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Using natural seeding material to generate nucleation in protein crystallization experiments

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The nucleation event in protein crystallization is a part of the process that is poorly controlled. It is generally accepted that the protein should be in the metastable phase for crystal growth, but for nucleation higher levels of saturation are needed. Formation of nuclei in bulk solvent requires interaction of protein molecules until a critical size of aggregate is created. In many crystallization experiments sufficiently high levels of saturation are not reached to allow this critical nucleation event to occur. If an environment can be created that favours a higher local concentration of macromolecules, the energy barrier for nucleation may be lowered. When seeds are introduced at lower levels of saturation in a crystallization experiment, nucleation may be facilitated and crystal growth initiated. In this study, the use of natural materials as stable seeds for nucleation has been investigated. The method makes it possible to introduce seeds into crystallization trials at any stage of the experiment using both microbatch and vapour-diffusion methods.

1. Introduction

A great deal of interest has been focused recently on ways to improve the success rate in protein crystallization. This interest has mainly been fuelled by the many structural genomics programs, where major efforts have been directed towards finding more efficient ways to screen for initial crystallization conditions. Progress in the area of miniaturization and automation of the crystallization experiments has made it possible to set up thousands of crystallization trials in a single experiment (Stevens, 2000; Luft *et al.*, 2001). Despite the large number of proteins and screening conditions that have been tested, the success rate has been lower than might be expected (Dale *et al.*, 2003). In our experience using commercially available crystallization screens, typically up to 50% of the drops will remain clear throughout the crystallization experiment, which can vary from weeks to months. This may be because the protein and precipitating agents do not reach levels of saturation that are high enough for nucleation to occur spontaneously. Many investigators have observed the phenomenon of proteins nucleating on contaminants within the crystallization drop such as dust, fibres *etc.* As early as 1987, the idea of introducing heterogeneous inorganic crystalline material to induce epitaxic growth of protein crystals was proposed (McPherson & Shlichta, 1987). In the study, crushed mineral materials were used to generate nucleation surfaces for protein crystals. In the past few years, there have been a number of reports on heterogeneous seeding. Punzi *et al.* (1991) studied crystal growth in the presence of polyvinylidene difluoride and the use of porous silicone to influence nucleation was reported by Chayen *et al.* (2001). Pechkova & Nicolini (2001, 2002) have described the use of the Langmuir–Blodgett technique to coat cover slides with a thin protein film, resulting in an acceleration of the growth of lysozyme crystals. Fermani *et al.* (2001) have used polymeric films containing ionizable groups to influence the nucleation of concanavalin A. More recently, the use of nanostructured heterogeneous nucleants based on compositionally modulated superlattices was proposed for the nucleation of protein crystals (Haushalter *et al.*, 2002).

We have routinely observed many examples of crystals growing spontaneously on fibres and hairs (Fig. 1). The use of hairs for streak-seeding is well established (Leung *et al.*, 1989; Stura & Wilson, 1991). With these observations in mind, we decided to investigate the possibility of using this type of material to influence nucleation in a crystallization experiment. In our present study, we present a simple method of inducing nucleation at lower concentrations of protein or precipitating agent using stable and readily available natural materials.

2. Experimental procedures

2.1. Materials

The proteins used in the study were lysozyme (Merck cat. No. 1.05281), glucose isomerase (Hampton Research cat. No. HR7-100) and trypsin (Sigma T-8003 and Roche cat.

No. 1 027 891) prepared according to the manufacturer's instructions or in the case of trypsin in the presence of 50 m*M* benzamidine. *Escherichia coli* malonyl coenzyme A-acyl carrier protein transacylase (Fab-D) was expressed and purified as described previously (Serre *et al.*, 1994). Protein concentrations were between 10 and 100 mg ml^{-1}. For microbatch experiments with glucose isomerase, lysozyme and trypsin, a standard IMPAX I-5 robot (Douglas Instruments) was used, dispensing volumes of 1 µl protein plus 1 µl precipitating agent into Nunc 72-well HLA plates covered with 6 ml of paraffin oil (Chayen *et al.*, 1990). Fab-D was crystallized using vapour diffusion in a hanging-drop system (McPherson, 1982). Hair was obtained from the manes of two horses, 5 and 13 years of age, and from the whiskers of a laboratory rat; seaweed was obtained from a Tunisian beach in a naturally dried form.

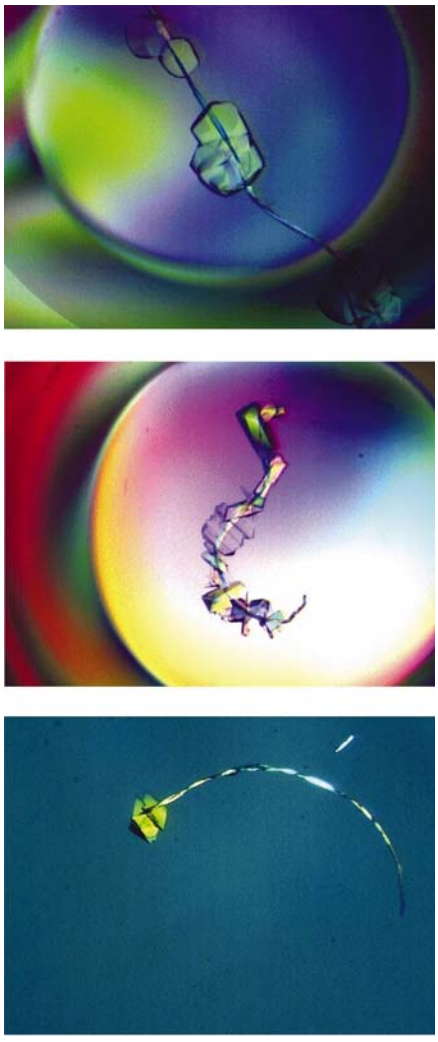

Figure 1
Examples of spontaneous nucleation of protein crystals in fibres.

Table 1
Conditions used for crystallization of the proteins tested.

Protein	Concentration (mg ml^{-1})	Crystallization solution
Glucose isomerase	5–8	30% PEG monomethyl ether 550, 100 m*M* HEPES pH 7.0, 50 m*M* $MgCl_2$
Trypsin	17	25% PEG 3350, 200 m*M* Li_2SO_4, 100 m*M* bis-tris pH 6.5
Lysozyme	80	25% PEG 3350, 200 m*M* NaCl, 100 m*M* HEPES 7.5
Fab-D	21	2.2 *M* ammonium sulfate, 100 m*M* Na acetate pH 5.0, 1% PEG 400

Table 2
Effects of different hair types on nucleation of glucose isomerase.

Type of hair	Total No. of crystals observed in 6 drops	Total No. of crystals growing on hair
Rat	4	1
Horse 1 (13 y)	4	0
Horse 2 (5 y)	4	3
Dried seaweed	4	1

2.2. Crystallization and seeding experiments

Crystallization experiments were set up using the test proteins mentioned previously including Fab-D, which was chosen as a protein known to be difficult to nucleate under certain conditions (Table 1). A series of dilutions of the test proteins were made in order to establish at which concentrations spontaneous nucleation would not occur. In a typical experiment, equal volumes of protein and pre-determined precipitating agents were mixed and either a microbatch (D'Arcy *et al.*, 1996, 2003) or hanging-drop vapour-diffusion technique (McPherson, 1982) was used. The experiments were observed on a daily basis to determine the date of appearance and number of crystals over a period of one week.

2.3. Seed preparation

For initial experiments, horse hair, rat whiskers and dried seaweed were cut into small pieces with a scalpel and placed in the crystallization drop. For subsequent experiments, approximately 0.5 g of horsehair was crushed with a porcelain mortar in a pestle filled with liquid nitrogen until a crude powder was obtained. For the final experiments, the crushed hairs were sonicated in 10 ml of aqueous solutions to produce a more homogeneous suspension; these stayed suspended for several hours. The hair stock was mixed in the appropriate crystallization solutions at various dilutions to determine their effect on nucleation.

3. Results

Having established protein concentrations and precipitating agent conditions where little or no spontaneous nucleation occurred, a number of different fibrous materials that could have an effect on nucleation were tested (rat whiskers, horse hair and dried seaweed) with glucose isomerase. For these initial experiments, a single piece of each type of hair was placed in the crystallzation drop after mixing protein and precipitating agent in a microbatch experiment. Six identical drops were set up for each hair type, plus control drops without hair. Hair from one of the horses chosen appeared to have a more pronounced influence on the nucleation of the protein (Table 2). The fact that this was the younger of the two horses

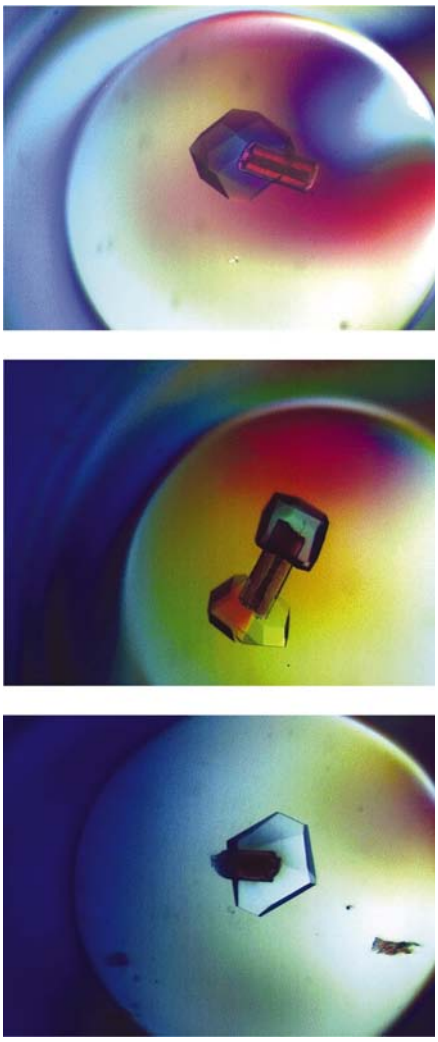

Figure 2
Examples of nucleation of protein crystals on horse hair.

Table 3
Experiment to show the effect on the number of crystals produced with and without the introduction of hair seeds (each value represents the average number of crystals for at least 3 drops).

Protein	Control (0 seeds)	Seed dilution 1/5	Seed dilution 1/25
Trypsin	0	75	8
Glucose isomerase	1	125	64
Lysozyme	1	9	6
Fab-D	0	8	2

may or may not be significant and this will need to be examined in future experiments; however, to simplify the experimental setups this type of hair was used for all subsequent tests. When this experiment was repeated using single pieces of hair from horse 2, crystals often appeared to grow at the extremities of the hairs (Fig. 2). This led us to postulate that the inner part of the hair may be a more suitable surface for nucleation than the exterior. Animal hairs have three layers with different constituents, which might feasibly interact differently with the protein. We therefore used a preparation of hair as described previously, which was subsequently sonicated to produce a more homogeneous suspension and to expose more of the interior structure. This suspension was diluted in the respective crystallization solutions to determine the extent of nucleation on the test proteins; a dilution of the stock solution from between 1/5 and 1/25 proved suitable for most of the proteins without introducing too much extraneous material into the crystallization drop. The effect on the different proteins with and without seeds is shown in Table 3. An example of glucose isomerase grown in the presence and absence of hair seeds is shown in Fig. 3. In this illustration it is still possible to identify pieces of hair at the centre of the crystals. Surprisingly, the effect is less pronounced with lysozyme and a more concentrated hair stock is required to induce nucleation. Having established a correlation between the introduction of hair seeds and crystal nucleation on the test proteins, we decided to test the effect on a protein known to be difficult to nucleate. In our experience, Fab-D from *E. coli* produced only spherulites under the published conditions for the tetragonal form (Serre *et al.*, 1994). Streak-seeding from these microcrystalline aggregates generally produced crystals, which can be used for subsequent seeding experiments to produce large well formed bipyramid crystals. The standard conditions for this protein were set up with and without hair seeds at different dilutions. In the control experiment without seeds no crystals were observed; in the trials with hair seeds nucleation had occurred and crystals grew. The crystals obtained from these seeds had identical morphology to those produced from the homogeneous crystal seeds. In the initial experiment a fairly crude and concentrated hair suspension was used and in some cases hairs can once again be identified as inclusions within the crystal (Fig. 4).

4. Conclusions

This preliminary investigation has shown that a very simple method introducing horse hair at the beginning of the crystallization experiment can have an effect on nucleation of the test proteins glucose isomerase, lysozyme and trypsin. The method was also shown to be useful in a case where nucleation was known to be problematic (Fab-D).

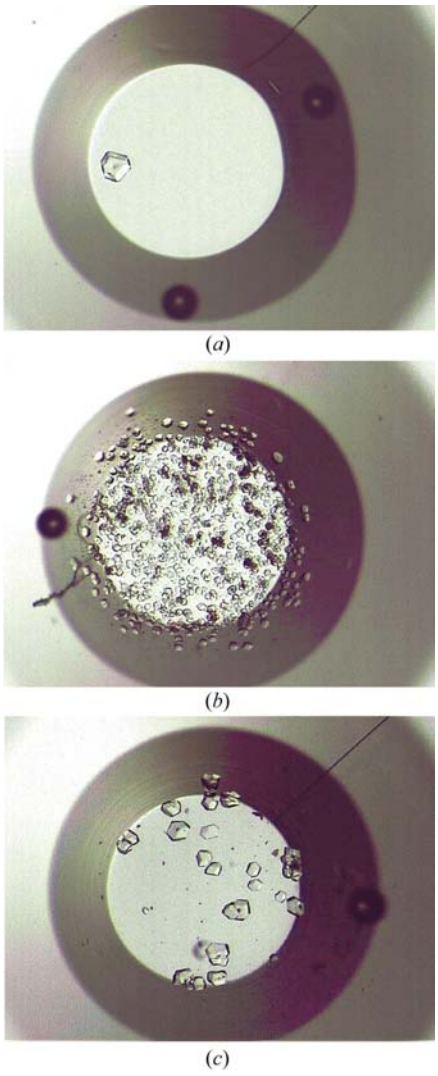

Figure 3
The effect of introducing the crushed hair seeds on glucose isomerase: (*a*) no seeds, (*b*) concentrated seed stock, (*c*) diluted seed stock.

The fact that seeding is one of the most important tools for obtaining protein crystals is not novel, but the possibility of having a stable stock of seeding material that is not protein-specific in its nucleation behaviour is quite attractive. In addition, this method simplifies the experimental side of a seeding experiment, with regard to the time point at which seeds are introduced. When protein crystal seeds are used at the beginning of a crystallization, they may dissolve at the low concentrations of precipitating agent and need to be reintroduced once the drops have equilibrated. In addition, fresh crystal seeds need to be made for each new seeding experiment. With hair seeding neither of these problems are encountered: the hairs are stable in any buffer system and can also be frozen. These types of seeds can be introduced at any time during the experiment at different dilutions. The method will

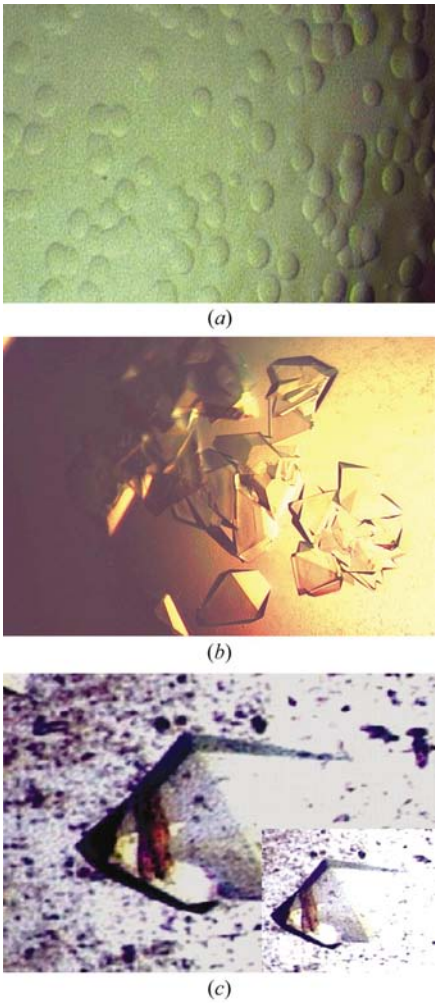

Figure 4
Fab-D crystallization in ammonium sulfate, acetate pH 5.0 and PEG 400: (*a*) no seeds added, (*b*) crystal seed added, (*c*) horsehair seeds added showing hair inclusion in crystal.

also allow the introduction of seeds during initial screening trials for crystallization conditions, which should increase the number of hits that can be expected in such screens; we are at present investigating the validity of this approach. A number of questions remain to be answered to show the general applicability of this approach. Different types of animal hair from animals of varying ages will need to be tested. Experiments will be needed to identify whether the 'nucleation agent' present in the seeding stock is solid or a soluble extract. However, we feel this initial study has shown an effect on more than one protein and that further investigation of the phenomenon is justified.

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