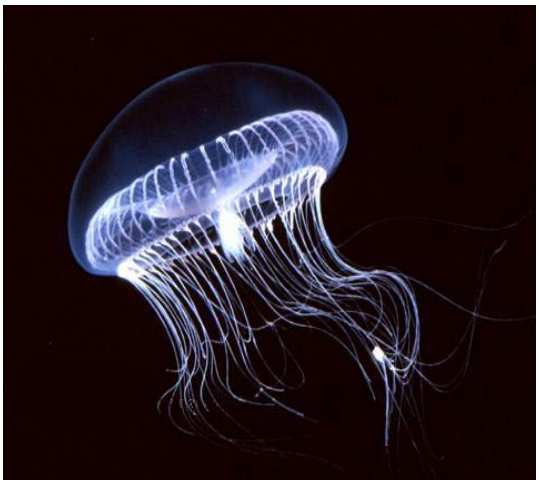
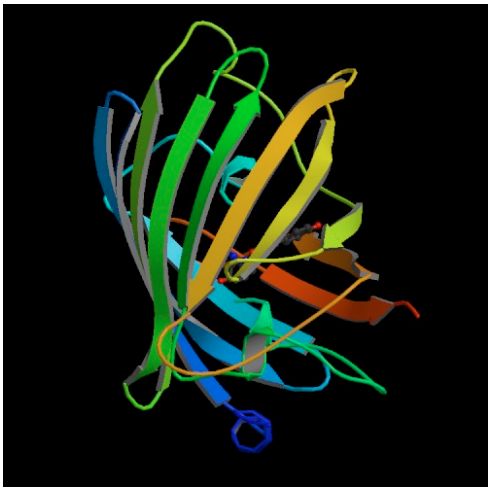
Molecular Biology Lab Name _____

Lab #7
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 most 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 high salt buffer. The salt causes the three-dimensional structure of proteins to 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 high salt 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 centrifuge and spin at 6000 x g for 5 minutes. Discard the supernatant by decanting it into the beaker labeled as bacterial waste. Take care not to disturb cell pellet.
3. To cell pellet, add 500 µl of TE buffer. Resuspend cells by pipetting the fluid up and down in tube until the pellet is gone and cells are 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 µl 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. Buffers and washes used for the chromatography experiment are located at the teacher's station. Use empty microcentrifuge tubes or glass culture tubes as vessels for storing the solutions you need.
2. Obtain your pGlo-transformed cell lysate from last week. It should be labeled as pGlo and with your initials.
3. Fully thaw the cell lysate by holding it in your hand.
4. Pellet the cell lysate by centrifugation at 13,000 RPM for 10 minutes.
5. Decant the supernatant to one clean microcentrifuge tube. Label the tube as CL (for crude lysate) and with your initials. Place on ice.
6. Label a clean microcentrifuge tube as SBB (for supernatant-binding buffer). Pipet 400 µL of supernatant into this tube. Add 400 µL of binding buffer. Mix well by inverting 6 times. Keep your sample on ice
7. Label 3 glass test tubes 1-3 and place the tubes in a test tube rack.
8. Place the HIC column into test tube #1. Be certain to let the solid and mobile phases of the column separate.

9. 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.
10. 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.
11. Apply 500 µL 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).
12. 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.

13. Add 1000 µL 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).
14. Mix tube #2 well, then transfer 300 µL of this sample to a clean microcentrifuge tube. Label the tube as fraction #2 and with your initials. Place on ice.
15. Mix tube #3 well, then transfer 300 µL of this sample to a clean microcentrifuge tube. Label the tube as fraction #3 and with your initials. Place on ice.

Day 2 - Further Purification of GFP by SDS-PAGE

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). Typically, polyacrylamide gels are poured in 2 stages; first the resolving gel is poured and allowed to polymerize and then the stacking gel is poured. The stacking gel is quite porous and functions to concentrate the proteins into a tight band before they hit the resolving gel. The resolving gel is denser and functions to separate proteins on the basis of size. When electrophoresis is complete, 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. Assemble glass plates as demonstrated by the instructor.
2. Insert a comb between the plates and use a permanent marker to mark a line 1 cm below the bottom of the wells. This will leave enough space for the stacking gel.
3. Put on a pair of vinyl gloves to protect yourself from the acrylamide. Polymerized acrylamide gels are safe to handle, however liquid acrylamide is toxic.
4. In a small flask, mix components for the resolving gel in the order listed below. Polymerization will begin after addition of TEMED. These solutions are located at the teacher's station.

   3.3 ml deionized water
   4.0 ml 30% acrylamide mix
   2.5 ml 1.5 M Tris (pH 8.8)
   0.1 ml 10% SDS
   0.1 ml 10% APS
   4 µl TEMED

5. Swirl mixture and, using a plastic Pasteur pipet, immediately pour the gel up to the line you drew earlier.
6. Carefully overlay the gel with a thin layer of deionized water.
7. Allow mixture to polymerize at room temperature, 30 min. Also allow any excess acrylamide in the mixing vessel to polymerize before it is thrown in the trash.
8. Rinse top of resolving gel with water, and blot with Kimwipes.

In a small flask, mix components of the stacking gel in the order listed below, swirl to mix and, using a plastic Pasteur pipet, add the mixture to the gel until it reaches the top of the plate. Insert the comb and let the gel polymerize at room temperature for 45 min. These solutions are located at the teacher's station.

3.4 ml deionized water
0.83 ml 30% acrylamide mix
0.63 ml 1.0 M Tris (pH 6.8)
0.05 ml 10% SDS
0.05 ml 10% APS
5 µl TEMED

Sample Preparation:

1. You will be loading a size marker and 3 protein samples on your gel. The samples are crude lysate (CL), the flow through (fraction 2) and GFP (fraction 3).
2. Prepare sample buffer by adding 50 µl β-mercaptoethanol to 950 µl sample buffer (found at your bench).
3. Label 4 new microcentrifuge tubes as 1-4.
4. Add 20 µl of sample buffer to each tube.
5. Add 10 µl of ladder to tube #1, add 10 µl of crude lysate (CL) to tube #2, add 10 µl of fraction 2 to tube #3, add 10 µl of fraction 3 to tube #4. Mix each tube well.
6. Poke a hole in the top of each tube with a pin.
7. Heat at 100ºC in a boiling water bath for 3 min.

Loading Gels:

1. Once stacking gels have polymerized completely, remove the comb and place the gel cassettes into the clamping frame. The short plates face inwards, so that the spacer plates form the outer walls of the assembly. The instructor will demonstrate.
2. Fill assembly with 1x running buffer so that the buffer is above the short glass plate.
3. Rinse the wells with running buffer using a syringe. Remove any extraneous acrylamide pieces from the wells.
4. Fill the wells with 25 µl sample. Fill any empty wells with an equal volume of sample buffer.

Running Gels:

1. Place assembly into the tank, making sure that the electrodes line up correctly.
2. Fill tank with running buffer to the mark on the front of the tank. For 2 gels, this will require approximately 700 ml running buffer. This includes the amount poured into the gel cassette assembly.
3. Place lid on the tank, aligning the color-coded banana plugs and jacks.
4. Plug into power source and run at 150 V for 60-75 min.
5. Remove gels from between the glass plates as demonstrated by the instructor.
6. The instructor will stain the gel in coomassie blue overnight.

Staining:
Place gels in a sturdy, shallow tray. Pour stain over gel until it is completely covered and let sit overnight, covered and refrigerated.

Day 4 Analysis of Results of SDS-PAGE

1. Observe the SDS-PAGE gel directly if available
2. Staple a photograph of the gel to the back of your lab exercise
3. Label the photograph to show the location of GFP in the gel

Questions:

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1. GFP is derived from what organism?

2. What is fluorescence?

3. How does the HIC column function to purify GFP away from most proteins in the cell?

4. What is the function of the enzyme called lysozyme?

5. Describe the original discovery of lysozyme (1 point extra credit).

6. What is the function of SDS in the SDS-PAGE technique?

7. How does the stacking gel differ from the resolving gel and what are their functions in PAGE?

8. What is the approximate molecular weight of GFP?

9. What is the function of coomassie blue in PAGE?

10. How is GFP used in molecular biology?