Colorimetric Measurements of Amylase Activity: Improved Accuracy and Efficiency with a Smartphone

By

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Supporting Information

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Name ____ID__________ Date______________

Experiment: UV–Vis and Smartphone Colorimetric Measurements of Amylase Activity

Objectives:

1. To determine the activity of amylase as a function of temperature.
2. To compare the results from UV–Vis and smartphone colorimetric analysis.
3. To observe the impact of light scattering in UV–Vis measurements.

Introduction:

Enzymes are protein molecules that act as catalysts by increasing the rates of chemical reactions without affecting the amount of products obtained. In enzymatic reactions, the reactants are called substrates, and enzymes catalyze reaction that converts them into different molecules, called products. Enzymes are crucial in all living organisms as they control their metabolic processes. The breakdown of food into smaller molecules and extracting energy from it requires various enzymes. Each enzyme is specific for a certain reaction. It acts on particular substrates to produce particular products. The high specificity of enzymes makes them powerful catalysts. Only a certain set of specific reactions are enhanced, while chemical catalysts are less specific and a wide variety of products can be obtained.

The activity of enzymes can be altered by various conditions, such as temperature, pH, and the concentration of substrate. There are also molecules that increase or decrease enzyme activity. Inhibitors are molecules that decrease enzyme activity; activators are molecules that increase activity. Many drugs and poisons are enzyme inhibitors. Enzymes are also used in commercially, such as the synthesis of antibiotics. Some consumer products also contain enzymes to speed up biochemical reactions, such as enzymes in laundry detergent for breaking down of protein or fat stains on clothes.

Amounts of enzymes can either be expressed as molar concentration, as with any other chemicals. However, since enzyme has to be active to perform their function as catalyst; therefore, it is more appropriate to describe how active an enzyme is in terms of activity. Enzyme activity is a measure of the quantity of active enzyme present and is thus *dependent* on conditions, *which should be specified*. For amylase, 1.0 unit of enzyme activity is defined as the amount of enzyme producing of 1.0 μmol of reducing sugar, equivalent to glucose per minute at certain assay conditions. A unit may be defined differently for some enzymes. While the term *activity* refers to the total units of enzyme in a solution, the number of enzyme units per milligram of total protein is the *specific activity*, expressed in units/mg. The specific activity is a measure of enzyme purity: it increases during purification of an enzyme and becomes maximal and constant when the enzyme is pure.[1] In this experiment, students will measure the activity of amylase as a function of temperature through spectroscopic and colorimetric measurements.

Amylase is a group of enzymes that breaks down starch into disaccharide maltose, which is a reducing sugar. Amylases hydrolyze α-1,4-glycosidic bonds. The substrate of amylase enzyme is starch, which is a turbid slurry solution. Determination of the amylase activity can be done by measuring the amount of starch (substrate) remained by either observing the decrease in turbidity of

the starch solution, or by the iodine test of starch.

Alternatively, the activity can be determined by measuring the reducing sugar (the amylase digested product) obtained from the reaction in a certain period of time. In this study, amylase activity in saliva is determined by colorimetric method to measure for the reducing sugar produced from the reaction. Reducing sugar, such as glucose, reduces 3,5 dinitrosalicylic acid (DNS, yellow) in an alkaline condition to 3-amino-5-nitrosalicylic acid (reddish brown).[2,3] The aldehyde group in glucose is oxidized to gluconic acid. The absorbance of the resultant solution is measured at 540 nm. We will study the activity of amylase as a function of temperature.

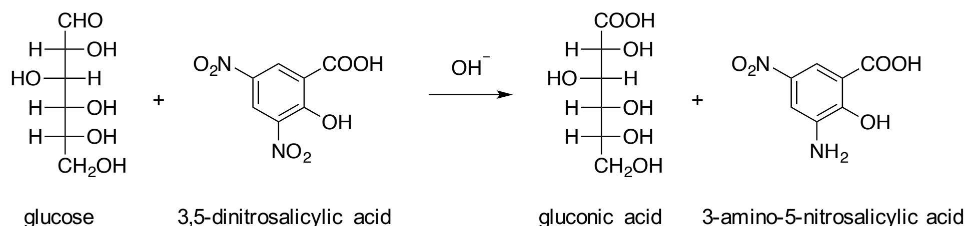

Figure 1: Glucose reduces 3,5-dinitrosalicylic acid (DNS) into 3-amino-5-nitrosalicylic acid

In addition to absorbance measurement, we will use a method of smartphone colorimetric analysis to measure the concentrations of glucose. Since the reactant, DNS, changes color to reddish brown of 3-amino-5-nitrosalicylic acid, we can measure the color intensity of the product to quantify the concentration of glucose presented. Similar to spectroscopic absorbance measurement, a standard calibration curve of color intensity vs. known glucose concentrations can be created. From the calibration curve, the concentration of unknown glucose from amylase reactions can be determined. The results from the two methods: UV–Vis and smartphone colorimetry can be compared to evaluate interferences and suitability of each method in amylase activity assay.

Hazard and Safety:

Dinitrosalicylic acid is corrosive and harmful if inhaled or swallowed. The DNS reagent also contains 0.40 M NaOH, which is an irritant. The reduced product, 3-amino-5-nitrosalicylic acid, is toxic if swallowed and causes eye irritation. The DNS reagent must be handled with care; laboratory gloves and eye protection must be worn at all times. DNS-containing waste must be separated for proper disposal. Phosphate buffer may cause eye and skin irritation. Saliva is a body fluid; thus, students should handle their own saliva, including disposal and rinsing equipment. Hot water can cause burns. Use heat resistant gloves when handling test tubes in a hot water bath. Do not heat test tubes on a direct flame.

Materials (per group):

- 3.0 mL of 1 mg/ml standard glucose solution
- 11.0 mL of 2% starch solution (2 g corn starch in 100 mL water; heated with swirling until homogeneous)
- 0.1 mL of saliva sample

- 7.0 mL of 0.1 M potassium phosphate buffer, pH 6.0
- 12.0 mL DNS reagent (1.0 g of 3,5-dinitrosalicylic acid in 50 mL of water, 30 g sodium potassium tartrate tetrahydrate, 20 mL of 2 M NaOH, dilute to 100 mL with DI water)
- Water baths at temperatures 0-60°C and ~90°C
- 25 small test tubes
- A Petri dish
- Whatman #4 filter paper and a hole puncher
- Glass Pipettes (1 mL and 5 mL) with rubber bulbs
- P1000 and P20 micropipettes
- A 50-mL beaker for saliva sample and a waste beaker

Method:

A. Preparation of the standard calibration curve:

1. Prepare 5 test tubes with the following solutions.

Tube no.	Standard Glucose (mL)	DI water (mL)	[glucose] (mg/mL)
1	0	1.00	0
2	0.25	0.75	0.25
3	0.50	0.50	0.50
4	0.75	0.25	0.75
5	1.00	0	1.00

2. Add 1.00 mL of DNS to each test tube. Mix the solution well.
3. Boil for 5 min, and immediately, transfer to ice bath. Allow the reactions to come to room temperature.
4. Pipette 0.50 mL of each solution into new test tubes and add 2.50 mL of water to each tube and mix well.
5. Measure the absorbance at 540 nm of the diluted solution using a spectrophotometer.
6. Plot graph of absorbance vs. concentration of glucose. This is your standard calibration curve for the spectroscopic measurement.
7. **Keep the undiluted solutions and Tube 1 (blank).** Dispose of the diluted tube #1-#5 in **a waste separate container**.

B. Amylase activity at different temperatures:

1. We will be working with a 50-fold diluted saliva in distilled water. Calculate the volume of saliva required in the experiment. Rinse your mouth. Spit in a small beaker and dilute it appropriately. This is your diluted amylase solution.
2. Prepare 7 reaction tubes as follows:

Solutions	Amount in each tube (mL)
2% starch solution	1.50
0.1 M phosphate buffer, pH 6.0	1.00

3. Mix the solution well after adding each solution. Incubate the test tubes at each temperature a few minutes before adding 0.50 mL of the diluted amylase. **Make sure you add amylase last, since the reactions start when amylase is added to the solution.** Start your timer at the same time that amylase is added to the solution, and immediately transfer to the water bath at specified temperature. Leave 30 sec to 1 min between each test tube for pipetting time.
4. Incubate each test tube, at the specified temperature for 5 min. While waiting, pipette 0.50 mL of DNS solution to 7 new test tubes.
5. After the incubation, pipette 0.50 mL of each reaction mixture to each DNS reaction tube.
6. Put all the DNS reaction test tubes in a boiling water bath for 5 mins.
7. Cool all the tubes in an ice bath. Allow the reaction to come to room temperature.
8. Dilute the DNS reacted solutions by pipetting 0.50 mL of the reactions, adding 2.50 mL of water. Mix the solution well. **Keep the undiluted reaction for part C.**
9. Measure the absorbance at 540 nm. Use the blank solution from the calibration curve as a blank.
10. Using the calibration curve from part A, find the concentration of glucose in each test tube and calculate the activity of amylase at each temperature. For amylase, 1.0 unit is defined as 1.0 μmole of glucose liberated from the reaction of 1.0 ml saliva in 1 min reaction time.

C. Smartphone colorimetric analysis:

1. On a Petri dish, arrange pre-cut filter papers into two rows: 5 pieces for the glucose standard from part A, and 7 pieces for the undiluted reactions from part B.
2. Carefully, pipette 20 μL of the undiluted samples onto each piece of filter paper. Make sure that you can identify your samples.
3. Take a photo of your Petri dish, containing the standards and samples under uniform lighting. Make sure that you take a photo directly above the Petri dish.
4. Import the photo into your computer and use ImageJ, a free image analysis program to measure the intensity of the green color. By clicking on Image > Color > Split channels, then select the green channel. Use a circle tool to select the most of the area without the edges of both the standard and the samples. Measure the area averaged gray intensity from each spot by clicking Analyze > Measure.
5. Plot a graph of Green Color Intensity as a function of the standard glucose concentrations. This is your standard calibration curve for part C.
6. Find the concentration of glucose in each test tube and calculate the activity of amylase at each temperature. For amylase, 1.0 unit is defined as 1.0 μmole of glucose liberated from the reaction of 1.0 ml saliva in 1 min reaction time.
7. Plot a bar graph to compare the activity of amylase at a function of temperature, as obtain from the absorbance measurement and the smartphone colorimetric analysis.

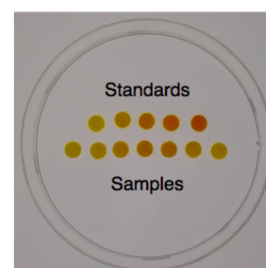

Figure 2: Standards and samples spotted on filter papers

Pre-lab questions

1. What is the purpose of adding DNS solution to the enzyme substrate solution?
2. From the literature, what is the optimum temperature for amylase?
3. From part B, assuming that the measured absorbance at 540 nm for a tube is A, the slope of your calibration curve is m and the y-intercept is c, derive an equation to calculate the activity of amylase in **unit** for this tube, 1.0 unit is defined as 1.0 μmole of glucose liberated from the reaction of 1.0 ml saliva in 1 min. Don't forget to take into account the dilution factors.
4. What are the factors that can affect the measurement? What are the factors that can affect the smartphone colorimetric measurement? Which method do you think will yield a more accurate result for this assay?

Post-lab questions

1. How does temperature affect the activity of amylase?
2. By comparing the results from the two methods of analysis (Absorbance measurements vs. Smartphone and Image Analysis), which method do you think is more suitable for the measurement of amylase activity and why?

Instructors' Notes

Chemicals Preparation

- 1.00 mg/mL Glucose
  - Dissolve 0.25 g of glucose in 250 mL of distilled water
- 2.0% Corn Starch
  - Dissolve 2.00 g of corn starch in 100 mL of distilled water
  - Heated on a hotplate with stirring until homogeneous
- 0.10 M potassium phosphate buffer, pH 6.0
  - 3.30 mL of 1 M K_2HPO_4 and 21.70 mL of 1 M KH_2PO_4
  - Dilute the combined 1 M stock to 250 mL
- DNS reagent
  - Dissolve 1.00 g 3,5-dinitrosalicylic acid in 50 mL of distilled water
  - Add 30.0 g sodium potassium tartrate tetrahydrate
  - Add 20.00 mL of 2.0 M NaOH
  - Dilute to 100 mL with distilled water

Chemicals, CAS No., and Vendors

Chemicals	**CAS No.**	**Vendors**
glucose	50-99-7	Ajax Finechem
corn starch	9005-25-8	Sigma-Aldrich
dipotassium hydrogenphosphate	7758-11-4	Ajax Finechem
potassium dihydrogenphosphate	7778-77-0	Ajax Finechem
3,5-dinitrosalicylic acid	609-99-4	Sigma-Aldrich
potassium tartrate tetrahydrate	6381-59-5	Ajax Finechem
sodium hydroxide	1310-73-2	Ajax Finechem
distilled water	7732-18-5	Thanthiya Technology

Image acquisition platform

- A 9-watt LED light bulb from a local convenient store
- A desk lamp
- A 2-mm solid white acrylic sheet
- Two sets of metal stand and clamps

Answer to pre-lab questions

1. What is the purpose of adding DNS solution to the enzyme substrate solution?
   The hydrolysis of starch by amylase yields reducing sugar, which reacts with DNS to produce a reddish-brown product of 3-amino-5-nitrosalicylic acid. Therefore, DNS allows quantification of reducing sugar and activity of amylase.
2. From the literature, what is the optimum temperature for amylase?
   37 °C (The literature value is between 30–40 °C.)
3. From part B, assuming that the measured absorbance at 540 nm for a tube is *A*, the slope of your calibration curve is *m* and the y-intercept is *c*, derive an equation to calculate the activity of amylase in **unit** for this tube, 1.0 unit is defined as 1.0 μmole of glucose liberated from the reaction of 1.0 ml saliva in 1 min. Don't forget to take into account the dilution factors.

$$Activity = [glucose]\left(\frac{mg\ glucose}{mL\ reaction}\right) \times \frac{1\ mmol}{180.16\ mg} \times \frac{10^3\ \mu mol}{1\ mmol} \times \frac{3\ mL\ reaction}{0.5\ mL\ dil.saliva} \times dil.factor \times \frac{1}{5\ min}$$

4. What are the factors that can affect the UV-Vis measurement? What are the factors that can affect the smartphone colorimetric measurement? Which method do you think will yield a more accurate result for this assay?
   Factors that can affect the UV-Vis measurement:
   Light scattering by suspended starch particles, scratches and fingerprints on the cuvettes, temperature, contaminants, etc.
   Factors that can affect the smartphone colorimetric measurement:
   Amount of solutions pipetted on the filter papers, thickness of filter papers, lighting when the image is taken, image quality, selecting circles during image analysis, etc.

Answer to post-lab questions

1. How does temperature affect the activity of amylase?
   At temperature lower than the optimal temperature of the enzyme, the energy of the enzyme-substrate is not enough to overcome the energy barrier; therefore, the reaction is limited. At high temperature, amylase is denatured and can no longer catalyze the hydrolysis reaction. Activity of amylase is reduced at extreme low and high temperatures.
2. By comparing the results from the two methods of analysis (UV-Vis Absorbance measurements vs. Smartphone and Image Analysis), which method do you think is more suitable for the measurement of amylase activity and why?
   In the case that amylase activity is limited, the smartphone colorimetry with image analysis is more suitable as the starch remained in the solution reflects and scattered the incident light in UV-Vis absorbance measurements.

Authors' data

Data shown in the main text are examples of the students' data. It is not possible to include error bars in those figures because students only run the experiments once. Authors' data including standard deviations from three trials are shown in Figure S1. From the smartphone method, the activity of the enzyme is highest and has a similar level at 30 and 40 °C. The absolute activity differs among student groups, as different saliva samples contain different amount of amylase. The activity obtained from the UV–Vis method is inaccurate due to the presence of starch particles in the samples.

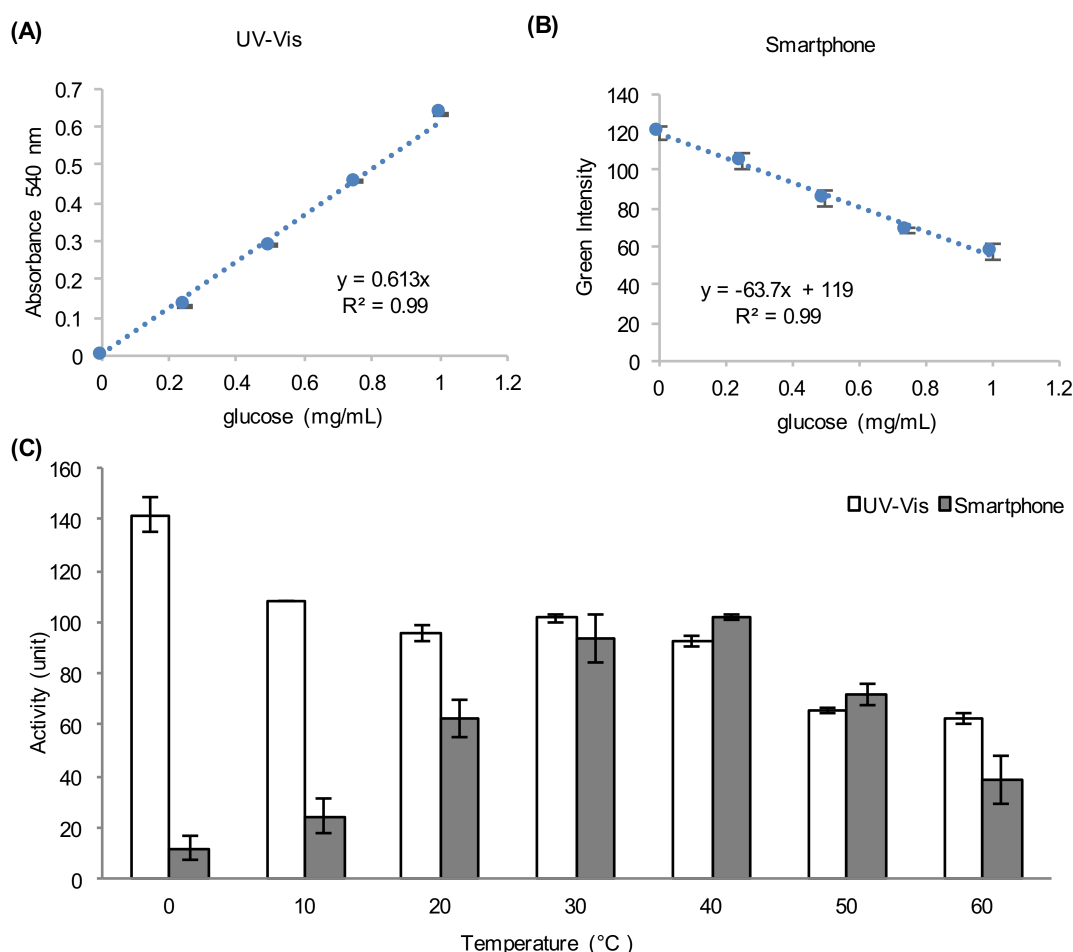

Figure S1. Authors' data from three measurements. (A) The standard calibration curve from UV-Vis absorbance measurements. (B) The standard calibration curve from the smartphone colorimetry. (C) The activity of amylase as a function of temperature. Error bars in all figures are standard deviations from three trials.

Student evaluation survey: Amylase activity at different temperatures Date____________________

1. List all chemistry classes you have taken ____

   List all biology classes you have taken ____

   List all chemistry/biology classes you are currently taking ____

2. Describe what you learn from conducting the experiment

   ____

   ____

   ____

   ____

   ____

   ____

3. Describe the aspects of the experiment that you like most and aspects that need improvements:

Aspects that you like most	**Aspects that need improvements**

4. Rate your agreement to the following statements. A rating of 1 (strongly disagree) to 5 (strongly agree). Please place an X in the table below.

Statements	**1**	**2**	**3**	**4**	**5**
I am satisfied with the contents of this experiment					
The experiment improves my understanding of spectrophotometry.					
The experiment improves my understanding of enzyme activities.					
Smartphone can be an adequate tool in colorimetric analysis.					
The experiment is suitable for the course.					
The instruction provides sufficient knowledge for me to conduct this experiment.					

Additional Comments____

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