Announcements

• No discussion next week – we’ll discuss the following lab today

• Schedule for the rest of the semester (pay close attention!!!)
  • Week of Chapter 5B normal – ends 11/12
  • Week of Chapter 5C normal – ends 11/19
  • Week of Chapter 6A Discussion begins 11/18 as usual
  • Monday’s Chapter 6A lab will meet on 11/25 (Thanksgiving week)
  • Tuesday’s Chapter 6A lab will not meet on 11/26 (Thanksgiving week), it will meet on 12/3
  • Chapter 6B discussion begins on 12/2 as usual

Chapter 5: Structural Characterization of LDH

Purpose:
Learn how to:
1) Pour an SDS Polyacrylamide gel
2) Prepare samples for SDS-PAGE
3) Determine purity and subunit MW of your LDH
Chapter 5: Overview

- **Week 1: Gel Filtration Chromatography**
  - Separate LDH from standard proteins by native size
  - Determine Native MW

- **Week 2: Denaturing Polyacrylamide Gel Electrophoresis (SDS-PAGE)**
  - Separate LDH from other proteins by subunit size
  - Determine Subunit MW

- **Week 3: Native Electrophoresis (Zymograms)**
  - Confirm the quaternary structure of LDH from Weeks 1 & 2

Calculation of $k_{cat}$ from Ch. 3-5 Data

- **Use $V_{max}$ from Chapter 4:**
  - Change in conc./min = mM/min
  - Realize that mM/min is same as Units/ml = μmol/(ml*min)
  - Therefore, 200 units/ml = 200 mM/min

- **For [E₁], use protein concentration from Chapter 3 & MW from Chapter 5:**
  - mg/mL protein = g/L protein
  - 50 mg/mL protein = 50 g/L protein

- **Multiply protein concentration by 1/Subunit MW from Chapter 5:**
  - (g protein/L)(1 mol/ MW of LDH g)(1000 mmol/1 mol) = mM protein
  - (50 g/L)(1 mol/25000 g LDH)(1000 mmol/1 mol) = 2 mM protein

- **Final Answer:**
  - $k_{cat} = \frac{V_{max}}{[E₁]} = (\text{mM/min})/(\text{mM}) = 1/\text{min}$
  - $k_{cat} = \frac{(200 \text{ mM/min})}{(2 \text{ mM})} = 100/\text{min}$

Note: can also calculate from specific activity (Units/mg x mg/μmole E (MWx10⁻³)) = min⁻¹
Separation of Isozymes by Electrophoresis

- Native electrophoresis separates proteins based on their **Native Charge**
- Direction and speed of movement in membrane is pH dependent
- Can be used to separate enzymes that catalyze the same reaction but are different protein molecules
  - Called **Isoenzymes or Isozymes**

Separation of Isozymes by Electrophoresis

- Separation based on small differences in charge
  - Different amino acid sequences
- Enzymes are not denatured before electrophoresis
  - Enzyme retains activity during gel
- Sample applied to cellulose-acetate “membrane” soaked in buffer
- Current applied to membrane, isozymes move toward particular ends depending on pH
Visualization of Isozymes

- Uses “Activity Stain”
- Requires use of Active LDH

\[
\begin{align*}
\text{Lactate} + \text{NAD}^+ & \rightleftharpoons \text{Pyruvate} + \text{NADH} + \text{H}^+ \\
\text{NADH} + \text{Phenazine methosulfate} & \rightleftharpoons \text{NAD}^+ + \text{Phenazine methosulfate} \\
\text{Phenazine methosulfate} + \text{Tetranitroblue tetrazolium} & \rightleftharpoons \text{Phenazine methosulfate} + \text{Tetranitroblue tetrazolium}
\end{align*}
\]

Purple Precipitate

- Final product can be observed on an agarose plate or cellulose-acetate membrane

Different Isozymes of LDH

- Two isozymes:
  - Heart
  - Muscle

- Have slightly different genes, charges and primary structure, and kinetic parameters

- Can be found in different combinations in different tissues based on quaternary structure of LDH:
  - 2 Subunits: M₂, HM, H₂
  - 3 Subunits: M₃, HM₂, H₂M, H₃
  - 4 Subunits: M₄, HM₃, H₂M₂, H₃M, H₄
Different Isozymes of LDH

- Possible Zymogram plot for Theoretical 3 Subunit LDH

<table>
<thead>
<tr>
<th>M₃</th>
<th>M₂H</th>
<th>MH₂</th>
<th>H₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Heart
- Muscle
- Lungs
- Kidney

Procedure: Chapter 5-Week 3

- Prepare Samples
- Prepare Membrane and Applicator
- Load Samples
- Run Electrophoresis
- Activity Staining and Imaging
Procedure: Chapter 5-Week 3

- Work with another group of the opposite isozyme!

**Prepare Samples:**
- Standard Heart
- Standard Muscle
- Pure Heart
- Pure Muscle

- *If you don’t have enough pure LDH, you can use Crude Extract*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity (Units/ml)</th>
<th>Volume to Load</th>
</tr>
</thead>
<tbody>
<tr>
<td>Std Heart</td>
<td>--</td>
<td>20 µl</td>
</tr>
<tr>
<td>Std Muscle</td>
<td>--</td>
<td>20 µl</td>
</tr>
<tr>
<td>Pure Heart</td>
<td>50-100</td>
<td>20 µl</td>
</tr>
<tr>
<td>Pure Muscle</td>
<td>50-100</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Dilute protein as necessary to load 1-2 total units

\[(50 \text{ units/ml})(0.02 \text{ ml}) = 1 \text{ unit}\]

---

**Procedure: Chapter 5-Week 3**

- *Example* for sample loading:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Activity (Units/ml)</th>
<th>Enzyme Volume (µl)</th>
<th>Buffer Volume (µl)</th>
<th>Total Units in Sample (1-2 total)</th>
<th>Final Activity (Units/µl)</th>
<th>Total Sample Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard Heart</td>
<td>---</td>
<td>--</td>
<td>--</td>
<td>~1</td>
<td>0.05</td>
<td>20</td>
</tr>
<tr>
<td>Standard Muscle</td>
<td>---</td>
<td>--</td>
<td>--</td>
<td>~1</td>
<td>0.05</td>
<td>20</td>
</tr>
<tr>
<td>Pure Heart</td>
<td>180</td>
<td>7</td>
<td>13</td>
<td>1.26</td>
<td>0.06</td>
<td>20</td>
</tr>
<tr>
<td>Pure Muscle</td>
<td>475</td>
<td>3</td>
<td>17</td>
<td>1.43</td>
<td>0.07</td>
<td>20</td>
</tr>
</tbody>
</table>

- Standard Heart and Standard Muscle will be given to you at the correct concentration!
Procedure: Chapter 5-Week 3

Prepare Membrane and Applicator:
- Soak cellulose-acetate membrane in barbitol buffer at least 20 min—Poisonous!!
- Clean applicator with water and check to make sure wells work properly
- Pipet your 20 μl of samples into wells
- Apply membrane to the running bridge—JUST BEFORE Loading samples!

Load Samples: Very Fast Process, > 1 min!
- Press applicator into sample wells ~15 sec
- Pick up applicator, press into membrane ~15 sec
- Repeat 2-3 times, should load 0.5 μl of protein onto membrane

Procedure: Chapter 5-Week 3

Run Electrophoresis:
- Move bridge with membrane to electrophoresis chamber at 4°C—Quickly!
- Attach to power supply and run at 200 V for 45 min
- Need to load and run all membranes at the same time for best results
- Clean applicator and wells with DI water
Procedure: Chapter 5-Week 3

**Activity Staining and Imaging:**

- During electrophoresis use test tube to mix reagents:
  - Lactate, NAD⁺, phenazine methosulfate, and tetranitroblue tetrazolium
- Add molten noble agar, pour into petri dish, let solidify and store at 4°C with foil until electrophoresis complete
- Remove membrane from bridge
- Put membrane top side down in noble agar plate at 37°C
- Let color develop, ~10 min
- Remove from agar plate, wash in acetic acid, then water
- Image membrane or draw bands