Kinetic Analysis of Amylase Using Quantitative Benedict's and Iodine Starch Reagents

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Carbohydrates are an important class of molecules used for food by all animals. Enzymes digest these complex sugars and convert them into simpler molecules, acting in a concentration dependent fashion without being changed themselves. One common enzyme is amylase, which breaks down starch into glucose. The protein is found in many places in the body and has been a staple of experiments designed to study enzymatic reactions for many years *(1, 2)*. α-Amylases break down the α(1–4) glycosidic bonds in starch into the simple sugar glucose that is consumed during glycolysis *(3, 4)*. This two-day laboratory uses spectroscopy or colorimetry to demonstrate the kinetics of substrate disappearance and product appearance during the breakdown of starch.

Two common techniques are used to quantify the activity of amylase in this laboratory: first, the quantity of starch is measured using KI/I_2 reagent *(5, 6)*; and second, the appearance of glucose is measured using Benedict's reagent *(7)*. This laboratory develops the student's ability to generate a standard curve, use that curve to calculate concentrations of an unknown, and to relate these concentrations to the activity of amylase. It also emphasizes that there are often multiple ways of approaching the same problem (i.e., the activity of amylase). These labs are used in a second-year cell biology class, although the concept of enzyme function and standard curves could be used at several different levels.

Materials and Reagents

The iodine reagent is made by dissolving 6.0 g of KI in 100 mL of 2% tincture of iodine. Benedict's reagent is made by dissolving 30.0 g of Cu(II) sulfate pentahydrate in 60 mL of hot water (may require extensive heating to fully dissolve) and in a second flask dissolving 20.0 g of sodium carbonate and 34.6 g of sodium citrate dihydrate in 120 mL of hot water. While still warm, these two solutions are mixed slowly as some bubbling will occur and then diluted to 200 mL with distilled water. Starch solution is made by dissolving 0.5 g soluble starch in 100 mL of distilled water (0.5% starch solution) by mixing it in cold water followed by gentle boiling. A 0.1% starch solution should be made by diluting the 0.5% stock into water. A 0.5% glucose solution is made by dissolving 0.5 g dextrose in 100 mL water. The concentrations of these reagents are important, as volumes are chosen to optimize spectrophotometry readings and to avoid saturating conditions.

Hazards

Iodine is often obtained as a solution in alcohol, which is flammable. Iodine also stains clothing and skin, so special care should be taken with this reagent. The Benedict's reagent assay requires that samples be boiled for at least 5 minutes. Tubes should be boiled in a slightly bubbling water bath and appropriate precautions against burns from the boiling water must be taken. Do not heat test tubes directly over a flame. Benedict's reagent should not be mixed with acids.

Results and Discussion

Spectroscopy

A standard curve for the starch reaction with iodine is generated using 0, 0.1, 0.3, 0.6, and 0.9 mL of 0.1% starch solution in a total volume of 3 mL water and one drop (50 μL) of iodine reagent is added and mixed by flicking the test tube (giving a concentration range of 0 to 0.3 mg/mL of starch). The purple color is read quickly at 620 nm in a spectrophotometer (the color fades over time). The linear standard curve obtained using these reagents is shown in Figure 1A, $R^2 = 0.9975$).

To test the reaction of amylase, 1 mL of a 1:100 dilution of human saliva is added to 9 mL of 0.1% starch solution in distilled water, a stopwatch started, and the solutions immediately mixed. Time points are collected immediately after mixing into tubes with 2 mL of water and one drop KI/iodine reagent already added to speed up sample processing. Absorbance readings are converted into starch concentrations using the standard curve and graphed versus time in Figure 1B. The average reaction velocity was calculated by taking the change in starch concentration and dividing by the change in time over that interval (typically 2 minutes). The reaction velocity was then graphed against time (Figure 1C). Early time points are problematic as mixing efficiency and timing of the dilution have a major effect on the intensity of the purple color. The Lineweaver–Burk plot for this reaction is generated by taking the reciprocal values of both axes (shown in Figure 1D) giving a linear response ($R^2 = 0.9961$). Note that this is not a typical method for performing a Lineweaver–Burk plot as the product concentration at later time points is not zero. A more accurate plot could be obtained by using different initial concentrations of starch and measuring the reaction rate in the first few minutes of the digestion.

On the second day of the experiment, A standard curve for the reaction of Benedict's reagent with glucose is made by diluting 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of 0.5% glucose into a total volume of 4.5 mL water and adding 0.2 mL of Benedict's reagent. Note that for this reagent, the absorbance reading of the spectrophotometer decreases and the "0 absorbance" of the machine is defined equal to 0 at 1 mL of 0.5% glucose. The mixture is then boiled for 6 minutes; the appearance of a red precipitate (and the disappearance of the blue color) is a positive indicator for reducing sugars such as glucose. The suspension is then filtered through superfine #42 ashless filter paper to remove the fine red precipitate, and the supernatant is read at 700 nm in a spectrophotometer. Filtration is critical for the absorption reading, as even a small quantity of precipitate will scatter exces-

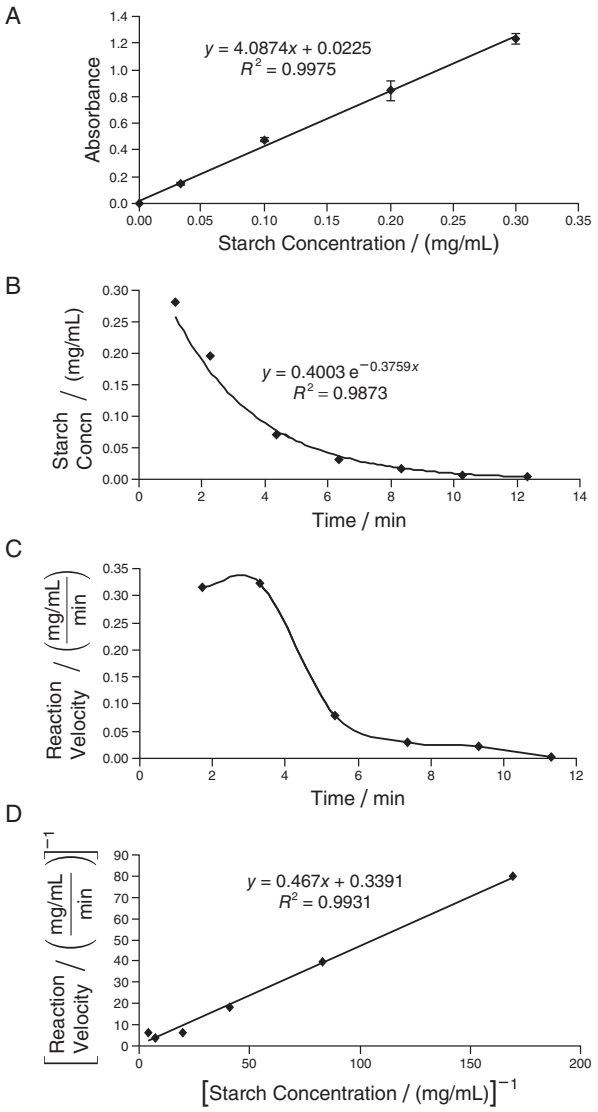

Figure 1. Reaction of starch with iodine: (A) The A_{620} increases linearly with starch concentration owing to the appearance of the purple reaction color. (B) Using the standard curve from 1A, the quantity of starch present at various times after amylase digestion can be plotted based on the A_{620} reading. (C) The plot of reaction velocity vs time shows an anomalous reading early before assuming the expected hyperbolic curve. (D) The Lineweaver–Burk plot for the same data points shows a close approximation to a straight line.

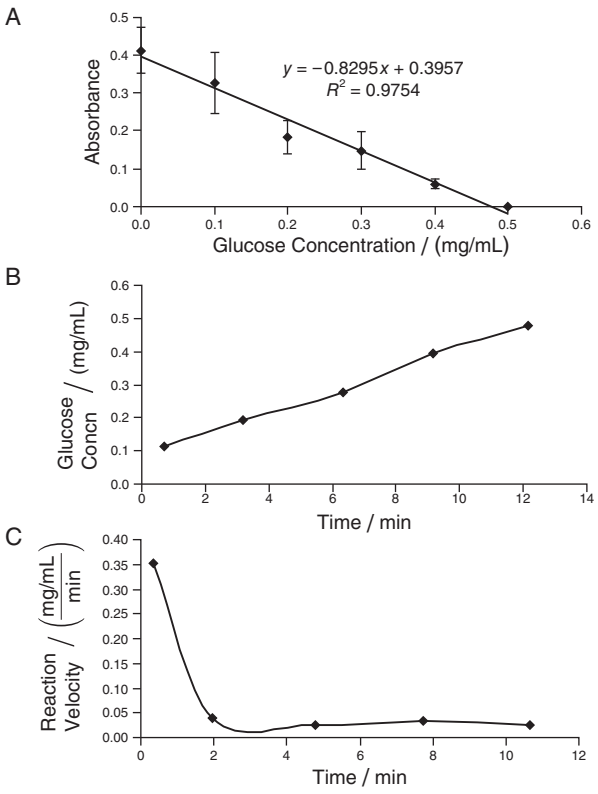

Figure 2. Reaction of glucose with Benedict reagent: (A) Using an arbitrary absorbance of 0 for a glucose concentration of 0.5 mg/mL, the A_{700} is inversely linear to glucose concentration. (B) Using the standard curve from 2A, the A_{700} is used to calculate the concentration of glucose produced by amylase during the digestion of starch over time. Glucose production is linear, consistent with the substrate being at a high concentration compared to the binding affinity. (C) Graph of reaction velocity for quantity of glucose produced vs time. Note that since the Benedict reaction does not follow the substrate Michaelis–Menten kinetics are not appropriate.

sive light. The linear standard curve for glucose concentration is given in Figure 2A ($R^2 = 0.9754$). To test the activity of amylase, 1 mL of a 1:20 dilution of human saliva is added to 9 mL of 0.5% starch solution in water, a stopwatch started, and the solutions immediately mixed. Time points of the amylase:starch reaction are collected immediately and every 3 minutes thereafter. Time points are then diluted in water, treated with 0.2 mL Benedict's reagent, boiled, and carefully filtered as above. Absorbance readings were converted into glucose concentrations using the standard curve and graphed versus time in Figure 2B. Because we are measuring the product of the reaction, and not the substrate, a Lineweaver–Burk plot is not appropriate. However, a graph of reaction velocity versus time (Figure 2C) again demonstrates a rapid dropoff of amylase activity over time as more product and less substrate is present in the reaction.

Colorimetry

This protocol is somewhat problematic as the filtration step to remove the red precipitate must be carried out extremely carefully. A recent article used a flatbed scanner to quantify the quantity of starch in a sample using colorimetry *(5)*. Since the Benedict reagent is blue and the reaction product with a reducing sugar is a red precipitate, a scanner could be used to quantify this reaction as well and avoid the necessity of filtering the red Cu(I) precipitate. The ratio of red to blue should provide an accurate measure of their relative intensities, with increasing concentrations of glucose increasing red intensity and decreasing blue, thus doubly increasing the ratio. This method will also avoid defining an arbitrary 0 point for the spectrophotometer.

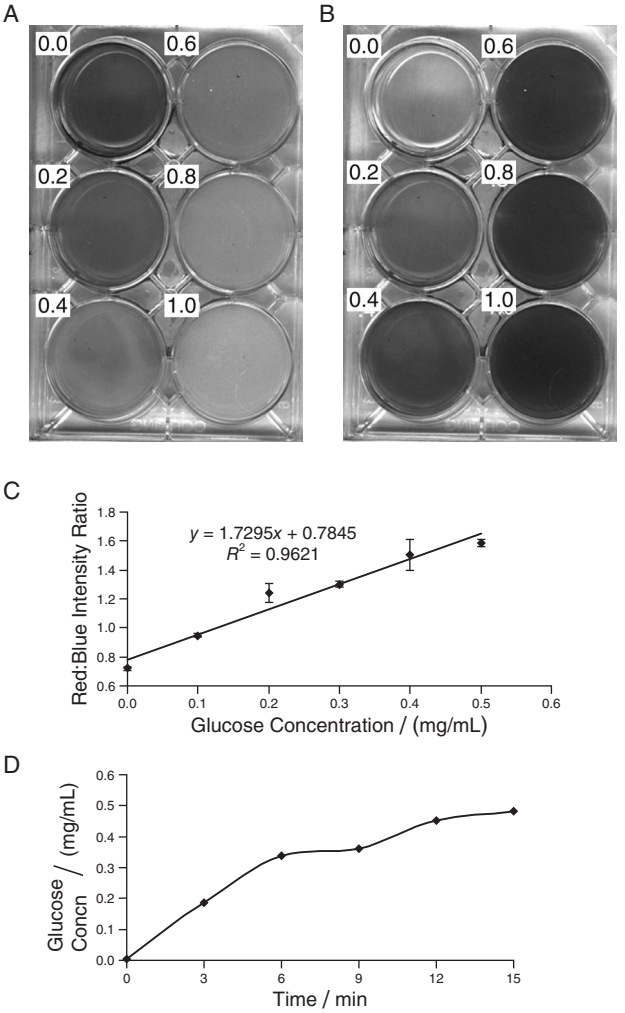

Figure 3. Colorimetry of the Benedict reaction using a flatbed scanner. (A) Grayscale image of the red channel of a scanned image using the listed volume (in mL) of 0.5% glucose solution. (B) Grayscale image of the blue channel from the same scanned image. (C) Graph of the red:blue ratio of the scanned images at various concentrations. This ratio provides a linear representation of glucose concentration detected using Benedict's reagent using a simpler and more available protocol than spectroscopy. (D) The enzymatic digestion of starch with amylase produces glucose detected by the Benedict reaction.

The standard curve was repeated using the same concentrations of glucose but including 0.2 mL Benedict's reagent. Tubes were boiled for 6 minutes, allowed to cool, and transferred without filtration into a 6-well flat bottom plate. Color images were obtained using a minimum of 150 dpi resolution, and images were opened in ImageJ, a freeware image analysis utility provided by the National Institutes of Health *(8)*. The color image was converted into red, green, and blue grayscale images by selecting the image menu, color sub-menu, and choosing RGB split. Grayscale images of the red and blue channels are given in Figure 3A and 3B (note that the more of a given color that an image has, the lighter that color must be in grayscale) showing the disappearance of the blue color and appearance of red. This modification makes the experiment accessible to laboratories without a spectrophotometer and in addition simplifies the glucose detection by removing the filtration step. Intensities were quantified from each of the red and blue images using the circle function (available from the main toolbar) and selecting most of one of the wells (avoiding the edges).

The measure function under the analyze menu was used to obtain the mean intensity of each well separately. In order to take into account both the decreasing blue and increasing red color in each well, we decided to use the red:blue ratio for determining the glucose concentration. Graphing the ratio of red:blue intensities of each well versus the glucose concentration gave a linear result (Figure 3C, $R^2 = 0.9621$) similar to spectroscopy but with less effort. The production of glucose from starch using amylase in saliva is equally quantifiable using colorimetry and glucose concentrations calculated from the standard curve (Figure 3D).

Summary

These experiments demonstrate several aspects of enzyme reaction kinetics, including the disappearance of substrate, changes in enzymatic reaction velocity, and the appearance of reaction product. In addition, the Benedict reaction product can be simply quantified using a flatbed scanner, allowing the quantity of reducing sugars in various samples to be quickly determined. Finally, students gain a better appreciation of the fact that several different methods may be used to obtain information regarding the same chemical reaction.

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