The effects of filtration on protein nucleation in different growth media

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Abstract

Filtration effects of turkey egg white lysozyme solution (TEWL) prior to subjecting it to crystallization conditions are investigated. Filtering TEWL solution and crystallizing it in ungelled media significantly decreased the number of conditions yielding crystals. This decrease depended on the membrane cut-off used for filtration. From this, the postulated factors aiding in nucleation are estimated to be 0.17 μm in diameter. The existence of these factors was verified by the procedure of reversed filtration: filtered solutions passed through their inverted filter membrane a second time lead to improved crystallization results.

The effect of aging of the TEWL solution prior to subjecting it to ungelled crystallization conditions was also verified. We did not find any time-dependent change in the size or the number of crystals per drop.

Repeating the filtration experiments in agarose-gelled crystallization media showed that the influence of filtration on the crystallization outcome was significantly diminished. Far better crystallization results were obtained compared to ungelled media. However, there is a certain aging effect linked to filtration in gelled media. Different crystallization results were obtained depending on whether filtration was performed before or after aging and subsequent crystallization. This suggests a secondary time-dependent effect.

Keywords: aging; filtration; gel crystallization; heterogeneous nucleation; impurity effects; nucleation, protein crystallization

Thanks to developments in data acquisition, X-ray generation, and computing in general, macromolecular crystallography has made significant progress in the last few years. One domain that has not followed this general trend is macromolecular crystallization, which is increasingly becoming the bottleneck in the determination of three-dimensional structures. The crystallization process is generally considered to depend critically on the purity of the protein solution (Giegé et al., 1994). Yet, it is well-known that protein solutions thought to be homogeneous still contain considerable levels of impurities (Vekilov et al., 1995) and that polyacrylamide gel electrophoresis on dissolved crystals often shows multiple bands. The impurities may stem from conformational heterogeneities, proteolytic fragments of the

original protein, isoforms containing a few replacements in the amino acid sequence, co-purified structurally unrelated proteins, or impurities other than proteins. They may interfere with both crystal growth and nucleation processes (Abergel et al., 1991; Sazaki et al., 1994). Previous findings using hen egg white lysozyme have also shown the influence of the aging of protein solutions prior to their crystallization (Chayen et al., 1993).

We have studied the incidence of filtration and aging of a protein solution on its crystallization using turkey egg white lysozyme as a model system. We have addressed these questions in terms of the number and size of resulting crystals. The filter membranes cut-offs used here correspond to the ranges routinely used in protein purification. Initially, we checked the influence aging had on our TEWL model system. For the sake of comparison, we applied the pretreatments already described by Chayen et al. (1993) for HEWL solutions to our TEWL solutions. This allowed us to assess the role filtration plays in the nucleation process.

Crystallization from gelled media does not only modify the growth process; it also influences the nucleation rates (Robert

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Abbreviations: TEWL, turkey egg white lysozyme; HEWL, hen egg white lysozyme; mwco, molecular weight cut-off of filter membranes; %cryst, successful crystallization scores; OD, optical density.

et al., 1994). We carried out the crystallization experiments both in ungelled and gelled media in order to determine whether the effects of aging and filtration were different in the two media. Agarose was used throughout as the gelling agent.

Results

Effects of aging and filtration

All experiments described here were performed using optimized TEWL crystallization conditions (see the Materials and methods). These conditions typically yield 1–5 well-shaped crystals of up to 1 mm in length. Occasional appearance of polycrystalline material could neither be suppressed by filtration through a 10-kDa molecular weight cut-off filter membrane nor by dialysis of the commercial-grade TEWL solution. For the aging experiments, the solution was subjected to the following treatments prior to crystallization: solution I, aged without filtration; solution II, first filtered, then aged; solution III, first aged, then filtered.

Table 1 shows the results of the crystallization of unfiltered TEWL solution in ungelled media after different aging periods. Our results indicate that aging had no systematic effect on the number or size of crystals obtained (not shown). Neither did the crystallization trials carried out using solutions II and III nor those carried out under gelled crystallization conditions show any evolution with time. On the other hand, large differences in the crystallization outcome were observed when comparing results from different kinds of pretreatments and crystallization media. Certain conditions suppressed the growth of well-shaped crystals altogether, producing either bundles of microcrystals or no crystals at all. Other conditions had no effect on the size, number, or habit of the TEWL crystals. From these results, we concluded that a different kind of evaluation was needed. We found that “%cryst,” the ratio between the number of crystallization trials giving rise to crystals and the total number of trials set up using the same conditions was a good indicator:

$$\% \text{cryst} = \frac{\# \text{ trials (crystals)}}{\left(\# \text{ trials (crystals)} + \# \text{ trials (microcrystals)} + \# \text{ trials (empty drops)} \right)}$$

Results of this reevaluation are shown in Table 2. Examination of experiments conducted in ungelled medium indicates a sharp decline in the crystallization scores of filtered solutions, no matter how long the solution has been aged. Both initial and final filtration of aged solutions reduced crystallization scores significantly as compared to unfiltered solutions.

Table 1. Average number of TEWL crystals per drop grown from unfiltered TEWL solution in ungelled crystallization media after different aging periods^a

Time of aging	0 h	9 h	1 day	1 week	1 month
Ave. number of crystal/drop	1.5	1.7	0.8	1.2	1.0

^a Sixfold set-up, threefold for 1 month's aging. Results change very little with aging time.

Table 2. Successful crystallization scores (%cryst) of differently filtered and aged TEWL solutions after various aging periods^a

TEWL pre-treatment	Aging time of the TEWL solution				
	0 h	9 h	1 day	1 week	1 month
A. %Cryst in ungelled crystallization media					
No filtration	71	100	83	83	100
First filtered, then aged	0 ^b	17	0	17	38
First aged, then filtered	0 ^b	0	17	43	0
B. %Cryst in gelled crystallization media (0.4% (w/v) gel)					
No filtration	100	100	100	100	100
First filtered, then aged	100 ^c	100	100	100	100
First aged, then filtered	100 ^c	67	33	0	67

^a 300-kDa filtration cut-off, corresponding to 0.0089 μm diameter. No direct aging effect exists for any of the applied pre-treatments. Part A shows the drop in %cryst for both filtered solutions compared to unfiltered solutions. Part B shows maximum %cryst for unfiltered as well as for first-filtered-then-aged solution, but lower %cryst for first-aged-then-filtered solutions.

^{b,c} Conducted under identical conditions (see the Materials and methods).

Optimized crystallization conditions for TEWL in gelled medium resulted in a slightly larger number of crystals (3–8, longest dimensions of 0.5–0.7 mm), displaying an even more regular habit than crystals grown in ungelled media. Gel growth also minimized the occasional growth of polycrystalline material found in ungelled media. Gel crystallization of TEWL solutions that were initially filtered and then aged gave maximal crystallization scores (Table 2B). This implies that filtration followed by aging no longer inhibited crystallization as it did in the case of ungelled media. Filtration after aging, on the other hand, still gave lower crystallization scores. They also showed strong fluctuations with the aging time (Table 2B).

Reverse filtration and filtration at various cut-offs

The results reported above indicate the presence of two effects. Firstly, an effect of filtration sharply reducing crystallization in ungelled solutions and, secondly, an effect of gelled media that partially compensates for the first effect. Both of these effects concern the nucleation process. They either lead to well-shaped crystals or to no crystals at all.

Because the drop in crystallization scores of filtered TEWL solutions could have been due to an artefact introduced by the filtration procedure, the observed effects had to be verified. The different effects could not be attributed to a change in protein concentration because OD measurements carried out before and after filtration were essentially constant. We concluded that the most likely explanation was that some crystallization-promoting elements were retained by the filter. In order to test this possibility, the filtered protein solution was passed through the same filter membrane used previously, but now inverted (see the Materials and methods), and subjected to standard ungelled crystallization conditions. With these reverse-filtered protein solutions it was actually possible to attain crystallization levels close to those obtained with unfiltered solutions (Table 3): unfiltered

Table 3. Successful crystallization scores (%cryst) of filtered and reverse-filtered TEWL solutions (see text) at different membrane cut-offs, crystallized in gelled and ungelled media^a

TEWL pre-treatment and cryst. media	Filter membrane cut-off [μm]				
	0.0089	0.0133	0.1	0.22	0.45
Filtered, ungelled	29	33	43	67	71
Reverse filtered, ungelled	63	—	71	86	—
Filtered, gelled	100	100	100	100	—
Reverse filtered, gelled	100	—	100	100	—

^a Corresponding %cryst score of unfiltered TEWL in ungelled media is 83%. Membrane cut-off conversion, 0.0089 μm , corresponding to 300 kDa mol; weight cut-off, 0.013 μm to 1,000 kDa mwco; —, experiment not carried out.

TEWL solutions yielded crystallization scores of 83%. After filtration through a 300-kDa cut-off membrane, this score dropped to 29%. It rose up to 63% upon reverse filtration. This shows that the filter had retained moieties responsible for the heterogeneous nucleation of TEWL crystals and that it is possible to recover these nucleation factors by reverse filtration. Filtration using membranes with higher molecular weight cut-offs also reduced crystallization scores, but to a lesser extent. In this case, crystallization scores could also be brought back to approximately pre-filtration levels by applying reverse filtration (Table 3). When plotting these results on a logarithmic scale, a sigmoidal curve is obtained (Fig. 1). The point of inflection indicates that the diameter of the mean nucleation factors is approximately 0.17 μm . Under the conditions applied here, these factors account for approximately half of the TEWL nucleation occurring in unfiltered solutions.

Fig. 1. Successful crystallization scores (%cryst) of TEWL solutions filtered at different cut-offs and crystallized in ungelled (circles) and gelled (triangles) media. %Cryst of crystallization after standard filtration are marked with open symbols; those after reverse filtration (see text) are marked with solid symbols (data shown in Table 3). The arrow marking the point of inflection indicates the predominant size of the nucleation factors to be about 0.17 μm . %Cryst for unfiltered TEWL solution in ungelled media is 83%. Reverse filtration increases %cryst of filtered solution to almost pre-filtration levels.

The same experiments of filtration and reverse filtration at different cut-offs were repeated using gelled crystallization media. They all resulted in maximum crystallization scores, regardless of the applied type of filtration or membrane cut-off (Table 3; Fig. 1). This indicates the same compensating effect of gels already found in the experiments involving aging and filtration.

In order to see whether the efficiency of the nucleation factors could even further increase the crystallization score of unfiltered TEWL solutions beyond their 83% value, the following experiment was performed. A freshly prepared TEWL solution was filtered through a 300-kDa cut-off filter; subsequently, another batch of unfiltered solution was passed through the same, inverted, filter. This solution now contained its inherent amount of nucleation factors plus the amount retained in the filter from the first solution. However, its crystallization score did not show any deviation from those obtained from standard unfiltered solutions (not shown). Neither the number of resulting crystals nor the overall crystallization score showed an increase.

We then investigated the compensating effect of gelled crystallization media on filtered TEWL solutions. This was accomplished by the crystallization of 300-kDa cut-off membrane-filtered TEWL solutions in gelled media of different gel concentrations. As can be seen in Figure 2, the crystallization score dropped as the gel concentration was lowered. The hyperbolic behavior of the curve indicates that there is no threshold value, as opposed to what was observed for the case of the filtration effect.

Discussion

We have not been able to observe a direct effect of TEWL solution aging in the results of numerous crystallization trials. This is true for ungelled as well as gelled media. Consequently, an effect of aging on the number and size of crystals, similar to the one found for HEWL crystallized under mineral oil (Chayen et al., 1993), can be excluded for TEWL under the conditions used here. Neither did we observe an effect of filtration on resulting crystal sizes or numbers, no matter which membrane cut-off was used for filtration. This agrees with results from Skouri

Fig. 2. Successful crystallization scores (%cryst) of filtered TEWL solutions (300 kDa cut-off) in gelled crystallization media of different concentrations. %Cryst drops continuously with decreasing gel concentration.

et al. (1995), who, after filtering an uncontaminated HEWL solution using a 0.22- μm cut-off, could not detect any significant changes in terms of crystal numbers or size.

Yet filtration reduced the crystallization score in ungelled crystallization media. This is valid for solutions that were filtered either before or after aging. These results suggested the existence of nucleation-promoting species retained by filter membranes that are invariant with time. Reversed filtration of filtered solutions increased crystallization scores to almost pre-filtration levels, confirming the existence of such nucleation factors. Exposing previously unfiltered solutions to these factors did not increase the crystallization score beyond its inherent level. Similar results were obtained when using different membrane cut-offs. From our results it can be concluded that these nucleation factors are more than 60 times larger in diameter than monomeric TEWL. Protein aggregates of up to 5,000 Å in diameter have been described in pre-crystallization conditions (Skouri et al., 1991; Sazaki et al., 1994), yet their existence is unlikely under the conditions used here because the protein solutions were filtered in the absence of precipitating agents, being dissolved in water only. The nucleation factors are most likely not structurally related to TEWL. They may be considered as dissimilar impurities of unknown nature, aiding in nucleation, possibly through a heterogeneous mechanism.

A first effect observed for gelled media is a general increase in unfiltered TEWL solutions crystallization scores. In addition, gelled media compensates for the absence of nucleation factors removed by filtration. This is true for TEWL solutions that were either not aged at all or aged after filtration. Because TEWL solutions that were first aged and then filtered showed lower crystallization scores, there must be a secondary, time-dependent, aging effect on crystal nucleation in gelled media. Because initial filtration had no effect on nucleation, filtration after aging must be retaining some species. These species were able to cross the membrane in the case of freshly prepared solutions but may have aggregated with time, leading to their retention by filtration after aging. Because this effect is only observed in gelled media, it must be somehow related to this crystallization environment.

We have observed that the crystallization scores of filtered TEWL solutions drop exponentially as a function of decreasing gel concentration. This shows that there is no threshold value of gel concentration beyond which total compensation of missing nucleation factors occurs (resulting in maximum crystallization scores). It has to be kept in mind, however, that the decrease in gel concentration of gelled crystallization conditions does not lead directly to the equivalent ungelled crystallization conditions due to differences in optimized crystallization conditions.

Conclusion

The impurities detected here play a decisive role in the nucleation process without influencing crystal characteristics such as size or morphology. If the nucleation results of TEWL are applicable to other proteins, then extensive protein purification prior to crystallization may not be essential, or even desirable. Furthermore, crystallization in agarose gel seems to be more advantageous than crystallization in solution, because the former reduces the influence of nucleation factors. A better knowledge of the role of contaminants in the crucial nucleation process may

be decisive in establishing rational approaches to successful protein crystallization protocols.

Materials and methods

Optimizing TEWL crystallizing conditions

Known crystallization conditions for TEWL in ungelled solution were initially used (Bott & Sarma, 1976; Abergel et al., 1991) and further optimized to give a smaller number of well-developed crystals under the following conditions: vapor-diffusion method using sitting-drop insets (DROP S.A. 38160 St. Marcellin, France) in Linbro plates with drops of 2 μL of commercial-grade TEWL (Sigma, crystallized, dialyzed, and lyophilized) at 30 mg/mL and 2 μL precipitant solution (2.40 M NaCl, 0.1 M HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid], Fluka BioChimica), 15 mM NaN_3) at pH 5.8, a reservoir volume of 1 mL of precipitant solution at 20 °C.

For TEWL crystallization in gel, low-gelling temperature Seaplaque agarose (Fluka BioChimica) was chosen. As opposed to silica gels, it does not release byproducts during solidification. Its low-temperature gelling point minimizes thermal stress on the protein. The gel crystallization conditions were separately optimized, taking the conditions described above for ungelled media as a starting point. A 1.5 % (w/v) aqueous solution of the agarose gel powder was boiled in a sealed Eppendorf tube for 1 h. After cooling to 50 °C, one part of it was mixed with three parts of the above described precipitant solution and one part water and maintained at 45 °C. The droplet now consisted of 2 μL TEWL at 30 mg/mL and 4 μL of the gel-precipitant-water solution. All other parameters of the crystallization conditions were the same as for ungelled conditions. The gel-precipitant-water solution was maintained in an Eppendorf tube at 45 °C. The protein solution was pipetted onto the sitting-drop inset as before. The gel-precipitant-water solution was taken from the Eppendorf and pipetted onto the protein droplet. All operations were performed at room temperature. The Linbro plates were stored at 20 °C.

Aging and filtration of the TEWL solutions

Aging of the differently pre-treated TEWL solutions was carried out in sealed Eppendorf tubes at room temperature under laboratory light exposure. The protein was dissolved in doubly distilled water without any further additives. TEWL solutions were filtered on a bench centrifuge at 2,000 $\times g$ through the following filter membranes: 0.0089 μm , Millipore Ultrafree MC 300 kDa; 0.0133 μm , Filtron Microsep 1,000 kDa; 0.1 μm , Millipore Ultrafree MC 0.1 μm ; 0.22 μm , Millipore Ultrafree MC 0.22 μm ; 0.45 μm , Millipore Ultrafree MC 0.45 μm .

Membrane cut-offs given in pore diameters (d , [cm]) were correlated to molecular weight cut-offs ([g/mol]) assuming a protein density of 1.35 g/cm³ and a spherical molecular shape:

$$\text{mwco} = (d/2)^3 * 4/3 * \pi * N_A * 1.35 \text{ g/cm}^3,$$

with $N_A = 6.022 * 10^{23}$ 1/mol.

Optical density measurements of the filtered solutions at $\lambda = 280$ nm were conducted in all cases to assure that no change in concentration had taken place. Reverse filtration was always car-

ried out immediately after normal filtration of the protein solution. The filter was removed, its upper collar cut off to fit into an Eppendorf tube, into which it was re-introduced upside down without any delay. The normally filtered protein solution was added in aliquots of 10 mL onto the reversed filter membrane and centrifuged as before. Reverse filtration through the 1,000 kDa cut-off membrane with the small amounts of protein used in these essays was impossible due to the large size of the Microsep filters. Aging conditions at $t = 0$ h of solutions II and III actually describe identical situations: both crystallization conditions were set up immediately after filtration (Table 3). All aging and filtration tests were carried out six times, except for aging experiments in gels, which were carried out three times.

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