**Cellular Stress Response – Electrophoresis**

**Background Information:** A typical eukaryotic cell contains tens of thousands of different proteins, each involved in specific cellular processes. The heat-shock proteins were originally identified because they are produced in large quantities when cells are subjected to heat-induced stress. Because subsequent studies have demonstrated that they are produced in response to a variety of stresses, these proteins are now often referred to as stress proteins. It is important to recognize that stress proteins have essential roles in non-stressed cells, where they function normally in the synthesis, transport and folding of non-heat-shock proteins.

Stress proteins are produced to protect the cell from situations that would lead to irreversible cell damage and ultimately to cell death. Under conditions of stress, the genes encoding the heat-shock proteins are activated and large amounts of the proteins are produced. When stress is relieved, the level of these proteins returns to normal. At the protein level, changes in the level of stress protein production can be observed by examining the total protein content of a cell using polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting (Western blotting) with an antibody that specifically recognizes one of these stress proteins. In *Drosophila*, the most prevalent heat-shock proteins belong to the HSP 70 family, so named because they have molecular weights of around 70,000 daltons.

In our lab this week, cellular proteins will be resolved by electrophoresis through polyacrylamide gels under denaturing conditions. This type of separation occurs based on protein size (i.e., molecular weight). Polyacrylamide is a gel-like polymer whose concentration can be controlled to influence the pore-size of the electrophoretic gel being produced. Higher concentrations of acrylamide result in a smaller pore/sieve size, where lower concentrations of polyacrylamide result in larger pore size. Proteins, being charged compounds, can then be placed on one end of a polyacrylamide gel, and pulled through the gel using electric current. As the current pulls this mixture of proteins through the acrylamide gel matrix, smaller proteins will be able to weave through this matrix further in any given period of time, relative to larger proteins. In this manner, a mixture of proteins can be separated by size as shown by the diagram (above right). In this diagram, the polyacrylamide is shown as a long squiggly line and the proteins are described as “macromolecules”. Once a mixture of proteins is applied to one end of a polyacrylamide gel (left panel) and current is applied, the current will pull these proteins through the matrix of polyacrylamide. In the right panel, the result of passing current through the mixture of proteins and gel matrix is demonstrated. Note the original location of this mixture (left panel), and the distribution of different sizes of proteins through the gel matrix (right panel).

Due to the fact that the concentration of polyacrylamide can be controlled during the initial gel-making process, the concentration of polyacrylamide in the gel matrix is important for your final goal. A low concentration of polyacrylamide makes a very ‘loose’ mesh of acrylamide molecules. Larger proteins are able to move without too much trouble through this loose mesh, and small proteins even more easily. Therefore, by the time a larger protein is able to reach the middle of a gel mesh, the smaller proteins have migrated beyond the end of the gel! The area of the gel surrounding the large protein of interest will likely contain fewer proteins permitting better resolution of your protein of interest. In the case of small protein analysis, one would have to use a very high percentage acrylamide gel. The high concentration of polyacrylamide makes a
‘tight’ mesh through which large proteins have a difficult time migrating. Small proteins, on the other hand, are able to move navigate through the tight mesh relatively easily; the large proteins get stuck near the top of the gel whereas the small ones reach the middle of the gel with relative ease. The table below shows common polyacrylamide percentages and their associated ranges of best resolution.

<table>
<thead>
<tr>
<th>Final % acrylamide</th>
<th>Size resolution (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.5</td>
<td>45-1000</td>
</tr>
<tr>
<td>10</td>
<td>22-300</td>
</tr>
<tr>
<td>12</td>
<td>13-200</td>
</tr>
</tbody>
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For separation based upon size exclusively, all proteins in a mixture must have the same shape (i.e., become unfolded or denatured) and charge. Before subjecting the extracted protein sample to electrophoresis, then, the proteins are denatured using a combination of a detergent, such as SDS, a reducing agent, such as mercaptoethanol, and heat. These treatments ensure dissociation (i.e., un-binding) of the covalent and non-covalent interactions maintained by amino acid side chains that are required for proper protein unfolding. Protein charge is also influenced by the SDS detergent as it is very negatively-charged, making all of the proteins with which it associates negatively-charged as well.

Finally, following separation of the proteins, it is necessary to visualize them to analyze their presence in your sample. Because proteins are colorless, visualization of the protein is enabled by staining of the separated proteins with a protein-binding dye. Although different dyes are used, the most commonly-used protein-binding dye is Commassie Brilliant Blue. Commassie blue (as it is most commonly called) is dissolved in an acid-alcohol solution into which an acrylamide gel can be submerged. The dye binds to proteins using charge, with the negatively-charged dye binding to positively charged proteins. Usually after several hours of staining, excess dye is washed from the gel matrix resulting in an image similar to that shown below:

The SDS-PAGE shown above has been stained with Commassie blue and “destained” in order to visualize every protein in the gel. The wells into which the protein mixtures were loaded are located at the top of the image. Because they have a more difficult time navigating the molecular acrylamide matrix, the largest proteins are located closest to these wells; the smallest proteins in each sample have migrated vertically down from the well to near the bottom. There are a number of items to note in the above image. From left to right, notice the distribution of proteins in the first 3 lanes and how each “band” (the word ‘band’ is often used to indicate the presence of a
single protein, but actually a ‘band’ represents collections of similarly-sized proteins) seems to be the same color density, therefore indicating similar amounts of each similarly-size protein collection in these 3 samples. Also note lanes 4, 8 and 9. The presence of many more bands indicates a larger number of proteins, and the strongly-intense band toward the bottom indicates a larger amount of this size of protein(s).

**Overall Objective:** To separate the proteins isolated from *Drosophila* larvae using polyacrylamide gel electrophoresis so that the amounts of total protein can be compared.

**Experimental Procedures:**

1. Label a *screw-cap* microcentrifuge tube for each of your three samples (control and experimental).

2. Last week, you calculated the volume of each of your three *Drosophila* extracts that contained 50 µg of total protein. Transfer the calculated volume to the appropriate labeled screw-cap microcentrifuge tube and bring the total volume to 25 µl.

3. Add 5 µl of sample loading buffer (provided by your instructor) into each tube.

4. Loosen the screw caps a bit and place your three samples in a heating block set at 100°C and incubate for 4 minutes.

5. While your samples are heating, prepare the polyacrylamide gel apparatus as described by your instructor.

6. When the samples have finished heating, place them on ice briefly (~1 minute) to cool, and briefly (~30 seconds) spin in the microcentrifuge to bring all the liquid down to the bottom of the tube.

*To demonstrate the proper loading procedure, your instructor will add 10 µl of a size standard to the first well of each gel. The size standard (“protein ladder”) contains bands of between 10,000 and 200,00 daltons.*

7. Carefully load 20 µl of each denatured sample in adjacent empty wells. Load the sample slowly in a steady stream so that it has time to sink to the bottom of the well. Make sure you record the lane number and the identity of your samples, as well as their positions relative to the size standard used.

8. After all of the samples in the class have been loaded, carefully connect the leads to the electrophoresis chamber and run the gel at 100 volts until the loading dye has migrated completely off of the gel (about 1 hour). Note the time and continue running the gel for an additional 30 minutes.

Staining and destaining take a minimum of two hours and are preferably done overnight. Since the gels are stable indefinitely, it may be convenient to look at the results on another day.
Post-lab assignment:

Preparing and Interpreting the Data

a. Using distances that the proteins in your molecular weight standards migrated, create a ‘standard curve’ by plotting migration distance (use whatever units are appropriate) versus the $\log_{10}$ of the molecular sizes of the protein standards. Given this, extrapolate the sizes of largest and smallest protein sizes in your *Drosophila* samples.

b. Compare the results of your electrophoresis procedure with the results shown in the Background information above. Are all of the equivalently-sized bands in your samples present in similar amounts (like the first 3 lanes in the example)? If there are some bands that appear in one sample that do not appear in another, hypothesize why these differences exist.

c. Similarly, are there any bands in your gel that are present in different amounts when comparing one sample to another? If so, hypothesize why these differences exist.

Points to Consider

The sample buffer that is added to the protein before loading on the gel contains a detergent (SDS) and a reducing agent (mercaptoethanol). What is the purpose of these two compounds?

Why are the protein samples heated before loading on the gel?

Would you expect the other (non-heat shock) proteins in a cell to be produced at the same, reduced, or elevated levels during heat shock when compared to their normal levels? What did you observe when comparing the heat-shock and control protein samples?

What is the relationship between the normal function of heat-shock proteins in a cell and their function during the stress response?

The heat-shock response and the associated proteins have been observed in all cells, from bacteria, to fungi, to plants, to animals. What does this suggest about the evolutionary origin of the stress response?