LABORATORY OF ELEMENTARY BIOPHYSICS

Experimental exercises for III year of the First cycle studies

Field: “Applications of physics in biology and medicine”

Specialization: “Molecular Biophysics”

PROTEIN CRYSTALLISATION

PB9 v2

HUMAN CAPITAL
NATIONAL COHESION STRATEGY

EUROPEAN UNION
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SOCIAL FUND

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Crystallisability is inversely proportional to biological interest
Murphy's crystallization law

1. Introduction

Proteins maintain most of important functions in our bodies. Knowledge about their three dimensional structure very often is essential for understanding biological processes at atomic level. How goes chemical reaction? How proteins interact with other proteins and small molecules? Konowlegde obout three dimensional structure of actives and binding sites, allows to understand substrate specificity of an enzyme and designing specifically interacting ligand, a potential cures.

The most basic optical rule says, that the smallest object that can be seen, has dimensions similar to the length of wavelength used for observation. Average distances between atoms in proteins molecules are 0,16 – 0,35nm. So, in order to "see" a protein with atomic resolution it has to be "observed" with X-rays. The easiest way to "see" a protein, would be, to build X-ray microscope. This is impossible, due to problems in constructing lenses focusing the light. But what can be done , is measurement of X-rays diffraction of molecules and calculation the shape of an object that reflected the light. The X-rays diffract on electrons, due to very fast movements of electrons, the shape obtained from calculations is a shape of electron density cloud in the molecule. As electrons are centred around atoms and bonds this gives good estimation of the shape of molecule itself, but due to relatively low electron density, in comparison with carbon and oxygen, hydrogen atoms are rarely visible in diffraction images of proteins.

Besides electro-magnetic waves, the object can be "viewed" also by means of matter waves. Neutrons and electrons accelerated to the proper speed produces a wave which wavelength is comparable with distances between atoms in protein molecule: $\lambda = h/p$, where λ – wavelength, h – Planck constant, p – momentum.

The diffraction of neutrons, as they are electrically neutral, is quite similar to X-rays diffraction. The main difference, is that, neutrons scatter on atoms nuclei, so hydrogen atoms are well visible. But electrically charged electrons can be focus with magnetic lenses, thus electron microscopes(EM) can be build. Main problem with both methods is damage of the sample by high energy particles scattering on it. So in EM the weak electron beams are used, but this causes low signal to nose ratio and need for averaging of many images. Like in X-rays crystallography, to prolong sample survival in the beam, the sample is frozen – cryogenic electron microscopy (Cryo-EM). Accuracy of the best Cryo-EM is approaching atomic resolution. As far as now, this method allows for resolution around 10Å what allows to research large complexes like ribosome or virus capsids, thus in the area when X-ray crystallography reaches its limits. X-ray crystallography and EM complements each other well.

Another important, in protein structure determination, method is Nuclear Magnetic Resonance (NMR). NMR bases on quantum-mechanic features of atoms, its nuclear spin. By means of designed impulses of electro-magnetic waves from the radio wavelengths range (λ ~1m) spins are diverted from its equilibrium positions. Then decay of the signal of spin coming back to equilibrium is observed. Haw atoms responds to the magnetic field carried by the impuls, depends of their chemical environment. The measurement of decay profiles allows to determine close chemical environment of each atom responding to applied magnetic field, and calculate the interatomic distances and torsion angles.

The main quality of NMR is that sample in in water solution, thus attempt to recreate physiological

conditions can be made, providing conditions in which the molecule in question can be as close to native state as it is only possible. As it gives native protein, NMR also allows tu study protein behaviour under stimulus like titration with ligand, temperature or pH changes, protein complexes association, in realistic time scale. The main limitation of the method is the size of the molecule. As the number of atoms rises, the peaks from atoms start to overlap. Standard, one dimensional hydrogen experiment allows to determine structure of protein of maximum weight about 10kDa. Two and three dimensional techniques using proteins labelled with ^{13}C i ^{15}N allows to move the limitation up to about 35kDa. Total deuterisation(exchanging hydrogens to deuters) of such labelled protein can move the limitation further up to about 50kDa. Application of even more advanced techniques allows to cross 100kDa borderline[1].

As a consequence of limitations of EM and NMR, the X-rays crystallography is still a champion in the field of protein structure determination. There is over one hundred thousand structures of proteins deposited in Protein Data Bank(PDB), among which almost 90% is determined with X-ray crystallography.

experimental technique	**number of structures deposited in PDB (access 12.09.2014)**
PX	91 554
NMR	10 629
EM	841

But protein X-ray crystallography has also its limitation, and the are not little ones. The bottleneck of the method, is its phase one: obtaining well diffracting crystal of studied protein.

2. *Mechanisms of crystallisation.*

Crystallisation is not a spontaneous process, from thermodynamic point of view, is all but spontaneous process. Almost all processes occurring during crystallisation are thermodynamically unfavourable, because they lead to decreasing entropy.

$$\Delta G_{crys} = \Delta H_{prot} - T\Delta S_{prot} - T\Delta S_{solv}$$

where: ΔG_{crys} - change in Gibbs potential related to crystallisation,

ΔH_{prot} - enthalpy change due to bonds creation between proteins molecules. This process favours crystallisation, but is little in comparison with entropy drop,

T - temperature

ΔS_{prot} - entropy change due to decrease number of degrees of freedom, caused by arranging molecules into regular and rigid structure of crystal, stabilisation of flexible loops and amino acids side chains placed at the surfaces of molecules interacting with each other inside crystal,

ΔS_{solv} - entropy change of solvent molecules. Releasing solvent molecules from protein molecules solvatation shells leads to entropy rise.

This means, that to enhance protein crystallisation interaction between solvent and protein molecules must be modified.

This can be achieved by adding to the protein solution precipitating agent, so called precipitants. Mechanism of action of these agents is not very well known, but this is widely accepted, that they compete with proteins molecules for solvent molecules. Adding proper kind and amount of precipitant into protein solution, may lead to favour protein-protein interaction over protein-solvent molecules interaction. This may lead to forming a protein crystal.

Nucleation is a stochastic process. It starts with an aggregate of few protein molecules bundled together. If this chaotic bundle starts to organise itself into regular structure, the crystallisation nucleus will appear. If more protein molecules will settle on the nucleus extending the initial ordered pattern, the macroscopic protein crystal can grow.

The phase diagram of protein solubility in the presence of precipitant is shown at Fig. 1. When the multiplication of protein concentration and precipitant concentration is low, the protein is soluble, and the solution is undersaturated. Increasing concentration of protein, or precipitant, or both lead to supersaturated solution. Moving from undersaturated to supersaturated state, the solution first enters metastable zone, where the crystals may grow, but no crystallisation nucleus are formed. Further increasing of concentration/concentrations lead solution to nucleation zone, where crystallisation nuclei may appear. The longer the solution spends in nucleation zone, the more crystallisation nuclei will appear. If the solution with large number of nuclei moves to metastable zone there will be so called crystal shower, or microcrystalline precipitation. Further increasing of protein and precipitant concentration will lead to precipitation zone, where the protein fall out of solution forming amorphous precipitat.

The process described above is crystallisation of protein by **salting out** from solution. Phase diagram for this process is presented on Fig. 1. If the precipitant is salt, protein crystallisation may be achieved also by decreasing precipitant concentration. Such a process is called **salting in**. In that case there is additional area on the phase diagram for low precipitant concentration, and the solubility curve has maximum.

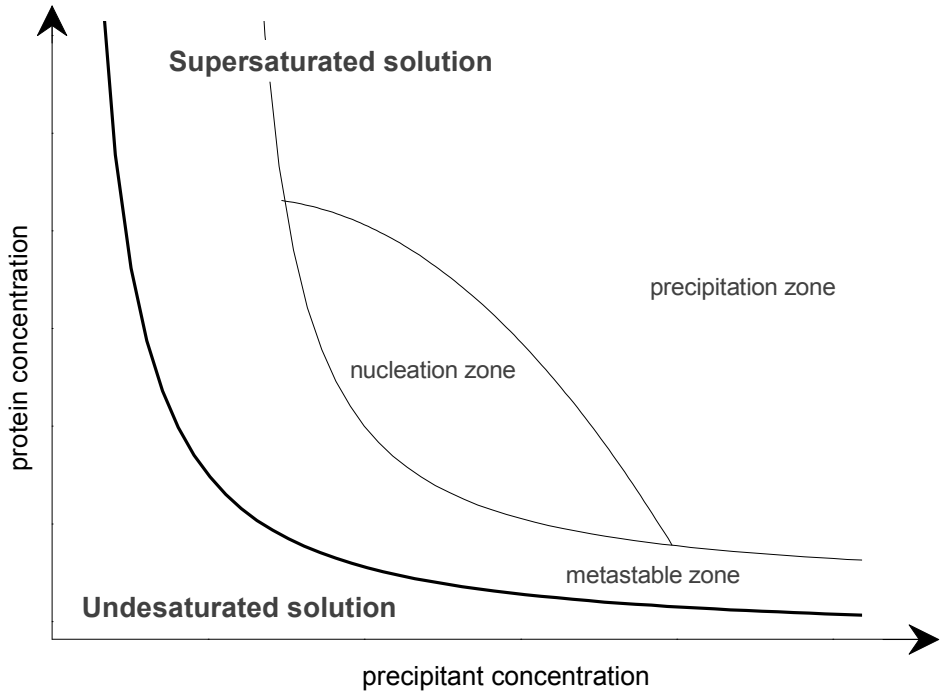

Fig. 1. Phase diagram of protein solubility in bicomponent solution of protein and precipitant, the salting out zone.

For a crystallographer the ideal situation is as follows: the solution of protein and precipitant reaches nucleation zone, stays there short enough to allow forming of just few crystallisation nuclei, causing decreasing of protein concentration in solution and moving solution to metastable zone where crystal may grow.

How to achieve such phenomena? Below, there are described the most common crystallisation techniques.

2.1. Protein crystallisation techniques

2.1.1. Batch crystallisation

In this method crystals are obtained straight from the solution of protein. The protein solution is gently mixed with a crystallisation liquid containing all other necessary ingredients, including precipitant, creating supersaturated solution. Then the mixture is placed in sealed container, and left undisturbed. If the concentration had been chosen properly, solution may be at the nucleation zone, or move there, as the solvent evaporate reaching equilibrium. Main disadvantages of this technique is large protein consumption and poor control over solution reaching nucleation zone. The advantage is possibility to grow really big crystals.

Modification of this method, that is less protein consuming is microbatch crystallisation. The mixture of protein and all crystallisation ingredients is prepared like in the above described procedure, and then, little drop of the mixture is placed under the layer of oil, which limits evaporation.

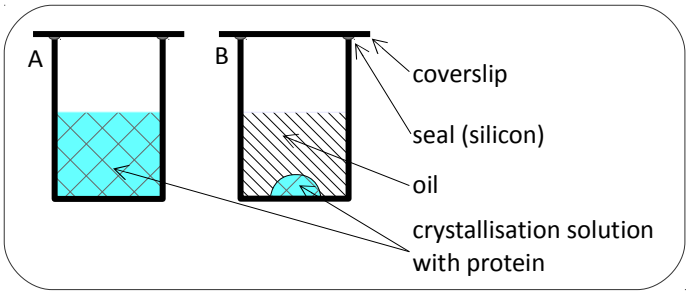

Fig. 2. Experimental set-up for batch(A) and microbatch(B) crystallisation

2.1.2. *Vapour diffusion*

Little volume of protein solution (few microliters) is mixed with equal or less volume of crystallisation solution to form a little drop and placed in the sealed container, already containing larger volume of crystallisation solution (few hundred microliters). The drop with protein and the reservoir of crystallisation solution don't touch each other, they exchange only volatiles. At the beginning the evaporation is fast, then it slows down reaching equilibrium. Protein and other ingredients of the drop get concentrated, and this process is relatively gentle. Evaporation may be further slowed by decreasing crystallisation temperature. Final concentration of drop ingredients are close to concentration of ingredients in the reservoir, thus, setting a drop in the volume ratio 1:1, the protein gets concentrated around two times. Setting drop with higher volume of protein, it can be concentrated more.

If the initial concentration are chosen properly, the drop of solution may enter nucleation zone, where crystallisation nuclei may appear. Further settling of proteins molecules on the nuclei would lead to decrease of protein concentration in solution, moving conditions in the drop to metastable zone, where the crystals may grow.

The path of solution in the drop is plotted on the phase diagram (Fig. 5), shown path is optimal one, leading to obtaining crystals.

There are two experimental set-ups for vapour diffusion crystallisation, so called sitting drop method and hanging drop method, see Fig. 3

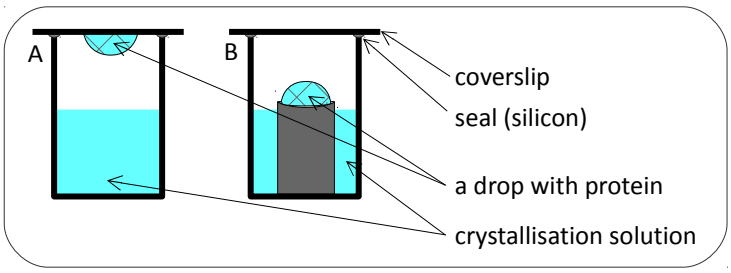

Fig. 3. Experimental set-up for vapour diffusion crystallisation: hanging drop method(A), sitting drop method(B).

2.1.3. *Dialysis*

The protein solution is in contact with crystallisation solution through dialysis membrane, which allows ions and small molecules flow, but the protein concentration remains constant. By means of dialysis one can perform crystallisation by salting out and salting in.

The schematic image of experimental set-up for crystallisation with dialysis is shown on Fig. 4.

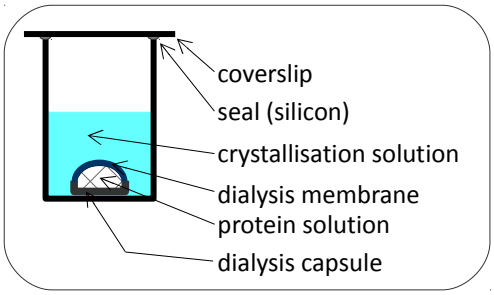

Fig. 4. Experimental set-up for crystallisation with dialysis.

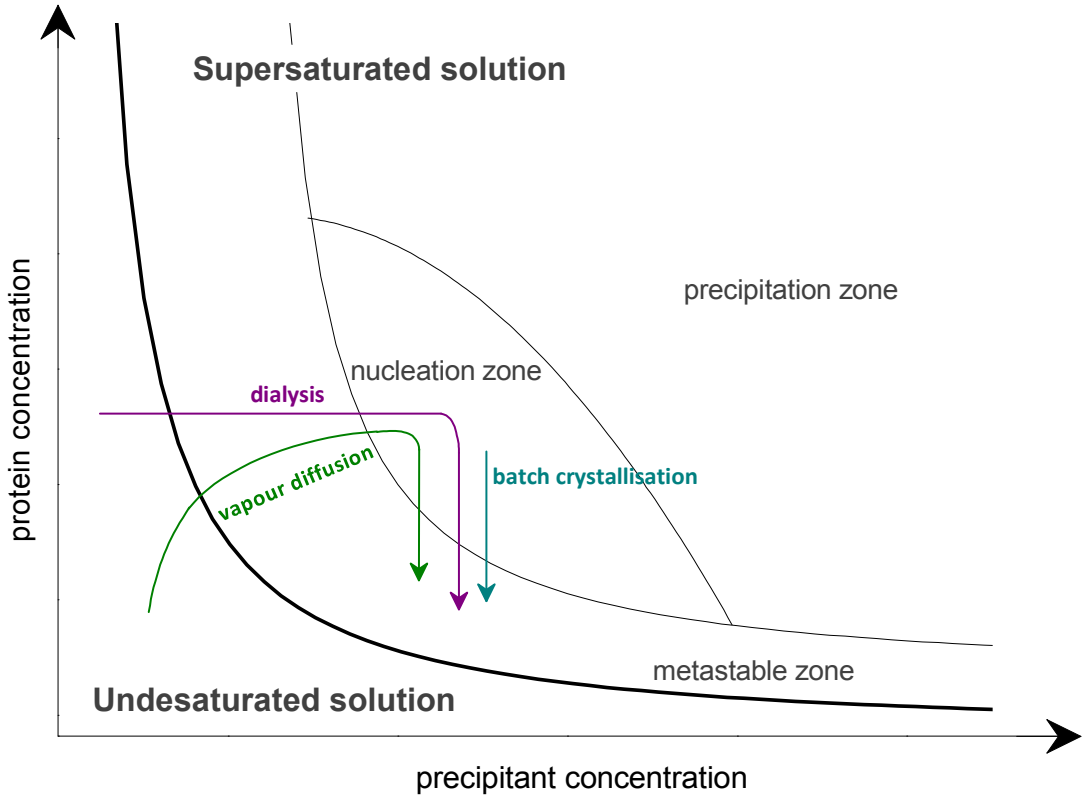

Fig. 5. Phase diagram with "paths" leading to crystallisation success

2.1.4. Seeding

Seeding is a method of growing crystals from a "template". If crystals obtained, by means of any of the above described methods, are ugly(irregular, concrescent, or...) or are in fact microcrystals, they can be used as seeds, to grow big crystals. Microcrystals must be taken as they are, bigger crystals must be crushed. New crystallisation must be set in identical or similar conditions, in typical manner, and then seeds must bee placed in drops.

The general idea is simple: make your protein and precypitant solution supersaturated, make it to appear just few crystallisation nuclei, which then grow to macroscopic crystals.

It's not that easy in practise.

3. How to obtain well diffracting protein crystal?

The key problem in protein crystallisation is impossible to predict what factors may induce crystallisation of chosen protein. Similarity in sequence or structure doesn't mean similarity of crystallisation conditions.

In the table below(table 1) is a, probably incomplete, list of factors that affect crystallisation process.

Physical factors	Chemical factors	Biochemical factors
Temperature	Precipitant type	Sample purity
Pressure	Precipitant concentration	Sample homogeneity
Gravity	pH	Sequence modifications
Magnetic fields	Buffer type	Posttranslational modifications
Electric fields	Ionic strength	Chemical modifications
Dielectric properties	Sample concentration	Aggregation
Viscosity	Metal ions	Proteolysis
Vibrations and sound	Detergents	Sample pI
Time	Small molecule impurities	Ligands, co-factors, inhibitors
Equilibration rate	Crosslinkers	
Nucleants	Reagent source	
Methodology	Reagent formulation	
Surface of crystallization device		

Table 1. Factors affecting crystallisation[2].

Next important aspect is homogeneity of protein sample. The protein for crystallisation has to be pure, properly folded and stable. Sample has to be as uniform as it is only possible.

Protein has to be pure, because all kind of impurities may act as crystallisation nucleus, and on the other hand impurities may disturb uniform crystal growth. The protein has to be properly folded and active in order to crystallised structure was close to native one, if it is supposed to be biologically relevant. All other conformation of protein should be removed. Unstable proteins, which looses its conformation over time, often has tendency to aggregate, and as crystallisation experiments may last many months, it will decrease chances of obtaining crystals. Also every flexible part of protein makes crystallisation difficult or impossible. Flexible loops, or natively unstructured domains must be stabilised prior crystallisation, or removed. Flexible parts of proteins may be stabilised by creating complexes with ligands or other proteins, or removed by biochemical or genetic engineering methods.

Another important factor is composition of the buffer protein is solved in. High concentration of buffer itself, or salt may interfere with crystallisation condition, changing final pH or causing precipitation of salt crystals (like magnesium phosphate or calcium phosphate). On the other hand protein has to be stable and acitve so the pH and ionic strength has to be maintained. The compromise is tu use the lowest possible concentration of buffer, salts, and other additives, that can keep protein stable.

Crystallisation usually demands high protein concentrations, at least 10mg/ml. For unstable or prone to aggregating preteins this range of concentration may be unachievable.

3.1. Screening for crystallisation conditions.

With this mount of factors, it is impossible to perform systematic search for best crystallisation conditions. So over years another approach has been work through: the crystallisation screens. Analysis of collections of conditions in which various proteins crystallised over years showed that there are substances and group

of substances which promotes crystallisation better then others. The most commonly used precipitants are ammonium sulphate and polyethylene glycols(PEGs) of various molecule mass[3].

Typical crystallisation solution is composed of buffer, precipitant and optionally additional substances (called additives) which may promote crystallisation or protein well-being. For example detergents may suppress proteins tendency to aggregate, salts ensure high ionic strength necessary for some proteins to be stable. Some proteins need specific metal ions to crystallise. Glicerol, by increasing viscosity of solutions, decreases nucleation rate, thus enhancing growth of fewer but larger crystals.

Screening for crystallisation conditions usually starts from sparse matrix screens*[4]* probing not very densely the best crystallisation conditions selected over the years. A number of such basic screens are available commercially. This kind of screens allows to narrow the selection of substances which promotes crystallisation of chosen protein, allowing to start experiment in more specialised screens, more densely probing selected areas.

Typical screen consist of 48 or 96 conditions, and experiments are carried out in 96 or even 386-well crystallisation plates. Handling this amount of samples manually is nearly impossible, the crystallisation experiments are set by robotic systems. Despite saving time, robotic systems allow also to save protein, as the drop set by machine can be few to tens times smeller in volume than that set manually. Inspecting and documenting results also can be carried out by robotic systems.

Crystallisation screening allows to initially estimate condition in which protein can crystallise, even if no crystals were obtained, these estimation includes pH, kind and concentration of precipitate and protein concentration.

Results of crystallisation can be as follows:

– clear drop (Fig. 6A) – the solution is in undersaturated state.

– crystals (Fig. 6B) – that is what we looking for! Crystals on Fig. 6B are quite large and well separated from each other – that's an ideal situation, and of course not very often, especially in preliminary screening. Crystals in tiny drop set by robot are often not large enough to diffract well, and obtaining crystals good enough for diffraction experiment usually requires further optimisation.

- microcrystallic precipitation (Fig. 6C) – its not what we want, but it is not bad outcome, as it is crystallic status solid. Sometimes crystals may grow from this kind of precipitation, but this is very slow process. This amount of very little crystals suggest that concentration of protein or precipitant or both are to high, or the sample is trapped in nucleation zone. Conditions need further optimisation

- amorphous precipitation (Fig. 6D) – its usually shapeless, beige or light brown precipitation, indicating that protein is desaturated. It is not good outcome, but it gives directions for further optimisation.

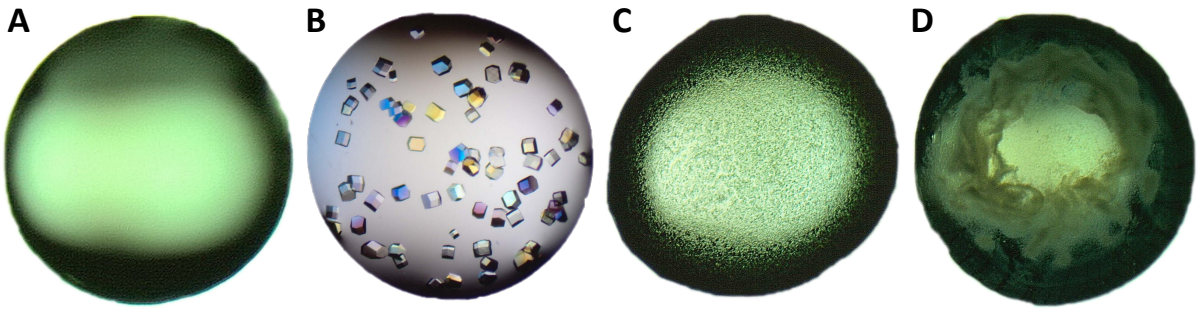

Fig. 6. Some of typical crystallisation outcomes: A: clear drop, B: large, well separated crystals – ideal!, C: microcrystallic precipitation, D: amorphous precipitation.

3.2. Optimization of crystallisation conditions

Optimization usually involves screening densely conditions around conditions obtains from previous stages of screening. Parameters to variate at first shot are:

– precipitant concentration – screen conditions usually suggest into which direction modify concentration
– protein concentration – screen conditions usually suggest into which direction modify concentration
– buffer pH – this should be applied carefully, sometimes change pH of 0,1 may cause huge change in crystallisability
– temperature – lowering temperature decrease nucleation rate, thus may lead to obtain fewer but larger crystals, and decrease crystal growth rate, thus may lead to better ordered and better diffracting crystals
– drop size – larger drop usually allows grow larger crystals, simply because there is more protein to form crystal from.

At this stage one can also try to start playing with additives like detergents, metal ions, glicerol.

When the conditions for crystals to grow are quite well established, large, well diffracting crystals are usually obtained from large drop set manually. For that purpose 24-well crystals plates are used, and drops of 4-6µl or sometimes even 10µl or 20µl are set.

Screening of crystallisation results is performed with stereoscopic, optical microscope. By visual inspection one can determine shape and size of crystals, assess if crystals have any visible cracks, if the edges are well visible and sharp. But in the world of protein crystals beauty might be deceitful. The most beautiful, regular crystals may do not diffract at all, and ugly, irregular crystals with ragged edges may diffract excellent. It may also be a salt crystal!

The only definite proof of a good crystal is its diffraction pattern

4. Bibliografia

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5. Further reading

1. M. Benvenuti, S. Mangani *Crystallization of soluble proteins in vapour diffusion for X-ray crystallography* Nature Protocols (2007) Vol.2 No.7 1633-1651
2. I.R. Krauss et. all *An Overview of Biological Macromolecule Crystallization,* 2013, International Journal of Molecular Sciences 14, 11643-11691
3. Terese Bergfors: Protein Crystallization/Crystallization Tutorial: http://xray.bmc.uu.se/~terese/tutorials.html - a gallery of crystallisation results with very good explanation how to interpret crystallisation results.

6. Topics for preliminary test

1. Experimental techniques used in protein structure research, their advantages and disadvantages.
2. Why proteins have to be crystallised?
3. Why the protein might “not want” to crystallise?
4. Phase diagram for bicomponent protein-precipitant solution.
5. Protein crystallisation techniques, and their explanations in regards to phase diagram
6. Mechanisms of crystallisation.
7. Conditions for protein crystallisation, typical ingredients of crystallisation solutions.
8. What are crystallisation screen, why are they used?

7. Performing exercise

The purpose of the exercise is obtaining lysozyme crystals, theirs visual inspection towards selecting best for diffraction measurement, and discussion of the results towards improving crystallisation conditions.

As an annex to this instruction, there is a paper describing lysozyme crystallisation conditions. Basing on these results students will plan and perform their own crystallisation experiment.

Provided equipment and chemicals:

- 24-well crystallisation plate

- lysozyme solution in water at concentration of 100mg/ml
- 30% solution of NaCl in water
- 0,2M acetate buffer
- computer with LibreOffice for performing necessary calculations

The first visual inspection of the plate should be performed just after setting plate. Then plate has to be placed in the incubator maintaining stable temperature. Next inspection of the plate has to be done after 1,2 and 5 days.

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