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Optimizing protein crystal growth through dynamic seeding

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A dynamic seeding method that is different from the conventional method of seeding drops that have been equilibrated is described. The method basically consists of two steps. Firstly, microseeding was used in association with adjustment of the seeding-drop components, including buffer, additive and concentrations of the precipitants and protein, in order to screen suitable seeding conditions under which microseeds are seeded into a new non-equilibrated drop as the dynamic macroseed drop for the following step. Secondly, after being equilibrated for various times against the reservoir solution, the macroseed drops were used to prepare a dilution series with which the qualified crystals could be harvested using macroseeding. Compared with a conventional seeding technique, this method is distinct with a dynamic situation of macroseed drops before macroseeding and a non-equilibrium serial seeding where all the seeds are seeded into new non-equilibrated drops and the micro/macroseeding are efficiently combined into a whole system. The method simplifies control of the number of microseeds because an excess of microseeds has little effect on the final result. The method also simplifies the manipulation of macroseeds by optimizing the equilibration time and the dilution multiple of the macroseed drops before macroseeding. This dynamic seeding technique has been used in the crystallization of novel protein CutCm, which has a fast crystal-growth rate, and proved that the method is useful for optimizing protein crystallization.

1. Introduction

Seeding provides a template for the separation of the crystal nucleation and growth phases, where previously nucleated crystals are used as seeds and introduced into new drops to grow. Recently, seeding has been critical for obtaining diffraction-quality crystals for many structures. Several methods of seeding are currently used. Macroseeding techniques introduce a single crystal (macroseed, usually 5–50 μm) into a pre-equilibrated protein solution, which involves repeatedly washing the single crystal (Stura, 1999; Bergfors, 2003). In microseeding, submicroscopic crystals (microseeds) are introduced into the protein solution, which traditionally involves pulverizing the existing crystals to prepare the seed stock and then diluting the seed stock for seeding (Stura, 1999; Bergfors, 2003). The advantages and disadvantages of different pulverization methods have been discussed (Luft & DeTitta, 1999). Streak seeding techniques, technically a microseeding method, usually use an animal whisker to touch or stroke over the surface of the protein crystal and then draw through the protein drop (Stura & Wilson, 1991; Stura, 1999). Other seeding techniques include epitaxic seeding, where the nucleating agents are introduced into the growth condition (D'Arcy *et al.*, 2003; McPherson & Shlichta, 1988), and *situ* seeding, where the original drops are diluted into the metastable zone after incubating them for a given time so that no new nuclei will be formed but the existing ones will continue to grow (Saridakis *et al.*, 1994; 2002). Lately, a seeding technique termed 'microseed matrix screening' has been reported (Ireton & Stoddard, 2004), in which microseeds from the nucleation step are systematically seeded into new conditions where all components of the drop are allowed to vary so as to screen for subsequent growth of well ordered crystals. The preparation and subsequent three-dimensional growth of the seeds are commonly

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involved in all seeding methods described above. Therefore the quality of the seeds is very critical for each seeding method, which could be improved by serial seeding (also called repeated seeding), where crystals resulting from the first round of experiments are used as the seeds of the next round (Bergfors *et al.*, 1989; Rouvinen *et al.*, 1990*a*,*b*).

In practice, the microseeding or streak seeding and macroseeding are often used separately as the general seeding techniques, in which seeding drops have been equilibrated. Usually, microseeding or streak seeding is considered as an easy and efficient method and therefore normally tried first in crystallization, while it is difficult to control the number of seeds that are transferred in the procedure (Bergfors, 2003). Macroseeding is a powerful tool for enlarging the size of the crystal. However, it needs much more labor in handling because it requires tedious transfers of the parent crystal through multiple washes and other manipulations. In addition, during these manipulations of macroseeding, the macroseed could get damaged sometimes, causing unintentional showers of microcrystals (Stura, 1999). Here, we report an extension method termed 'dynamic seeding' which is different from conventional seeding techniques and has successfully been used in optimizing the crystallization of the novel protein CutCm with a fast crystal growth rate. The dynamic seeding can be divided into two sequential steps: firstly, using microseeding to refine the seeding condition with which the macroseed drop was prepared; secondly, using macroseeding to obtain the qualified large single crystals. Briefly, the method is a non-equilibrium serial seeding where all the seeds are seeded into newly non-equilibrated drops and the equilibration times of the dynamic macroseed drop produced by microseeding are optimized before macroseeding. Compared with the conventional seeding techniques, the method simplifies the control of the number of microseeds and the manipulation of macroseeds. As a result, the method significantly improves the diffraction quality of the CutCm protein crystal and accelerates the optimizing speed.

2. Experimental procedures

2.1. Materials

The putative copper homeostasis protein (CutCm) used in crystallization was expressed from the CutCm gene of *Shigella flexneri* 2a.301, which consists of 248 residues and belongs to the CutC family (Jin *et al.*, 2002; Bateman *et al.*, 2004). The protein was purified with Ni-NTA (Novagen) and AKTA purification system (Amersham Pharmacia) (Zhu *et al.*, 2005). The protein was concentrated to approximate 15 mg ml^{-1} in 50 m*M* Tris–HCl pH 8.0 and stored at 253 K. Protein concentrations were determined by absorbance at 280 nm, assuming an A_{280} of 0.506 for a 1.0 mg ml^{-1} solution. A probe for seeding is easily made with a cat whisker mounted with wax to the end of a glass tube (Stura, 1999). The Crystal Screen and Crystal Screen 2 used in the initial crystallization (Jancarik & Kim, 1991) and the utensil used for smashing crystals were from Hampton Research, USA. The polyethylene glycols were from Fluka, Switzerland. All other reagents were from Sigma–Aldrich, Germany.

2.2. Initial crystallization

The initial screening conditions were Crystal Screen and Crystal Screen 2 (Hampton Research). The crystallization experiments were conducted using the hanging-drop vapor-diffusion method at 293 K (McPherson, 1999), with 2 µl drops containing 1 µl 10 mg ml^{-1} protein and 1 µl reservoir solution suspended over 0.5 ml reservoir solution. After two days, crystals were observed in three different conditions: Crystal Screen No. 9 [30%(*w*/*v*) polyethylene glycol 4000, 0.2 *M* ammonium acetate, 0.1 *M* tri-sodium citrate dihydrate pH 5.6], 15 [30%(*w*/*v*) polyethylene glycol 8000, 0.2 *M* ammonium sulfate, 0.1 *M* sodium cacodylate pH 6.5] and 46 [18%(*w*/*v*) polyethylene glycol 8000, 0.2 *M* calcium acetate hydrate, 0.1 *M* sodium cacodylate pH 6.5]. The No. 9 and 15 conditions produced needle-shaped clusters of crystals, while No. 46 resulted in many small block-shaped crystals with heavy precipitates (Fig. 1*a*). The precipitants of the three conditions were polyethylene glycol (PEG). These conditions were optimized at 277 or 293 K by varying the precipitants (PEG 400–10000), the additives, pH values (4.0–9.0) and the protein concentration. The additives used for screening include organic solvent (glycerol, 1,6-hexanediol, ethanol and 2-propanol), chelator (EDTA), reducing agents (β-mercaptoethanol, DTT) and a series of salts which are composed of alternative anions (chloride, formate, acetate, phosphate, sulfate, citrate, nitrate and tartrate) and cations (sodium, potassium, ammonium, lithium, magnesium, calcium, zinc). In the prime optimization, all concentrations of the organic solvent and salts are 5%(*v*/*v*) and 0.2 *M*, respectively. But the concentrations of the salts are varied from 0.05 *M* to 0.4 *M* in the further optimization. It is found that the salts, EDTA, reducing agents (β-mercaptoethanol, DTT) and the buffer pH could significantly influence the crystal growth of CutCm, while the organic solvent did not effect it. With EDTA or reducing agents, it is difficult to produce the crystals but easy to produce heavy precipitates. When the cations of salts are single valence, the crystals produced by these conditions often were needle-shaped and difficult to grow to single crystals. Even some which looked like single needle-shaped crystals under the microscope were not able to diffract to X-rays. When the cations of salts are magnesium or calcium with pH (5.4–8.0), many small single block-shaped crystals were often quickly produced accompanied by heavy precipitates and were difficult to grow large. The larger single crystals (Fig. 1*b*) with heavy precipitates at a very low reproducibility were obtained from the following refined conditions: 14–16%(*w*/*v*) PEG 8000, 0.1–0.25 *M* calcium chloride/acetate and 0.1 *M* sodium cacodylate pH (6.0–6.3) with 5 mg ml^{-1} protein. However, these large block–shaped crystals also diffracted very poorly (below 5 Å). Subsequently either precipitates or many small crystal clusters were produced through the general microseeding technique (Stura, 1999).

2.3. Dynamic seeding

In order to improve the crystal quality, a dynamic seeding method derived from the conventional seeding technique was used for optimizing the crystallization of protein CutCm with the fast crystal growth rate. Briefly, the method is a non-equilibrium serial seeding method that can be divided into two sequential steps. In the first step, named the microseeding stage, microseeding was used in association

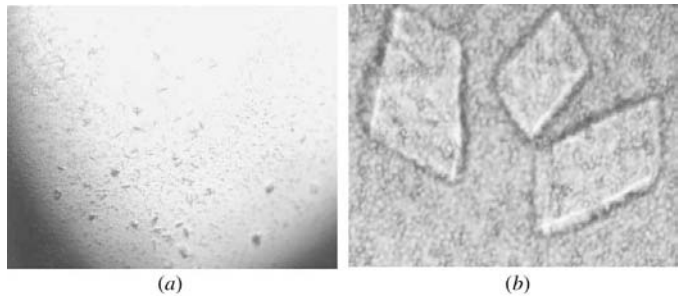

Figure 1
Crystals of CutCm before (*a*) and after (*b*) optimization (approximate dimensions 0.25 × 0.20 × 0.04 mm)

with adjusting the seeding drop components, including buffer, additive, concentrations of the precipitants and protein, to screen a suitable condition under which the microseeds are seeded into a new non-equilibrated drop, which will then be chosen as the dynamic macroseed drop for the following step. In the second step, named the macroseeding stage, after equilibrating for different times against the reservoir solution, the macroseed drops were used to prepare a dilution series, with which the qualified crystals were obtained by using macroseeding. The experiment temperature is set to 293 K since the crystals often twin at 277 K.

2.3.1. Microseeding stage. The needle and block-shaped crystals from the refined conditions were crushed separately by the microneedle tool (Hampton Research) after transferring to 25%(*w*/*v*) PEG 8000 solution, which were chosen as the microseed stocks. Then the cat whisker correspondingly was drawn across the microseed stocks and soon through the newly mixed non-equilibrated drops containing 1 µl of 5–15 mg ml^{-1} protein and 1 µl of the different test solutions (7–22% PEG 8000, 0.1 *M* buffer pH 5–8, 0.05–0.4 *M* ammonium, calcium and magnesium salts). Judged by the shape of the crystals produced and the reproducibility, it was found that the block-shaped crystals were a better seed source and the suitable seeding conditions were 6–8 mg ml^{-1} protein, 12–15%(*w*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.1, 0.1–0.2 *M* calcium chloride. Under these conditions ordered crystals (Fig. 2*a*) were produced with a good reproducibility. Especially, small single crystals (>5 µm) appeared 1 h later with the seeding recipe of 6 mg ml^{-1} protein, 14%(*w*/*v*) PEG 8000, 0.1 *M* sodium cacodylate pH 6.1, 0.15 *M* calcium chloride, thus these are taken as the optimizing seeding conditions. The optimizing reservoir solution was chosen for all the following steps. Then with the cat whisker the submicroscopic seeds were transferred from the microseed stock described above into new mixed non-equilibrated drops containing 2 µl of 6 mg ml^{-1} protein and 2 µl of the reservoir solution to give rise to the macroseed drops for the following step.

2.3.2. Macroseeding stage. The macroseed drop was suspended over 0.5 ml reservoir solution and equilibrated for different times. When the equilibration times of the macroseed drop were respectively at 1, 2, 4, 12, and 24 h, 0.5 µl of the macroseed drop was picked up to mix with 4.5 µl reservoir solution and then diluted into the range 10^{-1} to 10^{-8} by a repeated procedure with picking up 0.5 µl of the drop from the foregoing mixed solution to continuously mix with the next 4.5 µl reservoir solution. After the dilutions, the pipette tip with the 0.5 µl drop with small crystals was reversed, and then its top was made to slightly touch across the surface of the new mixed non-equilibrated drop containing 2 µl of 6 mg ml^{-1} protein and 2 µl of the reservoir solution on the cover slip. Finally, the cover slip was covered on the well with 0.5 ml reservoir solution.

3. Result and discussion

The larger perfect single crystals were obtained within 2–3 d (Fig. 2*b*) when the equilibration time of the macroseed drop was 2 h and the macroseed drop was diluted to 10^{-4}-fold. These crystals diffract to better than 2.5 Å resolution on an in-house X-ray source (Rigaku R-AXIS IV^{++} image plate, Cu $K\alpha$ radiation from a rotating anode operating at 40 kV and 20 mA with 0.1 mm cofocus incident beam diameter) and higher than 1.8 Å resolution with synchrotron radiation (BL6A, PF, Japan).

In the dynamic seeding technique, all the seeds are seeded to newly non-equilibrated drops and the macroseed drops are also dynamic before macroseeding. If the seeding drops were pre-equilibrated before seeding, either precipitates or many small crystal clusters were produced in the first step. In this case the macroseeds would be difficult to grow up to be larger or be covered by other small crystals in the second step. In this method, micro- and macroseeding are efficiently combined into a whole system. In fact, the microseeding is very important in the procedures, which mainly have three functions, including screening the seeding conditions for optimizing, improving the quality of seeds and preparing the dynamic macroseed drops for the following step.

Evidently, the quality of crystals finally produced by the method depended on the equilibration times and the dilution multiple of the dynamic seed drop before seed transfer. The best equilibration times are around 2 h and the best multiple range for the dilution is 10^{-4}–10^{-5}. The experiments showed that either precipitates (the crystal seeds dissolved) or the crystals with heavy precipitates (Fig. 3*a*) were finally produced when the equilibration time $\leq$1 h because the seeds were so 'young' at the time. While the crystals grown slowly with a poor diffraction ability (about 3.5 Å resolution) when the equilibration time $\geq$4 h because the seeds were so old (Fig. 3*b*). When the multiple for the dilution $\geq 10^{-3}$, many small crystals were easily produced, which may be because many seeds were seeded into the new drop by macroseeding. When the multiple for the dilution $\leq 10^{-6}$, the precipitates or the crystal clusters with heavy precipitates were produced because the number of macroseeds transferred was not enough and the macroseeds were easily etched by the newly no-equilibrated drop after more transfers. Furthermore, the origin of the microseeds should also be taken into consideration in the first step of the method. When the needle-shaped crystals were chosen as the microseeds source, it was very difficult to obtain good ordered single crystal, though we have tried to make seeding with many additional conditions to that in optimization of initial crystallization described in §2.2.

Compared with conventional seeding techniques, the method is distinct with a dynamic situation of macroseed drops before macro-

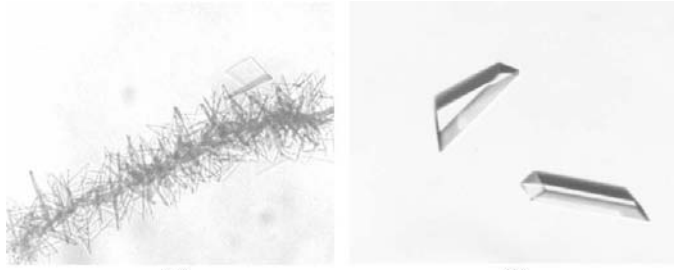

Figure 2
(*a*) The small crystals produced by microseeding; (*b*) the crystals qualified for X-ray diffraction produced by the dynamic seeding method (approximate dimensions 0.15 × 0.07 × 0.05 mm)

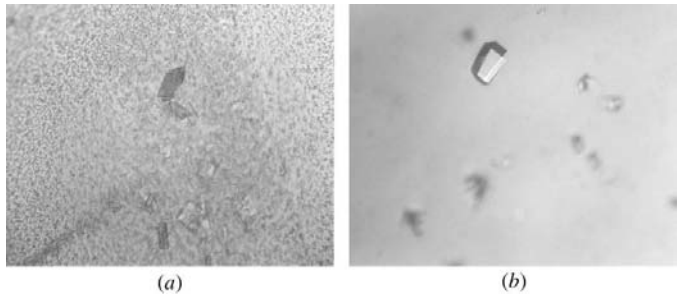

Figure 3
The crystals of CutCm produced by the dynamic seeding method at the different equilibration times of macroseed drop. (*a*) The equilibration time was 1 h; (*b*) The equilibration time was 4 h.

seeding and a non-equilibrium serial seeding where all the seeds are seeded into new non-equilibrated drops and the micro/macroseeding are efficiently combined into a whole system. The method simplifies the control of the number of microseeds because an excess of microseeds to a certain extent has little effect on the final result, and thus the result of the microseeding can be evaluated fast. The method also distinctly simplifies the manipulation of macroseeds by optimizing the equilibration time and the dilution multiple of the macroseed drops before macroseeding, and thus saves the labor of washing for macroseeds.

The dynamic seeding can be successfully used in optimizing the CutCm protein crystal due to the fast rate of the protein crystal growth. We can usually obtain the qualified crystals within 2–3 d as scheduled. Certainly, some more proteins need to be tested with this method so as to assess whether it is generally useful for the effective optimization of protein crystals.

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