The Crystallization of Hen Egg White Lysozyme

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The functions of many macromolecules have been able to be studied due to knowledge about their structure. Crystallization is a method often used to obtain the structure of many macromolecules, especially proteins. In the course of this project, hen egg white lysozyme was crystallized with the hanging drop method, and the crystals were then analyzed to determine the optimal conditions for crystal growth. Many trials were conducted with varied conditions to achieve this purpose. Some crystals were then exposed to a high-intensity X-Ray to obtain the diffraction pattern. If it were not for time constraints, the electron density diagram and the structure of the crystals could then have been determined.

1. Introduction

Hen egg white lysozyme is also called Mucopeptide N-acetylmuramdylhydrolase. Discovered by Sir Alexander Fleming in his search for an antibiotic, which ended in penicillin, lysozyme has been studied ever since.

Lysozyme is also found in human tears and mucus. It protects against bacterial infection by breaking down the bacterial cell walls. This lyses the cells and kills them. In eggs it protects proteins and fats, necessary nourishment for the chick. Lysozyme used in experiments is from hen egg whites because it appears in such large concentrations and is easy to harvest. This protein would have great potential as an antibiotic if not for its large size which prohibits it from traveling between cells.

A common method for the preparation of lysozyme crystals is the hanging drop technique. In this procedure the wells of a microplate are filled with a mother liquor (prepared from buffer and precipitant). A drop, made up of lysozyme and the well solution, is suspended from a

siliconized cover slip over the well, which is then sealed shut with a layer of grease. The difference between the well solution concentrations in the drop and in the well creates a transfer of water from drop to well and vice versa until equilibrium is reached. After a few days, crystals form on the cover slip.

Lysozyme is most often studied using crystallization and X-Ray diffraction. Optimizing crystal growth conditions allows for a more accurate diffraction pattern and structural map. The crystals researchers desire to grow are those large enough not to degrade as quickly during X-Ray diffraction.

2. Materials and Methods

2.1. Solution preparation for crystallization

A 0.1 M sodium acetate buffer of pH 4.6 was prepared by dissolving 5.4432g of sodium acetate in 400ml of distilled water. The pH was adjusted using aliquots of concentrated hydrochloric acid. A 10% weight per volume sodium chloride precipitant was prepared by adding 5g of sodium chloride to 50ml of the buffer. The pH was adjusted to 4.2 using hydrochloric acid and sodium hydroxide. The stock protein was prepared by mixing 100mg of lysozyme in 2ml of distilled water. This protein solution was then vortexed to stir the solution and filtered to remove all impurities. The stock protein was serially diluted to 17.5mg/ml, 5mg/ml, and 1.5mg/ml and these dilutions were stored in three microfuge tubes.

2.2. Preparation of trays 1 and 2

A layer of grease was applied to the rims of the wells in both micro-plates.

2.2.1. Well solution

To the wells in columns 1& 4, 1.0ml of precipitant was added. To the wells in columns 2 & 5, 0.65ml of precipitant and 0.35ml of buffer were added. To the wells in columns 3 & 6, 0.5ml of precipitant and 0.5ml of buffer were added.

2.2.2. Hanging drops

For each of the two trays being prepared, the 50mg/ml lysozyme was used in row A, the 17.5mg/ml in row B, the 5mg/ml in row C, and 1.5mg/ml was used in row D. To each cover slip, 10µl of protein were added, as were 10 µl of the solution from the well the cover slip was going to seal.

The cover slips were then inverted and placed drop-down over the grease; light pressure was applied to ensure the seal was complete. The trays were labeled and stored in a sealed cabinet at room temperature for five days.

2.3. Preparation of trays 3 and 4

A layer of grease was applied to the rims of the wells in both microplates.

2.3.1. Well solution

To all wells, 0.65ml precipitant and 0.35ml buffer were added.

2.3.2. Hanging Drops

For each of the two trays being prepared, the 60mg/ml protein concentration was used in columns 1 and 4, the 50mg/ml was used in columns 2 and 5, and the 40mg/ml was used in columns 3 and 6. In row A the drops were made up of 10µl protein solution and 10µl well solution. The drops in row B were made up of 7.5µl protein solution and 10µl well solution. The drops in row C were made up of 5µl protein solution and 10µl well solution, and the drops in row D were made up of 2.5µl protein solution and 10µl well solution.

The cover slips were then inverted and placed drop-down over the grease; light pressure was applied to ensure the seal was complete. The trays were labeled and stored in a sealed cabinet at room temperature for five days.

2.4. Preparation of trays 5 and 6

A layer of grease was applied to the rims of the wells in both microplates.

2.4.1. Well Solution

To all wells 0.65ml precipitant and 0.35ml buffer were added.

2.4.2. Hanging Drops

For each of the two trays being prepared, the 60mg/ml protein concentration was used in columns 1 and 4, the 50mg/ml was used in columns 2 and 5, and the 40mg/ml was used in columns 3 and 6. In row A the drops were made up of 10µl protein solution and 10µl well solution. The drops in row B were made up of 5µl protein solution and 10µl well solution.

The cover slips were then inverted and placed drop-down over the grease; light pressure was applied to ensure the seal was complete. Tray 5 was labeled and stored in a sealed cabinet at 16.4 degrees Celsius for five days, while tray 6 was in similar conditions for the same amount of time at 4 degrees Celsius.

3. Results

These tables correspond to the wells prepared in the course of this experiment

3.1. Tray 1

	1	2	3	4	5	6
A	Needle clusters, lots of crystals	2 crystals	----------	Needle clusters, lots of crystals	1 crystal	----------
B	1 tiny crystal, lots of needle clusters	----------	----------	1 tiny crystal, lots of needle clusters	----------	----------
C	----------	----------	----------	----------	----------	----------
D	----------	----------	----------	----------	----------	----------

3.2. Tray 2

	1	2	3	4	5	6
A	1 needle cluster, medium amount of crystals	1 crystal	----------	Medium amount of crystals	4 crystals	----------
B	Several needle clusters	----------	----------	Lots of needles	----------	----------
C	----------	----------	----------	Some crystals and needle clusters	----------	----------
D	----------	----------	----------	----------	----------	----------

3.3. Tray 3

	1	2	3	4	5	6
A	Some needle clusters, 29 crystals	6 large crystals	14 medium crystals	17 large crystals and 1 small	9 large crystals	17 medium crystals
B	30 medium crystals	5 medium crystals	9 medium crystals	45 medium crystals	6 medium crystals	15 medium crystals
C	54 medium crystals	1 medium crystal	5 medium crystals	30 medium crystals	3 medium crystals	2 medium crystals
D	34 small crystals	----------	----------	5 small crystals, 1clump	----------	----------

3.4. Tray 4

	1	2	3	4	5	6
A	30+ small crystals	5 large crystals	13 crystals of varied size	40+ small, clumping crystals	1 small, 2 large crystals	11 medium and small crystals
B	20+ small, some clumping crystals	13 medium-large and small crystals	20+ total. Lots of small, clumping on the side, some medium crystals	30+ small and medium crystals	2 large crystals	1 large and 1 medium crystal
C	20+ small-medium crystals	1 small and 2 medium crystals	1 small crystal	30+ small crystals, 3 needle clusters	1 large, 1 medium, and 1 small crystal	6 medium crystals
D	35+ tiny crystals	----------	35+ tiny crystals	----------	----------	----------

3.5. Tray 5

	1	2	3	4	5	6
A	Lots of little crystals	15 crystals total. Some medium and small crystals	20+ small crystals, more spread out	A great deal of small crystals (more than A1)	20 medium crystals	30 crystals total. Medium crystals mixed with small
B	A lot of small crystals (especially on the edge)	8 crystals total. Most are medium, a few are small	15 medium crystals	A lot of small crystals	Large needle cluster, 5 small crystals	20 small crystals

3.6. Tray 6

	1	2	3	4	5	6
A	Hundreds of very tiny crystals	Hundreds of very tiny crystals (a little less than A1, more spaced)	Hundreds of very tiny crystals (a little less than A2, more spaced)	Hundreds of very tiny crystals	Hundreds of very tiny crystals (a little less than A4, more spaced)	Hundreds of very tiny crystals (a little less than A5, more spaced)
B	Hundreds of very tiny crystals	Hundreds of very tiny crystals (a little less than B1, more spaced)	Hundreds of very tiny crystals (a little less than B2, more spaced)	Hundreds of very tiny crystals	Hundreds of very tiny crystals (a little less than B4, more spaced)	Hundreds of very tiny crystals (a little less than B5, more spaced)

4. Discussion

In trays 1 and 2, crystal growth was optimized in columns 2 and 5 which had 0.65ml precipitant and 0.35ml buffer. This was illustrated by the growth of a few large crystals. The ideal protein concentration appeared to be about 50mg/ml.

In trays 3 and 4, protein concentrations of 60mg/ml, 50mg/ml, and 40mg/ml were used. Based on the success of the first tray's results, we attempted to see where in the range of 50mg/ml protein concentration crystals grew best. The ratio of buffer and precipitant concentrations was held constant for the remainder of this procedure at 0.65ml precipitant and 0.35ml buffer. Another goal of these trays was to determine the optimal drop size, which was

found to be 10µl of protein solution and 10µl of well solution. Again it was seen that a protein concentration of 50mg/ml produced optimal crystals.

In trays 5 and 6, the effect of temperature on crystallization was determined. It was found that as temperature decreased, the number of crystals increased and the size of individual crystals decreased. Neither of these trays produced optimal crystals as they were would be too small to analyze using X-Ray diffraction.

After the development of these crystals, the next step is to begin the analysis of their structure using X-Ray diffraction. From this diffraction pattern, the electron density diagram and the actual structure can be determined.

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