Bi 1x
Enzyme Catalysis

1 Overview

Enzymes are a critical part of life. They act as catalysts for a vast majority of the biochemical reactions that take place in living organisms. Without these essential proteins, reactions would not be able to happen at the high rates necessary to sustain life. In this module, you will explore the activity of the enzyme amylase by harvesting it from your own saliva. You will learn about the role that this enzyme plays in digestion, as well as work with the Michaelis-Menten model of enzyme kinetics.

2 Background

2.1 Enzymes in Our World

In his Great Ideas of Biology, Nurse put forward the idea of "life as chemistry." Indeed, the living world around us is made up of chemical reactions—there are billions of billions of reactions happening in just your own body at any given second! However, many of these reactions would be impossible without catalysts under normal cellular conditions; the temperature and pressure in cells are too mild to speed up a reaction. Furthermore, the reactants involved in biological processes may also be present at such low concentrations that it is unlikely that a reaction will occur. This is why enzymes, which act as biological catalysts, are an essential part of life. They are able to increase the rates of reactions by several orders of magnitude, potentially reducing the time scale of a process from years to fractions of a second.

Enzymes are involved in a vast variety of reactions. For example, ATP synthase is crucial for the formation of ATP molecules (the unit of energy for life). Alcohol dehydrogenase helps break down the ethanol in your body after a night out on the town. Reverse transcriptase is used by RNA viruses to form DNA and initiate replication inside a host cell, while DNA ligase joins DNA strands together and is an important part of a bioengineer's toolkit. RuBisCO is the perhaps most abundant protein on Earth, and it is massively important to plants because of its role in carbon fixation. In this module, you will be working with amylase, an enzyme that helps break down starch in the digestive process.

2.2 Michaelis-Menten Kinetics

Since enzymes are catalysts, they speed up reactions without altering the chemical equilibrium between products and reactants and without being consumed in the reaction. This is done by lowering the activation energy for a reaction.

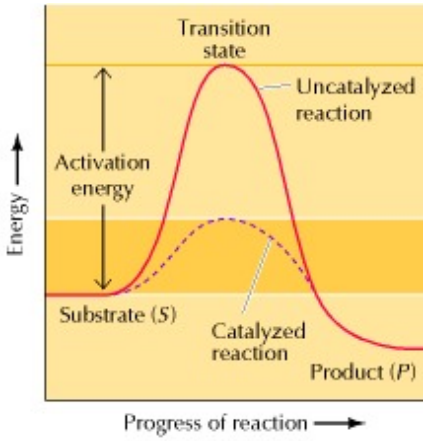

Figure 1: For the reaction to occur, a high energy transition state must be passed. This activation energy acts as the rate-limiting step for the reaction. Enzymes increase the reaction rate by lowering the activation energy. Figure 2.22 from *The Cell: A Molecular Approach, 2nd edition*.

The Michaelis-Menten model is one of the most well-known models of enzyme kinetics. It models enzyme kinetics as a set of chemical reactions between an enzyme, E, and a substrate, S, to give product P.

$$E + S \underset{k_-}{\overset{k_+}{\rightleftharpoons}} ES \xrightarrow{v} P + E \tag{1}$$

Here, k and v are rate constants. The enzyme reversibly binds the substrate with binding rate constant k_+. The substrate may also unbind with unbinding rate constant k_-. When bound, the enzyme can serve to convert the substrate to product with rate constant v.

We can explore how the concentration of each species changes over time by using mass action kinetics, which says that the rate of a reaction is proportional to the product of the concentrations of the reacting species. Applying this rule, we can write the dynamics as a system of ordinary differential equations.

$$\frac{\mathrm{d}c_e}{\mathrm{d}t} = -\frac{\mathrm{d}c_{es}}{\mathrm{d}t} = -k_+ c_e c_s + (k_- + v)\, c_{es}, \tag{2}$$

$$\frac{\mathrm{d}c_s}{\mathrm{d}t} = -k_+ c_s c_e + k_- c_{es}, \tag{3}$$

$$\frac{\mathrm{d}c_p}{\mathrm{d}t} = v c_{es}, \tag{4}$$

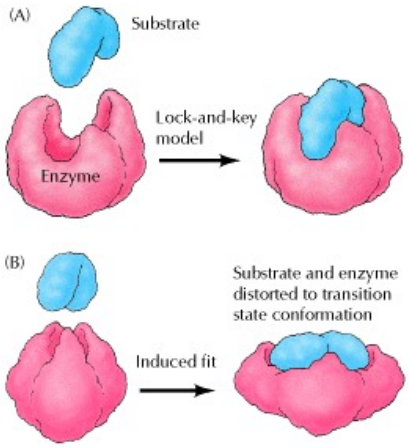

Figure 2: There are two models which describe the binding of substrate to enzyme. In the lock-and-key model, the substrate is able to fit directly into the enzyme's active site. In the induced-fit model, binding of substrate to the enzyme changes the conformations of both species. This causes the substrate's configuration to more closely match the conformation of the transition state, which increases the reaction rate. Figure 2.24 from *The Cell: A Molecular Approach, 2nd edition*.

where c_i denotes the concentration of species i. Note that

$$\frac{\mathrm{d}c_e}{\mathrm{d}t} + \frac{\mathrm{d}c_{es}}{\mathrm{d}t} = 0 \tag{5}$$

which is a statement of conservation of enzyme. This means that we need to specify a total enzyme amount to fully specify the problem. We define this to be c_e^0 such that $c_e^0 = c_e + c_{es}$. With this conservation law, we can write the ODEs as

$$\frac{\mathrm{d}c_{es}}{\mathrm{d}t} = k_+ \left(c_e^0 - c_{es}\right) c_s - \left(k_- + v\right) c_{es}, \tag{6}$$

$$\frac{\mathrm{d}c_s}{\mathrm{d}t} = -k_+ \left(c_e^0 - c_{es}\right) c_s + k_- c_{es}, \tag{7}$$

$$\frac{\mathrm{d}c_p}{\mathrm{d}t} = v c_{es}. \tag{8}$$

These equations describe the full dynamics of the enzyme-catalyzed system. To simplify the analysis, we often make the quasi-steady state approximation that the bound substrate intermediate ES does not see appreciable change in its concentration on the time scale of the production of the product P. That is,

$$\frac{\mathrm{d}c_{es}}{\mathrm{d}t} = k_+ \left(c_e^0 - c_{es}\right) c_s - \left(k_- + v\right) c_{es} \approx 0 \tag{9}$$

This enables us to solve for the quasi-steady state fraction of enzyme that is bound to substrate.

$$\frac{c_{es}}{c_e^0} \approx \frac{c_s/K}{1 + c_s/K} \tag{10}$$

where we have defined the Michaelis constant

$$K = \frac{v + k_-}{k_+}. \tag{11}$$

It has dimension of concentration and is analogous to a dissociation constant in that it is the ratio of the total rate constant for dissociation of the enzyme from the catalyst to that of binding the enzyme to the catalyst.

Finally, substitution of this expression gives the rate of product formation,

$$\frac{\mathrm{d}c_p}{\mathrm{d}t} \approx v c_e^0 \frac{c_s/K}{1 + c_s/K} \tag{12}$$

If $\mathrm{d}c_{es}/\mathrm{d}t \approx 0$, then the rate of substrate consumption is

$$\frac{\mathrm{d}c_s}{\mathrm{d}t} \approx -\frac{\mathrm{d}c_p}{\mathrm{d}t} \approx -v c_e^0, \frac{c_s/K}{1 + c_s/K} \tag{13}$$

2.3 Starch Hydrolysis by Amylase and the Starch-Iodine Complex

In this module, you will be working with the enzyme amylase obtained from your own saliva. For most humans as well as many animals, starchy foods make up a majority of their diet. Starches are naturally synthesized in a variety of plants, including potatoes, rice and corn. In the human body, glucose is used for carbon and energy. Since starch molecules are glucose polymers, your digestive system must break down starch into sugars in order to derive energy from starchy foods. These sugars are then broken down by other enzymes into glucose. This is done with the enzyme amylase, which catalyzes the hydrolysis of starch into sugars. This enzyme is present your saliva, and is the start of the process of digestion.

Aside from being an important part of the digestive system, amylases are also sometimes used in brewing and fermentation (which you have done earlier in the term when brewing kombucha!). For example, amylase is used in the process of brewing barley-based beers, since the grain contains starch. Adjusting the temperature of brewing can then be used to control the activity of the amylase enzyme, which helps brewers control the taste of the final product. In fact, ancient brewing methods have even been found to have included a step where grains are chewed in order to convert starch to sugar via salivary amylases!

In the experiment you will do, you will be mixing iodine with a starch-amylase solution. You will be able to track the progress of the resulting enzyme catalysis

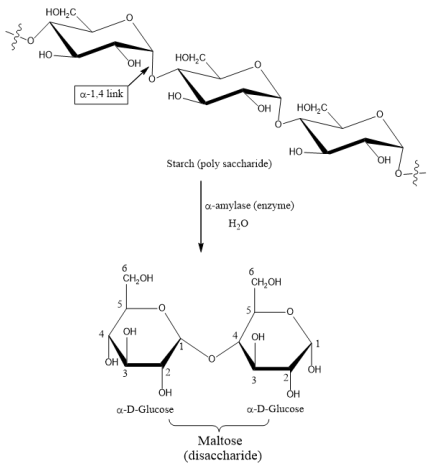

Figure 3: Amylase catalyzes the breakdown of starch, a polysaccharide, into sugars. From Chapter 21 of *Fundamentals of General, Organic, and Biological Chemistry, 7th edition*.

reaction by observing a color change in the solution. This is thanks to the presence of amylose (this is where the name amylase comes from, since -ase is used as a suffix to name enzymes) in starch, which forms a dark blue color in the presence of iodine. As shown below, amylose is a coiled, helical polymer, and iodine molecules are able to slip inside of this coil, forming a complex with a dark blue color.

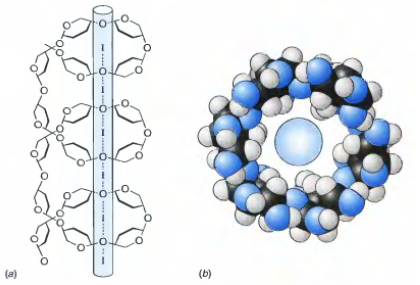

Figure 4: Starch is a coiled, helical polymer, into which an iodine molecule can fit. This complex has a dark blue color. Figure from `knowledgepayback.blogspot.com`.

3 Protocol

For this experiment, you will be using your homemade spectrophotometers to track the progress of the starch-amylase reaction by measuring the OD of a solution containing iodine.

3.1 Diluting the iodine solution

Because the starch-iodine complex produces a very dark blue color, you will need to dilute your solutions in order to obtain a solution that is in the accurate range of OD measurements (0-1 OD) later on.

1. Dilute your iodine solution. You will want iodine up to the first black tick mark of your 15mL Falcon tube, and water up to the last black tick mark.
2. Mix the diluted iodine solution by shaking gently.

If you are finding that your OD measurements still exceed the accurate range in later steps, you may want to dilute these solutions further. If that is the case, please mention what dilution you used in your homework write-up.

3.2 Preparing the amylase solution

You may want to brush your teeth or rinse your mouth before doing this step!

1. Take a sip of water, and try to secrete saliva for 2-5 min. You should collect the saliva in a small cup or other container. Just like for CCSL!
2. Filter the saliva through a coffee filter to obtain a clear saliva solution.

You will only need a small amount of filtered saliva (maybe aim for half of a CCSL tube :)).

3.3 Measuring the reaction curve

You will need to work as quickly as possible (without spilling or making mistakes, of course) for these next steps. Be sure to read them all carefully *before* performing them.

1. Using a pipette, dispense a small amount of iodine into an empty cuvette. You will not need much; around 20-50 µL should be sufficient (you may need to experiment with the amount, adjusting so the OD readings fall into the accurate range of <1). Repeat this for a second cuvette. With one of these cuvettes, you will measure the OD using the spectrophotomer. The other is simply for you to observe the reaction and corresponding change in color by eye.
2. Have your spectrophotometer ready to acquire measurements. You should use the Bokeh app, and blank the device with water.
3. In a separate container, mix 2 mL of starch solution with saliva solution. You will want to fill the pipette with saliva up to the first segment, the same volume as we used with iodine in the previous step. Quickly mix by pipetting up-and-down a couple of times.

4. Working quickly, pipette approximately 1 mL of starch-saliva solution into each cuvette. Place one of the cuvettes into the spectrophotometer, and immediately start acquiring measurements. Near the beginning of the reaction, you will want to acquire quickly, perhaps every 5-15 seconds. As the reaction slows down, you may want to acquire a data point every one minute. Take measurements until the solution reaches 0 OD, or until there is no more observable decrease in OD, and save your data to a CSV file.

   Note 1: Automation has recently been a hot topic in science. Thus, we encourage "hacking" for this last step! If you are comfortable with coding, you might edit the Arduino or Python code for the spectrophotometer app to acquire data automatically. Alternatively, you may want to use an auto-clicker program (an automation tool that allows for automation of mouse clicks, readily available online) to click the "Acquire" button for you! (I HIGHLY recommend this.) Please be sure to mention if you used one of these methods (or if you used/can think of any other methods) in your write-up. If you do hack some automated acquisition, you should test that it works with blank (or soy sauce, or whatever) cuvettes before doing the "real thing" with your starch solution.

   Note 2: You may wish to run the reaction outside of the spectrophotometer to see the color changes by eye separately from the measurement sample *before* making spectrophotometric measurements to enable you to work quickly.

   Note 3: Can you think of why these color changes, and corresponding decreases in OD, are happening?

3.4 Cleaning up

You may dispose of your starch and saliva solutions down the drain. It is not recommended to pour iodine (even dilute) down the drain, and so your best option would be to seal it in a container (such as a water bottle or one of the used Falcon tubes) and dispose of it in the trash.

4 Assignment

Problem 0 (Summary)**.**
Write a summary of this experiment, its objectives, and conclusions between one paragraph and one page in length.

Problem 1 (Spectrophotometer instructions and automation (prelab))**.**
Before the lab, review the spectrophometer User Guide so you are refreshed on how to operate the instrument and Python app. Also make sure the Python app launches properly. You may also want to create or download an automator program (such as an autoclicker) for more convenient data acquisition. You don't have to turn anything

in for this problem, but you can note in your lab notebook if you opted to use an automator program.

Problem 2 (Order of magnitude estimate for RuBisCO)**.**
Come up with an order of magnitude estimate of the total mass of RuBisCO (the world's most abundant enzyme) on Earth. You may want to familiarize yourself with the role of RuBisCO; where is it found? You may want to consider estimates like how much plant mass you think there is on Earth and how much carbon these plants fixate annually. You will want to consider both the mass in terrestrial plants, as well as in aquatic life such as algae. We encourage you to limit the amount of Googling you do for parameters and to answer as much as you can with the prior knowledge you have. There is no right answer to the problem, so feel free to go wild with estimates for parameters even if you are not completely sure they may be accurate! However, if there is a parameter that you need to know, the BioNumbers website is a fantastic resource.

Problem 3 (What are we measuring?)**.**
We will use Michaelis-Menten kinetics to model the enzymatic reaction in this experiment. Which biochemical species correspond to the enzyme, substrate, and product? The OD measurements we are making (potential calibration issues aside) are proportional to the concentration of which species?

Problem 4 (Limits of the Michaelis-Menten equation)**.**
Using the Michaelis-Menten equation we derived, derive an expression of $\mathrm{d}c_s/\mathrm{d}t$ in the limit of high substrate concentration at the beginning of the reaction. Also derive an expression for $\mathrm{d}c_s/\mathrm{d}t$ in the limit for end of the reaction, where substrate concentration is low. Solve the resulting ODE for $\mathrm{d}c_s/\mathrm{d}t$ in each of the two limits to write expressions for the substrate concentration c_s as a function of time t. In what regime does the rate of substrate consumption approximately follow a linear decay, and in what regime does it approximately follow an exponential decay?

Problem 5 (Time course of an enzyme-catalyzed reaction)**.**
Plot the substrate concentration in units of OD versus time for your data. The time output of the Bokeh app is the number of milliseconds passed since the Arduino board began running the current program, and you should shift these values such that the time of the first measurement you acquired is zero. Does the window you observed with your measurements follow a linear or exponential decay, or were you able to capture both regimes? What does this tell you about the speed of the reaction?

Problem 6 (Estimating parameters via curve-fitting)**.**
Using your analytical result, fit a curve to your data to obtain the parameters for your observed reaction. Do these values look reasonable? If not, why do you think we were unable to obtain accurate estimates using only the data we collected? (Hint: You may want to read up on the problem of identifiability in statistics and think about the functional form of the Michaelis-Menten equation. Can accurate estimates for our two parameters which are involved in a ratio really be obtained in our case?)

In the laboratory, enzyme assays are often done in which the initial rate of product formation (where the reaction is in the linear regime) is measured for many different substrate concentrations. The relevant constants are then estimated using these data. Compared to the data we obtained, how might this additional information help you obtain better parameter estimates for Michaelis-Menten kinetics?