Protein crystal growth with a two-liquid system and stirring solution

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We developed two novel methods for growing large, high-quality protein crystals. A two-liquid system enables the convenient extraction of protein crystals without causing mechanical damage due to growth at the interface between two liquids. Since this system does not require limitations on solution volume, it is also suitable for the seed technique, and for growing large crystals. Another new concept is the mild stirring of the solution using the Floating And Stirring Technique (FAST) and the Micro-stirring technique. When compared to conventional techniques, both techniques result in a reduced number of crystals, as well as the growth of large crystals.

Keywords: crystal growth, floating, stirring solution, seed technique, large crystal.

1. Introduction

Interest in growing protein crystals is related to determining the three-dimensional (3D) structure at atomic resolution by X-ray diffraction (XRD). The increasing need for this technique continues to intensify with rapid advances in biotechnology. However, an obstacle to structural determination by XRD is production of suitable diffraction-quality protein crystals, since it is difficult to grow and extract high-quality, but often very soft crystals.

Many protein crystallization techniques, such as hanging-drop and sitting-drop vapor diffusions, batch, microbatch, and dialysis, have been developed to meet the restricted conditions of protein crystal growth (McPherson, 1982, 1999; Chayen *et al.*, 1990; D'Arcy *et al*., 1996; Chayen, 1999). Each of these techniques has its merits and demerits. Therefore, we developed two novel methods, a two-liquid system and a stirring solution technique, to grow protein crystals.

2. Two-liquid system

2.1. Introduction

Protein crystals are very soft and fragile. When a protein crystal adheres to its growth vessel, it is impossible to remove individual crystals without causing mechanical damage, such as cracks and scratches. Additionally, the crystallinity of the adhered crystal is low (Higuchi *et al*., 1996). Although the hanging-drop method is favored over the sitting-drop method, it is not without drawbacks (Adachi & Takano *et al*., 2003a). Only small crystals can be obtained due to the limited drop volume. It is also difficult to automate hanging-drop experiments, whereas, the sitting-drop technique is easily automated. Nevertheless, both methods result in protein crystals that adhere to the growth vessel, making it difficult to remove them without causing mechanical damage. Therefore, an ideal technique would allow the growth of protein crystals that never contact the growth vessel.

We developed the two-liquid system, which easily achieves this ideal condition and can be applied to both the sitting-drop vapor-diffusion method and the batch method. We call these methods the "floating-drop vapor-diffusion technique" and the "two-liquid batch method" (Fig. 1).

The principle of the method is very simple, since it employs an insoluble and highly dense liquid. We selected Fluorinert^{TM} FC-70 (3M, USA), which has a high density (FC-70 is 1940 kg/m^3) and does not dissolve in most solvents. The solubility of FC-70 to water is less than 1%. It is transparent and colorless, and therefore, does not influence the observation of grown protein crystals. When a protein solution was added, it separated from this high-density liquid and floated on top of it, forming an interface. The protein crystals that grew at the interface did not contact the vessel, resulting in crystals with structurally perfect formation and improved crystallinity. Fluorinert^{TM} also facilitated easy extraction of the protein crystals after growth, thus avoiding any mechanical damage. In addition, a seed crystal could easily be moved from the interface of one vessel into another, if required. A containerless technique in a batch method (Lorber & Giege, 1996; Chayen, 1996, 1999) is similar to the two-liquid system. However, the two-liquid system is more suitable for seed method than the containerless technique.

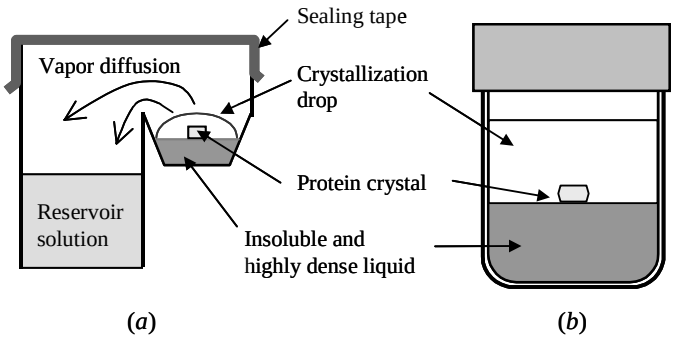


Figure 1

Schematic illustration of (a) the floating-drop vapor-diffusion technique and (b) the two-liquid batch method.

2.2. Floating-drop vapor-diffusion technique

Hen egg white lysozyme (HEWL), A32S mutant human lysozyme, and glucose isomerase crystals were grown using the floating-drop technique in the same manner as the sitting-drop technique. HEWL and glucose isomerase were purchased from Seikagaku Kogyo and Hampton Research, respectively. A32S mutant human lysozyme was prepared by the procedure previously described (Takano *et al*., 1995, 2001). The crystallization plate and sealing tape were purchased from Emerald BioStructures. All other chemicals were reagent grade. The protein solution was passed through 0.22 μm filters prior to crystallization.

HEWL was crystallized at a protein concentration of 25 mg/ml by the vapor diffusion in 0.6 to 1.0 M sodium chloride, 0.1 M sodium acetate, at pH 4.5. A32S mutant human lysozyme was crystallized at a protein concentration of 10 mg/ml by vapor diffusion in 1.5 to 3.0 M sodium chloride, 0.05 M sodium acetate, at

pH 4.5 (Takano *et al*., 1995; Yamagata *et al*., 1998). Glucose isomerase was crystallized at a protein concentration of 10 to 30 mg/ml by vapor diffusion in 5 to 15% PEG 6000, 0.2 M ammonium sulfate, pH 7 (Carrell *et al*., 1989).

Crystallization trials were set up using both the sitting-drop and floating-drop vapor-diffusion techniques at 20 °C. The volume of protein drops was 6 to 10 µl in both techniques, and the volume of Fluorinert™ was 5 to 10 µl in the floating-drop technique. Figure 2 shows the obtained glucose isomerase crystals (Adachi & Takano *et al*., 2003a). The shape of the crystals depended on the crystallization conditions, but the results exhibited no difference in crystal growth between the sitting-drop and floating-drop vapor-diffusion techniques. This indicated that Fluorinert™ did not affect crystal growth in either vapor-diffusion method. Additionally, Fluorinert™ did not contaminate the crystals, since the protein solution and Fluorinert™ liquid were not miscible. The floating-drop technique solves the problem of crystals adhering to the growth vessel that occurs in the sitting-drop technique, which in turn leads to the production of high-quality protein crystals suitable for XRD.

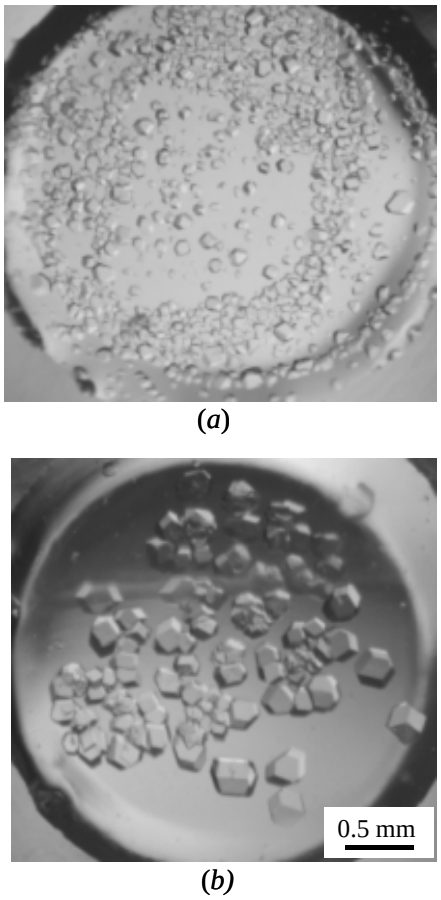


Figure 2

Crystallization results from the (a) sitting-drop and (b) floating-drop vapor-diffusion experiments for glucose isomerase (Adachi & Takano *et al*., 2003a).

2.3. Two-liquid batch method

We demonstrated how to grow a large HEWL crystal through the combination of a seed technique and slow cooling. The two-liquid system is particularly suitable for growing large, high-quality protein crystals because the volume of the protein solution can be increased considerably. Although small protein crystals are sufficient for XRD measurement, large protein crystals are needed for measurement of detailed XRD data, analysis of supramolecules (giant or complex molecules), and neutron diffraction. For example, measurement by neutron diffraction requires a crystal size of at least 1 mm^3 using current neutron sources. The two-liquid system enables seed growth that is already widely used to grow large crystals. However, it is not common in the field of protein crystal growth due to difficulty in handling protein crystals.

Using the two-liquid batch method, seed crystals were prepared by spontaneous nucleation (Adachi & Watanabe *et al*., 2002). The seed crystal (1.5 mm in length) was introduced into the solution at 22 °C and placed centrally at the interface between the two liquids. The concentration of HEWL in solution at this temperature was slightly higher than the solubility concentration (Sazaki *et al*., 1996). The seed crystal grew slowly in such a low supersaturation region, leading to high crystallinity in the grown crystal (Adachi *et al*., 1999). A constant crystal growth rate can be achieved by controlled cooling. The proper cooling rate was determined by observation of the crystal growth, and estimation of the supersaturation from the solubility data (Howard *et al*., 1988; Ataka *et al*., 1988; Sazaki *et al*., 1996). The crystal grew at a rate of 0.03 to 0.04 mm/day when the cooling rate was changed from 0.1 to 0.5 °C/day. A large crystal (2.5 mm in length) was obtained after 25 days of growth.

In summary, large protein crystals can be successfully obtained when the seed technique is employed in a two-liquid system and combined with the slow cooling method.

3. Solution stirring

3.1. Introduction

Stirring is widely used to control crystal growth by minimizing concentration gradient across the crystal surface, therefore one has more even growth and in theory higher quality crystals (Kamimura *et al*., 2001; Ono *et al*., 2002; Adachi *et al*., 1999). Without stirring, the growth solution may become inhomogeneous, resulting in problems. For example, excess solute that is not supplied to the seed crystal leads to additional spontaneous nucleation in the seed growth, which makes it difficult to control the growth rate of the seed crystal. We also have to stop the growth in order to prevent the seed crystal from forming poly-crystals. These phenomena make it inefficient to grow large protein crystals because of the uncontrollable short-period growth.

Crystallographers maintain that protein crystal growth must occur under "still" conditions, which is performed under microgravity conditions in order to reduce convective flow (Rosenberger *et al*., 1996; Chayen *et al*., 1997; McPherson *et al*., 1999; DeLucas *et al*., 2002). The effect of forced solution flow on the growth kinetics of protein lysozyme was also reported (Vekilov & Rosenberger, 1998). We assert that the protein solution must be homogenized to grow large, high-quality protein crystals, similar to the growing techniques to obtain inorganic and organic crystals. This is an entirely different approach than the conventional theory for growing protein crystals.

3.2. Floating and stirring technique

To promote protein crystal growth, it is necessary to stir the protein solution gently, since directly stirring the solution causes problems such as spontaneous nucleation, protein denaturation, and damaged crystals. We previously developed a unique stirring technique, which we call the Floating And Stirring Technique (FAST) (Adachi & Takano *et al*., 2002). FAST is a mild, indirect stirring method in

which the liquid of the lower layer is stirred (Fig. 3). This enables the growth of large, high-quality protein crystals using the two-liquid system and a magnetic stirrer.

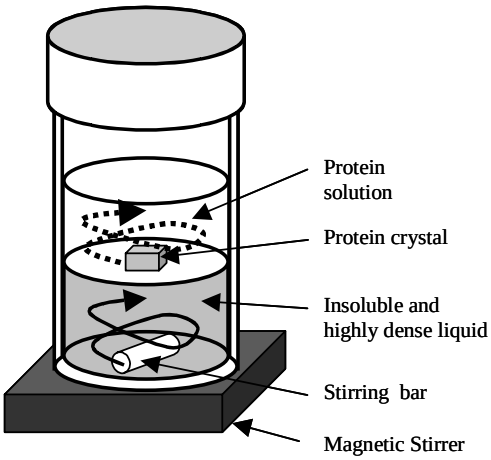


Figure 3

Schematic illustration of the Floating And Stirring Technique (FAST).

3.3. Micro-stirring

We propose another novel approach to mildly stirring both protein solution and multiple micro-scale samples. We call this method the "Micro-stirring" technique (Adachi & Takano *et al*., 2003b). The principle is very simple. A rotating or vibrating shaker shakes crystallization plates in the sitting-drop or floating-drop vapor-diffusion technique (Fig. 4).

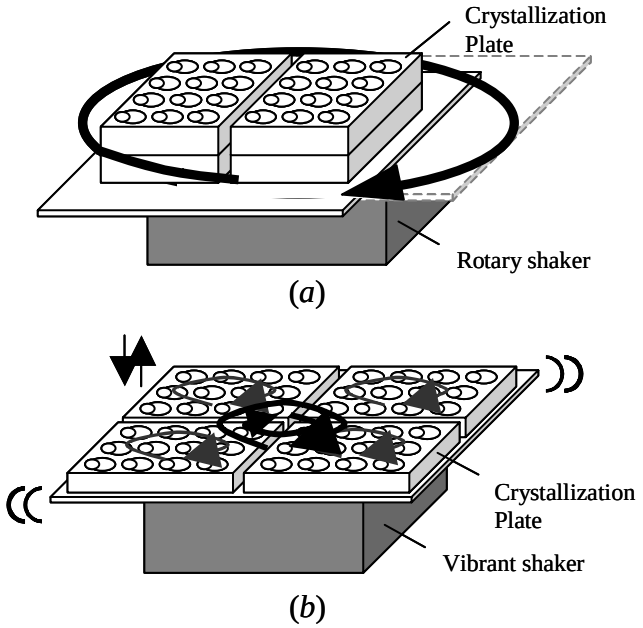
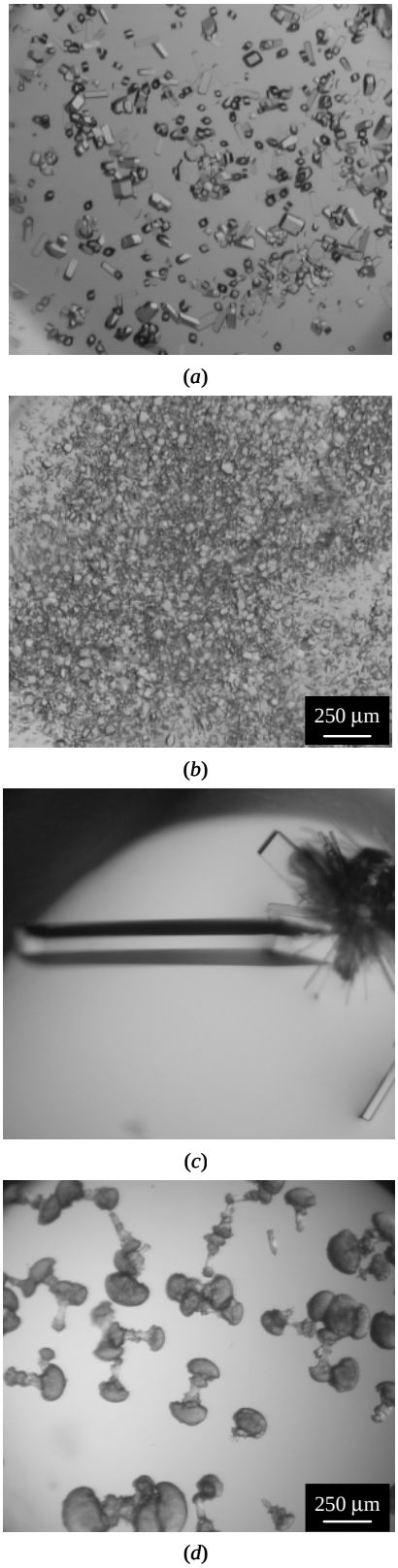


Figure 4

Schematic illustration of the Micro-stirring technique showing (a) rotating and (b) vibrating methods.



(*a*) (*b*) (*c*) (*d*)

Figure 5

Crystallization results from the sitting-drop vapor-diffusion experiments for HEWL and wild type human lysozyme. (a) With rotary shaking. (b) Without shaking for HEWL. (c) With vibration shaking. (d) Without shaking for wild-type human lysozyme.

We applied the Micro-stirring technique to the growth of HEWL and wild-type human lysozyme crystals. The crystallization condition of HEWL was the same as described in section 2.2 above. Wild-type human lysozyme was crystallized at a protein concentration of 10 mg/ml by vapor diffusion in 4.0 M sodium chloride, 0.05 M sodium acetate, at pH 4.5. The trials were set up in both shaken and unshaken conditions of the sitting-drop and floating-drop vapor diffusion techniques. For Micro-stirring, we used a 50 rpm rotation (175 rpm vibration) speed to mildly stir the protein solution in the rotating (vibrating) shaker. Figure 5 shows the resulting crystals obtained within a couple of days using a 3 μl protein solution. The results show differences in shape, number and size of nucleated crystals for shaken and unshaken plates. These observations indicate that homogenizing the protein solution by shaking the plates tends to prevent spontaneous nucleation. When compared to the conventional vapor-diffusion technique, the Micro-stirring technique is extremely effective in reducing the number of crystals and encouraging the growth of large crystals.

We measured the XRD data of wild-type human lysozyme crystals. Figure 6 shows the XRD pattern of the wild-type human lysozyme crystal grown by the Micro-stirring technique. The data were collected at 100 K at the SPring-8 on beam line 44XU. The crystal to detector distance was set at 290 mm, and the crystal oscillation angle per image was set at 1°. The crystal diffracted beyond 1.5 Å resolution. Conventional theory asserts that stirring a solution is useless for growing protein crystals, and will result in inferior crystallinity. However, we obtained excellent resolution that was nearly the same as the crystal grown using the conventional technique (Adachi & Matsumura *et al*., 2003). We anticipate that stirring the protein solution will improve crystallinity, as shown in the case of growing inorganic and organic crystals.

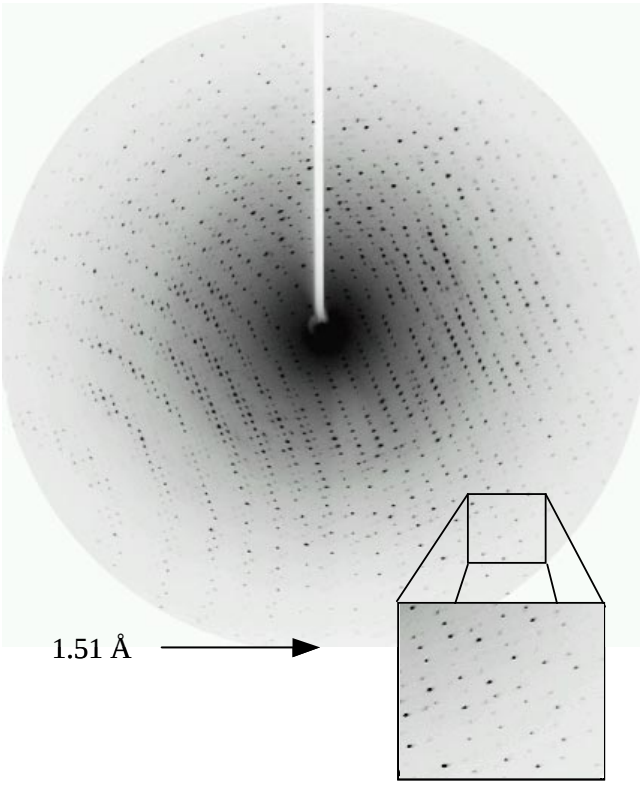


Figure 6

XRD pattern of the wild-type human lysozyme crystal grown by the Micro-stirring technique. The crystal diffracted to a resolution of 1.5 Å.

4. Conclusion

In this study, we demonstrated an easier and more effective protein-crystal growth technique by developing a two-liquid system and the Micro-stirring technique. These new methods enabled the production of large, high-quality protein crystals for XRD measurement. We therefore predict that these techniques will become the more popular methods for protein crystal growth and will accelerate structural genomics and structure-based drug discovery in the near future.

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