Molecular Biology
Lab 5
February 23rd , 2010

Purification of pGLO **protein** from *E.coli* transformants.

Today we will branch out again from our pGLO transformation exercise to use the *E.coli* transformants as a source, not of pGLO plasmid DNA, but of the Green Flourescent Protein itself. As we learned from our first experiment, the GFP gene is *inducibe*, that is the mRNA and protein are only produced if a sugar arabinose, is present in the media. Therefore, any *E.coli* which have acquired the pGLO plasmid should be able to produce the GFP protein when given arabinose as a nutrient.

We observed the expression of GFP protein in our first transformation exercise by shining UV light on colonies of transformed *E.coli*. Often, as scientists we are interested in recovering "bioengineered" proteins and purifiying them for research or medical use. For example, human insulin is mass produced in this way for treatment of diabetes. The insulin *gene* was incorporated into a plasmid, by a process called recombinant DNA technology or DNA cloning. Bacteria were transformed with the plasmid and allowed to produce the protein.

Protein purification simply means separation of a given protein from all/most other molecules in a cell. This process requires knowing something about the protein and designing a separation apparatus based on a unique quality of the protein. We will separate GFP from other *E.coli* proteins based on its high proportion of hydrophobic amino acids. We use a process called **affinity chromatography** to "pull" GFP out of a mixture of many proteins.

In any type of chromatography, proteins are run through a vertical column containing some type of matrix (porous substance). Some protein purification is based on size of proteins (**size-exclusion chromatography**). Large proteins cannot make their way through a certain size matrix and pass instead around it coming out of the column quickly, whereas smaller proteins are retained longer in the matrix and elute later.

Today's procedure uses a different type of separation procedure called **affinity chromatography**. This process allows separation of a protein based on its ability to bind to something. GFP is a very hydrophobic protein, having an abundance of hydrophobic amino acids. Hydrophobic amino acids will naturally interact with other hydrophobic molecules in an attempt to avoid interacting with water. This plays a large part of determining the folded structure of GFP. The chromatography columns we use today are lined with beads coated with hydrophobic molecules. We will thus attempt to separate GFP from all the other bacterial proteins by its attraction to these hydrophobic beads. A series of binding and wash steps will be used to "stick" GFP to the column and then release it after removal of other proteins.

The basic steps of today's exercise include:

1. Growing a culture of pGLO transformed *E.coli* and inducing the production of many GFP molecules by including arabinose in the media.

2. Concentrating the bacteria using centrifugation

3. Lysing the bacteria (breaking them open) and allowing the proteins of the *E.coli* to be released into solution.

4. Separating GFP from other proteins using Hydrophobic Interaction (affinity) Chomatography.

The advantage of purifying a protein like GFP is the ability to follow its location by shining UV light on it. In most cases protein purification requires further analysis (such as testing the enzymes activity on its specific substrate) to be sure one has a pure sample of the desired protein.
*** Be sure you understand where the protein is in each step of the procedure**.

Molecular Biology

Lab 6 Green Fluorescent *Protein* Purification

Procedure

***Several procedural steps have accompanying questions. Try to answer as you go along. You will be given a UV light to track GFP. Try to predict where GFP will be in each of the steps. Confirm by using the UV light.*

Day 1

(2 p.,m or later)

1. Prepare two 5-ml cultures of bacteria as directed for the lab 2. LB-A-A broth will be made for you. Use colonies from your original plates from Lab 1. Use sterile technique. Dr. Super will place cultures in the incubator late in the afternoon.

Day 2

Preparing the bacterial lysate
(Source of GFP)

1. Obtain a tube of fresh culture of *E.coli* (pGLO transformants). Review how this was prepared. What media was used? What was added to the media to *select* for transformants? What was added to the media to *induce the expression* of GFP?

2. Centrifuge the sample @ 2000 RPM for 15 minutes in Dr. Super's tabletop centrifuge in CM217. Balance the tubes!!

3.What is in the supernatant? What is in the pellet? Check this using your UV light.

4. Carefully decant (pour off) the supernatant and blot on paper towel to remove most of the media. (paper towel becomes biohazard)

5. Loosen the pellet by tapping and flicking the tube. Add 250 μl of Tris-EDTA (TE) to each tube and carefully pipette up and down to resuspend the bacteria evenly. Tris is a buffer to maintain a range of pH in which proteins remain folded in their active form. EDTA is a chelator of divalent cations? What is EDTA for? Transfer the bacteria sample to a microfuge tube.

6. Add 25 μl of lysozyme to each tube. Lysozyme is an enzyme we secrete in tears, and mucous. It is a natural antibacterial agent and acts by lysing bacterial cell walls. Why are we using it here?

7. Place your tubes in the -80 C freezer for 20 minutes. Freezing and thawing of cells of any type is very hard on cell membranes. Why are we freezing our sample?

8. After 20 minutes, remove your sample from the freezer and thaw it in your hands. Check for lysis with your UV light. Spin in the centrifuge for 10 minutes at 13,000 RPM. What is in the pellet? What is in the supernatant? Is GFP where it should be at this step? If not repeat the freeze/thaw step.

9. Transfer 250 µl of the supernatant (if you are sure the bacteria were well lysed) to a new tube. Add 250 /-!l Binding Buffer to the lysate. Transfer the remaining lysate to a new tube and save. Binding buffer is a high salt buffer that is insures that all proteins are initially bound to the column, by exposing any hydrophobic amino acids.

Preparing the HIC column

1. Obtain an HIC column and place it in a clear collection tube. Remove the cap and snap off the bottom of the column. Allow the buffer to drain from the column. The rate of drainage can vary considerably.

2. Carefully add 2 ml (not µl) of Equilibration Buffer to the HIC column. Use care not to disturb the surface of the column a great deal. Slowly dribble the equilibration buffer down the side of the column so that the bed of the column can remain as flat as possible.

Protein Purification

1. Label 4 collection tubes 1,2, and 3, 4 and place them in the rack at your lab bench. Place the HIC column in tube 1.

2. . Carefully/gently load the prepared bacterial lysate (with binding buffer added) to the column. (Place the pipette tip along the side of the column just above the surface and let the lysate drip onto the column bed.)

3. Allow the lysate to drip through and observe the position of the GFP in/on the column.

4. Move the column to tube 2. Add 250-500 µl Wash Buffer to the column in the same careful manner. Wash buffer is a medium salt buffer which will restore more normal folding of the proteins in the column. Proteins that are not particularly hydrophobic will be released from the column. Allow all of the wash buffer to drip through the column. Check the position of GFP with the UV light.

5. Move the column to tube 3. Add 750 µl TE (elution buffer) to the column in the same careful manner. TE is a low salt buffer has the highest concentration of

water of the 3 buffers used. It acts in 2 ways to release highly hydrophobic proteins from the column. 1) It changes the conformation of some proteins to expose the more hydrophilic regions which will want to interact with water and move through the column with the water 2) The high water concentration will compete with the hydrophobic beads and will in a sense "chase" the more . hydrophobic proteins out of the column. Watch for movement of GFP in the column using the UV light.

6. Quickly move the column to tube 4 when the GFP appears to start flowing out of the column. Allow it to completely elute from the column.

Discuss what is present in your 4 tubes. Place parafilm around tube 4 and save.

GFP Purification PART II

While you are purifying GFP using affinity chromatography, we will also separate *E.coli* proteins using electrophoresis. The concept is similar to DNA electrophoresis in that proteins are separated based on molecular weight and charge. However you should recall that proteins are not nearly as uniform in charge and shape as DNA. At physiological cell conditions, some proteins carry a net negative charge (as does DNA) whereas some carry a net positive charge or are neutral. More importantly proteins vary extensively in overall shape, which influences how they migrate through a matrix used in electrophoresis. (recall how relaxed DNA circles migrate slower than, compact, supercoiled circles)

There are 2 basic types of protein electrophoresis:

A. Denaturing electrophoresis----puts all proteins on the same level in terms of molecular weight since most protein structure is eliminated. Proteins migrate solely based on their molecular weight. Proteins within the gel would not be expected to be active.

B. Native electrophoresis----Proteins are NOT denatured but are maintained in their normal shape during the separation. Proteins of the same molecular weight may run differently depending on their "bulk" due to normal folding. Proteins should remain active since they are not changed in shape.

***in most types of electrophoresis we combine our protein sample with a detergent that makes the proteins overall negatively charged so that they all run toward the positive pole.

Dr. Super will prepare an acrylamide gel for your samples today. Following electrophoresis we will detect GFP within the gel and then stain the gel to note all of the *E.coli* proteins we started out with in the lysate.