Chapter 5: Structural Characterization of LDH

Purpose:
Learn how to:

1) Use gel filtration chromatography
2) Determine native molecular weight of proteins
3) Assay a mix of different standard proteins
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
Gel Filtration Chromatography

- Size-Exclusion Chromatography
- Technique to separate molecules by size
- Resin: Gel Matrix with particles or beads of a particular size with small pores
- Can be used for preparative or analytical separations
Gel Filtration Chromatography

- Very small molecules enter many pores in the gel, equilibrating between the gel and the moving buffer, and so travel slowly and are eluted later.

- Medium-sized molecules enter some pores in the gel, equilibrating between the gel and the moving buffer.

- Large molecules enter few pores in the gel, and so travel rapidly and are eluted sooner.
Types of Gel Filtration Resins

- **Cross-linked Dextran** – Sephadex
- **Cross-linked Agarose** – Bio-Gel A, Sepharose, Superose
- **Cross-linked Polyacrylamide** – Bio-Gel P
- **Combination** –
  - Dextran with Polyacrylamide – Sephacryl
  - Dextran with Agarose – Superdex
- **We are using Sephadex G-150**
  - Dry resin has pores with a maximum size of 150,000 Da
  - Resin will swell in water and aqueous salt solutions
  - Can fractionate proteins from 5,000-300,000 Da
Determining Molecular Weight from Gel Filtration

Linear Relationship between Log MW and $\frac{V_e}{V_o}$

$V_e$ = Elution Volume of Compound
$V_o$ = Void Volume of Column, volume of very large material that does not bind to any of the resin

$y = -1.06x + 6.77$

$R^2 = 0.97$

LDH Elution Point
Calculate MW from equation for graph
Column Packing and Running Review

1. Pipet in all resin on side of column, open stopcock, let settle, but DO NOT let column dry out!
2. Equilibrate column with buffer using buffer reservoir
3. Drain Buffer to surface, close stopcock
4. Apply sample with pipet to side of column, open stopcock
5. Let sample drain to surface, close stopcock

See p. 129 for more diagrams

Add buffer to top, attach buffer reservoir, open stop cock, collect with fraction collector
Procedure: Chapter 5-Week 1

- TF’s will pack and equilibrate column for you
- We will use buffer reservoirs and fraction collectors for this lab
- Using your final concentrated LDH sample, calculate volume needed to load 50-100 units
  
  \[
  \frac{2000 \text{ units}}{1 \text{ mL}} = \frac{50 \text{ units}}{x \text{ mL}}
  \]
  
  \[x = 0.025 \text{ mL}, 25 \mu \text{L}\]

- Add LDH to Calibration Proteins – total volume ~ 0.5 mL
- Drain buffer in column to surface and load protein down side of column

**Do not dry column! You will have very poor separation and possibly lose all your protein!**
After protein almost completely loaded, add buffer to top and attach buffer reservoir.

Collect fractions with fraction collector:

- About 25 drops/fraction (~0.5 mL/fraction, measure exact volume)

As calibration proteins move through column they will separate:

- **Ferritin** – Light yellow (MW = 440,000)
- **Catalase** – (MW = 220,000)
- **LDH**
- **Ovalbumin** – (MW = 45,000)
- **Myoglobin** – Light brown (MW = 17,300)

Dilute each fraction with 2.7 mL, 0.02 M KPO$_4$ Buffer
Procedure: Chapter 5-Week 1

- Start Analyzing Column Fractions – Find Peaks!
  - **Ferritin** – $A_{360}$ in your spectrophotometer
    - Peak of ferritin is your $V_o$
  - **Catalase** – Assay with KMnO$_4$
    - Time sensitive assay, $\downarrow$ # of aliquots = $\uparrow$ catalase activity
      
      \[
      \text{Catalase} \\
      2 \text{H}_2\text{O}_2 \rightleftharpoons 2 \text{H}_2\text{O} + \text{O}_2
      \]
      
      \[
      2 \text{KMnO}_4 + 5 \text{H}_2\text{O}_2 + 4 \text{H}_2\text{SO}_4 \rightleftharpoons 2 \text{MnSO}_4 + 5 \text{O}_2 + 2 \text{KHSO}_4 + 8 \text{H}_2\text{O} \\
      \text{Purple} \rightarrow \text{Colorless}
      \]
  - **LDH** – Activity Assay ($\Delta A_{340}$/min)
    - Use 50 µl of diluted fractions for assay
  - **Ovalbumin** – $A_{280}$ in UV-spectrophotometer
  - **Myoglobin** – $A_{410}$ in your spectrophotometer
Procedure: Chapter 5-Week 1

- First: Find **Ferritin** and **Myoglobin**
- Next: **LDH** Activity Assay, then **Ovalbumin**
- Last: **Catalase** Assay – Start at ferritin peak and assay to LDH peak

  - 50 µL of diluted fraction + 4.0 mL H₂O₂, mix, 6 min incubation, quench with 2.0 mL H₂SO₄
  - Add 100 µL aliquots of KMnO₄ until reaction stays pink
  - Fewest aliquots of KMnO₄ = Highest Activity of Catalase

\[
\text{Catalase} \quad 2 \text{H}_2\text{O}_2 \underset{\text{Purple}}{\overset{\text{Colorless}}{\rightleftharpoons}} 2 \text{H}_2\text{O} + \text{O}_2
\]

\[
2 \text{KMnO}_4 + 5 \text{H}_2\text{O}_2 + 4 \text{H}_2\text{SO}_4 \underset{\text{Purple}}{\overset{\text{Colorless}}{\rightleftharpoons}} 2 \text{MnSO}_4 + 5 \text{O}_2 + 2 \text{KHSO}_4 + 8 \text{H}_2\text{O}
\]

- *Need to invert catalase values for graphs*
Procedure: Chapter 5-Week 1

- Normalize All Peaks to 1.0 and Graph on 1 Axis

- Determine Native MW from $V_e/V_o$ vs Log MW graph
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Ferritin
Catalase
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