Recombinant Expression, Protein Purification, and Crystallization of *Thermococcus Thioreducens STR. OGL-20*

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Introduction

One of the leading methods to gain protein structural information is through protein crystallization. However, getting the protein to crystallize (and a crystal that will diffract X-ray) tends to be very challenging and thus far has limited the rate at which structures can be solved through this technique. Pursuing the crystallization of archaeal proteins for protein structural information has benefits over proteins from other domains of life. For one, the high stability of thermophilic proteins leads to a longer shelf life which extends the viability of obtaining a biological relevant structure from a protein crystal that may take weeks to months to form. Its high stability has proven useful to test new approaches to crystallization techniques and for teaching crystallization techniques to students (because it is less sensitive to less than perfect handling).The goal of this project was to purify and crystallize proteins from *Thermococcus thioreducens* for the purpose of obtaining structural information via X-ray crystallography. As a side-product of this exercise, the feasibility of creating capillary counter-diffusion screens with cheap plastic tubing and wax (rather than expensive glass setups) was also tested.

Procedure

Background

Previous to this summer, the entire genome of *Thermococcus thioreducens* was sequenced and the genome was probed by identifying potential start codons. The open reading frames processed through bioinformatic programs like BLAST to identify any homologous known proteins. Another program, Expasy Proparam, was used to gather information such as theoretical molecular weight, pI, and UV extinction coefficient. Then selected open reading frames were cloned into a pCR-T7-TOPO expression vector and transformed *Rosetta E. coli.*

Recombinant Expression

This summer, glycerol stocks of successfully transformed *Rosetta E. coli* were used to create an overnight starter culture in selective media. The plasmid of interest also coded for chloramphenicol and carbenicillin resistance. Therefore, *E. coli* that lost the lost the plasmid was selected against. The starter culture was then used to inoculate a larger scale growth (4 – 8 mL). When the OD_{600} reached 0.60, overexpression of recombinant protein was induced by addition of IPTG (lactose antagonist).

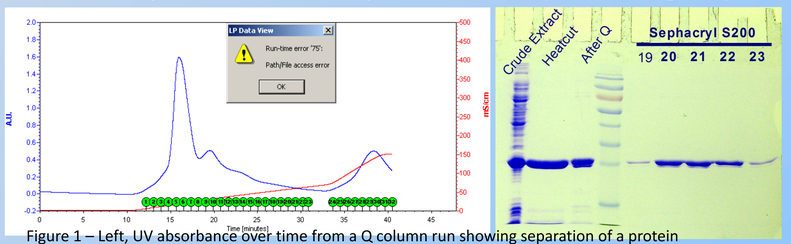

Figure 1 – Left, UV absorbance over time from a Q column run showing separation of a protein peak (left) verses a DNA peak (right). Right, SDS-page results from protein # 81.

Purification

Later, the crude cellular extract was harvested by sonication of the recombinant cells. A heat cut step was applied (30mins @ 75˚C) that denatured non-thermostable proteins and centrifugation separated non-thermostable protein out of solution. The heat cut step was vital to purification because it removed most of the proteins other than the hyperthermophile protein of interest. Then the typical purification scheme involved an ion-exchange column (High Q or S depending on the pI of the protein) followed by size-exclusion column. The purity of the protein from other proteins was analyzed by SDS-page. The protein to nucleic acid ratio and protein concentration was analyzed through UV spectral analysis.

Trace Fluorescent Labeling

If the protein was sufficiently pure and existed in adequate milligrams then it was used in crystallization trials. Before setup of crystallization trails, the proteins were fluorescently labeled for ease of identification of protein congregation later. The most popular fluorescent label was carboxy rhodamine G6 (absorbs light at 525 nm and emits at 547nm. To perform the labeling, a portion of the protein was transferred to buffer 50 mM Borate pH 8.75 via desalting columns and the carboxy rhodamine G6 dye was added and allowed to react for 30 mins. After 30 mins, the labeled protein was transferred back to the original buffer and was added back to the original protein solution. The result of this was a trace labeling which made it possible to distinguish congregation of protein verses protein dispersed in solution and also protein crystals verses salt crystals.

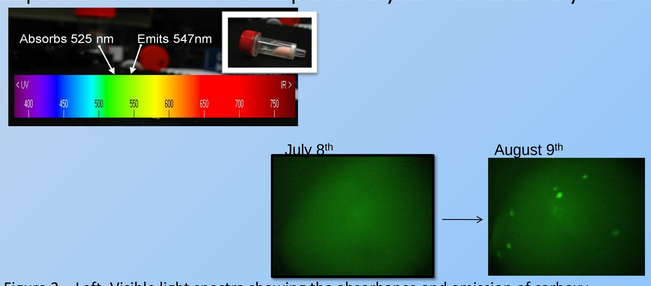

Figure 2 – Left, Visible light spectra showing the absorbance and emission of carboxy rhodamine. Center and Right, the trace labeled protein solution appears evenly green. As protein crystals form, the intensity of returned light increases.

Crystallization: Vapor Diffusion

The approach to crystallizing a new protein was to test broad scale crystallization conditions using vapor diffusion techniques and then follow up with optimization conditions tested with capillary counter-diffusion. The conditions for the broad vapor diffusion screen consisted of a 96 well screen using hit conditions provided by Hampton Research *Crystallization Screen HT*. The general screen of 96 condition was further expanded to 288 by testing 3 different ratios of protein solution to precipitant solution per condition.

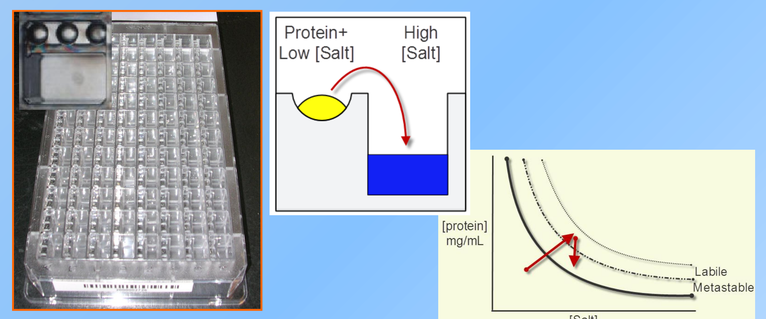

Figure 3 – Left, vapor crystallization plate showing the 3 sitting drop wells for further differentiation. Center, Water diffuses from low salt solution to high salt solution. Right, phase solubility diagram.

Acknowledgements

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Crystallization: Capillary Counter-Diffusion

The vapor diffusion screen resultswere used to make inferences on optimization conditions. The hit condition was modified with altered ratios of salt to polymerconcentrations. The optimized precipitant conditions mixed with 1% agar was loaded into 96 labeled tubes. Then plastic tubing was loaded with 30mg/mL concentration of labeled protein solution. Small 2 cm straws of protein loaded tubing could be cut off with a simple pair of scissors and one end was sealed with wax. The open end of straw was inserted into the precipitant condition to allow counter-diffusion to begin. The capillary counter-diffusion setup creates a restricted geometry which inhibits the disturbance of growing crystals by convection of surrounding solution. Also, because the counter-diffusion is a dynamic between high protein concentration verses high salt concentration, a large area of the phase diagram possibilities can be tested within one setup.

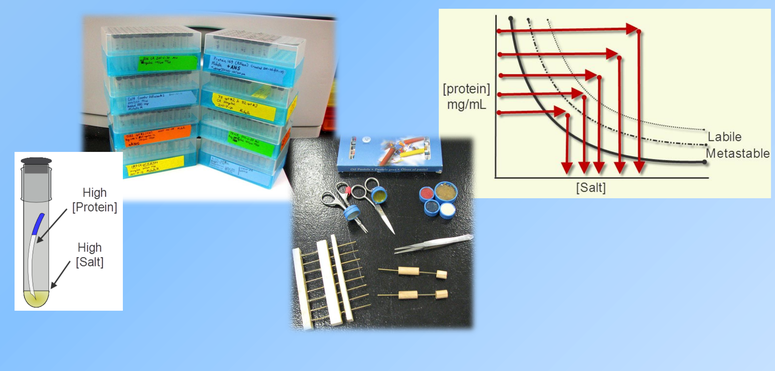

Figure 4 – Left to right, illustration of a capillary counter-diffusion setup, boxes of optimization experiments I set up, tools that were experimented with for loading capillaries, phase solubility diagram showing the larger range of possibilities tested.

Conclusions and Ideas for Future Work

This method has often produced a very quick way of obtaining proteins sufficient for use in crystal screens. The trace fluorescent labeling of proteins made protein crystals (developed by Marc Pusey) easier to spot and false positives (salt crystals) less likely. And now, the transition of using plastic (rather than glass; also developed by Marc Pusey) for capillary counter-diffusion has made the setup costs cheaper. During the course of the summer, tools for aiding the setup of plastic capillary counter-diffusion experiments were also tested and the time required to create an experiment went from a day and a half to only 4 hours. Of the optimization experiments I helped to set up, promising crystals have developed from two proteins (phosphoglycolate phosphate and protein #82).

In the future, I would like to attempt to repeat the crystal growth of phosphoglycolate phosphate and protein #82 in order to increase the odds of acquiring well X-ray diffracting crystal. Also, in was noticed that in some of the counter-diffusion setups, heavy precipitant formed in the precipitant rather that the straw of protein. This suggests that possibly the protein was diffusing faster than desirable and was “leaking” into the well of salt solution. A small scale test to rectify this was made by adding 1% agar to the protein solution as well as the precipitant solution. It is theorized that adding the agar to both the protein and salt will slow the rate of diffusion down and also inhibit disturbance of forming crystals. The small scale test of adding agar to both the protein and precipitant solution produced the largest crystals of 82 thus far.