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A Gel-Mediated Feeding Technique for Protein Crystal Growth from Hanging Drops

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Abstract

A procedure which allows an investigator to supply a crystal with fresh mother material without inducing significant growth defects is described. This technique requires that the crystal is grown in a gelled hanging or sitting drop. An example concerning a model macromolecule, hen egg-white lysozyme, is given. Extension of this procedure to other macromolecules is discussed.

Introduction

Most protein crystal growth experiments are performed using the widespread hanging- (or sitting-) drop technique. This technique requires limited quantities of mother material solutions (20–30 μl) so that many trials can be made, covering a large range of nucleation and crystal growth conditions. The consequence of such a parsimonious supply of solute is the limited size of crystals which can be expected, especially when more than one crystal has nucleated in the same droplet. One is tempted to replenish the droplet with solute by adding a new protein solution droplet but, due to perturbating effects induced by the coalescence of old and new droplets and subsequent mixing, growth defects can be generated at the seed/new crystal interface. Furthermore, secondary nucleation can be a problem.

Recently, it has been shown that biological macromolecule crystals can be grown in gel media by the hanging-drop technique (Provost & Robert, 1991). The main advantage of this method is the presence of a gel layer surrounding the crystal which shelters the growing interface against perturbation and, consequently, provides better quality crystals (Miller, He & Carter, 1992). It is proposed to take advantage of this protecting effect by supplying the crystal with fresh mother material. An experimental procedure is illustrated here for the case of hen egg-white (HEW) lysozyme crystals.

Experimental

Fig. 1 shows how the composition of the growth solution of the hanging drop varies at the different steps of the feeding process.

Step 1

The initial drop is formed by coalescence of a cold (293 K) droplet of HEW lysozyme solution (30 mg ml^{-1}) and a warm (313 K) droplet of NaCl solution (0.8 *M*) containing 0.1%wt agarose. Both solutions have been prepared with 0.05 *M* acetate buffer solution (pH 4.75). In the present case, the volume of each droplet is 10 μl. Practical details on crystallization protocol (gel preparation, removal of crystals out of the gel) are given in Robert, Provost & Lefaucheux (1992). The drop, which rapidly gels as the temperature is decreased to room temperature, is then suspended over a reservoir filled with the 0.8 *M* NaCl solution.

Step 2

After dehydration owing to water-pressure equilibration between drop and reservoir, the volume of the droplet is reduced by one half and the solution becomes strongly supersaturated so that crystals nucleate and begin to grow.

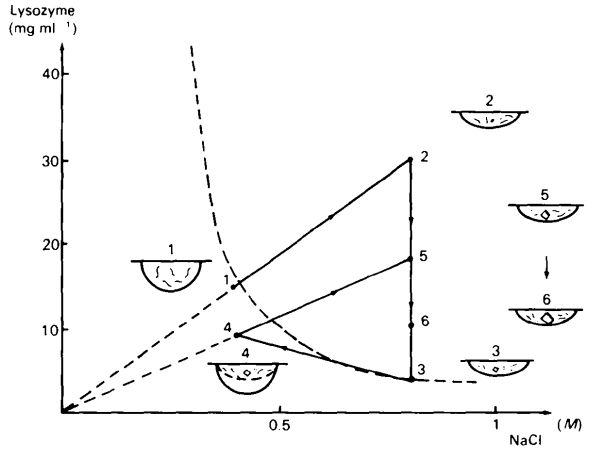

Fig. 1. Solubility variation of HEW lysozyme as a function of NaCl content at room temperature (dotted line); data are taken from Riès-Kautt & Ducruix (1989). In this diagram, the path followed during the different steps of the feeding process is drawn (full line) together with a schematic representation of the crystal in the gelled hanging drop.

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Step 3

About one week later, the crystals stop growing. Fig. 2(*a*) presents an optical view of such a crystal. In the solubility diagram (Fig. 1) the position which corresponds to step 3 has been placed on the saturation curve, assuming that, at that time, the solution is exhausted but, in fact, the correct position could be somewhat above this. Growth cessation may have several causes (Giegé & Ducruix, 1992). For example, growth could become much slower at lower supersaturation, or even be stopped due to the poisoning effect of some adsorbed impurities. Most of the time, a rough estimation of the amount of protein which has been crystallized, from measurements of the crystal sizes, shows that the solution might not be exhausted when growth cessation is observed; however, this does not take into account the fraction of protein which denatures during the growth process, or which acquires a perturbed conformation when lying in the surface layer of the droplet. In any case, growth cessation requires the supply of fresh mother solution.

Step 4

A 10 mg ml^{-1} protein gel-free solution droplet (10 µl) is then added onto the surface of the gel containing the crystal to be fed. This droplet does not contain sodium chloride so that, at step 4, the global content of salt is the same as for step 1 and the same reservoir can be used for the second dehydration leading to step 5.

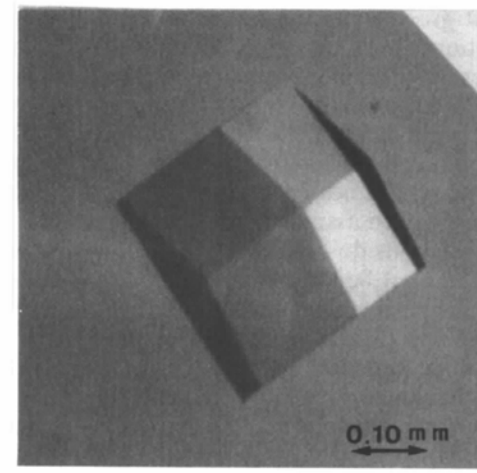
As the volumes of the gelled droplet (C_{NaCl} = 0.8 *M*) and of the added droplet (C_{NaCl} = 0.0 *M*) are small, the diffusion distances are very short and it is reasonable to assume that the homogenization of concentrations (C_{NaCl} = 0.4 *M*) occurs very rapidly (step 3–step 4) compared with the time required for dehydration (step 4–step 5). The actual path is not known and it is probably a loop inscribed in a 3–4–5 triangle.

Step 5

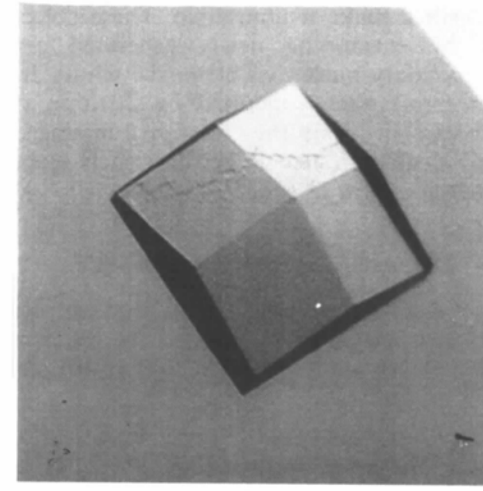
Fig. 2(*b*) shows the same crystal as in Fig. 2(*a*) at the beginning of regrowth when dehydration has proceeded; the presence of a large macrostep indicates that, in the present case, growth cessation was associated with accumulation of impurities on the crystal surface (Durbin & Feher, 1990).

Step 6

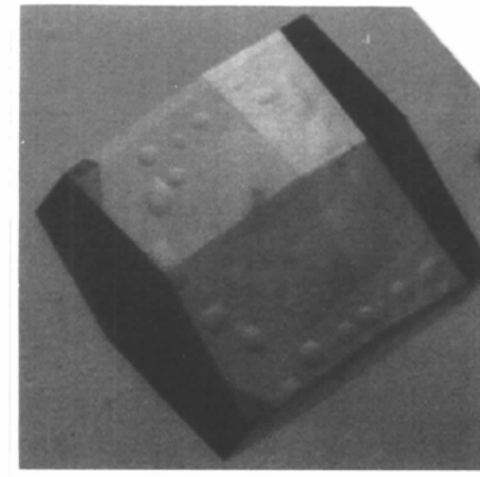
Within a few days, growth resumes as shown in Fig. 2(*c*). The crystal presents numerous growth hillocks which probably correspond to the emergence of screw dislocations (Monaco & Rosenberger, 1993). These growth hillocks were not observed in



(*a*)



(*b*)



(*c*)

Fig. 2. *In situ* micrographs of the growing crystal: (*a*) at the end of the initial growth (step 3), (*b*) at the beginning of regrowth (step 5), (*c*) when regrowth has proceeded for a couple of days (step 6). The three images are presented with the same magnification.

the first growth step (Fig. 2*a*); this shows that such dislocations have probably been generated at the seed/new crystal interface which is rather usual when a crystal experiences a modification of its growth medium (Lefaucheux, Robert & Authier, 1973). The relative diffracting power of the crystals before and after feeding has not been investigated in the present case but one can assume from previous studies that a few dislocations do not change the diffracting power. This would not be the case when an accumulation of dislocations inducing a grain boundary has increased the mosaicity (Mikol & Giegé, 1992).

The same experiment has been performed with a gel-free hanging drop. Fig. 3 presents an optical view of the whole droplet at the end of the feeding process. At step 3, the largest crystal was unique and did not present visible defects. At step 6, this crystal is significantly larger but macroscopic defects are present which make it unusuable. These defects are created by entrapping newly generated crystals. These secondary nuclei, visible in the whole droplet, have probably been generated by attrition of the primary crystal during the convective mixing of old and new droplets (step 4) because such secondary nuclei never appear in gelled droplets.

Discussion and concluding remarks

From the example presented here, one can imagine many other possibilities which, by varying the volume and the composition of the added droplet, would provide different '3–4–5' paths. However, to use this method to advantage with different materials, it is necessary to know the solubility curves in order to locate the '3–4–5' triangle in a region where the solubility variation with the precipitating agent content is low. For example, in the case of HEW lysozyme, it would not be possible to work at low salt content because the solubility variation is too sharp in this domain; even for a very small added droplet step 4 would correspond to a large undersaturation and the crystal would rapidly dissolve.

Feeding of gel-grown crystals using the so-called 'concentration-programming technique' has been proposed by Henisch and coworkers as far back as 1967, but this technique has only been applied in a limited number of cases for mineral crystals (Henisch, 1988). There are at least two reasons for such a limitation; first, it is not easy to provide a local supply of solute in the vicinity of the crystal without disturbing the gel structure; second, concentration variations may induce polynucleations on the growth front. These difficulties are circumvented here; first, the growth medium is naturally confined to the small volume of the droplet surrounding the growing crystal so that there is no loss of mother material in the bulk; second, the concentration increase occurs after a short undersaturation period which is unfavourable for the formation of polynucleation. This period induces a slight dissolution of the crystal surface which eliminates the impurities trapped on the crystal faces, but the path in the undersaturated region must be adjusted carefully because the dissolution rate is all the more rapid when the size of the crystal is small.

In the same way, too high a supersaturation should also be avoided in step 5 in order to reduce the number of dislocations that could be generated. It seems advisable to iterate the process using small concentration variations.

In the case of biological macromolecules, the solubility curves of the majority of crystals are not known; application of such a feeding technique is then risky, but one can imagine the use of trial-and-error procedures. For example, when a series of similar droplets containing crystals are available, one can add increasing volumes of a rather concentrated protein solution to the gels. The present study shows that gel growth affords new possibilities for changing growth conditions in the case of a very fragile material.

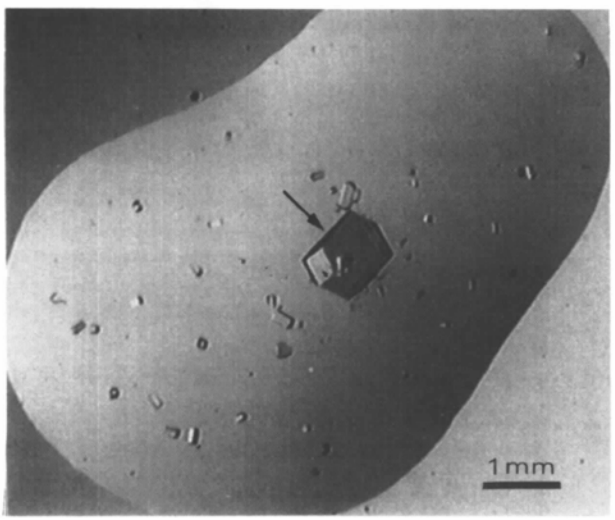


Fig. 3. Optical view of a crystal (marked by an arrow) growing in a gell-free hanging drop. This crystal has been submitted to the same feeding process as for the gel-grown crystal presented in Fig. 2. Secondary nuclei have appeared in the whole droplet. Some of them have sedimentated on the primary crystal inducing important defects. These secondary nuclei never appear in a gelled droplet.

References

DURBIN, S. D. & FEHER, G. (1990). *J. Mol. Biol.* **212**, 763.

GIEGÉ, R. & DUCRUIX, A. (1992). *Crystallization of Nucleic Acids and Proteins. A Practical Approach*, edited by A. DUCRUIX & R. GIEGÉ, pp. 1–18. Oxford: IRL Press.

HENISCH, H. K. (1988). *Crystals in Gels and Liesegang Rings*, p. 106. Cambridge Univ. Press.

LEFAUCHEUX, F., ROBERT, M. C. & AUTHIER, A. (1973). *J. Cryst. Growth*, **19**, 329–337.

MIKOL, V. & GIEGÉ, R. (1992). *Crystallization of Nucleic Acids and Proteins. A Practical Approach*, edited by A. DUCRUIX & R. GIEGÉ, pp. 219–239. Oxford: IRL Press.

MILLER, T. Y., HE, X. M. & CARTER, D. C. (1992). *J. Cryst. Growth*, **122**, 306.

MONACO, L. A. & ROSENBERGER, F. (1993). *J. Cryst. Growth*, **129**, 465–484.

PROVOST, K. & ROBERT, M. C. (1991). *J. Cryst. Growth*, **110**, 258–264.

RIÈS-KAUTT, M. & DUCRUIX, A. (1989). *J. Biol. Chem.* **261**, 1969.

ROBERT, M. C., PROVOST, K. & LEFAUCHEUX, F. (1992). *Crystallization of Nucleic Acids and Proteins. A Practical Approach*, edited by A. DUCRUIX & R. GIEGÉ, pp. 127–142. Oxford: IRL Press.