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Enzyme Kinetics

Prior to lab you should:

- o Know how enzymes catalyze chemical reactions
- Know which enzyme will be studied in this laboratory exercise as well as its substrates and products.
- Explain how the spectrophotometer can be used to monitor the reaction catalyzed by this chemical reaction.
- Explain how the saturation kinetics of an enzyme can be studied.
- Explain the relationship between Km and Vmax.

I. Objectives:

- To analyze the reaction catalyzed by Adh using a spectrophotometer.
- Analyze the saturation kinetics of Adh for its substrate ethanol.

II. Background:

Enzyme Reaction

Alcohol Dehydrogense (Adh) catalyzes the chemical reaction outline below (Fig1). During the reaction ethanol loses an electron (and hydrogen) and becomes acetaldehyde. The electron is transferred to NAD+ to form NADH. This enzyme is found in almost all prokaryotic and eukaryotic organisms. In vertebrates, it helps detoxify ethanol. Adh oxidizes ethanol to form acetaldehyde. The acetaldehyde is then further oxidized by a second enzyme to form non-toxic acetic acid.



Figure 1: Chemical Reaction catalyzed by Adh

Measuring Enzyme Activity

The enzyme activity catalyzed by Adh is measured by monitoring the accumulation one of the products of the chemical reaction, NADH. Basically the enzyme reaction is started by mixing ethanol, NAD+ and Adh in a buffer. This mixture is placed into a spectrophotometer set to measure the absorbance of light at 340nm (Fig 2). The product, NADH, is the only molecule in the reaction that absorbs light at this wavelength. Therefore, as NADH is generated by the reaction, the amount of light (at 340nm) absorbed by the sample increases.



The faster the rate of the chemical reaction, the faster NADH will be formed, and the greater the change in absorbance as measured by the spectrophotometer. The rate of the reaction can be reported as change in the absorbance of light (340nm) per minute (ΔA_{340} /min) This rate can be measure most easily using the following steps.

- 1. Mix the substrates for the reaction (ethanol and NAD+) along with a buffer (Tris) in a cuvette.
- 2. Add the enzyme to the reaction. Rapidly mix the cuvette by inverting it and then place the cuvette in the spectrophotometer.
- 3. Immediately record the absorbance (Abs at T_0).
- 4. Record the absorbance after 1 min (Abs at T_1).
- 5. The rate of the reaction = (Abs at T_1) minus (Abs at T_0).
- 6. The unit for rate is $(\Delta A_{340}/\text{min})$.

Saturation Kinetics

An important way to characterize enzyme catalysis is to study its saturation kinetics. For a saturation experiment, the enzyme reaction is carried out using varying concentrations of one of the enzyme's substrates. At low substrate concentrations, as the substrate concentration increases the rate of the chemical reaction increases. However at higher substrate concentration, an increase in the substrate does not substantially increase the rate of the chemical reaction. This is because the active site of the enzyme as been "saturated". To graphically illustrate saturation, a Michaelis-Menton graph is generated. The data from the saturation experiment is plotted (substrate concentration on X axis and rate of the reaction (V) on the Y axis (Fig 3).



<u>x-axis:</u> concentration of substrate (ethanol) <u>y-axis:</u> rate of the reaction (change in absorbance/minute) V_{max} : the theoretical maximum rate of the chemical reaction approaches as the substrate concentration approaches infinity. <u>K_m</u>: an indicator of the affinity of the substrate for the enzyme. It corresponds to the concentration of substrate when the rate of the reaction is half of V_{max}

In this exercise, you will investigate the saturation kinetics of yeast Adh using its substrate ethanol. You will setup a series of enzyme reactions containing different concentrations of ethanol. For each concentration, you will measure the rate of the chemical reaction. Using this information you will prepare a Michaelis-Menton saturation plot. You will use this plot to estimate two characteristics of yeast Adh, its Vmax and its Km.

III. Materials:

- Spectrophotometer
- Micropipette (p200)/(p1000)
- Stop Watch
- Wash Bottle
- Parafilm
- Cuvettes
- Kimwipes

- Solutions:
 - o 15mM NAD+
 - Ethanol (.025M/.05M/.1M/ .375M/.75M/1.5M/3M)
 - Yeast Adh (20 units/ml)
 - o 20mM Tris Buffer (pH 8)

IV. Procedure:

1. Turn on the spectrophotometer. Make sure that it is set on absorbance mode and adjust wavelength to 340nm.

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2. Place a cuvette with distilled water into the chamber (**clear side should line up with arrows) and push the ZERO button.

3. To prepare your first enzyme assay

- Add 900 µl of Tris buffer to an empty cuvette
- Add 33 µl NAD+
- Add 33 μ l ethanol (3M)

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- 4. Working quickly but smoothly
 - Add 33 µl Adh to cuvette, mix by inverting, and place cuvette into spectrophotometer.
 - <u>Immediately</u> record the absorbance (Enter value into table 1 as the first trial for 3M ethanol, Abs at T₀)
 - After 1 minute record the absorbance. (Enter it into table 1, Abs at T₁)

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5. Repeat steps 3-4 for a total of three trials using the 3M ethanol.

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5. Repeat steps 3-4 using the other ethanol concentrations: 1.5M, 0.75M, 0.375M, 0.1M, 0.05M, and 0.025M (Record the data in table 1.



Figure 4: Setup enzyme reaction by adding Tris, NAD+, Adh, and a varying Ethanol concentration.

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V. Short Report:

1. Record data in table 1.

[Ethanol]	[Ethanal]	Trial	Initial Absorbance	Abcorbonce at 1min	Data (AA /min)	Ava
		Iriai	Initial Absorbance	Absorbance at 11111	Rate (ΔA_{340} /min)	Avg.
Stock Conc	Final Conc		(Abs at T_0).	(Abs at T_1)	(Abs at T_1) - (Abs at T_0)	Rate
3M	100m <u>M</u>	1				
		2				
		3				
1.5M	50mM	1				
		2				
		3				
.75M	25mM	1				
		2				
		3				
.375M	12.5mM	1				
		2				
		3				
.1M	3.3m <u>M</u>	1				
		2				
		3				
.05M	1.67m <u>M</u>	1				
		2				
		3				
.025M	0.83m <u>M</u>	1				
		2				
		3				

Table 1: Absorbance Measurements

2. Prepare Michaelis-Menton graph by plotting final concentration of ethanol against the rate of the reaction. Tape that graph in the space provide below. Be sure to label both axis and include units. (Indicate Vmax and Km on your graph.)

3. Explain whether your Michaelis-Menton graph shows the typical pattern expected for a saturation experiment. If not, explain possible errors involved in this type of investigation.

4. Explain why using higher concentrations of substrate does not produce a significant change in the rate of a chemical reaction after a certain point.