

Amylase: a sample enzyme

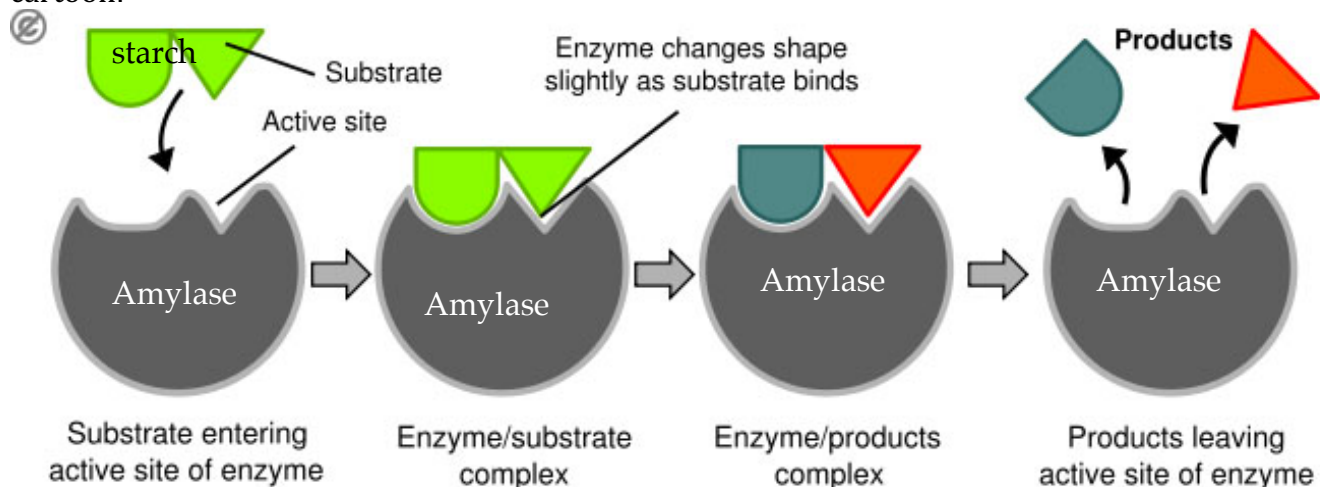
Objectives: After completion of this laboratory exercise you will be able to:

1. Explain the importance of enzymes in biology.
2. Explain the basic properties of an enzyme as a catalyst.
3. Discuss the effect of enzyme concentration and various inhibitors on the rate of an enzymatic reaction.
4. Evaluate the efficacy of nutritional supplements called “starch blockers”

Introduction:

Enzymes are biological catalysts that constitute the largest and most highly specialized class of protein molecules. Enzymes act as catalysts to increase the rates of chemical reactions, but they do not cause a reaction to occur that would not proceed spontaneously without the enzyme; that is, the reaction must have $\Delta G < 0$. The reactions of metabolism would occur at extremely slow rates at normal body temperature and pH in the absence of enzymes. An appreciation of the catalytic efficiency of enzymes can be gained by realizing that under optimal conditions, most enzymatic reactions proceed 10^8 to 10^{11} times more rapidly than the corresponding reactions without enzymes. Without the enzymes in our digestive tract for example, it would take us about 50 years to digest a single meal!

One very important property of enzymes is their specificity. Any one enzyme will only catalyze a single class of chemical reactions. Some enzymes act on one substrate only; other enzymes act on a family of related molecules. Enzymes participate in the reaction that they catalyze, but they emerge unchanged at the end of the reaction, i.e., they are not used up. Thus, a few enzyme molecules can go a long way. This reaction can be represented by the following cartoon:



<http://www.phoenixhealth.me>

Most enzymes are proteins with specific three dimensional shapes necessary for the proper function of the enzyme (some RNA molecules also have catalytic activity). As with any protein, the shape of an enzyme can be affected by different environmental conditions. An increase in temperature can speed up the rate of an enzymatic reaction but it can also change the shape of the enzyme causing it to become inactive because the substrate can no longer bind to it. The pH of the environment is also important to the proper functioning of an enzyme. Enzymes work best at a

specific pH and a change in this value can also cause the enzyme to denature (change its shape) and become inactive.

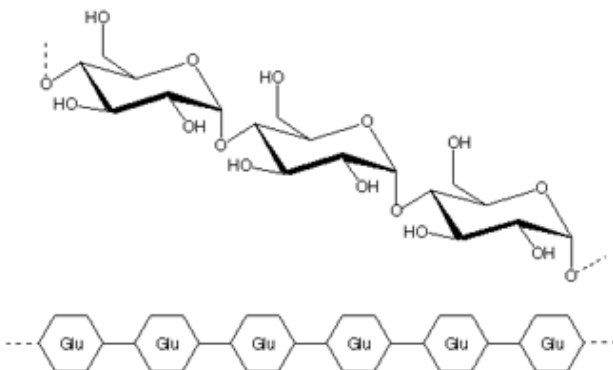
In this lab we will demonstrate the hydrolysis of starch to glucose using the enzyme amylase which is found in saliva and in secretions from the pancreas. Starch (also known as amylose) is a polymer made from many glucose molecules connected together in a long chain via covalent bonds. It is produced by dehydration synthesis and is used by many organisms as a way to store glucose for later use. Its structure is shown in figure 1 (right).

For organisms to be able to use the energy in starch the polymer must be broken down into its simpler glucose units. In animals, this hydrolysis reaction is catalyzed by the enzyme amylase. In fact, amylase converts starch to glucose dimers (called maltose) and trimers (called maltotriose); these are then converted by other enzymes to glucose which can then be used for cellular respiration, etc. We will study the effect of enzyme concentration on the rate of the conversion of starch to maltose.

There are a variety of nutritional supplements that call themselves “starch blockers”. Their manufacturers claim that they contain extracts from the common bean (*Phaseolus vulgaris*) that inhibit amylase. Beans are known to contain amylase inhibitors – these are proteins that bind to the active site of amylase and prevent it from catalyzing the breakdown of starch. However, the supplement industry is very lightly regulated, so manufacturers are able to make claims of efficacy without having to provide evidence to support these claims. As a result, not all supplements claiming to be “starch blockers” will necessarily contain amylase inhibitors. In this lab, we will measure one or more “starch blockers” to see if in fact they do inhibit amylase.

The action of amylase on starch can be readily followed with the IKI (a mixture of iodine and potassium iodide) test. IKI stains starch a blue-black color, but it does not stain maltose or maltotriose. When all of the starch has been hydrolyzed to these sugars, the solution will remain the yellowish-brown color of IKI. As hydrolysis is taking place and some of the starch has been hydrolyzed and some has not, the solution will show intermediate shades of brownish-black. The diagram below shows these changes. Please note that a positive test for starch means that the enzyme has not broken down all of the starch in the sample yet and a negative test for starch means that the enzyme has broken down all of the starch in the sample to sugars.

Figure 1–Structure of amylose



Adapted from BeMiller, J. N., and Whistler, R. L. (1996). Carbohydrates. In *Food Chemistry* (3rd ed., pp. 157-223.) New York, New York: Marcel Deker.

- | | |
|------|---|
| Time | 1. START: only un-hydrolyzed starch (BLUE-BLACK) |
| | 2. {some starch hydrolyzed to maltose} |
| | 3. MIDDLE: Mixture of starch and glucose (BROWN-BLACK) |
| | 4. {finally, all the starch has been hydrolyzed to maltose} |
| | 5. END: No starch; only maltose (YELLOW-BROWN) |

Procedure

PART 0: PIPETTE PRACTICE

You should practice using pipettes to measure precise volumes of water into a test tube until you're sure you can do it quickly and repeatably. The best practice is to show that you can add and subtract volumes accurately – for example, put two samples of 2.5ml each into a test tube and be sure that you can pipette out exactly 5ml.

PART I: REACTION RATE VS. ENZYME CONCENTRATION

0. Guide to colors: Each group should prepare one spot plate with a drop of IKI test solution in each of two of the depressions. To one of the IKI drops, add a drop of starch solution. Record the colors and make it clear in your notes what happened in each case. The depression with the IKI only is the standard or reference that shows the color of the solution when no starch is present and the depression with the drop of starch is the standard that shows the color of the solution when high amounts of starch are present.

1. Label 3 test tubes “SS” for “Stock Starch” and your group name. Pipette 4 ml of the stock starch solution into each test tube. Be as accurate as possible in your measurements. **BE SURE TO SWIRL THE STARCH SOLUTION BEFORE EACH PIPETTING TO GET THE PROPER CONCENTRATION OF STARCH.** Place the tubes in the 37°C water bath.

2. Prepare a series of 3 different test tubes, labeled 10%, 5%, 1% and your group name for the different concentrations of amylase that we will test. Pipette one ml from each of the amylase dilutions into the appropriate test tube. Swirl the amylase flasks before pipetting your sample. Place these 3 test tubes in the 37°C water bath and allow five minutes for the samples to reach the water bath temperature (equilibration); keep them in the bath until you are ready to use them.

You should now have 6 tubes:

- 3 tubes each with 4ml starch stock solution; labeled “SS”
- 1 tube with 1ml 10% amylase; labeled “10%” and your group name
- 1 tube with 1ml 5% amylase; labeled “5%” and your group name
- 1 tube with 1ml 1% amylase; labeled “1%” and your group name

3. Obtain a spot plate and place one drop of IKI solution in each depression. The top left-hand depression will be called the first depression, and you will be testing samples starting with the first depression and moving to the right with successive samples. Number the depressions if you think you might lose track of which ones have been used.

4. One student in the group should record the time while another student in the group pours the contents of one of the “SS” test tubes from the water bath into the test tube labeled 10%. Swirl this tube and return to your table to test this solution.

5. Remove ONE drop immediately using a plastic pipette and place it on the first IKI drop in your spot plate. If there is any blue-black color in this depression, put a check mark next to the “time 0” line in the 10% amylase column showing that there is still starch in the solution.

6. Continue taking one drop of the solution and putting it in successive depressions of the spot plate every 30 seconds until you have a negative starch reaction which will show you the original color of the IKI solution (your negative standard). Put a check mark next to each line corresponding to the time that continues to show any blue-black color. Stop testing at 10 minutes.

7. When your drop tests negative for starch, put an “x” in the line next to the time when it happened. Take two more drops at 30-second intervals to be sure that the test is correct. Then determine how much time it took for your 10% solution to “reach end point” – that is, how long did it take for all the starch to be digested?

Concentration of Amylase Data Table

Put a check (√) in the box when any blue-black color is visible. Put an “x” when there is a no color change (the color looks like the “no starch standard” from (0) above). Show 3 Xs in a row to be sure the reaction is complete.

AMYLASE CONCENTRATION			
Time (min:sec)	10%	5%	1%
0:00			
0:30			
1:00			
1:30			
2:00			
2:30			
3:00			
3:30			
4:00			
4:30			
5:00			
5:30			
6:00			
6:30			
7:00			
7:30			
8:00			
8:30			
9:00			
9:30			
10:00			

8. Determine the end point for your other amylase concentrations using the same techniques (steps 4-7).

9. Summarize your data as follows:

AMYLASE CONCENTRATION	TIME TO REACH ENDPOINT	RATE OF REACTION (100/TIME TO ENDPOINT)
10%		
5%		
1%		

Note: We are calculating the rate of the reaction (3rd column above) by assuming that we started with 100 units of starch in each tube, and then dividing 100 by the number of minutes that it took to digest all the starch. For instance, if it took 5 minutes, the rate would be $100/5 = 20$ units per minute – that is, 20 units of starch were digested each minute.

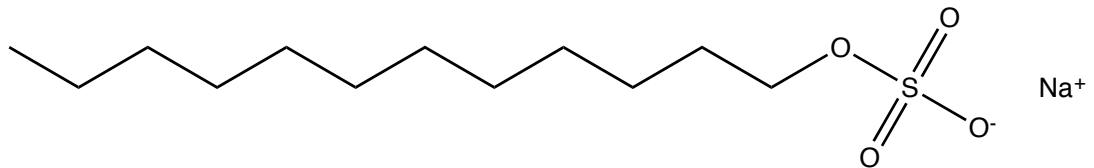
10. Pool the data from your class (average data when needed).

PART II: ALTERING THE ACTIVITY OF AMYLASE

Enzymes like amylase are proteins whose activity depends on their shape and structure. There are many chemicals and treatments that could potentially alter amylase's structure and function. Your group will pick one of these and investigate its effect on the activity of amylase.

These are the potential inhibitors of amylase you can choose from and their modes of action:

- **High Temperature** – at high temperature, molecules have more energy. This is a “double-edged sword”. As more molecules of the substrate have more energy, more of them will be able to react. However, as the energy of the enzyme molecule increases, weaker non-covalent bonds can break. You will use two high temperatures: 50°C and 75°C.
- **Low temperature** – at low temperature, molecules have less energy. As fewer molecules of the substrate have enough energy to react, fewer will react in a given time. You will use two low temperatures: 22°C and 10°C
- **Sodium Dodecyl Sulfate (SDS)** – this is a detergent; its structure is shown below. Detergents disrupt the hydrophobic effect. You will use two concentrations of SDS: 0.05% and 0.025%.



- **EDTA** – this is a chelating agent. It binds ions like Ca²⁺ and Mg²⁺ so tightly that they cannot bind to any other molecules. You will use two concentrations of EDTA: 2mM and 5mM.
- **Amylase Inhibitors** – these are nutritional supplements that claim to contain proteins that bind to the active site of amylase. You will need to prepare them as follows:
If you choose to use this, prepare a solution of the amylase inhibitor by opening or grinding one tablet in a mortar and pestle. Then dissolve the resulting powder in 10ml phosphate buffer solution. This will be the “full-strength inhibitor” solution. Take 1ml of this solution and add it to 9ml phosphate buffer; this will be the “1/10-strength inhibitor” solution.

The “control” reaction

You will be doing several reactions with different levels of inhibitors. In order to be sure that any differences in activity are due to the inhibitor and not differences in the concentration, age, etc. of the amylase solution, you need to compare the rates of reaction to un-inhibited amylase. This un-inhibited reaction is the “control” against which you will compare the reactions with inhibitor present to determine the effect, if any, of the inhibitor.

1. Determine which treatment you are going to use. Obtain the necessary reagents. Note that, for the chemicals, the stock solutions are at 10-times higher concentration than they will be in the reaction. This is because the stock solutions will be diluted 10-fold (0.5 ml into 5ml) in the final reaction.

Set up your spot plates with IKI as in step (3).

The next steps differ depending on the type of inhibitor you are using:

• If you are testing temperature:

1. Obtain 3 test tubes.
 - a. Label them "SS" and your group name.
 - b. Pipette 4ml stock starch solution into each (be sure to swirl the stock first!).
 - c. Leave one at 37°C – this is the control – and let the other two equilibrate for 5 minutes at the temperatures you will be testing.
2. Obtain 3 more test tubes.
 - a. Label one with "Control" (37°C) and the others with the temperature at which each will be tested along with your group name.
 - b. Pipette 1ml 10% amylase into each tube.
 - c. Leave one at 37°C – this is the control – and let the other two equilibrate for 5 minutes at the temperatures you will be testing.
3. Use the same techniques as in Part I, steps 4-7.
 - a. Bring your materials to the water baths and keep your test tubes in the water bath during the experiment.
 - b. Start the timer while one student pours the contents of the SS tube at a given temperature into the tube containing 10% amylase at the same temperature.
 - c. Take one drop immediately and put it in the first depression of a clean spot plate.
 - d. Put this tube back into your temperature condition and keep it there for the duration of the experiment.
 - e. Continue taking drops at 30 second intervals to test for the presence of starch and take 2 test drops after you have a negative reaction to be sure it is complete.

- If you are testing a chemical inhibitor:
 1. Put drops of IKI into wells of a clean spot plate as before.
 2. Obtain 3 test tubes
 - a. Label all three with the name of the inhibitor you chose and your group name
 - b. Further label one with “Control”, one with “reduced-strength inhibitor”, and the last with “full-strength inhibitor”.
 3. Put in starch and inhibitor as appropriate:
 - a. Pipette 4ml stock starch solution into each (be sure to swirl the stock first!).
 - b. Pipette 0.5ml phosphate buffer into the “control” tube and mix gently.
 - c. Pipette 0.5ml of reduced-strength inhibitor into the “reduced-strength inhibitor” tube and mix gently.
 - d. Pipette 0.5ml of full-strength inhibitor into the “full-strength inhibitor” tube and mix gently.
 - e. Put all 3 tubes at 37°C to equilibrate for 5 minutes.
 4. Using the same techniques as in Part I, steps 4 to 7.
 - a. Bring your materials to the water baths and keep your test tubes in the water bath during the experiment.
 - b. When you’re ready to begin, add 0.5 ml of 10% amylase solution to a tube.
 - c. Immediately begin timing the experiment and take a drop now for time “0” and put it in the first depression of a clean spot plate.
 - d. Continue taking drops at 30-second minute intervals and recording the presence or absence of starch as you have done before.
 - e. Use the chart below to record your results. Repeat this procedure with the other 2 conditions.

With either chemical inhibitors or temperature:

4. Enter your data into the table below. Put a check (√) when any blue-black color is visible. Put an “x” when there is a complete negative result. Show 3 Xs in a row to be sure the reaction is complete.

CONDITION			
Time (min:sec)	Control (no special treatment)	Treatment 1:	Treatment 2:
0:00			
0:30			
1:00			
1:30			
2:00			
2:30			
3:00			
3:30			
4:00			
4:30			
5:00			
5:30			
6:00			
6:30			
7:00			
7:30			
8:00			
8:30			
9:00			
9:30			
10:00			

5. In the following table, put your group's data.

CONDITION	TIME TO REACH ENDPOINT	RATE OF REACTION (100/TIME TO ENDPOINT)

Lab Report

Information about your lab reports:

- The report must be typed; handwritten reports will not be accepted. Hand-drawn and labeled drawings are fine.
- Lab reports are due the next week at the start of lab. This is a firm deadline.
- Although you will perform these activities as a group, each member of the group must turn in an individual lab report. Each person's report must be in his or her own words as much as possible.

Your lab report must contain the following items:

1. Data table from Part I (columns: Amylase concentration; time to endpoint; rate of reaction).
2. Line graph of rate of reaction (y-axis) vs concentration of amylase (x-axis).
3. Data table from Part II step 5.
4. Answers to the following questions for your treatment:
 - a. Which treatment did your group choose?
 - b. Explain briefly why you would expect that treatment to inhibit amylase.
 - c. Did your treatment inhibit amylase? Explain your interpretation briefly using the data from (3).
 - d. Provide a plausible explanation for your result from 4c – why did (or why didn't) the inhibitor inhibit amylase.