Student Manual

Background

Enzymes

Enzymes are typically proteins (some nucleic acids have also been found to be enzymes) that act as catalysts, speeding up chemical reactions that would take far too long to occur on their own. Enzymes speed up the vast majority of the chemical reactions that occur in cells. Reactions that break down molecules (such as those involved in digestion and cellular respiration) and those that build up molecules (such as the ones involved in photosynthesis and DNA replication) all require enzymes. Each type of enzyme has a specific shape that compliments the structure of its substrate (Figure 5). The substrate is the molecule or molecules that the enzyme converts into product. The substrate fits into an indentation in the globular protein called the active site. The shape and chemical properties of this active site are critical to the enzyme's function.

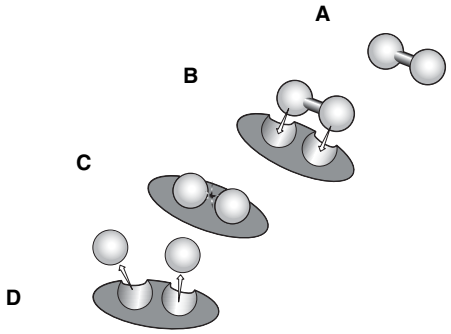


Fig. 5. A schematic of cellobiose and cellobiase in solution. A. Cellobiose in solution is composed of two glucose molecules covalently connected by a β 1–4 linkage. **B**. Cellobiase has a pocket that fits the cellobiose molecule. **C**. Cellobiase helps stabilize the cellobiose in a shape so that the bond between the two glucose molecules can be broken. **D**. Once the β 1–4 bond in cellobiose has been broken, the two glucose molecules are released from the cellobiase, and the enzyme is free to bind to another molecule of cellobiose and begin the cycle again.

Many chemical reactions that enzymes speed up can occur at a much slower rate without the enzymes. Enzymes speed up reactions by positioning the substrates, adjusting their bonds so that they become unstable and reactive. Let's use the analogy of a friend setting up a blind date. The two people may have found each other on their own and made the connection, but the matchmaker sped up the process by putting the two people in the same room at the same time. The matchmaker may have also influenced the couple by pointing out the good points about each individual. Like enzymes, the matchmaker did not change and he/she was able to go on and make further matches. In chemical terms, the enzyme lowers the energy of activation of a reaction. This is the amount of energy required to get the reaction going. Enzymes also stabilize the transition state of the reaction. The transition state is the structure in the reaction with the highest energy. By lowering this energy, the reaction can take place much more easily.

Enzymes are "picky" about the conditions at which they work best. The temperature and pH must be ideal for the enzyme to catalyze reactions efficiently. For any chemical reaction, raising the temperature will increase the movement of the molecules and cause more collisions to occur. It increases the average kinetic energy (energy of movement) of the molecules so that more of them will be able to react. However, in an enzymatic reaction,

too much heat is a bad thing. You may recall from studying about proteins that the non-covalent interactions within the protein, such as hydrogen and ionic bonds, can break at high temperatures. This will change the shape of the enzyme. If the enzyme changes shape, then the active site will not fit the substrate properly and the enzyme will not be able to function.

Cellobiase Enzyme

In this laboratory experiment, you will be studying cellobiase. Cellobiase is involved in the last step of the process of breaking down cellulose, a molecule made up of bundled long chains of glucose that are found in plant cell walls, to glucose. This is a natural process that is used by many fungi as well as bacteria (some present in termite guts, others in the stomachs of ruminants and also in compost piles) to produce glucose as a food source. Breaking down the cellulose from plants into sugar is also an important step in the creation of ethanol for fuel.

Cellobiase Substrates

The natural substrate for the enzyme cellobiase is cellobiose (Figure 6). This is a disaccharide composed of two beta glucose molecules. However, when scientists study enzyme function, it is best if there is an easy way to detect either the amount of substrate that is used up or the amount of product that is formed. Solutions of cellobiose (substrate) and glucose (product) are clear, and there are not many simple, inexpensive, fast methods to detect these molecules quantitatively.

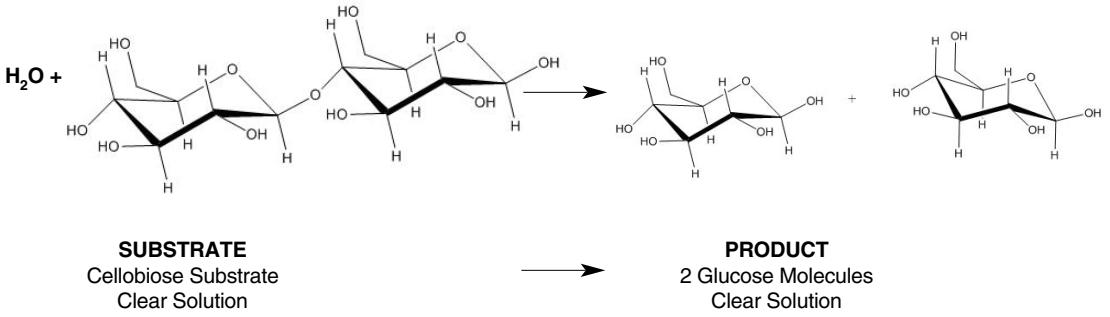


Fig. 6. Breakdown of cellobiose by cellobiase. The natural substrate of cellobiase is the dissacharide cellobiose. When cellobiose is bound by cellobiase, the cellobiase breaks apart the β 1–4 bond that connects the two glucose molecules and then releases two glucose molecules.

So, to make this reaction easier to follow, an artificial substrate, *p*-nitrophenyl glucopyranoside, will be used. This artificial substrate can also bind to the enzyme and be broken down in a manner similar to the natural substrate cellobiose. When the artificial substrate, *p*-nitrophenyl glucopyranoside, is broken down by cellobiase, it produces glucose and *p*-nitrophenol (Figure 7). When *p*-nitrophenol is mixed with a solution that is basic in pH (such as the stop solution provided in the kit), it will turn yellow. The amount of yellow color is proportional to the amount of *p*-nitrophenol present. And for every molecule of *p*-nitrophenol present, one molecule of *p*-nitrophenyl glucopyranoside is broken apart. For the cellobiase reactions being run, another advantage of using a basic solution to develop the color of the *p*-nitrophenol is that the basic pH will also denature the enzyme and stop the reaction.

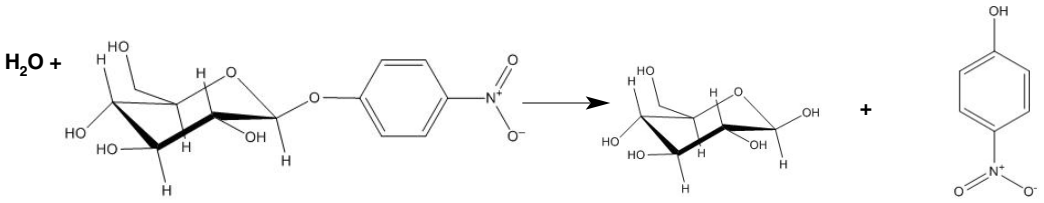


Fig. 7. Breakdown of *p*-nitrophenyl glucopyranoside into glucose and *p*-nitrophenol by cellobiase. When the *p*-nitrophenyl glucopyranoside is broken apart by cellobiase, one molecule of glucose and one molecule of *p*-nitrophenol are released. If the *p*-nitrophenol is put into a basic solution, it will produce a yellow color, which is detected by a simple colorimetric quantitative method.

Measuring the Amount of Product Produced

Since the product (*p*-nitrophenol) of the artificial substrate reaction turns yellow once base is added, you can tell how much product is being produced. The deeper the color, the higher the amount of product made. One simple method of estimating how much product is formed is to compare the yellowness of enzyme reaction samples to a set of known standards, which contain a known amount of colored product. You can estimate which tube in the set of standards most closely matches your samples in color. This will give you an estimated amount of product. Alternatively, you can use an instrument called a spectrophotometer (or a colorimeter), which quantitatively measures the amount of yellow color by shining a beam of light (wavelength of 410 nm) through the sample. The spectrophotometer measures the amount of light that is absorbed by the sample. The darker the color of yellow the sample is, the more light that is absorbed, and thus the more concentrated the sample. The absorbance values of a set of standards can first be measured to create a standard curve, a plot of the absorbance values of samples of known concentration of *p*-nitrophenol. The absorbance values of the reaction samples can then be measured, and the standard curve can be used to convert the absorbance value to a concentration value.

Measuring the Rate of Cellobiase Activity

In order to determine what factors influence an enzyme's ability to break down its substrate, the rate of reaction or how much product is formed in a set amount of time is determined. For studying cellobiase activity, you will measure the rate of reaction by adding enzyme to the artificial substrate *p*-nitrophenyl glucopyranoside. The enzyme and substrate are dissolved in a buffer that is at an ideal pH (pH 5.0) for the reaction to occur. At set times, a sample of the enzyme reaction will be removed and added to a high pH stop solution which will help develop the color of the product *p*-nitrophenol, as well as stop the reaction by increasing the pH to above the range where the enzyme can work. By calculating how much *p*-nitrophenol is produced over time, the rate of reaction can be calculated. By looking at small increments of time, you will be able to determine whether the rate of the enzyme is constant or whether it slows down toward the end as the amount of substrate decreases. You will also be able to detect any effects pH, temperature, substrate concentration or enzyme concentration have on the initial rate of reaction.

Pre-lab Questions

1. What type of molecule is an enzyme?

2. Why is an enzyme's shape important to its function?

3. How does an enzyme speed up chemical reactions?

4. What is the name of the enzyme involved in this laboratory experiment?

5. What is one practical, industrial application of this enzyme?

6. What is the natural product of this enzyme?

7. What is the natural substrate of this enzyme?

8. How will you be able to determine the amount of product that is produced at each time period?

9. How can you measure the rate of product formation?

Activity 1: Determine the Reaction Rate in the Presence or Absence of an Enzyme

In this activity, you will compare the rate of breakdown of *p*-nitrophenyl glucopyranoside into glucose and *p*-nitrophenol in the presence and absence of cellobiase. Enzymes are molecules that increase the rate of a reaction, but are not used up in the reaction. Because the enzyme can keep processing the substrate over and over again, very few molecules of enzyme are needed relative to the number of molecules of substrate.

Because it is difficult to add really small volumes, your instructor has diluted the enzyme with a buffer solution — this will allow you to easily add the required volume that still contains a very small number of molecules of enzyme. However, to ensure that the buffer in which the enzyme was diluted does not affect the rate of formation of the product, a control reaction containing just the buffer will be run alongside the reaction containing the diluted enzyme.

To the first reaction tube, you will add enzyme into a solution of substrate and determine the initial rate of reaction (product formation). The second reaction, which is the control reaction, will have the same buffer added to the same substrate, but does not include enzyme. This way, you will be able to compare the breakdown rate of *p*-nitrophenyl glucopyranoside to glucose and *p*-nitrophenol in the presence of enzyme and the presence of a control buffer.

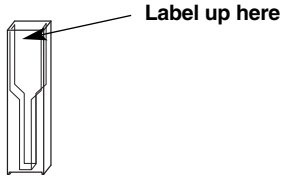
Student Workstation	**Quantity**	**(✔)**
1.5 mM substrate	1	❒
Enzyme	1	❒
1x stop solution	1	❒
Buffer	1	❒
DPTPs	4	❒
15 ml conical tubes	2	❒
Colorimetric standards (S1–S5) in cuvettes	1 of each	❒
Cuvettes	7	❒
Marker	1	❒
Beaker with deionized or distilled water to rinse DPTPs	1	❒
Stopwatch or timer	1	❒

Instructor's Workstation (Optional)	**Quantity**	**(✔)**
Spectrophotometer	1	❒

Protocol

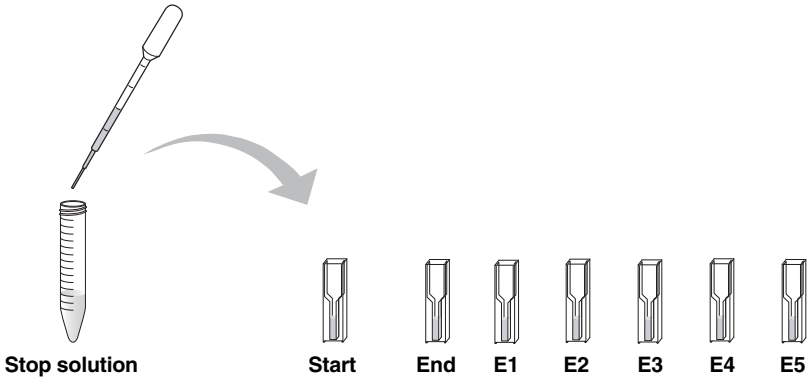
1. Locate the 15 ml conical tubes labeled “Stop Solution”, “1.5 mM Substrate”, “Enzyme” and “Buffer”. Label each of the tubes with your initials.

2. Label five cuvettes E1–E5 (for five time points). Label only the upper part of the cuvette face.



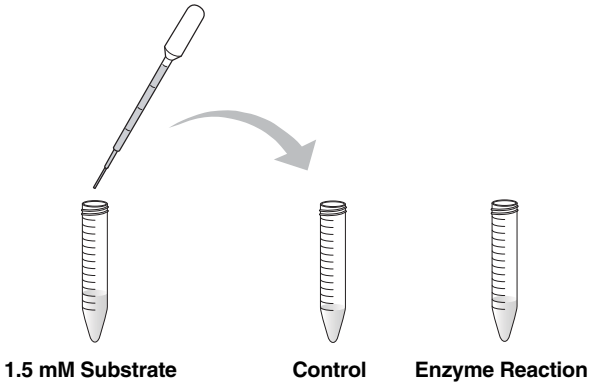
3. Label the two remaining cuvettes “Start” and “End” on the upper part of the cuvette. The cuvettes will serve as control time points at the start and end of the reaction and neither cuvette will contain enzyme.

4. Using a clean DPTP, pipet 500 µl of stop solution into each of the seven labeled cuvettes. The stop solution is a strong base, so avoid getting it on your skin or clothes. Rinse the DPTP well with water and save it for future activities.



5. Locate two empty 15 ml conical tubes. Label one “Enzyme Reaction” and the other “Control”.

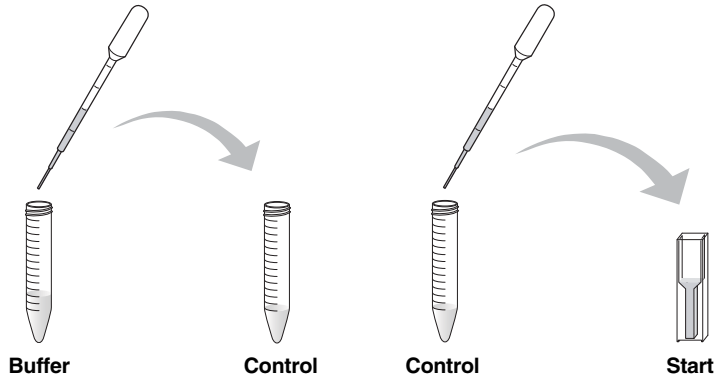
6. Using a clean DPTP, pipet 2 ml of 1.5 mM substrate into the 15 ml conical tube labeled “Enzyme Reaction”. Use the same DPTP and pipet 1 ml of 1.5 mM substrate into the conical tube labeled “Control”. Rinse the DPTP well with water and save it for future activities.



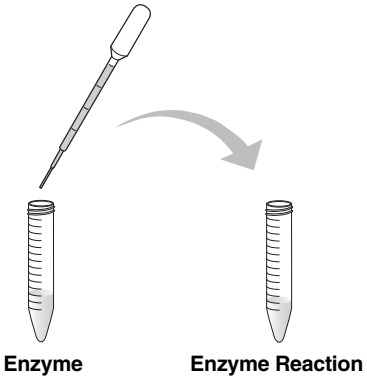
7. Label one DPTP “E” for enzyme and the other “C” for control. **Only use the DPTP labeled “E” for the enzyme reaction tube and the DPTP labeled “C” for the control reaction tube**.

Read and understand steps 8–11 fully before proceeding. These steps are time sensitive!

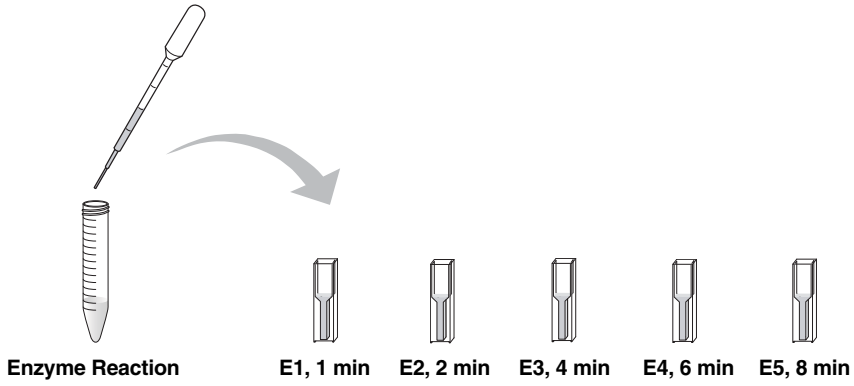
8. Using the DPTP labeled “C”, pipet 500 µl of buffer into the 15 ml conical tube labeled “Control” and gently mix. Once you have mixed the buffer with the substrate, remove 500 µl of this solution and add it to your cuvette labeled “Start”.



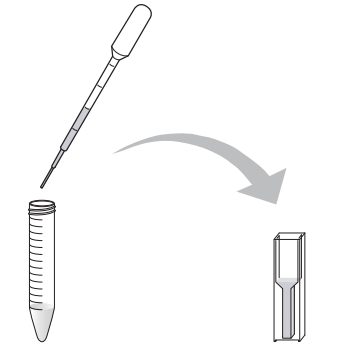
9. Using the DPTP labeled “E”, pipet 1 ml of enzyme into the 15 ml conical tube labeled “Enzyme Reaction”. Gently mix, then **START YOUR TIMER**. This marks the beginning of the enzymatic reaction.



10. At the times indicated below, use the DPTP labeled “E” to remove 500 µl of the solution from the “Enzyme Reaction” tube and add it to the appropriately labeled cuvette containing the stop solution.



11. After all the enzyme samples have been collected, use the DPTP labeled “C” to remove 500 µl of the solution from the “C” reaction tube and add it to the cuvette labeled “End”.



Time (Min)	Enzyme Cuvette	Control Cuvette
0 (Start)		Start
1	E1	
2	E2	
4	E3	
6	E4	
8	E5	End

12. Rinse out all DPTPs with copious amounts of water and save them for later activities. After you have finished your analysis, rinse out your reaction (conical) tubes and cuvettes with copious water and save them for later activities.

Note: Do not discard the unused stock solutions or cuvettes containing standards. They will be used for the next activity.

RESULTS

Qualitative Determination of the Amount of Product Formed

1. Locate the five cuvettes of standards labeled S1–S5 at your lab bench; their concentrations are noted in Table 1. Compare all 7 cuvettes (control and reaction cuvettes) to the standard cuvettes by holding them against a white background. Record in Table 2 the standard that is most similar to your control and enzyme reaction cuvettes.

Table 1. *p*-Nitrophenol standards.

Standard	Amount of *p*-Nitrophenol (nmol*)
S1	0
S2	12.5
S3	25
S4	50
S5	100

*1 nmol = 1 nanomole = 1 x 10^{-9} mol = 0.000000001 mol

Table 2. Comparison of reaction cuvettes to standard cuvettes.

Time (minutes)	Cuvette	Standard That Is Most Similar	Amount of *p*-Nitrophenol (nmol)
0	Start		
8	End		
1	E1		
2	E2		
4	E3		
6	E4		
8	E5		

2. If you are not using a spectrophotometer, please skip ahead to Analysis of Results.

Quantitative Determination of the Amount of Product Formed

1. Locate the five cuvettes of standards labeled S1–S5 at your lab bench; their concentrations are noted in Table 1. Blank your spectrophotometer at 410 nm with the cuvette labeled S1. Then measure and record the absorbance at 410 nm for the remaining standards in Table 3. You will use this information to generate a standard curve that correlates the absorbance at 410 nm with the amount of *p*-nitrophenol present.

Table 3. Absorbance values for standards.

Standard	Amount of *p*-Nitrophenol (nmol)	Absorbance at 410 nm
S1	0	0.00
S2	12.5	
S3	25	
S4	50	
S5	100	

2. Measure the absorbance of your enzyme-catalyzed reaction cuvettes (E1–E5) and your control cuvettes (Start, End) at 410 nm, and record your results in Table 4. You will use this information to determine the amount of product, *p*-nitrophenol, formed in the reaction cuvettes.

Table 4. Determining *p*-nitrophenol produced using a standard curve.

Time (minutes)	Cuvette	Amount of *p*-Nitrophenol (nmol) from the Standard Curve	Absorbance at 410 nm
0	Start		
8	End		
1	E1		
2	E2		
4	E3		
6	E4		
8	E5		

3. Determine nanomoles (nmol) of product formed from absorbance values. The absorbance of the product, *p*-nitrophenol, is directly related to the amount of *p*-nitrophenol present in the cuvette. In other words, the more yellow a solution appears, the more *p*-nitrophenol in the solution and the higher its absorbance value at 410 nm. By plotting the absorbance values for the standards with known amounts of *p*-nitrophenol (called a standard curve), you can determine how much *p*-nitrophenol is present in your enzyme assay samples.

In the example shown in Figure 8, the solid filled diamonds represent the absorbance values for the five standards. The best line connecting all the data points is then drawn. The circle symbol represents the E3 data point; its absorbance was measured to be 0.73. To determine the amount of product corresponding to this value, locate the absorbance value of 0.73 (approximately) on the y-axis and then follow the value horizontally until it intersects with the standard curve. From this point, draw a line down to the x-axis (amount of *p*-nitrophenol) and read the value directly from the graph. In this case, it is approximately 44 nmol. This same process is done with the remaining data points.

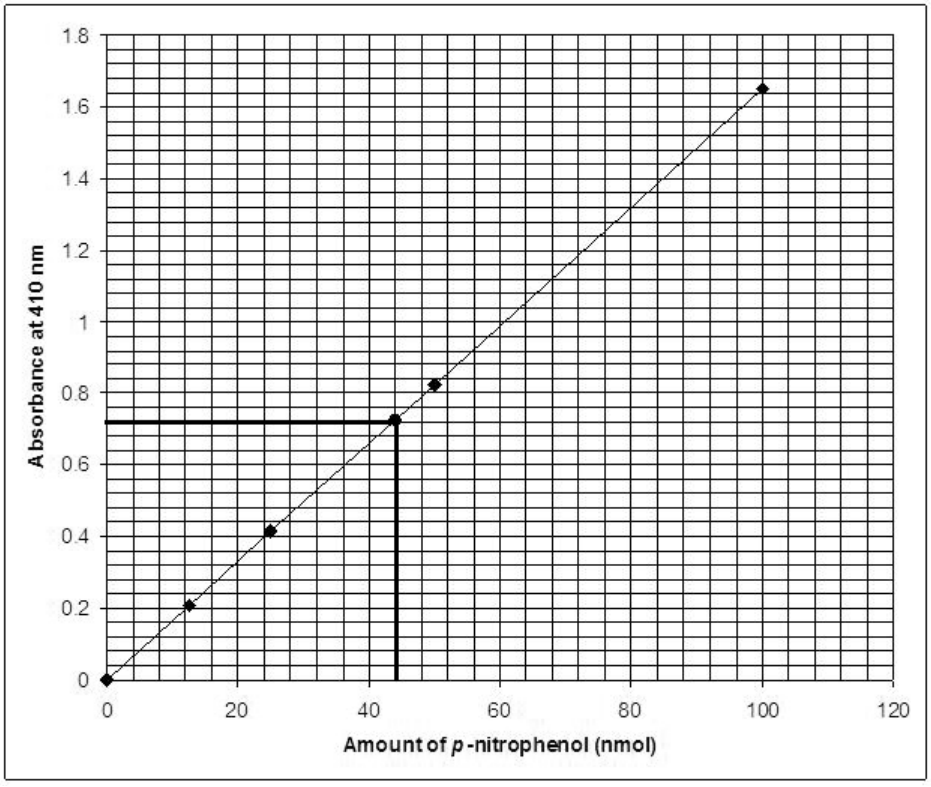


Fig. 8. Example of a standard curve. Absorbance of *p*-nitrophenol is plotted against known quantities of *p*-nitrophenol standards, S1–S5.

Use the data in Table 3 to create a standard curve. Plot the absorbance values for each standard in Figure 9, and then draw the line that best goes through all the data points. As described in the example on page 47, plot the absorbance values for the five time points (E1–E5) and non-catalyzed reaction (Start and End), then determine the corresponding amount of product for each time point. Record this information in Table 4.

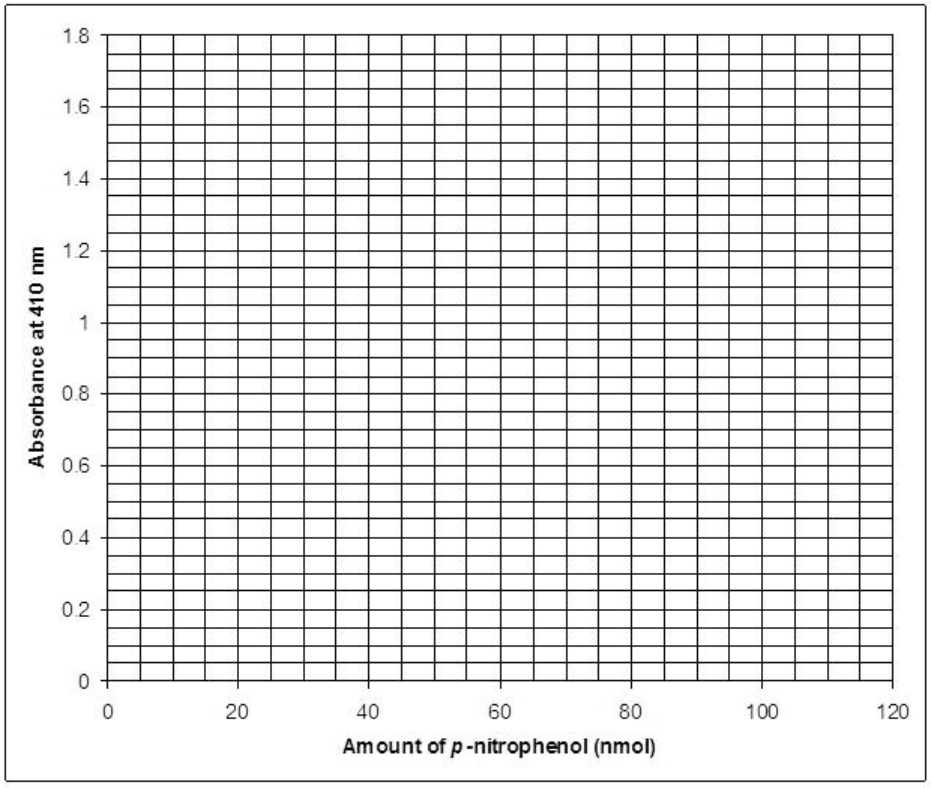


Fig. 9. Creating a standard curve by plotting absorbance of *p*-nitrophenol against known quantities of *p*-nitrophenol standards, S1–S5.

Analysis of Results

1. Initial rate of product formation

At the beginning of the reaction, there is plenty of substrate available for the enzyme to encounter. However, as the reaction progresses, there is less substrate readily available, because it is being converted to product. If you graph the amount of product formed at each time point, the data can be used to calculate the initial rate of product that is formed in the presence or absence of enzyme.

In Figure 10, the amount of product, *p*-nitrophenol, is plotted over time to determine the initial rate of product formed. The unit of rate is nmol/min.

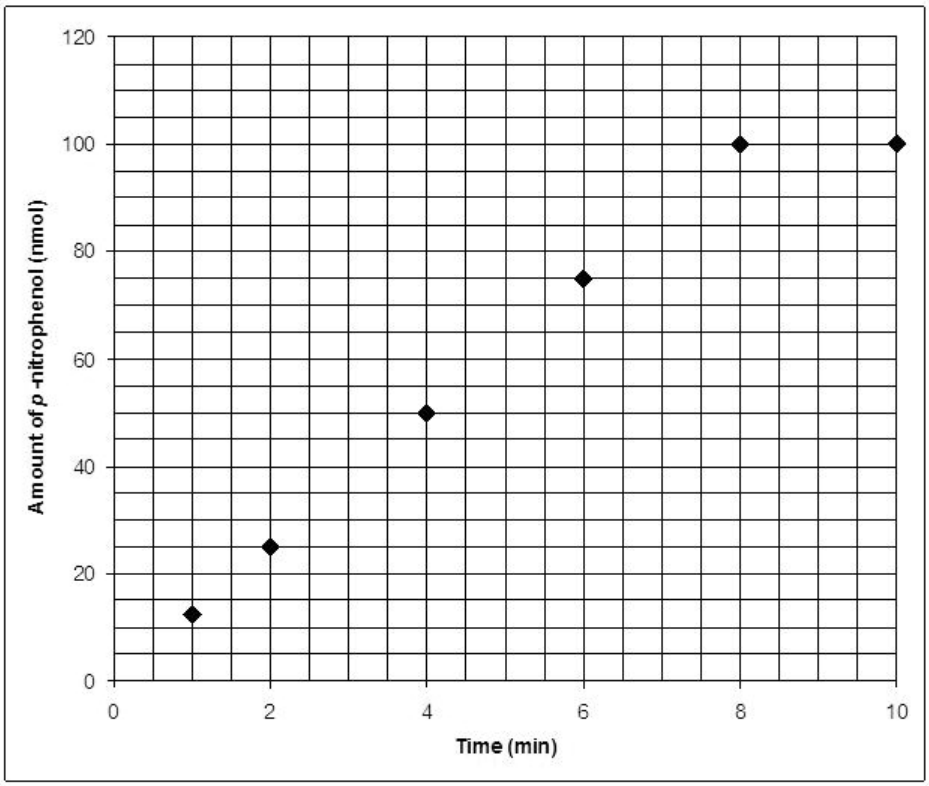


Fig. 10. Example of a rate curve for an enzyme reaction. The amount of product made is plotted against time to determine the initial rate of reaction.

There is a region where the amount of product formed increases in a linear fashion. This is called the initial rate of reaction. In the graph above, this linear region is between 0 and 8 minutes.

Initial rate of product formation = slope of the line = change in y/change in x

Initial rate of product formation = (100 nmol – 12.5 nmol)/(8 min – 1 min) = 12.5 nmol/min

2. Conversion of substrate to products

As illustrated in Figure 10, plot the amount of *p*-nitrophenol produced over time on Figure 11 using data from either Table 2 or Table 4. Draw a line that best fits through the data points.

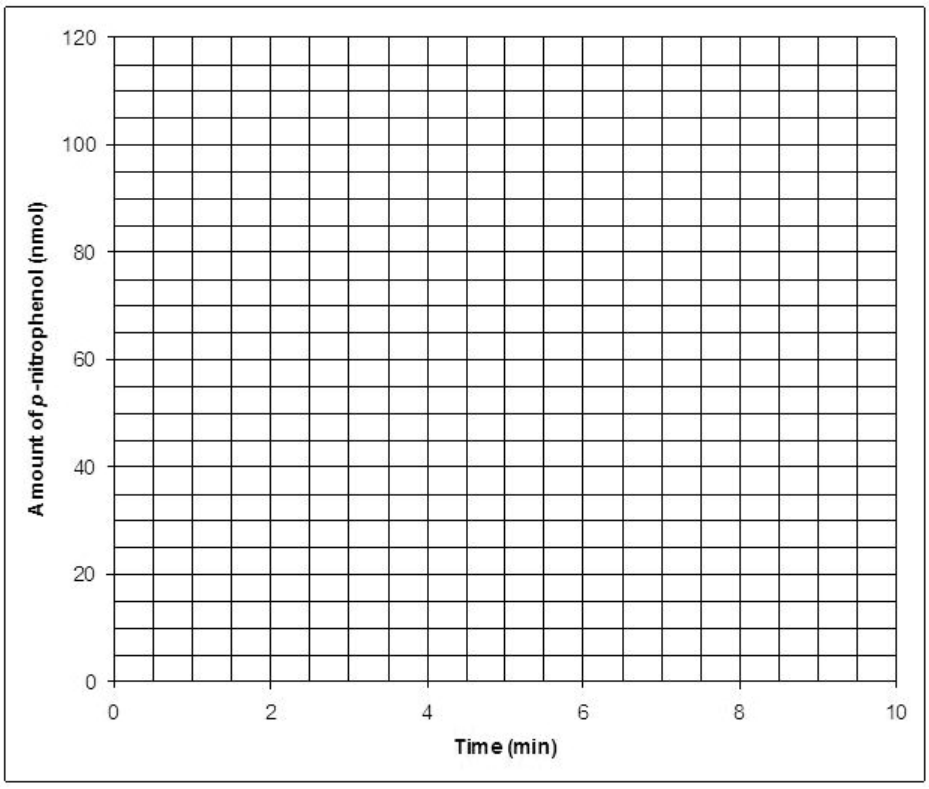


Fig. 11. Reaction rate curve for cellobiase. The amount of product made is plotted against time to determine the initial rate of reaction.

At the beginning of the reaction, there is plenty of substrate available for the enzyme to encounter and create product. Locate the linear region where the concentration of the product increases linearly.

Using the graph you generated for concentration of product as a function of time, you will be able to determine the rate that the product is produced when there is plenty of substrate.

Perform these calculations for your data

Initial rate of product formation with enzyme present = _____ nmol/min

Rate of product formation with no enzyme present = _____nmol/min

Activity 1 Analysis Questions

1. Did you observe any changes in the enzyme reaction and control reaction conical tubes during the time that the reaction was occurring?

2. What happened to the solution in each cuvette after you added the enzyme/substrate mixture to the stop solution?

3. Describe the chemical reaction that occurred in this experiment.

4. Describe the amount of product produced in the enzyme-catalyzed reaction compared to the control where no enzyme was added.

5. If you took a time point at 15 minutes, do you think more product would be produced than at 8 minutes? Explain your answer.

6. How did you estimate the amount of product (in nmol) produced by the enzyme?

7. Why is the amount of light absorbed by the sample proportional to the amount of product produced?

8. Determine the initial rate of product production from your absorbance measurements. **Hint**: The rate of product production is measured in absorbance units/min or nmol/min and it is the slope of the line between the zero and 1 minute time points.

9. Is the rate of product production constant over time? **Hint**: Is the slope of the line constant or does it change?