Concentration Determination and Activity Confirmation

Outline:

- **Using SDS-PAGE, determine which chromatography technique yields a pure protein.**
- **Determine the Abs_{280} of your enzyme. Use the molar absorptivity to determine the concentration**
- **Confirm that your enzyme is active and identify the best dilution for kinetic experiments.**

Purpose and Theory:

The experiments this week constitute the final pieces to the protein purification. We will be using two different techniques to determine the concentration of your pure LDH enzyme. Additionally, we'll be making sure that your enzyme is active and assessing the best concentration to use in the kinetics experiments that will be done during the last several weeks of the term.

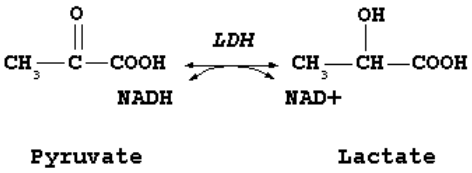
New Techniques:

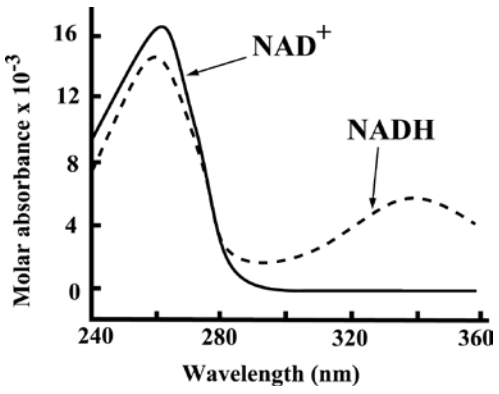
Concentration Assay

Beer's Law ($A=\varepsilon cl$) can be used to accurately determine analyte concentrations if the molar absorptivity is known. When dealing with proteins, it is most common to use the absorbance of 280 nm light by aromatic side chains – Trp (ε_{280} = 5690 M^{-1} cm^{-1}) and Tyr (ε_{280} = 1280 M^{-1} cm^{-1}). The ε_{280} of a protein can be approximated by adding up the total contribution from Trp and Tyr. This can become quite the tedious process, so fortunately automated algorithms are available online to do it for you (as you saw in your Bioinformatics assignment).

Activity Assay

Lactate dehydrogenase catalyzes the reversible reduction of pyruvate to lactate with the NADH/NAD+ cofactor used as a redox couple.





As seen here, the UV absorbance spectrum of nicotinamide is sensitive to the oxidation state. This feature is commonly exploited to monitor reaction progress of redox enzymes using NADH as a cofactor. Indeed, LDH kinetic parameters will be determined using this as our spectroscopic probe. If pyruvate, and NADH are mixed together with active LDH, NADH should be oxidized to NAD^+ with a corresponding decrease in Abs_{340}.

Procedure

1. **Set up the SDS-PAGE gel with instructor.**

2. **Retrieve the enzyme sample. Keep it on ice at all times!**

3. **Using the NanoDrop Spectrometer, determine the Abs_{280} of your enzyme.**
    a. Make sure to use cuvette mode.
    b. Blank the instrument with 100 μL of buffer.
    c. Carefully remove 10 μL and replace it with 10 μL of your protein. Determine Abs_{280}.
    d. If $Abs_{280} > 1$, you'll need to dilute your enzyme.
    e. Using ε_{280} (you should already know this), calculate the concentration of your enzyme.

4. **Activity Assay**

Your goal is to find a protein concentration where $\frac{\Delta abs}{\Delta t} \approx 0.5\ Abs/_{min}$

    a. Obtain one tube of pyruvate (300 mM) and one tube of NADH (6.6 mM).
    b. In separate tubes, make 100 μL of each dilution (use the Assay Buffer you made 2 weeks ago) – make sure to keep them on ice the whole time:
        i. 1:2
        ii. 1:5
        iii. 1:10
        iv. 1:20
    c. In five separate tubes, mix together
        i. 900 μL Assay Buffer
        ii. 33 μL LDH (make sure to label appropriately so that you know which dilution you're using.
        iii. 33 μL Pyruvate
    d. In a UV cuvette, blank the spectrophotometer (340 nm) with the mixture from part c.
    e. Make sure the spectrophotometer is set up to monitor the Abs340 every 2 seconds over the period of 2 minutes
    f. Add 33 μL NADH, quickly aspirate, and click "go". Repeat for the other LDH dilutions

5. **Kinetics of LDH (continue what you don't finish next week)**

Your goal is to determine k_{cat} and k_{cat}/K_M for LDH.

    a. Prepare serial dilutions of Pyruvate (300, 150, 75, 37.5, 18.75, 9.3725, and 4.69 mM)
    b. Using the best dilution that you found in part 4, repeat the kinetic assay for each [pyruvate].
    c. Plotting the initial rates vs. [pyruvate] will allow you to do a full kinetic analysis.