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JBS Crystallization Freshman Kit – Junior/Scholar

Get a head start crystallizing your protein

Introduction to protein crystallization

There are currently two main methods for determining the three-dimensional structure of a protein: Nuclear Magnetic Resonance (NMR) Spectroscopy and X-ray crystallography. The NMR method is able to resolve the atomic structure of proteins with an upper molecular weight limit of approximately 25000 Da (25 kDa, or approximately 220 amino acids), whereas the X-ray method is more suitable for determining the structure of larger proteins or macromolecular complexes. The pioneering studies on the X-ray crystal structures of myoglobin (1950) and hemoglobin (1955) were honored with the Nobel Prize in Chemistry in 1962. This recognition not only underscored the importance of X-ray crystallography but also signaled the emergence of structural biology as an essential field in the life sciences.

The first step in the determination of an X-ray crystal structure, which is often also the most difficult step, is the growth of sufficiently large protein crystals. A crystal is a three-dimensional periodic arrangement of building blocks; in our case, these building blocks are protein molecules. How does one manage to grow crystals from such complicated molecules like proteins?

In order to arrive at a crystalline form starting from a dissolved form, the solubility of the protein molecules must be reduced. As a general rule, reducing the solubility of a protein solution will result in the formation of an amorphous protein precipitate. However if the appropriate conditions are selected so that complementary patches are present on the surfaces of adjacent protein molecules in solution, specific attractive interactions can occur between the protein molecules. If these interactions are also geometrically favorable, this can lead to protein crystallization.

The process of crystallization can be differentiated into two steps: (1) the nucleation process, and (2) crystal growth. With correctly selected conditions, crystal nucleation and growth can occur within the supersaturated regions of the phase diagram. (see Fig. 1).

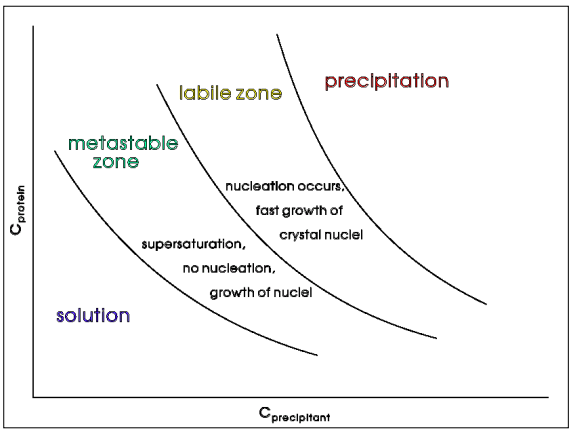

Figure 1: *Schematic phase diagram of a protein-precipitant mixture*

As can be implied from the Fig. 1, crystallization requires the formation of a supersaturated protein-precipitant solution, i.e. a solution which contains more protein molecules than would dissolve under normal conditions. Unfortunately, one needs a higher degree of supersaturation for nucleation as for growth, and therefore the different processes of crystal nucleation and growth are often difficult to individually control. The nucleation range is also called the labile zone, while the growth range is known as the metastable zone. For crystal nucleation to occur, the experimenter must push the protein solution into the labile zone, which is also the region where fast growth of crystal nuclei occurs. Herein lies the danger: maintaining the protein solution within the labile zone for too long will result in rapid growth of too many crystal nuclei and produce an excess of small crystals. Since one is interested in obtaining large protein crystals (ca. 0.5 mm in length), it's important that not too many nuclei are formed. This means that for a successful experiment, the protein/precipitant mixture must approach the nucleation zone very slowly so that the developing nuclei have enough time to grow.

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The transition from a stable solution to a supersaturated solution can be achieved by adjusting the position of the protein-precipitant mixture in the example phase diagram. This can be achieved by increasing either the concentration of the protein or the precipitant (vertical and horizontal axes in Fig. 1).

The physical process of causing a change in concentration can be carried out through dialysis or diffusion. Both are characterized by the transport of material. The method of vapor diffusion is particularly suitable for protein crystallization.

The experimental setup for a typical vapor diffusion experiment is illustrated in Figure 2. This particular setup is also known as the "hanging drop" method.

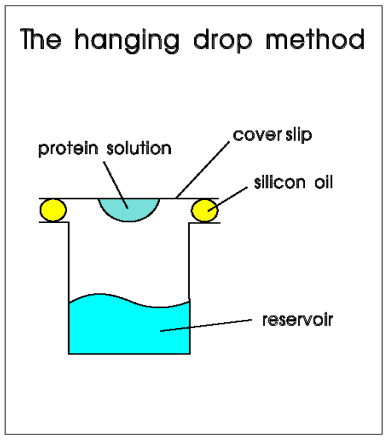

Fig. 2: *The Hanging-Drop Method*

The protein solution is located within a drop hanging on the underside of a microscope cover slip. The drop size can vary from 0.5 µl up to approximately 20 µl. The reservoir solution (ca. 1 ml), which is located beneath the hanging drop, contains a high concentration of precipitant and thus has a lower vapor pressure than the protein solution. Over the course of time, water from the drop diffuses as water vapor into the reservoir solution. This raises the concentration of the protein and precipitant in the drop. With the correctly chosen conditions, crystallization of the protein will occur over the course of days or weeks. In principle one therefore uses a system (drop plus reservoir) in which thermodynamic equilibrium is slowly adjusted over time.

Which materials are useful as precipitants?

In principle any material which has an influence on the solubility of the protein can be used as a precipitant, provided that a high concentration of the precipitant does not denature the protein.

One differentiates the different precipitants which are often used in protein crystallization according to the effect they have on the solution. Commonly used precipitants include salts, organic polymers, alcohols, and occasionally pure water.

Salts such as $(NH_4)_2SO_4$, NaCl, LiCl, KH_2PO_4, etc., change the ionic strength of the solution. The solubility of proteins as a function of ionic strength is shown in Figure 3.

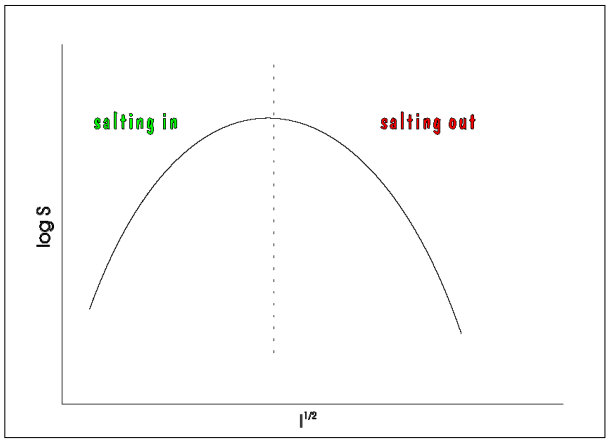

Fig. 3: *Dependence of the solubility (S) on the ionic strength (I)*

The zone to the left of the protein solubility maximum is the 'salting-in' zone, and to the right is the 'salting-out' zone. Solubility increases within the salting-in range due to the elevation of the dielectric constant of the solvent. This results in the charges on the protein surface interacting better with the environment. Within the salting-out range the solubility is reduced again because the charges of the precipitant compete for water molecules with the charges on the protein surface, thereby effectively lowering the overall hydration state of the protein.

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Organic precipitants such as ethanol, methanol, propanol, MPD (2-methyl-2,4-pentanediol) or acetonitrile reduce protein solubility by lowering the dielectric constant of the solvent. Organic polymers work the same way, such as PEGs (polyethyleneglycol), which are available in different molecular weights from 400 to 20000 Da.

Another important parameter which affects protein solubility is the pH-value of the solution. As a general rule, solubility is lowest at the protein's isoelectric point (IEP), since at the IEP the protein carries a net charge of zero.

Due to the enormous number of possible parameters that can affect crystallization, and because of the relatively small quantity of protein which is usually available, it is practically unfeasible to test all parameter combinations over their entire range. It is therefore important to develop a strategy to arrive at your goal as quickly as possible and without using too much material.

Characteristics of Protein Crystals

Protein crystals are built up in principally the same manner as crystals of small molecules or salt. The same rules of molecular packing and symmetry apply. However, there are also some fundamental differences. These differences extend to mechanical and optical characteristics of crystals as well as composition. Protein crystals are very soft and generally contain between 30% and 70% water, most of which is relatively unordered within the crystal.

The integrity of protein crystals is maintained by interactions between the individual protein molecules, and the remaining space within the crystal consists of large cavities containing water and/or buffer molecules. Consequently, the protein molecules within the crystal exist in a quasi natural, i.e. aqueous, environment. The native structure (the proper folding of the protein molecule which essentially confers its function or activity) remains, and can often be demonstrated by carrying out enzymatic activity tests on the protein in the crystalline form. In some cases the crystalline form is even a natural storage form, such as in the case of insulin.

Acknowledgements

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