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Noncovalent Polymerization of Mesogens Crystallizes Lysozyme: Correlation between Nonamphiphilic Lyotropic Liquid Crystal Phase and Protein Crystal Formation

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Abstract

Crystallization of proteins is important for fundamental studies and biopharmaceutical development but remains largely an empirical science. Here, we report the use of organic salts that can form a class of unusual non-amphiphilic lyotropic liquid crystals to crystallize the protein lysozyme. Certain non-amphiphilic organic molecules with fused aromatic rings and two charges can assemble into stable thread-like noncovalent polymers that may further form liquid crystal phases in water, traditionally termed chromonic liquid crystals. Using five of these mesogenic molecules as additives to induce protein crystallization, we discover that molecules that can form liquid crystal phases in water are highly effective at inducing the crystal formation of lysozyme, even at concentrations significantly lower than that required for forming liquid crystal phases. This result reveals an example of inducing protein crystallization by the molecular assembly of the additives, and is consistent with a new mechanism by which the strong hydration of an assembly process provides a gradual means to compete for the water molecules to enable solvated proteins to form crystals.

INTRODUCTION

While many new methods for efficient protein crystallizations have been developed,[1–4] the science of protein crystallization is still largely empirical.[5–9] The mechanisms of how organic and inorganic additives promote protein crystallization are still under intense study.[10,11] For instance, the concept of depletion interactions has been applied to the system for using poly(ethylene glycol) (PEG) to crystallize proteins.[10,12,13] However, direct interaction between polymer additives and proteins has been suggested to cause different size and shape of the protein crystals.[11] Most of the additives used for inducing protein crystallization, such as PEG or sodium chloride, do not self-associate in aqueous solutions, and the individual additive molecule sequesters water molecules on its own. In fact, PEG is known to repel itself.[14,15] Here, we report the use of a class of nonamphiphilic organic molecules that can form liquid crystal phases in water to induce the crystallization of proteins. These nonamphiphilic organic molecules consist of fused aromatic rings with two charges, and may form stable noncovalent thread assemblies that result in an unusual nonamphiphilic lyotropic liquid crystal – traditionally termed chromonic liquid crystals. [16–21] We discover that there is a strong correlation between whether a molecule

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Supporting Information Experimental details and crystal structure of lysozyme induced by 5′DSCG. This material is available free of charge via the Internet at http://pubs.acs.org.

forms the liquid crystal phase and the ability for that molecule to induce protein crystallization. These results suggest that the assembly process of molecules in water can be used to induce the crystallization of proteins. New mechanisms involved in this approach for protein crystallization are also discussed.

Molecular crowding effects are recognized to promote a wide range of assembly phenomena including the formation of liquid crystal phases,[22] protein denaturation and aggregation,[23,24] and are also considered to be involved in protein crystallization.[25,26] For instance, crowding and confinement can increase the stability of folded proteins.[25,26] However, whether the protein is stabilized or destabilized in a crowded environment also depends on whether the additives are kosmotropic or chaotropic in nature. The mechanism of how a kosmotropic additive stabilizes or a chaotropic additive destabilizes protein structures is still a subject of active study[27] since its discovery in the late 1800s.[5,28–30] Interestingly, some of the additives used for protein crystallization are actually chaotropic in nature and, at high concentrations, tend to destabilize folded proteins.[5,27,30–32] These phenomena make it more challenging for achieving a rational understanding that may converge to predictive guidelines or theories. Folded proteins, in general, do not have well-separated large areas of hydrophilic and hydrophobic regions on their surfaces, and thus are not amphiphilic in nature. Therefore, protein crystallization can be considered as a nonamphiphilic assembly process that involves the association of protein components with exclusion of large amounts of water molecules and solvated electrolytes, and perhaps some impurities.

In addition to the conventional thermotropic and lyotropic liquid crystals, there is a class of water-soluble nonamphiphilic molecules with fused aromatic rings that also form liquid crystal phases when dissolved in water at certain concentrations. This class of nonamphiphilic lyotropic liquid crystals, traditionally termed chromonic liquid crystals, consists of two types of organic molecules. One type includes molecules with two charged chromonyl groups connected by a water-soluble linker.[16–21] The other type includes charged organic dye molecules.[33–36] While the assembly structure of this unusual class of lyotropic mesogens has been thought to be of H-stacking,[19,37] we recently discovered that dichromonyl molecules in this liquid crystal form noncovalent polymers with linear thread structures that are connected by salt bridges stabilized by aromatic groups in close promixity (Figure 1).[38] These noncovalent polymers exhibit phase separation with many types of covalent polymers when mixed in water.[16,18] In an earlier study, Abbott and co-workers discovered that the specific binding of surface-bound human immunoglobulins (IgG) to antibodies of human IgG was retained even when the anti-human IgG was dissolved in this type of liquid crystal phases.[39] Together, these works suggest that nonamphiphilic lyotropic liquid crystals may also exhibit phase separation with biomacromolecules, and do not denature proteins.

Prompted by the above-mentioned knowledge, we explore in this work the use of mesogens that may or may not form nonamphilphilic lyotropic liquid crystals as additives to induce the crystallization of the protein lysozyme. Here, we used a series of structurally similar dichromonyl molecules that consist of two charged chromonic groups tethered by different linkers.[20] While bearing two charges, these molecules exhibited a wide range of solubility in water, and only one out of the twelve new molecules can form nonamphiphilic lyotropic liquid crystals.[20] This work examined four from this class of molecules (5′DSCG, 5′DSCG-diviol, 5′DSCG-EG2 and 5′DSCG-diglycerol), a dye-based molecule (Sunset Yellow dye), and a mono-charged molecule (5′MSCG) as additives for their ability to induce protein crystallization (Figure 1). While aqueous solutions of 5′DSCG, 5′DSCG-diviol and Sunset Yellow dye form liquid crystal phases, the others do not. Thus, we also examined the correlation between the existence of liquid crystal phase and the ability to induce protein crystallization.

EXPERIMENTAL

Materials and methods

Disodium cromoglycate (5′DSCG) was purchased from MP Biomedicals (Solon, OH), Lysozyme and sodium acetate was purchased from Sigma (St. Louis, MO), VDX48 Plate with sealant, and 12mm × 0.22mm Siliconized circle cover slides were purchased from Hampton Research (Aliso Viejo, CA), and Sodium Chloride (NaCl) was obtained from Fisher Scientific (Fairlawn, NJ). Monosodium cromoglycate (5′MSCG), 5′DSCG-EG2, 5′DSCG-diviol were synthesized previously.[20] Samples were viewed under an Olympus BX51 Polarizing Microscope (without polarizer) while images were taken using Olympus C-5060 Zoom digital camera.

Crystallization of lysozyme

Lysozyme from chicken egg white (M.W. = 14,307 Da) was crystallized via vapor diffusion methods as hanging drops (~5 μL) in 48 well VDX plates (Hamilton Research, Aliso Viejo, CA) with sealant. All solutions were prepared using 50 mM sodium acetate (pH 4.59) at 25°C. A stock solution of lysozyme (75 mg/mL) was prepared and syringe filtered (0.22 μm). Five reservoir solutions that included sodium chloride (958 mM, 10.93 mM), 5′DSCG (5.48 mM), 5′DSCG-EG2 (5.34 mM), 5′MSCG (10.53 mM), and 5′DSCG-diviol (5.47 mM) were separately prepared and filtered.

Equal volumes (2.5 μL for each solution) of the filtered lysozyme and the reservoir solution were mixed in an Eppendorf tube. The solution consisting of lysozyme and reservoir solution was suspended on a siliconized cover slip as an inverted drop over a well containing 350 μL of the corresponding reservoir solution. Crystals were grown in for a period of 2–4 d at ambient conditions, and were observed under a polarizing light microscope.

Data Collection

Data were collected at the Cornell High Energy Synchrotron (Ithaca, NY) using beamline F1 with a wavelength of 0.917 Å resolution. Images were processed using HKL-2000 for integration and scaling[40] and refined using rigid-body refinement and restrained coordinate and B-factor refinement using REFMAC 5.4.[41] We refined using data from 25.5 – 1.15 Å and with highest resolution shell 1.17 – 1.15 Å. The structure determined by molecular replacement was carried out using MOLREP.[42] Using a previously reported hen egg-white lysozyme as a model (2W1Y.pdb)[43] pictures of the crystal structure were prepared with Coot[44] and PyMOL (http://www.pymol.org).

RESULTS AND DISCUSSION

The four dichromonyl molecules are chosen because of their water-solubility, and their differences in the ability to form liquid crystals.[20] Molecule 5′DSCG forms liquid crystals at about 11–12 wt% in water at ambient temperature. Molecule 5′DSCG-diviol, consisting of a mixture of 48 mole % meso compounds and 52 mole % racemic mixture, forms liquid crystal phase at 15–18 wt% in water below the ambient temperature (15–18 °C). Molecule Sunset yellow (SSY) dye forms liquid crystal phase at 30 wt % in water at ambient temperature. The synthesis of 5′DSCG-diviol, 5′DSCG-EG2, 5′DSCG-diglycerol, and monosodium cromoglycate was reported in an earlier work.[20]

Lysozyme is a protein that can be readily crystallized, for which the effects of additives are well studied,[45,46] and thus provides a model system to decipher the effect of systematic structural change of the additives on protein crystallization. To study the effectiveness of the organic salts listed in Figure 1 at crystallizing lysozyme, we used the hanging drop

methods.[45–47] Equal volume of stock solutions containing additives and lysozyme in 50 mM sodium acetate buffer (pH 4.6) were mixed to make drops (~3–5 μL) containing 37.5 mg/mL of lysozyme and various concentrations of the additive. The drops, dispensed on a cover slip, were suspended over a reservoir solution (350 μL) containing twice the concentration of the additive in the same buffer. Sealing the cover slip over the reservoir with grease, the water content in the hanging drop was allowed to approach equilibrium with that of the reservoir, and the crystallization was monitored over time.

Molecule 5′DSCG can be precipitated in the presence of lyoszyme at acidic conditions (< pH 4.59). Started at concentrations around 5.6 wt% (~109 mM) of 5′DSCG in the buffer, we observed precipitation of 5′DSCG (confirmed using 1H NMR) upon addition of an equal volume of lysozyme solution (75 mg/mL). We reduced the concentration of 5′DSCG until no precipitate was observed when lysozyme solution was introduced. We found that using a stock solution of 5′DSCG at 0.28 wt% (or lower), which corresponds to 0.14 wt% (~2.74 mM) in the drop, did not cause precipitation when lysozyme solution was added. After about 72 h, lysozyme crystals were observed in the drops containing 5′DSCG (Figure 2A). These crystals were relatively small (61.3±8.2 μm × 233±22.9 μm) and of tetragonal shape. The control using NaCl (~5.5 mM), which doubles the concentration as that of 5′DSCG to match the ionic strength, did not induce crystallization of lysozyme. Only when the concentration of NaCl was increased to ~479 mM did lysozyme crystals (273.0±21.2 μm × 308.5±6.4 μm) appear (See supporting information). Comparing to using NaCl (~479 mM) to crystallize lysozyme, only low concentration of 5′DSCG (~2.74 mM) was required. We note that in a 10-μL hanging drop containing ~2.74 mM of 5′DSCG, the number of 5′DSCG molecules (2.74×10^{-8} moles) is comparable to the number of lysozyme proteins (2.62×10^{-8} moles) in the drops.

The needle-like crystals of lysozyme induced by 5′DSCG alone (Figure 2A), however, did not give sufficient X-ray diffraction for a structure determination. To obtain a single, diffraction quality crystal, we further *reduced* the concentration of 5′DSCG from ~2.74 mM to ~1.37 mM and added relatively small amount of sodium chloride (~128 mM) (Figure 2C). This mixture provided large tetragonal crystals (Figure 2C) of lysozyme that afforded full X-ray diffraction (h,k,l = 68, 48, 32) with 100% homology to the lysozyme structure reported in the literature.[43] This high homology indicates that the presence of 5′DSCG does not affect the structure of the lysozyme and is not included in the crystals, but with the assistance of other additives promotes effectively the formation of compact and well-ordered protein crystals. We note that using the same concentration (~128 mM) of sodium chloride alone without 5′DSCG did not result in lysozyme crystallization.

The effectiveness of 5′DSCG at inducing lysozyme crystallization enables a systematic study of the effect of the additives' structure on protein crystallization. We examined 5′DSCG-diviol, 5′DSCG-EG_2, 5′DSCG-diglycerol, Sunset Yellow dye, as well as 5′MSCG, as additives to crystallize lysozyme. In contrast to 5′DSCG, ~2.7 mM of 5′DSCG-diviol caused precipitation when mixed with lysozyme (Fig. 3A). When the concentration of 5′DSCG-diviol was reduced by half to ~1.37 mM, needle-like lysozyme crystals were observed after ~96 h (Fig. 3B). For other molecules (5′DSCG-EG_2, 5′DSCG-diglycerol, Sunset yellow dye, and 5′MSCG), either precipitate or isotropic solution was obtained in the broad concentration range studied. At 2.67 wt%, 5′DSCG-EG_2 caused precipitation. Molecules 5′DSCG-diglycerol, Sunset yellow dye, and 5′MSCG did not induce the formation of crystals or precipitates over these concentrations (5.26 wt% or lower). Further increase in the concentrations of these additives caused precipitation.

Table 1 summarizes the properties of the molecules studied and their abilities to induce lysozyme crystallization. We observed that, except for Sunset Yellow dye, molecules

5′DSCG and 5′DSCG-diviol that can form nonamphiphilic lyotropic liquid crystal phases in water crystalized lysozyme. Molecules that do not form liquid crystals (5′DSCG-EG_2, 5′DSCG-diglycerol, 5′MSCG) do not induce protein crystallization. This strong correlation between liquid crystal formation and protein crystallization support the notion that the process of thread assembly of dichromonyl molecules involves a water-sequestering process that promotes the crystallization of solvated proteins.

The protein crystallization induced by these nonamphiphilic lyotropic mesogens appears to be enabled by two characteristics of the system. *First*, the assembled noncovalent polymer of the nonamphiphilic molecules does not have strong interactions with protein structure, and does not mix well with proteins at a molecular level. We believe that this thermodynamic incompatibility is due to the strong affinity for self-assembly by the nonamphiphilic mesogens. It is well known that, when mixed in a solution, different polymers,[48–50] including proteins,[51–56] can phase separate into volumes enriched with each component. However, this phase separation does not necessarily lead to the crystallization of either component. In fact, precipitates are often observed when the concentration of either (or both) component is high. *Second*, the noncovalent polymerization of nonamphiphilic lyotropic mesogens requires the strong hydration of the assembled noncovalent polymers, which provide a slow means to sequester water molecules in the bulk solution.

We studied the phase separation between 5′DSCG and lysozyme at high concentration of 5′DSCG, for which the readily observable liquid crystal phase provided a means to observe the phase separation. We found that liquid crystal phase of 5′DSCG readily phase separate with the proteins resulting in a mixture of liquid crystal and isotropic phases in a solution (see supporting information). The initial concentrations of 5′DSCG (0.25 wt %) and 5′DSCG-diviol (0.07 wt %) that are capable of inducing lysozyme crystallization, however, are significantly lower than that required for the formation of liquid crystal phase (~12 wt% for 5′DSCG and ~18 wt% for 5′DSCG-diviol). However, the concentrations of additives when proteins crystallize were likely higher than the initial concentrations due to the vapor transfer from the hanging droplets to the reservoir. Nevertheless, the assembly of molecules in this class of nonamphiphilic lyotropic liquid crystal is believed to occur by an “isodesmic” process, for which all molecules are in equilibrium with monomers, dimers, trimers,…, oligomers, and polymers, and are governed by the same equilibrium constant, K.[57–59] The concentrations of monomers (c_1) and any n-mers (c_n) at equilibrium are characterized by $c_n = K^{n-1}c_1^n$; $c_o = \sum_{n=1}^{\infty} nK^{n-1}(c_1)^n = \frac{c_1}{(1 - Kc_1)^2}$, where c_0 is the total concentration of the molecules.[57,58] This isodesmic assembly is fundamentally different than the assembly by a micelle formation in that the isodesmic assembly does not have a critical concentration for assembly. Early studies suggested that 5′DSCG in this assembly has a strong hydration shell. For example, for a 10 wt% solution of 5′DSCG, about 260 water molecules are associated with each 5′DSCG.[60] Because the molecules are in close contact with each other in the thread assembly, the whole oligomer or thread assembly is heavily hydrated. Thus, even at low concentrations, the assembly of oligomers also sequesters water molecules that can deplete the water molecules in the bulk solution to promote protein crystallization.

Some nonamphiphilic molecules may form temporal assembly in water, but not liquid crystal phases.[20] For those that do form liquid crystal phases in water (5′DSCG and 5′DSCG-diviol), the hydrate shells of the threads must also take part in the alignment for the liquid crystal phases. Thus, if a nonamphiphilic molecule forms liquid crystal phase in water, the assembled threads should be heavily hydrated. If the assembled threads are not hydrated, precipitates will likely form instead of an entirely birefringent liquid crystal

sample. This logic is consistent with the observation that molecules capable of forming liquid crystal can induce protein crystallization; and those that cannot, do not induce protein crystallization. We note that it is not certain whether more hydration is required for the threads than for the individual molecules that do not form liquid crystal phases, such as 5′DSCG-diglycerol or 5′MSCG. However, the hydration of individual molecules in water is unlikely a slow process, whereas the self-assembly of thread will be a dynamic and relatively slow process due to the requirement of the molecular diffusion.

For 5′DSCG, 12 wt% in water is required for liquid crystal formation, whereas for SSY dye, 30 wt% is required. Thus, threads formed by 5′DSCG are more heavily hydrated than threads formed by SSY dye because 5′DSCG requires a lower concentration (12 vs. 30 wt%) than SSY dye to form liquid crystals. This information also reveals the correlation between the inability of Sunset Yellow dye to sequester water and its inability to induce lysozyme crystallization.

Protein crystallization involves nucleation[10,61] and crystal growth. As a large number of small protein crystals were obtained when low concentration of 5′DSCG (~2.74 mM) alone was employed, we believe that the noncovalent polymerization of 5′DSCG induces the nucleation of lysozyme crystals, rather than promotes the growth of the crystals. In the presence of high concentration of proteins, the competition for water for solvation will cause an increase in the effective concentration of nonamphiphilic mesogens and promote their assembly to form noncovalent threads through an isodesmic process. Their assembly requires molecular diffusion, and provides a gradual means to compete for the water molecules in the bulk and to crystallize proteins. At high concentration of the additives of nonamphiphilic molecules, proteins precipitate out of solution. These observations are consistent with the molecular crowding effect,[22,62] but are promoted by the assembly process of the additives.

Considering the molecular crowding effects for inducing protein crystallization, we believe that at least two conditions need to be fulfilled. *First*, the additives must have the ability to sequester water molecules by hydration. *Second*, the additives should not denature protein under the crystallization conditions. The water sequestering ability of salts is directly proportional to the square of the valence of the ions,[63] and that multivalent anions seem to be more effective at crystallizing protein crystals. For example, divalent anion, malonate, has been found to crystallize more proteins than commonly used sulfates and chlorides.[46] The 5′DSCG derivatives used in this study all contain two carboxylates and have good water solubility, but exhibit different ability to crystallize lysozyme and form liquid crystal phase. These results thus suggest an additional mechanism for sequestering water molecules – self-assembly processes that also require strong hydration. Furthermore, the potential direct interactions between the assembled threads and the proteins may also contribute to the crystallization of protein, which is an ongoing subject of our study.

To summarize, this study reveals the enabling of protein crystallization by a dynamic assembly process of nonamphiphilic additives. As many small molecules form hydrates and exhibit polymorphism, results from this study suggest that these assembly processes can be explored to induce protein crystallization, and to further test the requirement that the assembly should sequester water and not denature proteins. Because these nonamphiphilic molecules are entirely water soluble, they will not have strong interactions with the oily part of a surfactant molecule. For this reason, we are studying the phase separation between these nonamphiphilic mesogens and surfactants, and exploring their ability to crystallize surfactant-protected membrane proteins.[64]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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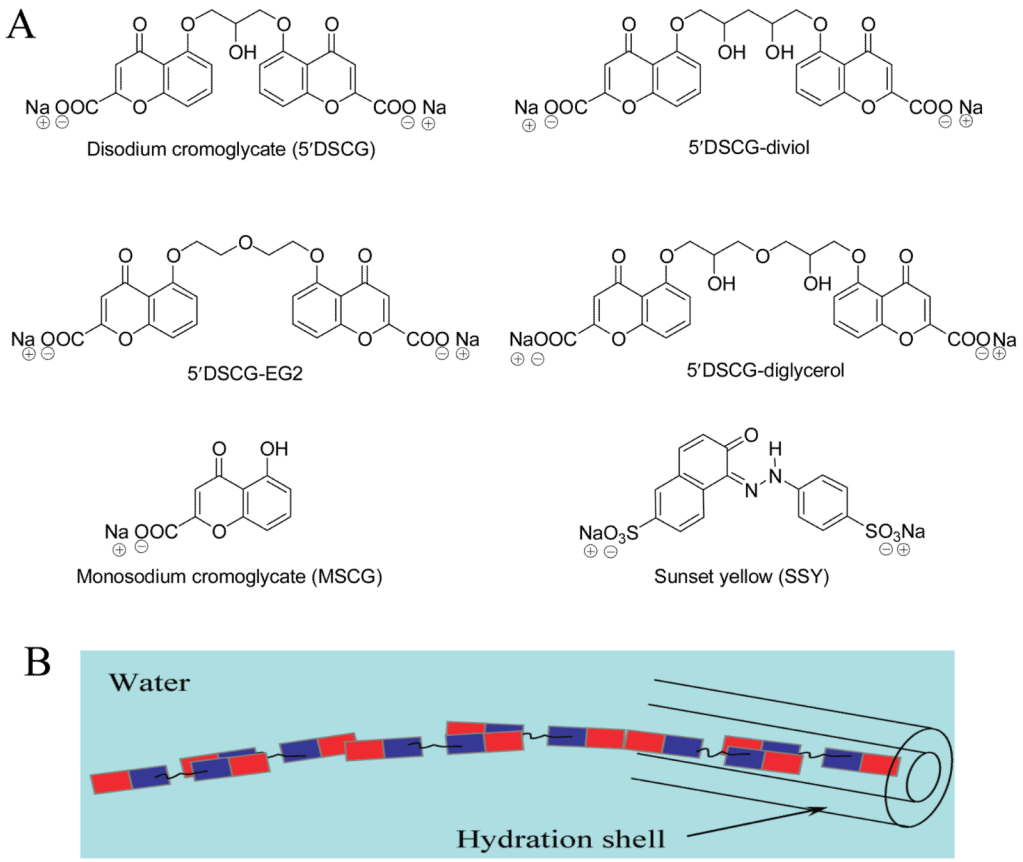

Figure 1.

(A) Molecular structures of additives. Molecule 5′DSCG-diviol consists of a mixture of 48 mole % meso compound and 52 mole % racemic mixture. (B) Schematic representation of the thread assembly formed by 5′DSCG molecules in water.

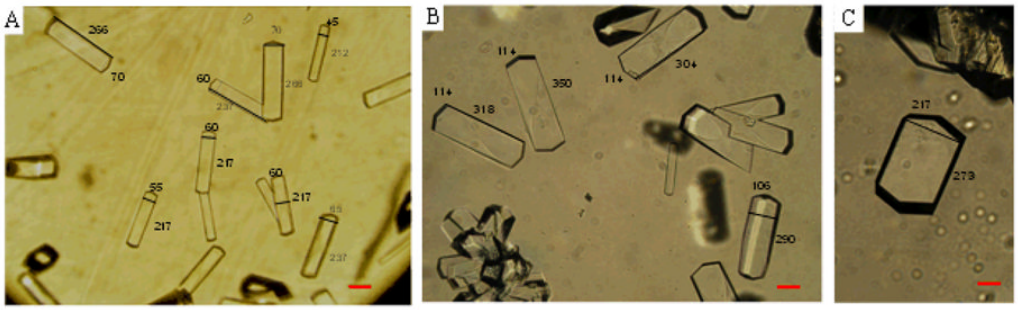

Figure 2.

Lysozyme crystals induced by 5′DSCG additives in hanging droplets. The droplets (5 μL) contained 37.5 mg/mL of lysozyme with (A) ~ 2.74 mM 5′DSCG, (B) ~2.74 mM 5′DSCG and ~128 mM NaCl, and (C) ~1.37 mM 5′DSCG and ~128 mM NaCl. The reservoir solution contained 350 μL of (A) ~ 5.5 mM 5′DSCG, (B) ~ 5.5 mM 5′DSCG and ~ 256 mM NaCl, and (C) ~ 2 M $(NH_4)_2SO_4$. Hanging drops kept at ambient temperature were observed over 3–5 days. All solutions were prepared using 50 mM sodium acetate buffer (pH 4.6). Scale bar = 76 um.

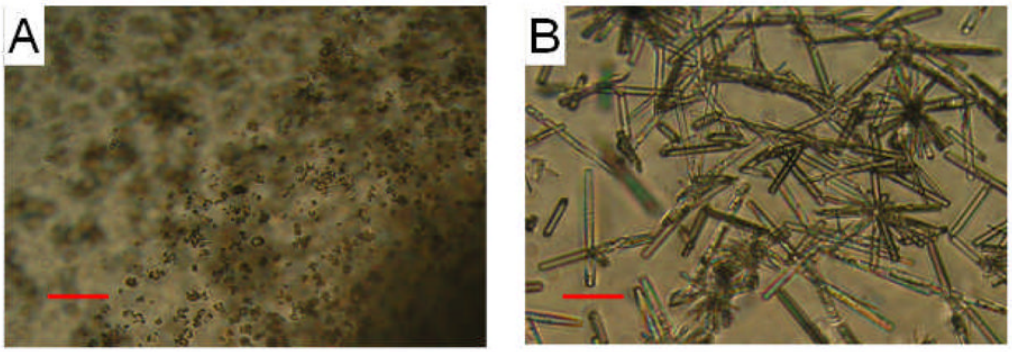

Figure 3.
Crystallization of lysozyme using 5′DSCG-diviol. The hanging drops (5 μL) contained 37.5 mg/mL of lysozyme with (A) 0.14 wt% (~2.73 mM), or (B) 0.068 wt % (~1.37 mM) of 5′DSCG-diviol in 50 mM sodium acetate buffer (pH 4.59). Reservoir solutions (350 μL) contained the same additives with twice the concentration as that in the respective drops. The drops were kept at 25°C for about 3 to 5 days. Scale bar = 76 um.

Table 1

Correlation of the existence of liquid crystal phases and the ability to induce protein crystallization by different additives.

Additives	Existence of LC phase	Formation of Protein Crystals
5′DSCG	Yes (12 wt%)[a]	Yes (0.25 wt%)[c]
5′DSCG-diviol	Yes (18 wt%)[b]	Yes (0.07 wt %)[c]
5′DSCG-EG2	No	No
5′DSCG-diglycerol	No	No
5′MSCG	No	No
Sunset Yellow dye	Yes (30 wt%)[a]	No

[a] Concentration at which liquid crystal forms at ambient temperature.

[b] Concentration at which liquid crystal forms at 15 °C.

[c] Concentration that induces protein crystallization.