Effectiveness of Varying pH Levels and Salt Concentration on Lysozyme Crystallization

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ABSTRACT:

The outcome of applying different salt concentrations and pH levels during the formation of lysozyme crystals were shown through this experiment. Through the hanging drop method, the largest crystals formed in solutions that contained 1M of sodium chloride, or NaCl at a pH of 4.5. Increasing the salt concentration lowered the amount of lysozyme crystals. In addition, more crystal formation occurred in the various solutions that were at a pH of 4.8. However, these crystals were noticeably smaller. Raising the pH results in more crystallization, but noticeably smaller crystals. NaCl was used as the salt of choice and pH was varied using acetate during crystallization. After 3 weeks, lysozyme crystals were observed through a microscope before and after applying Izit dye to the solutions to confirm protein crystal growth.

INTRODUCTION:

Changes in a solution's composition can greatly affect the process of protein crystallization. Salt concentration and pH levels are two examples of components that can affect the formation of a lysozyme crystal (Alderton et al., 1946). By increasing or decreasing salt concentration or pH, the amount of crystallized lysozyme can also be increased or decreased. This knowledge can be used in the laboratory to maximize protein crystal yield. However, it is important to realize that not all salts will interact with protein formation in the same way (Ries-Kautt et al., 1988). Different salts will have different optimal concentrations at which the greatest amount of lysozyme crystals form. This is due to the salt creating a super saturated solution for

the lysozyme protein causing it to aggregate together and crystalize. In the same way that varying salts will interact with lysozyme formation in different ways, pH of different buffer types will have a different optimal pH at which lysozyme formation is the greatest (Hodgson et al., 2008).

Based on previous studies done, if the solution is at a low pH of 4.5 and a salt concentration of 1M, then the greatest amount of lysozyme crystals will be formed. This is due to observations that protein crystal formation begins to retard at pH of 4.89 (Hodgson et al., 2008) and at salt concentrations larger than 1M (Ries-Kautt et al., 1988). A second study also shows that lysozyme crystal growth is greater at a pH of 4.6 than 4.8, but is less at a pH of 4.0 (Judge et al., 1999). These seems to be the conditions of pH level and salt concentration that will produce the largest and most defined lysozyme crystals.

MATERIALS AND METHOD:

The crystallization of lysozyme in different pH levels and concentrations of salt was done by using stock solutions of NaCl and lysozyme. The two stock solutions of NaCl used were at a concentration of 5 M in 0.05 M acetate buffer at a pH of 4.5 and 4.8.The stock solution of lysozyme used was at 100 mg/ml in 0.05 M acetate buffer. In order to create varying salt concentrations the stock solution of NaCl had to be diluted using additional acetate buffer at the same pH as the stock solution being diluted.

To properly calculate the proper amount of stock NaCl solution (X) to use to attain the desired concentration (D), Equation (1) was used. A total of 1.2 ml reservoir solution was to be placed in each well on the crystallization tray.

$$(X) = (D)(1.2\ \text{ml})/(5\ \text{M NaCl}) \qquad \text{Equation (1)}$$

The desired salt concentrations for this experiment are 1 M, 2 M, 3 M, 4 M, 5 M and 0 M as a control group. The amount of NaCl calculated for each concentration were then placed into wells. If a well did not have 1.2 ml of reservoir solution, acetate buffer was added until the reservoir had 1.2 ml solution, therefore completing the dilution process.

To see how different pH levels affected crystal formation, all of the dilutions were performed using stock solutions at a pH of 4.5. A second set of solutions containing the exact same concentrations were then made using stock solutions at a pH of 4.8. In this way, the effect of the difference in pH levels can be observed at several different concentrations. After each well was filled with 1.2 ml reservoir solution, the rims of the well were greased in order to create a seal when the glass cover is placed over the well. This prevents the solutions inside the well from evaporating away.

In order to introduce lysozyme protein to the well, it needed to be placed on a glass coverslip. This is known as the hanging drop method. Since the lysozyme was provided at a concentration of 100 mg/ml and the desired concentration was 50mg/ml, it had to be diluted. This was achieved by placing 4 µl of lysozyme on the coverslip and then adding 4 µl of a reservoir solution to the cover slip. The cover slip was then placed on the respective well solution side down. This creates an environment where external interference with lysozyme crystal formation is kept to a minimal. The crystallization tray was then placed on a table in the lab room at room temperature for 3 weeks.

After the crystallization period was over, the crystals were then observed from the tray through a microscope at 40x magnification. After viewing and recording the sample from each well once, 1 µl of Izit dye was introduced to the sample of each well. This was done in order to help distinguish the difference between a salt crystal and a protein crystal since the protein

crystal will allow the dye to penetrate while the salt crystals do not. This is due to salt crystals packing tighter than protein crystals. After the dying process was completed with all of the samples, the samples were then observed under the microscope again at 40x magnification and recorded. Each sample was recorded by capturing a picture of the sample via digital camera.

RESULTS:

When the samples of lysozyme crystals were observed salt crystals were present in some and made distinguishing the protein crystals from the salt crystals slightly difficult (Fig. 1). In order to better distinguish crystals a dye was used that stained protein crystals blue (Fig. 2).

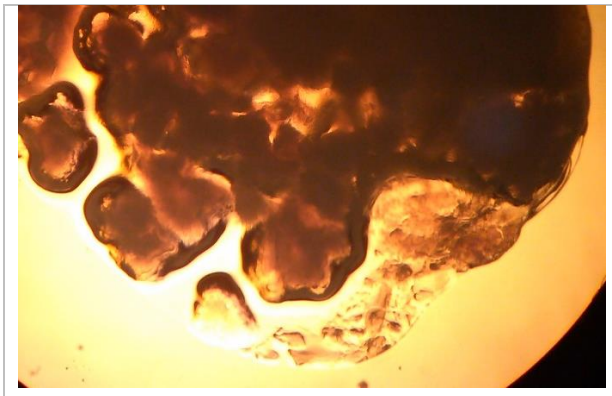

FIGURE 1. Lysozyme crystals and NaCl crystals in 3.0 M NaCl and 4.8 pH solution without Izit dye

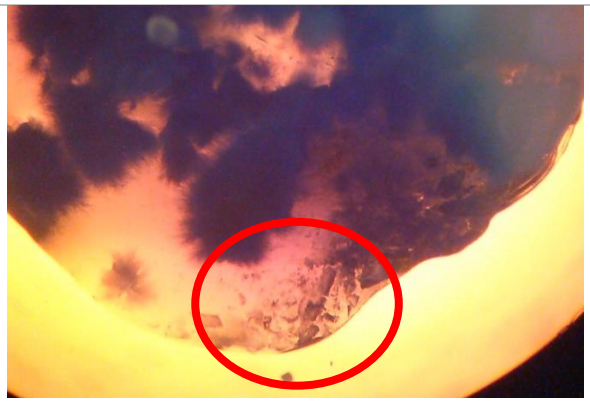

FIGURE 2. Lysozyme crystals and NaCl crystals in 3.0 M NaCl and 4.8 pH solution with Izit dye.

After the dying process it was observed that neither of the control groups, which contained no NaCl, at either pH formed any crystals. As summarized in Table 1 and Table 2, the rest of the wells were observed in order to determine whether lysozyme crystals form in the solution's pH level and salt concentration. At 1.0 M NaCl solution the sample at a pH of 4.5 formed a single, large, well defined lysozyme crystal while the sample at a pH of 4.8 formed two medium sized,

well defined lysozyme crystals. The term large is indicated by Figure 3 and the term medium indicated by Figure 4.

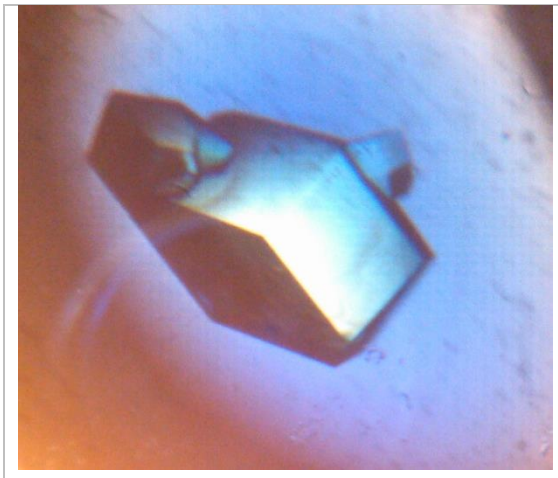

FIGURE 3. Lysozyme crystals in 1.0 M NaCl and 4.5 pH solution without Izit dye

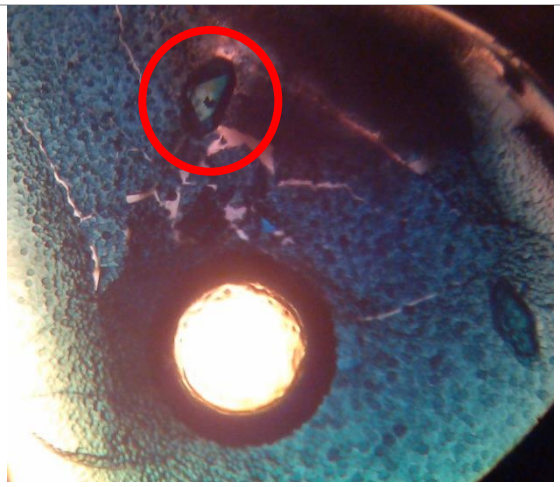

FIGURE 4. Lysozyme crystals and NaCl crystals in 1.0 M NaCl and 4.8 pH solution with Izit dye.

A single, medium sized lysozyme crystal was found in the sample containing 2.0 M NaCl at a pH of 4.5. In contrast, two small protein crystals were found in the sample containing 2.0 M NaCl at a pH of 4.8. The term small is indicated by Figure 2. In the 3.0 M, 4.0 M, and 5.0 M NaCl solutions at a pH of 4.5, no protein crystal formation was found. The 2.0 M NaCl in a pH of 4.8 sample contained two small crystals. The sample in the 3.0 M NaCl at a pH of 4.8 contained many crystals, but they were very small. Only salt crystals could be observed in the 4.0 M NaCl solution at a pH of 4.8. Finally, three small lysozyme crystals were observed in the sample with 5.0 M NaCl at a pH of 4.8.

TABLE 1. Summary of the lysozyme crystallization at each concentration of NaCl at a pH of 4.5.

Lysozyme Crystallization in NaCl at a pH of 4.5		
NaCl Concentration (M)	Crystal Amount	Crystal Size
0.0	No crystals	No crystals

1.0	1 Crystal	Large and Defined
2.0	1 Crystal	Medium
3.0	No crystals	No crystals
4.0	No crystals	No crystals
5.0	No crystals	No crystals

TABLE 2. Summary of the lysozyme crystallization at each concentration of NaCl at a pH of 4.8.

Lysozyme Crystallization in NaCl at a pH of 4.8		
NaCl Concentration (M)	Crystal Amount	Crystal Size
0.0	No crystals	No crystals
1.0	2 Crystals	Medium and defined
2.0	2 Crystals	Small
3.0	Many Crystals	Very small
4.0	No crystal	No crystals
5.0	3 Crystals	Small

In order to calculate the proper amounts of stock 5.0 M NaCl to use for dilution to produce each concentration, Equation (1) was used. The amount calculated was then subtracted from 1.2 ml to find the total amount of stock 0.05M acetate buffer needed to fill up the well. The exact calcuated numbers for the experiment are found in Table 3.

TABLE 3. Summary of calculated values to dilute stock 5.0 M NaCl solution

Amount of 5.0 M NaCl and 0.05 M acetate buffer needed to produce desired salt concentration		
Desired Concentration of NaCl (M)	Amount of NaCl needed (ml)	Amount of Acetate Buffer needed (ml)
0.0	0.0	1.2

1.0	0.24	0.96
2.0	0.48	0.72
3.0	0.72	0.48
4.0	0.96	0.24
5.0	1.2	0.0

DISCUSSION:

As observed in the results, lysozyme crystal formation did occur in several wells containing different concentrations of NaCl at either a pH of 4.5 or 4.8. However, a NaCl concentration of 1.0 M lead to the formation of the largest protein crystal at each pH of 4.5 and 4.8. This result fits both the hypothesis and is similar to the results of a previous study (Alderton et al., 1946). The concentration that lysozyme becomes super saturated enough to aggregate amd form the largest in a NaCl salt solution is about 1.0 M. It is at this point where super saturation of the protein is the greatest without the NaCl becoming super saturated itself and begin hinder lysozyme crystallization. This is observed in this experiment because the samples at 1.0 M NaCl have very little to no salt crystals. Looking at only pH, solutions at a pH of 4.5 had larger crystals than solutions at a pH of 4.8. This also fits in with the results from a previous study a well as the hypothesis (Hodgson et al., 2008). The lower pH of 4.5 produces the larger lysozyme crystals when compared to a sample with a pH of 4.8 at 1.0 M and 2.0 M NaCl, which are the salt concentrations where both samples had crystals. This matches the previous study's findings that crystal formation began to slow down at a pH of 4.8 (Hodgson et al., 2008).

In opposition to crystal size, a higher pH of 4.8 allowed for more crystals to form in a solution, but they were considerably smaller. This can be observed in salt concentrations of 1.0

M (Fig 3., Fig 4.) and 2.0 M. As seen from the results, lysozyme crystals were also able to form in NaCl concentrations that it could not form in while at a pH of 4.5. This can be seen at salt concentrations of 3.0 M and 4.0 M. This was unexpected may have been due to the higher pH, the lysozyme as well as the NaCl had different saturation capacities allowing aggregation of the protein at different NaCl concentrations. The NaCl concentration of 3.0 M at a pH of 4.8 had the greatest number of crystals but they were also the smallest. This unforeseen observation may have been due to the higher concentration of the salt which hindered the protein from aggregating together and crystallizing into fewer, but larger crystals.

Salt concentrations above that of 1.0 M of NaCl all produced large masses of salt crystals. This is due to the solution becoming super saturated relative to the NaCl. Thus, the NaCl begins to aggregate and crystallize out of solution. The salt crystals were easily identified after the dying process because the crystals did not take up any of the dye due to the tight packing of the crystal.

After analyzing the results of this experiment, a good follow up experiment would be to test the effect of temperature on protein crystallization. This parameter could possibly increase the size of crystals formed from the salt concentration of 1.0 M NaCl and the pH level of 4.5 that produced the largest crystal in this experiment. This is due to temperature having a direct effect on salt and protein solubility. While too great of an increase in temperature may denature a protein and cause the protein to become more soluble in the solution, an increase in temperature generally leads to an increase in salt solubility. Perhaps an increase in temperature would decrease lysozyme crystal size while a decrease in temperature would increase the protein crystal.

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