Review

Methods to grow better diffractive protein crystals acquired through space experiments

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Abstract: We summarize how to obtain protein crystals from which better diffraction images can be obtained. In particular, the quality evaluation of the protein sample, the crystallization method and crystallization conditions, the freezing protection of the crystal, and the crystallization in the microgravity environment are described in detail.

Keywords: protein crystallization; protein sample qualification; JAXA PCG; microgravity

1. Introduction

We have been providing protein crystallographic services, especially for the Japan Aerospace Exploration Agency's High-Quality Protein Crystal Growth Experiment (JAXA PCG) for many years. Crystallization with a sample from a user often encounters the following issues and challenges: (1) crystals cannot be obtained with good reproducibility; (2) crystals showing good reflection are not reproducible; (3) crystals are obtained but do not give any good reflections; (4) users request us to grow larger crystals; and (5) users expect better crystal quality from space experiments. We have overcome many of these issues and accumulated experience from over 500 samples from users. Many methods have been reported for obtaining protein crystals from which good diffraction images can be obtained, but in fact it is a comprehensive technology from sample preparation to obtaining diffraction images. This paper explains our experience, which is useful for not only space experiments, but also for crystallization in the laboratory.

From our experience, we are targeting more rational technology by referring to what has been reported as the mechanism of crystal growth [1-4].

It is believed that protein crystals are obtained by reducing the solubility of an aqueous solution of proteins by adding a crystallization agent, which is called a "precipitant". However, the protein molecules in the crystals are not precipitates, but are well-arranged in the water inside of the crystal, which is a high−density solution. In addition, it seems that the concentration of the precipitant inside the crystal is significantly lower than outside which balances the chemical potential [1].

Between the protein molecules aligned in the crystal, there are various forces such as hydrogen bonds, ionic and van der Waals interaction, some of which are attractive and others which are repulsive [5]. In addition, at the crystal interface, a macroscopic force called interfacial tension also works (Figure 1).

In a good crystal, it is important to have a good alignment, but to do so, these forces need to be uniform. If there are defects in the crystal, it will cause stress and strain, etc., resulting in disordered packing. Because the plasticity of the crystals is not large, once a defect occurs, a mosaic occurs in the crystals [1]. One of the causes of misorientation is homologous impurity [6, 7]. Impurities also greatly reduce the growth rate of crystals [8]. For this reason, it is important that the protein sample to be

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used for crystallization is uniform, and therefore, sample preparation is essential to obtaining good crystals from the outset.

The second important factor is related to the crystallization conditions. It is important to control the force between protein molecules to achieve better alignment by controlling the optimal balance of intermolecular attraction and repulsion as well as macro interfacial tension.

2. Improvement of the sample quality

In order to obtain good crystals with good reproducibility, a good protein sample is the first requirement. A 'good protein sample' in this case is, of course, a sample which can produce good crystals. In addition, the sample must stay stable for a long time. It is also important that samples of equivalent quality can be obtained with good reproducibility. The sample preparation method for this purpose is not covered in this paper. In this paper, the evaluation method of the prepared protein sample and the countermeasures based on the evaluation results are explained.

It is important to perform accurate evaluations of protein samples with a relatively easy method. We apply SDS-PAGE, Native-PAGE, two-dimensional electrophoresis, high-resolution chromatography and Dynamic Light Scattering (DLS), then comprehensively evaluate the results. From our experience, it is likely that a sample with high uniformity and good consistency with the calculated value of electric charge density [9] is empirically good and has a high possibility of growing crystals of good quality. If the molecules taken in the crystals are different in molecular weight or the electric charge is non-uniform even though the molecules are equal in molecular weight, it directly leads to a disturbance of the arrangement of molecules in the crystal because molecules close to each other interact due to an electrostatic dipole moment in the crystals. As a result, it has serious effects on a crystal's formation and its quality.

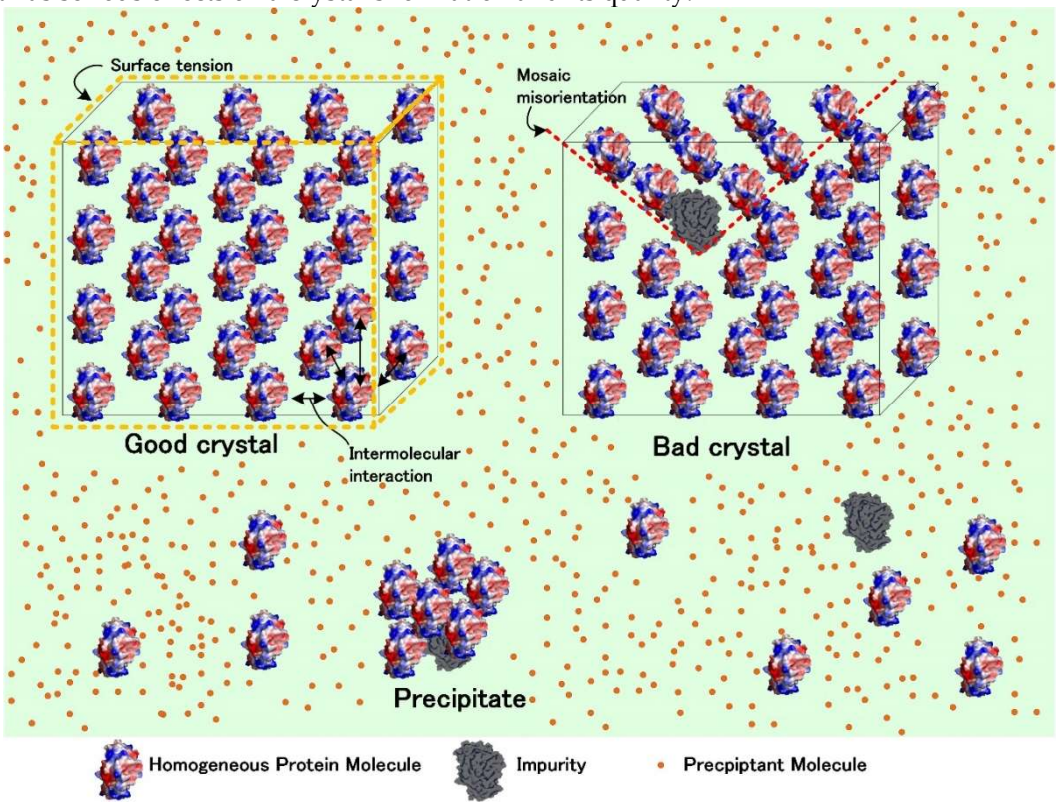

Figure 1. Conceptual diagram of crystals with good molecular alignment (left) and bad molecular alignment (right) and precipitation (bottom). In the crystal, molecules that are homogeneous with one another can be arranged regularly. If non-uniform molecules are incorporated, it affects the alignment of molecules around them, and it is difficult to obtain a good X-ray diffraction image.

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2.1. Physical property values of protein molecules

Empirically, whether protein samples can produce good crystals can be evaluated by the deviation between the physical property values of protein molecules assumed from the amino acid composition and the analysis results described below. From the amino acid composition of the protein sample, not only the molecular weight but also the electric charge number at a specific pH can be calculated [10]. Furthermore, electric charge density obtained by dividing this electric charge number by the estimated lattice volume [9] can be obtained by calculation (C-Profile, Confocal Science Inc.) (Figure 2). The Grand Average of Hydropathy (GRAVY) value can also be calculated.

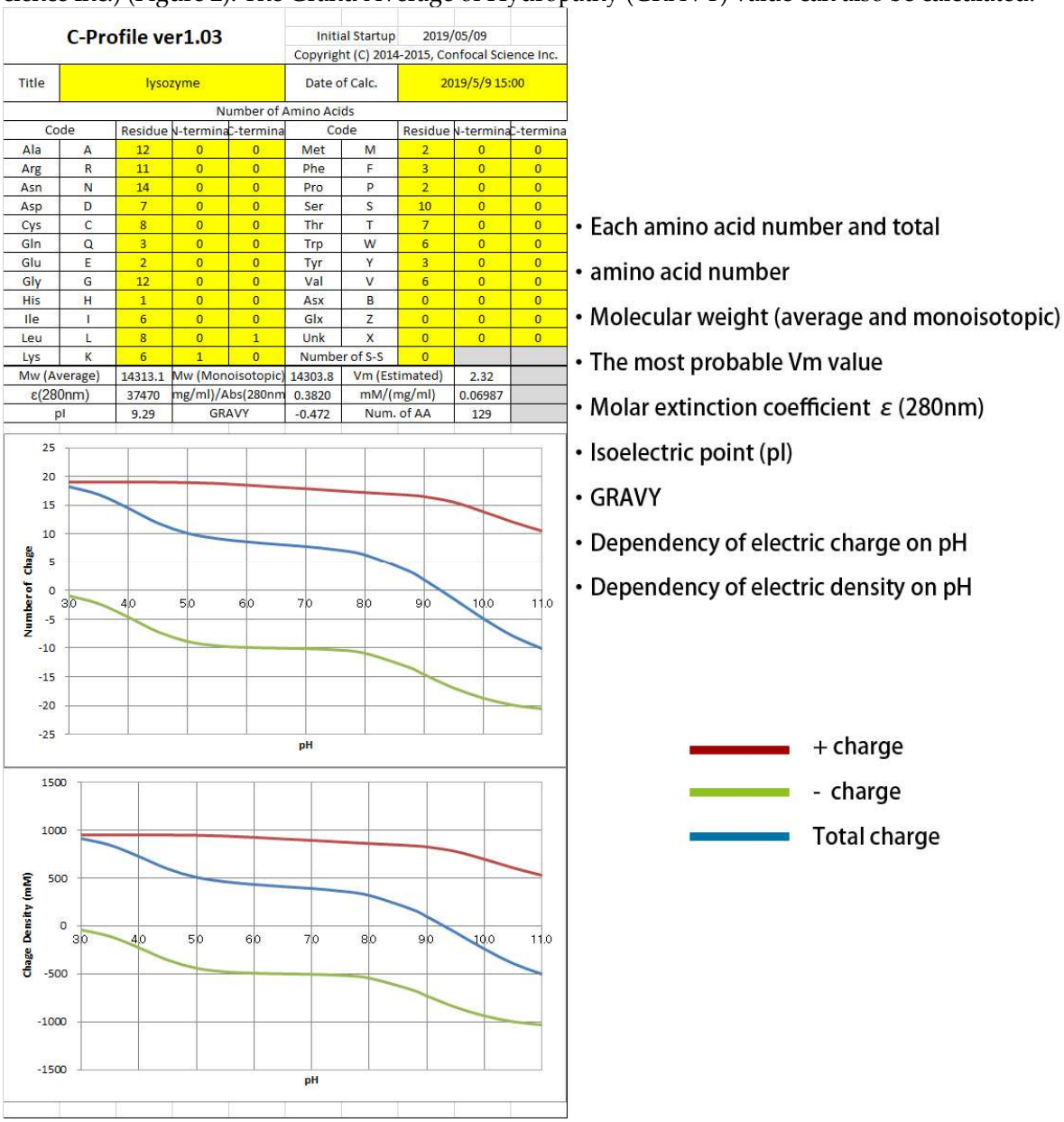

Figure 2. Physical property values calculated from amino acid composition. The physical property values of proteins can be calculated from amino acid composition (UniProtKB/Swiss-Prot [10]). C-Profile (Confocal Science Inc.) is an application available for personal computers.

2.2. SDS-PAGE

Analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is the most common method to check the purity of protein samples prepared as crystallization samples [11]. It is important to check whether the band clearly migrates to a position corresponding to the molecular weight calculated from the target protein sequence (Figure 3a). When some minor bands emerge or

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when the main band seems doubled (Figure 3b), this shows that a heterogeneous component of molecular weight exists in the prepared sample. In such cases, it is difficult to obtain crystals because other protein has contaminated the sample as an impurity or the target protein itself may be heterogeneous and have some different molecular weights. The various causes of this phenomenon can be estimated [12]. It is most likely that the expressed protein undergoes some modification, so that the molecular weight becomes heterogeneous or the target protein is partially digested during the expression / purification process.

2.3. *Analysis by Native-PAGE*

The electric charge of a protein molecule varies in accordance with the pH in the solution, and the amount of electric charge can be estimated by calculation. When analyzing with Native-PAGE [11], it is possible to know whether the target protein migrates in accordance with the calculated electric charge (Figure 3c, d). The Native-PAGE mentioned here is a simple PAGE analysis in which only SDS is removed from the solution and the gel. Protein samples used here are those without SDS treatment or boiling treatment. We usually consider two kinds of pH, acidic and basic conditions. Empirically, it is often observed that even a sample showing a very clear single band on SDS-PAGE gives multiple bands on Native-PAGE.

Furthermore, on Native-PAGE it is often observed that the band migrates to a smear or ladder shape or does not enter the gel. Sometimes electric charges estimated from the above calculation do not match with the mobility of the band. Perhaps this is caused by some unexpected aggregation of the protein molecules in the solution. Empirically this affects the possibility of crystal formation and quality.

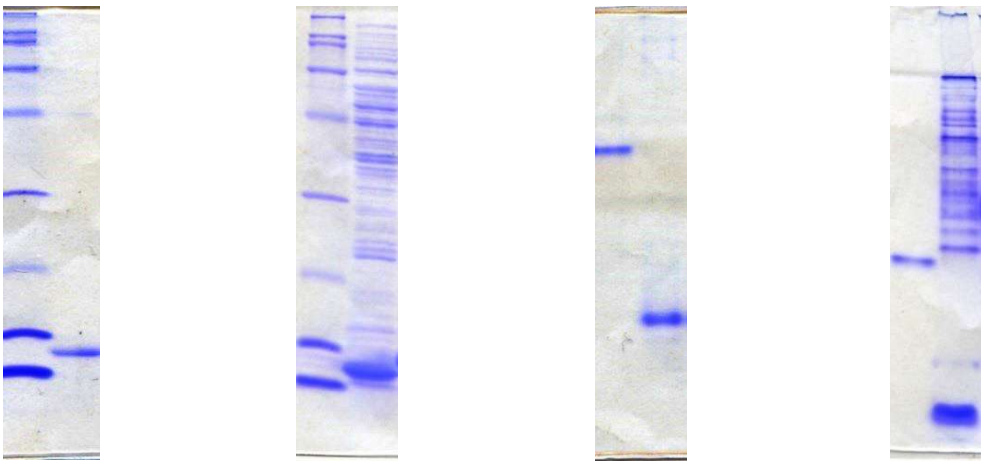

Figure 3. PAGE analysis of typical protein samples. In all gels, the left lanes are for molecular markers. (a) Homogenous sample migrates as a single band on SDS-PAGE; (b) Sample containing contaminated protein gives many bands other than target protein on SDS-PAGE; (c) Sample migrates in proportionate to its total electric charge. Sample with electrical homogeneity migrates as a single band on Native-PAGE; (d) Aggregate sample migrates as a smear and ladder on Native-PAGE.

2.4. *Two-dimensional electrophoresis*

Another method for confirming the uniformity of electric charge is two-dimensional electrophoresis [13], combining SDS-PAGE etc. with isoelectric focusing (Figure 4). By using the method where protein samples are separated with isoelectric focusing prior to SDS-PAGE, the homogeneity of the protein molecule can be shown more clearly. We sometimes see samples which have the same molecular weight but give slightly different spots, as much as half of the pH unit on isoelectric focusing. These samples often have problems for crystallization.

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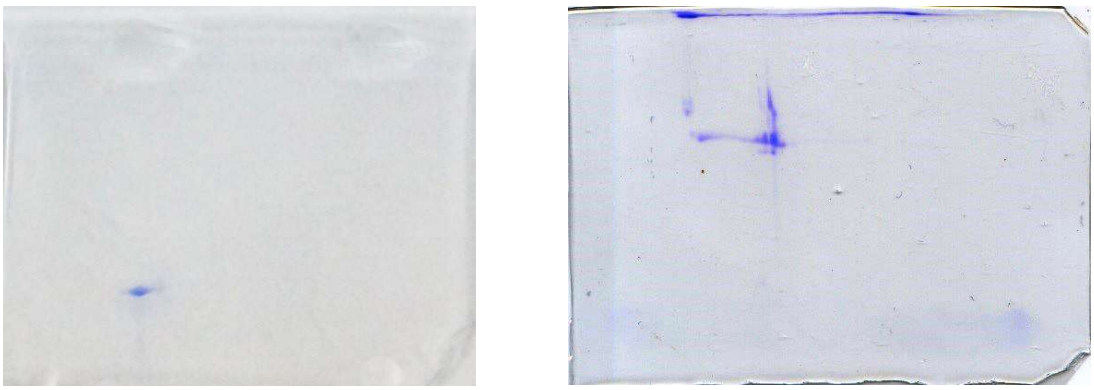
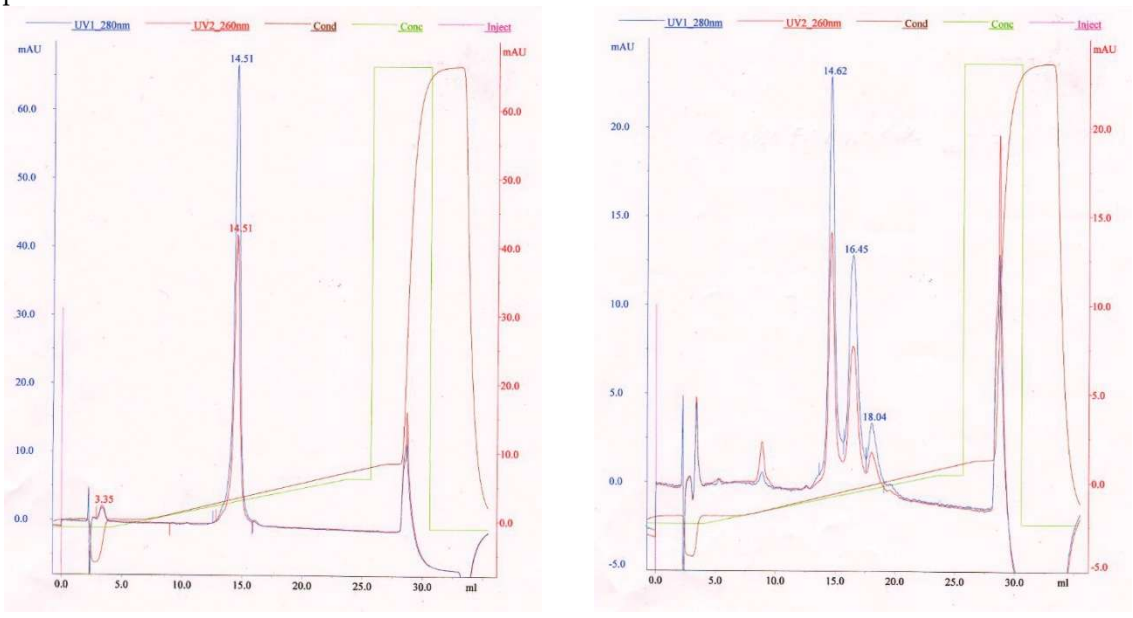


(**a**) (**b**)

Figure 4. Two-dimensional electrophoresis analysis of: (a) homogenous sample migrates as a single spot; (b) heterogeneous sample migrates as plural spots.

2.5. *High-resolution ion exchange chromatography*

In high-resolution ion exchange chromatography, an NaCl-gradient for elution from the column is commonly used [14]. Samples which can give a sharp, clear peak and elute at an expected concentration of NaCl (Figure 5a) often give good crystals. On the other hand, samples eluted at largely different NaCl concentrations (Figure 5b) contain unexpected problems, and crystallization tends to be difficult. When the heterogeneity of a sample is shown on Native-PAGE and two-dimensional electrophoresis, these samples rarely give one sharp peak on a chromatogram. In such a case, the sample elutes in some different peaks or in asymmetrical peaks with leading or tailing. On the contrary, in spite of the sharp peaks in chromatography, non-uniformity of the electric charge may be confirmed from the results of Native-PAGE and two-dimensional electrophoresis. It is conceivable that the forming aggregate will result in a great difference between its NaCl concentration eluted and its calculated value from electric charge density, so that good crystals are hard to grow. In the high-resolution ion exchange column, there are various kinds of ion exchange resins with different chromatographic particles (quaternary ammonium group and carboxymethyl group, etc.) [15]. Because the difference in separation results is derived from the difference in the surface charge of protein molecules, it is important to choose the appropriate resin for better separation.



(**a**) (**b**)

Figure 5. Typical chromatogram of Ion-exchange chromatography: (a) homogeneous sample; (b) heterogeneous sample.

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2.6 Dynamic light scattering method

With dynamic light scattering (DLS) measurement, it is possible to know what the molecular size distribution is like in the solution. The points to be confirmed from the obtained results are that estimated molecular weight is equal to the integral multiple of its own molecular weight with a narrow distribution, and there are no large sized particles (mono-modal distribution) [16]. In many cases, samples in which the distribution width of its radius is in the latter half of 20% will give good crystals (Figure 6a). X-ray crystallographic analysis shows that the maximal size of a protein molecule of about 50kDa (450 aa) is 4 to 5 nm. If on the analysis of the DLS measurement, a particle, whose size is more than 50 nm emerges, the possibility that protein molecule will form irregular aggregates must be considered. From our experience, it is difficult to obtain crystals from such a sample (Figure 6b).

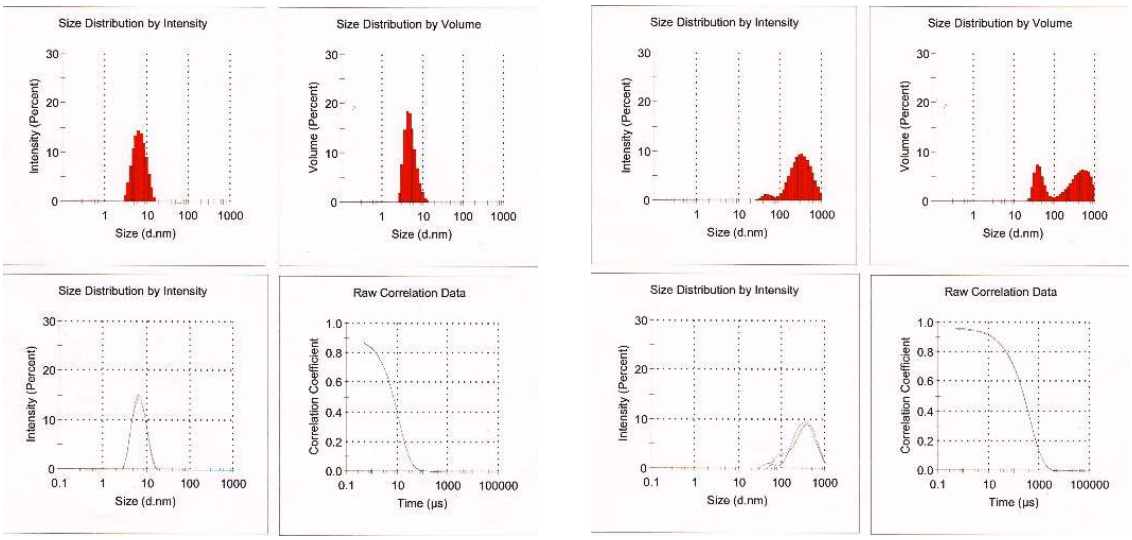

Figure 6. Typical results of dynamic light scattering (DLS): (a) Good sample shows narrow and mono-modal distribution; (b) Bad sample shows broad and poly-modal distribution.

2.7. Measures to improve protein samples

For samples with problems, we need to consider measures for improvement.

2.7.1. Uniformity improvement

When multiple bands or spots are found on Native-PAGE, two-dimensional electrophoresis, and high-resolution chromatography, it is often possible to separate them with high-resolution chromatography. When you see peak shoulders or asymmetrical peaks, such as leading or tailing, those peaks often may be separated by reconsidering the elution condition (chromatography particle, resins, buffers and gradient programs) to give high-resolution separation.

In such a case, the components to be separated are as follows:

- Other proteins, lipids etc. failed to be removed through other purification steps
- Those N or C terminal ends not processed, correctly
- Those residues modified irregularly

Sometimes, when these contaminants are removed, the total amount of target protein is reduced, so it is necessary to design an expression host and expression plasmid carefully.

2.7.2. Aggregate removal

If irregular aggregates are present in the sample, it is difficult to overcome. It is necessary to find out what caused such irregular aggregates through its purification process. From our knowledge, the probable causes are as follows:

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- Denatured proteins caused by the concentration process in the crude state or ammonium sulfate precipitation. Avoiding these processes might improve the sample.
- Aggregate proteins associated with contaminants or isozymes with different pI through hydrophobic or hydrophilic interactions. Dialysis, gel filtration, or high-resolution chromatography sometimes remove such aggregates, and this might dramatically improve the sample quality.

2.7.3. Improvement of sample quality deterioration over time

It is often observed that samples for crystallization undergo degradation. Quality changes, associated with time (sample degradation and increase of aggregates), can be evaluated by the methods mentioned above. From our experience, effective countermeasures are as follows:

- For unstable proteins, construction of mutants to improve their stability is advised.
- In the case of protease degradation, adding protease inhibitors followed by removing proteases in the following chromatography step is usually effective.
- In the case of damage caused by oxidation, purification, storage, and crystallization under a deoxygenated state is advised.

3. Crystallization

3.1. Reagent

It is well known that protein can be crystallized by mixing protein samples and crystallization reagents. Generally, protein solubility is decreased by this operation [1, 2, 4]. A component in the crystallization reagent which dramatically reduces the solubility of the protein is called a precipitant. However, it should be noted that a "precipitant" is not expected to produce true protein precipitate but to separate protein molecules in a highly crowded phase inside a crystal, the outside of which is in a freely dispersed phase. Frequently used precipitants are as follows [17]:

- Salts-combination of mono- or multi-valent anions and cations, for example, $(NH_4)SO_4$, Na-malonete, etc. The tendency of lowering the solubility is listed in the Hofmeister Series [18]. In general, anions and cations have various effects other than simply reducing solubility. Therefore, determining a proper salt for crystallization is accompanied by some difficulties.
- Polymers-high molecular weight polymers, for example, polyethylene glycol (PEG). The mechanism of reducing solubility is explained as an excluding volume effect [1]. In general, the preferable molecular weight of PEG is related to the target protein [19], although a molecular weight of 400 to 20,000 is frequently used. Lower molecular weight PEG, such as PEG 400, has characteristics similar to alcohol. But higher molecular weight PEG does not have significant side-effects other than reducing solubility. Thus, PEG is easier for controlling the crystallization process and is frequently used in crystallization.
- Organic solvents-alcohol, for example, 2-methyl-2,4-pentanediol (MPD), isopropanol, etc. The mechanism is explained as reducing the dielectric constant of the solution [1]. Some hydrophobic proteins sometimes prefer organic solvents.

In general, these precipitants are used in significantly higher concentration, such as several tens of weight per volume percentage.

In addition to these main precipitants, some amounts of additives are frequently used. They are summarized in Table 1.

In the case of a crystallization solution consisting of a large number of components, it is quite difficult to accurately estimate each component's effects on crystallization although there may be some synergetic effects [17]. However, by understanding the protein concentration and phase diagrams of these components as coordinate axes, it is possible to grasp the effect on crystallization, and it is useful for more rational optimization of crystallization conditions. In the batch method, the fixed crystallization condition is one point on the phase diagram and would not be changed, which

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Table 1. Frequently used additives in crystallization solution.

Effect	Classification	Reagent and usage example	Explanation
Electrostatic interaction	Counter ion	10-1000 mM NaCl	Reduces the electrostatic repulsion between protein molecules by creating an ion pair on the protein molecule surface [18]. Na^+ and Cl^- are the most conventional ones.
	Organic solvent	5-20% MPD 5-20% Dioxane	Reduces the electrostatic repulsion between protein molecules by reducing the dielectric constant of the solvent [1].
Specific intermolecular interactions	Multivalent acid	10-200 mM Tartrate	Intervenes and attracts between protein molecules
	Multivalent metal ion	10-200 mM $MgCl_2$	
	Multivalent base	10-200 mM Bis Tris Propane	
pH buffering	Weak acid	10-100 mM Acetate	Buffer pH of solution
	Weak base	10-100 mM Tris	
Solubilizing	Detergent	0.1-2% DDM (n-Dodecyl-beta-D-maltopyranoside)	Solubilization of protein with strong hydrophobicity of membrane protein [20, 21]

is suitable for this investigation. In other methods, the condition varies with time on the phase diagram, which induces some complicated phenomena. So, it is necessary to understand the time change for each method.

A few heuristics are as follows.

- In the case of PEG, as the concentration increases, the number of generated crystals increases once, but decreases as the concentration of PEG further increases. It is thought that the nucleus formation probability decreases as the viscosity increases [22, 23, 24]. Further, in a state where the nucleation formation probability is lowered, the degree of supersaturation is high, so that secondary nucleation on the crystal surface is likely to occur and cluster crystals are likely to be formed.
- When there is no reagent that enhances intermolecular interaction, reduction of electrostatic repulsion is necessary for crystallization. Neutralization by Na^+ and Cl^- as counterions of divergent groups ($-COO^-$, $-NH_4^+$) of proteins is one of the methods. In this case, it is necessary to add NaCl at a concentration in relation to the electric charge density [9, 18].
- Ions such as Na^+ and Cl^- not only interact with divergent groups on the protein surface but also interact with other acids and bases and affect their effects. Therefore, when they coexist with a reagent that enhances intermolecular interaction, conversely, the effect is diminished.

3.2. Crystallization method

Methods of protein crystallization have been devised which allow a large number of conditions to be studied with a small amount of sample. Typical crystallization method are as follows:

3.2.1. Batch method

The batch method is the oldest and the simplest method for protein crystallization [25]. In the batch method, a protein sample and a crystallization reagent solution (reservoir solution) are mixed at an appropriate ratio and left to stand. As long as crystallization does not start, the concentration of

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the components in the solution does not change. Therefore, it is a good method to study crystallization after fixing the concentration of each reagent in the solution precisely.

3.2.2. Vapor-diffusion method

In the vapor-diffusion (VD) method [26, 27], a solution prepared by mixing a protein sample and a crystallization reagent solution (reservoir solution) in a ratio of 1: 1 interacts with a reservoir solution via an air layer.

By 1:1 mixing, the concentration of other components coexisting with the protein sample; the concentration of the protein sample and the crystallization reagent derived from the reservoir; and their coexisting components become half at the beginning, but water migrates due to the interaction with the reservoir and concentrates [26, 27]. As a result, the protein sample, the other components coexisting with the protein sample, the crystallization reagent, and the coexistent component derived from the reservoir all increase toward the original concentration, and crystallization occurs when reaching the concentration at which crystals are formed in the process. In many cases, it is more likely that crystals are grown if the concentrations of the protein sample, the other components coexisting with the protein sample, the crystallization reagent or the coexistent component derived from the reservoir become higher at the same time. Therefore, crystals are easy to grow by the VD method. On the other hand, with this method, it is not possible to individually control the concentration of protein sample, other components coexisting with the protein sample, the crystallization reagent or coexistent component derived from the reservoir. For this reason, there are cases where crystals cannot be obtained, for example, when other components coexisting in the protein sample are not favorable for crystallization.

The VD method is the most common method used by many researchers. A large number of screening kits for searching for crystallization conditions are also on the market, but it should be taken into account that all components are concentrated in this method.

3.2.3 Counter-diffusion method

The counter-diffusion (CD) method [28, 29] generally fills a capillary with a protein sample and diffuses the crystallization reagent components from the capillary end. At the same time, the protein sample in the capillary and other coexisting components diffuse outside the capillary. Therefore, the coexisting components in the capillary are replaced by the components in the reservoir solution. As a result of such bidirectional diffusion, a combination of wide concentration regions of the crystallization reagent and the protein sample is scanned.

The Granada Crystallization Box (GCB) places an agarose gel layer between a protein solution and a crystallization reagent to achieve relatively mild solution diffusion. This method enables screening of infinite crystallization conditions in one capillary [30]. We modified this method and used a gel-tube instead of the agarose gel layer to simplify this method [31].

This time lapse is not easy to measure. Therefore, we prepared a one-dimensional (1-D) diffusion simulation program so that various concentration components in the capillary can be estimated [31]. It is necessary to consider the correlation between this diffusion time course and the crystallization start time on the phase diagram when studying the crystallization condition.

The component with the smaller molecular weight diffuses quickly and the component with the larger molecular weight, such as the protein, slowly diffuses. Therefore, among the other components coexisting with the protein molecules filled in the capillary, a component having a low molecular weight diffuses faster and leaks out of the capillary. On the other hand, the main crystallization reagent component of the reservoir and the coexisting components diffuse into the capillary.

In the CD method, usually, the volume of the reservoir is much larger than the capillary content, so that components other than the protein molecules in the capillary are replaced with reservoir components. Therefore, the concentrations of the reagent components related to the crystallization conditions can be individually controlled, and more advanced crystallization conditions can be set. For example, in cases where crystals are not obtained by the VD method, such as when some of the

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components coexisting in a protein sample are not favorable for crystallization, good crystals have often been obtained by the CD method.

By the way, diffusion of protein molecules is greatly reduced in PEG, whereas diffusion of low molecular weight compounds does not slow-down in PEG [32]. Therefore, when a PEG type crystallization reagent is applied to the CD method, diffusion leakage of protein from the capillary can be suppressed, which is preferable. There are not many researchers using the CD method. However, having a good understanding of the mechanism as described above when setting crystallization conditions is a good way to obtain crystals with more optimal crystallization conditions than other methods.

We have obtained a lot of fine crystals with the CD method. Some of the crystals grown in capillaries are shown in Fig. 7.

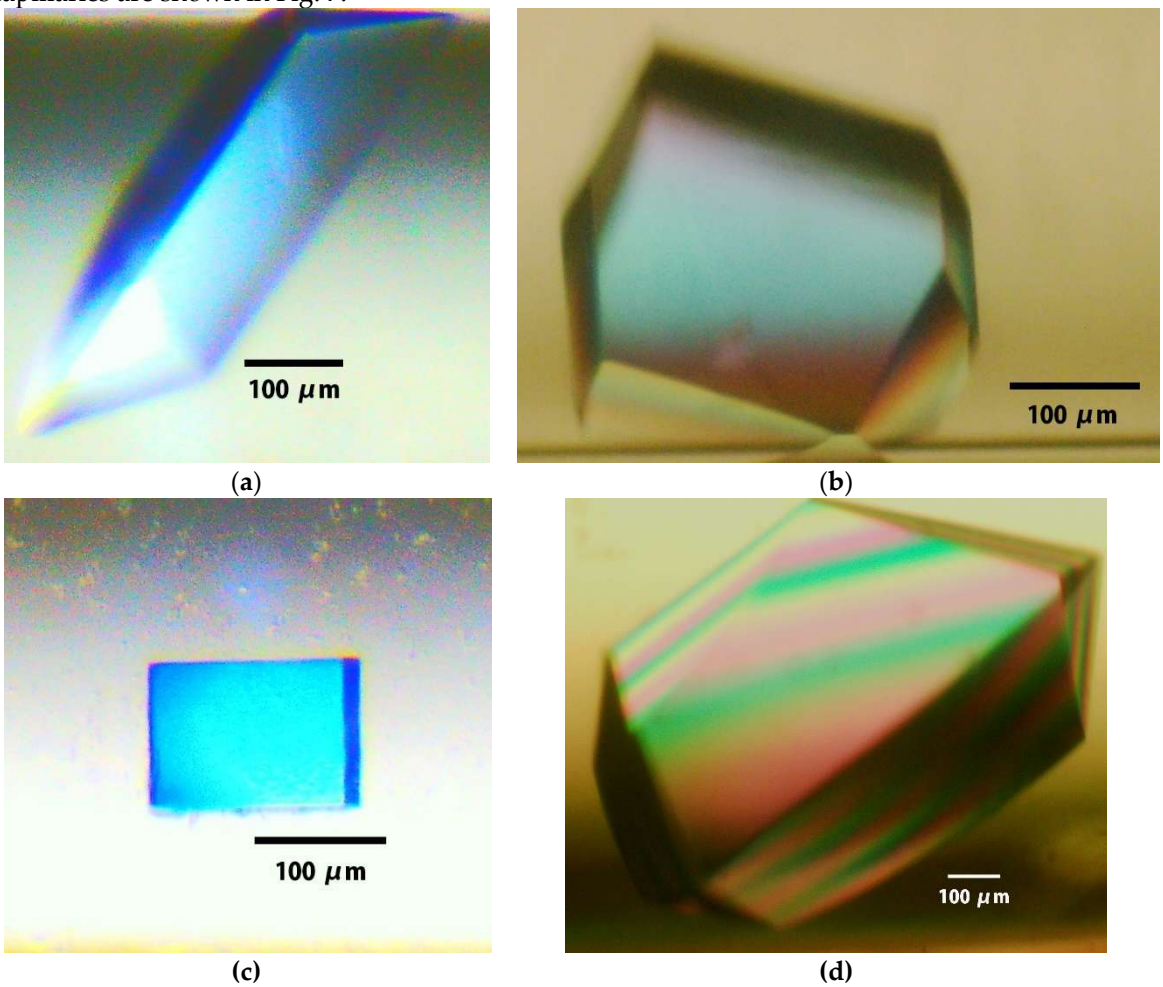

Figure 7. Examples of crystals grown with the CD method: (a) an Alpha-amylase crystal grew as a single crystal in optimized PEG/salt condition, while it tended to grow as a clustered crystals before optimization; (b) a large lysozyme crystal grew in optimized PEG/salt condition, while many but small lysozyme crystals tended to grow in salt condition; (c) an H-protein single crystal grew in optimized PEG/ammonium sulfate/salt condition, while clustered H-protein crystal tended to grown in ammonium sulfate/salt condition; (d) a large PcCel6A crystal grew in PEG solution in which salt concentration was optimized.

3.2.4 Dialysis method

The dialysis (DL) method replaces the components coexisting with the protein sample into the reservoir solution components while suppressing diffusion leakage of the protein molecules by the dialysis membrane [33, 34]. Therefore, the concentration of the reagent components related to the crystallization condition can be individually controlled, and similarly to the CD method, more advanced crystallization conditions can be set. In the general DL method, the Button method has

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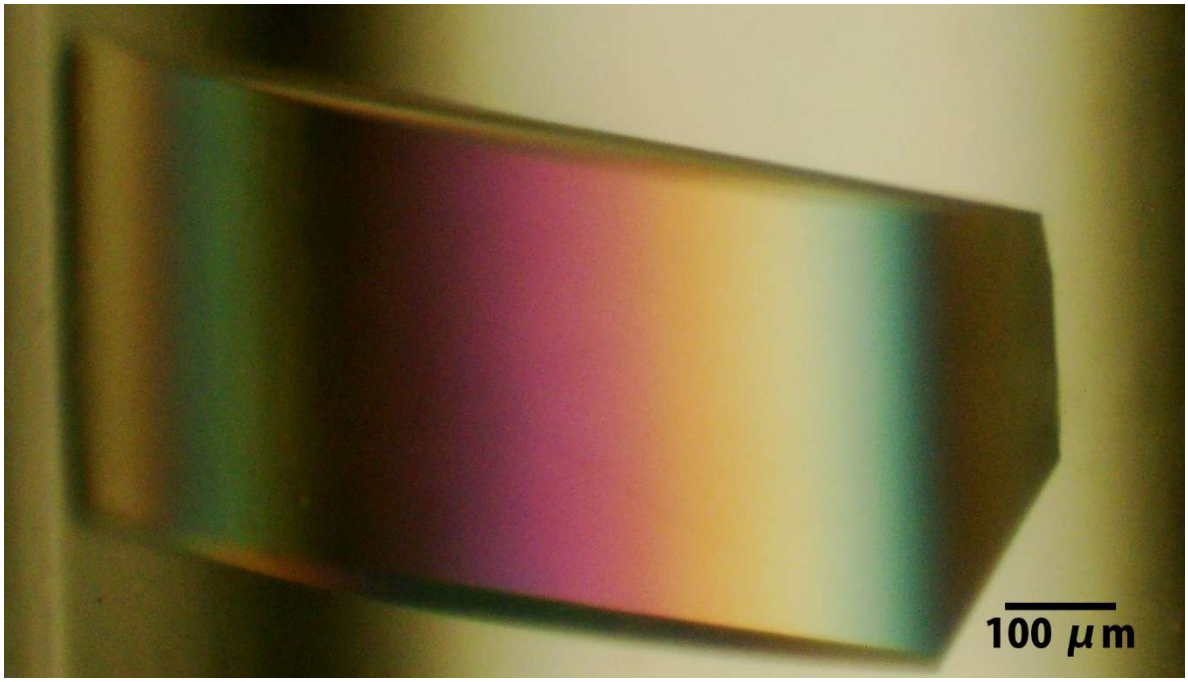
been used. In this method, since the reservoir solution diffuses into the container immediately, problems such as the generation of bubbles accompanying a sudden change in osmotic pressure are likely to occur. Recently, we have developed a dialysis method with a diffusion path with a dialysis membrane attached to the opening end of the CD method capillary, achieving the dialysis method under mild conditions [35]. A crystal obtained by the DL method in a capillary is shown in Figure 8. There are not many researchers using the DL method. However, having a better understanding of the mechanism above when setting conditions, as in the CD method, is a good way to set more optimal crystallization conditions.



(a)

(b)

Figure 8. An example of crystal grown by DL method. (a) A large lysozyme crystal was grown in optimized PEG/salt condition (reference 39); (b) a large thaumatin crystal was grown in optimized PEG condition, that was originally crystallized in sodium/potassium tartrate condition. It was grown near the dialysis membrane (lower end of the photo). The crystal looks shimmered because another large crystal is growing in front.

3.3. Improvement of crystal quality

When there are some issues in the crystallization, we consider the following possibilities.

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335 *3.3.1. Reproducibility of crystal*

336 Often a good quality protein sample does not produce crystals with high reproducibility. In this
337 case during the protein sample preparation, special care should be taken not to vary the salts, buffers,
338 and pH in the protein sample. In general, it is thought that crystallization occurs due to a main
339 crystallization reagent, but other coexisting components greatly influence it. Particularly in the VD
340 method, it should be noted that the components contained in the protein sample are concentrated
341 like the reservoir components during crystallization.

342 *3.3.2. Obtaining appropriate size and number of crystal*

343 Often many, but small crystals are obtained or a crystal is extremely difficult to obtain. In
344 general, the three-dimensional nucleation probability in crystallization is expressed by the following
345 equation [22, 23].

346 $$\mathrm{I} = \frac{const}{\eta} \times C \times exp\left(-\frac{16\pi\gamma^3}{3kT(\overline{\Delta\mu})^2}\right) \cdots\cdots\cdots (1)$$

347 η : viscosity, C : solution concentration, γ : surface tension, $\Delta\mu$: chemical potential difference
348 between the crystal and the solution of a unit volume.
349 As can be seen from this equation, the probability of three-dimensional nucleation becomes
350 higher as the protein concentration (C) is higher, lower when the viscosity (η) is higher, and lower
351 when the interfacial tension (γ) is higher. When the solubility is low, $\Delta\mu$ becomes high, and the
352 nucleation probability increases. Actually, when the protein concentration (C) is high, the number of
353 crystals tends to increase. As the concentration of the main crystallization reagent is increased, $\Delta\mu$
354 becomes larger as the solubility decreases, and the number of crystals increases once. However, as
355 the interfacial tension (γ) increases further, the number of crystals tends to decrease. With a highly
356 viscous crystallization reagent like PEG, when the viscosity (η) further increases, the crystal number
357 significantly decreases. For a crystallization method in which the concentration of the main
358 crystallization reagent is increased with the lapse of time, it is better to be conscious of how the three-
359 dimensional nucleation probability has passed. Not only the main crystallization reagent but also
360 counterion, multivalent cation, polyvalent anion and the like may possibly lower the solubility. It is
361 necessary to choose the reagents to be added, considering how much the plus or minus electric charge
362 the protein has at the pH for crystallization.

363 *3.3.3. Obtaining appropriately shaped crystals for diffraction experiment*

364 When a crystal can be obtained, but becomes needle-shaped or thin plate-shaped and not
365 suitable for X-ray diffraction experiments, there are usually some problems with the homogeneity of
366 the protein sample. In many cases, it is better to improve the sample quality to overcome this.
367 However, there are cases where protein samples are not problematic, yet an appropriate shape is not
368 obtained. In such a case, adding reagents that affect anisotropic forces between molecules is effective,
369 that is, electrostatic interactions, hydrophobic interactions, and specific intermolecular interactions.
370 It is also possible to change the crystal shape by changing the pH to the side opposite to the isoelectric
371 point and changing the polarity of the electrostatic repulsion.

372 *3.3.4. Avoiding crystal clustering*

373 In a cluster crystal, very fine crystals are gathered, or a single crystal of a certain size seems to
374 grow from the surface of another single crystal. In the former case, there are usually problems with
375 protein samples, and the first thing to do is to improve the sample quality. In the latter case, although
376 the degree of supersaturation is high, the probability of three-dimensional nucleation is low, so that
377 two-dimensional nuclear growth is occurring from the crystal surface as the nucleus. In this case as
378 well, the problem may be caused by heterogeneous components contaminating the protein sample
379 and can be solved by improving the protein sample. However, this may happen even if the sample
380 has no problem. In this case, reducing the supersaturation degree by lowering the protein

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concentration, or lowering the interfacial tension or viscosity by decreasing the concentration of the main crystallization reagent are effective.

3.3.5. Resolution

Although crystals are obtained, the resolution of the X-ray diffraction may not meet the requirements. If the uniformity of the protein sample is not sufficient, the improvement of sample quality is necessary first. Generally, when the sample has no problem, the diffraction resolution improves as the interaction between adjacent protein molecules increases. That is, it is effective to increase the main crystallization reagent concentration to increase the interfacial tension, decrease the counterion concentration and enhance the electrostatic interaction.

3.3.6. Molecular packing

Although crystals are obtained, packing is not always suitable for structural analysis. While it is difficult to respond to this problem properly, there have been some successes with the countermeasures explained in Section 3.3.3 and in Section 3.3.5.

3.4. Growing large crystals

Today, a crystal of 1 mm^3 or more is necessary for neutron diffraction experiments [36]. Compared to crystals for general X-ray diffraction experiments, one side is 10 times or more larger, creating a bottleneck.

In order to obtain large crystals, it is necessary to find a condition in which the number of crystals produced per unit volume is approximately one and to grow the crystal while controlling appropriate crystallization conditions. These include experimental strategies utilizing solubility diagrams, ripening effects, classical crystallization techniques, microgravity and theoretical considerations [36].

Nakamura et al. have succeeded in obtaining large crystals by determining such crystallization conditions using phase diagrams and shifting to appropriate crystallization conditions [37]. Niimura and colleagues have devised a device capable of reversibly dissolving the crystals once produced and have successfully obtained large crystals by reducing the number of crystals [38].

The authors succeeded in producing lysozyme crystals with a long side of about 1 mm using the DL method in a capillary in which a dialysis membrane was attached to a gel tube (Figure 8a) [39]. The crystallization condition was optimized by using a three-dimensional phase diagram in which the main crystallization reagent (PEG) concentration and the counter ion (NaCl) concentration were changed [24].

4. Harvesting crystal and cryo-protection

When removing crystals from the crystallization vessel, we often experience crystal damage. For X-ray diffraction experiments with synchrotron radiation, it is necessary to cryoprotect crystals so that there is no damage to the crystal when frozen.

4.1. Optimization of harvest solution

In the batch method, since there is no concentration change of the solution component with time, it is often preferable to harvest crystals in the same solution as the batch solution. Using the VD method and the DL (with Button) method, the time to reach equilibrium is short, and it becomes almost the composition of the reservoir solution, so the reservoir solution can be used for the harvest solution. On the other hand, in the CD method and DL method with a diffusion path, it takes time to diffuse the crystallization reagent inside the capillary. This tendency is particularly remarkable when high molecular weight PEG is used as a reagent. Even after two to three months have elapsed since filling in the capillary, the concentration of crystallization reagent inside the capillary is not in equilibrium, and its concentration varies depending on the location in the capillary. Due to the difference in reagent concentration at the location where crystals were grown, if the wrong harvest

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solution is used, crystals are destroyed or damaged due to osmotic shock, and the quality of crystals is markedly degraded.

In order to avoid this phenomenon, the time change of the reagent concentration at each point inside the capillary is calculated in advance with a 1-D simulation program [31]. From the place where the crystal was actually obtained and the elapsed time after the setup, the crystallization reagent concentration can be estimated for the preparation of the harvest solution.

4.2. Treatment of crystals obtained in a capillary

In the batch method, VD method and DL (with Button) method, crystals can be taken out directly from a crystallization drop with a cryo-loop. On the other hand, when crystals are generated in a capillary such as the CD method and DL (with a diffusion path) method, it is necessary to carry out considerably detailed work under a stereoscopic microscope in order to remove crystals intact. It is necessary to observe the capillary where crystals are formed in detail, and decide which crystal is best for extraction. The capillary is cut with a range of about 5 mm in front of and in back of the crystal. The cut capillary segment that contains the targeted crystal is held by tweezers, and the harvest solution is poured into one side of the capillary with a micropipette. Hopefully, the crystals will come off the capillary wall and get pushed out to be scooped up with a cryo-loop.

If crystals stick to the inner wall of the capillary and will not come out even after applying the harvest solution, a thin wire is used to grind the crystal and remove it from the capillary wall.

4.3. Cryo-treatment of crystals

During a diffraction experiment using synchrotron radiation, crystals should usually be frozen for protection from radiation damage. When freezing crystals, it is necessary that the solution around the crystal solidify into a glassy state [40]. For this reason, crystals are passed through a cryoprotectant solution prepared by adding cryoprotectant to the harvest solution, and the solution around the crystal is replaced. Empirically,

- Ensure that the drops of cryoprotectant solution can be frozen into a glass form in advance.
- In the case of PEG alone as a cryoprotectant, a concentration of about 35% or more is desirable.
- In the case of a PEG type of lower concentration in the harvest solution, it is preferable that PEG or glycerol is added to a total concentration of about 35% or more. However, as the amount of glycerol to be added increases, the osmotic pressure difference becomes large, so be careful.
- In the case of salt in the harvest solution, when glycerol cannot be added, sucrose or trehalose are the next choice.

5. Optimization for microgravity condition

5.1. Introduction

In the crystallization of proteins in space experiments, the problems of crystallization may be alleviated by the effects of microgravity [1, 41-44]. Crystal clustering and disordering are suppressed, and the resolution of X-ray diffraction is improved. Also, although the reason is unknown, nucleation formation is suppressed, crystals are increased in size, and in some cases crystals having different space groups are grown.

In the solution around the growing crystal, protein molecules are incorporated into the crystal surface and the density of the solution is lowered [1, 45, 46]. In the terrestrial environment, a density-driven flow occurs. As a result, protein molecules are continuously transported by this flow from a place far away from the crystal in the solution. Impurities and minute crystals in the solution are also carried and taken into the surface of the crystal. On the other hand, in the microgravity environment, protein molecules, impurities, minute crystals and the like approach the crystal surface only by

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thermal motion. As a result, their concentration on the crystal surface decreases compared to the terrestrial environment.

Indeed, Otálora et al. [45] confirmed by optical interferometry that a protein depletion zone (PDZ) was formed in the vicinity of growing lysozyme crystals in space experiments (STS-95) using the 1998 Space Shuttle mission. It is believed that if the protein concentration on the growing crystal surface decreases due to the formation of PDZ, the supersaturation degree decreases, the growth rate decreases, and the disorder of the protein molecule taken in the crystal decreases. Thomas et al. [46] also revealed that impurity uptake is greatly suppressed in lysozyme crystals grown in the microgravity environment in the space experiment (STS-95). It is thought that this is due to the formation of an impurity depletion zone (IDZ) around the crystal which decreases impurity incorporation in the crystal. For the same reason, if the adhesion of minute crystals decreases, not only disorder reduction but also the suppression of clusters may be expected. On the other hand, according to Vekilov et al. [47], fluctuation occurs in the crystal growth rate due to the interaction between density-driven convection and the molecular uptake process into the crystal, and as a result it conceivably causes disorder by step bunching. Therefore, suppression of density-driven convection also solves this problem. Incidentally, even in the terrestrial laboratory, a method and apparatus for growing crystals with less disorder has been devised by suppressing the degree of supersaturation or conversely giving a flow actively to the crystal surface which may suppress step bunching [48].

5.2. Space experiment model

For the diffusion field formed around the growing crystal, numerical analysis can be performed with a simplified model system, assuming that the crystal is a sphere, ignoring the dissociation of protein molecules and impurity molecules from the crystal. The effect of formation of PDZ is defined as DFR (Driving Force Ratio), and the effect of suppressing impurity uptake by formation of IDZ is IR (Impurity Ratio) [49-51].

$$DFR = \frac{DFR_{0G}}{DFR_{1G}} = \frac{1}{1+\frac{R\cdot\beta}{D}} \cdots\cdots(2)$$

$$IR = \frac{IUR_{0G}}{IUR_{1G}} = \frac{1+\frac{R\cdot\beta}{D}}{1+A\cdot\frac{R\cdot\beta}{D}} \cdots\cdots(3)$$

$$A = \frac{\beta i\cdot D}{\beta\cdot Di}$$

Where $C(\infty)$, $C(R)$, *and* Ce are protein concentrations away from the crystal, at the crystal surface and the saturated solution, respectively. $Ci(\infty)$ *and* $Ci(R)$ are impurity concentration away from the crystal and that on the crystal surface. β *and* βi are kinetic constants of crystal growth of protein molecules and impurity molecules. D *and* Di are diffusion constants of protein molecules and impurity molecules. R is the crystal radius. *IUR* stands for impurity uptake ratio.

It can be seen that when the equations (2) and (3) are plotted on the abscissa with the $R\beta$ / D value, the effect of formation of the concentration depletion zone increases as the $R\beta$ / D value increases. For details, see Ref. 50. A positive effect can be expected as R increases, as β increases, or as D decreases. Therefore, when diffusion is slow in the solution and the crystal growth is fast and the crystal grows large, the more effects of the space experiment can be expected.

The authors devised an approximate expression [32] which roughly estimates D, and an experimental method which roughly estimates β, [51, 52]. In evaluating the results of the space experiment in JAXA using this index, when D/β was 3 mm or less, there was an effect of microgravity in about 70% of the crystals such as improvement of clustering and improvement of X-ray diffraction resolution [53].

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Based on the average value of the sizes of the generated crystals and the values of D and β, the $R\beta/D$ is calculated to be 0.035 or more. From this, it is considered that the effect of *IDZ* (suppression of impurity uptake) is dominant in crystals having sizes used for X-ray diffraction experiments. On the other hand, when R is large (about 1 mm or more) like in crystals for neutron diffraction experiments, both *PDZ* and *IDZ* effects can be expected.

5.3. Measures to enhance the effects of space experiments

From this estimation of the concentration depletion zone around the crystal, in order to positively enhance the effect of the space experiment, it is understood that D should be decreased and β should be increased. Since D depends on the viscosity of the solution, it is possible to use a highly viscous reagent such as PEG. Regarding the crystallization conditions including PEG, optimizing the salt concentration in the solution is very important when the condition is applied to various kinds of protein samples [9]. On the other hand, β is increased by refining the protein sample and increasing uniformity. For example, if lysozyme is purified by ion exchange chromatography to increase homogeneity, β becomes several times larger [52]. From these results, it is possible to determine in advance whether or not the effects of the space experiment can be expected by determining the values of D and β beforehand. It is also possible to improve the usefulness of space experiments by improving samples and crystallization conditions that are inappropriate for space experiments with these promotional measures [54, 55].

5.4. Analysis of transient crystal growth process

The solution concentration around the crystal in the actual protein crystallization is a transient process which decreases as the crystal grows. That is, when nuclei are formed and crystal growth starts, the degree of surface supersaturation is high, but at the end of crystal growth the protein concentration in the solution drops to the concentration of solubility. As a result, crystals will grow from center to surface all under different supersaturation degrees. In addition, the amount of impurities taken in varies depending on the location in the crystal.

The authors devised a numerical calculation model to understand the crystal growth process closer to actual crystallization [56]. For the sake of simplicity, partial differential equations describing both the diffusion process in the virtual sphere and the crystal growth process in the center of the sphere are described.

By applying various constants of the crystallization process of lysozyme to this model, the impurity concentration is low in the portion close to the center in the crystals grown in microgravity, and on the contrary, in the peripheral portion, the impurity concentration is higher than the crystals grown on the ground [56, 57]. In this simulation, β is set to a constant value, but, in reality, β increases as the impurity concentration decreases as described above. In fact, based on the results of the in-situ observation of the NanoStep project by Tsukamoto et al. [58], in the microgravity environment where IDZ is formed, as the impurity concentration decreases, it was shown that the crystal growth rate became faster than in the terrestrial environment and β became large. Therefore, the effect of PDZ and IDZ seems to be further enhanced in microgravity. From this result, it is suggested that there is a difference in the quality of the X-ray diffraction pattern of a crystal due to the difference in position in the crystal. Therefore, the authors investigated the local reflection of the crystal by growing crystals from a lysozyme sample containing some impurities and performed X-ray diffraction experiments to the crystals with a grid scan [57]. Although a preliminary experimental result, it was observed from the crystals obtained in the terrestrial experiment that the a and b axes of the crystal lattice become slightly larger going outward from the center of the crystal. On the other hand, such a phenomenon was not observed in the crystals obtained from the space experiment. Probably, crystals grown on the ground seem to have a large amount of impurities taken up around the center of the crystal, and these impurities have a larger influence on the lattice of the crystal toward the outside of the crystal.

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5.5. Other phenomena

Ng et al. reported on crystallization in a microgravity environment for 6 months using the CD method, and 2 mm square crystals were grown in a capillary with an inner diameter of 2 mm [36]. In this experiment, Ostwald ripening, the larger the crystal, the lower the solubility, resulted in the phenomenon that small crystals were dissolved and absorbed into large crystals. Therefore, it is necessary to investigate the extent to which this phenomenon can be applied to the formation of large crystals for structural analysis and the optimum conditions.

6. Finally

This paper summarizes the evaluation of the quality of protein samples and crystallization conditions and the handling of the obtained crystals based on decades of experience, especially that acquired from the JAXA PCG space experiments. These technologies still have room for improvement, and progress is being made daily. We hope that they will lead to the realization of practical and useful crystallization experiments.

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