Physical Chemistry Laboratory [CHEM 335]
[Enzyme Kinetics]

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ABSTRACT

Enzyme kinetics is the study of the rate at which an enzyme works. Enzymes form a bound complex to their reactants (i.e. substrates) during the course of their catalysis and prior to the release of products. In this case, the enzyme was glucose oxidase, which catalyzed the conversion of glucose to gluconolactone, using KI (potassium iodide) as the catalyst. The reaction was repeated a total of 8 times, varying the volumes of both glucose oxidase and glucose and recording the absorbance readings of each assay, plotting this value against time to determine the rate of each reaction. The data obtained was then used to calculate the total enzyme concentration, Km, Ratemax, and the enzyme's turnover number. The values were **1.5625*10^-8(M), 0.0173 (M), 2.0072 (M/s),** and **1.28*10^8 molecules/s**.

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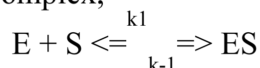
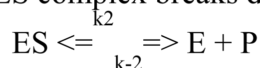
INTRODUCTION

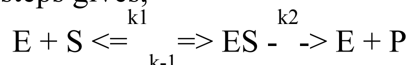
Living systems depend on chemical reactions which, on their own, would occur at extremely slow rates. Enzymes are protein catalysts that, like all catalysts, speed up the rate of a chemical reaction without being used up in the process. They achieve their effect by temporarily binding to the **substrate** and, in doing so, lowering the activation energy needed to convert it to a product. The study of the rate at which an enzyme works is called **enzyme kinetics**. There are several factors that influence the rate of reaction of an enzyme namely, pH, substrate concentration, the presence of inhibitors and temperature.
The catalyst-dependant reaction that converts substrate to product involves the formation of a transition state. The complex that forms, when substrate and enzyme combine is called the enzyme substrate (ES) complex,



Reaction products arise when the ES complex breaks down releasing the free enzyme,



Combining both elementary steps gives,



Therefore, we can describe the rate of product formation to be,

$$R = d[P]/dt = k2[ES]$$

The steady state approximation tells us that,

Rate ES formation = Rate of ES destruction

which means that d[ES]/dt is negligible, and because the enzyme is not used up,

$$[E]_T = [E] + [ES]$$

where [E]T, [E], and [ES] are the total enzyme concentration, free enzyme and bound enzyme respectively. Rewriting the steady state equation, solving for [ES], and assuming that [S] is very low gives us the following equations,

$$k1\{[E]_T-[ES]\}[S] = k\text{-}1[ES] + k2[ES]$$

$$[ES] = [E]_T[S] / \{[S] + Km\}$$

$$R = k2[E]_T[S] / Km$$

However, if [S] becomes very large, the rate approaches its limiting value,

$$R = k2[E]_T$$

and k2 becomes the turnover number. This is the number of substrate molecules the enzyme is able to process into product per unit time.

METHOD AND MATERIALS

The following materials and apparatus were required for this experiment:

- Glucose oxides (Sigma catalog #G-6125)
- Horseradish peroxidase (Sigma catalog #G-6140)
- 2-2`-azino-bis(3-theylbenzthiazoline-6-sulfonic acid)(Sigma catalog #A-1888)
- analytical grade B-D-glucose and D-mannose
- Sodium phosphate buffer materials
- Spectrophotometer with computer interface or spectrophotometer + timer
- Cuvets
- Micropipettes 5 ul ~ 1000 ul

The enzyme assays were to be carried out on solution mixtures of 1.0 ml total volume.

- A 0.1 M sodium phosphate buffer was prepared and adjusted to pH 7.0.
- A HRP/ABTS solution of concentration 50 mM ABTS and 25 Sigma units of HRP per ml were prepared.
- The glucose oxidase stock was about 0.5 sigma units per ml.
- The sugar stock, consisting of glucose and mannose, was made to 1.0 M
- The assays were prepared in the following way;
- Assay a1/B1 consisted of 700 ul of buffer, 100 ul of glucose oxidase stock, 100 ul of ABTS/HRP stock, and 100 ul of glucose stock. This gave us the final concentrations of glucose, glucose oxidase and ABTS/HRP.
- To prepare assay a2, we mixed 725 ul of buffer, 100 ul of ABTS/HRP, 100 ul of glucose and 75 ul of glucose oxidase.
- Likewise, to go from a1 to a3 we mixed 750 ul of buffer and 25 ul of glucose oxidase.
- Similar steps were taken to vary the substrate concentrations, keeping the enzyme concentration constant for B2-B4.

RESULTS

Total Enzyme Concentration

Sigma catalog for glucose oxidase => 25 ug/ml
But a10-fold dilution brings this to => 2.5 ug/ml. Since, mw of 160 kDa = 160,000 g/mol

the total enzyme concentration = $\frac{2.5 \times 10^{-6} \text{ g/ml} \times 1000 \text{ ml/L}}{160{,}000 \text{ mol/g}}$ = $\mathbf{1.5625 \times 10^{-8}}$ **mol/L**

Conversion from Absorbance units to Molar Concentration

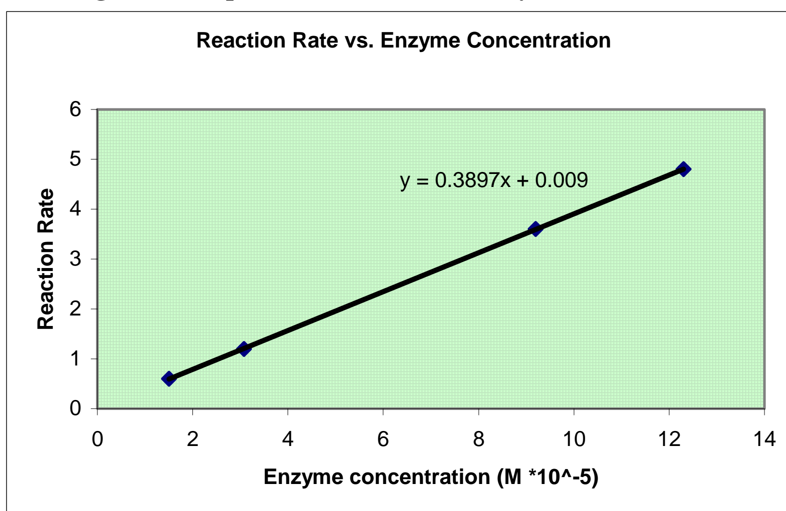
Using the Beer-Lambert law $A = \varepsilon cl$, we calculate molar concentration as

$c = A/2\varepsilon \Rightarrow A/3.9 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}$ (1 cm). The values for changing [E] are in table below. The value $3.9 \times 10^{4} \text{ M}^{-1} \text{ cm}^{-1}$ was used for the extinction coefficient because the stoichiometry of the reaction is two molecules of ABTS oxidized for every molecule of glucose oxidized.

Table 1. Molar concentrations for enzyme and substrate

	[E]	**[3E/4]**	**[E/4]**	**[E/8]**
Rate	4.8	3.6	1.2	0.6
Concentration (M/s)	1.26×10^{-4}	9.47×10^{-5}	3.16×10^{-5}	1.57×10^{-5}
	[S]	**[3S/4]**	**[S/4]**	**[S/8]**
Rate	4.8	3.9	3.5	2.0
Concentration (M/s)	1.26×10^{-4}	1.03×10^{-4}	9.21×10^{-5}	5.26×10^{-5}

Figure 1. Graph of reaction rate vs. enzyme concentration.



Calculation of changing [E] and [S] concentrations

Using this formula and substituting for each trials [E] or [S] volume gives the table below:

$$\frac{\text{Standard concentration} \times \text{trial [E] volume}}{\text{Total trial concentration}}$$

For example, in calculating [S] we use,

$$\frac{1.0\ (\text{mol/L}) \times 100 \times 10^{-6}\ (\text{L})}{1000 \times 10^{-6}\ (\text{L})} = 0.1\ (\text{M})$$

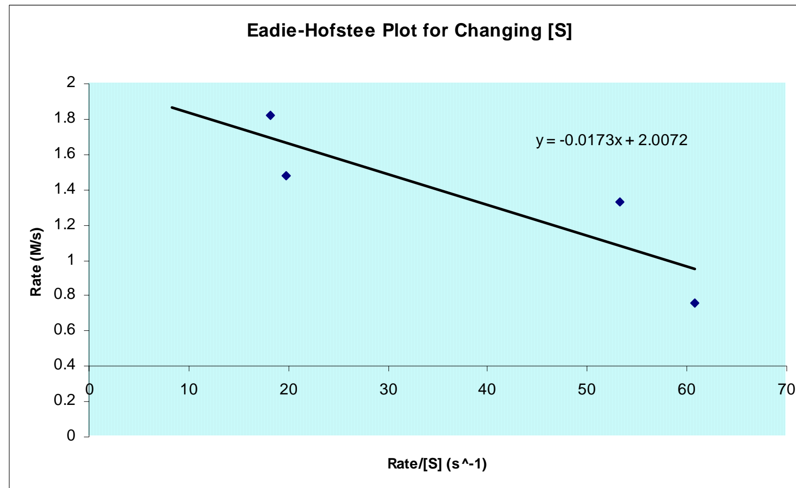
Table 2. Changing [E] and [S] concentrations

	[E]	**[3E/4]**	**[E/4]**	**[E/8]**	**[S]**	**[3S/4]**	**[S/4]**	**[S/8]**
Volume (ul)	100	75	25	12.5	100	75	25	12.5
Conc (M)	1.26E-4	9.47E-5	3.16E-5	1.58E-5	0.1	0.075	0.025	0.0125

Table 3. The values for Rate and Rate/[S] for the Eadie-Hofstee plot.

	[S]	**[3S/4]**	**[S/4]**	**[S/8]**
[S] (M)	0.1	0.075	0.025	0.0125
Rate * 10^-3	1.824	1.482	1.330	0.760
Rate/[S]	18.24	19.76	53.20	60.80

Figure 2. The Eadie-Hofstee plot used to calculate km, $Rate_{max}$, and K_2.



The slope of the above graph gives –km values, therefore by simply looking we can see that the km value is **0.0173 M**. The y-intercept should give the $Rate_{max,}$ which comes out to be **2.0072 M/s**. Therefore, using the $K_2 = Rate_{max} /[E]_{T,}$ K_2 (turnover number) comes out to be **1.28 x 10^8** molecules/s**.** This figure refers to the number of molecules of substrate that the enzyme processes per second.

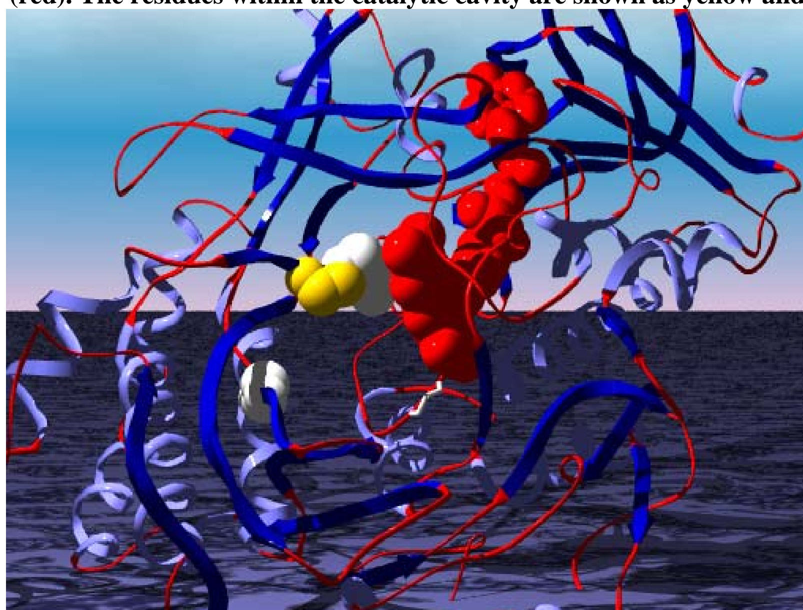
Responses to questions.

1. Glucose oxidase is very specific for the stereo isomer D-glucose. However, it does oxidize other reducing sugars like mannose, albeit at a much slower rate. This is because, although mannose and glucose have the same molecular formula, they have different orientations and so can not both fit into a structure specific site equally. Remember that mannose is a stereoisomer of glucose.
2. Recall that under neutral conditions the reaction process follows that glucose oxidase removes protons and electrons from its substrate glucose. This leads to the formation of gluconolactone, which hydrolyzes spontaneously into gluconic

acid. Basically, glucose is acting like a base, if you will. In basic buffer conditions (pH), the buffer can now deprotonate the gluconic acid formed, causing a forward shift in the production of gluconic acid from gluconolactone as well as a depletion of gluconolactone. This in turn causes a forward shift in the production of gluconolactone by glucose oxidase from glucose. In summary, the rate of the reaction is increased.

Under acidic buffer conditions, according to Harold Bright, the combining of the substrate to the oxidized enzymes is dependent on a basic group in the enzyme, so the buffer would create a sort of shielding effect, reducing the enzymes affinity for the substrate glucose. This of course, reduces the reaction rate

Fig. 3. A POV-RAY rendered image of the enzyme showing the proximity of the active site to the FAD cofactor (red). The residues within the catalytic cavity are shown as yellow and white spheres.



References

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