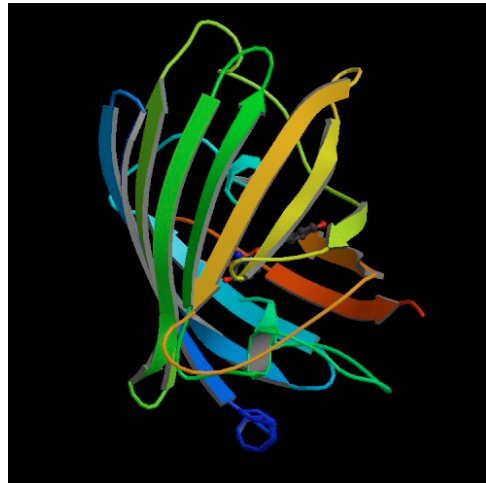


Lab #6**Isolation of Green Fluorescent Protein (GFP) by Chromatography and PAGE**

Green fluorescent protein (GFP) originally comes from the jellyfish *Aequorea victoria*. It's a relatively moderate-sized protein with 238 amino acids, and fluoresces green when exposed to UV light. In fluorescence, a photon of light is absorbed by the fluorophore and as the fluorophore relaxes to the ground state, light is emitted at a different wavelength (hence the fluorescence you see). GFP absorbs light at 395 nm and emits green light at 475 nm.

GFP has a multitude of uses in molecular biology. It is often used as a reporter protein to indicate the levels of gene expression from a particular promoter in the nucleus. It is also used as a fusion protein marker, which allows for the localization of a second protein (the fusion partner) in the cell.



In this experiment, you will purify GFP from *E. coli* cells transformed with pGLO™ by hydrophobic interaction chromatography. GFP contains many hydrophobic amino acid residues and is therefore more hydrophobic than other proteins in the cell. In the first step, you will pass the supernatant containing the bacterial proteins and GFP over a HIC column in a highly salty buffer. The salt causes the three-dimensional structure of proteins to actually change so that the hydrophobic regions of the protein move to the exterior of the protein and the hydrophilic regions move to the interior of the protein. The chromatography column contains a matrix of microscopic hydrophobic beads. When your sample is loaded onto the matrix in very salty buffer, the hydrophobic proteins should stick to the beads. The more hydrophobic the proteins, the tighter they will stick. The more hydrophilic the proteins, the less they will stick. As the salt concentration is decreased, the three-dimensional structures of proteins change again so that the hydrophobic regions of the protein move back into the interior and the hydrophilic regions move to the exterior.

After isolating GFP by column chromatography, we will further purify the protein through Polyacrylamide Gel Electrophoresis (PAGE).

Day 1 Production of Cell Lysates

Procedure:

1. You have at your bench 5 ml of pGlo-transformed E. coli overnight culture. Label the tube with your initials.
2. Place the tube in a bench-top centrifuge and spin at setting 4 for 5 minutes. Discard the supernatant by decanting it into the beaker labeled “bacterial waste”; take care not to disturb cell pellet.
3. To cell pellet, add 500 μ l of TE buffer. Resuspend cells by pipetting the fluid up and down in tube until the cells are uniformly dispersed in buffer.
4. Transfer the cell suspension to a 1.5 ml microcentrifuge tube. Label the tube as pGlo and with your initials.
5. Add 200 μ l of lysozyme to the cells; mix well by inverting tube 8-10 times. Place in 37°C water bath for 60 minutes. Lysozyme lyses bacterial cells by digesting their cell walls.
6. Place culture tube in -20°C freezer until next week. Ice crystal formation helps disrupt cell walls, membranes, and intracellular vesicles.

Day 2 Isolate GFP by Column Chromatography

Column Chromatography Hints – Read before beginning the experiment

- NEVER let your column go dry. Always have a meniscus of mobile phase above your column bed.
- Try not to disrupt the column bed once you’ve added the sample. When you add liquid to the column, run the liquid down the side of the column in a slow trickle.
- The columns are designed to drip slowly, so be patient, but don’t space out and forget to keep watch.
- It is important not to move the column more than needed as motion can cause major disturbance to the column bed. When you move the column from one test tube to another, do so slowly and carefully.

Procedure

1. Obtain your cell lysate from last week. It should be labeled as pGlo and with your initials.
2. Fully thaw the cell lysate by holding it in your hand.
3. Pellet the cell lysate by centrifugation at 13,000 RPM for 10 minutes.
4. Decant the supernatant to one clean microcentrifuge tube. Label the tube as CL (for crude lysate) and with your initials. Place on ice.
5. Label a clean microcentrifuge tube as SBB (for supernatant-binding buffer). Pipet 400 μ L of supernatant into this tube. Add 400 μ L of binding buffer. Mix well by inverting 6 times. Keep your sample on ice
6. Label 3 glass test tubes 1-3 and place the tubes in a test tube rack.
7. Place the HIC column into test tube #1. Be certain to let the solid and mobile phases of the column separate.

8. Remove the top cap and snap off the bottom cap from the HIC column. Allow all the liquid buffer to drain from the column into test tube 1, but LEAVE A MENISCUS ABOVE YOUR STATIONARY PHASE. To stop flow of your column, put a yellow cap on the bottom of the column.
9. Holding your pipet against the side of the column, being careful not to disrupt the column bed, add 2 mL of equilibration buffer to the top of the column in small portions. Let the equilibration buffer drain until the meniscus is just above the stationary phase but not touching it. Cap the bottom of the column until you are ready for the next step.
10. Apply 500 μ L of supernatant-binding buffer (SBB) to the column. Hold the pipet tip against the side of the column wall, just above the upper surface of the matrix and let the supernatant drip down the side of the column wall. Examine the column using a hand-held UV light. Note your observations. When your cell lysate has completely adsorbed onto the column, but the column isn't dry (still a meniscus), transfer the column to the second test tube (fraction 2).
11. Add 4 mL of wash buffer to the column. Examine the column using UV light. Note your observations. When your entire wash buffer has adsorbed onto the column but the column isn't dry (still a meniscus), cap the bottom of the column and read through the next step before proceeding.

The next step is to elute GFP from the column. Our objective is maximal purification of GFP. You are able to assess the position of GFP with the UV light. When you collect the final fraction into tube #3, attempt to collect only fluorescent green drops of sample.

12. Add 1000 μ L of elution buffer to the column. Let your column drip into test tube 2. Examine the column using UV light. As soon as you see GFP come out of the column then move the column to test tube #3 and collect GFP. Add more elution buffer to the column if needed to ensure that the column does not go dry before you elute all the GFP from the column. When the last drop of green GFP has been eluted from the column, remove the column from test tube #3 (we don't want to dilute the GFP with elution buffer that does not contain our protein).
13. Mix fraction #2 well, then transfer 300 μ L of this sample to a clean microcentrifuge tube. Label the tube as fraction #2 and with your initials. Place on ice.
14. Mix fraction #3 well, then transfer 300 μ L of this sample to a clean microcentrifuge tube. Label the tube as fraction #3 and with your initials. Place on ice.
15. Give your samples (CL, fraction #2, fraction #3) to the instructor to be stored at -20°C until next week.

Day 3 Further Purification of GFP by SDS-PAGE

Last week you purified GFP by column chromatography. Now we will further purify GFP by SDS-PAGE. In this technique, proteins in your column fractions are first denatured by a detergent, sodium dodecyl sulfate (SDS). SDS, which is negatively charged, coats the protein throughout giving each protein a uniform charge to mass ratio. Proteins treated in this way can be separated on the basis of size by polyacrylamide gel electrophoresis (PAGE). Next we will visualize the proteins by staining the gel with Coomassie Brilliant Blue, a dye that binds tightly to proteins. GFP is approximately 27 kilodaltons (KDa) in size. We should be able to identify GFP based on its position in the gel relative to size marker proteins of known size. Furthermore, the results of this experiment should allow us to assess the degree of purification of GFP relative to the crude lysate.

Procedure

1. Fill a 500 ml beaker half way with water and get it boiling on a hot plate.
2. Obtain your samples (CL, fraction #2, fraction #3) from the last week's chromatography experiment.
3. Thaw and mix each sample well.
4. Transfer 20 μ l of CL (crude lysate) to a clean microcentrifuge tube. Add 20 μ l of protein loading buffer to this tube and mix well. Label the tube as CL-B (for crude lysate – buffer). Using a pin, poke a hole through the top of the tube. Store on ice.
5. Transfer 20 μ l of fraction #2 to a clean microcentrifuge tube. Add 20 μ l of protein loading buffer and mix well. Label the tube as #2-B. Using a pin, poke a hole through the top of the tube. Store on ice.
6. Transfer 20 μ l of fraction #3 to a clean microcentrifuge tube. Add 20 μ l of protein loading buffer and mix well. Label the tube as #3-B. Using a pin, poke a hole through the top of the tube. Store on ice.
7. Place tubes CL-B, #2-B, and #3-B in a floating rack and boil for 2 minutes to denature the proteins.
8. Load 25 μ l of each sample on the gel from left to right in order: CL-B, #2-B, and #3-B. The instructor will demonstrate.
9. Turn on the power supply and run the gel for approximately 2 hour at 60 mAmps.
10. Jim will stain the gel, destain the gel, photograph the gel and store the gel in the refrigerator until the next period. The general procedure is as follows:
 - a. Carefully remove the gel from the glass plates and place in a Tupperware container
 - b. Add enough Coomassie Brilliant Blue stain to cover the gel.
 - c. Gently agitate the gel for approximately 1 hour
 - d. Dispose of the stain in the "used stain" jar
 - e. Rinse the gel with DI water and dispose of this down the drain.
 - f. Destain the gel overnight with shaking in methanol/acetic acid solution
 - g. Dispose of destain in the "used destain" jar
 - h. Rinse gel in DI water
 - i. Photograph the gel
 - j. Wrap gel in saran wrap/zip lock bag
 - k. Store gel in refrigerator until next week

