
A. Introduction

Enzymes are one of the largest groups of proteins in cells. They perform two fundamental and extremely important tasks in the cell: a) they are biological catalysts, that is, they increase the rate of chemical reactions to a useful level, and b) they provide a point where chemical reaction rate can be regulated to meet the moment-to-moment needs of the cell.

One very important characteristic of an enzyme is its specificity, i.e., an enzyme's inherent nature to bind with a specific substrate, or a family of similar substrates, to form a specific product or family of similar products. An enzyme's specificity is based on the 3-D structure and chemistry of its active site, where a few critical R groups are positioned to interact with a few specific functional groups on the substrate.

There are five distinct types or degrees of specificity:

- **Absolute specificity** - the enzyme will bind only one specific substrate and catalyze only one reaction.
- **Relative specificity** - the enzyme binds with a group of similar substrates and catalyzes a group of similar reactions.
- **Group specificity** - the enzyme will act only on molecules that have specific functional groups, such as amino, phosphate and methyl groups.
- **Linkage specificity** - the enzyme will act on a particular type of chemical bond regardless of the rest of the molecular structure.
- **Stereochemical specificity** - the enzyme will act on a particular steric or optical isomer.

Understanding an enzyme's or another protein's specificity is key to understanding the reaction it catalyzes and the cellular process it mediates. The type or degree of specificity of a given enzyme can be determined through a **kinetic analysis** of the enzyme-catalyzed reaction. The
purpose of today’s exercise is to characterize the substrate specificity of the enzyme glucose oxidase by determining the substrate binding affinity of glucose oxidase for two monosaccharides, D-glucose and D-xylose.

B. Glucose Oxidase

The Enzyme

Glucose oxidase was discovered by D. Muller, 1928, in the molds *Aspergillus niger* and *Penicillium glaucum*. The enzyme is a dimer (contains two subunits) that catalyzes the oxidation of D-glucose to gluconic acid using molecular oxygen (see reaction below). Note that hydrogen peroxide (H$_2$O$_2$) is also a product of the reaction.

\[
\text{D-Glucose} + \text{H}_2\text{O} + \text{O}_2 \rightarrow \text{Gluconic Acid} + \text{H}_2\text{O}_2
\]

The enzyme requires the prosthetic group FAD (flavin adenine dinucleotide) to carry out the reaction. FAD is commonly used in biological oxidation-reduction reactions as an intermediate electron acceptor / donor. In the figure to the right, FAD is shown in pink.

The normal biological function of glucose oxidase is to produce hydrogen peroxide. Hydrogen peroxide is a toxic compound that is used by cells to kill bacteria. Glucose oxidase is found on the surface of various fungi, where it helps protect against bacterial infection, and it is also found in honey, where it acts as a natural preservative.

While the natural function of glucose oxidase seems minor, the enzyme is the most critical part of blood glucose meters, an industry estimated to be worth over $5 billion annually. In a typical blood glucose meter, the enzyme is trapped inside a membrane. Blood enters the meter, and the blood glucose is converted to gluconic acids and hydrogen peroxide by the enzyme. The hydrogen peroxide is sensed by a platinum electrode. The more glucose there is in the sample, the more peroxide is formed, and the stronger the signal at the electrode.
The experimental question we are asking in this lab is *How specific is Glucose Oxidase for D-glucose?* Will the enzyme productively use empirical isomers of D-glucose, such as D-xylose, as substrates? These questions deal with the concept of substrate specificity of the enzyme. We will be analyzing the substrate specificity of glucose oxidase by performing a kinetic analysis of the enzyme in the presence of D-glucose and D-xylose.

**The Glucose Oxidase Assay**

In a kinetic analysis, the velocity of a reaction is usually measured by measuring the rate of consumption of the substrate or the rate of production of the product of the reaction. As is frequently the case in kinetic analyses, measuring substrate consumption or product production is challenging because they often require laborious analytical procedures.

A relatively easy way to measure the velocity of a reaction is by modifying the reaction so that the reaction leads to a change in the color of the reaction medium. This is usually done by adding an artificial substrate, the consumption of which leads to the color change. Reaction velocity can be then determined by measuring the velocity of the color change, which can be done with a simple spectrophotometer. This type of assay is called a *colorimetric assay.*

A colorimetric assay has been developed for glucose oxidase. In this assay, a second enzyme-catalyzed reaction, one that utilizes the enzyme *peroxidase* and an artificial substrate, *ABTS,* is added to the glucose oxidase reaction described above. In this second reaction (see reaction below), the reduced form of ABTS, which is colorless, is oxidized by the hydrogen peroxide generated in the glucose oxidase reaction. The final oxidized product, the oxidized form of ABTS, is blue-green in color, and has an absorption maximum of 420nm. The oxidation of ABTS and the associated color change can easily be measured spectrophotometrically.

\[
\text{H}_2\text{O}_2 + \text{reduced ABTS (clear)} \rightarrow \text{H}_2\text{O} + \text{oxidized ABTS (blue-green)}
\]

In the glucose oxidase assay, then, our measure of the rate of the glucose oxidase reaction is accomplished through a measure of the rate of the peroxidase reaction, which is determined by measuring the rate of appearance of the oxidized ABTS. We will measure the rate of appearance of oxidized ABTS by spectrophotometrically measuring the rate of blue-green color formation.
C. Enzyme Kinetics

Basic Michaelis-Menten Kinetics & the Michaelis-Menten Reaction

The specificity of an enzyme is determined by determining the **substrate-binding affinities** of an enzyme for several different substrates. These substrate-binding affinities show how selective an enzyme is for a particular substrate or group of similar substrates.

To determine substrate-binding affinities of an enzyme for different substrates, a kinetic analysis must be performed on the enzyme in the presence of varying concentrations of each substrate. The specific objective of a kinetic analysis is to determine the quantitative relationship between the initial velocity of the reaction ($v_i$ or $v_0$) and substrate concentration [$S$]. From this relationship, the substrate-binding affinity can be determined.

The graph showing the quantitative relationship between $v_i$ and [$S$] in a simple, generic enzyme-catalyzed reaction is shown to the right. This graph is called a **Michaelis-Menten Graph**. As the Michaelis-Menten graph shows, the relationship between $v_i$ and [$S$] in a basic enzyme-catalyzed reaction is defined by a **rectangular hyperbola** that exhibits a **maximum velocity** ($V_{max}$). The rectangular hyperbola and the presence of a $V_{max}$ indicates that in an enzyme-catalyzed reaction, the substrate must first bind to the enzyme to form an enzyme-substrate complex for product to be formed. Or, in other words, the enzyme is equipped with a **substrate-binding site**, a.k.a **active site**.

This means that an enzyme-catalyzed reaction is, in its simplest form, a two-step reaction. This reaction, known as the **Michaelis-Menten Reaction**, is summarized below.

\[
[E] + [S] \rightleftharpoons [ES] \rightleftharpoons [E] + [P]
\]

Reaction #1 of this two-step reaction is known as the **substrate-binding reaction**. During this reaction, substrate binds to the active site of the enzyme.

Reaction #2 is **catalysis**. During catalysis, substrate activation and product formation occurs.
KM and Vmax

Two important parameters that help quantitatively describe the performance of an enzyme can be obtained from kinetic data:

1) The maximum velocity of the reaction ($V_{max}$). The $V_{max}$ of a given reaction is equivalent to the maximum velocity of the catalytic event, which occurs in reaction #2. Thus, $V_{max}$ is a measure of the velocity of reaction #2.

2) The Michaelis-Menten constant ($K_M$). The $K_M$ is an index of substrate-binding affinity, i.e., how strongly the substrate binds to the active site on the enzyme, and is thus a measure of enzyme specificity. Arithmetically, $K_M$ is the substrate concentration ([S]) that produces $\frac{1}{2} V_{max}$. $K_M$ and substrate-binding affinity are inversely related, i.e., the smaller the $K_M$, the greater the substrate-binding affinity.

Both of these parameters appear as terms in the generic equation for the rectangular hyperbola one gets from a kinetic analysis: $V_i = (V_{max} \times [S]) / (K_M + [S])$
Determining the KM and Vmax from Kinetic Data

The Vmax and the KM are fairly easy to determine from the results of a kinetic analysis. There are two ways of determining the numbers. One way is to determine the numbers directly from the equation for the rectangular hyperbola defined by your data (see fig. below). For this you need a computer and software capable of accurately calculating the equation for the hyperbola. This is preferred by enzyme biochemists and statisticians. If you do not have a software package capable of accurately calculating the equation for the hyperbola, you are forced to plot a double-reciprocal plot of the data (1/vi vs. 1/[S]). When the data are plotted in this way, the straight line results, and the Vamx and Km can be determined from the X and Y intercepts.

D. This Week’s Experiment

Introduction

The normal biological function of glucose oxidase is to produce hydrogen peroxide from glucose. The hydrogen peroxide produced is a toxic compound that is used by cells to kill bacteria.

The scientific questions we are asking in this lab are

- How specific is glucose oxidase for D-glucose?
- Will the enzyme productively use other monosaccharides, such as D-xylose, as substrates?
- How does the substrate-binding affinity of glucose oxidase for D-glucose compare with that of another sugar, like D-xylose?

The specific objective of today’s experiment is to characterize the substrate specificity of the enzyme glucose oxidase by determining the substrate binding affinity of glucose oxidase for two monosaccharides: D-glucose and D-xylose.

Substrate-binding affinity will be measured by determining the Michaelis-Menten Constant (Km) for the enzyme in the presence of each of the two substrates.
Experimental Design

This week’s experiment is an observational study designed to characterize the substrate specificity of the enzyme glucose oxidase by determining the substrate binding affinity of glucose oxidase for two monosaccharides: D-glucose and D-xylose, with no application of an experimental treatment. As such, there are no treatment or control groups. To learn more about observational studies and experimental design, students should refer to the appendix page on Experimental Design.

In this exercise, each pair of students will perform two kinetic analyses of glucose oxidase. One in the presence of the substrate D-glucose, and one in the presence of the substrate D-xylose.

Both D-glucose and D-xylose are monosaccharides. D-glucose is a hexose (C₆H₁₂O₆). D-xylose is a pentose (C₅H₁₀O₅).

The kinetic analyses will employ the colorimetric glucose oxidase assay, which involves measuring the formation of the oxidized form of the artificial dye ABTS.

Each kinetic analysis will involve measuring the reaction color change at 7 different substrate concentrations, ranging from 20mM to 200mM. Three independent sets of assays will be performed for each substrate concentration.

Statistics and Analysis

The descriptive statistics used to describe the data are mean, as a measure of central tendency, and standard deviation, as a measure of data dispersion around the mean. For each of the two substrates, each pair of students will calculate the mean initial velocity (Vi) and the standard deviation of each of the 7 substrate concentrations used in the study. The raw data and descriptive statistics will be presented in a data table. The mean Rf will also be graphically displayed on a scatter plot with error bars equal to 2 X SE.

Equipment & Materials

1. A series of 7 solutions of different concentrations of glucose (A - G)
   1. glu-a: 20 mM glucose
   2. glu-b: 30 mM glucose
   3. glu-c: 40 mM glucose
   4. glu-d: 50 mM glucose
   5. glu-e: 100 mM glucose
   6. glu-f: 150 mM glucose
   7. glu-g: 200 mM glucose
2. A series of 7 solutions of different concentrations of xylose (A - G)
   1. xyl-a: 20 mM xylose
   2. xyl-b: 30 mM xylose
   3. xyl-c: 40 mM xylose
   4. xyl-d: 50 mM xylose
   5. xyl-e: 100 mM xylose
   6. xyl-f: 150 mM xylose
   7. xyl-g: 200 mM xylose

3. A bottle containing Assay Cocktail A (see formulation below).
4. A bottle containing Assay Cocktail B (see formulation below).
5. 5 ml pipets
6. Blue automatic pipettors (100 - 1000 ul)
7. pipet tips
8. A Genesys 20 spectrophotometer

The Assay Cocktails

Two assay cocktails have been formulated. Each contains all essential ingredients for the reaction except the substrate.

Cocktail A will be used in the analysis with glucose.

Cocktail B will be used in the analysis with xylose.

The ingredients for each cocktail are listed below. Note that both cocktails are identical, except for the glucose oxidase concentration, which is higher in cocktail B to insure the reaction goes fast enough when xylose is used as substrate.

<table>
<thead>
<tr>
<th>Cocktail A</th>
<th>Cocktail B</th>
</tr>
</thead>
<tbody>
<tr>
<td>used when glucose is the substrate</td>
<td>used when glucose is the substrate</td>
</tr>
<tr>
<td>1. Glucose Oxidase: 0.004 mg/ml</td>
<td>1. Glucose Oxidase: 0.4 mg/ml</td>
</tr>
<tr>
<td>3. ABTS: 2.78 mM</td>
<td>ABTS: 2.78 mM</td>
</tr>
</tbody>
</table>
Experimental Protocol

Preparation

A. Turn on the spectrophotometer and allow it to warm up.

Before the start of the experiment, make sure the spectrophotometer is turned on. Allow the spectrophotometer to warm-up for at least 30 minutes before using it.

Note: A spectrophotometer should always be turned on 30 minutes before its use. The lamp that serves as the source of incident light must warm up to produce a stable incident light.

Set the measurement mode of the instrument to absorbance.

Set the wavelength to 420 nm, the absorption maximum for oxidized ABTS.

B. Prepare the assay cuvettes.

1. Obtain 44 disposable cuvettes and divide them into 2 sets of 22.

2. Label each of the 22 cuvettes in one set with a "G" for glucose. These 22 will be used when glucose is the substrate in the assay. Label each of the 22 cuvettes in the other set with an "X" for xylose. These will be used when xylose is the substrate in the assay. Remember to always label the cuvette on the top frosted half of the cuvette.

3. Add 0.9 ml of assay cocktail A to each of the 22 cuvettes labeled "G".
4. Add 0.9 ml of assay cocktail B to each of the 22 cuvettes labeled "X".

With your preparation complete, you are ready to run the assays.

**Assay Protocols**

Before you begin the following assays, type your name into the data Table 1 in workbook 1.

**A. Kinetic analysis of glucose oxidase with glucose as the substrate.**

1. Take one of the cuvettes labeled "G" and label it to identify it as the blank. Then add 0.1 ml dH2O to it, invert it 2X to mix, then set it aside. You will use this blank through your kinetic analysis with glucose as the substrate.

2. Arrange the 21 remaining cuvettes in three sets of 7. With these 3 sets of 7 cuvettes you will be assaying the 7 glucose solutions (A through G) in triplicate (replicates 1, 2 and 3).

3. With the spectrophotometer set to read the absorbance of 420 nm light, perform the following assay on each of the 7 glucose solutions in triplicate. For each assay, do the following:
   
   a. Zero the spectrophotometer with the blank. This will correct for any nonspecific background absorption of the 420 nm light.
   
   b. Perform the following steps rapidly and carefully.
      
      1. With a pipettor, add 0.1 ml of the desired glucose solution to one of the cuvettes, immediately cover the cuvette with your gloved finger and invert it 2X to mix (Timer: start the timer at the second inversion.)
      
      2. Place the cuvette in the spectrophotometer and close the cuvette holder.
      
      3. Record the absorbance (A$_{420}$) at 15-second intervals for 60 seconds (that is, at 15s, 30s, 45s, and 60s) in Table 1, Workbook 1.
      
      4. Repeat this assay on the next cuvette until you have performed the assay on each of the 7 glucose solutions in triplicate.

**B. Kinetic analysis of glucose oxidase with xylose as the substrate.**

1. Take one of the cuvettes labeled "X" and label it to identify it as the blank. Then add 0.1 ml dH2O to it, invert it 2X to mix, then set it aside. You will use this blank through your kinetic analysis with glucose as the substrate.
2. Arrange the 21 remaining cuvettes in three sets of 7. With these 3 sets of 7 cuvettes you will be assaying the 7 xylose solutions (A through G) in triplicate (replicates 1, 2 and 3).

3. With the spectrophotometer set to read the absorbance of 420 nm light, perform the following assay on each of the 7 xylose solutions in triplicate. For each assay, do the following:

   a. Zero the spectrophotometer with the blank. This will correct for any nonspecific background absorption of the 420 nm light.

   b. Perform the following steps rapidly and carefully.

      1. With a pipettor, add 0.1 ml of the desired xylose solution to one of the cuvettes, immediately cover the cuvette with your gloved finger and invert it 2X to mix (Timer: start the timer at the second inversion.)

      2. Place the cuvette in the spectrophotometer and close the cuvette holder.

      3. Record the absorbance (A<sub>420</sub>) at 15-second intervals for 60 seconds (that is, at 15s, 30s, 45s, and 60s) in Table 1, Workbook 1.

      4. Repeat this assay on the next cuvette until you have performed the assay on each of the 7 xylose solutions in triplicate.

**C. Save the Excel Worksheet**

When all of the data have been collected & recorded, save Workbook 1 to your computer. When you save the file, it is recommended you change the filename to include your name to distinguish the file from the original.

**D. Homework Assignment**

The raw data you collected today requires a significant amount of further calculation and analysis to acquire the final kinetic results. Your instructor will give you a homework assignment that will require you to determine the Vmax and KM of the glucose oxidase reaction in the presence of each of the substrates. Make sure you understand the assignment before you leave lab today.

**E. Clean Up**

Once you saved the Excel table to you computer, today's experiment is complete. Before you shut down, you should make sure the Excel file is saved to your computer. You can logout of the lab manual. Before you leave you must clean up your place so it looks the way it did when you walked into lab today.
E. Bibliography