A simplified counter diffusion method combined with a 1-D simulation program for optimizing crystallization conditions

Hiroaki Tanaka,[1,2] Koji Inaka,[3] Shigeru Sugiyama,[3] Sachiko Takahashi,[2] Satoshi Sano,[1] Masaru Sato,[1] and Susumu Yoshitomi[1]

[1]Space Utilization Research Center, National Space Development Agency of Japan, Tsukuba, Ibaraki, 305-8505, Japan, [2]Japan Space Utilization Promotion Center,Shinjyuku-ku, Tokyo, 169-8624, Japan, [3]Maruwa Food Industries, Inc., Yamatokoriyama, Nara, 639-1123, Japan. E-mail: PXW01674@nifty.ne.jp; FAX: +81-3-5273-0705

We developed a new protein crystallization method using a simplified counter-diffusion method for optimizing crystallization condition. It is composed of only a single capillary, the gel in the silicon tube and the screw-top test tube, which are readily available in the laboratory. The one capillary can continuously scan a wide range of crystallization conditions (combination of the concentrations of the precipitant and the protein) unless crystallization occurs, which means that it corresponds to many drops in the vapor-diffusion method. The amount of the precipitant and the protein solutions can be much less than in conventional methods. In this study, Lysozyme and alpha-Amylase were used as model proteins for demonstrating the efficiency of this method. In addition, 1-dimensional (1-D) simulations of the crystal growth were performed based on the 1-D diffusion model. The optimized conditions can be applied to the initial crystallization conditions for both other counter-diffusion methods with the Granada Crystallization Box (GCB) and for the vapor-diffusion method after some modification.

Keywords: counter-diffusion technique; capillary; gel; silicon tube; 1-D simulation.

1. Introduction

New crystallization technique, called the counter-diffusion method inside a capillary inserted into gel layer was introduced (Garcia-Ruiz & Moreno, 1994). It is an excellent crystallization method, since diffusion of precipitant solution into and through the gel layer regulates the delivery of precipitant to the protein solution and forms a precipitant concentration gradient from end to end in the capillary (McPherson, 1999). Diffusion of the protein solution into the gel layer also helps form a protein concentration gradient in the capillary, though diffusion of the protein solution usually much slower than that of the precipitant solution. Thus, the concentration gradients of the precipitant and the protein solutions are formed in one capillary from opposite directions. The optimum crystallization conditions in the capillary could be found slowly, and crystallization would occur gently. This type of experiment can explore a wide range of crystallization conditions in one single experiment, so that there is a higher possibility of obtaining crystals in a single experiment than with conventional vapor-diffusion methods.

Recently the device whose name is Granada Crystallization Box (GCB) became commercially available for crystallization experiments using this technique (Garcia-Ruiz, 1991, Garcia-Ruiz *et al*., 2002). It consists of a simplified flat polystyrene box with six capillaries. A main feature of GCB is that there is a gel layer between the protein solution and the precipitant solution. The melted gel solution is poured into the bottom of the GCB, and the protein solutions in the capillaries (six for one GCB) are thrust into the gel layer, on which the precipitant solution is poured. Both the protein and the precipitant solutions diffuse through the gel over a period of time, and the crystallization occurs in the capillary. However, it is better if we can optimize the crystallization conditions beforehand, because the GCB with a long capillary takes more time to crystallize, requires larger amounts and higher concentrations of precipitant solution than the conventional methods, and requires melting the gel for each preparation. It is also inconvenient to use the same precipitant solution for six capillaries in one box.

In this study, we introduce a simplified experimental device that includes a shorter capillary, the gel in the silicon tube, and a screw-top test tube. All the materials we use in this method are readily available in the laboratory. Lysozyme and alpha-Amylase were chosen as model proteins for the crystallization experiments because Lysozyme is the most studied protein for crystal growth experiments and alpha-Amylase is a representative of many physiologically significant proteins. Before carrying out the preliminary experiments, we performed 1-D simulation of the continuous concentration change in the capillary using a computer program we developed. Comparing the experimental results of the crystallization in the capillary with this 1-D simulation, enables estimating the optimal condition in which the crystallization occurs. This optimized condition can be applied for the preliminary experiment for the GCB as well as vapor-diffusion devices.

2. Materials and methods

2.1. Simplified counter-diffusion technique

Fig. 1 depicts the configuration of the simplified counter-diffusion method. The device is composed of a 60mm capillary, 1.0% (w/v) agarose gel in a 15mm silicon tube and a 15ml screw-top test tube.

2.2. Sample preparation

2.2.1. Gel-tube preparation

First, agarose (Agarose-III: Wako Pure Chemical Industries) was prepared by mixing the appropriate volume of distilled water with agarose powder with continuous stirring to obtain a final agarose concentration of 1.0% (w/v) with 0.04% NaN_3. The mixture was then heated to 368K, while stirring, at which point the solution became clear. It was then cooled to 313K. The prepared agarose solution was sucked into the silicon tube (Kaneka Medics; bore diameter 1mm, external diameter 2mm) as shown in Fig. 2a. After the agarose gel is fixed in the silicon tube, the tube can be stored for further experiments. When the experiments were ready to be set up, the tube was cut to a 15mm length. The gel can be equilibrated by soaking in a suitable buffer beforehand

2.2.2. Protein solution preparation

We prepared 100mg/ml Hen egg white lysozyme in a 50mM acetate buffer at pH 4.5 and 90mg/ml alpha-Amylase derived from *Aspergillus oryzae* in a 50mM acetate buffer at pH 6.0 for the model experiments.

2.2.3. Precipitant solution preparation

Approximately 3ml of the precipitant solution was poured into the 15ml screw-top test tube. We then prepared a 20% (w/v) NaCl in a 50mM acetate buffer at pH 4.5 as a precipitant for Lysozyme crystallization. Next we prepared a 40% (w/v) PEG8000 in a 50mM

 © 2004 International Union of Crystallography • Printed in Great Britain – all rights reserved

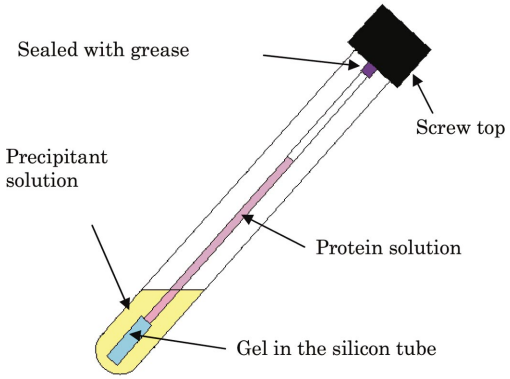

Figure 1 Configuration of the simplified counter-diffusion method. This device includes the shorter capillary, gel in a silicon tube and a screw-top test tube. All the materials in this device are readily available in the laboratory.

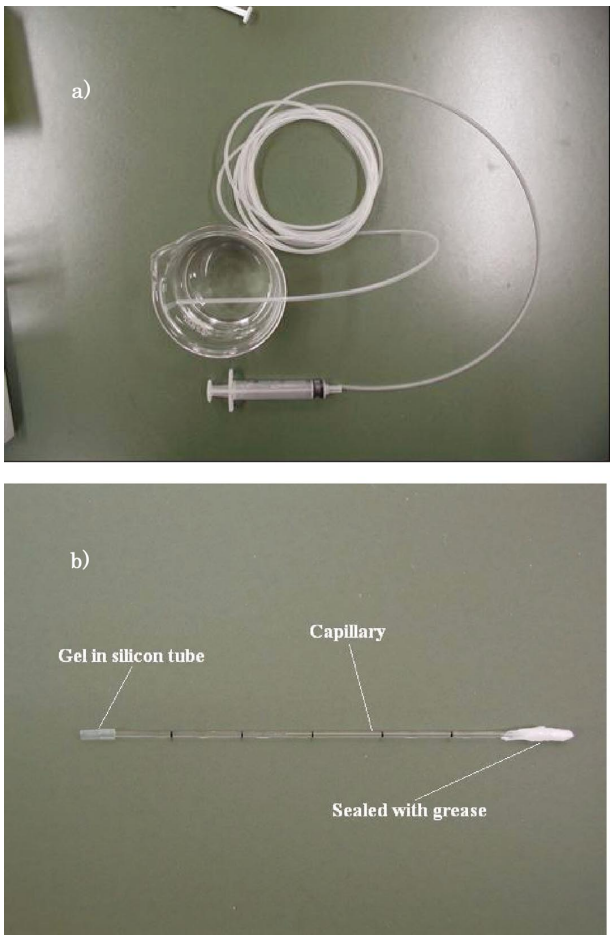

Figure 2 Preparing gel in the silicon tube. a) The end of the silicon tube was dipped into the melted agarose gel in the beaker. The other end of the tube was connected with a syringe through which the agarose gel solution was sucked. The gel was fixed in the tube and the tube was cut to an appropriate length. b) Capillary inserted into the gel-tube. The end of the capillary filled with protein solution was inserted into the gel in the silicon tube. The other end of the capillary was sealed with grease.

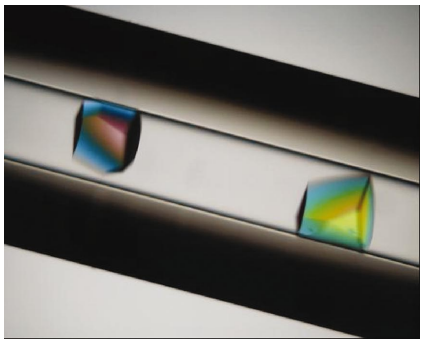

Figure 3 Crystals of Lysozyme in the capillary. 100mg/ml Lysozyme was crystallized in a 50mM acetate buffer at pH 4.5 with a 20% (w/v) NaCl as a precipitant.

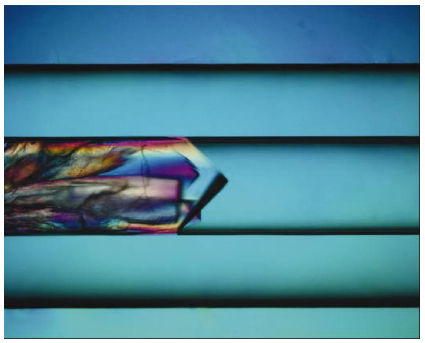

Figure 4 Crystals of alpha-Amylase in the capillary. 90mg/ml alpha-Amylase was crystallized in a 50mM acetate buffer at pH 6.0 with a 40% (w/v) PEG8000 as a precipitant.

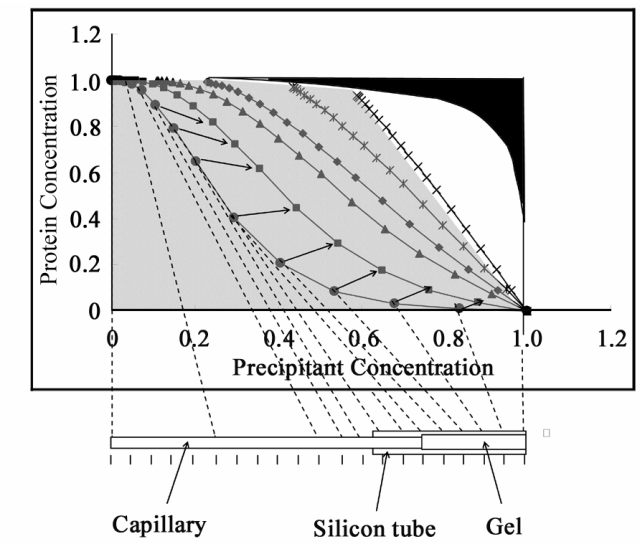

Figure 5 Schematic diagram of 1-D simulation. The circles, squares, triangles, diamonds, asterisks and crosses show the time evolution. The shadowed area represents scanned crystallization conditions in one capillary unless the crystallization occurs. The diagram curve transfers from left to right with time. The black area cannot be covered by this method.

acetate buffer at pH 6.0 as a precipitant for alpha-Amylase crystallization.

2.3. Crystallization

The 0.3mm diameter capillary was filled with protein solution to a height of 30mm. The upper end of the capillary was sealed with grease. The capillary was inserted into the gel-tube, which was equilibrated with the buffer, taking care to prevent air bubbles between the gel and the capillary. When the capillary was placed in the gel-tube, some of the gel was pressed out from the tube. This portion of the gel must be cut away from the tube using a razor blade. The lengths of the gel were adjusted to 10mm for Lysozyme and 3mm for alpha-Amylase. The capillary was then placed into the screw-top test tube, as shown in Fig. 1. The precipitant solution was poured into the tube. The crystallization was performed at 293K for several weeks. The experimental set up of the capillary inserted into the gel-tube is shown in Fig. 2b.

2.4. 1-D simulation

We developed a simulation program to predict the time-course of concentration changes of the precipitant and the protein solutions in each position of the capillary. Partial differential equations of the one-dimensional diffusion process were used to simulate the diffusions of the protein and the precipitant in the capillary and in the gel-tube.

The Lysozyme-NaCl system was simulated at intervals of 10sec and 0.4mm length steps of the capillary with 10mm gel and 30mm protein solution length. The alpha-Amylase-PEG8000 system was simulated at intervals of 10sec and of 0.4mm length step of the capillary with 3mm gel and 30mm protein solution length.

The diffusion coefficients of the proteins and the precipitants in the buffers and in the 1% agarose gel were obtained experimentally for the 1-D simulation. For PEG 8000, the diffusion coefficient at an arbitrary PEG8000 concentration was extrapolated from the concentration dependence of the PEG8000 viscosity.

3. Results

3.1. Lysozyme crystallization

Many small crystals appeared at the bottom of the capillary (about 10mm above the open end of the gel-tube) within six hours. After 24hours, crystallization had occurred in all the capillaries. After 120hours, crystallization was observed at the top of the capillaries. Crystals were found in some gel-tubes within 5mm of the capillaries (Fig. 3).

3.2. Alpha-Amylase crystallization

The first crystals appeared at the bottom of the capillary (about 3mm above the open end of the gel-tube), as a cluster of rod shaped crystals after 2 to 5 days. Crystals grew continuously with a cluster-like morphology, but after two weeks, some single crystals grew at the top of the cluster. The crystals grew quite slowly. Most of the crystal clusters grew to 5mm from the bottom of the capillary within two weeks (Fig. 4).

3.3. 1-D simulation

The diffusion coefficients obtained experimentally were substituted in the partial differential equations. A schematic diagram of the time course of the concentration changes in the capillary is shown in Fig. 5. Points indicate the concentrations of the precipitant and the protein solutions. The curve of the diagram transferred from left to right and scanned the shadowed area as a function of time.

For Lysozyme, if no crystallization occurred, the simulation showed that the capillary could scan about 82% of the precipitant-protein area shown in Fig. 6 within five days. For alpha-Amylase, if no crystallization occurred, the simulation showed that the capillary could scan 91% of the area shown in Fig. 7 within 20 days.

We applied the experimental result of Lysozyme crystallization obtained from our method to the 1-D simulation in Fig. 6, and estimated the crystallization condition to be about 20mg/ml Lysozyme with 5% NaCl. This result was consistent with the result in Fig. 6, which showed the crystallization occurred one day after the experiment started (dotted line) (Yoshizaki *et al*.).

For alpha-Amylase, the crystallization condition was estimated to be about 10mg/ml alpha-Amylase with 35% PEG 8000 based on the experimental result in our method and 1-D simulation. This result was almost consistent with our crystallization experiment by batch method shown in Fig. 7 (cross) though crystal appeared later than expected.

4. Discussion

Our results in this study show that the simplified counter diffusion method, which is convenient and easy, produces good crystallization and that the diffusion processes in the capillary are simulated by the 1-D simulation for Lysozyme and alpha-Amylase crystallization. The crystallization region and timing obtained by this method was consistent with the 1-D simulation with previous data of crystallization conditions by the batch method, especially for Lysozyme. Alpha-Amylase, crystallized a little later than expected, possibly because alpha-Amylase nucleated in the PEG8000 solution rather slowly and it took more time to start crystallization after acquiring the optimized crystallization condition (shown as cross in Fig. 7). We recommend using a longer gel-tube, so that it will be scanned more slowly over the optimized crystallization conditions in the capillary. Based on these results for the preliminary experiment, we can predict the pair of protein and precipitant concentrations for the crystallization. These concentrations can be used both in the GCB method and in the drop of vapour-diffusion method.

Two essential points determine the optimal experimental crystallization condition, the proper precipitant agents and the proper concentrations of the precipitant and the protein solutions. Once an appropriate precipitant is chosen, optimizing the crystallization condition is one of the most time-consuming steps in the protein crystallization experiment.

For the vapor-diffusion method, it is necessary to prepare many drops with various concentrations combination of the protein and the precipitant solution. Although GCB is an excellent device for protein crystallization and does not require using so much of the sample as the vapor-diffusion method, the crystallization conditions should be optimized beforehand to reduce the time consumed and the amount of the precipitant and the protein solutions. Furthermore, in GCB, the final concentration of the precipitant in the capillary will be diluted by the volume of the gel layer. Our method, however, uses a shorter capillary and screw-top test tube, so it requires less precipitant and protein solutions and reaches the optimal crystallization conditions faster. Also the precipitant will be less diluted because the volume of the gel is very small in our method.

By using our new method, one can easily perform the crystallization experiment using only one capillary with the precipitant at very high concentration, so that it will scan a range of concentration combinations of the precipitant and the protein and will provide the optimal crystallization conditions. If crystals are obtained, it is easy to estimate at least one pair of the precipitant and the protein

concentrations for crystallization by 1-D simulation. These values can be used as the optimized condition and can be introduced to determine the initial condition for other crystallization methods including GCB. Thus, our method in this study can definitely shorten the time for finding optimal experimental crystallization conditions. Using our method for GCB, the initial condition for crystallization and the penetration length in the gel layer can be modified to pass through the optimum concentrations of the protein and the precipitant at an appropriate timing while approaching the final concentration. In the vapor-diffusion method, the initial condition for crystallization can be modified by our method to pass through the optimum concentrations of the protein and the precipitant while approaching the final concentration.

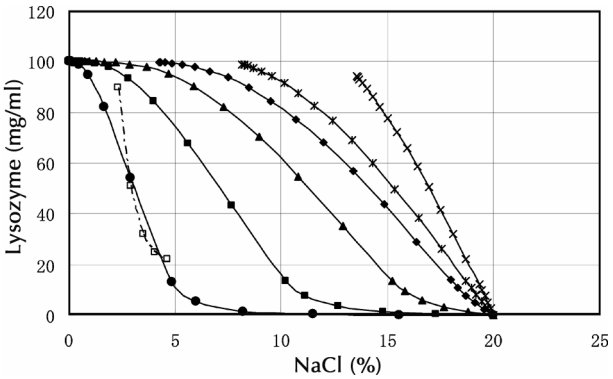

Figure 6 Result of 1-D simulation of the Lysozyme experiment. Each curve shows the NaCl-Lysozyme relation along the capillary and gel-tube length. Results are shown for 0.25days (dots), 0.5days (squares), 1day (triangles), 2days (diamonds), 3days (asterisks), and 5days (crosses). The point on the abscissa indicates the open end of the gel-tube. The points on the curve represent points along the capillary and the gel-tube length every 2 mm. The dotted line with open squares shows the conditions at which concentration crystallization occurred one day after the experiment started using the batch method (Yoshizaki *et al*., 2003).

Diffusion coefficients must be obtained to perform 1-D simulation. From the experimental results, the diffusion coefficient value of PEG8000 in agarose gel was much lower than that of salts, which meant that it would take much more time to start the crystal growth using PEG8000 as a precipitant. The diffusion rate did not seem to increase when we used a lower concentration of agarose gel. The diffusion coefficient is correlated with the solution viscosity. Since the viscosity of the PEG8000 solution is very high, the diffusion coefficient of alpha-Amylase in PEG8000 was significantly decreased (Inaka et al., 2003).

However, in general, it is difficult and time-consuming to obtain diffusion coefficients experimentally. In addition, it takes additional protein samples and experimental proceed to measure the coefficients. Therefore, we are now investigating a convenient method for estimating the diffusion coefficients both in the solution and in the gel from the molecular weights of the precipitant and the protein. If we can establish a method for estimating diffusion coefficients and can apply our simplified counter diffusion crystallization method for optimizing the crystallization conditions, we may significantly contribute to the high-throughput and the essential protein crystallization experiments for the structure determination.

As shown in Fig. 5, the concentration area in the upper right corner of the figure cannot be scanned by our method. This uncovered area increases as the height of the protein solution in the capillary

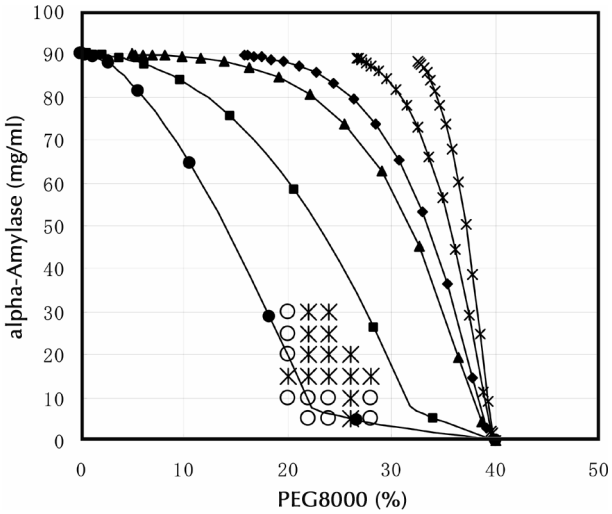

Figure 7 Result of 1-D simulation of the alpha-Amylase experiment. Each curve shows the PEG8000-alpha-Amylase relation along the capillary and gel-tube length. Results are shown for 0.5days (dots), 1day (squares), 4days (triangles), 8days (diamonds), 14days (asterisks), and 20days (crosses). The point on the abscissa represents the open end of the gel-tube. The points on the curve show the points along the capillary and the gel-tube length every 2 mm. The crosses show the conditions under which concentration crystallization occurred using the batch method. (Inaka *et al.*, 2003). The open circles show conditions under which concentration crystallization did not occur.

decreases. Thus, it is important to set up the initial condition and the protein solution length in the capillary to scan the optimized concentration area for crystallization.

Our new method introduced here may also be used in the crystallization for X-ray diffraction data collection. However, to obtain high quality crystals, GCB may still be superior to our simplified method because of the gel volume in GCB. The larger volume of the gel makes the diffusion of the precipitant into the capillary slower, so that the crystal grows slower than in our method and high quality crystal tends to grow in the capillary.

However, if the longer gel-tube is used, it may be possible to extend the time for crystal growth. If capillaries with larger diameters are used, it may be possible to obtain larger crystals suitable for X-ray diffraction. Thus, our method may be recommended both for optimizing crystallization conditions and for obtaining high quality crystals with some modification. Furthermore, a technique for removing the grown crystals from the capillary without any damage is crucial.

References

Garcia-Ruiz, J.M. (1991). Key Engineering Materials, 58, 87-106
Garcia-Ruiz, J.M. & Moreno, A. (1994). Acta Cryst., D50, 484-490
Garcia-Ruiz, J.M., Gonzalez-Ramirez, L.A., Garvia, J.A. & Otalora, F. (2002). Acta Crystal. D58, 1638-1642
McPherson, A. (1999). Crystallization of Biological Macromolecules, Cold Spring Harbor Laboratory Press
Inaka, K., Sugiyama, S., Tanaka, H., Takahashi, S., Sano, S., Fukuyama, S., Yoshizaki, I., Nakamura, H., Sato, M. , Motohara, M., Kobayashi, T., Yoshitomi, S., Sazaki, G. & Komatsu, H. (2003). International Symposium on Diffraction Structural Biology 2003, Symposium Booklet, 179
Yoshizaki, I., Nakamura, H., Fukuyama, S., Komatsu, H., & Yoda, S. (2003). Microgravity Transport Processes in Fluid, Thermal, Biological and Materials Sciences III