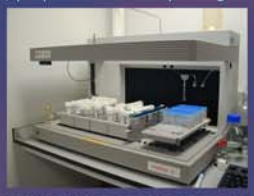
Protein crystallisation at York.

Practical developments and personal stories,

by Shirley Roberts on behalf of the group.

Implementation of Robotics

1) preparation and dispensing of screens



Tecan Miniprep
(Tecan UK Ltd)

stocks (york and commercial) in sterilin pots with customised lids

Screens mixed by Tecan :-

- screen 1 based on Jancarik and Kim
- screen 2 (J. Appl. Cryst. (1991) 24, 409-411)
- Clear Strategy 1 and 2
- Peg Ion
- Stura footprint 1

The Index, Salt Rx and Pact commercial screens are purchased in bulk from Hampton Research and Molecular Dimensions Ltd and dispensed by the Tecan.

2) transfer of screen solutions

This is done manually using a multichannel pipette, the labtech robot is in trial but it requires customised expensive tips, the search is on for economic tips that work well.

3) setup of crystallisation trials - sitting or hanging drop trays

Mosquito
(TTP LabTech Limited, Cambridge UK)

Greiner 3-well crystal quick and single well low profile plates are routinely used for sitting drops 100-400nl final drop size. Hanging drops are dispensed onto adhesive sheets supplied by Molecular Dimensions Ltd and TTP labTech Ltd. These sheets (with 96 circles containing no adhesive) fit onto standard 96 well tissue culture plates.

4) image capture of crystallisation trials

BioStore robot
(BioTOM, Evry, France)

This is a recent acquisition not in general usage yet due to various problems. The long term plan is to interface BioStore with the crystal recognition software being developed by Julie Wilson and colleagues in YSBL. For now, manual examination of trays is necessary. In the future, detection of crystals and automatic alerts to owners of trays should become a reality!

Changes in practice brought about due to robotics:

A 96 well screen can be set up with 18µl protein solution in minutes compared to 60µl protein solution in 1 hour, using a tenth of the screen solution volume. The tendancy now is to set up many more screens. This has prompted a revue to rationalise those on offer as many solutions are very similiar. Consumable cost is comparable:- 4 linbro 24-well plates, cover slips and grease or sitting drop equivalent - £8 pounds per 96 well screen, 96 well microplate set up with the mosquito - £12 pounds. The robots however do need someone to spend a significant time on them, training, programming, maintenance and ordering consumables, especially if they arrive in a budget "develop on site" deal!

The quicker equilibration rates obtained with the micro set up seems to result in more salt crystal growth. Scaling up to the larger 24-well format can be troublesome. In many cases optimisation is best done using the greiner low profile plates either using the mosquito or by hand. Larger drops can be set up in these plates and retrieving crystals for data collection is relatively straightforward.

Shirley Roberts

Some facts and figures

In YSBL a group of researches are part of the SPINE (structural proteomics in Europe) programme. They are determining protein structure by X-ray crystallography from the human pathogenic bacteria *Bacillus anthracis*, *Campylobacter jejuni* and *Mycobacterium tuberculosis*. The following statistics are from an ongoing project started in January 2003.

Target Status

Progression	Totals
Selected	219
Cloned	172
Expressed	142
Soluble	102
Crystallised	50
data sets	25 (+3NMR*)
structures solved	19 (+2 NMR*)
in PDB	12

* In conjunction with partner 5 Bijovet Center for Biomolecular Research (Utrecht)

Mark Fogg

Personal crystallisation stories

Leishmania major dUTPase - dUPNP complex

Purification and Crystallisation

Three- step purification using AS cut, ion-exchange and hydrophobic chromatography resulted in low yield, not pure enough protein and very poor crystals.

Re-cloned into YSBLic plasmid (Mark Fogg) which added a N-terminus 6xhis-tag. Initial crystals were obtained using the mosquito and the index screen. Final crystals were obtained in 20% PEG 3350, 0.2M MgCl2, Bis-tris pH5.5 after several rounds of optimisation by hand, mainly seeding.

Seeding

Crystal structure

Data collected to 2.3Å at ESRF, space group P_i522. This is the third dimeric dUTPase to be determined in York, all possess the similar all α-fold in contrast to human enzymes (α and β). This makes them a suitable as drug targets.

Olga Moroz

Favourite additives with proteins crystallised from *Bacillus anthracis*

4mM TCEP

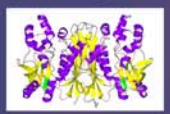
alanine dehydrogenase TCEP additive improved diffraction of crystals from 8Å to 3Å, refinement of data is in progress.

10% glycerol

Thymidylate synthase, glycerol in the well buffer improved diffraction of the crystals to 3Å enabling data to be collected.

100mM TMAO

phosphoribosyl formaylglycinamidine cycla-ligase TMAO additive improved crystal diffraction from 8Å to 2.4Å and enabled data to be collected and the structure to be solved.



Elena Blagova, Olga Moroz and Mark Fogg

GP6: A protein that prefers the right!

Introduction

- GP6 is a bacteriophage protein.
- *In vivo* the protein forms rings of 11, 12, 13 and 14 subunits.
- DNA is transported through the central channel into the viral capsid.

Protein purification

- Size exclusion chromatography is the final step in the purification.
- crystals formed when all fractions were pooled were small and did not diffract (picture 1).
- when the peak fraction and those that eluted after were pooled the resulting crystals diffracted to 3.2Å (picture 2).

Conclusions

- for GP6, better diffracting crystals are produced when protein from the peak fraction and those to the right is used.
- this may be because the molecules in these fractions are more tightly packed.

Better crystals!

Jo Turner, Pip Seavers and Fred Antson

Seeding and tags in the crystallisation of lichenase 26A and its complex.

- When the native protein structure was solved the his tag of one molecule was in the active site of a neighbouring molecule so no ligand complexes could be studied. Only poorly diffracting crystals (7Å) of untagged protein were obtained that could not be improved (figure 1a and 1b).

- One poor crystal of the nucleophile mutant was obtained and, after removal for testing, many small crystals subsequently formed in the drop (figure 2). Crushing these crystals with a hypodermic needle and seeding into several protein drops without reloading the needle resulted in larger crystals (figure 3). Several rounds of seeding were required, crystals of protein-ligand complexes were obtained in the final round by inclusion of ligand in the protein solution.

Seeding

crystalline precipitate formed initially with crystals forming over several days

- Seeds from the nucleophile mutant were used to seed the wild type protein and again sequential seeding and cocrystallisation resulted in the formation of diffraction quality crystals. The best crystals were obtained in the same conditions as the nucleophile mutant (figures 4a, b, and c).

Victoria Money

And finally, two unusual crystallisation stories!

- ThiI contains a N-terminal THUMP domain, an RNA binding domain for which no structure is available in the PDB.

- The protein requires high salt concentrations to remain soluble (1M NaCl). Initial crystals from screening (figure 1), were not reproducible. It was decided to try substituting chloride for phosphate, a more effective ion in the Hoffmeister series.
- The protein was dialysed into 150mM potassium phosphate. A simple peg screen resulted in crystals in very low PEG conc. Meanwhile protein solution left in the fridge in 100mM phosphate was found to contain crystals!

- The large crystal in figure 2 grew from a hanging drop containing 10mg/ml protein in 150mM phosphate sealed over a reservoir of 50mM potassium phosphate, the drop grew in size as the salt concentration decreased and the crystal grew with it.
- Cryo-protection was achieved by diluting the protein with glycerol before setup and then transfering to 30% glycerol plus additives before flash-cooling.
- Native data to 2.5Å have been collected and the structure solved using selenomethionine prepared protein. The structure is currently being refined.

David Waterman and Fred Antson

The *Bacillus anthracis* chaperone PrsAB is thought to be important in folding the anthrax protective antigen molecule. No crystals were obtained during screening apart from in one drop where mother liquor was absent from the well. These crystals have formed during direct evaporation of the protein in buffer, they are not salt but do not show any diffraction at this size unfortunately!

Mark Boyle

A few recent crystallisation-related thoughts from those setting up the trays.

- If your protein is stored frozen in aliquots, warm up quickly, eg in your hand before setup rather than leaving on ice
- His-tag proteins often don't need a final "polishing" gel-filtration step. Some react badly to the concentrating required. Ion-exchange chromatography may be a better choice if further purification in necessary. Sometimes getting proteins screened quickly is more important than further purification. Removal of salt can be achieved by a second pass down the Ni column.
- Some screens (eg natrix) can be rearganised, for example into pH blocks, giving a rational template and trends that can be more easily identified.
- Some proteins require a lower concentration than the popular 10mg/ml for screening.
- Seeding is always worth a try in optimisation protocols.

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