ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Journal of Structural Biology 142 (2003) 66–76

Journal of Structural Biology

www.elsevier.com/locate/yjsbi

Seeds to crystals

Terese Bergfors*

Department of Cell and Molecular Biology, Biomedical Center, Uppsala University, Box 596, 751 24 Uppsala, Sweden

Received 5 February 2003

Abstract

Seeding has been critical for obtaining diffraction-quality crystals for many structures. In this article, applications and recommendations for seeding are presented based on examples from our laboratory and other groups. The implementation of seeding in high-throughput crystallization, robotics, and other emerging technologies is also discussed.

Keywords: Crystallization; Macroseeding; Microseeding; Nucleation; Ostwald ripening; Seeding; Streak-seeding

1. Introduction

One of the paradoxes in crystallization is that the optimal solution conditions for nucleation of the crystals are not the ideal ones to support their subsequent growth. This is because spontaneous nucleation is quite simply more likely to occur when the levels of supersaturation are high, whereas slow, ordered growth of large crystals is favored by lower levels. The ideal experiment therefore must somehow uncouple nucleation from growth to satisfy the distinctly different requirements of the two events.

Seeding is a powerful tool for the separation of nucleation and growth. In this technique, previously nucleated crystals are used as seeds and introduced into new drops equilibrated at lower levels of supersaturation. Seeding techniques can be classified into two categories based on the size of the seeds:

- Microseeding—transfer of submicroscopic seeds, too small to be distinguished individually.
- Macroseeding—transfer of a single crystal, usually 5–50 μm.

Seeding can be used in conjunction with other optimization measures such as fine-tuning the precipitant and protein concentrations, adjusting the pH, and screening additives. The course of one such optimization is shown in Fig. 1.

Apart from a role in optimization, seeds can also be used as heterogeneous nucleants to facilitate the crystallization of similarly related proteins. This is often done to ensure that the target molecules will crystallize isomorphously with the native crystals. When one molecule not only nucleates on another but also shares some of the same structural features as the substrate (e.g., the same or very similar crystallographic lattice), the growth is said to be epitaxial. Proteins that have been crystallized by cross-seeding include mutants, selenomethionyl-substituted proteins, heavy atom derivatives, complexes with DNA- and RNA-mers, substrates, inhibitors or other ligands, different monoclonal antibody fragments, and homologous proteins from a different organism.

2. How to seed

Microseeding is an easy method and therefore the type of seeding to try first. Macroseeding (also called seed transfer) is much more labor intensive. It requires tedious transfers of the parent crystal through multiple washes, although the effort can be worth it, especially in terms of increasing the size of the crystal. However, in most seeding situations, a whole seed is not required; microseeds will suffice. The disadvantage to microseeding is that it is difficult to control the number of seeds that are transferred.

* Fax: +46-18-53-69-71.
E-mail address: terese@alpha2.bmc.uu.se.

1047-8477/03/$ - see front matter
doi:10.1016/S1047-8477(03)00039-X

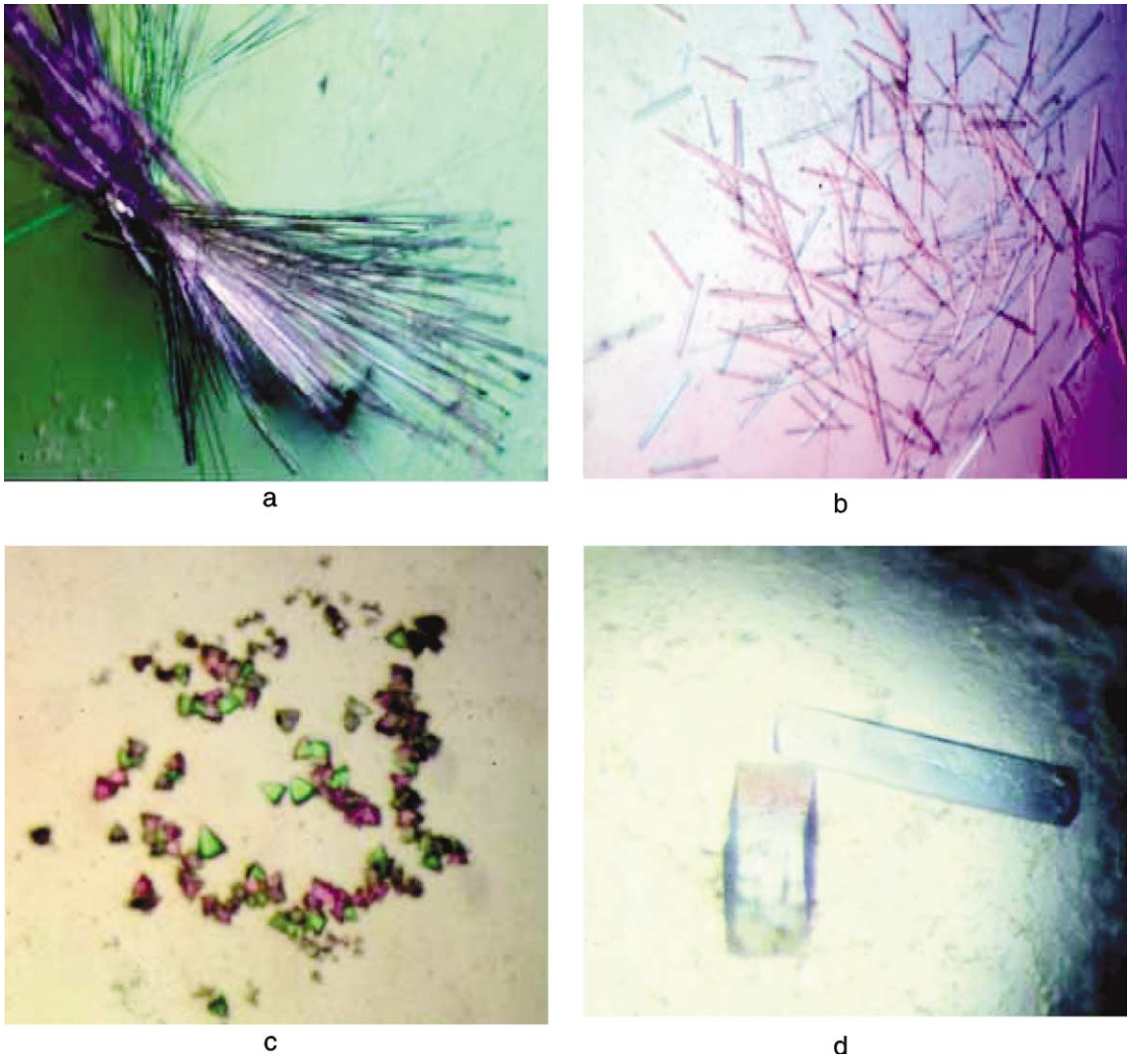

Fig. 1. Optimization of bovine acyl-CoA binding protein crystals (van Aalten et al., 2001). Microseeding was necessary in all the optimization steps (b–d) to obtain crystals that grew singly. Protein concentration in (a) is 18 mg/ml and 10 mg/ml in (b–d). (a) Crystals from the initial screen. (b) Single, tetragonal crystals after microseeding with the crystals in (a). (c) Bipyramidal crystals, $0.2 \times 0.3 \times 0.3$ mm, as a result of including nickel sulfate in the crystallization conditions. (d) Orthorhombic crystals of the complex of the protein and its ligand palmitoyl-CoA.

2.1. Handling microseeds

Microseeds can be made and introduced into the new drop in many different ways. The crystals can be pulverized (smashed) into crystalline particles by tissue homogenizers, sonication, vortexing, seed beads, glass rods, or other utensils. An article which specifically discusses the advantages and disadvantages of different pulverization methods is one by Luft and DeTitta (1999). Preparing a dilution series of the seed stock in a stabilizing mother liquor will give solutions with different amounts of nuclei. The dilution with the optimum number of nuclei has to be determined experimentally but is usually somewhere between 10^{-2} and 10^{-7}. The nuclei can be pipetted from the stock as a small aliquot or transferred with a seeding wand which is dipped into the microseed mixture to pick up seeds and then touched, stirred, or streaked across the surface of the new drop.

Preparing a dilution series of the seed stock makes it more quantitative, but by far the easiest and fastest method for seed transfer is streak seeding (Stura and Wilson, 1990). This method uses an animal whisker (usually cat or rabbit) as a seeding wand, which is touched or stroked over the surface of the parent crystal to dislodge and trap the nuclei. The whisker is then drawn through the new drop, depositing the seeds in a streak line. Although seeding wands have been made from thin glass rods, platinum-wire inoculation loops, and acupuncture needles, animal whiskers or hairs offer a definite advantage. The overlapping cuticles (Fig. 2a) capture the seeds effectively. Here we would like to bring attention to an animal source (Fig. 2b) of material for making seeding wands which, at least to our knowledge,

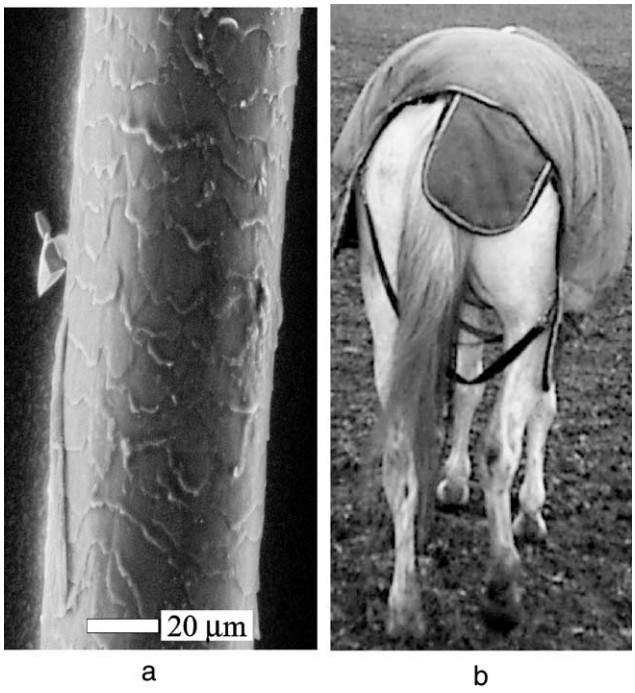

Fig. 2. Material for seeding wands. (a) Scanning electron micrograph of a horse tail hair showing the overlapping cuticles. Note the entrapped crystal on the left. (Micrograph courtesy of G. Vrdoljak, University of California at Berkeley Electron Microscope Laboratory.) (b) An abundant source of material for seeding wands can be found at the flexible C-terminus of this animal.

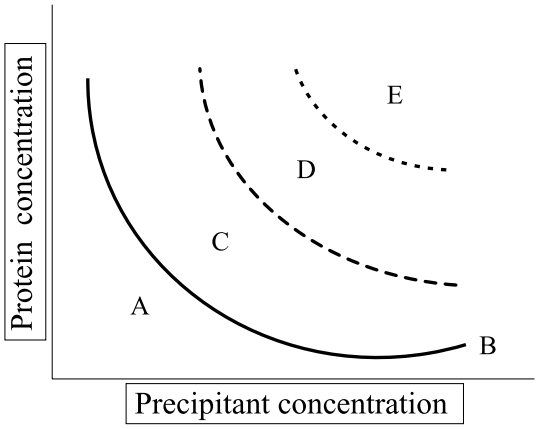

Fig. 3. Two-dimensional, theoretical phase diagram. (A) Undersaturation zone. Undersaturated drops remain clear. (B) Limit of solubility. The solid phase of the protein is in equilibrium with the liquid phase. (C) Metastable zone of supersaturation. The level of supersaturation is high enough to support growth of the solid phase of the protein but not high enough to initiate spontaneous nucleation. This is the best zone for seeding. Note that drops in the metastable zone are also clear, making it difficult to distinguish them from undersaturated drops. One diagnostic is to add a seed: seeds will melt if the solution is undersaturated. The metastable zone pictured here is wide. When the metastable zone is narrow it is difficult to accurately seed into it. (D) Labile supersaturation. Ordered aggregation (spontaneous nucleation) can occur here. (E) Precipitation zone. This region is highly supersaturated, leading to disordered aggregation (amorphous precipitate).

has not been exploited much by the crystallization community. Horse tail hair is much more abundant than most animal whiskers, which makes it useful when organizing large-scale laboratory sessions. In addition, horse tail hairs maintain the same thickness throughout their length (which is why violin bow makers use it), and this property may be useful in its own right for reproducibility in seed transfer. On the other hand, a cat whisker can be cut into three or four segments to obtain wands of different thickness. We use both horse tail hairs and cat whiskers because of their complementary properties. We find that rabbit whiskers, being thinner than both horse hair and cat whiskers, are too flexible.

2.2. Finding the metastable zone

After the dilution series of microcrystals has been made or a parent seed selected for macroseeding, the seeding wand or pipette (if using the aliquot method) is used to place the seed(s) into a new experiment equilibrated at the metastable zone of supersaturation. This is the area in the phase diagram where crystal growth is supported but no nucleation occurs (Fig. 3). A protocol for the determination of a phase diagram for microseeding can be found at http://www.douglas.co.uk (Research Report 1, August 1995). This microseeding method was used for the successful crystallization of two alcohol dehydrogenases (Korkhin et al., 1996).

In practice, most seeding experiments are done without knowledge of the protein's phase diagram since determining the phase diagram is expensive in terms of the researcher's time and protein consumption. If it is not convenient to determine the phase diagram for the protein, some rules of thumb, derived from our own empirical observations, can be applied instead. To move to the metastable zone of supersaturation, the concentration of at least one of the components (precipitant, protein, or both) must be lowered. (See Figs. 1 and 4 for examples.) Our first choice is to begin by lowering the protein concentration. Protein concentration is inversely and linearly proportional to its solubility. Hence, if lowering the protein concentration too much puts the experiment below the solubility limit and the seeds dissolve, only a simple linear interpolation is required for the next round of drops. The protein concentration is raised to some level between where spontaneous nucleation occurred in the first drop and where the seeds melted in the second experiment. The relationship between protein solubility and the concentration of polymeric precipitants (of which polyethylene glycol (PEG) is the most commonly used) is also linear, i.e., protein solubility decreases proportionally as the polymer concentration increases (Atha and Ingham, 1981). This is not true for salt precipitants for which the relationship between salt concentration and protein solubility is

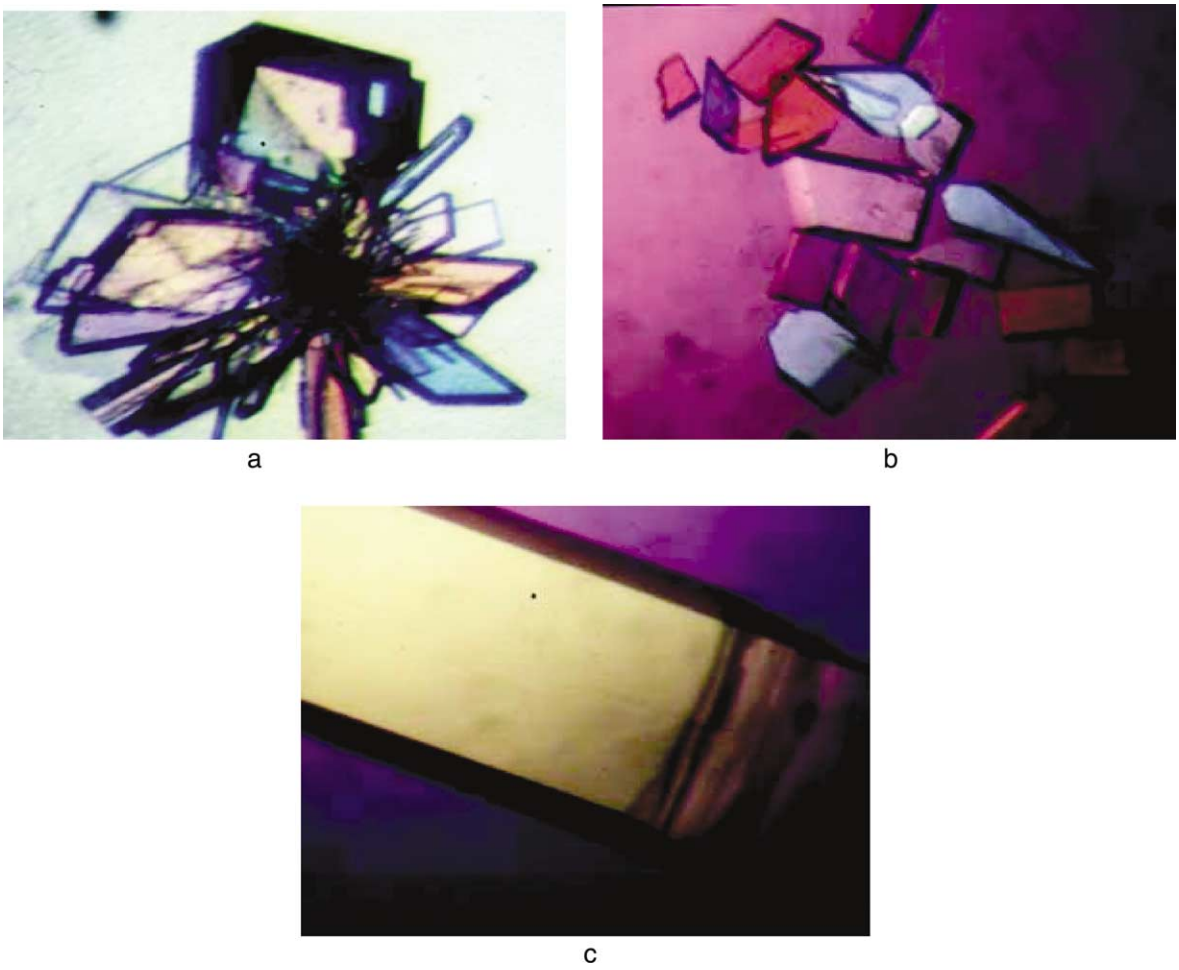

Fig. 4. Improvements through serial seeding. (a) Crystals of CBH2 resulting from spontaneous nucleation after 1 month. (b) Microseeding with the crystals of (a) into a new drop at lower protein concentration gave crystals that grew singly. (c) Result from the second round of seeding, using the crystals in (b) as microseeds. Magnification is the same in (a–c).

more complex, and the nature of the salt affects the solubility, independent of the ionic strength. Thus, it can be more difficult to correctly localize the metastable zone by lowering the salt concentration and we therefore prefer to lower the protein concentration instead.

Lowering the protein concentration also gives a more efficient use of protein. The structure of the complex of tissue factor, factor VIIa, and an inhibitor (Tf.VIIa. 5L15) was obtained with only 1.5 mg of protein (prior to the advent of nanocrystallization methods) (Stura et al., 1996). By judicious use of streak-seeding, the crystals which self-nucleated at a protein concentration of 21 mg/ml could be grown at 7 mg/ml instead.

Nevertheless, it should be pointed out that different results can be obtained depending on which of the concentrations is lowered: protein or precipitant. For example, the cell dimensions and diffraction quality of Tf.VIIa. 5L15 complex crystals were different depending on whether the seeds were grown in the metastable zone at high-protein, low-precipitant concentrations or at low-protein, high-precipitant concentrations. Protocols are available for obtaining polymorphs by changing the precipitant concentration with or without seeding (Stura, 1999).

An option completely different from lowering the protein/precipitant concentration(s) is to seed into a mother liquor totally unrelated to that in which the seeds originated. Transfer of crystals to solvents drastically different from the original mother liquor has been done in many cases out of necessity to facilitate heavy atom or substrate binding. For instance, triose phosphate isomerase crystals grown at 2.4 M ammonium sulfate were moved to 44% PEG 6000 (Wierenga et al., 1992). In our laboratory we use whatever crystals are obtained in the initial screen to streak-seed any of the drops that may have remained clear. We also typically streak-seed the screens for cryoconditions.

2.3. In situ seeding in the metastable zone

Developments in dynamic light scattering technology (DLS) may soon make it possible to decouple nucleation from growth in a more quantitative fashion, be it for

seeding or other optimization technique. The formation of the first nuclei is an event which occurs long before they become visible in the microscope. Saridakis et al. (2002) have recently reported using DLS to identify this onset of nucleation. This information is then used to determine the optimal time at which to dilute the drops. Dilution of the drops lowers the supersaturation, thereby shifting the experiment from the labile to the metastable zone, where no nuclei form but the existing ones will continue to grow.

In a sense this could be described as in situ seeding, i.e., rather than transferring seeds to a new drop at metastable supersaturation, the drop itself is altered so that the nuclei are in a metastable environment. The drops are diluted with water, buffer, or mother liquor (minus the protein). Carboxypeptidase G_2 crystals were grown in this way (Saridakis et al., 1994). In situ seeding is also used for growing crystals for atomic force microscopy (AFM), which requires that the crystals are fastened to a surface that can be inserted into the AFM sample chamber (Kuznetsov et al., 1997).

Other ways to manipulate the solution instead of the seeds include feeding and weeding. In feeding, more protein is added to the drop. The effects are twofold: the drop becomes diluted, lowering the supersaturation, while at the same time more protein becomes available to the growing nuclei. This is an effective approach if the crystals nucleated in the labile zone have exhausted all the dissolved protein. In weeding (Han and Lin, 1996) excess crystals are removed from the drop so that the protein in solution is available to the selected few crystals that remain. Care must be exercised to avoid evaporation in the drop during the weeding. Since this is difficult to do, weeding is not often successful.

3. Some variations and applications

3.1. Improving the seed quality

The selection of the seed crystal is critical in macroseeding, but the origin of the seed should also be taken into consideration for microseeding. Defects accumulate in crystals as they grow and so the oldest, largest crystals should be avoided as sources of seeds. If no small, freshly grown crystals are available, a seed can be chipped from the growing edge of the parent crystal. The fragment can be used as a macroseed or pulverized into microcrystals. If the fragment is to be used as a macroseed, it must be soaked briefly in an etching solution (i.e., undersaturated conditions). This will slightly melt the crystal and generate a fresh surface. The procedure for macroseeding has been described in detail for ribose binding protein and can be used as a guideline in designing a macroseeding protocol for other proteins (Mowbray, 1999).

The quality of the seed crystals, whether for macro- or microseeding, can be improved by serial seeding (also called repeated seeding). In serial seeding, crystals resulting from the first round of experiments are used to seed new drops. When crystals have appeared in these second drops, they are now used as seeds in their own turn for a third set-up, and so on. The seeds can be introduced into the new drops as macroseeds, streak-seeded, or crushed and introduced as microseeds, although the latter two are the most common methods. Fig. 4 shows how the quality of cellobiohydrolase 2 (CBH2) crystals improved with two rounds of seeding. The spontaneously nucleated crystals (Fig. 4a) appeared after 1 month in drops with 20 mg/ml protein, as rosette formations, i.e., many different crystals growing from a single nucleus. To eliminate both the long nucleation time and the tendency of these crystals to grow in rosettes, the crystals were crushed and used as microseeds in new drops at 10 mg/ml protein concentration. At this protein concentration no nucleation ever occurred spontaneously. Single crystals appeared after some days (Fig. 4b). These crystals were then used in turn as microseeds for yet another series of drops. The protein concentration was maintained at 10 mg/ml. This second round of seeding yielded crystals that grew to over 2 mm in 1 week and the 2.0 Å structure was solved with them (Bergfors et al., 1989; Rouvinen et al., 1990).

Here only 2 rounds of reiterative seeding were necessary, but sometimes as many as 7–10 rounds may be required (Thaller et al., 1981). If any improvement is apparent after 1 round of seeding, a second or more passes should always be attempted. This has been found to be of benefit in many of our projects.

3.2. If no crystals are available—seeding with oils, spherulites, and precipitates

3.2.1. Oils

If no crystals or microcrystals are forthcoming in the initial crystallization screening, seeding can be attempted with any solid phase that has resulted. It is obvious that spherulites represent some kind of semi-ordered aggregation, but even oils and precipitates can exhibit short-range order and therefore act as nuclei (ordered aggregates) for further crystal growth (Stura and Wilson, 1991). For these solid states of the protein, streak-seeding is the method of transfer.

The crystallization of the cytochrome domain of cellobiose dehydrogenase (CDH) can be used as an example (Hallberg et al., 2000). This protein was particularly recalcitrant in crystallizing, with the only result being an oily precipitate (Fig. 5), which was found after 1 year in the screens. Despite their semiliquid appearance, these protein oils are quite distinct from free protein in solution. Their exact nature is still under investigation but they are known to be a protein-rich

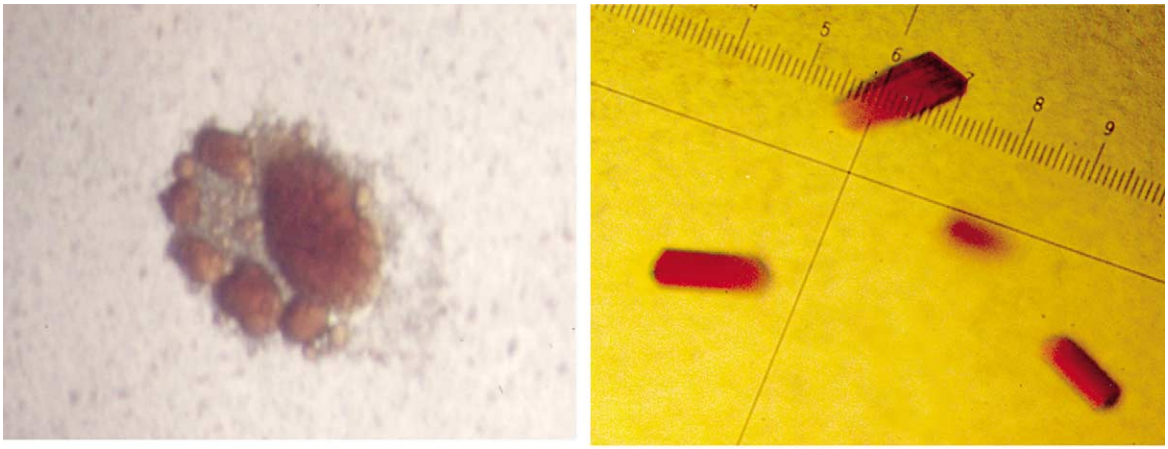

Fig. 5. Crystallization of CDH. (a) CDH precipitated from solution as the oil seen here. (b) The crystals used in determining the structure. Longest dimension is 0.1 mm (courtesy of B.M. Hallberg).

phase (Kuznetsov et al., 2001). The CDH protein is inherently red (due to its heme group), making it easy to identify the red oil as concentrated protein. The oil was used to streak-seed new drops, resulting in microcrystals. Although these were not the crystals ultimately used to solve the structure, they provided the necessary impetus to continue what initially seemed a hopeless project.

3.2.2. Precipitates

Even precipitates can be utilized in combination with seeding:

- Precipitate can be assayed for microcrystallinity by streak-seeding (Figs. 6a and b).
- Drops that have precipitated can be seeded to provoke Ostwald ripening (Fig. 6c).

Not all precipitates are equal: it is important to distinguish the precipitates of denatured protein from amorphous and nonamorphous precipitates and recognize submicroscopic crystals which look like amorphous precipitate. One quick assay for microcrystallinity of precipitates is streak-seeding (Stura and Wilson, 1991). In this method, the precipitate is streak-seeded into a new drop and the results are examined after 2–7 days. Amorphous precipitates will generate only more precipitate along the streak line (Fig. 6a), whereas crystalline precipitates will generate microcrystals. Sometimes, as in Fig. 6b, the seeds generated along the streak line are large enough to use without further refinements.

Precipitation occurs at high levels of supersaturation, far from the metastable zone, so generally seeds are not introduced into precipitate. For some proteins, however, the metastable and labile zones are so narrow that it is difficult to pinpoint the area between precipitation and undersaturation. The seeds are therefore introduced, either inadvertently or of necessity, into the precipitate or the labile phase. This can sometimes provoke a positive response via a mechanism known as Ostwald ripening.

This interesting process is a result of the thermodynamic advantage which large crystals have with respect to smaller ones. Kinetically, it is easier to form many small crystals, i.e., they nucleate more easily. However, large crystals, with their greater volume-to-surface area ratio, represent a lower energy state. In Ostwald ripening the many small crystals are driven thermodynamically to obtain a lower energy state by becoming incorporated into larger crystals (Boistelle and Astier, 1988; Ng et al., 1996). Thus, a seed crystal introduced into a shower of many small nuclei or a precipitate can grow at their expense via Ostwald ripening.

Sousa et al. (1991) report that crystals of T7 RNA polymerase seeded into an amorphous precipitate phase deteriorated into precipitate. This was not expected since the precipitate phase is at a level of supersaturation high enough to support a solid phase of the protein. On the other hand, precipitate that they put into drops in the metastable zone transformed into crystals. The labile and precipitated zones in the phase diagram are both states far from the limit of solubility, i.e., equilibrium, and it is not always possible to predict what will happen kinetically when seeds are introduced. It may provide some comfort to researchers looking at precipitated crystallization drops to keep in mind that "in systems far from equilibrium, order can arise from chaos" (Prigogine, 2001).

3.3. Seeding and additives

Another way to manipulate the level of nucleation through seeding is suggested by the work of Baker and Stewart (2000). Needle crystals of Mog1p (a protein that binds GTPase Ran/Gsp1p) were obtained under several conditions and therefore an array of additives was

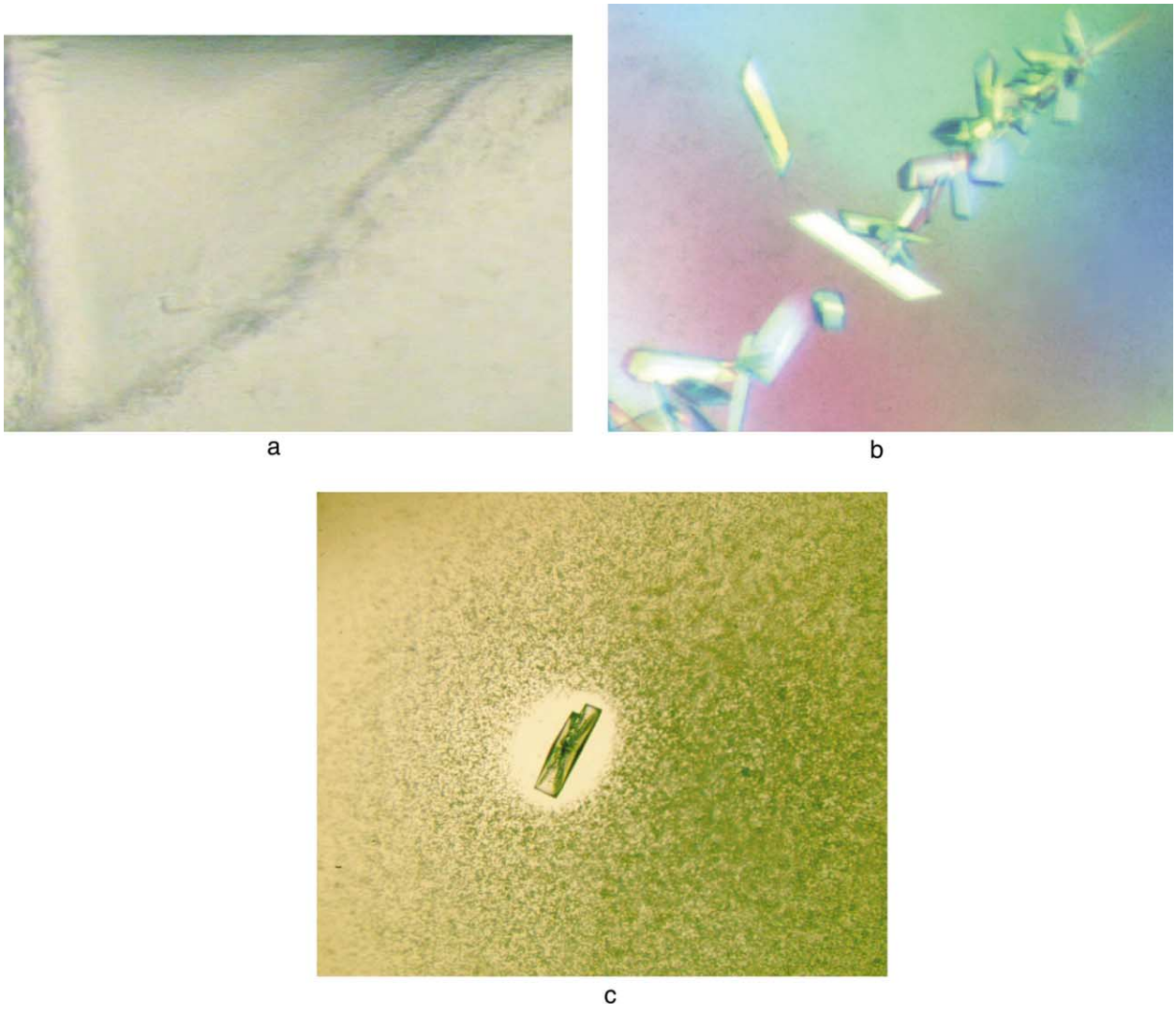

Fig. 6. Seeding and precipitates. (a) Precipitate obtained in the initial crystallization screen for this protein (*Mycobacterium tuberculosis* RV 2465c) was assayed for microcrystallinity by streak-seeding it into a new drop. Only more precipitate grew along the streak line. (b) By contrast, if the precipitate is microcrystalline, crystals will appear along the streak line in the drop. These crystals of *Aspergillus niger* epoxide hydrolase were obtained after several rounds of improvement by streak-seeding (Zou et al., 2000). (c) Ostwald ripening. As the crystal grows, a local concentration gradient with respect to the protein develops. Precipitated protein redissolves, creating the halo or depletion zone seen around the growing crystal.

screened in the subsequent round of optimization. One of the additives, 0.1–0.25% β-octylglucoside, was found to inhibit nucleation completely, i.e., the drops remained clear. These clear drops were streak-seeded to supply the nuclei and crystals appeared within 12 h. After 1 week they had achieved their ultimate size, producing large crystals which diffracted to 2.5 Å. Here the additive functioned as a "poison" in the drop to inhibit nucleation, then seeds were added to provide a controlled number of nuclei.

Glycerol is another commonly used additive in crystallization mother liquors since it offers cryoprotection and also increases the stability of many proteins (Sousa, 1995). (It should be noted that another effect of glycerol is that it increases protein solubility, which then affects the phase diagram of the protein.) The viscosity of glycerol can be exploited in combination with microseeding to titrate the number of nuclei. For example, in the crystallization of T7 RNA polymerase, Sousa et al. (1991) injected microseeds into one end of a large drop (100 μl). The normal convective mixing which occurs in liquids was reduced because of the viscosity of the glycerol and therefore a concentration gradient of microcrystals developed across the drop. Many small crystals appeared at the seed inoculation site but they became fewer and larger at the far end of the drop (Fig. 7). Obviously this effect can be utilized with other viscous mother liquors if the drop is large enough to permit a significant gradient to develop.

Another variation for titration of microseeds which we use in our laboratory is the following. We add the microseeds as a 0.2-μl droplet beside the new drop to be

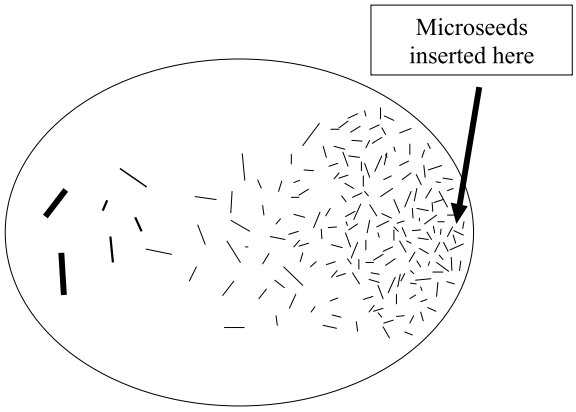

Fig. 7. Effect of microseeding into a viscous mother liquor. Schematic representation of the concentration gradient of microseeds that develops when they are introduced at one end of a 100-μl drop containing 30–40% glycerol in the mother liquor. Adapted from Sousa et al. (1991); used by permission of the publisher.

seeded. A thin liquid bridge is then drawn between the two. This works with or without high-viscosity mother liquors. A greater titration effect can be achieved though, when the liquid is viscid and when there is a large difference between the volumes of the two drops.

Crystallization in gels can also filter away excess nuclei, whether they have occurred spontaneously or have been introduced by seeding.

3.4. Filtration

If excess nuclei are a problem, the simplest recourse is to filter the protein through a 0.22 μm filter to remove dust, precipitated protein, and other particles. (Usually the stock solutions for the mother liquor components will have been filtered previously, but often the proteins are not.) This will help ensure that the seed or seeds are placed in an environment free of unintentional nucleants. In fact, filtering the solutions may be sufficient in itself to improve the quality of the crystals without seeding (Blow et al., 1994).

3.5. Cross-seeding selenomethionyl-substituted proteins: Synergism between microseeds and a heterogeneous nucleant

Incorporation of anomalous scatterers such as Se, Xe, or Br into proteins for multiwavelength anomalous diffraction (MAD) is the phasing method most used in high-throughput protein crystallography. Selenomethionyl proteins crystallize under essentially the same conditions as their native counterparts, although the protein concentration may be different because the Se proteins are sometimes less soluble. Seeding with native protein crystals is frequently done (Doublie and Carter, 1992).

An example from our lab, Fig. 8, shows crystals of selenomethionyl limonene epoxide hydrolase (SeMet-LEH) (Arand et al., submitted). Although it was possible to obtain crystals of the native protein, the selenomethionyl protein was difficult to crystallize. Microseeding with the native crystals sometimes helped but was unreliable. Interestingly, it was found that adding a single grain of sand in combination with the microseeds somehow enhanced the effect of the microseeding so that crystals did grow in most drops. (Sand alone without microseeds had no effect.) The first description of a structure solved with MAD, *Escherichia coli* thioredoxin, also reports using a grain of sand with the microseeds to promote the crystallization of the SeMet-protein (Hendrickson et al., 1990).

The main ingredient in sand is SiO_2. It may be that there are no unique properties of sand which account for its success in the SeMet-LEH and SeMet-thioredoxin crystallizations. Perhaps the addition of any nucleant would have enhanced the microseeding but this was not tested. The effects of heterogeneous nucleants in synergism with homogeneous nucleants (i.e., seeds of the native protein) have not been much investigated, although the use of minerals alone has been the subject of a paper (McPherson and Shlichta, 1988). Currently there is an intense investigation of materials such as silicons (Chayen et al., 2001; Sanjoh et al., 2001), Langmuir–Schaeffer films (Pechkova and Nicolini, 2001, 2002), and polymeric films (Fermani et al., 2001) as surface catalysts. These surfaces promote nucleation but without epitaxy, i.e., there is no lattice match between the nucleant and the protein as in cross-seeding. Synergistic effects between homogeneous and heterogeneous nucleation will need to be explored as this research area develops.

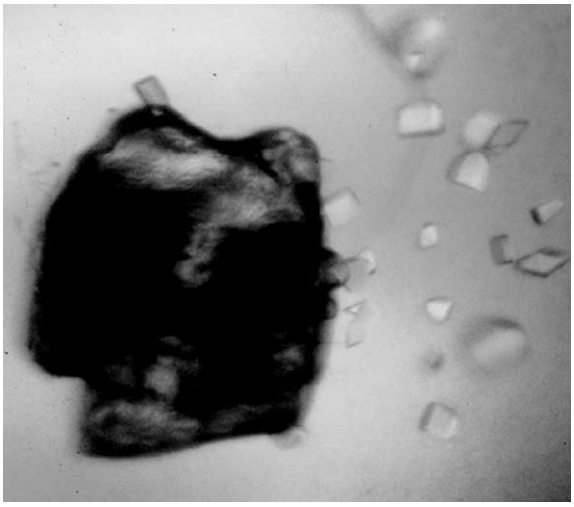

Fig. 8. Crystals of SeMet-LEH growing on and in the vicinity of a grain of sand. The large object to the left is the grain of sand. It is speculated that the crystals seen growing to the right may have nucleated on the sand and floated away later. The typical crystal size is 0.25 mm^3.

4. When seeding will not or does not work

4.1. If the protein is not pure: Effects of seeding into impure solutions

Crystallization is often used in the process industry as a purification step to recover proteins from the highly impure fermentation broth. That this is possible is evidence of the crystal surface's remarkable capacity to select appropriate molecules for incorporation. Nonetheless, purity of the protein solution is among the most critical factors in determining the outcome of the crystallization experiment and many studies have been conducted on the effects of contaminants on crystal quality. Caylor et al. (1999) have specifically looked at the role of seeding and protein purity for hen egg-white lysozyme solutions contaminated with (a) a structurally unrelated protein and (b) a structurally similar one. The result of their study (see Table 1) is probably equally applicable to seeding experiments with other proteins. It seems to indicate that problems resulting from microheterogeneity in the protein solution are not likely to be resolved by seeding.

4.2. Alternatives to seeding

Seeding is only one of many possible optimization methods. Some alternatives to separate nucleation from growth, or to slow down the growth after nucleation has occurred, are presented in Table 2. This table should not be regarded as a comprehensive list of all optimization methods. Rather, the suggestions have been selected on the basis of their simplicity of implementation.

5. Perspectives

The advent of microfocusing synchrotron beams now makes it possible to collect data from crystals of less than 100 μm but the need for large crystals still exists (see Rayment, 2002, for a discussion). Seeding can be used to grow larger crystals or get them to grow singly, improve their diffraction quality, save protein, reduce long waits for spontaneous nucleation, and cross-seed other proteins. We foresee that the usefulness of seeding will continue in the era of high-throughput crystallography. New developments that will affect how seeding is implemented in the future include automation of crystallization, use of laser beams for seed handling, DLS for determining when nucleation has occurred, and new materials for the promotion of nucleation.

5.1. Automation

Large-scale screening for initial crystallization conditions discovers more polymorphs. To some extent the

Table 1
Impurity effects with and without seeding (adapted from Caylor et al., 1999)

Type of contaminant added to the hen egg-white lysozyme (HEWL)	Effect without seeding	Effect when seeded with a well-ordered HEWL seed
20% ovotransferrin (an unrelated protein)	Spontaneous nucleation is difficult	The seeded crystal grows without defect
	Disordered crystal core resulting in cracking and disorder	Spherulites appear in the same drop
20% turkey egg-white lysozyme (structurally similar protein)	Lengthened *c* axis	No improvements in crystal quality
	Crystal cracking	

Table 2
Easy optimization alternatives to seeding

Method	Comments	Reference
Filter the protein	Removes dust, precipitated protein, and particles that could be unintentional nucleation sites	Blow et al. (1994)
Cover the reservoir with oil (in vapor diffusion)	Slows the rate of equilibration	Chayen (1997)
Evaporative dialysis	Useful for proteins with a narrow metastable zone	Bunick et al. (2000)
Temperature shifts	Can work for proteins that exhibit temperature-sensitive solubility	Blow et al. (1994)
"Backing off" (in vapor diffusion)	After nucleation has occurred, the coverslip is moved to a different reservoir for equilibration at metastable conditions	Saridakis and Chayen (2000)

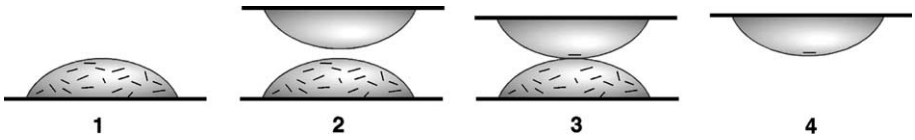

Fig. 9. Sandwich drop set-up for seed transfer (macroseeding) with a laser beam, adapted from Bancel et al. (1998); (1) Sitting drop with microcrystals. (2) A new drop is suspended over the sitting drop. (3) A sandwich interface is made to connect the two drops. A laser beam of some few milliwatts is used to select and propel a single seed to the surface of the sitting drop. (4) After the seed is transferred, the sandwich interface is interrupted to leave a hanging drop with the single seed. (Graphics by Pierre Filing, Biomedical Center Photo Department, Uppsala University.)

availability of more crystals to choose from in the initial screen may lessen the need for optimization. Still, most of the initial trials will produce only microcrystals or crystals that require further improvement. Optimization is difficult to automate since it must be adapted for each case individually. Among the efforts to automate optimization is the program Streak, developed by Douglas Instruments (London, UK). Streak uses a cat whisker mounted into the Douglas Instruments robot Impax, to inoculate microbatch drops with seed dilutions ranging from 10^{-1} to 10^{-6}. It has been tested on racGDP, a small G protein (Haire et al., 2001).

Even without automation, microseeding and streak-seeding are simple, quick, and cheap.

5.2. Laser beams for single-seed transfer (macroseeding)

Macroseeding, however, is more difficult because it requires many manipulations. During all these manipulations, it can happen that the macroseed gets damaged, causing unintentional showers of microcrystals. To address this problem, Bancel et al. (1998) constructed a tabletop laser beam for macroseeding individual seeds (1–25 μm) of tomato bushy stunt virus. The beam levitates the seed crystal from a sitting drop and propels it via a sandwich interface to the new drop. After the transfer is completed, the sandwich interface is interrupted, leaving a hanging drop with the single macroseed (Fig. 9). This method greatly simplifies macroseeding and makes it possible to use extremely small, but individually distinguishable, seeds.

5.3. DLS to determine the metastable zone

Applications using DLS will make it easier in the future to pinpoint the metastable zone or to find the optimal nucleus size (Saridakis, 2000) and thereby increase the accuracy of the seeding.

5.4. Heterogeneous nucleants

The research on nucleation-inducing materials for protein crystallization is currently just getting under way. The role of these heterogeneous nucleants in promoting nucleation alone and in synergism with seeding needs to be explored.

6. Summary

Spontaneous nucleation is a rare and unusual event. No other step in crystallization requires as much energy as the formation of a nucleus. In the experiment that uses a seed, this difficult kinetic barrier to spontaneous nucleation is bypassed. It is hoped that the techniques and applications which have been presented here will make it easier for crystal growers to exploit the valuable resource of seeds. "From a small seed a mighty trunk may grow" (Aeschylus, 526–456 BC).

7. Technical note: obtaining whiskers or horse tail hairs for seeding

Cat whiskers are shed naturally by cats and can be recovered from the floors and carpets of cat owners. Under no circumstance should they be yanked from a living cat: the whiskers are extremely sensitive sensory organs in the feline. Whiskers from dead cats can be bought from Charles River Suppliers. Horse tail hair can be simply cut off using scissors. Do not use horse tail from mares since estrous females spray urine.

Acknowledgments

I thank Irina Zaitseva for her suggestion regarding *Equus caballus*, Jeroen Mesters for locating the whisker supplier, Gordon Vrdoljak at the Electron Microscope Department of the University of California at Berkeley for Fig. 2a, Stefan Odestedt for Fig. 2b, Martin Hallberg for Fig. 5b, Pierre Filing at the Uppsala University Biomedical Center Photo Department for making Fig. 9, Naomi Chayen for helpful discussions, and Gerard Kleywegt, Elisabeth Sauer-Eriksson, Johanna Sandling, and Matti Nikkola for critically reading the manuscript.

References

Atha, D.H., Ingham, K.C., 1981. Mechanism of precipitation of proteins by polyethylene glycols. Analysis in terms of excluded volume. J. Biol. Chem. 256, 12108–12117.

Baker, R., Stewart, M., 2000. Crystallization and preliminary X-ray diffraction analysis of the *Saccharomyces cerevisiae* Ran-binding protein Mog1p. Acta Crystallogr. D 56, 229–231.

Bancel, P., Cajipe, V., Rodier, F., Witz, J., 1998. Laser seeding for biomolecular crystallization. J. Cryst. Growth 191, 537–544.

Bergfors, T., Rouvinen, J., Lehtovaara, P., Caldentey, X., Tomme, P., Claeyssens, M., Pettersson, G., Teeri, T., Knowles, J., Jones, T.A., 1989. Crystallization of the core protein of cellobiohydrolase II from *Trichoderma reesei*. J. Mol. Biol. 209, 167–169.

Blow, D.M., Chayen, N.E., Lloyd, L.F., Saridakis, E., 1994. Control of nucleation of protein crystals. Protein Sci. 3, 1638–1643.

Boistelle, R., Astier, J., 1988. Crystallization mechanisms in solution. J. Cryst. Growth 90, 14–30.

Bunick, C., North, A., Stubbs, G., 2000. Evaporative microdialysis: an effective improvement in an established method of protein crystallization. Acta Crystallogr. D 56, 1430–1431.

Caylor, C., Dobrianov, I., Lemay, S., Kimmer, C., Kriminski, S., Finkelstein, K., Zipfel, W., Webb, W., Thomas, B., Chernov, A., Thorne, R., 1999. Macromolecular impurities and disorder in protein crystals. Proteins: Struct. Funct. Genet. 36, 270–281.

Chayen, N., 1997. A novel technique to control the rate of vapour diffusion, giving larger protein crystals. J. Appl. Crystallogr. 30, 198–202.

Chayen, N., Saridakis, E., El-Bahar, R., Nemirovsky, Y., 2001. Porous silicon: an effective nucleation-inducing material for protein crystallization. J. Mol. Biol. 312, 591–595.

Doublie, S., Carter, C., 1992. Preparation of selenomethionyl protein crystals. In: Ducruix, A., Giege, R. (Eds.), Crystallization of Nucleic Acids and Proteins. Oxford Univ. Press, Oxford.

Fermani, S., Falini, G., Minnucci, M., Ripamonti, A., 2001. Protein crystallization on polymeric film surfaces. J. Cryst. Growth 224, 327–334.

Haire, L., Vasisht, N., Hirshberg, M., Li, J., Taylor, I., Smerdon, S., 2001. Experiences in microbatch crystallization. Poster presented at Recent Advances in Macromolecular Crystallization, Le Bischenberg, France.

Hallberg, B., Bergfors, T., Bäckbro, K., Pettersson, G., Henriksson, G., Divne, C., 2000. A new scaffold for binding haem in the cytochrome domain of the extracellular flavocytochrome cellobiose dehydrogenase. Structure 8, 79–88.

Han, Q., Lin, S.-X., 1996. A microcrystal selection technique in protein crystallization. J. Cryst. Growth 168, 181–184.

Hendrickson, W., Horton, J., LeMaster, D., 1990. Selenomethionyl proteins produced for analysis by multiwavelength anomalous diffraction (MAD): a vehicle for direct determination of three-dimensional structure. EMBO J. 9, 1665–1672.

Korkhin, Y., Frolow, F., Bogin, O., Peretz, M., Kalb (Gilboa), A., Burstein, Y., 1996. Crystalline alcohol dehydrogenases from the mesophilic bacterium *Clostridium beijerinckii* and the thermophilic bacterium *Thermoanaerobium brockii*: preparation, characterization and molecular symmetry. Acta Crystallogr. D 52, 882–886.

Kuznetsov, Y., Malkin, A., Land, T., DeYoreo, J., Barba, A., Konnert, J., McPherson, A., 1997. Molecular resolution imaging of macromolecular crystals by atomic force microscopy. Biophys. J. 72, 2357–2364.

Kuznetsov, Y., Malkin, A., McPherson, A., 2001. The liquid protein phase in crystallization: a case study—intact immunoglobulins. J. Cryst. Growth 232, 30–39.

Luft, J., DeTitta, G., 1999. A method to produce microseed stock for use in the crystallization of biological macromolecules. Acta Crystallogr. D 55, 988–993.

McPherson, A., Shlichta, P., 1988. Heterogeneous and epitaxial nucleation of protein crystals on mineral surfaces. Science 239, 385–387.

Mowbray, S., 1999. Macroseeding: a real-life success story. In: Bergfors, T.M. (Ed.), Protein Crystallization: Techniques, Strategies, and Tips. International University Line, La Jolla, CA.

Ng, J., Lorber, B., Witz, J., Theobald-Dietrich, A., Kern, D., Giege, R., 1996. The crystallization of biological macromolecules from precipitates: evidence for Ostwald ripening. J. Cryst. Growth 168, 50–62.

Pechkova, E., Nicolini, C., 2001. Accelerated protein crystal growth by protein thin film template. J. Cryst. Growth 231, 599–602.

Pechkova, E., Nicolini, C., 2002. Protein nucleation and crystallization by homologous protein thin film template. J. Cell. Biochem. 85, 243–251.

Prigogine, I., 2001. Speech cited in Horisont Uppsala, Uppsala University Year Book, April 2002, p. 3.

Rayment, I., 2002. Small-scale batch crystallization of proteins revisited: an underutilized way to grow large protein crystals. Structure 10, 147–151.

Rouvinen, J., Bergfors, T., Teeri, T., Knowles, J.K., Jones, T.A., 1990. Three-dimensional structure of cellobiohydrolase II from *Trichoderma reesei*. Science 249, 380–386 (Published erratum appears in Science 249, 1359, 1990).

Sanjoh, A., Tsukihara, T., Gorti, S., 2001. Surface-potential controlled Si-microarray devices for heterogeneous protein crystallization screening. J. Cryst. Growth 232, 618–628.

Saridakis, E., 2000. Optimization of the critical nuclear size for protein crystallization. Acta Crystallogr. D 56, 106–108.

Saridakis, E., Chayen, N., 2000. Improving protein crystal quality by decoupling nucleation and growth in vapor diffusion. Protein Sci. 9, 755–757.

Saridakis, E., Dierks, K., Moreno, A., Dieckmann, M., Chayen, N., 2002. Separating nucleation and growth in protein crystallization using dynamic light scattering. Acta Crystallogr. D 58, 1597–1600.

Saridakis, E., Shaw Stewart, P., Lloyd, L., Blow, D., 1994. Phase diagram and dilution experiments in the crystallization of carboxypeptidase G2. Acta Crystallogr. D 50, 293–297.

Sousa, R., 1995. Use of glycerol, polyols and other protein structure stabilizing agents in protein crystallization. Acta Crystallogr. D 51, 271–277.

Sousa, R., Lafer, E., Wang, B., 1991. Preparation of crystals of T7 RNA polymerase suitable for high-resolution X-ray structure analysis. J. Cryst. Growth 110, 237–246.

Stura, E., 1999. Microseeding. In: Bergfors, T.M. (Ed.), Protein Crystallization: Techniques, Strategies, and Tips. International University Line, La Jolla, CA.

Stura, E., Ruf, W., Wilson, I., 1996. Crystallization and preliminary crystallographic data for a ternary complex between tissue factor, factor VIIa and a BPTI-derived inhibitor. J. Cryst. Growth 168, 260–269.

Stura, E., Wilson, I., 1990. Analytical and production seeding techniques. Methods: Companion Methods Enzymol. 1, 38–49.

Stura, E., Wilson, I., 1991. Applications of the streak seeding technique in protein crystallization. J. Cryst. Growth 110, 270–282.

Thaller, C., Weaver, L., Eichele, G., Wilson, E., Karlsson, R., Jansonius, J., 1981. Repeated seeding technique for growing large single crystals of proteins. J. Mol. Biol. 147, 465–469.

van Aalten, D., Milne, K., Zou, J., Kleywegt, G., Bergfors, T., Ferguson, M., Knudsen, J., Jones, T., 2001. Binding site differences revealed by crystal structures of *Plasmodium falciparum* and bovine acyl-CoA binding protein. J. Mol. Biol. 309, 181–192.

Wierenga, R., Zeelen, J.P., Noble, M., 1992. Crystal transfer experiments carried out with crystals of trypanosomal triosephosphate isomerase (TIM). J. Cryst. Growth 122, 231–234.

Zou, J., Hallberg, B., Bergfors, T., Oesch, F., Arand, M., Mowbray, S., Jones, T., 2000. Structure of *Aspergillus niger* epoxide hydrolase at 1.8 Å resolution: implications for the structure and function of the mammalian microsomal class of epoxide hydrolases. Structure 8, 111–122.