Journal of Crystal Growth ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Journal of Crystal Growth

journal homepage: www.elsevier.com/locate/jcrysgro

A crystallization technique for obtaining large protein crystals with increased mechanical stability using agarose gel combined with a stirring technique

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ARTICLE INFO

Communicated by T. Paskova

Keywords:
A1. Fluid flows
A1. Nucleation
B1. Proteins
Nucleation control
Crystallization technique

ABSTRACT

We developed a protein crystallization technique using a 0.0–2.0 w/v% agarose gel solution combined with a stirring technique for the purpose of controlling the crystal number in the gelled solutions. To confirm the stirring effect in the gelled solution, we investigated the nucleation probability and growth rate of the crystals produced using this method. The stirring operation by a rotary shaker affected the behavior of protein molecules in the gelled solution, and both a significant decrease in the nucleation rate and an enhancement of the crystal growth rate were achieved by the method. As a result, we concluded that the proposed technique, the stirring technique in a gel solution, was effective for generating protein crystals of sufficient and increased mechanical stability.

1. Introduction

Determination of the three-dimensional (3D) structures of protein molecules is crucial for structure-guided drug design and controlled drug delivery. X-ray diffraction studies and neutron diffraction studies are widely used to determine the 3D structures of protein molecules, but cannot be applied to crystals of insufficient size or quality. Therefore, the difficulty of generating large high-quality protein crystals remains a bottleneck in the field of molecular structure study. In addition, the fragility of protein crystals limits the success rate of structure analysis, because fragile crystals can be damaged during the preparation process before X-ray or neutron diffraction analysis. To overcome these problems, there is need of a crystallization technique that achieves protein crystals of sufficient quality, size and physical strength.

Many crystallization techniques have been investigated, including the batch method, vapor diffusion method [1], the counter-diffusion method [2], the microseeding method [3], crystallization in hydrogel [4–10], or crystallization under microgravity [11–13], a magnetic field [14,15], or forced solution flow [10,16–28]. When using these protein crystallization techniques, controlling the solution flow is one of the most important parameters [17,18,24,29], and several groups have developed practical techniques for this purpose.

Establishing further practical and effective crystallization techniques, we have to get protein crystals to overcome handling damages before X-ray diffraction experiment including the soaking process using cryo-protectant solutions and radiation damage during X-ray diffraction experiment. Crystallization techniques using hydrogel are useful to overcome these problems. Among

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http://dx.doi.org/10.1016/j.jcrysgro.2015.11.008
0022-0248/

typical gels used for protein crystallization, agarose gels are the most employed hydrogels in protein crystallization because of its stability, easy for use and high familiarity for biochemists [6]. Crystallization using agarose gel has some advantages; the nucleation enhancement effect [7,30], the impurity filtering effect during crystal growth [31], the suppression of heat convective transport and crystal sedimentation [32], and the enhancement of crystal strength by incorporation of agarose gel fibers into protein crystals [6,8]. Especially the enhancement of crystal strength by agarose gel has great advantages for overcoming handling damage before X-ray diffraction studies or osmotic shock damage during ligand soaking experiments [8,9]. In fact many crystals including model proteins had been improved by the agarose gel crystallization techniques [8,33]. Contrary to the very useful aspects, the batch method combined with agarose gel occasionally increases nucleation probability too much, as a result problems such as size degradation of each crystals and/or generation of polycrystals occur. The agarose gel method combined with the counter diffusion method is one of a useful technique to get fine crystals, though there arise some crystal size variation due to the gradation of protein and precipitant concentration [34]. In this study, resolving the problem that too much nucleation and the size degradation, we combined the hydrogel crystallization with the stirring technique [35–37] which enables us to decrease the nucleation probability and increase the size of each crystal. Gel existence must inhibit solution convection and forced solution flow as it reported [32]. However, from the view point of the pore size of agarose gel, it is enough larger than protein single molecules [38,39] and that is why protein crystals can grow in high concentrated agarose gel media. Considering the relation between the agarose gel pore size and protein molecule size, we thought that there is possibility to affect the protein molecules in agarose gelled solution by stirring operation. To test the effect of the stirring technique on gelled solution, we used the findings that appropriate solution stirring made delay of nucleation and the number of crystals was finally lowered. Using this newly developed crystallization technique incorporating the stirring of a gel solution, we achieved effects comparable to those from either of the previous methods used singly.

2. Experiments

2.1. Materials

Three-times recrystallized hen egg-white lysozyme (HEWL) from Wako Co. (Catalog no. 120-02674) was used without further purification. Sodium chloride and sodium acetate were purchased from Wako Co., Japan (Catalog nos. 192-13925 and 192-01075, respectively). Agarose was purchased from Wako (Catalog no. 50101). The gelling temperature of the agarose was 299–303 K. We used 1 ml glass vials for crystallization. The 0.2 μm pore size filters were purchased from Advantec (Catalog no. 25CS020AS).

2.2. Crystallization

Six percent (w/v) agarose solutions were prepared by slowly stirring agarose powder in water at room temperature and then heating at 373 K. The 6% (w/v) agarose solutions were stored at 277 K. Before setting up the crystallization trials, the agarose gel was reheated at 368 K and diluted by 2–5% using a buffer containing 0.1 M NaAc (pH 4.5), and then the liquids were kept at 318 K. Lysozyme solution was prepared by dissolving the HEWL powder in the 0.1 M NaAc buffer, then filtering using 0.2 μm pore size filters. The concentration of the lysozyme solution was checked by absorbance measurement at 280 nm once, then diluted using the 0.1 M NaAc buffer by the concentration of 75–125 mg/ml. NaCl solutions (12–15 wt%) were also prepared by dissolving sodium chloride in the 0.1 M NaAc buffer. The protein solutions in 0.1 M NaAc buffer and the NaCl solutions in 0.1 M NaAc buffer were kept at 318 K using the block incubator just before mixing. We mixed these solutions appropriately for adjusting the final crystallization solutions (Table 1).

Table 1
The summary of solution conditions.

Solution no.	Agarose gel conc. (%)	HEWL conc. (mg/ml)	NaCl conc (%)	Buffer
1	0.0	25.0	3.0	0.1 M NaAc (pH 4.5)
2	0.5			
3	1.0			
4	2.0			

2.3. Experimental setup

All of experiments were conducted by batch method. The crystallization solutions were dispensed into 1 ml glass vials. The lysozyme solutions were kept at 318 K during dispensing it, then once replaced in room temperature. The volume of each solution was 0.3 ml. We prepared two vials for each condition. The vials were placed on a specially designed rotary shaker (SH-BD04 by SOSHO Inc.), and fixed using a pad with sticky, then the rotary shaker was rotated at either 0, 25, 50 and 75 rpm. The rotation radius is about 2 cm. The rotary shaker can give the glass vials mild rotation movement in a horizontal direction. We have to emphasize that each solution in glass vial was not stirred directly, but the glass vials were set in mild rotation movement in a horizontal direction. This protocol will be referred to as “the stirring operation” hereinafter. The picture and the schematic of the experimental setup are shown in Fig. 1(a) and (b). All parts of the experimental setup, including the crystallization solution in vials, were incubated at 21 ± 0.5 °C. The rotary shaker does not give serious temperature increase by its motor movement. Fig. 1(c) is time dependences of temperature just on the rotary shaker in 75 rpm operated in the incubator. The time dependence of temperature of inside of the incubator without rotary shaker movement is shown as a reference. The time dependence on temperature is stable and it is obvious that the motor movement does not increase temperature around the crystallization glass vials.

As a next try, we conducted a scale down experiment using another crystallization plate (Imp@ct plate Catalog no. HR3-100 by Hampton Research) for the use of sample-limited case. The basic protocol of the gelled protein solution preparation is same as mentioned above. The agarose gel concentration also ranged from 0–2.0 w/v%. The solution volume for each batch was 6 μl. The batch number of each condition was 20–24. We used the same rotary shaker and the incubator. The rotary shaker speed was 0 and 50 rpm.

2.4. Evaluation of the number of crystals

At five days after the start of the crystallization, we stopped the rotary shaker and moved each vial to the stage of a microscope. In the case of agarose gel solutions (0.5%, 1.0% and 2.0%), crystal sedimentation was suppressed by agarose gel and crystals stay almost at each nucleation position [32]. Thus we observed each vial from top and lateral directions and got some cross section pictures. Using all the images, we can count the number of crystals which were focused, then we summed up the total number of crystals. In the case of solutions without agarose gel, the crystals