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When less is more: a more efficient vapour-diffusion protocol

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Reducing protein consumption during crystallization screening is of utmost importance to crystallographers because of the time, effort and money that goes into producing pure protein. One approach is to reduce sample volumes with robotics, but a patent and the high cost of equipment limits access. Here, it is shown that the same result can be obtained by reducing the sample concentration in a modified vapour-diffusion protocol, the dilution method. In this protocol, the protein and mother liquor in the crystallization drop are both diluted, while the mother liquor in the well remains undiluted. Vapour diffusion will shrink the initial volume of the crystallization drop, *e.g.* 1 µl or more, to a drop size equivalent to one dispensed by a robot. This new crystallization method circumvents some of the current problems associated with robotic crystallization screening trials. Because of the large initial volume of the crystallization drop, the evaporation problem is eliminated and dispensing accuracy is improved. In addition, the likelihood that the crystallization experiment starts in the undersaturated region is increased.

1. Introduction

1.1. Reducing protein consumption

Reducing protein consumption during crystallization screening is an important challenge for crystallographers (Rupp, 2003*a*). This is because the availability of sufficient amounts of highly purified protein, in particular for those proteins that are difficult to overexpress, is frequently a limiting factor. For traditional vapour-diffusion crystallization screening, the desired amount of protein before screening is started is usually 5–10 mg, although often much greater amounts are used before a diffraction-quality crystal is obtained.

There are three factors that determine the total amount of protein used in a crystallization screen. These factors are the number of crystallization trials, the volume of protein sample used per trial and the concentration of protein sample. This is illustrated in the equation

$$\text{total protein} = \text{No. of trials} \times \text{volume per trial} \times \text{concentration}.$$

As a consequence, there are three possible means of reducing protein consumption during crystallization screening. One involves reducing the number of crystallization trials by more efficiently sampling crystallization space. There are a couple of means of achieving this: (i) using a random-sampling screening technique, which has been found to be the most efficient screening method (Segelke, 2001), and (ii) performing a small pre-screen to determine the protein solubility over a range of

Table 1
The effect of the dilution method on lysozyme crystal growth.

Dilution	No. of crystals per drop†	Longest crystal edge† (μm)
1	1150 (300–2000)	65 (30–100)
2	70 (60–80)	125 (110–140)
3	25	120 (110–130)
4	15 (10–20)	140
5	20 (15–25)	120 (110–130)
6	15 (10–20)	130 (110–150)

† The mean value for the quadruplicate experiment is listed first, followed by the lowest and highest values in parentheses. For lysozyme, all of the quadruplicate experiments yielded crystals.

pH values. This is useful so that an optimal choice of screening conditions may be made (Rupp, 2003*b*). In future, the results from data-mining operations that correlate properties of proteins and their crystallization conditions may allow the rational prediction of crystallization conditions for previously uncrystallized proteins (Luft *et al.*, 2003), thus reducing the number of crystallization trials required.

The second method of reducing protein consumption is decreasing the volume of protein solution used per trial. As the limitation of manual pipetting is approximately 0.5 μl, robots are required for dispensing smaller volumes. Santarsiero *et al.* (2002) have reported producing diffraction-quality crystals using a hanging-drop size of as little as 40 nl. However, 100 nl volumes more routinely produce diffraction-quality crystals (Santarsiero *et al.*, 2002). This still represents a tenfold improvement over manual pipetting. Unfortunately, the robots are very expensive and the creation of crystallization drops with volumes less than 1 μl has been patented (Regents of the University of California, 2001).

The third method of reducing protein consumption involves dilution of the protein solution. Here, we demonstrate a modified vapour-diffusion method in which the protein and mother liquor in the crystallization drop are diluted.

1.2. The dilution method

The theoretical differences between the traditional vapour-diffusion method and the dilution method are illustrated in Fig. 1. In a traditional experiment, the initial concentration of protein ($C_{P\ \mathrm{traditional}}$) and mother liquor ($C_{\mathrm{ML\ traditional}}$) in the crystallization drop are half that of the protein sample and mother liquor (black dot). By vapour diffusion, both the protein concentration and mother-liquor concentration increase (bold black line) until the concentrations pass into the nucleation region, where a nucleation event can provide the core for crystal formation. During the equilibration process, the concentration of the mother liquor in the drop doubles. The protein concentration also rises until the nucleation event, at which point the protein concentration in solution decreases as the crystal is formed.

In the dilution method, both the protein and precipitant solution are diluted *n*-fold. The initial conditions are given by $C_{\mathrm{ML\ diluted}}$ and $C_{P\ \mathrm{diluted}}$ in Fig. 1. Owing to vapour diffusion, the protein and precipitant concentration will increase as indicated by the dashed line. When the concentrations have reached $C_{\mathrm{ML\ traditional}}$ and $C_{P\ \mathrm{traditional}}$, a situation identical to the traditional experiment is obtained but with an *n*-fold lower drop volume. Here, we explore the practical use of this new strategy.

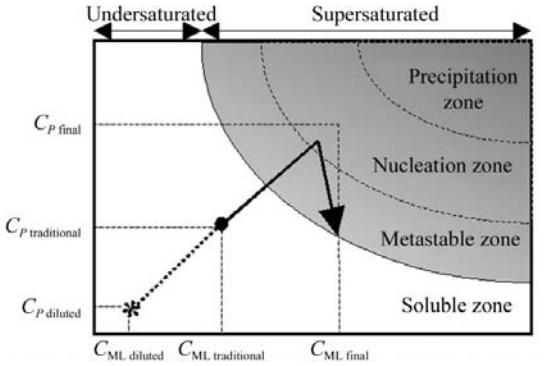

Figure 1
Crystallization space diagram showing the path of mother-liquor and protein concentrations during a vapour-diffusion crystallization experiment. The black circle at $C_{\mathrm{ML\ traditional}}$ and $C_{P\ \mathrm{traditional}}$ represents the initial mother-liquor and protein concentrations for traditional vapour-diffusion crystallization experiments. The asterisks at $C_{\mathrm{ML\ diluted}}$ and $C_{P\ \mathrm{diluted}}$ represent the initial concentrations of protein and mother liquor for an *n*-fold diluted vapour-diffusion experiment. The dashed and solid lines indicate the concentrations during the equilibration process.

2. Experimental

2.1. Protein preparation

Lysozyme (hen egg-white, Sigma; MW = 14.3 kDa) was dialyzed against 0.1 *M* sodium acetate pH 4.8, whereas glucose isomerase (Hampton Research; MW = 173 kDa) was dialyzed against water. The final concentrations determined by UV–Vis spectroscopy (ε_{280} values of 1.042 and 2.68) were 50 and 26 mg ml^{-1}, respectively.

2.2. Crystal growth

The crystals were grown in quadruplicate at 295 K by sitting-drop vapour diffusion using previously determined crystallization conditions. For lysozyme, the reservoir solution consisted of 1 ml of 8%(*w*/*v*) sodium chloride and 0.1 *M* of sodium acetate at pH 4.8 (modified from Drenth, 1994). For glucose isomerase, the reservoir solution consisted of 1 ml of 0.7 *M* sodium citrate pH 6.3 (Hampton Research, 2001).

To form the crystallization drop, 1 μl of the diluted protein solution and 1 μl of the diluted mother-liquor solution were pipetted into the sitting-drop post. 1 ml of undiluted reservoir solution was pipetted into the reservoir.

3. Results and discussion

3.1. Effects of the dilution method on crystallization

As explained in the introduction, crystallization theory predicts that the dilution method is equivalent to ultrasmall-volume crystallization experiments after the evaporation of the excess water from the drop. In a preliminary test of the method, we created crystallization experiments for lysozyme

and glucose isomerase with dilutions up to sixfold. Tables 1 and 2 give the results of these experiments.

For lysozyme, crystals were obtained at all dilution levels. The crystals all had the same morphology; however, dilution had an effect on two crystal properties: size and number. The size of the crystals increased almost twofold upon a twofold dilution. Further dilution had no effect on the size of the crystals. The number of crystals formed decreased upon increasing dilution between onefold and threefold; however, at dilutions greater than threefold the number remained constant (Fig. 2*a*–2*f*).

For glucose isomerase, crystals were also obtained at all dilution levels, but dilution caused the appearance of two new crystal forms. The crystal form depends on the extent of dilution. Form *A* (Fig. 2*g*) belongs to space group *I*222 (unit-cell parameters a = 101.81, b = 98.05, c = 92.62 Å) and crystallized at onefold and twofold dilutions. In drops containing crystal form *A*, no other crystal form was present. Form *B* (Fig. 2*h*) belongs to space group $P2_12_12$ (unit-cell parameters a = 78.21, b = 99.30, c = 129.92 Å) and crystallized in drops at dilutions of twofold or greater. Structures for both form *A* and *B* crystals have been solved previously. Form *C* (Fig. 2*i*) crystals were too small to be characterized and crystallized in drops at dilutions of fourfold or greater. Crystal forms *B* and *C* could appear as a single form in a crystallization drop or as a mix of these two forms. The form *B* crystals were larger when they were not mixed with form *C*.

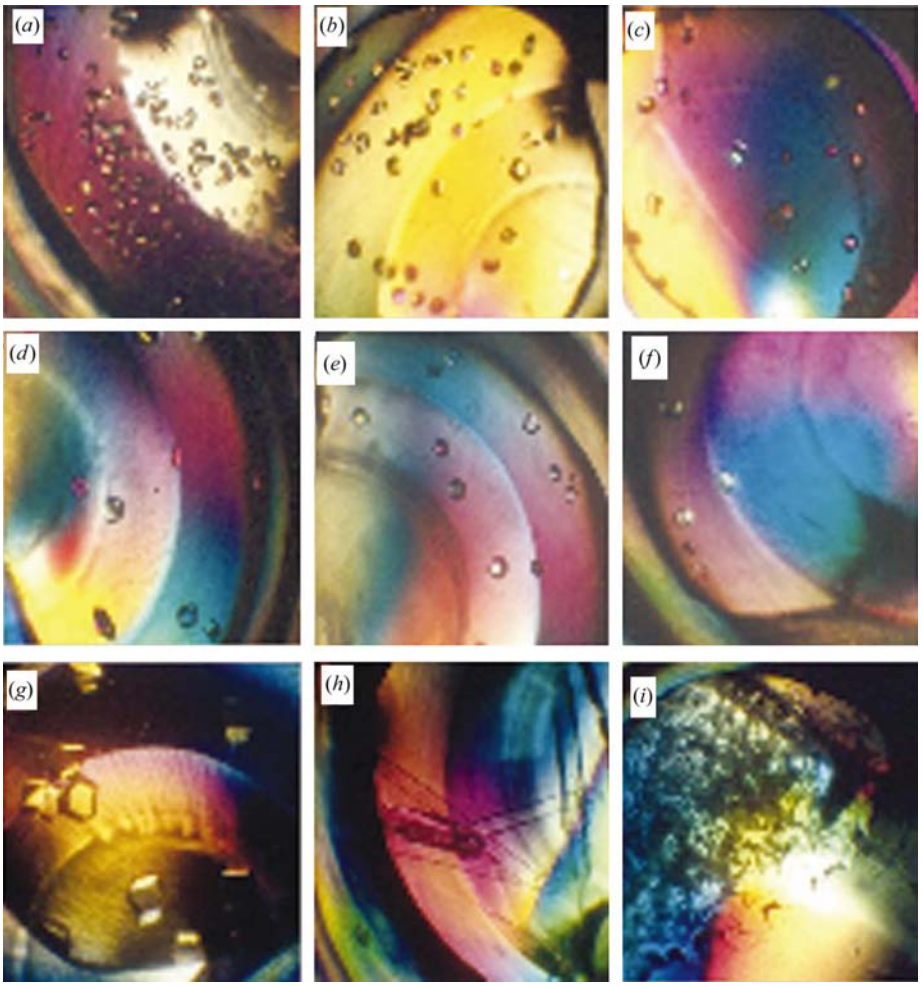

Figure 2
Crystals of lysozyme and glucose isomerase: (*a*–*f*) lysozyme crystals at onefold to sixfold dilutions, respectively, (*g*) glucose isomerase crystal form *A*, (*h*) glucose isomerase crystal form *B*, (*i*) glucose isomerase crystal form *C*.

The time taken for crystal growth in the dilution method was slightly longer than for the traditional method. The extra time taken depended on the dilution of the crystallization drop, but was never more than 1 d. In this respect, nanolitre-volume experiments have an advantage as they have been reported to actually speed up crystal formation.

3.2. Dilution and traditional vapour-diffusion method comparison

As predicted by theory, the dilution method in comparison with the traditional method does not affect crystal detection in our two test cases. There are, however, clear differences between the diluted and undiluted experiments most likely caused by processes occurring during the evaporation of the excess water from the drop (corresponding to the dashed line in Fig. 1). For instance, if there are volatile components other than water in the mother liquor then they may diffuse to the crystallization droplet and affect crystallization behaviour. More interestingly, if the starting conditions of the undiluted experiments already fall in the nucleation zone, then the diluted experiment will traverse a region of lower supersaturation not explored by the undiluted experiment. Fewer nucleation sites and therefore larger crystals could be expected in this situation. This is indeed what we observe for lysozyme in the undiluted to threefold-diluted range. This suggests that at least the undiluted and twofold diluted starting conditions are already in the nucleation zone. For glucose isomerase, dilution actually leads to different crystal morphologies. Again, this could occur if the initial conditions are already in the nucleation zone and the different crystal forms need different levels of superaturation; crystal form *A* would need the highest supersaturation and crystal form *C* the lowest.

3.3. Advantages and concerns of the dilution method

Although more research is required, our preliminary results suggest that the dilution method is a practical alternative for

Table 2
The effect of the dilution method on glucose isomerase crystal growth.

Dilution	Crystal form†	No. of wells‡	No. of crystals per drop§	Longest crystal edge (µm)§
1	*A*	4	16 (10–32)	202 (110–250)
2	*A*	1	1	210
	B	2	4 (1–7)	700 (600–800)
3	*B*	4	7 (1–10)	940 (800–1000)
4	*B*/*C*	4	10	300 (200–400)
5	*B*	1	20	1000
	B/*C*	3	9	470 (400–500)
6	*B*	1	5	1000
	B/*C*	2	9 (8–10)	650 (600–700)

† *A*, *B* and *C* represent the three different crystal morphologies of glucose isomerase shown in Figs. 2(*g*), 2(*h*) and 2(*i*), respectively. ‡ The number of wells in the quadruplicate experiment that yielded this crystal form. § The mean value is listed first, followed by the lowest and highest values in parentheses.

ultrasmall-volume experiments. Apart from the fact that it is amenable to both manual and automated procedures, there are a few other practical advantages over ultrasmall-volume drops. Firstly, small nanolitre-volume drops can evaporate during the time between dispensing and sealing the plate. Current solutions involve base-plate cooling and humidifying the robotic setup environment (Bodenstaff *et al.*, 2002). The dilution technique will greatly reduce or eliminate this problem because of the very low initial precipitant concentrations of the drops and the large volume-to-surface area ratio. Secondly, dispensing accuracy decreases as dispensing volumes decrease, especially for viscous or low-surface-tension solutions (Bodenstaff *et al.*, 2002). The dilution method improves accuracy by both increasing dispensing volumes and decreasing viscosity. Thirdly, dilution ensures that almost all experiments will start in the undersaturated zone. As a result, there is an increased likelihood of crystal formation. Fourthly, since the dilution method can give different crystallization behaviour, it may be considered as an optimization method when preliminary conditions have already been found. Based on theory and the observations for lysozyme, dilution may lead to fewer and larger crystals. Alternatively, the dilution method may yield different crystal forms that may be more suitable for diffraction studies or can facilitate structure solution by cross-crystal averaging.

One concern when using the dilution method is that dilution may cause protein instability if salt is required for its stability. There was no such problem in our experiments, but the two test proteins are highly soluble. Tests on a broader range of proteins will be undertaken to determine whether this potential concern occurs in practice.

4. Conclusions

A simple but innovative vapour-diffusion protocol has been developed to reduce protein consumption in crystal screens. The method can be used in manual or automated modes and does not require special equipment. The primary focus of the method is to conserve protein, but our theoretical considerations and our observations suggest that it may also have some practical advantages over ultrasmall-volume experiments. Crystallization experiments on two test proteins have confirmed and refined our theoretical expectations and constitute a proof of concept. A much more extensive study is planned to evaluate the general applicability and to make a more quantitative comparison with existing methods.

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