Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Application of a two-liquid system to sitting-drop vapour-diffusion protein crystallization

Hiroaki Adachi,[a] Kazufumi Takano,[b,c]* Masaaki Morikawa,[b] Shigenori Kanaya,[b] Masashi Yoshimura,[a] Yusuke Mori[a] and Takatomo Sasaki[a]

[a]Department of Electrical Engineering, Graduate School of Engineering and Venture Business Laboratory, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan, [b]Department of Material and Life Science, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan, and [c]PRESTO, JST, Japan

Correspondence e-mail: ktakano@mls.eng.osaka-u.ac.jp

Received 1 August 2002
Accepted 28 October 2002

A new method of protein crystallization, the floating-drop vapour-diffusion technique, has been developed. The method combines the traditional sitting-drop vapour-diffusion technique and a novel two-liquid system. A crystallization drop composed of a mixture of sample and reagent floats on an insoluble and very dense liquid. Protein crystals are grown by vapour diffusion at the interface of the two liquids. The method makes it possible to remove the crystals easily without causing mechanical damage. This approach also significantly reduces the time and cost compared with the hanging-drop technique, which is presently the most popular method for protein crystallization.

1. Introduction

X-ray crystallography is a powerful tool for determining the three-dimensional structure of proteins at atomic resolution. There are several protein crystallization techniques, including sitting-drop vapour-diffusion, hanging-drop vapour-diffusion, batch and dialysis methods (McPherson, 1982, 1999). The hanging- and sitting-drop techniques are presently the most popular methods. However, the process of producing suitable diffraction-quality protein crystals is a rate-limiting step. It is difficult to predict the optimal conditions for crystal nucleation and growth and therefore crystallographers must investigate many conditions. The hanging-drop technique requires somewhat more time to set up than the sitting-drop technique. In contrast, the sitting-drop technique often presents problems in handling the crystals since they adhere to the growth vessel.

A two-liquid system for protein crystallization has recently been reported (Adachi, Takano *et al.*, 2002; Adachi, Watanabe *et al.*, 2001). A protein solution floats on an insoluble and very dense liquid in this system. Crystals grow at the interface of the two liquids under optimized growth conditions by a slow-cooling method; the crystals thus do not contact the growth vessel (Adachi, Watanabe *et al.*, 2002). The crystals are easily removed without causing mechanical damage. In addition, the seeding crystal can be easily picked up and dropped onto the interface in a new vessel. A containerless technique used in a batch method (Lorber & Giegé, 1996; Chayen, 1996) is similar to the two-liquid system. The searchable space of precipitant-sample concentration in the batch method, however, is narrower than that in the vapour-diffusion method.

In this paper, we apply the two-liquid system to the sitting-drop vapour-diffusion technique and call the new method the 'floating-drop vapour-diffusion' technique. Glucose isomerase, xylanase and A32S mutant human lysozyme crystals were grown by the floating-drop technique in the same manner as in the traditional sitting-drop technique. The crystals in the floating drop did not adhere to the vessel. We will further describe the floating-drop method and discuss its advantages.

2. Experimental procedures

2.1. Materials

Glucose isomerase and xylanase were purchased from Hampton Research. A32S mutant human lysozyme was prepared by a previously described procedure (Takano *et al.*, 1995, 2001). Fluorinert was purchased from SUMITOMO 3M. All other chemicals were reagent grade. The crystallization plate and sealing tape were purchased from Emerald BioStructures.

2.2. Crystallization

Glucose isomerase protein solution was passed through 0.22 μm filters prior to crystallization. Glucose isomerase was crystallized at a protein concentration of 10–30 mg ml^{-1} by vapour diffusion in the following reagents: (i) 1.5–2.5 *M* ammonium sulfate pH 7, (ii) 5–15% PEG 6000, 0.2 *M* ammonium sulfate pH 7 and (iii) 1.0 *M* sodium formate pH 6 (Carrell *et al.*, 1989). Xylanase was crystallized at a protein concentration of about 15 mg ml^{-1} by vapour diffusion in 0.7 *M* sodium potassium phosphate pH 7 (Torronen *et al.*, 1993). A32S mutant human lysozyme was crystallized at a protein

concentration of about 10 mg ml^{-1} by vapour diffusion in 1.5–3.0 *M* sodium chloride, 0.05 *M* sodium acetate pH 4.5 (Takano *et al.*, 1995; Yamagata *et al.*, 1998). All proteins were crystallized at 293 K.

3. Results and discussion

3.1. Floating-drop vapour-diffusion technique

Fig. 1 schematically illustrates the floating-drop vapour-diffusion technique. The method combines the traditional sitting-drop vapour-diffusion technique and the novel two-liquid system (Adachi, Watanabe *et al.*, 2002). The principle is very simple. The method uses an insoluble and very dense liquid. A suitable liquid is Fluorinert, which is transparent with a high density (Fluorinert FC-70 has a density of 1940 kg m^{-3}) and does not dissolve in most solvents. When a protein solution and Fluorinert are mixed, the protein drop immediately separates from the Fluorinert and floats on top of it. The volume of the protein drop can be changed depending on the size of the crystallization vessel. We utilized sealing tape to seal the growth vessel in this work. The advantages of the sealing tape include simplicity, speed and economy. It is not necessary to use glass cover slides and grease for sealing. Furthermore, it is easy to wash the crystallization plate and reuse it.

3.2. Crystallization by the sitting- and floating-drop vapour-diffusion techniques

Crystallization trials were conducted on three different test proteins to demonstrate the utility of the floating-drop vapour-diffusion technique. The proteins used were glucose isomerase (tetramer; 173 kDa), xylanase (21 kDa) and A32S mutant human lysozyme (15 kDa). The crystallization conditions using vapour diffusion for these proteins are well known. Trials were set up using both the sitting- and floating-drop vapour-diffusion techniques. The volume of the protein drops was 2–10 µl in both techniques and the volume of Fluorinert was 2–10 µl in the floating-drop technique. Fig. 2 shows the crystals obtained. The shape of the crystals was dependent on the crystallization conditions, but the results exhibited no difference in crystal growth between the sitting- and floating-drop vapour-diffusion techniques. This indicates that Fluorinert does not affect crystal growth in vapour-diffusion methods.

The crystals obtained by the sitting-drop method adhered to the growth vessels. In contrast, the crystals obtained in the floating-drop method did not contact the growth vessels. Changes in Fluorinert volume from 2 to 10 µl did not influence the floating crystals. It was also possible to rid the crystals of Fluorinert liquid because they were completely separated. These results indicate that the floating-drop vapour-diffusion technique leads to easy extraction of crystals without any mechanical damage. This is important for X-ray crystallography because damaged crystals are useless.

3.3. Advantages and disadvantages of each vapour-diffusion technique

Sitting- and hanging-drop vapour-diffusion techniques are very popular since they facilitate crystallization, including screening and optimization, and require a small sample amount. We have introduced a

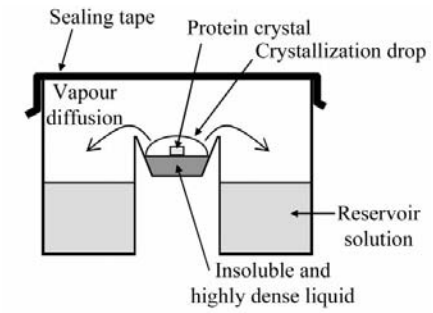

Figure 1
Schematic illustration of the floating-drop vapour-diffusion technique. A crystallization drop composed of a mixture of sample and reagent floats on an insoluble and highly dense liquid. Protein crystals can be grown at the interface between the two liquids without contacting the vessel.

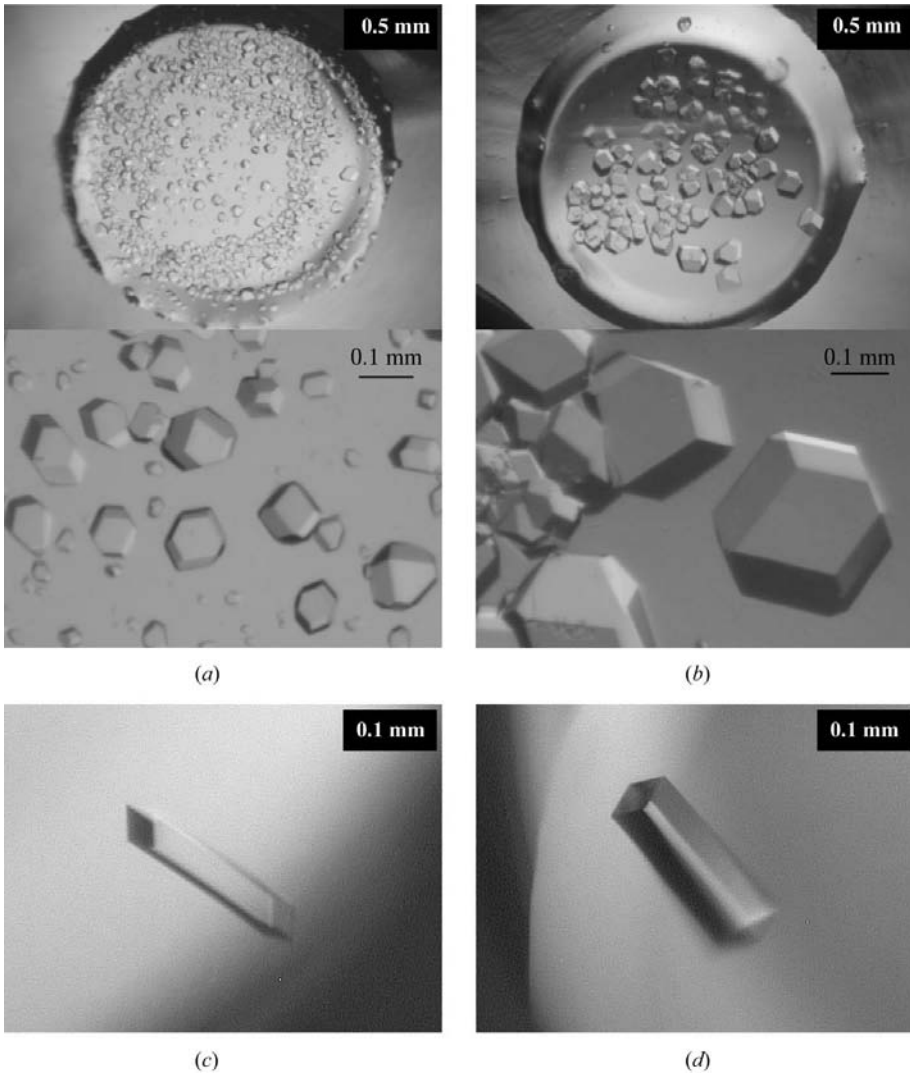

Figure 2
Crystallization results from the sitting-drop (*a* and *c*) and floating-drop (*b* and *d*) vapour-diffusion experiments for glucose isomerase (*a* and *b*) and A32S mutant human lysozyme (*c* and *d*). The reagents are 5–15% PEG 6000, 0.2 *M* ammonium sulfate pH 7 (*a* and *b*) and 1.5–3.0 *M* sodium chloride, 0.05 *M* sodium acetate pH 4.5 (*c* and *d*). These were crystallized at 273 K. Although the crystal sizes in (*b*) and (*d*) are larger than those in (*a*) and (*c*), the differences have no meaning statistically in this experiment.

Table 1
Advantages and disadvantages of each vapour-diffusion technique.

	Hanging drop	Sitting drop	Floating drop
Drop volume	Limited	No limitation	No limitation
Time	A little extra time is required	Speedy	Speedy
Handling crystals	Easy, but crystals sometimes adhere to the siliconized glass cover	Difficult; crystals adhere to the vessel	Easy
Other supplies	Siliconized glass cover and grease	Sealing tape	Sealing tape and Fluorinert
Reusing plate	Difficult; greasy	Easy	Easy
Cost	Uneconomic	Economic	Economic
Automation	Difficult	Easy	Easy

new method: the floating-drop vapour-diffusion technique. Table 1 summarizes the advantages and disadvantages of the various vapour-diffusion techniques. The hanging-drop method is currently the most popular method for protein crystallization, but it has some disadvantages. Speed and automation in particular are necessary in structural genomics and structure-based drug discovery (Chayen & Hilgenfeld, 2002). The hanging-drop method may fall from favour because it is difficult to automate hanging-drop experiments. In contrast, the sitting-drop technique, including the use of a floating drop, is suitable for automation. However, the conventional sitting-drop technique causes crystals to adhere to the vessel. It is impossible to separate a protein crystal adhering to the vessel without causing mechanical damage. The floating-drop technique solves this problem, leading to production of high-quality protein crystals suitable for X-ray diffraction. This suggests that the floating-drop vapour-diffusion technique may become the most popular method for protein crystallization in the near future.

References

Adachi, H., Takano, K., Yoshimura, M., Mori, Y. & Sasaki, T. (2002). In the press.

Adachi, H., Watanabe, T., Yoshimura, M., Mori, Y. & Sasaki, T. (2002). *Jpn J. Appl. Phys.* **41**, L726–L728.

Carrell, H. L., Glusker, J. P., Burger, V., Manfre, F., Tritsch, D. & Biellmann, J. F. (1989). *Proc. Natl Acad. Sci. USA*, **86**, 4440–4444.

Chayen, N. E. (1996). *Protein Eng.* **9**, 927–929.

Chayen, N. E. & Hilgenfeld, R. (2002). *Trends Biotechnol.* **20**, 320–321.

Lorber, B. & Giegé, R. (1996). *J. Cryst. Growth*, **168**, 204–215.

McPherson, A. (1982). *Preparation and Analysis of Protein Crystals*. New York: John Wiley & Sons.

McPherson, A. (1999). *Crystallization of Biological Macromolecules*. New York: Cold Spring Harbor Laboratory Press.

Takano, K., Ogasahara, K., Kaneda, H., Yamagata, Y., Fujii, S., Kanaya, E., Kikuchi, M., Oobatake, M. & Yutani, K. (1995). *J. Mol. Biol.* **254**, 62–76.

Takano, K., Yamagata, Y. & Yutani, K. (2001). *Biochemistry*, **40**, 4853–4858.

Torronen, A., Rouvinen, J., Ahlgren, M., Harkki, A. & Visuri, K. (1993). *J. Mol. Biol.* **233**, 313–316.

Yamagata, Y., Kubota, M., Sumikawa, Y., Funahashi, J., Takano, K., Fujii, S. & Yutani, K. (1998). *Biochemistry*, **37**, 9355–9362.