Affinity Chromatography and SDS-PAGE

1. Ni-column electrophoresis (already discussed in lecture)
2. Sodium dodecyl sulfate - polyacrylamide gel electrophoresis = SDS PAGE (today’s lecture).

SDS PAGE:
   Experiment #4 of text.

Electrophoresis

- Electrophoresis is the process whereby charged molecules migrate through a solution under the influence of an applied electric field.

- In gel electrophoresis, a gel matrix of an aqueous solution is subjected to an applied electric field. Samples are loaded into one end of the gel, and ‘pulled through the matrix by the attraction between their charge and the oppositely-charged electrode.

- The sample molecules must have charge. DNA and RNA have negative charge and the charge to mass ratio is constant: one negative per base. Nucleic acids therefore migrate towards positive charge.

\[
\begin{align*}
\text{CH}_3(\text{CH}_2)_{11}\text{OSO}_3\text{Na}
\end{align*}
\]
**Background**

- There are two common types of gel electrophoresis: those based on an agarose gel matrix, and those based on a polyacrylamide gel matrix.

- Polyacrylamide gels can give much greater resolution than agarose gels, but are much more cumbersome to make.

- Protein separations typically use polyacrylamide gels. Nucleic acid separations typically use agarose for low resolution and polyacrylamide for high resolution separations.

- Effective pore size of the matrix can be manipulated by altering the degree of crosslinking within these gels. This is done to achieve the desired separation range.

**Separation Ranges**

<table>
<thead>
<tr>
<th>Table 4-3</th>
<th>The Effective Separation Range of Polyacrylamide Gels of Various Percent Acrylamide Monomer for Use With SDS-PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Acrylamide in Resolving Gel</td>
<td>Effective Separation Range (Da)</td>
</tr>
<tr>
<td>7.5</td>
<td>45,000–200,000</td>
</tr>
<tr>
<td>10</td>
<td>20,000–200,000</td>
</tr>
<tr>
<td>12</td>
<td>14,000–70,000</td>
</tr>
<tr>
<td>15</td>
<td>5,000–70,000</td>
</tr>
<tr>
<td>20</td>
<td>5,000–45,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 4-5</th>
<th>The Effective Separation Range of Agarose Gels of Various Composition for Separation of Nucleic Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Agarose (wt/vol)</td>
<td>Effective Separation Range (base pairs)</td>
</tr>
<tr>
<td>0.8</td>
<td>700–9000</td>
</tr>
<tr>
<td>1.0</td>
<td>500–7000</td>
</tr>
<tr>
<td>1.2</td>
<td>400–5000</td>
</tr>
<tr>
<td>1.5</td>
<td>200–3000</td>
</tr>
<tr>
<td>2.0</td>
<td>100–500</td>
</tr>
</tbody>
</table>

3 4
Electrophoresis

- Under native conditions, a protein can have a positive or a negative charge, which can be large or small. To eliminate this source of variability, the anionic detergent SDS (sodium dodecyl sulfate) is used to fully coat the proteins in a sample. Now every protein has a relatively uniform charge-to-mass ratio.

- Proteins also have differing shapes and rigidities, they can also be constrained by disulfide bonds or complexed together in large oligomers. To eliminate these differences between proteins that mask their true molecular weights we denature them using heat, the SDS and β-mercaptoethanol. Now they will experience resistance from the polyacrylamide gel simply in proportion to their size, and can be separated (resolved) on that basis.

- SDS-PAGE is described on pages 65-71.

The effect of SDS and β-mercaptoethanol on proteins

- ≈ 1 dodecyl sulfate / 2 amino acids

\[ \beta\text{-mercaptoethanol} = \text{HS}_2\text{CCH}_2\text{OH} \]
Relative mobility in PAGE

- The mobility of a molecule increases with increasing applied voltage, increasing net charge on the molecule, and decreasing friction of the molecule.
- Mobility = (voltage x charge)/(friction)
- Hence, information on SIZE.
- A MW 'standard' curve is created by simultaneously running proteins of known MW. Plot log(MW) vs. Rf.

How stacking gels improve resolution.

- At pH 8.3 glycine has a charge of -1, but at pH 6.8 it is neutral (slow).
- Dodecyl sulfate has a charge of -1 at both pHs, so does Cl⁻.
- Glycine is very small (fast when charged), but the proteins are big (slow).
- The resolving gel has a smaller pore size and more greatly restrains the proteins.
SDS-PAGE, visualization

- Dyes are incorporated with samples to analyze gel migration properties.
  - Is the gel working?
  - How far have the sample run?

- After the gel is run, two methods are used to visualize the individual protein banding patterns.

- Acetic acid and methanol precipitate proteins and ‘fix’ them in place.
  - Coomassie Brilliant Blue R250 binds non-specifically to all proteins, turning protein bands blue. Binds to Arg (and Trp, Tyr, Phe, His & Lys). 0.1 - 0.5 µg protein can be detected.
  - Alternately you can stain with substance that specifically binds a particular protein, and on binding somehow generates a visual signal.

- A western blot is when antibodies are used to specifically bind a protein, which then is used to generate a visual signal.
### E. coli total cell protein

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
<th>MW (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Prestained SDS-PAGE standards, low range</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>SOD2-pEL002 cells without addition of IPTG</td>
<td>28829</td>
</tr>
<tr>
<td>3</td>
<td>SOD2-pEL002 cells with addition of IPTG</td>
<td>19455</td>
</tr>
<tr>
<td>4</td>
<td>SOD-pET23d cells without addition of IPTG</td>
<td>34900</td>
</tr>
<tr>
<td>5</td>
<td>SOD-pET23d cells with addition of IPTG</td>
<td>26100</td>
</tr>
<tr>
<td>6</td>
<td>Kaleidoscope polypeptide standards</td>
<td></td>
</tr>
</tbody>
</table>

**Figure:** SDS-PAGE gel (stained with Coomassie blue) showing overexpression of SOD2-pEL002 and SOD-pET23d. Lane 1, prestained SDS-PAGE standards, low range; lane 2, SOD2-pEL002 cells without addition of IPTG; lane 3, SOD2-pEL002 cells with addition of IPTG; lane 4, SOD-pET23d cells without addition of IPTG; lane 5, SOD-pET23d cells with addition of IPTG; lane 6, kaleidoscope polypeptide standards.

### The Experiment

- We will isolate His-tagged nitroreductase, using immobilized metal ion affinity chromatography (Ni column).
- Day 1: Run Ni column.
- Day 2: Analyze fractions saved from IMAC by SDS PAGE.
Day 1

- Obtain cell extract from T.A.
- Isolate His-tagged nitroreductase from the lysate, using IMAC over a Ni column (protocol follows).
- Save fractions: Pre-IPTG, Post-IPTG, Crude extract, Column flow-through, Column wash, Eluate.

**Questions** for your post-lab write-up:
- Could there be non-specific binding to the Ni column (proteins other than the His-tagged nitroreductase)?
- If so, what properties would proteins have to have in order to bind the column?
- How can we separate non-specifically bound proteins from His-tagged nitroreductase?
- What could cause an IMAC column to not function?
- Name two ways in which you could dislodge His-tagged nitroreductase from the column.

Day 2: SDS PAGE

(see experiment 4 in text)

- The text describes the principles and apparatus.
- We will use purchased (precast) gels.
- You will simply need to add samples into the wells, add buffers, hook up to power and supervise.
- Gels will be removed from glass plates and stained with our old friend Coomassie brilliant blue.
- All materials will be made for you. Your execution plan simply needs to provide a sketch of your planned loading arrangement (what samples in which lanes).
- We WILL use the mobilities of molecular weight standards to determine the molecular weight of our test protein.
- Also DO exercises 1 and 2 (pages 74-77)
Changes From the Book

- In your prelab, sketch what you expect your gel to look like, assuming staining with Coomassie brilliant blue, which detects all proteins. Don’t forget that you will have the samples you made on Tuesday PLUS a sample of cells harvested before induction with IPTG, and molecular weight standards.
- The basic procedure of running SDS gels is outlined in experiment #4. Running at 200 V increases the migration speeds.
- Skip steps 1-7. Assemble the running assembly as instructed by T.A. (approximately as in Figure 4-10).
- In step 9, the concentrated protein sample buffer is 2x, not 4 x. Therefore, use 10 µl of a protein sample (from Tuesday) + 10 µl of 2x protein sample buffer.
- We will load our gels using pipetmen not syringes.
- Our Molecular Weight standards are from Biorad, use 10 µl MW standards without dilution (Biorad part #161-0324).

Molecular Weights of standards

<table>
<thead>
<tr>
<th>Protein</th>
<th>Color</th>
<th>Calibrated MW (daltons) on 4-20% Tris-HCl gel</th>
<th>Calibrated MW (daltons) on 4-12% Bis-Tris gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>Blue</td>
<td>202,594</td>
<td>196,235</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>Magenta</td>
<td>113,770</td>
<td>110,676</td>
</tr>
<tr>
<td>BSA</td>
<td>Green</td>
<td>80,582</td>
<td>59,594</td>
</tr>
<tr>
<td>Carbonic anhydrase</td>
<td>Violet</td>
<td>38,644</td>
<td>30,517</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>Orange</td>
<td>30,062</td>
<td>25,095</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Red</td>
<td>15,453</td>
<td>12,902</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>Blue</td>
<td>5,631</td>
<td>6,528</td>
</tr>
</tbody>
</table>

Molecular Weights of standards
**Safety Considerations**

- Observe all normal laboratory safety practices.
- Dispose of all waste into the proper waste containers.
- Be cautious around electricity.
- Acrylamide is a neurotoxin !!
- Are ALL the compounds we will use familiar to you with respect to their safety implications ?

**Chemical Structures**

- IPTG
- Acrylamide