Sample preparation

The purity of the sample is critical. If particulate or amorphous matter is present centrifugation or micro-filtration is advisable. A sample concentration of 5 - 25 mg/ml is recommended.

Terese Bergfors

Uppsala University, Uppsala, Sweden

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It is always advantageous to purify the protein yourself because you learn a great deal about its behavior and stability in the process. If some-one else purifies the protein for you, it can be hard for them to know which considerations are important when handling a macromolecule that is intended for crystallization trials. The following guidelines may be helpful.

Avoid Lyophilization

There are many examples of proteins that crystallize after lyophilization, lysozyme being one of them. Lyophilization is often used as a way to concentrate or store proteins, but it is not a gentle method and should be avoided if possible when the protein is intended for crystallization.

If the protein you receive is lyophilized, it is important to **dialyze** it against the target buffer. It is not sufficient to **dissolve** the protein in the buffer, for several reasons. The protein chemist may have lyophilized the protein in a non-volatile buffer. The buffer residue will then be a major contaminant when the protein is redissolved. It can also happen that the protein chemist changes the buffer in which the protein is lyophilized and does not inform you (because they are unaware of the significance for your experiments). This can result in a long series of irreproducible experiments while the cause of the problem is traced. Therefore, dialyze the lyophilized protein thoroughly, measure the concentration, and check the pH.

Ammonium Sulfate Precipitation

Be careful when using ammonium sulfate precipitation as a purification or concentration step.

As an early step in the purification, ammonium sulfate precipitation is usually perfectly acceptable, but it is not advisable as a final step when the protein is going to be crystallized. One type of problem is that it is virtually impossible to dialyze away, or remove all traces of the ammonium sulfate on a short desalting column. Even minute amounts are sufficient to react with the components in the crystallization screen, giving rise to salt crystals or irreproducible results. Ask the protein chemist to replace this step with other concentration methods where possible.

Keep Purification Batches Separate Whenever Possible

Do not mix different purification batches in crystallization trials. Neither the growth conditions nor the purification are ever identical, so each new batch should be considered and screened separately.

Characterize the Protein Immediately upon Arrival

The more ways in which you can characterize your protein, the better; although restrictions on time and material will determine to what extent this is possible. Always run SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and native PAGE or dynamic light scattering as soon as the protein arrives. If possible, complement with an IEF (iso-electric focusing) gel and mass spectroscopy. Store a small quantity of the protein from each batch at -70°C as archive material.

For the initial screening trials, the protein should be at least 90% pure when stained with Coomassie Blue on an SDS gel. Always consider further purification of the protein 1) if the initial screen does not produce any promising results, or 2) to improve crystal quality when optimizing.

The initial gels are important for documentation of the homogeneity of the protein and possible batch variations. In addition, proteins degrade with time. If you notice that a certain batch of protein is no longer producing good crystals, run new gels (preferably native PAGE or IEF) on the protein. Comparison of these gels with the original ones will establish if the protein has deteriorated in some way. Similarly, gels can be run on failed crystallization drops or ones that suddenly produce crystals after a suspiciously long time.

Storage of Protein

Not all proteins tolerate freezing at -20°C. Most proteins can be kept at 4°C or -70°C, but the activity and stability must be checked for each new protein. Freezing and rethawing of the sample should be avoided, so store the protein in aliquots. Sometimes glycerol (10-50%) is added to the protein to help it tolerate freezing better. This can be a problem in itself because removing the glycerol by dialysis is slow and difficult.

Cells or bacteria tolerate freezing (-70°C) better than many purified proteins. It can be better to store the cells or other expression hosts, then thaw them and purify the target expression product (protein) freshly. Use of protease-deficient hosts can be expedient here.

My preference is to freeze the protein (without any glycerol) directly in liquid nitrogen and store it at -70°C. The method is as follows:

1. Take a 500 ml glass beaker and fill it with 100 ml liquid nitrogen. Do not use plastic (because the frozen pellets of protein tend to stick). Safety glasses should always be used when handling liquid nitrogen.
2. Add the concentrated protein solution drop-wise with a pasteur pipette into the liquid nitrogen from a height of 10-20 cm.
3. The frozen droplets should be about the size of green peas. Continue until all the solution is frozen. It is quite possible to freeze some 25 ml of protein in 5-10 minutes. Pour or boil off the remaining nitrogen, then transfer the "peas" into a container (a 50 ml Falcon tube works fine) and place immediately at -70°C.
4. Aliquots ("peas") can be thawed as needed. Confirm the homogeneity and activity after long-term storage, regardless of the storage temperature.

As a general rule, it is better to store proteins concentrated than diluted. For example, the losses due to adsorption, etc., will be less if the protein is stored at 5 mg/ml than at 0.5 mg/ml. However, storing the protein *too* concentrated can lead to some of it precipitating.

Be Gentle

Handle the protein solution gently. Avoid foam. Do not vortex or shake it. If your protein stock solution is stored at a temperature different from the one at which crystallization trials will be set up, the best practice is to remove an aliquot and allow it to equilibrate to the target temperature. In practice, most people simply keep the protein stock on ice while setting up room temperature experiments and then return it to the cold room. In any event, avoid subjecting the protein stock to temperature variations unnecessarily.

Keep Good Records

Ask for a copy of the purification protocol. Is it the same for each batch? Collaborators often make changes without passing the information on, simply because they are unaware that it may have any significance. Make a note of everything you do to the protein.

Learn All about the Protein

Find out as much as you can about your protein. Some questions to ask are:

- Does it have free cysteines?
- Are there any known substrates/ligands/inhibitors?
- Is it sensitive to proteolysis?
- Does it bind metals or share homology with other metal-binding proteins which might indicate that it needs metals?
- Have similar proteins been crystallized?
- At what pH range is the protein stable/active?
- At what temperature is the protein stable/active?
- Is the protein glycosylated? phosphorylated? N-terminal methylated?
- Has sodium azide (or other bacteriocide) been added?

Read the literature on your protein and ask your collaborators questions. Important clues can emerge which will help obtain or optimize crystals.