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Mini Review

Important Factors Influencing Protein Crystallization

Abstract

The solution of crystallization problem was introduced around twenty years ago, with the introduction of crystallization screening methods. Here reported some of the factors which affect protein crystallization, solubility, Concentration of precipitant, concentration of macromolecule, ionic strength, pH, temperature, and organism source of macromolecules, reducing or oxidizing environment, additives, ligands, presence of substrates, inhibitors, coenzymes, metal ions and rate of equilibration. The aim of this paper to give very helpful advice for crystallization.

Introduction

X-ray crystallography has provided 3D structures of thousands of proteins. In spite of these advances, many factors continue to be problem that can lead to unsuccessful proteins crystallization. We always know theoretical pI, molecular weight and amino-acid composition, while pH and salt concentration are some of the variables that can be expected from other similar known structure. Yet, a protein behavior depends very much on the environment it is in.

Proteins are generally present in a biological sample as their native state. They are very often associated with other proteins and integrated into large complexes. In most cases they are not soluble in their innate state after isolation as result of that must be denatured to help solubilization and Crystallization. Initial protein crystal commonly need to screen conditions using little as possible to improve the optimization methods for protein crystallization.

Crystallization is thermodynamically process. Once begin, will continue under kinetic control until super saturation is lost. The crystallization process affected by physical conditions of the solution, solution solubility, the presence of impurities, nucleation, solution saturation and degree of super saturation, crystal growth, including solution composition, pH and temperature, and to date is not fully understood.

Prediction of secondary structure, solubility, domain organization, stability, signal peptide, hydrophobicity and pH, can provide useful information for crystallization strategies and salt prediction tools it need to be develop. in general proteins has low molecular weight easier for crystallization than high molecular weight, single-domain easier for crystallization than multi-domain and an oligomeric state multimer more likely to crystallize than a monomer high molecular weight.

Buffer is most straight forward way to make a crystallization problem by effect the protein behave. There are many rules and different protein crystallization methods, some of it has been developed during the recent years. However, Protein crystallization still represents a great challenge for bio crystallography. The cumulation of crystallization information in crystallization databases and in structural articles it allow us to design crystallization experiments depending on the character of the protein. Because there is no ideal procedure for crystallization, our article discusses the crystallization problem try to simplify the composition of the crystallization and give some solution advice.

Protein solubility

Protein solubility is a common complex interaction problem between the physiochemical nature of the proteins, rate of protein synthesis, amino acid composition, the Protein concentration, type of salt present in the buffer, the concentration of the salt used, ionic strength, pH, temperature, osmotic pressure, cellular location of expression, and cellular tools or chaperones are all important in protein solubility. Protein solubility may decrease in the presence of complexes with lipids, nucleic acids or other nonprotein.

It is important to select better option for tag in the target proteins. Unfortunately, there is no consolidated system for rapid screening to differentiate the best tag.

Addition L-Arg and L-Glu at 50 mM to the buffer can increase solubility up to 8.7 times, because they can in preventing protein aggregation and precipitation as well as increase the long-term stability also it did not prevent specific protein-protein and protein-RNA interactions [1].

Normally when there is solubility issues, the first thing we need to check it the buffer composition, varying pH and salt strength it can make a big difference to solubility. Then try solubilising agents, e.g NDSBs or detergents. Protein solubility can improved by selecting only a soluble domain for expression, and delete the hydrophobic domain. Moreover there are solubility prediction tools in the internet it can be useful.

In order to be sure target protein is soluble culture need to be lysate (lysozime 1mg/ml on ice 1hour, it should be under non

denaturation condition) and centrifugation at 35000 x g. then use an aliquot of supernatant for SDS-PAGE. In case of protein is soluble well found in the supernatant.

Detergents

Detergents are commonly used at concentrations of 1–4%. It useful in solubilizing by disrupt hydrophobic interactions between and within proteins. The detergent used for solubilization does not requisite to be the same for purification and crystallization, also the presence of various detergents perhaps inhibit the activity of different proteases. When despite all efforts not work then it needs denaturation and solubilization of the target protein. This is done by a denaturing agent e.g. guanidine or urea under reducing conditions (~ 20 mM DTT). A western blot necessary for the supernatant before and after the high-speed centrifugation will give an indication of how much detergent was effect in the supernatant (membrane).

Reducing agents

Reducing agents are generally used in sample preparation to cleave disulfide bond DTT, DTE and β-ME are more commonly reducing agents used. The aim of using these agents to protect proteins from precipitating and aggregating due to oxidative crosslinking and to reduce protein aggregation that may inhibit crystallization, Reducing agents can interact with metals within protein sample and that is not good for crystallization in this case. BME is particularly sensitive to cobalt, copper and many phosphate buffers while DTT is sensitive to nickel. But until now no specific article show different between the DTT, DTE and β-ME as well as in which crystallization step is better to be use.

Isoelectric point (pI), pH & Temperature

The pH influence on protein solubility, different proteins are soluble at different pH values, at high pH protein soluble (deprotonate), low pH protein soluble (Protonated) and at isoelectric point: protein aggregates. Acidic proteins has pI lower than 7, more likely to crystallize around one pH unit above their pI, while basic proteins more likely to crystallize lower than their pI about 1.5–3 pH units [2]. Generally, protein aggregation or precipitation when the solubility decrease at pH close to the isoelectric point (pI). It better to move pH away from the pI to increase protein solubility and Super saturation. At low salt concentration the electrostatic repulsions well decrease when pH is different from pI, this well likely induce PEG to effect depletion attraction. The depletion attraction due to polymer is more effective when the protein net charge is lower.

To screen favor attraction between macromolecules and protein charges it better to increasing ionic strength or gently moving pH closer to pI. The most critical things is pI we need to make Sure the protein having net charge close to zero and it soluble, and this depend on some paper advice.

Actually, temperature is important parameter for crystallization, it is better be screened. Temperature influences crystal growth and nucleation by changing super saturation as well as solubility of the sample. For instance, pH of Tris buffer is susceptible to temperature changes. However, temperature can useful at low ionic strengths by effects the amplified. Proteins very often show several crystal polymorphs as result of different temperature. Lowering the growth inducing temperature, this lessening the rate of protein synthesis and usually more soluble protein is obtained. Because the temperature can affects solubility is better to cool the Crystal solutions to prevent protein degradation.

Optimum salt concentration

Different NaCl concentrations with different pH levels it was use for crystallization. The effective of salt concentration in a crystallization solution can be prognosticate before performing in the experiment, because it is depended on the buffer pH and the pI of the protein. It is important for successful crystallization to keep the salt concentrations neither too low nor too high in the protein solution, highly salt concentration may stabilize the protein buffer or decrease the protein solubility lead to precipitation. Then crystallization happens in the drop in which the components are concentrated through water loss. If the original proteins as well as reservoir solution do not contain enough salt, the concentration in the drop does not reach the marginal concentration level.

Commonly, the increase of precipitant concentration in crystal solutions is an effective technique adopted to lowering the solubility of proteins. The salt it can stabilizes the water structure (Kosomtropic) or disrupts the water structure (Chaotropic). In addition, the solubility of the proteins drop at high concentrations of salt as well as solubility of the proteins increases at low concentrations of salt. The optimal salt concentration will slowly dehydrate our drop as well as increase the concentration of our protein slowly.

The marginal ionic strength become high when the difference between the pI of the proteins and the pH of the buffer is large, which is agreement with the idea of the electrostatic screen effect of a salt [3].

Multi subunit proteins or virus it is large macromolecules, highly salt concentrations are not effective to promote enough interactions to induce crystallization while the Polyethylene glycol (PEG) is a commonly used as precipitant in protein crystallization is indispensable to induce crystallization.

Crystallization of iron protein under anaerobic condition may facing precipitation problem, if the oxidation of the protein causing the precipitation try to add sodium dithionite to preserve the reduced state, also is better to change the ratio in the crystallization drop by higher the amount of the precipitant.

Expression system

E coli is first choice for structural biologists to produce protein for X-ray crystallography, but sometime unsuitable to express membrane proteins in bacterial expression systems because the system cannot provide the post-translational modifications, the folding machinery and specific lipid environment. For eukaryotic membrane protein better to express in *Saccharomyces cerevisiae, Pichia pastoris*, Sf9 insect cells [4] and human embryonic kidney.

Improve the expression level

In case of poor expression it need to be sure the protein is not toxic for the cell. Choosing a smaller fragment of the target protein can improve the expression levels and solubility, because

E. coli may not express well very large proteins > 70 kDa. Using strains carrying mutations which remove the production of proteases can occasionally enhance accumulation by diminution proteolytic degradation, also reducing the growth temperature will produce protein slow but at the same time slower proteolytic degradation. Using special media containing trace metals (high density culture media) it can increase protein yield 10 time over LB.

Co-expression of foldases proteins e.g. peptidyl prolyl cis/trans isomerases (PPI's), disulfide oxidoreductase (DsbA), disulfide isomerase (DsbC) and protein disulfide isomerase (PDI) with the target protein may lead to highly levels of protein solubility. The other advantages for this enzymes assist the formation of disulfide bonds which does not happen in the reducing environment an also reduced proteolysis

Commonly challenging and important task, protein aggregation and precipitation is often happen when increasing a protein concentration in the buffer to the required level.

Size exclusion chromatography

Is useful for further purification but some time is better to use new column for purification. The shape of the chromatogram and retention time provides.

High homogeneity and purity of the sample are crucial for the crystallization to be successful, the presence of different aggregates or oligomeric forms in the protein solution it well effect, we need to use Dynamic light scattering (DLS) to check that or small-angle X-ray scattering (SAXS).

Folding and stability

'Natively unfolded' proteins are some proteins unfolded in any physiological conditions, and this kind may better to co crystallize with other protein. UN folding, while highly beta strand are more likely to form amyloid-like aggregates, but both of them can be crystallize PDB (1aos--1by3). There is no direct correlation between percentage of helix secondary structure as well as beta strand and stability. However, proteins with high alpha-helical protein habitually are less problematic upon. There are more hydrophobic contacts in Beta sheets, misfolding or mutation might result in exposure of hydrophobic residues. In this case, proteins aggregate to inhibit hydrophobic exposure as well as avoid entropic penalty. Beta sheets are stabilized through hydrogen bonding as well as hydrophobic contacts, while Alpha helices are predominantly stabilized through hydrogen bonding. Thus sheet is a little inferior in terms of stability.

Addition of co-factors or prostethic groups (such as a vitamin, lipid, or inorganic like a metal ion) which are essential for protein stability or for proper folding, but must be not fluctuation the pH in the medium during growth, this leads to the accumulation of the target protein osmoprotectants in the cell, which stabilize the protein structure.

Biophysical methods like CD spectroscopy and other differential scanning fluorometry (DSF) can be used for characterizing the stability of the protein in different buffers, pH and in the presence of different ligands to make sure that the protein is correctly folded. There is several fold recognition websites (FOLDpro, RF-Fold, SSHMM, THREADER, BLASTLINK, SSEARCH, PSI-BLAST and HMMER) used to predict the protein fold.

Mutation

When the native protein fails to crystallize against ~ 300500 crystallization conditions it was propose genetically modified proteins whether by truncations or point mutations might better for crystallization.

Mutants of Tyr →Phe and Thr→Val more stable than the wild-type protein, but have little effect on the conformation of the protein [5]. However, it has been reported that point mutations spectacular affect the amount of aggregate formation in number of protein systems; these include the interferon-γ, P22 tailspike protein, colicin A, single-chain antibodies, immunoglobulin domains, and interleukin-1β [6]. However, it is still not clear what mutations are likely to be helpful in crystallization (Figure 1).

Cysteine and histidine predominantly react with reagents prepared for the other and for other amino acid side chains as well, that why it is problematic during crystallization. Some domain and cloning are affecting the solubility this checking before expend time on buffer optimization.

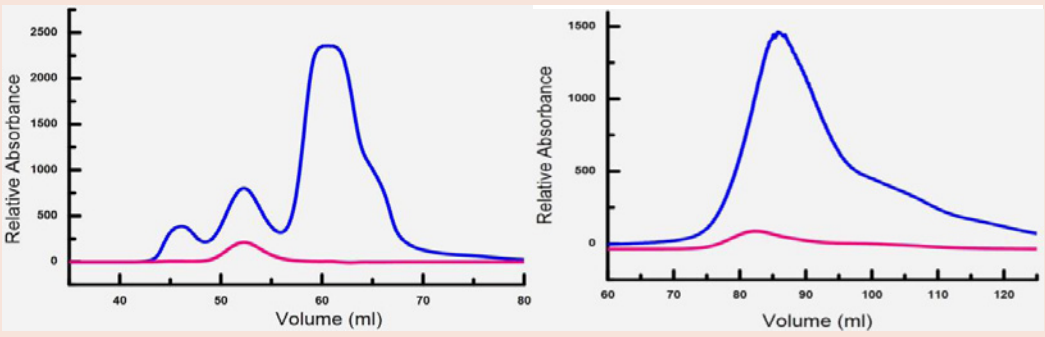

Figure 1: superdex 75 A. New superdex 75 B. Old superdex 75 as we can see new superdex 75 separate the protein to three pick, one is aggregation protein, two is dimer protein, three is monomer, while the old superdex 75 cannot separate them and in this case is difficult to be crystal because the crystal condition for dimer is not same to the monomer condition rather then that aggregation it can be crystal.

Nucleation

Sometime one protein can create different crystal, but that not mean all of them they are stable, it can just be kinetically favored. There is two kind of nucleation: heterogeneous is the most common, occurs by interface of different composition and homogeneous as high purity crystal. The nucleation rates it can be increased by increasing the super saturation, solubility, viscosity and decreases temperature and solid–liquid interfacial tension.

Ethanol

In ethanol, the assist of peptide group burial to protein stability would be increase and the contribution of non-polar group burial would be decrease. Because α-helices are expected to be stable structures in ethanol, ethanol used to increase α-helix formation in proteins and peptides [7], also ethanol and methanol can lowers the thermal denaturation temperature.

Glycerol

Often use in crystallization to protect the proteins as cryosolvent because antifreeze properties and to enhance their solubility by stabilizing the conformation, sugar protein specially affected by glycerol because of ribonuleotides bonds. But some time glycerol work as an antinucleation agent in crystallization.

TRIS & HEPES buffers

The differences in the influence of the buffers could be attributed neither to disparity in the amounts of de-protonated buffer ions nor to disparity in ionic strength. Tris and HEPES being positively charged at pH 7.0. Protein activity increased in different buffer by following order: citrate < Tris ≈potassium phosphate <HEPES. While oligonucleotide initiator decreased in the following order: cacodylate > MES > HEPES > TRIS > phosphate [8]. Tris as well as HEPES prevent the auto-oxidation. HEPES accelerated the degeneration rate of the oxidant and peroxynitrite. The exist of as much as 100 mM NaCl enhance the reversibility and stability of unfolding transitions in Hepes buffer. The crystal structure obtain from HEPES buffer was more similar to the active conformation.

Proteins in L-arginine and Tris buffer at pH 7.4 stay stable against aggregation for longer periods of time. Tris is Good's buffers bind to the DNA not only by electro static interactions but also by hydrogen bonds primarily to the pyrimidine or purine rings [9]. Tris decrease the metal ion–catalyzed oxidation of Cys, consequently, Cys being available for interaction with the other reagent.

Conclusion

In this review presents important factors that affect protein crystallization and ways to improve the results, but more needs to be done to better understand. Some proteins have ability to make crystal within initial concentration less than 1 mg per ml. Right working sequence and buffer well make all the difference.

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