MW of Acid Phosphatase by SDS Gel Electrophoresis

Electrophoresis is a technique in which charged molecules migrate in electric fields. In zonal electrophoresis, the molecules are carried in an aqueous solution through a solid support or matrix. Samples are applied as zonal spots or bands. Zonal systems include paper electrophoresis and gel electrophoresis.

In all types of electrophoresis, the mobility of a molecule \((U)\) is proportional to the applied voltage \((E/d)\), the net charge on the molecule \((q)\), and inversely related to the friction on the molecule \((f)\):

\[
V = \frac{(E/d)}{(q/f)}
\]

by definition,

\[
U = \frac{V}{(E/d)}
\]

so,

\[
U = \frac{q}{f}
\]

The total movement of the molecules increases with time; mobility involves rate of movement. Most electrophoresis systems employ an equal and constant voltage on all the cross-sectional areas of the matrix or support system. However, current is often used to define voltage requirements for separation of components. It is possible to specify either voltage or current because Ohm’s law relates the two at constant resistance.

When separating small molecules, one can predict their electrophoretic mobility by considering the full or partial charges on their acidic and basic groups at the desired pH. Consequently, electrophoresis can separate molecules of similar character, e.g., nucleotides. For macromolecules, prediction of the charges on all the acidic and basic groups may not be possible. In addition, the relative sizes of the molecules to be separated are also important (due to friction). Mobility of macromolecules, therefore, is due to both the net charge and the size of the molecule. Shape of the macromolecule can also affect mobility, as molecules with high axial ratios (length to width) are very long and usually have less electrophoretic mobility than more spherical molecules.

Paper Electrophoresis

Paper is the most common electrophoretic system of analysis of small molecules, but is rarely used for macromolecules because adsorption and surface tension cause denaturation and poor resolution. In this method, a sample is placed on damp paper and an electric field placed across the paper.

Gel Electrophoresis

Molecules separate in aqueous buffers supported within a gel matrix. Gels can handle large volumes of sample and can be used both analytically and preparatively. A wide variety of matrices and pore sizes can be used. By altering the pore sizes, the effects of friction are altered, hence the separation of macromolecules is altered. The two most common gel matrices used are
agarose (a polymer of polygalactose) and polyacrylamide. Polyacrylamide gels are formed by the crosslinking of acrylamide and N,N'-methylene-bisacrylamide in the presence of free radicals. There are two general methods of free radical generation: chemical and photochemical (using riboflavin). In the chemical method, a free radical initiator, ammonium persulfate (APS) is added to the gel solution along with a catalyst, N,N,N',N’-tetramethylenediamine (TEMED). TEMED catalyzes free radical propagation. By altering the amounts of TEMED and APS, you can alter the polymerization time. In the photochemical method, riboflavin, which replaces APS, generates free radicals upon exposure to UV light.

**Formation of polyacrylamide gels**

\[
\begin{align*}
\text{H}_2\text{C}=&\text{CH} & \text{H}_2\text{C}=&\text{CH} & \text{-CH}_2\cdot\text{CH} &\cdot \text{CH} &\cdot \text{CH}_2\cdot \text{CH} \\
| & | & | & | & | & | & | \\
\text{C}=&\text{O} & \text{C}=&\text{O} & \text{C}=&\text{O} & \text{C}=&\text{O} \\
| & | & | & | & | & | & | \\
\text{NH}_2 & \text{NH} & \text{NH}_2 & \text{NH}_2 & \text{NH} \\
| & | & | & | & | & | & | \\
\text{CH} & \text{NH} & \text{NH} & \text{NH} & \text{NH} \\
| & | & | & | & | & | & | \\
\text{C}=&\text{O} & \text{C}=&\text{O} & \text{C}=&\text{O} & \text{C}=&\text{O} \\
| & | & | & | & | & | & | \\
\text{CH}=\text{CH}_2 & \text{C}=&\text{O} & \text{C}=&\text{O} & \text{C}=&\text{O} & \text{C}=&\text{O} \\
\end{align*}
\]

**SDS Gel Electrophoresis**

Sodium dodecyl sulfate (SDS) gel systems are used to resolve and characterize the number and size of protein chains or subunit chains in a protein solution. The matrix is polyacrylamide. The sample of protein is treated with an excess of thiol reagent (reducing reagent), e.g., β-mercaptoethanol, in the presence of SDS (a detergent) and heat. The reducing or thiol reagent reduces the disulfide bonds in the protein. The SDS and heat, then, completely denatures the reduced protein. This will disrupt all intermolecular bonds but will not degrade the chains. SDS contains a negative charge from the sulfate, hence will give each protein chain a nearly constant net negative charge to mass ratio. When placed in an electric field, the proteins will have the same charges and shapes, with mobility differing solely due to size differences (due to friction). The relative mobility of each chain is a log function of its molecular weight.

**Laemmli SDS gel electrophoresis**

In 1970, U.K. Laemmli described an SDS gel electrophoresis technique that included a stacking gel. This system is very similar to the one first described by Ornstein and Davis in 1964. The system uses two different gels and three different pH’s to “stack” or shrink the sample size, which increases the resolution of the gels.
The reservoir buffer is Tris-glycine, pH 8.3. The upper or stacking gel is at pH 6.8, and the lower or running gel is at pH 8.8. When the sample starts electrophoresing, there are 3 negatively charged ions present, chloride, SDS-protein, and glycine. At pH 6.8, glycine has only a very small negative charge, chloride has a -1 charge, and the protein has a moderate negative charge due to the SDS. The order of mobility is chloride fastest, than the protein, with glycine slowest. The protein is “stacked” between the other ions. Since, according to the Kohlrausch principle, all regions of the gel must have the same current, the voltages needed to move each ion at the same rate must vary. Glycine needs lots of voltage to move as fast as the chloride. Therefore, if a protein molecule moves too fast, it reaches a region of lower voltage and the protein slows back down. If the protein moves too slowly, it reaches a region of higher voltage and the protein speeds up. This shrinks the sample to a small volume. When the sample reaches the running gel, the pH decreases to 8.8. This pH change causes the glycine to become much more negative, hence speed up. The chloride still moves fastest, but now the glycine moves through the protein layer. This allows the proteins to slow down and move at their own rate, hence the mobility of the protein is now a function of their size differences.

Materials

Pre-cast gels (e.g., Any-kD Mini-PROTEAN® TGX™ Precast Gel, 10 lanes)
10% SDS (5g SDS in 50 ml)
5X or 10X SDS gel Running buffer (1X is 6.0 g Tris OH, 28.8 g Glycine, 0.1 g SDS in 1 liter)
Laemli sample buffer [or 0.02% Bromphenol blue in 50% glycerol (Loading dye)]
β-mercaptoethanol stock
MW protein standards
95°C water bath
Acid phosphatase standard (2 mg/ml) [this is not pure]
Gel electrophoresis system and power supply
Millimeter ruler
0.25% Coomassie blue in 40% methanol, 10% acetic acid [stain]
5% Methanol, 10% Acetic Acid [40% Methanol, 10% Acetic Acid if in a hurry]

Procedure

Note: We will be running our samples on Laemmli SDS gels.

Preparing the samples

1) Prepare your samples as follows:

a) Prepare Supernatants I, II, IV, V, and VI as follows: Mix together the following: up to 8 µl of the samples (containing up to 10 µg protein), 1.0 µl β-mercaptoethanol, and 1.0 µl 10% SDS. Bring each sample up to 10 µl with H₂O. [Note: if you haven’t calculated
protein concentrations of your samples, use 1 \( \mu l \) of supernatants I and II and 8 \( \mu l \) of the others.

b) For the protein standards, use 10 \( \mu l \) of the standard, 1.0 \( \mu l \) \( \beta \)-mercaptoethanol, and 1.0 \( \mu l \) 10\% SDS.

c) For the commercial acid phosphatase, use 5 \( \mu l \) of the standard, 3 \( \mu l \) of H\(_2\)O, 1.0 \( \mu l \) \( \beta \)-mercaptoethanol, and 1.0 \( \mu l \) 10\% SDS.

d) Heat each protein sample at 95\°C in a H\(_2\)O bath for 3 min. Do not heat too long, as it allows reoxidation of the disulfide bonds!

e) After cooling the samples to room temperature, add 6 \( \mu l \) Laemli sample buffer.

Note: You should prepare up to 10 samples: the MW standard (protein ladder); the standard Acid phosphatase, up to six supernatants from the Acid phosphatase purification; and, if you purified Acid phosphatase from another source, those supernatants.

Setting up the apparatus

2) One or two groups per gel.

3) Make 600 ml of 1X SDS gel running buffer from the stock.

4) Get a pre-cast gel and remove it from the storage pouch.

5) Gently remove the comb from the gel and rinse the wells with deionized water.

6) Expose the bottom edge of the gel by pulling the clear tape.
   Note: If only one gel will be used, use a dam for the other one.

7) Place a gel cassette sandwich into the slots at the bottom of each side of the assembly. The short plate should face inward toward the notches of the U-shaped cassettes.

8) Lift the gel cassette into place against the green gaskets. Slide it into the clamping frame.

9) Press down on the electrode assembly while closing the two cam levers of the frame. This forms the inner chamber and insures a proper seal. The short plate should be aligned with the notch in the gasket.

10) Lower the inner chamber assembly into the mini tank. Fill the inner chamber with 1X running buffer. The level should reach almost to the top of the taller plate of the gel cassettes. Do not overfill! If it is leaking, you have assembled it incorrectly.

11) Add around 1X running buffer to the main tank. Make sure the level is above the bottom of the gel plate.

Loading the samples and running the gel

12) GENTLY layer the entire prepared samples into the wells with a micropipette; the dense protein solution should layer easily. You should use the sample loading guide for loading the samples.
13) Place the lid on the unit and electrophorese the samples at about 200 V for about 25 minutes. The current should be about 50-60 mA per gel at the start and 30 mA at the end (due to the differences in the two gels). Note that as the protein is negatively charged, the anode (positive electrode) is on the bottom.

14) After the run is finished, remove the inner core and the gels by releasing the cams. Gently force the plates apart by twisting the plates. Place the gel and plate on a flat surface and measure the front edge of the tracking dye.

15) Rinse the gel apparatus and let it dry.

**Staining and destaining**

We stain the proteins in the gel with Coomassie blue dye. After staining, we need to remove the dye that is not bound to protein.

**Note:** Wear gloves when working with Coomassie blue, as it stains everything blue (hands, clothes, etc.).

16) Place the gel in a glass or plastic pan, add Coomassie blue dye in 50% methanol-acetic acid, and remove the gel from the plate.

17) Stain for at least 30 minutes. It can stain overnight (or indefinitely).

18) To destain the gel, decant and save the Coomassie blue. To destain, add 5% methanol-acetic acid. [For quick destaining, add 40% methanol-acetic acid to the gel and let sit at least 1 hour.] Remove the solution and place the gel in 5% methanol-acetic acid to remove more dye. If necessary, repeat with the same solution until the gel has no significant background. It usually takes two destain steps to remove enough of the unbound dye.

19) Place the gel on a **visible** light box (white light) and take a picture using the camera.

20) On each gel, measure the distance the bands have migrated in the lower or running gel, as well as the overall length of the gel. Calculate the relative mobilities for each band (distance the band migrated divided by the distance the dye migrated). Calculate the molecular weight(s) of your unknown(s) and for acid phosphatase (assume the most prominent band is the enzyme) by plotting the log MW vs. relative distance migrated for your standards and reading off the graph the log MW of the unknown, based upon their migration.

**Analysis**

1) Plot the log molecular weight vs. mobility for your known proteins. Read the molecular weight of acid phosphatase and the unknowns off the graph. What are their MWs?
2) From your results, how pure did the acid phosphatase appear to be? How many bands did you see for your most pure sample? How many did the standard acid phosphatase have (if run)?
3) Don’t forget to suggest improvements to this experiment!
Appendix

MW of some proteins as determined by SDS gels (the first set is our marker set, the other is another set that we sometimes use):

Bio-Rad Precision Plus protein standards

<table>
<thead>
<tr>
<th>Protein MW (subunits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250,000</td>
</tr>
<tr>
<td>150,000</td>
</tr>
<tr>
<td>100,000</td>
</tr>
<tr>
<td><strong>75,000</strong></td>
</tr>
<tr>
<td><strong>50,000</strong></td>
</tr>
<tr>
<td>37,000</td>
</tr>
<tr>
<td><strong>25,000</strong></td>
</tr>
<tr>
<td>20,000</td>
</tr>
<tr>
<td>15,000</td>
</tr>
<tr>
<td>10,000</td>
</tr>
</tbody>
</table>

*Note: Those in bold have darker bands after staining.*

<table>
<thead>
<tr>
<th>High MW proteins</th>
<th>MW (subunits)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>200,000</td>
</tr>
<tr>
<td>Phosphorylase b</td>
<td>97,400</td>
</tr>
<tr>
<td>Bovine Serum Albumin</td>
<td>68,000</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>43,000</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>29,000</td>
</tr>
<tr>
<td>β-lactoglobulin</td>
<td>18,400</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>14,300</td>
</tr>
</tbody>
</table>

References


