Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

An improved protocol for rapid freezing of protein samples for long-term storage

Junpeng Deng,[a,b] Douglas R. Davies,[a] Goragot Wisedchaisri,[a] Meiting Wu,[a] Wim G. J. Hol[a,b,c]* and Christopher Mehlin[c]

[a]Department of Biochemistry, University of Washington, USA, [b]Howard Hughes Medical Institute, USA, and [c]Structural Genomics of Pathogenic Protozoa (SGPP), Biomolecular Structure Center, University of Washington, USA

Correspondence e-mail: wghol@u.washington.edu

© 2004 International Union of Crystallography
Printed in Denmark – all rights reserved

Received 6 September 2003
Accepted 23 October 2003

Freezing of purified protein drops directly in liquid nitrogen is a convenient technique for the long-term storage of protein samples. Although this enhances reproducibility in follow-up crystallization experiments, some protein samples are not amenable to this technique. It has been discovered that plunging PCR tubes containing protein samples into liquid nitrogen results in more rapid freezing of the samples and can safely preserve some proteins that are damaged by drop-freezing. The PCR-tube method can also be adapted to a PCR-plate freezing method with applications for high-throughput and structural genomics projects.

1. Introduction

Long-term storage of protein solutions is an important technical issue for many crystallographic structure-determination projects. Storage of purified protein in solution at 277 K can be problematic owing to the potential for proteolysis, oxidation or other degradation over extended periods of time. Such difficulties can be overcome by freezing protein solutions as soon as purification is complete. The common practice of freezing proteins in a glycerol solution is also problematic because the cryoprotectant can possibly interfere with crystallization and may need to be removed by dialysis after thawing. A simplified procedure is to merely drop small aliquots of protein ($\sim$20–50 μl) directly into an open container of liquid nitrogen (see, for example, Bauer *et al.*, 1997; Gulick *et al.*, 1998). In this procedure, the small pellets of frozen protein are removed from the liquid nitrogen with a pair of forceps and stored in plastic tubes at 193 K. Frozen aliquots preserve the freshly prepared protein and can be thawed in small batches; this allows the investigator to perform several reproducible crystallization experiments over a long period of time with an essentially unchanging sample. Since the drop-freezing method was implemented in our laboratory, several different proteins have been preserved this way and these have been observed to crystallize as well as freshly purified samples.

However, we have encountered a number of cases in which the drop-freezing method was unsuccessful. In these instances, the proteins precipitated out of solution upon thawing. In order to overcome this problem, we began freezing aliquots of protein in thin-walled PCR tubes. Here, we demonstrate that protein solutions freeze more quickly in PCR tubes than when they are dropped freely into liquid nitrogen and attribute the increased success rate of freezing to this more rapid process. Furthermore, we describe an adaptation of this method using PCR plates that is well suited for structural genomics and other high-throughput applications.

2. Experimental

Drop-freezing is carried out by dispensing the protein sample as a single drop from a Gilson P200 Pipetteman into a short cylindrical Dewar flask filled with liquid nitrogen from a height of approximately 10 cm. Dropped aliquots of protein solutions form spherical pellets which eventually sink to the bottom of the container. These pellets are retrieved from the bottom of the Dewar with a pair of forceps and placed into an Eppendorf tube or cryo-vial for long-term storage at 193 K. Freezing in PCR tubes was performed by first placing the protein sample into 0.2 ml ultrathin-walled PCR tubes (Island Scientific, cat. No. IS-430), submerging the tubes under the surface of the liquid nitrogen with a pair of forceps until completely frozen and then transferring the tubes to a 193 K freezer for long-term storage. For comparison, protein was also frozen in standard 1.7 ml microcentrifuge tubes (Island Scientific, cat. No. 20-Z). Timed experiments for comparing the two methods (Fig. 1) were carried out with a solution of hen egg-white lysozyme at a concentration of 10 mg ml^{-1} in 200 m*M* NaCl and 10 m*M* Tris pH 7.0. The time for freezing was measured from the first contact of the protein solution (or tube) with the liquid nitrogen until the protein solution changed from clear and colorless to cloudy and semi-opaque.

 DOI: 10.1107/S0907444903024491

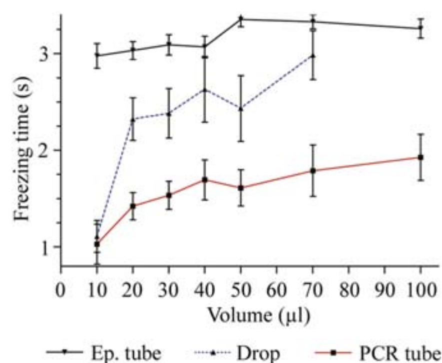

Figure 1
The freezing time of a protein solution either dripped directly into liquid nitrogen (Drop), frozen in PCR-tubes (PCR tube) or frozen in Eppendorf-type microcentrifuge tubes (Ep. tube) is shown in a representative experiment. Drops tended to break apart at volumes greater than 70 μl. Values are the mean of ten replicates ± s.d.

For high-throughput and structural genomics applications, a PCR-plate method has been developed. Individual PCR tubes are replaced with thin-walled unskirted PCR plates (MJ Research MLP-9601). Protein solution is distributed into the plate wells in 20–50 μl aliquots, the plate is sealed with foil (VWR 29445–080) and the entire plate is flash-frozen by immersing the wells in liquid nitrogen. Individual wells may later be cut free from the plate without thawing the entire plate. As the PCR plates become somewhat brittle at low temperatures, they are stored in standard lidded plastic PCR-plate trays (GeneMate–ISC Bioexpress R-7909-2). These trays are labeled and pre-chilled in a 193 K freezer before the PCR plate is added. Dozens of proteins have been shipped in this manner without incident and standard 193 K freezer racks are available which will accommodate multiple trays (ISC Bioexpress UPCR-66), allowing facile storage and retrieval of frozen samples.

3. Results and discussion

Two proteins under crystallographic investigation have been found to precipitate upon thawing following drop-freezing, but survive thawing after freezing in PCR tubes. During our studies on *Mycobacterium tuberculosis* iron-dependent repressor (Pohl *et al.*, 1999; Feese *et al.*, 2001), a truncated variant was frozen in liquid nitrogen at 7.5 mg ml^{-1} (in 10 m*M* Tris–HCl buffer pH 7.0, 50 m*M* NaCl and 1 m*M* EDTA) by the drop-freezing method and by the PCR-tube method at 10 and 20 μl drop volume. When frozen by the drop-freezing method, the protein precipitated out after thawing on ice and also after thawing more rapidly by being held in the investigator's hand. When frozen by the PCR-tube method, the protein solution after thawing in hand remained clear. Dynamic light-scattering results revealed no significant change of the protein polydispersity upon freezing and thawing by the PCR-tube method. When the volume of the protein solution drop was increased to 50 μl and frozen by the PCR-tube method, some cloudy precipitate was observed in the protein solution after thawing in the hand.

The other problematic case in which PCR-tube freezing proved superior to drop-freezing involved Tbmp57, a terminal uridyl transferase from *Trypanosoma brucei* (Ernst *et al.*, 2003). Purified protein was kept in a buffer containing 20 m*M* Tris, 500 m*M* NaCl, 5 m*M* DTT and 10% glycerol. The protein started to precipitate out of solution at 277 K in less than a week. After drop-freezing a 20 μl volume of protein in liquid nitrogen, Tbmp57 would precipitate upon thawing. However, using the PCR-tube freezing method with the same volume, the protein was successfully recovered after thawing. Size-exclusion chromatography and dynamic light scattering demonstrated that Tbmp57 remained in a monomeric state with constant polydispersity (19%) before and after PCR-tube freezing.

Timed experiments demonstrate that protein samples freeze more quickly in PCR tubes submerged in liquid nitrogen than protein samples dropped directly into liquid nitrogen (Fig. 1). Several key factors are likely to contribute to the more rapid heat transfer from PCR tubes. Owing to their conical shape, PCR tubes have a greater surface area-to-volume ratio than free drops in liquid nitrogen, which assume a spherical shape. Free drops in liquid nitrogen also glide across the surface during the freezing process, partially in contact with the relatively warm air above the surface of the nitrogen. These drops are further insulated from the liquid nitrogen by a thin film of nitrogen vapor created by the Leidenfrost effect (Leidenfrost, 1966). This effect is minimized for PCR tubes that are plunged below the surface, where nitrogen vapor created by the initially relatively hot PCR tube bubbles away rapidly. Freezing can damage proteins through pH changes and concentration of protein and other solutes owing to exclusion from water ice crystals that can lead to denaturation and aggregation (Franks, 1985). Extremely fast freezing such as occurs in PCR tubes may minimize these stresses on proteins, as ice formation may occur too quickly to allow local concentration of solutes. In contrast, Fig. 1 shows that the freezing of protein samples is slowest in 1.7 ml microcentrifuge tubes. The walls of the microcentrifuge tubes are approximately 1 mm thick, nearly the thickness of standard screw-capped cryo vials, and apparently act as an insulator to slow the rate of freezing of protein samples.

The high-throughput demands of structural genomics have pushed this flash-freezing process a step further. The Structural Genomics of Pathogenic Protozoa (http://www.sgpp.org) consortium produces a large number of proteins that must be frozen, labelled, shipped on dry ice and systematically archived. Individual PCR tubes are impractical for such applications, but the use of one-piece PCR plates has proven to be a convenient and robust adaptation of the PCR-tube freezing method.

We would like to thank Kenneth Stewart and coworkers of the Seattle Biomedical Research Institute for providing the clone for Tbmp57 and Claudia Roach at the Howard Hughes Medical Institute and Randall Holmes and collaborators at the University of Colorado in Denver for the IdeR gene. This work was supported by NIH grant No. P50 GM64655 and NIH grant No. 1P50 GM64655-01 to WGJH.

References

Bauer, C. B., Kuhlman, P. A., Bagshaw, C. R. & Rayment, I. (1997). *J. Mol. Biol.* **274**, 394–407.

Ernst, N. L., Panicucci, B., Igo, R. P. J., Panigrahi, A. K., Salavati, R. & Stuart, K. (2003). *Mol. Cell* **11**, 1525–1536.

Feese, M. D., Ingason, B. P., Goranson-Seikierke, J., Holmes, R. K. & Hol, W. G. J. (2001). *J. Biol. Chem.* **276**, 5959–5966.

Franks, F. (1985). *Water Science Reviews.* Cambridge University Press.

Gulick, A. M., Song, H., Endow, S. A. & Rayment, I. (1998). *Biochemistry*, **37**, 1769–1776.

Leidenfrost, J. G. (1966). *Int. J. Heat Mass Transfer*, **9**, 1153–1166.

Pohl, E., Holmes, R. K. & Hol, W. G. J. (1999). *J. Mol. Biol.* **285**, 1145–1156.