

Lab 3: Protein Determination and Enzyme Assay of Crab and Onion Samples

Khandi Coffman, Satoshi Sagami, Tramanh Do, Michaela Smith and Melissa Kindhart

Lab 3: Protein Determination and Enzyme Assay of Crab and Onion Samples

Introduction

The objective of this experiment is to determine the protein concentration of an onion sample and a crab meat sample, and to quantify the activity of the enzyme malate dehydrogenase (MDH). Spectrophotometry will be used to determine the protein concentration of the crab and onion samples using a dye reagent, Bio-Rad containing Coumassie blue dye, which binds to proteins. The absorbance at 595 nm is enhanced by protein binding making these readings a function of protein concentration. Absorbance readings for varying concentrations of BSA (bovine serum albumin) will be used to create a standard curve which will be used to determine the concentration of protein in the onion and crab samples.

Spectrophotometry will also be used to quantify the catalytic activity of malate dehydrogenase (MDH) by observing the formation of NADH. MDH catalyzes the interconversion of malate and oxaloacetate in the Krebs cycle with the help of the NAD/NADH co-enzyme system. NADH absorbs light at 340 nm so the absorbance values at this wavelength can be recorded over time to determine the rate of reaction.

Protein Determination

Preparing the Protein Standards (BSA)

1. Prepare 1 L of 10 mM TES buffer by weighing out 2.30 g of TES and diluting it to 1 L with deionized water.
2. Using a pH meter bring the pH of the TES buffer to 7.0 by adding the appropriate amount of acid or base.
3. Weigh 100.0 mg of BSA and place in a volumetric flask. Dilute to 100.0 mL USING the 10 mM TES buffer at pH = 7.0.
4. Swirl the mixture to dissolve all BSA. The resulting solution should have a protein concentration of 1.000 mg/mL.
5. Prepare 100 mL of dye reagent by diluting 1 part of the Bio-Rad concentrate with 4 parts of deionized water.
6. Make dilutions to prepare standards with the following concentrations: 1.000 mg/mL, 0.500 mg/mL, 0.100 mg/mL, 0.050 mg/mL, 0.010 mg/mL, and 0.005 mg/mL. Include a blank with only TES buffer as a reference for the spectrophotometer.
7. Prepare samples for standard protein determination by putting 0.100 mL of each of the standards into marked vials. You should have a total of 7 marked vials with protein

concentrations ranging from 0.000 mg/mL (buffer only) to 1.000 mg/mL.

8. Add 5.0 mL of prepared dye reagent to each of the vials containing standard protein.
9. Prepare samples for crab and onion protein determination by putting 0.100 mL of each extract into marked vials.
10. Add 5.000 mL of prepared dye reagent to each of the vials containing crab and onion.
11. Incubate all vials at room temperature for no less than 10 minutes and no more than 1 hour.
12. Determine absorbance of each sample at 595 nm. Obtain three consecutive absorbance readings for each sample and record the middle value on the data sheet.
13. Plot this data in Excel with mg of BSA on the x-axis and absorbance on the y-axis.
14. Use the trendline formula for the standard protein curve to determine the concentration of protein in the onion and crab samples.

Protein Extraction of Crab and Onion Samples

1. Weigh 2.50 g of onion and 2.50 g of crab and cut into small pieces.
2. Obtain 25.0 mL of TES Buffer at pH = 7.0 for **each** sample.
3. Grind each of your samples using a mortar and pestle and a small amount of the TES buffer.
4. Mix each of the ground samples in a beaker with the 25.0 mL of TES buffer.
5. Using cheese cloth, filter each sample into a clean beaker. When not using your samples, make sure to place them on ice.

Determination of Protein Concentration

1. Prepare 100 mL of dye reagent by diluting 1 part of the Bio-Rad concentrate with 4 parts of deionized water.
2. Prepare samples for standard protein determination by putting 0.100 mL of each of the standards into marked vials. You should have a total of 7 marked vials with protein concentrations ranging from 0.000 mg/mL (buffer only) to 1.000 mg/mL.

3. Add 5.0 mL of prepared dye reagent to each of the vials containing standard protein.
4. Prepare samples for crab and onion protein determination by putting 0.100 mL of each extract into marked vials.
5. Add 5.000 mL of prepared dye reagent to each of the vials containing crab and onion.
6. Incubate all vials at room temperature for no less than 10 minutes and no more than 1 hour.
7. Determine absorbance of each sample at 595 nm. Obtain three consecutive absorbance readings for each sample and record the middle value on the data sheet.
8. Plot this data in Excel with mg of BSA on the x-axis and absorbance on the y-axis.
9. Use the trendline formula for the standard protein curve to determine the concentration of protein in the onion and crab samples.

Determining the Catalytic Activity of MDH

1. Prepare 1L of TES buffer by diluting 2.30g of TES to 1L with Deionized water in a volumetric flask.
2. Using a pH meter, bring the TES buffer pH to 7.2 using NaOH.
3. Make 1L of MDH by mixing:
 - a) 1.3268g of NAD
 - b) 0.4000g Deoxycholic Acid
 - c) 2.6820g Malate

Dilute to 1L using Deionized water. The MDH substrate must be filtered until colorless. Multiple filtrations might be required.

4. Add 0.0027g Antimycin A to the substrate and dissolve. ****Wear gloves when handling Antimycin A ****
5. Ensure the pH is between 7.1-7.2.
6. Warm the MDH substrate in a hot water bath at 35°C until the temperature of the MDH substrate itself is 35°C.
7. On the Cary 50 UV-Vis spectrophotometer, zero it using TES buffer as the blank at 340nm.
8. Place 0.5mL of the crab or onion sample into separate vials.

9. Add 4.5mL of the heated MDH substrate into the crab or onion vials and invert.
10. Pour the samples into separate quartz cuvettes and read at 340nm on the spectrophotometer. Obtain three consecutive readings for each sample. Record the middle value on the data sheet.
11. Place the vials back in the 35°C water bath for 20 minutes.
12. Remove the vials from the water bath and swirl. Obtain three consecutive readings of absorbance again using the Cary 50 UV-Vis spectrophotometer at 340 nm. Record the time and the middle value on the data sheet.

Data Sheet and Calculations

Protein Determination

Absorbance Readings for BSA Standards, Onion Sample and Crab Sample									
Sample Number	1	2	3	4	5	6	7	8	9
mg BSA/tube	Buffer only	0.0005	0.001	0.005	0.01	0.05	0.1	onion	crab
A₅₉₅									

Using the equation from the standard protein curve, calculate the concentration of the onion and crab samples for your group.

Equation of the line from the graph _____

Protein concentration of onion _____

Protein concentration of crab _____

Calculations:

Data Sheet and Calculations

Determining the Catalytic Activity of MDH

Absorbance Readings for Onion and Crab Samples After Adding MDH Substrate

	Initial Time(min)	Initial Abs.	Final Time(min)	Final Abs.	Δ Time	Δ Absorbance
Onion						
Crab						

Using Beer's Law ($\Delta A = \epsilon b \Delta c$) calculate the MDH activity for the onion and crab. The units should be $\mu\text{mol}/\text{mg}/\text{min}$. The extinction coefficient, ϵ , for NADH is $6223 \text{ M}^{-1}\text{cm}^{-1}$.

Calculations: