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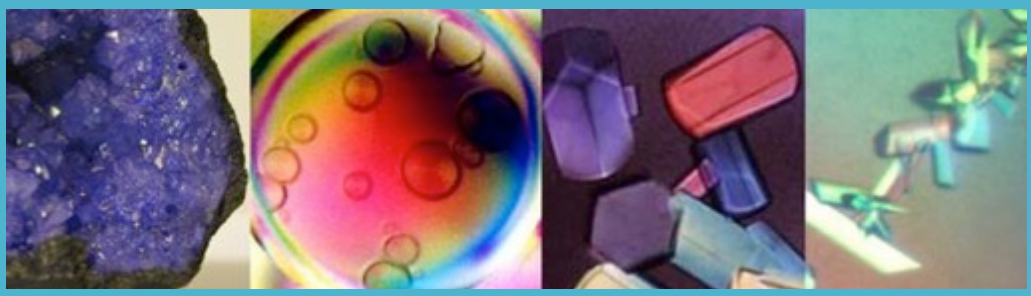
Laboratory exercises for protein crystallization

– 3 HEC PhD course PNS0158 Protein crystallization and X-ray data collection 9-20 April 2018



9-13 April 2018

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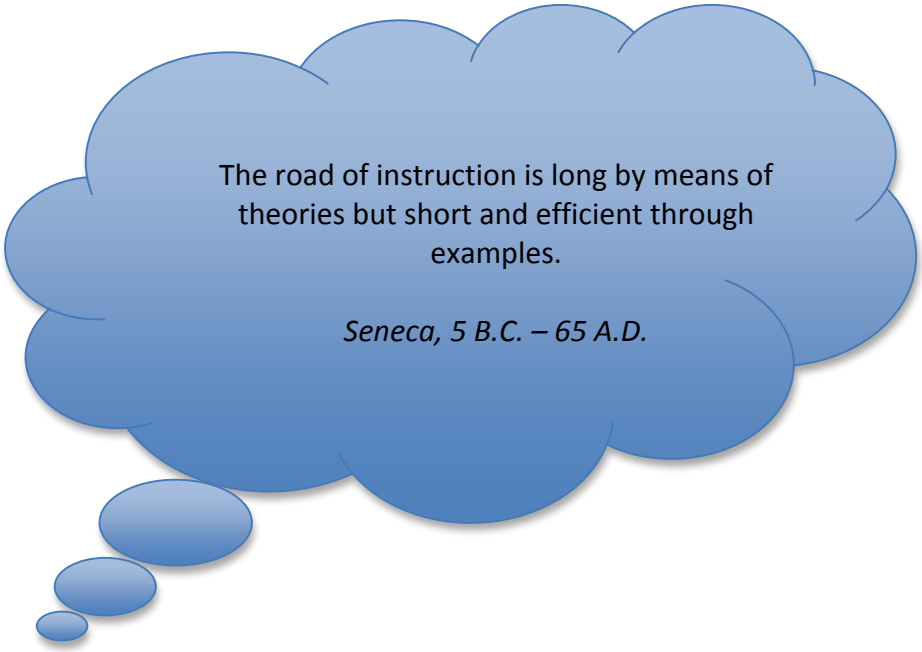


Tuesday

Exercise 1. Vapor diffusion with hanging and sitting drops: manual setup with lysozyme.

Materials

- One 24-well plate
- 6 cover slips for the hanging drops
- clear sealing tape for sitting drop experiments
- 6 sitting-drop bridges
- grease for the cover slips (for the hanging drops)
- pipettes

Proteins

- Lysozyme 20 mg/ml

Precipitant Solutions

- 5, 10, 15, 20, 25, 30% NaCl in 0.1 M sodium acetate buffer, pH 4.7

Here is the basic procedure for a manual setup in 24-well plates.

Hanging drops

1. Choose a 24-well plate with thick rims around the reservoirs and grease the rims. (It is also possible to buy pre-greased plates).
2. Pipete 0.5 ml of the precipitant solution into the reservoir of the plate.
3. Place a silanized cover slip on the benchtop. Carefully pipet 1-5 microliters of protein solution onto the cover slip.
4. Add 1-5 microliters of the reservoir solution to the protein. Avoid bubbles and make the drop as spherical as possible.
5. Optional : mix/stir the drop. Mixing increases the nucleation rate. This may or may not be an effect you want. The choice is yours, but be consistent. Don't mix some drops and not the others, or vice versa.
6. With a pair of tweezers, invert the cover slip and suspend it over the reservoir solution by placing the cover slip onto the greased rim. Press gently to ensure a good seal.
7. Prepare the rest of the plate in the same manner.

Sitting drops

1. Sitting drop plates are commercially available or you can insert sitting-drop bridges into an ordinary 24-well plate.
2. Put the screening solution into the reservoir.
3. Pipet the protein into the sitting-drop depression.
4. Add the reservoir solution as above.
5. Seal the experiment with Clear-Seal tape.

Today's experiment

The protein concentration will be kept constant (20 mg/ml) and we will vary the precipitant concentration from 5 to 30% NaCl in 50 mM sodium acetate, pH 4.7. Make drops of 2 microliters protein plus 2 microliters reservoir solution.

One row will be hanging drops.
The other row will be the same, but sitting drops. The layout will look like this:

	5%	10%	15%	20%	25%	30%
hanging drops						
sitting drops						

Questions for discussion

1. In an initial screening experiment, which makes more sense to do: set up the same kit as hanging and sitting drops or set up two different kits as sitting drops only?
2. Why and when do people use hanging drops? Which do you prefer?
3. Which kind of drop is better to set up at 4 degrees?

Tuesday

Exercise 2. Optimization of crystallization conditions for CBH1 by grid screening, and cocrystallization with ligand, using the hanging drop method in 15-well plates.

Materials and solutions

- One 15-well plate with screw caps (EasyXtal; Qiagen)
- stepper pipette with positive displacement tips
- pipettes, down to 1 ul volume
- 50% mPEG 5000 (polyethylene glycol monomethyl ether) = precipitant
- 1 M Na-MES (sodium 2-(N-morpholino)-ethanesulfonic acid), pH 6.0 = buffer
- glycerol (>95%) = cryoprotectant
- 1 M cobalt chloride, $CoCl_2$ = metal additive
- CBH1 protein, ~10 mg/ml, in 10 mM sodium acetate (NaAc) buffer pH 5.0
- ligand solution, 5 mM cellobiose (preliminary)

Procedure

Each group shall set up 9 crystallization drops in a small grid screen:

	1	2	3	4	5
	2 mg/ml protein	8 mg/ml protein	8 mg/ml + ligand	empty	empty
A: 15% mPEG 300 ul 50% 515 ul water					
B: 17.5% mPEG 350 ul 50% 465 ul water					
C: 20% mPEG 400 ul 50% 415 ul water					

1 ml reservoirs in the wells: 15%, 17.5% or 20% mPEG 5000
+ 50 ul 1 M NaMes pH 6.0 → 50 mM
+ 125 ul glycerol → 12.5%
+ 10 ul 1 M CoCl2 → 10 mM
Crystallization drops: 1 + 1 ul

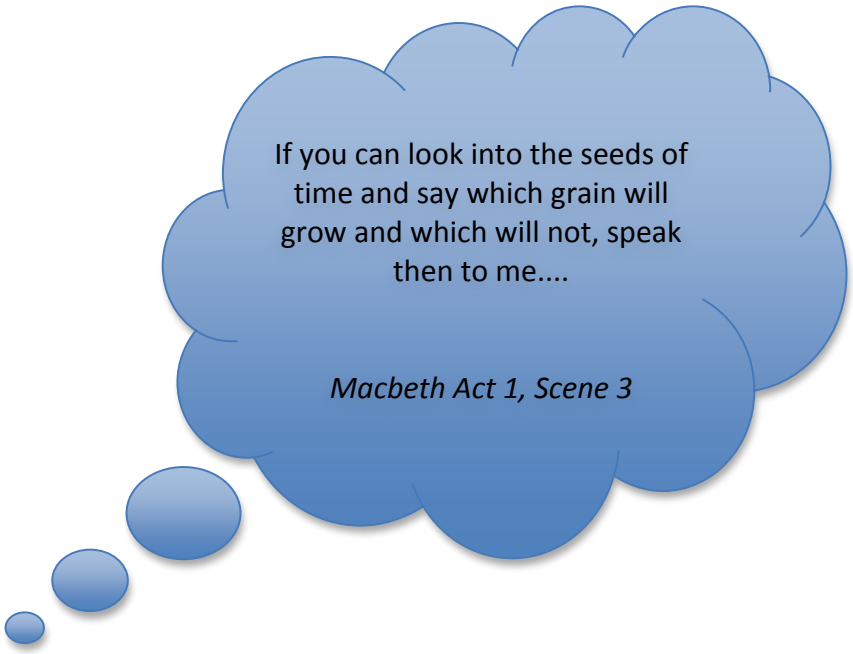
Mix reservoirs

1. Take one 15-well plate. Mark with date, experiment, group number and names. You may also scribble directly on the plate what you have in each row/column to quickly see what is where.
2. Pipet water to the wells, 515 ul in row A, 465 ul in row B, 415 ul in row C.
3. Add 10 ul $CoCl_2$ and 50 ul NaMES buffer to each well.
4. Use a stepper pipette to add glycerol (125 ul), and mPEG (see volumes above). NB! Ordinary pipettes do not work well with these viscous solutions.
5. Do not worry about precipitation of mPEG in the wells. Shake the plate some minutes on the plate shaker until reservoirs are clear.

Hanging drops

6. Pipet 8 ul water to an eppendorf tube. Add 2 ul CBH1 stock solution, to dilute to 2 mg/ml, to use for column 1.
7. Pipet 2 ul water to an eppendorf tube. Add 8 ul CBH1 stock solution, to dilute to 8 mg/ml, to use for column 2.
8. Pipet 2 ul **ligand solution** to an eppendorf tube. Add 8 ul CBH1 stock solution, to dilute to 8 mg/ml, to use for column 3.
9. Organize all you need for steps 10-12, so that you can set up the crystallizationn drops swiftly, in order to prevent substantial evaporation from the drops.
10. Place 1 ul of the correct protein solution on a screw cap. Avoid air bubbles.
11. Keep the pipette tip, and take 1 ul from the corresponding reservoir/well and add to the protein drop (no mixing is needed). Avoid air bubbles.
12. Turn the screw cap upside down so the drop will be hanging. Place above the corresponding well. Screw to fasten and tighten gently.
13. Prepare the rest of the drops in the same manner.
14. Examine your drops under the microscope and note if you have already got some crystals, or precipitate, or if the drops are clear. You may, e.g., use the table above for your notes.
15. Put the plate at the place dedicated for crystallization experiments until tomorrow.

Wednesday Seeding



Seeding Lab Exercises Background

1. What is seeding?

- The use of an existing nucleus (usually a small crystal or crystal fragment), introduced into a new drop, where it acts as a growth site.

2. Why seed?

- To separate nucleation from growth and bypass the need for spontaneous nucleation. It is easier to add onto an already existing nucleus than create one *de novo*. **See Fig. 1. Seeding and the phase diagram.**
- Improve the size of the crystals or control the number of crystals
- Get more consistent results when crystals don't always appear in known conditions
- Speed up results if spontaneous nucleation is slow
- To obtain a wider range of crystal forms (polymorphs) by seeding into totally different precipitants

3. What are the types of seeding?

- **Macroseeding** is the transfer of a single, pre-grown, washed crystal.
- **Microseeding** is the transfer of microscopic crystals, crushed up into fragments.
- **Streak seeding** is a form of microseeding that transfers the microseeds by a stroking motion with a whisker or hair of some sort. Can be used on its own or in combination with microseeds in a dilution series.
- **Jab seeding** is another variation of microseeding where the new drop is "innoculated" with a single jab of the seed transfer tool.
- **Matrix microseeding** is when microseeds are placed into a screen of conditions totally unrelated to the ones where the seed crystals originated.

Reference:

For a review article on seeding, see: Bergfors, T. "Seeds to Crystals" J. Structural Biol. 2003, vol. 142, 66-76

For an article on microseed matrix screening see: D'Arcy, Bergfors, Cowan-Jacob & Marsh. "Microseed matrix screening for optimization in protein crystallization: what have we learned?" Acta Cryst 2014, F70, 1117-1126. **This is the method we will demonstrate for you at the BMC B7:205 lab on Wednesday.**

Wednesday a.m.

Exp. 3. Demo of matrix microseeding

Visit to BMC B7:205, Husargatan 3

https://www.google.se/maps/@59.8418476,17.6348823,17.99z

Wednesday p.m.

Exp. 4. CBH1: manual microseeding

Materials and solutions

- Pipettes, down to 1 ul volume
- solution for seed preparation = precipitant solution with 20 % mPEG 5K, 50 mM Na-MES buffer pH 6.0, 10 mM cobalt chloride, and 12.5% glycerol (provided)
- one crystallization drop with CBH1 crystals (NB! Not crystals of active wildtype CBH1 if you are crystallising an inactive mutant)
- plate(s) with crystallization drops to be seeded, i.e. the CBH1 plate that you set up yesterday (on Tuesday)
- acupuncture needles (for seeding)
- pipettes

Microseed suspension

1. Examine the crystallization plate from yesterday under the microscope. Note if you have already got some crystals, or precipitate, or if the drops are clear.
2. Find and select one crystallization drop with crystals to use for preparing microseeds, among your own if you already got crystals, or pregrown crystals provided by the teacher. Do not take a drop where you have crystals that may potentially be used for data collection, but one where they are less nice-looking.
3. Pipet ~200 ul of the solution for seed preparation to an eppendorf tube. (Alternatively you can take ~200 ul of reservoir solution of the chosen drop).
4. Pipet some 5-10 ul from the eppendorf tube to the drop with crystals. Suck it all up again, with crystals, and add to the solution in the eppendorf tube.
5. Vortex the tube 30-60 seconds.
6. Centrifuge 1-2 minutes at max speed (e.g. 13,000 rpm) in an eppendorf centrifuge.
7. Transfer some 100-150 ul of the supernatant to a new eppendorf tube, being careful not to stir up any pelleted stuff from the bottom of the tube. The supernatant is your microseed solution (or rather suspension), ready for use.

Microseeding with needle

8. Pipet 100-500 ul of solution for seed preparation as reservoir in an empty well on a plate.
9. Pipet some 5-10 ul of your microseed solution on a new screw cap. Place on top of the well with the reservoir. Do not tighten the cap at this stage.
10. Screw off the cap with the crystallization drop to be seeded, turn upside down, and place in front of you (or hold with one hand while seeding with the other hand).
11. Dip an acupuncture needle in the microseed solution drop, then just touch the surface of the crystallization drop with the tip of the needle. The less of the needle that is immersed, the better.
12. Put back the screw cap above its well.
13. Repeat the procedure with all the crystallization drops. You can use the same needle for all drops. Should you rinse the needle off in between jabs? It's up to you, but a make a note of whether or not you do it. It may increase the number of seeds in the subsequent drops if you don't rinse between jabs. This may or may not be a good thing. It depends if you have too many or too few seeds to begin with. So, as always in lab work, keep careful notes of what you have done.
14. Take a quick look at the seeded drops in your plate. Note any “new” observations (e.g. precipitate, dust, dirt, air bubbles etc).
15. Put the plate at the place for crystallization experiments until tomorrow.

Wednesday

Exp 5. Phase diagram with lysozyme: Determination of the supersolubility curve in a microbatch setup.

Purpose of this experiment:
In this experiment we will construct a two-dimensional grid screen where we vary the protein concentration vs. the salt concentration. You want to find the nucleation zone, because this is where crystals first nucleate.

To find this region, we will empirically construct a two-dimensional phase diagram (protein concentration vs. precipitant concentration) for lysozyme. Within 30 minutes or less, we can establish the *precipitation boundary.* If you continue the experiment 24-48 hours, it is also possible to map the *nucleation zone*. Knowing both of these allows us to plot the *supersolubility curve.*

It is assumed you know the different regions of the phase diagram. If not, here they are again (see Fig. 1).

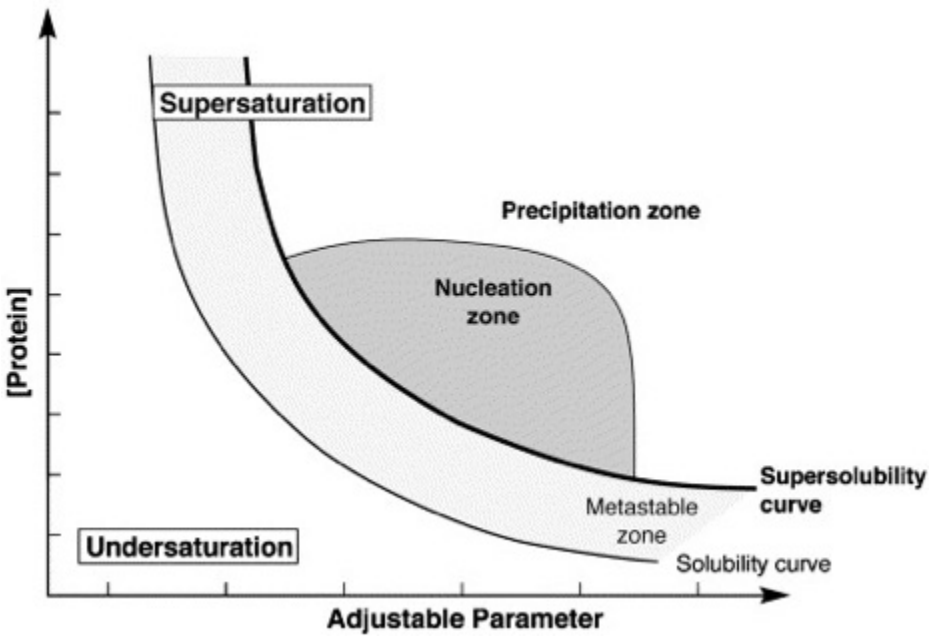


Figure 1. A schematic showing the different regions of the phase diagram. The exact locations of these zones must be determined empirically for each protein and precipitant. Reprinted here for teaching purposes with permission from the author, N.E. Chayen, reference 1.

Materials required:

- Terasaki plate
- Paraffin oil (about 8 ml is required to fill the Terasaki plate).
- P2 Gilson pipette or similar

Solutions and proteins required:

- 5%, 10%, 15%, 20%, 25%, and 30% NaCl buffered in 0.1M Na acetate, pH 4.7. Use the solutions from yesterday, Exp. 1.
- lysozyme with eosin red, at 10, 20, 40, 60, 80, and 100 mg/ml, dissolved in water. These dilutions have already been prepared for you.

Procedure:

1. Flood the Terasaki plate with the paraffin oil, i.e., about 8 ml. Make sure each well is flooded.
2. Pipette 2 microliters of each NaCl solution across as shown in the diagram below, Fig 2.
3. Pipette 2 microliters of each lysozyme concentration as shown in Fig. 2.
4. Stir the drops so that they mix.
5. Optional (if you have enough time): Repeat the grid (there should be enough room on the same Terasaki plate by using rows 7-12), but this time stir all the drops. Alternatively, you set up one experiment which you do not stir but your lab partner sets up the same experiment and stirs his/her drops.
6. Wait 5-30 minutes. Plot the results as a two-dimensional graph: protein vs. precipitant concentration.
7. Optional: Wait 24-48 hours. Plot the results again. (In today's experiment we will only do steps 1-6.)

Fig. 2. Terasaki plate setup

The droplets in rows 1-6 should be stirred.
Avoid introducing air bubbles if you aspirate the solutions up and down.

		5% NaCl	10% NaCl	15% NaCl	20% NaCl	25% NaCl	30% NaCl
Row 1	100 mg/ml						
Row 2	80						
Row 3	60						
Row 4	40						
Row 5	20						
Row 6	10						
		A	B	C	D	E	F

Expected results:

- You should see two clearly delineated zones where some drops stay clear and other drops precipitate more or less immediately (< 30 minutes). This gives you the precipitation zone.
- After 24-48 hours you should clearly see which conditions lead to nucleation with subsequent crystal growth and which ones remain clear. The zone with crystals is the nucleation zone.The clear drops are either undersaturated or in the metastable zone.
- If you plot the nucleation and precipitation zones, you can establish the *supersolubility curve.*

- To distinguish the undersaturated zone from the metastable one, one could streak seed all the clear drops. Drops that can support crystal growth, but do not give rise to it spontaneously are *metastable*. On the other hand, if the seeds dissolve, the drop is *undersaturated*. If you were to collect all this information you could now construct a *complete phase diagram*, showing all the different zones in Figure 1.

Discussion points:

Why determine the precipitation boundary and supersolubility limit?

1. Crystallization usually occurs close to these two zones. If you compare your first plot (the one made after 30 minutes) and again after 48 hours, you will clearly see that nucleation leading to crystal formation does in fact occur close to the precipitation boundary.

1. A supersolubility line can be determined with relatively little material and quite quickly. This is what Saradakis and Chayen call a "*working phase diagram*" (ref. 2). It is not a complete phase diagram, but it has enough information such that you can design useful experiments from it. You can use the information obtained for further screening setups or optimization. For more details on putting this experiment into practice, see references 3-5.

2. For a good explanation of WHY you want to do this, instead of carrying on with sparse matrix screening, see reference 6. To quote Hansen et al. (see reference below), **"…achieving optimal levels of supersaturation is more effective than broad sampling of chemical space."** If you are not getting results with your usual sparse matrix type screening, consider the method presented in this lab exercise as an alternative.

References

- Chayen, NE. 2005. Methods for separating nucleation and growth in protein crystallization. Prog Biophys Mol Biol 88, 329-337.
- Saridakis, E., Chayen, NE. 2003. Systematic improvement of protein crystals by determining the supersolubility curves of phase diagrams. Biophys J 84, 1218-1222.
- Shaw Stewart, PD, Khimasia, M. 1994. Predispensed gradient matrices - a new rapid method of finding crystallization conditions. Acta Cryst D50, 441-442.
- Douglas instruments application notes. Crystallization of a protein by microseeding after establishing its phase diagram. http://www.douglas.co.uk/rep1.htm
- Hansen, CL, Sommer, MO, Quake, SR. 2004. Systematic investigation of protein phase behavior with a microfluidic formulator. PNAS 101, 14431-14436.

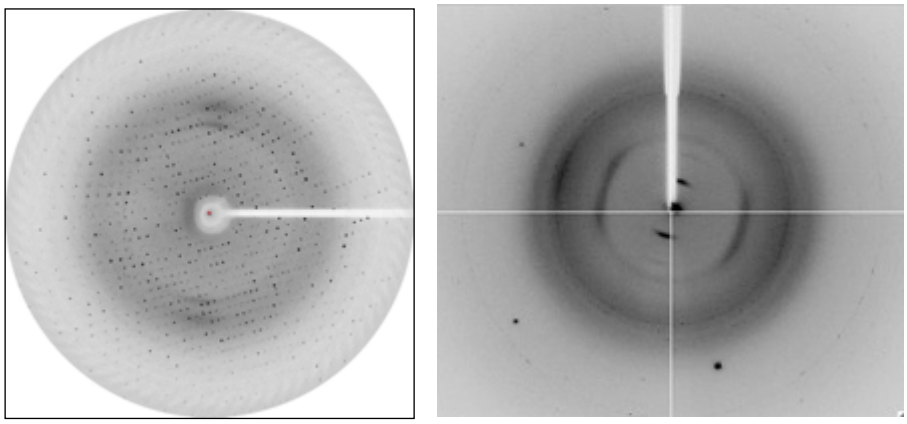
Wednesday
Exercise 6. Salt or protein crystal? Two simple tests

Background: *reprinted from: Bergfors, T. in Methods in Molecular Biology, vol. 363: Macromolecular Crystallography Protocols: Volume 1: Preparation and Crystallization of Macromolecules Edited by: S. Doublié © Humana Press Inc., Totowa, NJ.*

Many of the components commonly used for crystallizing protein can also give rise to salt crystals. Therefore no crystal should be celebrated until it is known if it is salt or protein.

Methods for testing if a crystal is salt or protein include:

1. X-ray diffraction This is the **definitive** method for determining if a crystal is salt or diffraction protein. If the crystal can be mounted, check the diffraction pattern on an in-house X-ray source. The answer will be immediately obvious because the diffraction spots for salt (at high resolu-
tion) are few and far apart, whereas they are many and close together for macromolecular crystals. Collect a 3–5°oscillation picture with the detector close to the crystal.



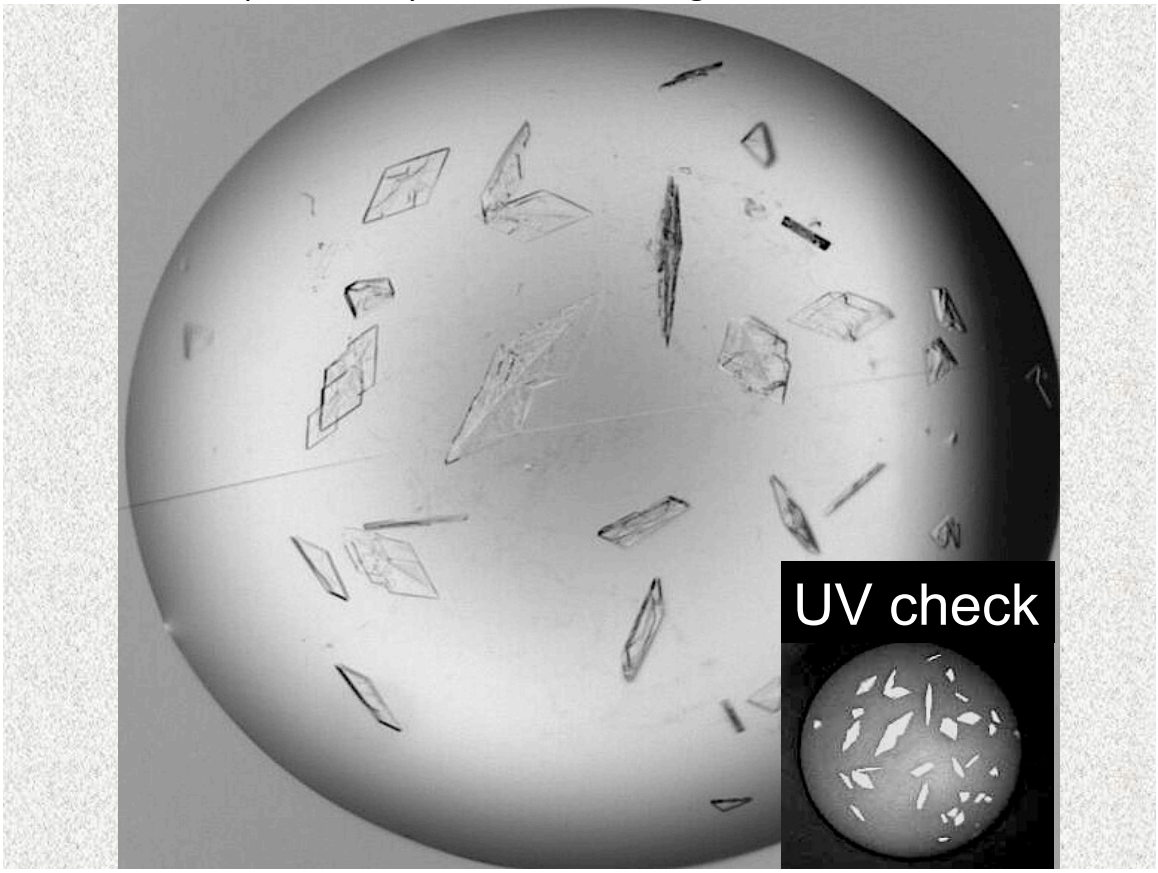
Left image: protein diffraction. Right image: salt crystal with ice rings.
(left image courtesy of C. Björkelid, Uppsala University)

2. Snap test With the drop at low magnification under the microscope, use an acupuncture needle or other sharp object to break the crystal. Salt crystals are extremely hard and can even be heard to snap upon breaking. Most protein crystals crumble and smash easily upon probing them.

3. IZIT dye from Hampton Research sells a proprietary blue dye, IZIT*, which binds to most proteins. The dye will concentrate in the crystals if they are protein and turn them blue, although there are reports of "false-negatives,"i.e., protein crystals that failed to turn blue. The IZIT dye itself can also crystallize, usually appearing as extremely long thin needles that form within minutes. Instructions for
using the dye accompany the product.

*Izit dye is 0.5% methylene blue.

4. Inherent UV fluorescence of tryptophans
This method requires that you have a UV imager.



Wednesday
Experiment 6: How to compare salt and protein crystals by the snap method and IZIT dye (methylene blue).

Purpose of this experiment: Often people look in their drops and wonder if the crystal they are seeing is protein or salt. The obvious test is to put it in the X-ray beam, but sometimes the crystals are too small to be mounted. Before optimizing to make larger crystals it can be useful to know if it is worth pursuing at all. One way is to snap the crystals, another way is to use a dye. Here we will use the "snap" method and the IZIT dye.

Materials required:

- Lysozyme crystals, prepared in advance.
- one microbatch Terasaki dish, filled with 5-8 ml paraffin oil
- a 50 ml Falcon tube with saturated NaCl (for the salt crystals)
- needles (acupuncture, sewing, or hypodermic needles) for the snap test
- IZIT dye from Hampton Research (= 0.05% methylene blue)

Today's experiment

- Fill a Terasaki plate with paraffin oil. Transfer a few NaCl crystals from the saturated solution to the plate.
- Take a needle and break a NaCl crystal, then one of the lysozyme crystals. NaCl will "snap". Salt crystals are very hard. Convince yourself of the difference in feel between smashing protein crystals and snapping salt crystals.
- Repeat with the lysozyme crystals. Notice the difference in "feel". Protein crystals are very soft, due to their high solvent content.
- **Instructions for testing the crystals for dye absorption:** (copied from Hampton's brochure)

"Place 1 microliter of Izit into the drop i.e. not the reservoir. (1 microliter IZIT per 10 microliter drop is sufficient, my comment). Protein, peptide, and nucleic acid crystals will absorb Izit within 1 to 4 hours, taking on a blue color. The blue color will intensify within the crystal over time, becoming a darker blue than the solvent. Precipitate and inorganic crystals will not absorb the Izit and will not become blue. If the blue background of the mother liquor is too dark, simply dilute Izit 1:10 or 1:100 with water and repeat."

Thursday
Exercise 7. Ligand soaking (eosin red) into pre-grown lysozyme crystals.

Materials required:

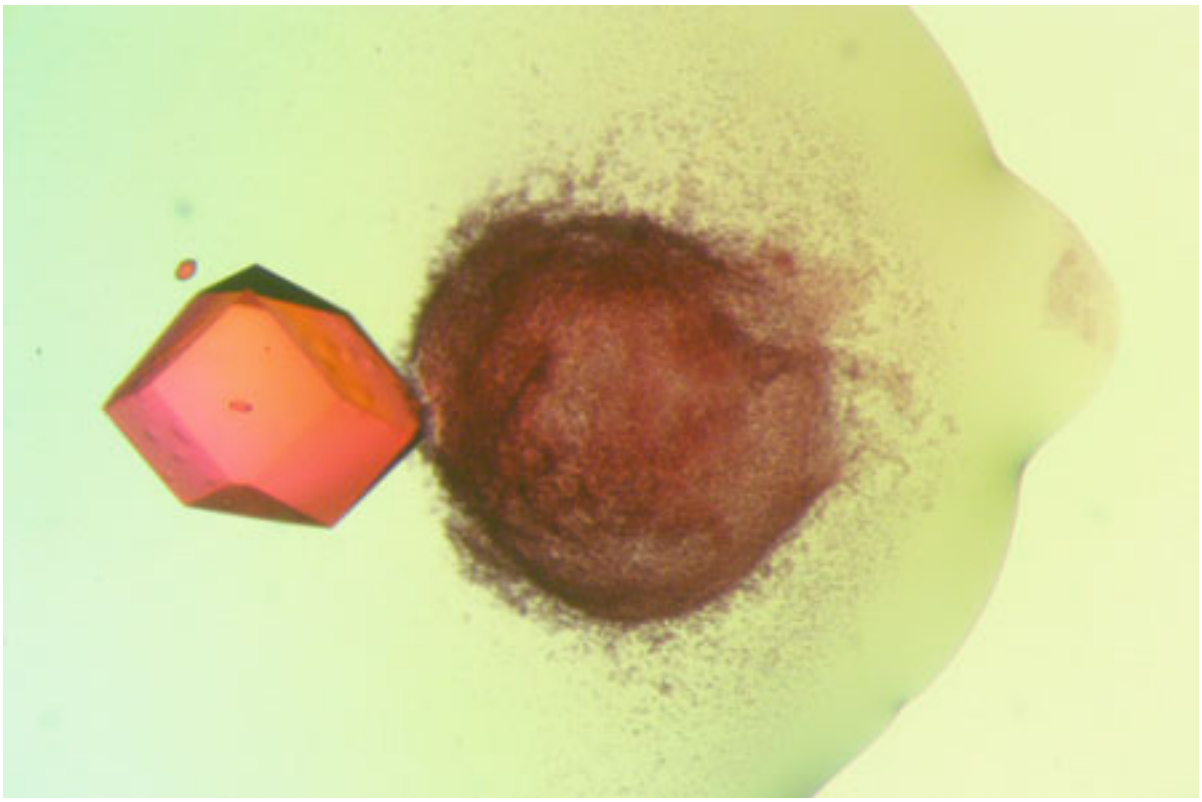
Dish with pre-grown lysozyme crystals.

Eosin Red, powder or a 1% solution

Procedure:

Carefully add the ligand to the drop. In the figure below, the eosin red precipitated heavily, but the dye has still penetrated the lysozyme crystal, turning it red.

Figure below. A crystal soaking experiment. This crystal of lysozyme, 0.3 x 0.3 x 0.3 mm, has been grown by vapor diffusion. The droplet volume (after equilibration against the reservoir solution) is approximately one-half of the initial volume of 4 microliters. A volume (0.2 microliters) of a red dye (1-10% Eosin scarlet) has been carefully pipetted into the droplet. Due to its limited solubility in the constituents of the droplet, much of the dye has precipitated, but some of it still appears to have diffused into the crystal. After about 4 h, the crystal had turned completely red.



Thursday

Exercise 8. CBH1 Ligand soaking by three methods

Materials and solutions

- Pipettes, down to 1 ul volume
- soaking solvent = precipitant solution with 20 % mPEG 5K, 50 mM Na-MES buffer pH 6.0, 10 mM cobalt chloride, and 12.5% glycerol (provided)
- crystallization drops with nice CBH1 crystals, your own or pregrown
- ligand powder(s) – to be decided
- ligand soak solution(s) – ligands to be decided, dissolved in soaking solvent
- small spatula
- acupuncture needles
- loops mounted on base, for picking up and transfer crystals
- magnetic wand

Choose crystals for soaking

1. Examine your CBH1 crystallization plate under the microscope. Note if/where there are crystals, or precipitate, or if the drops are clear.
2. Select some crystallization drops with nice-looking, single crystal(s) that may be used for data collection, among your own, or pregrown crystals provided by the teacher(s).

Method 1: Ligand powder soak

3. Take off the cap with the selected crystallization drop, turn upside down, and place in front of you.
4. Take as little as possible of ligand powder on the tip of a spatula (or similar), and place the powder at the edge of the drop, or near the edge of the drop. In the latter case, use a needle to make powder and drop come in contact.
5. Put back the screw cap above its well.

Method 2: Add ligand solution to crystals

6. Take 10 ul ligand soak solution in an eppendorf tube.
7. Take off cap with the crystallization drop to be soaked. Turn upside down and place in front of you.
8. Take 1 ul ligand soak solution and place near the edge of the crystallization drop, but not touching.
9. Use a needle to draw a line of liquid (i.e., a liquid bridge) between the two drops. The two drops will be connected and the ligand will gently diffuse into the drop with the crystals.
10. Put back the screw cap above its well. Tighten gently.

Method 3: Transfer crystals to ligand soak solution

11. Pipet 100-500 ul of soaking solvent as reservoir in two empty wells on a plate.
12. Place a 2 ul drop of ligand soak solution on new screw caps. Place on top of the wells with the soaking reservoirs. Do not tighten the cap at this stage.
13. Put a loop of suitable size on a magnetic wand.
14. Make sure that everything is ready for crystal picking and transfer. Take a deep breath, because the following steps need a steady hand and need to be done quickly.
15. Take one screw cap with crystals to be soaked and place it under the microscope.
16. Place one cap with ligand soaking drop close to the one with crystals.
17. Look through the microscope. Immerse the loop in the drop. Place the loop around one crystal. Drag the loop out, and hopefully the crystal follows.
18. Transfer the loop containing the crystal into the ligand soaking drop.
19. Return the caps above their respective wells.
20. Rinse the loop in a beaker of water. Let it dry in air.
21. Look at the ligand soaking drop under the microscope to see if you managed to transfer the crystal.
22. Repeat the procedure, using another crystal drop, and alternating between the soaking drops, to give them some time to equilibrate/recover above their reservoirs between exposures to air.
23. Take a quick look at all your soaks, before you put them away until tomorrow.

Thursday

Experiment 9. Cryocooling of crystals

Practice Session

Part 1: Read this safety warning first!

Liquid Nitrogen Demonstrations Safety Notes & Concerns

SOURCE: http://webs.wichita.edu/facsme/nitro/safe.htm

Liquid nitrogen is a dangerous material. The following is an excerpt from the Air Products Nitrogen Material Safety Data Sheet:

A back of the envelope calculation indicates that the entire contents of a 10 Liter dewar being spilled in a unventilated 274 square foot room with an 8 foot ceiling would reduce oxygen levels below the 19.5% level where Air Products recommends the use of a respirator. Since most classrooms are larger than this, suffocation does not represent a major danger. When transporting the liquid in a car, however, it is probably a good idea to open a window.

The possibility of freeze burns represents a much more serious danger and is therefore our first concern. This does not mean that the demonstration itself is dangerous, but it does mean you must be careful. Dangers include:

- Nitrogen can spatter (possibly in eyes) while being poured.
- Flying chunks of frozen objects could cause eye injury.
- Students might reach out and touch nitrogen or other cold objects. As mentioned above, contact with nitrogen can cause tissue damage, and this must be prevented.

Therefore specific safety precautions should include:

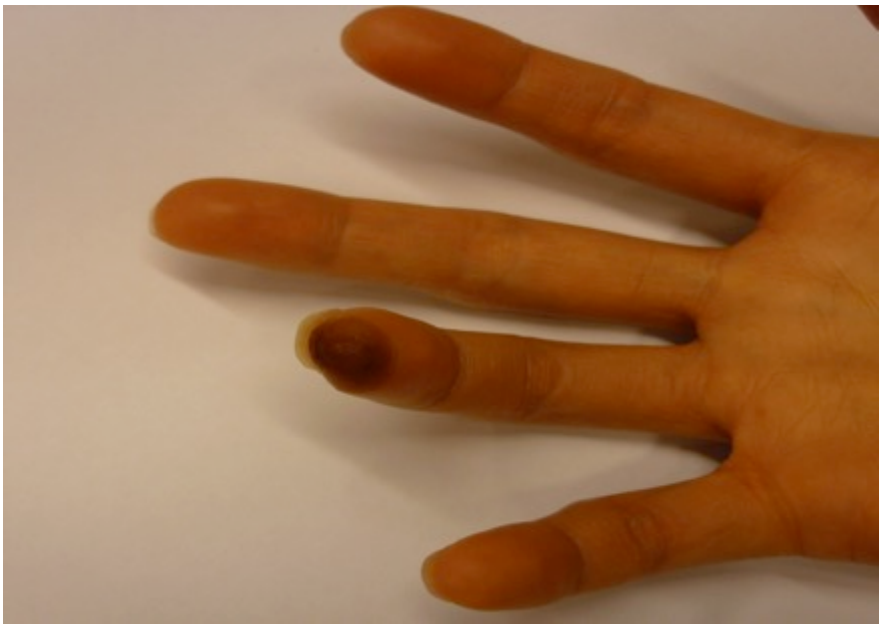
- Teachers must stress to their students the importance of not touching frozen objects or nitrogen.
- Wear goggles whenever pouring or dumping nitrogen. Nitrogen can spatter into the eyes, and potentially blinding pieces of frozen things can fly around when we drop it.
- Use a glove and / or tongs to handle any object going into or out of nitrogen and to carry the nitrogen dewar.

If cryogenic liquid or cold boil off contacts a worker's skin or eyes, frozen tissues should be flooded or soaked with tepid water (41-46C). DO NOT USE HOT WATER. Cryogenic burns which result in blistering or deeper tissue freezing should be seen promptly by a physician.

END OF COPIED MATERIAL FROM http://webs.wichita.edu/facsme/nitro/safe.htm

No warm water available? Put the frozen tissue (usually your fingers) under your armpit, skin-to-skin to warm it.

The figure below shows a frostbite accident from our own lab.



Part 2: Equipment required

a. Magnetic tongs
b. crystal caps, vials, and loops
c. foam dewar
d. liquid nitrogen
e. glycerol and mother liquor solutions
f. safety goggles when pouring the liquid nitrogen
g. cloth gloves covered with vinyl/latex gloves
h. microscopes
i. pipettes

Part 3: Method for cryocooling with pucks
Example for lysozyme crystals.

- Select the crystals you want to mount. What mother liquor did they grow from?
  If they grew in a cryoready solution, you can put them into LN2 directly.
- If the mother liquor is not cryoready, the crystal must be transferred to or soaked in a cryosolution. See example for lysozyme below.
- Preload the puck with empty vials and place in a foam container. Fill the foam container with liquid nitrogen. **See Fig 1a and b.** WEAR APPROPRIATE EYEWEAR.
- Select a loop size appropriate for the crystal size. **See Fig. 2.** Different loop types are available. **See Fig. 3.** Try different ones to find your favorite.
- PUT ON YOUR GLOVES.
- Pick up the magnetic cap containing the loop with the magnetic tongs.
- Loop the crystal from its original droplet **See Fig.4.** If a cryosolution is required, transfer the crystal to the cryodrop for 1 second.
- Put the crystal as quickly as possible into the nitrogen.
- Label/record which crystal is in which puck and the position.

Example for lysozyme cryosolution

- Make a 1 ml solution of 70% mother liquor:30% glycerol in an eppendorf tube. Tip: Warm the 100% glycerol in a heat block to make it easier to pipette.
- Put a 2-5 microliter droplet of the cryosolution next to your droplet containing the crystals. This is the “cryodrop”.
- Loop the crystal from its original droplet and transfer the crystal to the cryodrop for about 1 second.
- After the 1-second cryosoak in the cryodrop, put the crystal as quickly as possible into the pre-cooled vial in the liquid nitrogen.
- Alternative: you can also add the cryosolution (1 or 2 microliters) to the existing drop that contains the crystals.

Mounting the crystals in spine pucks

Fig 1a. Begin by filling the puck with empty vials. (No liquid nitrogen yet).

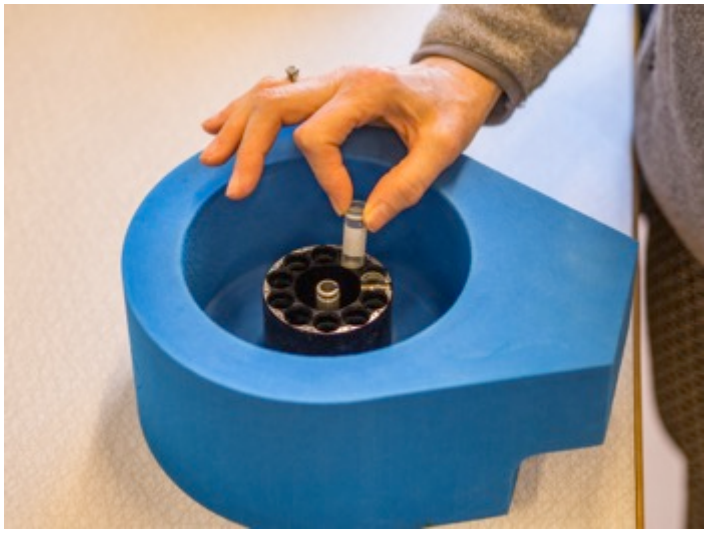
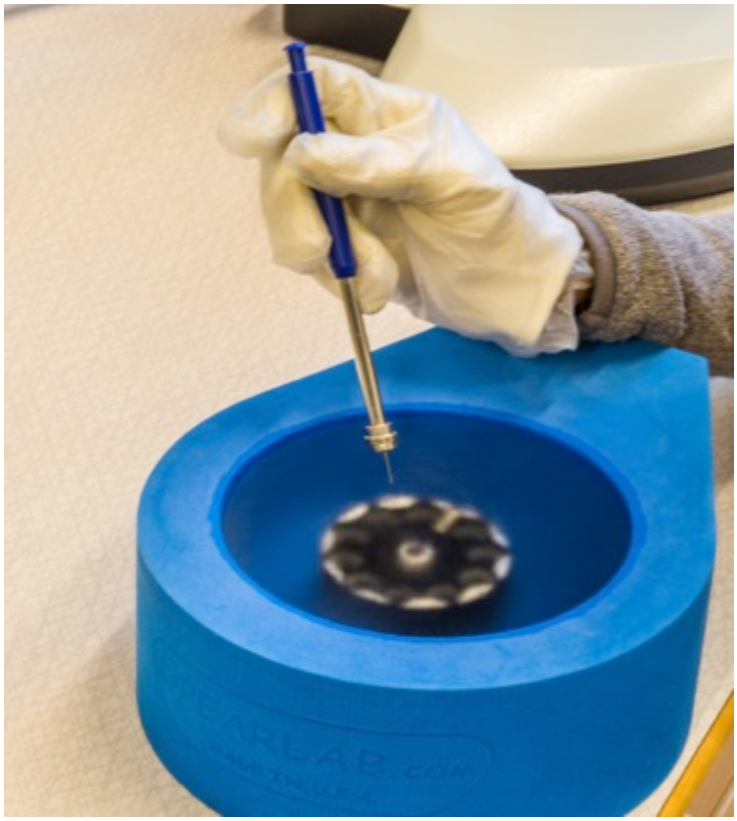


Fig 1b. Then fill the foam dewar with liquid nitrogen. Keep the nitrogen filled to the top at all times for better cryocooling.



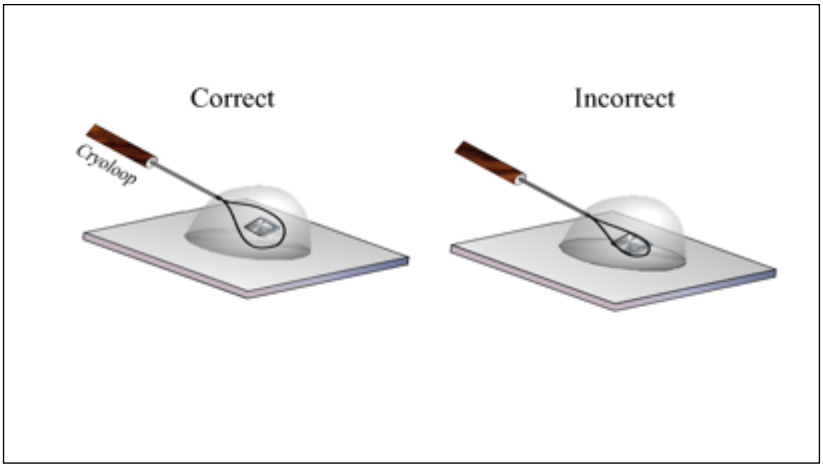


Figure 2: The cryoloop size should be appropriate for the crystal. Figure courtesy of Bergfors, T., Ed. "Protein Crystallization, 2nd Edition" 2009.

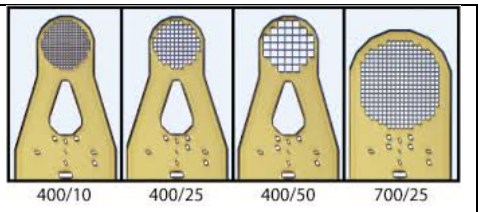
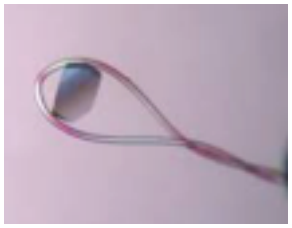
 **MiTeGen** **Dual thickness microloop**	 **MiTeGen** **mesh loops** for supersmall crystals (we do not have in this lab exercise).
 **Hampton nylon loop**	 **MiTeGen** **Dual thickness micromount**
 **Molecular Dimensions litholoop**	**and variants thereof.**

Figure 3. Types of cryoloops

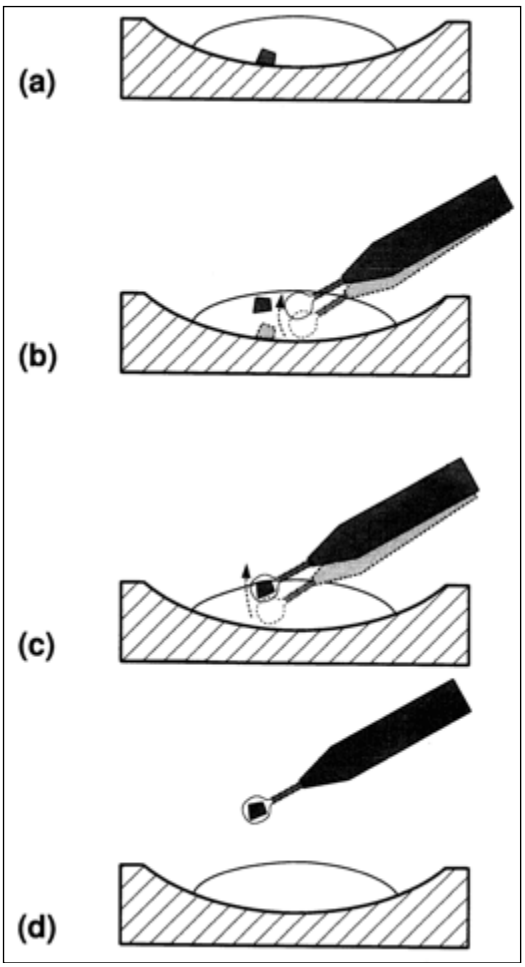
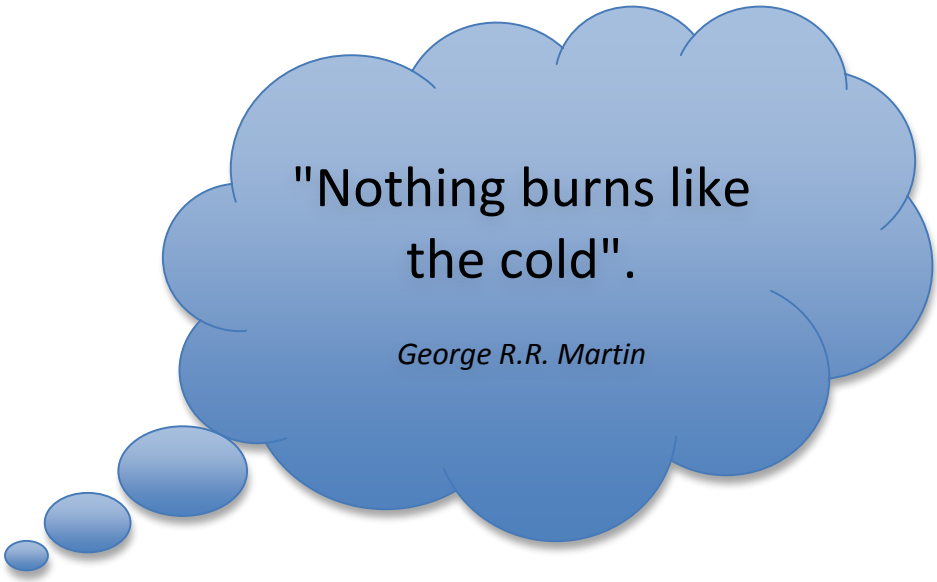


Figure 4: Looping the crystal out of the drop. Picture courtesy of Elspeth Garman, chapter 9 "Protein Crystallization" 2nd Edition, Edited by T. Bergfors, 2009.



Friday
Experiment 10. Cryocooling of crystals for transportation and data collection

Purpose of this experiment: Select, pick up, and cryocool crystals in liquid nitrogen, and arrange in dewar for transportation to the synchrotron for X-ray data collection. Each group will get one SPINE puck with room for 10 loops hanging in cryo vials, and shall cryocool and save at least two CBH1 and two Lysozyme crystals, and then try to fill the puck with further crystals, as time allows.

Materials

- Crystallization drops with nice, single crystals, your own or pregrown by teachers
- loops mounted on base, for picking up crystals
- cryovials for crystallization loops
- magnetic wand
- liquid nitrogen (LN_2)
- cryo pucks (SPINE pucks will be used this time)
- insulated container for keeping the sample puck immersed under LN_2
- dewar for transportation of the crystals to the synchrotron
- protection goggles (“skyddsglasögon”)
- gloves + mittens (to protect your hands from LN_2)

Preparation

1. Examine your crystallization plates and drops under the microscope, and evaluate each drop. Make good notes since this is the final documentation of your crystallization experiments.
2. Select some crystallization drops with nice looking, single crystal(s) that may be used for data collection, among your own and/or crystals provided by the teacher(s).

3. Fill one SPINE puck with cryovials and place on a puck support in a cryogenic foam dewar (or similar).
4. Before proceeding with liquid nitrogen, make sure that you are aware of all safety precautions and wear safety goggles and suitable gloves (and safe shoes in case you spill on your feet).
5. Carefully fill liquid nitrogen in the dewar to cover the puck. Top up when it has stopped boiling and settled somewhat. Keep it topped up with nitrogen, as discussed in the lecture. You want the dewar filled to the brim.
6. Collect and organize all stuff around the microscope that you will need for the cryocooling, i.e. crystal plates, magnetic wand, cryoloops, dewar with puck, and the list where you note which crystal goes from which plate to which puck position.

Cryocool crystals

7. Put on your gloves!
8. Pick up a loop of suitable size with the magnetic wand. Under the microscope you can compare the size of the loop with the crystal that you want to take.
9. CBH1 crystals are already cryoprotected (with 12.5% glycerol) and can be cryocooled directly, without ice crystal formation.
10. Take a deep breath, and be ready to work swiftly and with steady hands.
11. Place the crystal drop under the microscope. Loop up the crystal, transfer promptly into the liquid nitrogen, and place in a cryovial in the puck. Press the button on the wand, to release the cap with loop and leave in the cryovial. NB! Be careful to keep the loop with crystal under the surface once it is immersed in liquid nitrogen.
12. Put back the crystal drop immediately above its reservoir. Do not let the drop dry if it has crystals left that may be used.
13. Note in the list for the puck position what crystal is in the vial, i.e. from which plate and which position/droplet, and further details and observations regarding the crystal, e.g. size, shape, cracks, cocrystallized with ligand, ligand soak, etc. Form is on p. 30.
14. Pick and cryocool further crystals to try and fill your puck, as time allows.
15. NB! Lysozyme crystals must be soaked in cryoprotectant before crycooling, as described above, on p. 24.
16. Make sure that the transportation dewar is filled with liquid nitrogen, and that a puck holder is in place in the dewar and is cooled down and ready for use.
17. Transfer the puck to the puck holder and place in the dewar. NB! It is very important that the pucks are in the right order already when we pack the dewar here in Uppsala, so we can take them out in the right order when we shall collect X-ray diffraction data at the BioMAX beamline.
18. Check that the list of crystals in your puck is complete, and if not, add the missing information.

Puck System: label what's where

DEWAR_____

1 black TOP	1	6
	2	7
	3	8
	4	9
	5	10
2 black	1	6
	2	7
	3	8
	4	9
	5	10
3 black	1	6
	2	7
	3	8
	4	9
	5	10
4 black	1	6
	2	7
	3	8
	4	9
	5	10
5 black BOTTOM	1	6
	2	7
	3	8
	4	9
	5	10

Puck System: label what's where

DEWAR_____

1 red TOP	1	6
	2	7
	3	8
	4	9
	5	10
2 red	1	6
	2	7
	3	8
	4	9
	5	10
3 red	1	6
	2	7
	3	8
	4	9
	5	10
4 red	1	6
	2	7
	3	8
	4	9
	5	10
5 red BOTTOM	1	6
	2	7
	3	8
	4	9
	5	10

Summarize your plate results

Materials required:
Microscope
Your crystallization plates

Exercise:
Look at the drops and try to identify the different phenomena as:
Clear, precipitate, denatured precipitate **C, P, DP**
Crystals, crystalline, other **X, XN, O**
You can write your observations here:

Protein or plate number:
Method: Vapor-diffusion Hanging Sitting

A1	A2	A3	A4	A5	A6
B1	B2	B3	B4	B5	B6
C1	C2	C3	C4	C5	C6
D1	D2	D3	D4	D5	D6

Protein or plate number: **SCREEN:**

Method: Vapor-diffusion Hanging Sitting

A1	A2	A3	A4	A5	A6
B1	B2	B3	B4	B5	B6
C1	C2	C3	C4	C5	C6
D1	D2	D3	D4	D5	D6

Protein or plate number: **SCREEN:**

Method: Vapor-diffusion Hanging Sitting

A1	A2	A3	A4	A5	A6
B1	B2	B3	B4	B5	B6
C1	C2	C3	C4	C5	C6
D1	D2	D3	D4	D5	D6

Appendix 1: Reducing Agents in Crystallization Trials

When to use them:

— If your protein has free cysteines
— To prevent oxidation of Se-methionine-substituted proteins
— If you are not SURE your protein has free cysteines, add 1 mM beta-mercaptoethanol, the weakest of these reducing agents

How much to use?

1. Example: 1 mM protein solution (say, 20 kD protein at 20 mg/mL) with 6 free cysteines needs 6 mM DTT.
2. The large tetramer galactosidase required 70-140 mM BME in the crystallization conditions.

Which one to use?

You can check the DLS profile of the protein with the different reducing agents. It may be necessary to try different concentrations to find the optimum one.
All of these are more stable at 4 than 25 degrees C, but all of them will autooxidize eventually. Their ability to reduce existing S-S bonds is concentration dependent.

Reducing agent	#SH groups	How long does it last?	Comments
beta mercapto-ethanol	1	2-3 days	• Volatile and stinky (you can substitute MESNA, 2-mercaptoethanesulfonate, if you don't like the smell). • Add to the reservoir 1 microliter per 1 ml
DTT (or its isomer, DTE)	2	3-7 days; 20% oxidizes after 18 hr at pH 8-9	• Hard to renew in hanging drops, more useful in dialysis buttons. • Don't use on Ni columns, it reduces the Ni ions. • All metals degrade DTT, so include 1 mM EDTA. • Auto-oxidized DTT absorbs at 283 nm.
TCEP=tris 2-carboxyethyl phosphine	0	3-4 weeks	• Very powerful anti-oxidant • Cannot be used with phosphate • EDTA inactivates it • Can be used on Ni-IMAC columns • Will even cleave SH bridges, so don't use it if you have S-S bridges • It is very acidic; adjust the pH of TCEP to 7.0 (or buy it titrated from Pierce).

Appendix 2: CBH1 information

Why CBH1?

- We have ample amounts of protein.
- We can obtain crystals in days, i.e. within the time frame of the course.
- Crystals can be grown in cryoprotected conditions and can thus be directly cryocooled in liquid nitrogen.
- CBH1 crystals often give strong diffraction to high resolution.
- It is a more "real life" example than lysozyme, providing additional aspects:
- Optimized conditions are needed to get good crystals. It is easily seen how the crystal formation is affected by changes in ionic strength, buffer, pH, protein concentration, protein batch, precipitant concentration, metal ion, etc.
- Crystals may be big and beatiful, and yet give crappy diffraction, well illustrating the axiom that "Beauty is only skin deep!". Therefore you may have to screen several crystals until finding a good one.
- It has post-transcriptional modifications to look for in the solved structure.
- The N-terminal glutamine residue is cyclized to pyroglutamate (PCA).
- All cysteines should form disulfide bridges.
- The protein is glycosylated, and you may see sugar attached to the protein at one or two of the three N-glycosylation sites (Asn-Xxx-Ser/Thr).
- A divalent metal ion is required for crystallization (I222 form). Co^{2+} seems to be best. Ni^{2+}, Ca^{2+}, Mn^{2+} work too, but give less nice crystals.
- One metal ion sits on a 2-fold symmetry axis, which is of interest because it requires special treatment in structure refinement.
- The active site is easily seen in the structure, in the form of a cellulose binding tunnel that runs along the whole catalytic domain.
- Various ligands can bind at the active site.
- We have catalytic mutants and can thus soak in natural substrates.

Quick facts

Fullength CBH1: 497 aa, ~56 kDa, pI ~3.7; Catalytic domain (CD): 439 aa, ~47 kDa
Usual space group I222; unit cell 82-84 x 82-84 x 109-111 Å ($\alpha=\beta=\gamma=90°$)
Glycoside hydrolase family 7 (GH7). Retaining. Hydrolyzes β-1,4-glycosidic bonds with retention of anomeric configuration ($\beta \rightarrow \beta$), via covalent glycosyl-enzyme intermediate. Catalytic nucleophile Glu212; catalytic acid/base Glu217.

Background

CBH1 stands for Cellobiohydrolase 1. It is also known as CBH I, and Cel7A because it belongs to glycoside hydrolase family 7 (GH7) in the CAZY database (Carbohydrate Active enZYmes). It is the major cellulose-degrading enzyme produced by the ascomycete fungus *Trichoderma reesei* (also known as *Hypocrea jecorina*). CBH1 consitutes nearly half of the total amount of secreted protein when the fungus is grown on cellulose as carbon source. The native CBH1 enzyme is a single polypeptide chain of 497 amino acid residues. It consist of two independently folded modules: an N-terminal catalytic domain (CD) of ~430 residues, and a C-terminal cellulose binding module (CBM1) of ~35 residues, connected by an extended, unfolded and flexible linker peptide of ~30 residues. The structure of the CBM has been determined by

NMR (Kraulis *et al*, 1989, Biochemistry 28:7241-57). No one has yet managed to crystallize the complete fulllength CBH1 enzyme with both modules. However, by using the protease papain it is possible to cleave off the linker-CBM tail, and to crystallize the isolated catalytic domain (residues 1-439). The first structure was published in 1994 (Divne et al, 1994, Science 265:524–8). Today there are over 25 structure entries in the PDB, including several mutants and various ligand complexes. Most of them are from crystals of space group I222 with unit cell dimensions around 83 x 84 x 110 Å (α=β=γ= 90°).

Heterologous expression of CBH1 in *E. coli* has notoriously failed to produce either soluble or active enzyme. In yeast, expression levels are low, and the properties of expressed Cel7s have been unpredictable, probably as a result of both the lack of N-terminal glutamine cyclization of Cel7A expressed in yeast and the significant variation in protein glycosylation (Dana *et al*, 2014, Biotechnol Bioeng 111:842–847). Most CBH1 mutants have been made by homologous expression in *T. reesei*. Catalytically deficient mutants of CBH1, made by isosteric mutation of the catalytic nucleophile Glu212 or the catalytic acid/base Glu217 to glutamine, have been instrumental for the structure-function studies (Ståhlberg *et al*, 1996, J Mol Biol 264:337-349). The E212Q and E217Q mutants have enabled the determination of ligand complex structures with natural substrates bound at the active site without being hydrolyzed (Divne *et al*, 1998, J Mol Biol 275:309-325; Knott *et al*, 2014, J Am Chem Soc 136:321-329).

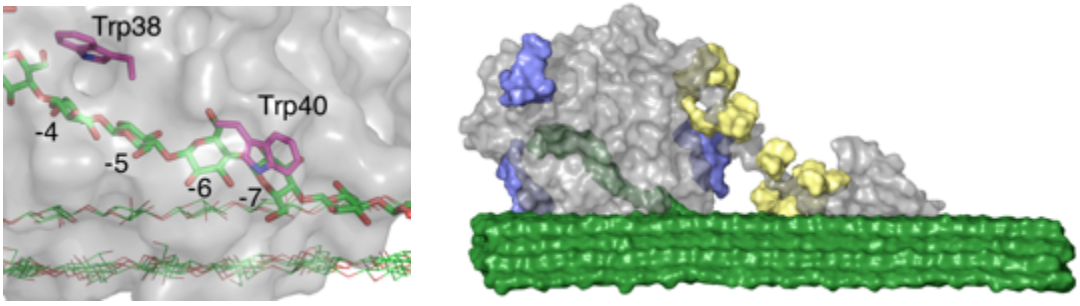


Figure 5: ***Trichoderma reesei*** **CBH 1 is a processive cellulase.** *Left:* A cellulose chain is threaded into the substrate binding tunnel. Tryptophan side chains form hydrophobic sugar binding platforms, Trp40 at subsite -7 and Trp38 at subsite -4. The cleavage site between subsites -1 and +1 is outside the view of this image. *Right:* Snapshot from an MD simulation of glycosylated, fullength CBH1, with linker and CBM, binding to a cellulose chain on the surface of a plant cellulose I microfibril. The protein and cellulose are rendered as surface models and the protein is partly transparent to show the cellulose chain bound in the active site tunnel. Amino acid residues are grey, N-linked sugars are blue, O-linked sugars are yellow, and the cellulose is green. From: Payne et al, 2013, PNAS 110:14646-51.

UPPSALA
UNIVERSITET

Uppsala University

Uppsala is Sweden's fourth largest city and one of its oldest. Ever since the Viking Age it has been an important cultural centre. Uppsala is a rapidly growing city and is becoming increasingly integrated with the wider Stockholm region, Sweden's most dynamic growth region. At the same time, Uppsala has kept many of its small-town characteristics. Offerings are myriad, but everything is close by – often within convenient cycling distance. The compact city centre with the cathedral, the River Fyris, and several small squares, parks, cafés, restaurants, and historic buildings lend the city its character.

Did you know?

Uppsala University has several museums. Large collections of art, science history and cultural history have been tended in Uppsala since the Middle Ages.

Uppsala has a rich musical scene on offer to both audience and musicians.

The Royal Academic Orchestra was founded in 1627.

The Botanical Garden is home to thousands of plants from the Scandinavian mountains to dry deserts and drenched rainforests.

The Silver Bible was donated to Uppsala University in 1669.

SWEDISH UNIVERSITY OF AGRICULTURAL SCIENCES

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Our university produces world-class research in several fields. Our degree programmes lead to important jobs, and our knowledge is sought-after by industry and society as a whole. We act locally and globally for a sustainable, thriving and better world.

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- SLU was founded in 1977, and celebrated 40 years as a university last year.
- SLU is the only Swedish university that trains foresters, landscape architects, veterinarians and agronomists.
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