Control of nucleation in the crystallization of lysozyme

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Abstract

This work investigates the influence of storage of lysozyme in solution on its crystallization. The crystallization of hen egg-white lysozyme exhibits a storage effect (aging) that depends on the length of time the lysozyme solution is stored, after dissolving from freeze-dried powder, before being brought to crystallization conditions. The number of crystals obtained increases, while their size decreases, as the solution ages. Observations suggest that this effect is due to the presence of fungi that multiply in the stored protein solution. This aging effect was used to control nucleation and determine the number and size of lysozyme crystals to be formed in a given sample.

Keywords: crystallization; fungi; lysozyme; microorganisms; nucleation

Hen egg-white lysozyme crystallizes beautifully, and with unusual ease, as tetragonal crystals first reported by Alderton and Fevold (1946). For this reason it has been chosen as a model system for many experiments on protein crystallization (e.g., Feher & Kam, 1985; Mikol et al., 1989; Ries-Kautt & Ducruix, 1989; Abergel et al., 1991). In studies on external influences on the crystallization of lysozyme, confusing results were observed. These led to the observations reported here on the influence of the aging (i.e., storage) of lysozyme solutions on the outcome of crystallization experiments.

Results

Effect of aging on the protein solution

Table 1 shows the results of lysozyme crystallization trials (2- μ L drops) comparing samples that were set up immediately after dissolving the protein in buffer (time 0) and after storage of 3.5 and 6 h. This procedure was repeated for various concentrations of protein and sodium chloride.

Over a range of conditions (12–24 mg/mL final concentrations of lysozyme and 60–100 mg/mL NaCl), a larger number of smaller crystals was observed in the crystallization trials of the aged protein solution compared to the

Table 1. *Effects of aging on the number of lysozyme crystals^a*

Concentration of lysozyme (mg/mL)	Age (h)	NaCl concentration (mg/mL)					
		50	60	70	80	90	100
24	0	2	2+ ^b	2++ ^b	2++	Cl ^b	Cl
	3.5	18	31	25	18	14++	Cl
	6	29	45	73	27	Cl	Cl
20	0	2	3	Cl	1++	Cl	Cl
	3.5	13	20	32	32	32+	Cl
	6	20	44	36	51	Cl	Cl
16	0	1	13	15	1++	Cl	Cl
	3.5	1	36	41	21	34	Cl
	6	2	35	26	26	21+	Cl
12	0	0	20	12	4+	Cl	Cl
	3.5	0	26	65	27	20	8++
	6	0	26	20	23	19	Cl

^a Total numbers of single lysozyme crystals in three identical trials are shown. Boxed section indicates the conditions used for all subsequent experiments.

^b Cl, clusters of tiny crystals; +, clusters of tiny crystals in one drop; ++, clusters of tiny crystals in two drops.

number of crystals formed from the fresh solution. The time for this aging effect to express itself was some hours (Table 1). Identical results were observed when performing the experiments manually as 4- μ L drops.

Table 2. *Effects of filtration and centrifugation on lysozyme crystals^a*

Time of aging	Sample									
	Unfiltered		Filtered then aged		Aged then filtered		Filtered then aged in sterile vial		Aged then centrifuged	
	No.	Size (μm)	No.	Size (μm)	No.	Size (μm)	No.	Size (μm)	Top fraction	Bottom fraction
0 h	3-7	400	0				0		0-1	400
6 h	2-6	400	0		0		0		0-2	400
9 h	2-5	400	0-1	400	0		0		0-1	400
24 h	3-4	400	0-2	400	0-1	400	0			
48 h	~20	200	200-400	50	0-1	400	0			~20
7 days	100s	20	200-400	50	0		0-1	400	0-2	400
2 months	1,000s	10	~300	50	0		0			100s

^a Numbers of lysozyme crystals in six identical trials are shown. The numbers represent the minimum and maximum numbers of crystals obtained in any one droplet, with average crystal size indicated. Centrifugation took place for 10 min at $2,000 \times g$.

When this test was repeated a year later, an aging effect was consistently found, but took 48 h or longer to express itself. In subsequent experiments, standard conditions were used such that freshly made-up lysozyme would give around three crystals in each sample measuring approximately $400 \mu\text{m}$ in length, whereas aged lysozyme yielded hundreds or thousands of crystals measuring $10\text{--}20 \mu\text{m}$ (Tables 2, 3). Once the solution had aged to give thousands of crystals under these conditions, it remained in this state even after months of storage.

Protein powders from different manufacturers may vary in their content of salt and small molecule contami-

nants. As these factors would affect crystallization results, the above experiments were repeated using lysozyme powder purchased from ICN Flow. The ICN Flow lysozyme also exhibited aging, similar to lysozyme from Sigma.

Effects of filtration and centrifugation

Experiments were carried out to test whether the aging effect could be prevented by removing particles from the protein solution by means such as filtration, centrifugation, or sterilization of the storage vials.

Table 3. *Effects of additives and desalting on lysozyme crystals^a*

Time of aging	Sample									
	Control (no additive)		+Ampicillin		+Thymol		+Antibiotic/Antimycotic		Desalted by column	
	No.	Size (μm)	No.	Size (μm)	No.	Size (μm)	No.	Size (μm)	No.	Size (μm)
A. Unfiltered samples										
0	0-2	400	0-4	350	1-5	400	0-6	400	1-5	400
24 h	0-2	400	0-1	400	1-4	400	0-1	400	3-6	400
48 h	0-2	400	0-1	250	2-4	400	0-2	400	1,000s	20
72 h	25-30	120	~120	100	1-4	400	0-2	400	1,000s	10
96 h	200	80	~120	100	3-9	400	1-3	400		100s
9 days	1,000s	10	1,000s	20	0-8	400	0-4	400	1,000s	50
B. Samples filtered prior to aging										
0	0-1	500			0		0-1	500	0-2	400
24 h	0-1	500			0		0-2	500	0-1	400
48 h	0-2	500			0		0-2	500	0-4	400
72 h	10-20	250			0-1	400	0-2	500	4-12	300
96 h	80	180			0-1	400	0-2	500		60-80
9 days	100s	20			0-1	400	0		100s	50

^a Numbers of lysozyme crystals in six identical trials are shown. The numbers represent the minimum and maximum numbers of crystals obtained in any one droplet, with average crystal size indicated.

A fresh stock solution of lysozyme was divided into two portions: one portion was stored and the other was filtered through a filter with 300,000 molecular weight cut-off immediately after dissolving and then stored. Some aliquots of the filtered solution were stored in sterile vials. At time 0 (immediately after dissolving) and at hourly and daily intervals, samples for crystallization were taken as follows:

1. From the filtered stock;
2. From the filtered stock stored in a sterile vial;
3. From the unfiltered stock;
4. From the unfiltered stock, centrifuged just prior to initiation of crystallization; and
5. From the unfiltered stock, filtered just before crystallization.

Table 2 shows the results of the crystallization trials with the lysozyme solution treated in various ways and aged for different times. The results show that both the unfiltered solution and that which was filtered prior to storage exhibited an aging effect, manifested as production of a larger number of smaller crystals after 48 h. Solutions that were either filtered or centrifuged just prior to initiation of crystallization showed no aging. However, the bottom fraction of the centrifuged samples gave identical results to the unfiltered samples. No aging was observed with the solution that was filtered and then stored in a sterile vial (even after a period of 2 months); practically no crystals appeared in the drops.

These observations show clearly that the agent causing aging of the protein solution could be removed by filtration or centrifugation. Earlier filtration experiments using 0.22- μ m filters showed a similar but less marked trend, indicating that the agent could pass through these coarser filters.

Effects of desalting and additives

In an attempt to identify the agent responsible for the aging effect, possible contaminants were removed from some aliquots of the stock solution by desalting the protein solutions, while certain additives were added to parallel aliquots.

Table 3 demonstrates the crystallization results of the various solutions sampled at a range of storage times. The solutions containing antifungal agents (thymol and amphotericin B [Merck, 1983]) did not exhibit an aging effect even when those solutions were not filtered (Table 3A). The control solutions and the samples including ampicillin and kanamycin (kanamycin not shown in Table 3) showed aging after 72 h, whereas the desalted solutions hastened aging and showed a more pronounced effect. The corresponding solutions filtered at time zero (Table 3B) displayed very similar results, the only differ-

ence being that the aging effect was lessened in terms of the number of crystals grown.

The results above (Tables 2, 3) demonstrate that the aging effect can be prevented if the solution was treated in any of the following ways:

1. Addition of antifungal agents;
2. Storage of a filtered solution in sterile vials; and
3. Filtration or centrifugation just prior to initiation of crystallization.

Control of nucleation

Having found that under our chosen conditions, filtration of lysozyme solutions immediately prior to crystallization results in hardly any crystal growth, an experiment was carried out on a large scale to find the exact distribution of crystals in samples after filtering. A distribution of the number of crystals in each of 130 drops is shown in Figure 1. The results gave an almost perfect Poisson distribution, with a mean (μ) of 0.30. The crystallization system behaves as though there is a low concentration of nucleation agents in the filtered crystallization samples, such that the mean number of nucleation agents in each sample is 0.3. Assuming the nucleation agents are introduced through the protein solution, their concentration would be 150/mL.

The above experiments show that by aging a protein solution on the one hand or by cleaning it on the other, two extreme situations were observed. One, where nucleation increased to a point at which thousands of crystals appeared, and the other where nucleation was almost eliminated. An experiment was designed to test whether the nucleation (and consequently the number of crystals to be grown in a sample) could be controlled and planned. This was achieved by diluting an aged lysozyme solution that

Fig. 1. Distribution of the number of crystals obtained in 130 samples (4- μ L drops) under standard conditions, using freshly filtered lysozyme. Line shows Poisson distribution with $\mu = 0.30$.

produced thousands of crystals in various proportions with a freshly made filtered solution that would give no crystals. A strong correlation was found between the amount (%) of aged solution present in the sample and the number of crystals grown (Fig. 2). In the presence of 20% aged solution, approximately 800 crystals were observed in each of six samples; at 0.2% there were only 20 crystals.

The size of the crystals was inversely related to the number in each sample. Where 20 crystals had grown they reached 260 μm . Where there were 800, the crystals measured 60 μm in each dimension. In six control experiments that used only fresh, filtered lysozyme solution, there were no crystals in five of the samples, and just one in one sample. In the controls that used only aged lysozyme solution, there were thousands of crystals in all the samples.

Similar results for all the experiments described were obtained when performed by several individual experimenters. Storage of the protein solutions in different refrigerators did not alter the aging effect either.

Summary of results

1. Under the particular conditions of these experiments, growth of lysozyme crystals depends on the presence of a nucleating agent.
2. Aging of lysozyme in solution gives rise to enhanced nucleation.
3. The aging effect is independent of the source of the lysozyme powder.
4. Filtration or centrifugation prior to aging does not eliminate the aging but lessens the number of crystals grown.
5. The agent responsible for the enhanced nucleation can be removed by centrifugation or filtration even after aging has occurred.
6. Desalting increases aging.
7. Aging is prevented by antifungal agents but is not affected by either class of antibiotics.
8. Aging is eliminated when a filtered solution is stored in a sterile vial.
9. Nucleation (and consequently the number of crystals grown) can be controlled by altering the ratio of freshly filtered and aged protein solution just prior to crystallization.

Discussion

The very large number of crystallization trials required to provide the results presented here was made economically possible by the microbatch technique under oil. The results of these trials are highly reproducible. A single small tissue culture plate accommodates 72 trials.

The results showing that addition of antifungal agents (Table 3) totally eliminated the aging suggest growth of

Fig. 2. Numbers of lysozyme crystals observed in crystallization trials (4- μL drops) using mixtures of aged lysozyme solutions with freshly filtered solutions. Each cross represents the mean number of crystals observed in six identical samples. The best straight line (dashed) through the results is shown.

fungi to be the cause of aging. Moreover, antibiotics did not prevent this effect, and storage of the filtered protein solution under sterile conditions stopped almost all nucleation even after months of storage (Table 2). The desalted solutions aged faster possibly because they offered more favorable growth conditions.

This contamination, which we interpret to be fungi, was independent of the source of protein, the experimenter, and the place of storage. It may have occurred because of prior contamination of the materials, because the vessels to which the liquid was transferred were contaminated, or due to air-borne contamination occurring in the laboratory. In different series of experiments, the initial level of contamination varied (compare, for example, the left-hand panel of Table 2 with that of Table 3A).

Apparently, growth of fungi introduced nucleation sites in the protein solutions, thereby generating growth of larger numbers of crystals from these solutions. It was not established which strains of fungi were present, nor whether the same strains were involved in each case. The rate of aging varied, presumably reflecting the multiplication rate of the fungi (e.g., a matter of hours during one year, a matter of days during another).

This aging factor should be taken into account especially with lysozyme, which is commonly used for a wide range of analytical experiments and also in recent microgravity research where launch can take place as late as 72 h after the preparation of samples. In the case of lysozyme, it is possible to exploit this aging phenomenon to control nucleation and consequently the number of lysozyme crystals grown.

In considering the general application of these results, it must be recognized that lysozyme represents a special case. Few proteins crystallize easily after freeze-drying, so the particular method used here to initiate the aging process (simply dissolving the freeze-dried powder) would not normally apply. The experiments do emphasize the importance of centrifugation of a protein solution immediately before initiating crystallization trials, which is recognized to be good practice. Centrifugation is often considered as a method of removing insoluble or denatured protein, but it has a much more important role in removing excess nucleation sites. The experiments also showed that the alternative procedure of filtration through an ordinary 0.22- μ m filter was unsatisfactory, as the nucleation agents were not eliminated fully by these filters.

Heterogeneous nucleation of lysozyme crystals on mineral crystals has been reported (McPherson & Schlichta, 1988). The study reported here suggests that these crystals can also be nucleated by a common microorganism. It is common practice to use antifungal agents in protein crystallization as a matter of routine. This is often done to eliminate the growth of visible aggregates in the nutrient provided by convenient buffers and protein solutions. The experiments emphasize that fungal growth can prevent the growth of large crystals by causing massive nucleation.

The experiments also highlight the possibility that contamination at an appropriately low concentration may have a beneficial effect on protein crystallization experiments by providing nucleation sites. It is significant that even with a protein that crystallizes as readily as lysozyme it is possible to fail to obtain crystals when the conditions eliminate all nucleation sites. Although there is no evidence presented here that such nucleation sites are essential to protein crystal growth under all conditions, the possibility is worth considering. In particular, consider the case where, whenever crystals are obtained, there is a mass of tiny crystals. Perhaps too many nucleation sites are present, and the solution needs more rigorous cleaning. Or, in cases where no nucleation sites are present, the addition of a few fungal spores might give large crystals under conditions that previously gave a clear solution.

Materials and methods

Preparation and storage

Hen egg-white lysozyme was obtained in the form of a freeze-dried powder and used to prepare a solution at 40 mg/mL in 10 mM sodium citrate buffer, pH 4.7. This enzyme stock solution was stored in a closed Eppendorf tube at 4 °C until it was brought to crystallization conditions. Some aliquots of the stock solution were stored in sterile (autoclaved) Eppendorf tubes, also at 4 °C. Lyso-

zyme was obtained from Sigma unless stated otherwise in the text. Sodium chloride, sodium hydroxide, citric acid, and paraffin oil were obtained from BDH Chemicals Ltd., UK, and stored appropriately but not autoclaved.

Crystallization

Aliquots of the same stock solutions were set up for crystallization at different times up to 2 months from the time of dissolving the freeze-dried protein powder into solution. All crystallization trials used the microbatch crystallization technique under oil described by Chayen et al. (1992). Crystallization samples were dispensed and stored under paraffin oil (BDH paraffin liquid, colorless light GPR cat. no. 29436) in 72-well tissue culture plates (Terazaki plates, Sterilin, UK).

First, a survey of crystallization conditions was conducted using sodium chloride as the precipitating agent. Two-microliter drops were set up in triplicate for crystallization by using the automatic dispenser described by Chayen et al. (1990). All subsequent experiments were set up manually, also in microbatch trials, as 4- μ L drops containing final concentrations of 20 mg/mL lysozyme solution and 60 mg/mL NaCl. Six identical drops were set up in each case. Crystallization always took place at 18 °C. Results were assessed 4 days after the initiation of crystallization. The rate of nucleation was indicated by the total number of crystals present in a drop after 4 days. The results reported represent typical values obtained from many repeat experiments.

Filtration and centrifugation

Fresh and stored stock solutions of lysozyme at 40 mg/mL were filtered through a filter designed to exclude particles exceeding 300,000 molecular weight (ultrafree-MC 300,000 NMWL Polysulfone Millipore UK, Ltd.). Aliquots from the original stock solutions were centrifuged in Eppendorf tubes for 10 min at 2,000 $\times$ g, using a small bench centrifuge (Tomy Seiko Co. Ltd., Japan). Separate samples were taken for crystallization from the top and bottom fractions of the Eppendorf tube.

In certain experiments specifically mentioned, 0.22- μ m filters (Millipore UK, Ltd.) were used.

Protein concentrations measured (after filtration or centrifugation) by absorption at 280 nm showed no loss of protein in any of the samples.

Desalting

Prior to storage, the protein solution was desalted by two different methods, one using a Sephadex G-25M desalting column (Pharmacia, Sweden) and the other by concentration of the protein solution in a 10,000 Centricon (Amicon Ltd. G.B.).

Additives

Stock solutions of lysozyme were made, each containing a different additive: (1) 4.5 mM thymol, an antifungal agent (Aldrich Chem. Co.); (2) 100 $\mu\text{g}/\text{mL}$ ampicillin (Sigma), an antibacterial agent effective against gram-positive and gram-negative bacteria; (3) 100 $\mu\text{g}/\text{mL}$ kanamycin (Sigma), an antibacterial agent with a mechanism of action different from that of ampicillin; (4) an antibiotic/antimycotic (Sigma) mixture containing 100 $\mu\text{g}/\text{mL}$ streptomycin, 0.25 $\mu\text{g}/\text{mL}$ amphotericin B, and 100 units of penicillin.

Mixing of fresh and aged lysozyme

A fresh solution of 40 mg/mL lysozyme in buffer was filtered and placed in a sterile Eppendorf tube. Eighty microliters of this fresh filtered solution was mixed with 20 μL of an aged lysozyme solution, thereby contaminating the fresh solution by 20% of aged material. A series of dilutions was done by adding fresh filtered solution to the contaminated one, thus decreasing the proportions of aged to fresh lysozyme solution from 20% down to 0.2%.

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