ADVANCED PLACEMENT BIOLOGY

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Regulation of Enzyme Activity

Introduction

Enzymes are proteins that catalyze biological reactions. Because virtually every vital function in the cell is dependent upon the activity of one or more enzymes, it is critical to life that the activity of these catalysts be precisely regulated. In this laboratory exercise, we will investigate four ways in which the activity of an enzyme can be regulated.

The activity of an enzyme is typically measured as either the rate at which the enzyme's substrate is utilized or the rate at which one or more of its products accumulate.

SUBSTRATE → PRODUCT 1 + PRODUCT 2 + ... etc.

In this laboratory exercise, we will measure the hydrolysis of soluble potato starch by the activity of the enzyme α-amylase. α-Amylase hydrolyzes internal α1-4 linkages in starch to produce the disaccharide maltose, that is composed of two glucose monomers covalently linked by a α1-4 glycosidic bond.

$$\textbf{starch} \xrightarrow{\alpha\text{-amylase}} \textbf{maltose}$$

$$\textbf{-glu-glu-glu-glu-glu-glu-glu-glu-glu-} \xrightarrow{\alpha\text{-amylase}} \textbf{glu-glu + glu-glu + glu-glu}$$

Thus, the starch polymer comprising many hundreds of glucose molecules is digested by α-amylase to produce the much smaller disaccharide, maltose.

We will take advantage of the unique interaction of iodine with starch, producing a characteristic blue-black color, to provide us with a means to quantify the activity of α-amylase using a spectrophotometer. When iodine is added to a solution of starch, the intensity of the blue-black color formed is proportional to the amount of starch present. As the amount of starch decreases, as would occur when it is digested by α-amylase, the intensity of the blue-black color will decrease proportionately. We can measure the intensity of the blue-black color by use of a spectrophotometer, which, as the name implies, measures light at various wavelengths or colors.

For this laboratory exercise, we will evaluate the effects of substrate concentration, enzyme concentration, pH, and temperature on the activity of α-amylase from barley, a type of grain.

Materials

Buffers: 1mM phosphate at pH 2 to 12
Starch Solution: Potato starch at a concentration of 2.5 mg/ml (2.5 μg/μl)
α-amylase: 2.5 mg/ml (2.5 μg/μl)
Ice and water baths at ~4, 15, 25, 37, 50, and ~100°C
10 ml pipettes and pipettor
Adjustable Eppendorf pipettors and tips
Spectrophotometer (Spec 20D) and tubes
Lugol's iodine solution

Procedure

A. Effect of Substrate Concentration on the Rate of the Reaction

The rate of an enzyme reaction is dependent upon the concentration of the substrate, which affects the ability of the enzyme to 'find' the substrate in solution and form the enzyme-substrate complex. It stands to reason that as the concentration of substrate decreases, the rate of formation of the enzyme-substrate complex will decrease, and hence, the rate of the reaction catalyzed by the enzyme will decrease. In this part of the lab exercise, we will vary the amount of substrate (starch) available to the enzyme (α-amylase) and measure the amount of starch remaining after a finite amount of time.

1. Prepare 11 spectrophotometer tubes with the indicated amount of each solution. NOTE: Use only pH 7.0 buffer.

Tube No.	µl Starch	ml of Buffer	Absorbance at 550nm
1	300	2.5	
2	200	2.6	
3	100	2.7	
4	50	2.8	
5	30	2.8	
6	0	3.0	
7	300	2.5	
8	200	2.6	
9	100	2.7	
10	50	2.8	
11	30	2.8	

2. Separate tubes 1-6 from tubes 7-11.
3. Rapidly, but with accuracy, add 20 µl of enzyme solution to each of tubes 7-11. Mix the reagents in the tube by vortexing. Note the time when enzyme was added to the last tube (#11). Incubate the tubes at room temperature.
4. Five (5) minutes after the addition of enzyme to the last tube (#11), add 50 µl of Lugol's iodine solution to all of the tubes, beginning with tubes 7-11. Again mix the reagents by vortexing. This addition of iodine effectively stops the enzymatic reaction and provides a measurable indicator of enzyme activity.
5. Determine the absorbance of light at 550 nm for each of the 11 tubes. The sample chamber should be empty and the digital readout indicating a transmittance of 0.0. If your spectrophotometer does not read 0.0, call your instructor to properly set the spectrophotometer. Begin with tube 6. Insert the tube in the sample chamber with the white line on the tube facing toward you and aligned with the line on the front edge of the sample chamber. Close the lid of the sample chamber and adjust the digital readout **using the right-hand knob on the lower front of the spectrophotometer**. The digital readout should indicate 100.0. Press the Mode button next to the digital readout until the absorbance light comes on. The digital readout should read 0.000 (0.001 is OK). The spectrophotometer now 'knows' how much light will pass through the tube if there was no starch in the solution.
6. Replace tube 6 with tube 5. Read and record the absorbance in the last column of the data table above. Continue to measure the absorbance of all of the tubes, recording the corresponding absorbance in the data table. Do not readjust the spectrophotometer with each new tube.
7. Tubes 1-6 give you a "Standard Curve" – known amounts of starch with known absorbance readings – from which you can determine the amount of unhydrolyzed starch in the tubes receiving enzyme (the experimental tubes).

B. The Effect of Enzyme Concentration on the Rate of the Reaction

Similar to the effect of substrate concentration, the concentration of the enzyme effects the efficiency at which the enzyme-substrate complex can form and the rate of product formation. In this part of the lab exercise, the amount of substrate is kept constant and the amount of enzyme is varied.

1. Prepare the following spectrophotometer tubes with the indicated amount of each solution. **Do not add the enzyme at this time**. NOTE: Use only pH 7.0 buffer.

Tube No.	µl Starch	ml Buffer	µl Enzyme	Absorbance at 550nm
1	300	2.5	50	
2	300	2.5	40	
3	300	2.5	30	
4	300	2.5	20	
5	300	2.5	10	
6	300	2.5	0	
7	200	2.6	0	
8	100	2.7	0	
9	50	2.8	0	
10	30	2.8	0	
11	0	2.8	0	

2. Rapidly, but with accuracy, add the indicated amount of enzyme to tubes 1-5. Mix the reagents in the tube by vortexing. Note the time when the enzyme was added to each tube. Incubate the tubes at room temperature.
3. Five (5) minutes after the addition of enzyme to the first tube (#1), add 50 µl of Lugol's iodine solution to tube 1. Mix by vortexing. Add iodine to each successive tube when 5 minutes have elapsed for each tube. The addition of iodine effectively stops the enzymatic reaction and provides a measurable indicator of enzyme activity. Finally, added iodine to tubes 6-11.
4. Determine the absorbance of light at 550 nm for each of the 11 tubes. The sample chamber should be empty and the digital readout indicating a transmittance of 0.0. If your spectrophotometer does not read 0.0, call your instructor to properly set the spectrophotometer. Begin with tube 11. Insert the tube in the sample chamber with the white line on the tube facing toward you and aligned with the line on the front edge of the sample chamber. Close the lid of the sample chamber and adjust the digital readout using the **right-hand knob on the lower front of the spectrophotometer**. The digital readout should indicate 100.0. Press the Mode button next to the digital readout until the absorbance light comes on. The digital readout should read 0.000 (0.001 is OK). The spectrophotometer now 'knows' how much light will pass through the tube if there was no starch in the solution.
5. Replace tube 11 with tube 10. Read and record the absorbance in the last column of the data table above. Continue to measure the absorbance of all of the tubes, recording the corresponding absorbance in the data table. Do not readjust the spectrophotometer with each new tube.
6. Tubes 6-11 give you a "Standard Curve" – known amounts of starch with known absorbance readings – from which you can determine the amount of unhydrolyzed starch in the tubes receiving differing amounts of enzyme (the experimental tubes).

C. The Effect of pH on Enzyme Activity

The enzymatic activity of an enzyme is exquisitely sensitive to changes in its environment that alter its tertiary structure. One of the most critical environmental parameters affecting an enzyme's activity is pH. The integrity of α-helical and β-pleated sheet regions of a protein is dependent upon intrachain hydrogen bonding. Interruption of these hydrogen bonds, especially by the addition of H+ ion, can significantly alter the tertiary structure of an enzyme and consequently its enzymatic activity. In this part of the laboratory exercise, we will examine the effect of pH on the activity of α-amylase.

1. Prepare 17 Spectrophotometer tubes according to the following table.

Tube	**µl**	**ml of Specific pH Buffers**											
No.	**Starch**	**2.0**	**3.0**	**4.0**	**5.0**	**6.0**	**7.0**	**8.0**	**9.0**	**10.0**	**11.0**	**12.0**	**Absorbance**
1	300	2.5											
2	300		2.5										
3	300			2.5									
4	300				2.5								
5	300					2.5							
6	300						2.5						
7	300							2.5					
8	300								2.5				
9	300									2.5			
10	300										2.5		
11	300											2.5	
12	300						2.5						
13	200						2.6						
14	100						2.7						
15	50						2.8						
16	30						2.8						
17	0						2.8						

2. Rapidly, but with accuracy, add 20 µl of enzyme to tubes 1-11. Mix the reagents by vortexing. Note the time when enzyme was added to the last tube. Incubate the tubes at room temperature.

3. Five (5) minutes after the addition of enzyme, add 50 µl of Lugol's iodine solution to all tubes. Again, mix by vortexing. This addition of iodine effectively stops the enzymatic reaction and provides a measurable indicator of enzyme activity.

4. Determine the absorbance of light at 550 nm for each of the 17 tubes. The sample chamber should be empty and the digital readout indicating a transmittance of 0.0. If your spectrophotometer does not read 0.0, call your instructor to properly set the spectrophotometer. Begin with tube 17. Insert the tube in the sample chamber with the white line on the tube facing toward you and aligned with the line on the front edge of the sample chamber. Close the lid of the sample chamber and adjust the digital readout using the **right-hand knob on the lower front of the spectrophotometer**. The digital readout should indicate 100.0. Press the Mode button next to the digital readout until the absorbance light comes on. The digital readout should read 0.000 (0.001 is OK). The spectrophotometer now 'knows' how much light will pass through the tube if there was no starch in the solution.

5. Replace tube 17 with tube 16. Read and record the absorbance in the last column of the data table above. Continue to measure the absorbance of all of the tubes, recording the corresponding absorbance in the data table. Do not readjust the spectrophotometer with each new tube.

6. Tubes 12-17 give you a "Standard Curve" – known amounts of starch with known absorbance readings – from which you can determine the amount of unhydrolyzed starch in the tubes receiving differing pH buffers (the experimental tubes).

D. The Effect of Temperature on Enzymatic Activity

Enzymatic reactions are ultimately chemical reactions and all chemical reactions are dependent upon the temperature of the system. An enzyme lowers the activation energy but does not eliminate the need for energy input. Where do you think the activation energy comes from in our test system? Although we are using a plant enzyme for this exercise, it too has an optimal temperature for its activity. In this part of the laboratory exercise, we will evaluate the enzymatic activity of α-amylase over a broad temperature range.

1. Prepare the following spectrophotometer tubes with the indicated amount of each solution. **Do not add enzyme at this time.** NOTE: Use only pH 7.0 buffer.

Tube No.	µl Starch	ml Buffer	Temperature (°C)	Absorbance at 550 nm
1	300	2.5	~4	
2	300	2.5	15	
3	300	2.5	25 (room temperature)	
4	300	2.5	37	
5	300	2.5	50	
6	300	2.5	~100	
7	300	2.5	25	
8	200	2.6	25	
9	100	2.7	25	
10	50	2.8	25	
11	30	2.8	25	
12	0	2.8	25	

2. Place each tube in the ice or water bath appropriate for its temperature for 7-10 minutes in order to equilibrate the buffer.

3. Rapidly, but with accuracy, add 20 µl of enzyme to tubes 1-6. Mix by vortexing. Note the time when enzyme was added to the first tube (#1). Incubate the tubes at their appropriate temperatures.

4. Five (5) minutes after the addition of enzyme to tube #1, add 50 µl of Lugol's iodine to all tubes. Mix by vortexing. The addition of iodine effectively stops the enzymatic activity and provides a measurable indicator of enzyme activity.

5. Determine the absorbance of light at 550 nm for each of the 12 tubes. The sample chamber should be empty and the digital readout indicating a transmittance of 0.0. If your spectrophotometer does not read 0.0, call your instructor to properly set the spectrophotometer. Begin with tube 12. Insert the tube in the sample chamber with the white line on the tube facing toward you and aligned with the line on the front edge of the sample chamber. Close the lid of the sample chamber and adjust the digital readout using the **right-hand knob on the lower front of the spectrophotometer**. The digital readout should indicate 100.0. Press the Mode button next to the digital readout until the absorbance light comes on. The digital readout should read 0.000 (0.001 is OK). The spectrophotometer now 'knows' how much light will pass through the tube if there was no starch in the solution.

6. Replace tube 12 with tube 11. Read and record the absorbance in the last column of the data table above. Continue to measure the absorbance of all of the tubes, recording the corresponding absorbance in the data table. Do not readjust the spectrophotometer with each new tube.

7. Tubes 7-12 give you a "Standard Curve" – known amounts of starch with known absorbance readings – from which you can determine the amount of unhydrolyzed starch in the tubes receiving differing pH buffers (the experimental tubes).

Data Analysis and Conclusions

You are to write a report on this laboratory exercise in which you address the following points.

A. Effect of Substrate Concentration in the Rate of the Reaction

1. Calculate the amount of starch hydrolyzed per minute (in μg/min) for each starting concentration of starch and plot these values against the starting starch concentrations.
2. Based upon your experimental results, explain the effect of substrate concentration on the rate of an enzymatic reaction?

B. Effect of Enzyme Concentration on the Rate of the Reaction

1. Calculate the amount of starch hydrolyzed (in μg/min) for each starting enzyme concentration and plot these values against the starting enzyme concentration.
2. Calculate the amount of starch hydrolyzed (in μg) per minute per μg of enzyme (μg starch/min/μg enzyme) added to the tube for each starting enzyme concentration. Plot these values against the starting enzyme concentrations.
3. Discuss the significance of the difference between these two graphs and what they tell you about the effect of enzyme concentration on enzymatic activity.

C. Effect of pH on Enzyme Activity

1. Calculate the amount of starch hydrolyzed per minute (in μg/min) for each pH and plot these values against the pH of the solution.
2. Part of the digestive process in humans is the addition of HCl to the food by the stomach. Based upon your experimental results and assuming human α-amylase has a pH optimum of 7.0, what would happen to the activity of your salivary amylase in the stomach. Explain your answer.

D. Effect of Temperature on Enzymatic Activity

1. Calculate the amount of starch hydrolyzed per minute (in μg/min) at each temperature tested and plot these values against the temperature.
2. Using your knowledge of the effect of temperature on enzyme activity, attempt to explain the distinctive coloration of Siamese cats in which brown pigment is only present at the extremities – paws, tip of tail and each ear. (Hint: The production of the brown pigment is an enzyme-catalyzed reaction.)