucleation; PEG forms microdroplets that reduce the solubility of the protein by steric exclusion, which helps to concentrate the protein locally and provide an interface for nucleation. The effectiveness this method is dependent on the concentration and its molecular weight of the PEG itself.^{6,7}

The molecular weight of PEG varies from 200-20,000 but PEGs of molecular weight in the range of 3,350-8,000 are considered to be the most effective when growing crystals for X-ray diffraction. High molecular weight PEGS are unlikely to penetrate into the crystals or to be co-crystallized, which is ideal for crystallography. The addition of PEG also results in decreased vapor diffusion equilibration rates, which extends the period of crystal growth.⁸ Overall, high molecular weight PEGs are the most popular additives to lysozyme structures uploaded to the Protein Data Bank.¹¹

1.0.4 Concentration of protein and precipitation agent

Generally, a protein will not crystallize below a certain concentration. On the other hand, increasing the protein concentration excessively leads to uncontrolled nucleation, rapid and disordered growth, and produces a large number of small crystals with visible and/or invisible defects. Lysozyme is readily soluble across a range of concentrations (10 - 100 mg/mL), but lower concentrations tend to yield fewer, larger crystals. Thus, a lysozyme solution of 10 - 20 mg/mL should produce suitable crystals for crystallization.⁵

1.0.5 Temperature and temperature fluctuations

Increased temperatures allow for more molecular diffusion collisions within the hanging drop and faster evaporation rates. Thus, higher temperatures tend to favor increased induction time and the degree of supersaturation, leading to larger numbers of crystals. Incubation temperatures generally lie between 4°C and room temperature; for crystallization of lysozyme, 20°C has been commonly used.

1.0.6 Purity of macromolecules

In general, higher purity samples produce more suitable crystals for diffraction studies. Contaminants, including lysozyme's substrates, inhibitors, and/or cofactors, can interfere with nucleation and/or crystal morphology.⁶

2 Materials and Methods

Lysozyme from chicken egg white and analytical-grade chemicals were purchased from Sigma-Aldrich.

Stock solution of 0.1M NaAc buffer was prepared in a 15 mL plastic centrifuge tube. The pH was adjusted to 4.9 by addition of 1M HCl or 1M NaOH. Lysozyme solution was prepared by dissolving protein powder in the buffer solution at 20 mg/mL in a 0.2–2-ml Eppendorf tube. Any remaining protein powder was dissolved by rocking the tube gently. Precipitant solutions A-D (Table 1) were prepared by dissolving solid additives in the NaAc buffer solution. Lysozyme and precipitant solutions E-F were obtained from the instructor.

The hanging-drop vapor diffusion crystallization experiments were performed at room temperature in 24-well Linbro plates (Hampton Research Corp., Aliso Viejo, CA, USA). About 400 μ L of reservoir (precipitant) solution was added to each well. For each drop, 2 μ L of protein solution was added to 2 μ L of appropriate buffer solution. Wells were sealed by hand using Dow Corning Vacuum Grease with siliconized circular cover slides type HR3-215 (Hampton Research Corp., Aliso Viejo, CA, USA). The trays were left undisturbed for approximately 39 hours at room temperature.

Microphotographs were made after 39h and 114h using a Leica EZ4 Stereo Microscope at 7X or 8X (lens diameter 18 mm) magnification and an Apple iPhone 7 Plus 12MP rear camera. Due to time constraints, the results of Trials E–F were not observed. After 114h, microphotographs for Trials A–F were made using a Leica EZ4 Stereo Microscope (with ruler in eyepiece) at 8X magnification and an Apple iPhone 7 Plus 12MP rear camera. Microphotographs were analyzed using a public domain image processing software (ImageJ, version 1.52p99, <https://imagej.nih.gov/ij/index.html>). The dimensions of the crystals were estimated by comparison with the diameter of the lens of the microscope or the ruler inside the eyepiece of the microscope.

PRECIPITANT SOLUTION				PROTEIN	
A.	0.1M NaAc pH 4.9	5.8% (w/v) NaCl			20 mg/mL
B.	0.1M NaAc pH 4.9	7.5% (w/v) NaCl			20 mg/mL
C.	0.1M NaAc pH 4.9	5.8% (w/v) NaCl	+ 10% (w/v) PEG 8000		20 mg/mL
D.	0.1M NaAc pH 4.9	5.8% (w/v) NaCl	+ 25% (w/v) PEG 8000		20 mg/mL
E.	50mM NaAc pH 4.5	1M NaCl	+ 30% (w/v) PEG 8000		16 mg/mL
F.	50mM NaAc pH 4.5	1M NaCl	+ 30% (w/v) PEG 8000		32 mg/mL

Table 1: **Precipitant solution recipes and respective protein concentration for each crystallization trial.** Preparation for Trials A-D is discussed in the text body. Precipitant and protein solutions for Trials E-F were assigned by the instructor.

3 Results and Discussion

A large number of birefringent tetragonal crystals had grown in well A when first observed at 39h. At 114h, the crystals had experienced further growth, but not to a significant extent. This was confirmed by comparison of their dimensions at each point in time (see Fig. 5). The majority of these crystals formed separately from one another in the droplet, and were defined by their $\lceil 110 \rfloor$ faces of similar height and width.

Next, the influence of an increase of ionic strength on crystal formation was tested by comparison of the crystals formed in precipitant solutions B to those in A. Precipitant solution B contained NaCl at 7.5% (w/v).² After 39h, only a single, extremely small, crystal was observed in well B, such that its morphology could not be adequately discerned. However, after 114h, the amount of individual crystals increased dramatically. Crystals were similar in size and dispersion to those produced in Trial A.

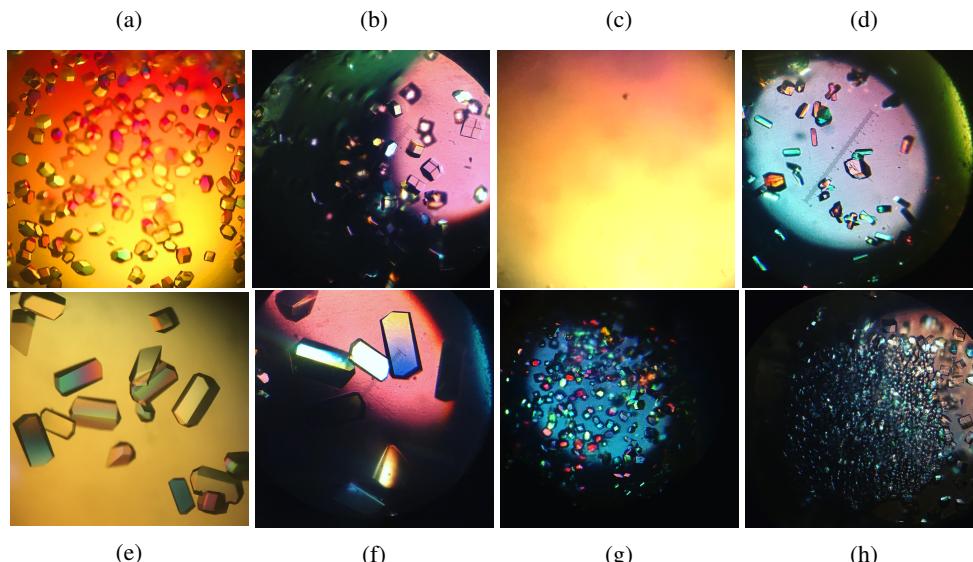


Figure 4: **Microphotographs of lysozyme crystals from hanging-drop crystallization.** Crystals obtained from an initial protein concentration of 20 mg/mL in 0.1 M NaAc buffer at pH 4.9 with additives at 28°C: (a) 5.8% (w/v) NaCl after 39h; (b) after 114h; (c) 7.5% (w/v) NaCl after 39h; (d) after 114h; (e) 5.8% (w/v) NaCl and 10% (w/v) PEG 8000 at 28°C at 7X magnification after 39h; (f) 8X magnification after 114h. Crystals obtained in 50mM NaAc buffer at pH 4.5 with 1 M NaCl and 10% (w/v) PEG 8000 at 28°C from an initial protein concentration of: (g) 16 mg/mL; (h) 32 mg/mL. Images taken at 8X magnification after 114h.

The majority of crystals produced by Trial B were tetragonal, but had three distinct appearances: (a) Defined $\lceil 110 \rfloor$ faces with much greater heights than lengths (resulting in a 'skinnier' appearance); (b) Defined $\lceil 110 \rfloor$ faces with similar

²About 1.28M NaCl.

heights and lengths; (c) Defined $\lceil 101 \rfloor$ faces with similar heights and lengths. The growth unit for the $\lceil 101 \rfloor$ and $\lceil 110 \rfloor$ faces of the crystal are tetramer and octamer, respectively (see Fig. 55c). In a solution at low supersaturation, the number of tetramers exceeds the number of octamers, and the $\lceil 101 \rfloor$ face grows at a faster rate. At high supersaturation, the number of octamers is greater than the tetramers, and the $\lceil 110 \rfloor$ face grows faster than the $\lceil 101 \rfloor$ face.⁷ Thus, the variation in morphology observed here may be partially attributed to the high supersaturation favored by the high salt concentration.

Additionally, some small crystals that had a triclinic morphology were observed. The formation of triclinic crystals occurs with increased ionic strength due to the increase in attractive supramolecular interactions.¹⁰

The effect of addition of PEG 8000 to the precipitant solution was investigated by a comparison of the results of Trials A and B to Trials C and D. Trial C (10% (w/v) PEG 8000) yielded the overall largest and most dispersed crystals, both at 39h and 114h. Individual crystals were of tetragonal morphology, but the relationship between the heights and widths of their $\lceil 110 \rfloor$ was unclear.

Trial D (25% (w/v) PEG 8000) yielded no crystals (that were visible at 8X magnification) throughout the course of the experiment. One possible explanation for this is the addition of PEG has resulted in the increased solubility of lysozyme to the extent that it remained in solution. This property of PEG is typically exploited to protect against the formation of salt-induced, disordered aggregates at high salt concentrations.^{7,11,12} As these conditions have been numerously reported to yield lysozyme crystals in the literature, it is unlikely that the increased concentration of PEG is the factor responsible for the lack of resulting crystals. Further crystallization trials would be necessary to determine the influence of increasing PEG 8000 concentration to 25% (w/v) in this case. In this experiment, it is more likely attributable to an unknown gross error that would be remedied by repeating the experiment.

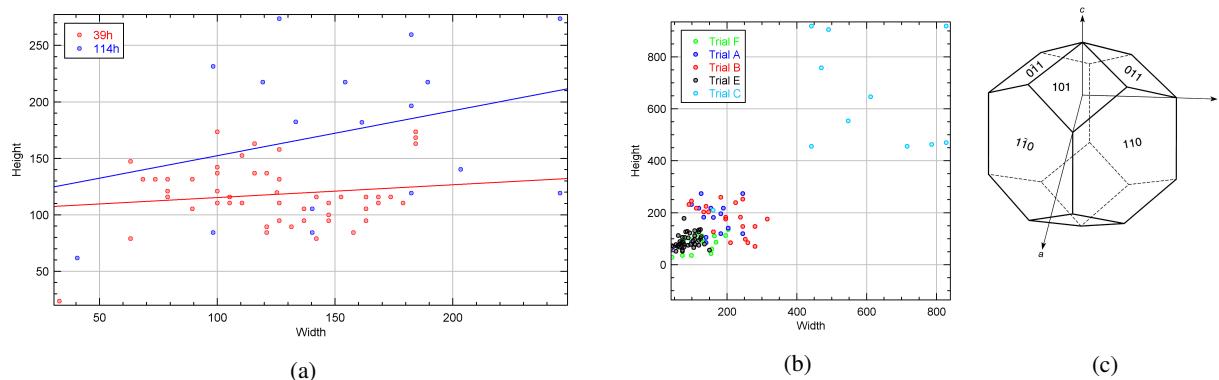


Figure 5: Measured dimensions of lysozyme crystals. (a) Example of data analysis done to confirm crystal growth between 39h and 114h for Trials A and C. Data shown is from Trial A. (b) All measurements taken at 114h. (c) The normal growth habit of tetragonal lysozyme crystals; here, the $\lceil 110 \rfloor$ face was considered for measurements.

	AFTER 39H		AFTER 114H	
	Length (μm)	Width (μm)	Length (μm)	Width (μm)
A.	40.88	62.18	161.40	181.90
B.	—	—	200.64	177.49
C.	212.10	94.40	611.30	646.40
D.	—	—	—	—
E.	—	—	88.56	92.12
F.	—	—	130	89.33

Table 2: Average measured length and width of crystals in Trials A-C.

The influence of varying the protein concentration was observed by comparison of crystals from Trials E and F to those from the previous trials. Trials E and F had the same concentration of PEG as in Trial C, but differed in the composition of the buffer, pH of the solution, and concentration of protein sample.

Trial E, with 16 mg/mL lysozyme, yielded tetragonal crystals at 114h that were much smaller and more concentrated in comparison to those produced in Trial C. On average, the heights and widths of their $\lceil 110 \rfloor$ faces were similar. While

Trial F (32 mg/mL lysozyme) also yielded lysozyme crystals, they were small and clustered together such that their individual sizes were difficult to measure. They were similar in morphology to those obtained in Trial E. However, these crystals would surely not be sufficient quality for use in X-ray diffraction studies. Even a visual comparison between Trials E and F insinuates that 32 mg/mL lysozyme is an excessive concentration of protein, at least for this precipitant solution. However, halving the protein concentration for Trial E failed to improve greatly on crystal quality, so the failure of both trials to produce diffraction-quality crystals can be more confidently attributed to the precipitant solution.

In comparison to precipitant solution C, the precipitant used in Trials E and F has a halved buffer concentration and lower buffer pH. The dramatic decrease in crystal quality can thus be attributed to either of these factors. The decreased pH of the buffer increases the solubility of lysozyme, which in turn promotes nucleation, while a lower buffer concentration results in more free space for nucleation to take place. These factors combined allow many more crystal nuclei to form simultaneously, thus leaving less sample to contribute to the growth of each crystal.

4 Conclusion

Of the precipitant solutions tested here, Trial C (0.1M NaAc pH 4.9 with additives 5.8% (w/v) NaCl + 10% (w/v) PEG 8000) produced the best quality crystals from a 20 mg/mL protein solution. The comparison of the crystals produced in trial C with those produced in the remaining trials further highlights the importance of detailed crystallization screenings; even small changes in buffer concentration, pH, and ionic strength have dramatic effects on crystal growth and quality. Additionally, crystal morphology is highly influenced by factors including the pH and ionic strength.

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Appendix I.

AFTER 39H		AFTER 114H	
Length (μm)	Width (μm)	Length (μm)	Width (μm)
45.06	40.11		
46.81	32.72		
58.29	41.34		
68.59	40.11		
72.29	45.73		
71.89	41.34		
72.31	44.84		
Avg.	40.88	62.18	

Table 3: Estimated dimensions of lysozyme crystals produced in Trial A.

AFTER 114H	
Length (μm)	Width (μm)
238.5	182.4
147.3	203.4
98.22	245.5
259.6	84.18
91.2	231.5
112.2	217.5
196.4	175.4
224.5	238.5
161.4	126.3
210.5	84.18
280.6	147.3
245.5	252.6
140.3	224.5
280.6	70.15
252.6	98.22
196.4	182.4
133.3	203.4
245.5	147.3
182.4	259.6
315.7	175.4
Avg.	200.636
	177.4865

Table 4: Estimated dimensions of lysozyme crystals produced in Trial B.

AFTER 39H		AFTER 114H	
Length (μm)	Width (μm)	Length (μm)	Width (μm)
222.6	98.1	491.1	905
226.7	99.7	827.8	470
231.9	101.9	547.2	554.2
232.6	97.8	470	757.7
234.6	83.7	785.7	463
184.5	101.9	442	919
243.3	109.7	715.6	456
175.7	81.4		
178.5	82.2		
233.3	90.3		
169.4	91.5		
Avg.	212.1	94.4	611.3
			646.4

Table 5: Estimated dimensions of lysozyme crystals produced in Trial C.

AFTER 114H	
Length (μm)	Width (μm)
81.85	126.3
107.6	112.2
74.83	86.52
123.9	135.6
72.49	72.49
60.8	112.2
119.3	74.83
65.48	79.51
65.48	88.86
77.17	67.82
123.9	100.6
77.17	177.7
149.7	56.12
119.3	133.3
93.54	121.6
126.3	107.6
119.3	126.3
107.6	109.9
105.2	65.48
135.6	79.51
60.8	74.83
107.6	79.51
88.86	74.83
107.6	131
119.3	93.54
46.77	70.15
88.86	102.9
77.17	79.51
70.15	84.18
79.51	105.2
63.14	67.82
72.49	51.45
53.78	77.17
86.52	128.6
107.6	95.88
53.78	51.45
95.88	77.17
102.9	91.2
72.49	56.12
58.46	70.15
42.09	72.49
109.9	77.17
72.49	105.2
Avg.	88.56
	92.11

Table 6: Estimated dimensions of lysozyme crystals produced in Trial E.

AFTER 114H	
Length (μm)	Width (μm)
163.7	109.9
154.3	42.09
128.6	109.9
168.4	86.52
77.17	98.22
98.22	95.88
105.2	88.86
135.6	88.86
112.2	114.6
72.49	60.8
79.51	100.6
98.22	35.08
156.7	60.8
203.4	135.6
196.4	112.2
Avg.	130
	89.33

Table 7: Estimated dimensions of lysozyme crystals produced in Trial F.