Enzyme assays are used to study the rates of enzyme catalyzed reactions.

## Introduction

Enzyme assays have many applications in enzyme kinetics. Understanding the rates of reactions can help determine the mechanism that the reaction follows (a single-substrate or multiple-substrate mechanism). Figure 1 below demonstrates how enzymes change a reaction's mechanism by lowering the activation energy.

![Potential Energy Profile](image)

Fig. 1 - A Potential Energy Profile demonstrating the affect adding an enzyme has in a reaction's mechanism.

### Michaelis-Menten Kinetics

#### Enzyme Activity

Enzyme activity is a measure of how much enzyme is present in a reaction. There are two ways to measure enzyme activity: monitoring the disappearance of substrate or the appearance of product. Measuring the appearance of product is usually more accurate because detecting small changes in [P] (when [P]=0) is easier to measure than detecting small changes in [S].

Through Michaelis-Menten kinetics, enzyme assays are used to calculate the enzyme's $K_m$ for a specific substrate, $V_{max}$, and $K_i$ for inhibitors. Enzyme assays can also reveal information about the substrates and inhibitors that may affect the enzyme. Figure 2 below uses a fixed-time assay to demonstrate the effect adding enzyme inhibitors have in a reaction. Details of enzyme-substrate reaction rates are further described by the Michaelis-Menten Kinetic Model.
Micromoles of substrate converted to product per minute (μmol P/min), or International Units (IU), are commonly used units to express reaction rates. However, different measurements are used to quantify enzyme activity, including:

- the specific activity of an enzyme: (# IU/mg of protein)
- the turnover number (k\text{cat}) of an enzyme: (IU/μmol enzyme)
- the catalytic center activity of an enzyme: (turnover #/# of catalytic sites)

**Assay Validity**

An enzyme assay must be considered valid to ensure accurate data and calculations. In order for an enzyme assay to be valid, it must meet multiple requirements:

- **Initial rates are being measured.** \(v_0\) must be both reproducible and dependent on [E]. Measuring initial rates demonstrates whether or not the product has reached a substantial concentration. Valid assay conditions exist if the product has consumed less than 5% of the substrate.
- **pH is constant:** Enzymes have specific pH ranges of activity due to structural sensitivity to proteins which cause enzymes to be sensitive to pH changes. A prepared buffer solution is used to keep the pH constant so that the pH resist changes. Good buffers do not cross membranes; do not absorb light; are chemically stable; and are biochemically inert.
- **\(V_0\) is proportional to the concentration of the enzyme, [E].** An assay is only valid when a plot of \(V_0\) vs [E] is linear because the enzyme must be the only limiting factor to the substrate concentration. Additionally, when \(V_0\) and [E] are proportional, the presence of effectors are measurable and one can determine if an inhibitor is competitive, noncompetitive, or uncompetitive.
- **\(V_0\) is corrected for non-enzymatic conversion:** \(V_0(\text{corrected}) = V_0(\text{+enzyme}) - V_0(\text{-enzyme})\). A control factor must be measured while conducting enzyme assays in order to ensure accurate calculations. For non-enzyme controls, buffers are used in place of enzymes.
Control Factors

When assaying enzyme activity many factors are considered to ensure proper results. Changes in these factors may lead to improper data and calculations.

- **Salt Concentration**: High salt concentration disrupts the weak bonds involved in enzyme folding and substrate binding. This can affect the enzyme’s ability to catalyze a reaction.

- **Enzyme and Substrate Concentration**: Enzyme activity is inhibited by enzyme saturation. Although increasing substrate concentration increases enzyme activity, the reaction rate will not increase after an enzyme has reached its saturation point. Precise enzyme and substrate measurements must be calculated to ensure optimum activity.

- **pH Dependence**: Most enzymes have an optimal pH at which their reaction is catalyzed the fastest. A pH-enzyme activity assay can show the likely amino acids of the catalytic site. Often enzymes operate at a maximum activity when the pH is close to the pKₐ of the enzyme’s active site.

- **Inhibition**: Inhibitors can decrease reaction rates via binding to the enzyme in the active site, or binding to a region of the enzyme that inactivates the substrate binding site. Since reactions are reversible, products of the reaction can often inhibit the enzyme by binding the active site. Dialysis is often used to remove enzyme inhibitors.

- **Activators**: Activators increase the activity of an enzyme. In the absence of a necessary activator, other enzymes can compete for substrates or used products. Additional chemicals may be needed to achieve necessary concentrations of activators, reactants, and co-factors.

- **Temperature dependence**: Many enzymes have an optimal temperature that can be found by measuring reaction rates with varying temperatures. Reaction rates usually increase with temperature, however high temperatures usually denature and give no activity of the enzyme.

TYPES OF ASSAYS

Enzyme assays measure either the disappearance of substrate over time or the appearance of product over time. Multiple methods have been developed to measure the concentration of substrates or products in a reaction, but all enzyme assays fall into two types: fixed-timed and continuous.

**Fixed-Timed**

The fixed-time (discontinuous) assay measures enzyme concentration in fixed periods of time. A common fixed-time assay method is using a microplate reader to read multiple solution concentrations. Multiple dilutions series are placed into microplate wells: dilution series for the substrate; dilution series for the enzyme; and dilution series for the substrate + enzyme. To start the fixed-time assay a start solution is added to all the wells. After the reactions start, the solutions are incubated for a fixed-period of time: this period of time can be determined from a continuous enzyme assay. To stop the reactions, a stop solution is added to prohibit the enzyme from reacting with the substrate. With fixed-timed assays, one can measure many assays simultaneously. Figure 3 below is an example of a microplate fixed-time assay procedure outline and Figure 4 demonstrates an example microplate layout of a substrate, enzyme, and enzyme + substrate dilution series.
**Performing a Fixed Time Assay**

**Preparation**

1. Obtain required amount of buffer
2. Obtain required amount of substrate
3. Obtain required amount of enzyme
4. Obtain required amount of stop solution
5. Obtain required amount of final volume

**Performing Substrate Standards**

1. Dispense 125 µl of buffer
   - Row A & B, Column 1
2. Dispense 125 µl of substrate
   - Row A & B, Column 1
3. Transfer 125 µl from Column 1
   - Rows A, B, C, 1-3
   - Use multichannel pipette
   - Mix well
   - Discard last 125 µl
4. Dispense 125 µl of stop solution
   - Rows A & B, Column 1
   - Use multichannel pipette
   - Mix well
   - Final volume is 250 µl

**Fixed Time Assay Preparation**

1. Dispense 125 µl of buffer
   - Rows C, D, E, F, G, 1-5
   - Use multichannel pipette
2. Dispense 125 µl of buffer
   - Column 6
3. Transfer 10 µl of enzyme dilution
   - Column 1-6, Row A, B, C, D, E
   - Use multichannel pipette
   - Mix well
   - Discard last 10 µl
4. Dispense 80 µl of stop solution
   - Columns 1-6
   - Use multichannel pipette
   - Final volume is 250 µl

**Microplate Reader**

1. Take plate to microplate reader
2. Start reading program
3. Print out results
4. Record data

**Fig. 3** - A procedure flow chart for performing a fixed-timed assay on a microplate. The amount of solutions transferred will vary between experiments, but the main concept is that the solutions are diluted.

**Fig. 4** - A sample fixed-time assay microplate layout. Column 7 will typically contain the blanks (controls).

The continuous assay uses a spectrophotometer to measure the appearance of product, or disappearance of substrate in real-time. With continuous assays, one can measure the linearity of the assay which can be used to conduct a fixed-timed assay. For best enzyme activity results, the optimum pH of an enzyme must be determined before conducting a continuous enzyme assay. The disadvantage of a continuous assay is that only one reaction can be measured at a time, but the advantage is the convenience of easily measurable reaction rates. Figure 5 below outlines basic procedures for performing a continuous assay and Figure 6 demonstrates how to determine linearity.
Fig. 5 - A continuous assay procedure flow chart. This flow chart demonstrates basic procedures for performing a continuous assay and using the spectrophotometer. Kinetics for the spectrophotometer vary between what enzymes are used. Note that maintaining temperature is important for the enzyme and substrate.

Fig. 6 - A spectrophotometer continuous enzyme assay readout of absorbance vs time. Blue indicates when the assay is valid (linear initial rates), red indicates when the assay is no longer valid (non-linear initial rates).

Spectrophotometric Assays

The spectrophotometric assay is the most common method of detection in enzyme assays. The assay uses a spectrophotometer, a machine used to measure the amount of light a substance's absorbs, to combine kinetic measurements and Beer's law by calculating the appearance of product or disappearance of substrate concentrations. The spectrophotometric assay is simple, non-destructive, selective, and sensitive. For example, the NADH/NAD$^+$ molecule is often used in enzymatic oxidation/reduction reactions. During these reactions NADH is often oxidized to NAD$^+$, or NAD$^+$ is reduced to NADH. NADH absorbs light at 340 nm, however NAD$^+$ does not hold that property. A spectrophotometer can be used to measure the change in absorbance of 340 nm light, thus indicating a change in amount of NADH.
Coupling Reactions

In many reactions, changes in substrates or products are not observable by spectrophotometric methods because they do not absorb light. These reactions can be measured by coupling them to enzymes that can be detected via a spectrophotometer. Light absorbing non-physiological substrates or products are synthesized for enzymes with substrates and products that do not absorb light. Visit the spectrophotometry page to learn more about the spectrophotometer assay.

Outside Links


References


Problems

1. An assay measuring conversion of pyruvate to lactate in the presence of lactate dehydrogenase with excess pyruvate gives a decrease in absorbance of 0.215 Abs/min. How many IU's are present in the assay?
   
   **Solution:**

2. An α-Amylase graph measures the initial velocity (IU) vs mg of enzyme. The graph yields an equation of $y=2.58x$; calculate the enzyme's specific activity and turnover number.
   
   **Solution:** specific activity=2.6 IU/mg, turnover #=270 IU/μmol.

3. An enzyme assay involves a 1/20 diluted enzyme solution (initial protein concentration of 0.086 mg/ml). 50 μl of an enzyme stock solution was added to start the reaction. Calculate the amount of protein in the assay if the stock solution had been diluted 3 times.
   
   **Solution:** 7.1 x 10^{-5} mg

4. An enzyme assay measuring the disappearance of product was conducted using different dilutions of the enzyme; results are shown in the following graph of absorbance vs time. Explain each curve and name what dilution should be used for further experiments.
Solution: A - The curve is represents an enzyme that is too diluted, yielding results that are too slow to measure. B - The curve represents a good enzyme dilution because it is linear. This dilution should be used for further experiments. C - The curve represents an enzyme that is too undiluted, yielding results that are too fast to measure.

5. A fixed time assay measured the same reaction 4 times, yielded the following absorbances: 1.59, 1.42, 1.64, and 1.54. If the assay ran over a span of 3 minutes and had a corrected absorbance of 0.90 (also measured for a duration of 3 minutes), calculate the average corrected absorbance.

**Solution:** 0.22

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