Experiment 9

NATURE OF $\alpha$-AMYLASE ACTIVITY ON STARCH

In Experiment 1 we described the action of $\alpha$-amylase on starch as that of catalyzing the hydrolysis of $\alpha$-1,4-glucosidic bonds at random in the interior of the starch molecule first giving large fragmentation to dextrins followed eventually by the accumulation primarily of diglucose (maltose) and triglucose (maltotriose) units. This action may be illustrated schematically on amylopectin as shown.

Action of $\alpha$-amylase on amylose, the straight chain isomer, gives primarily maltose with a small amount of maltotriose. The action of $\beta$-amylase and glucoamylase, on the other hand, releases maltose and glucose units, respectively, from the non-reducing ends of the starch molecules.

Since maltose and maltotriose still contain $\alpha$-1,4-glucosidic linkages one might inquire as to whether indeed $\alpha$-amylase stops at this level of hydrolysis or does it produce appreciable amounts of glucose. It is the purpose of this experiment to seek an answer to this question as well as to decide whether $\alpha$-amylase is indeed an endo-splitting enzyme.

To accomplish this we shall use three assays. The 3,5-dinitrosalicylic acid reagent will be used to determine the total number of glucosidic bonds hydrolyzed. The Statzyme reagent will be used to determine the total amount of glucose produced and an Ostwald viscometer will be used to determine the rate of decrease of viscosity. In Experiment 1 we indicated that reducing substances such as
glucose, maltose, etc. reduce the nitro groups of 3,5-dinitrosalicylic acid (yellow) forming a compound which is orange-red. By standardizing the reagent against known concentrations of a reducing compound, such as maltose, we can relate the change in absorbance to the number of glucosidic bonds hydrolyzed.

The Statzyme reagent contains, in addition to buffer, glucose oxidase, peroxidase and a chromogenic compound, 4-aminoantipyrine. The reactions involved are:

\[ \beta-D\text{-glucose} + O_2 \xrightarrow{\text{glucose oxidase}} \text{\delta-gluconolactone} + H_2O_2 \]

\[ \text{4-Amino-antipyrine} + p\text{-Hydroxybenzoate} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{Polymer} \ (\lambda_{\text{max}} \text{500 nm}) \]

In these reactions the limiting factor is glucose and the change in absorbance at 500 nm is directly related to the amount of glucose present. Glucose oxidase has primary specificity for \(\beta\)-D-glucose. \(\alpha\)- and \(\beta\)-D-glucose are in equilibrium; however, not all the \(\alpha\)-form is converted to \(\beta\)-form during the 10 min reaction time. Therefore, the standard curve and unknown determinations must be done under the same conditions and the time precisely controlled. (Note: 4-amino antipyrine has
been found to be a carcinogen and is no longer sold for this application. We will therefore use a related glucose assay, which Mike will explain at the beginning of lab class.)

There is no simple, direct correlation between decrease in viscosity and the number of bonds hydrolyzed. A rapid decrease in viscosity associated with a slow increase in number of bonds hydrolyzed is indicative of endosplitting of a polymer.

REAGENTS:

• Starch. Dissolve one gm (weighed accurately) of soluble starch in 100 mL of 0.02 M phosphate buffer, pH 6.9, containing 6.7 X 10^{-3} M NaCl.

• 3,5-Dinitrosalicylic acid reagent. Dissolve at room temperature 1 gm of 3,5-dinitrosalicylic acid in 50 mL of water to which 20 mL of 2 M NaOH is added after dispersing the 3,5-dinitrosalicylic acid in water. After complete solution add 30 gm of sodium potassium tartrate and make to a final volume of 100 mL with deionized water. Store in a brown bottle protected from CO$_2$.

• Statzyme reagent. Prepared according to directions furnished by Worthington Biochemical Co. Dissolve contents of the chromogen vial in 50 mL of water right in the vial. The solution should be prepared fresh daily, preferably just prior to use.

• α-Amylase solutions. Prepared from a commercial preparation (Nutritional Biochemical Corp.) of enzyme derived from $B.\ subtilis$. Solution A contains 0.5 grams of α-amylase per 100 mL; solution B contains 0.005 grams of α-amylase per 100 mL.

• Maltose standard solution. Prepare accurately to contain 1.0 mg maltose/mL in water.

• Glucose standard solution. Prepare accurately to contain 0.075 mg glucose/mL in water.

PROCEDURE:

**Enzyme reactions.** Preferably, the reactions should be run in a water bath. For convenience we shall use room temperature. Determine this temperature by placing a thermometer into a tube of water at room temperature; record temperature.

Viscosity: To 25 mL of 1% starch solution add 5.0 mL of buffer. Mix.

Transfer a 10 mL aliquot to a clean, dry viscometer. Measure the outflow time. This gives the outflow time at zero time of the enzyme reactions.
Reducing end and glucose determinations: To 25 mL of 1% starch solution add 5.0 mL of the α-amylase solution which contains 5 x 10^{-5} g α-amylase per mL. Rapidly transfer a 5 or 10 mL aliquot (depending on size of viscometer) to a clean, dry viscometer. Measure the outflow times at frequent intervals over 30 min. From the same reaction remove duplicate 1.00 mL aliquots of the reaction at 0, 10, 20 and 30 min into tubes containing 1.0 mL of 3,5-dinitrosalicylic acid reagent. At the same time intervals remove duplicate 1.0 mL aliquots into tubes, and immediately heat in boiling water for 2 min. These are for glucose assay.

Effect of higher enzyme levels: Repeat the procedure above including the viscosity determinations but use 5.0 mL of the 5 x 10^{-3} g/mL solution of α-amylase. Take duplicate 0.20 mL samples at 0, 10, 20 and 30 min into tubes containing 0.80 mL of water and 1.0 mL of 3,5-dinitrosalicylic acid reagent. At the same intervals take duplicate 0.20 mL aliquots into tubes containing 0.80 mL of water, and immediately heat in boiling water for 2 min. These are for glucose assay.

Reducing group assay.

Standard curve. To six test tubes add 0, 0.1, 0.3, 0.5, 0.8 or 1.0 mL of a maltose solution containing 1.0 mg maltose/mL. Add water to each tube to give a volume of 1.0 mL. Add 1.0 mL of the 3,5-dinitrosalicylic acid reagent. Heat the tubes for 5 min (exactly) in a boiling water bath. Cool in tap water. Add 5.0 mL of deionized water, mix and read at 540 nm in a spectrophotometer using tube No. 1 as the blank.

Enzyme assays. Place all the samples collected for reducing group determination into 3,5-dinitrosalicylic acid reagent above into a boiling water bath together. Heat for exactly 5 min, remove, cool, add 5.0 mL water, mix and read at 540 nm using tube No. 1 from the standard curve as a blank. This reaction would, of course, measure glucose or mannose, each of which has one reducing end. But since we have a specific assay for glucose (see below), we will attribute the appearance of new reducing groups in excess of the glucose determined with the Statzyme reagent to mannose production. A rapid loss in viscosity in the absence of glucose or mannose production will be attributed to endo cleavage.

Glucose determination.

Standard curve. To six test tubes add 0, 0.1, 0.3, 0.5, 0.8 or 1.0 mL of a glucose solution containing 0.075 mg glucose/mL. Add water to bring the volume to 1.0 mL in each tube. Add 2.0 mL of Statzyme reagent and mix. Exactly 10 min after adding the Statzyme reagent at 37°C, read at 500 nm in a spectrophotometer using tube No. 1 as the blank.
**Enzyme assays.** To each of the tubes collected above for glucose determination add 2.0 mL of Statzyme reagent. Mix. Exactly 10 min after adding Statzyme reagent and holding at 37°C, read at 500 nm using tube No. 1 from the standard curve preparation as a blank. Be sure to keep the reaction time constant; otherwise, the amount of product generated will not correspond to the amount of glucose present.
EXPERIMENT 9

Nature of α-Amylase Activity On Starch

Laboratory Report

Name: ___________________________ Date: _______________________

Temperature of enzyme reactions: ________________.

A. Standard Curves

1. Submit a graph of absorbance versus maltose concentration ($M$). Calculate the extinction coefficient.

2. Submit a graph of absorbance versus glucose concentration ($M$). Calculate the extinction coefficient.

B. Enzyme Activity

1. For the reaction containing $5.0 \times 10^{-5}$ g α-amylase/mL stock solution submit a graph in which maltose equivalents (measured by reducing group assay) are plotted versus time. Be sure to subtract the zero time value from the values at 10, 20 and 30 min in order to correct for contribution by the enzyme and substrate to the color. On the same graph also plot glucose equivalents and viscosity versus time.

2. Repeat item 1 for the reaction containing $5.0 \times 10^{-3}$ g α-amylase/mL stock solution.

3. Calculate the percent hydrolysis of the substrate at 10, 20 and 30 min based on the maltose equivalents and glucose equivalents and report in table form.

C. Evaluate your data in terms of the action of α-amylase on starch.