Characterization of Invertase from *Saccharomyces cerevisiae*

Experiment 2: Activity of Invertase with α-Glucosides

Experiment 2 Introduction

The method we used in *A Manual for Biochemistry I Laboratory: Experiment 7* worked well to detect any created reducing sugars because *sucrose* is not a reducing a sugar and *fructose* and *glucose* are reducing sugars. The fructose and glucose that were created react with 3,5-dinitrosalicylate to create an orange complex that is detectable with the spectrophotometer. The 3,5-DNS does not react with sucrose, so it does not contribute to the orange color.

The problem we have is that some of the sugars we want to test for reaction with invertase are reducing sugars already. For example, lactose is composed of one galactose unit and one glucose unit, but lactose is a reducing sugar, and the galactose and glucose units it decomposes into are also reducing sugars. That means that galactose, glucose, and unreacted lactose all will produce orange coloring.

However, we may be able to tell that the reaction has occurred, since if it proceeds, we now have 2 reducing sugar units produced from 1 reducing sugar unit, and each of the two units can react with 3,5-DNS to produce more orange coloring. The hope is that we can compare a ("blank") solution of lactose plus 3,5-DNS with a solution of lactose, 3,5-DNS, and lactase. The first solution will be orange, but the second solution should be *more* orange because there are more reducing sugar units available from the decomposition.

To test this modified method, we will use *lactose* (a disaccharide reducing sugar) and *lactase* (also known as β-galactosidase; it can hydrolyze lactose). We would rather use some disaccharide reducing sugar with invertase, but we don't know of any disaccharide reducing sugars that can be cut by invertase (it needs β-fructofuranosides).

Experiment 2 Outline

Part A: Detect lactose hydrolysis with lactase. This is successful if a tube containing lactose reacted with lactase produces a darker coloring (indicative of greater concentration of reducing sugars) than a tube containing lactose without lactase.

Part B: Use the same method with maltose and invertase, and cellobiose and invertase. It is expected that invertase cannot hydrolyze those sugars, so to prove this hypothesis, the tubes with enzyme should not be significantly darker than the tubes without enzyme.

Experiment 2 Materials

Stockroom:

10.00 mL volumetric flask
Lactase
Lactose • $1H_2O$
Maltose • $1H_2O$
Cellobiose
A hot water bath spec tube holder
Small magnetic stirring bar

In the lab:

3,5-dinitrosalicylate
pH 4.5 or 4.7 buffer
Invertase
Boileezers®
Spectrophotometer
Hot plate
Magnetic stirrer
Deionized water
Eppendorf tubes

In the lab drawer:

(12) Clean spec tubes
Beakers for water baths
(6) 50-mL beakers for:
- Lactase solution
- Invertase solution
- 3,5-dinitrosalicylate
- Buffer
- 0.0100 M lactose solution
- 0.0100 M maltose solution
- 0.0100 M cellobiose solution
- Deionized water

Experiment 2 Part A Procedure

Check the pH and temperature requirements for the lactase (see the bottle, or the entry on sigmaaldrich.com). For now, assuming that lactase from *Aspergillus oryzae* is used (pH 4.5, 30 °C, 8 units/mg solid).

The enzyme can be fairly concentrated, so it produces as much product as possible as quickly as possible. At 1 mg/mL, a 200 μL aliquot of enzyme should be enough to consume an entire 1.2 μmol aliquot of lactose in 45 seconds. This should nearly double the concentration of reducing sugars (from 0.000300 M to 0.000593 M) and produce an absorbance change from 0.200 to 0.473 A.

Prepare the enzyme solution, 1 mg/mL solution of lactase:
- Add 0.0100 g lactase to a clean 10.00 mL volumetric flask.
- Dilute with *cool* buffer solution (pH 4.5) to 10.00 mL.
- Transfer to a small clean beaker and use a magnetic stirrer to facilitate dissolution of the enzyme.
- Upon dissolution, transfer a 1000 μL aliquot to a fresh Eppendorf tube.
- Keep the tube on ice at 4 °C until ready to use.
- Vortex the Eppendorf tube thoroughly to redissolve solid just before using.

Start a warm water bath, with temperature of 30 °C. This might be tricky to get a hot plate to maintain the temperature. Or, figure out how long it takes to heat a known volume to 30 °C in the microwave.

If needed, prepare a stock of 0.0500 M acetate buffer solution, pH 4.5:
- Add 25.00 mL of 0.5000 M acetic acid to a clean 250.00 mL volumetric flask.
- Add ≈ 200 mL of water.
- 0.9567 g of 100% sodium acetate trihydrate (136.08 g/mol).
- Adjust to pH 4.5 dropwise with HCl or NaOH (if needed).
- Dilute (add remaining ≈ 25 mL) to 250.0 mL.

Prepare 0.0100 M lactose solution:
- 0.0331 g of α-lactose • $1H_2O$ (360.32 g/mol)
- Dilute with water to 10.00 mL

Prepare Blank 1:
- 480 μL of buffer
- 520 μL of water
- Heat for 5 minutes in a 100 °C water bath
- 3000 μL water

Zero the spectrophotometer's absorbance at 540 nm with Blank 1.

Prepare the lactose blank:
120 μL of 0.0100 M lactose solution
480 μL of buffer
400 μL of 3,5-DNS
Heat for 5 minutes in a 100 °C water bath to develop color
3000 μL additional water

Measure the absorbance at 540 nm. The amount of reducing sugar (120 • 0.0100 M = 1.200 μmol) has been chosen so that it should produce a measurable amount of absorbance that is neither too low (less than 0.100 A) or too high (greater than 0.700 A). It should have an absorbance around 0.200 A (and have [lactose] ≈ 0.000300 M in 4.00 mL). If not in the range of 0.100 to 0.700 A, create a new tube and reduce/increase the amount of lactose dissolved in solution until absorbance is in the range of 0.100 to 0.700 A.

Prepare the analytical sample:
120 μL of 0.0100 M lactose solution
280 μL of buffer
200 μL of enzyme

Produce additional identical samples, if possible.

After adding the enzyme, immediately move the sample to the warm water bath. Note the starting time. Let the reaction progress for 5 minutes. However, the reaction should be completed in 1 minute, when all the lactose has been exhausted.

Add 400 μL of 3,5-DNS solution, which contains NaOH that will denature the enzyme and stop the reaction. Place in a boiling water bath for 5 minutes to develop the color. Let cool.

Add 3000 μL of water.

Take absorbance readings of all samples and compare them to the lactose "blank" (actually, it is lactose without reaction by enzyme compared with lactose after reaction with enzyme). Hopefully the analytical sample will have a significantly *higher* absorbance than the blank, since it is hypothesized that reaction of lactose with lactase produces product monosaccharides that are reducing sugars (the reducing sugars react with 3,5-DNS to produce coloring which is detected by the spectrophotometer).

Experiment 2 Part B Procedure

The procedure developed in *Part A* is used with maltose and invertase, and with cellobiose and invertase. The only difference is that the absorbance readings of the blank and samples should *not* differ significantly, because the enzyme should not cleave those sugars.

Check the specifications for the invertase. For now, assuming that invertase from *Saccharomyces cerevisiae* is used (pH 4.5, 55 °C, 30 units/mg solid).

Start a warm water bath, with temperature of 55 °C. This might be tricky to get a hot plate to maintain the temperature. Or, figure out how long it takes to heat a known volume to 55 °C in the microwave.

Prepare the enzyme solution, 1 mg/mL solution of invertase:
- Add 0.0100 g invertase to a clean 10.00 mL volumetric flask.
- Dilute with *cool* buffer solution (pH 4.5) to 10.00 mL.
- Transfer to a small clean beaker and use a magnetic stirrer to facilitate dissolution of the enzyme.
- Upon dissolution, transfer 1000 μL aliquots to 2 or 3 fresh Eppendorf tubes.
- Keep the tubes on ice at 4 °C until ready to use.
- Vortex Eppendorf tubes thoroughly to redissolve solid just before using.

Prepare 0.0100 M maltose solution:
- 0.0360 g of maltose • $1H_2O$ (360.31 g/mol)
- Dilute with water to 10.00 mL

Prepare 0.0100 M cellobiose solution:
- 0.0342 g of cellobiose (342.30 g/mol)
- Dilute with water to 10.00 mL

Prepare a 1.000 M solution of sucrose:
- 3.4402 g of 99.5% sucrose (342.30 g/mol)
- Dilute with water to 10.00 mL

Prepare a 0.01000 M sucrose solution:
- 10 μL of 1.000 M sucrose into an Eppendorf tube
- 990 μL of deionized water

Leave the spectrophotometer zeroed at 540 nm with Blank 1 from *Part A*.

Prepare blanks for each sugar type (maltose, cellobiose, sucrose):
120 μL of 0.0100 M sugar solution
480 μL of buffer
400 μL of 3,5-DNS
Heat for 5 minutes in a 100 °C water bath to develop color
3000 μL additional water

Compare the sucrose blank to Blank 1. If it differs significantly, the sucrose solution is contaminated with reducing sugars.

Prepare and process the two blanks for each of maltose and cellobiose. Measure the absorbance at 540 nm. As with the lactose blank from *Part A*, they need to have absorbance readings between 0.100 and 0.700 A.

Prepare the analytical samples for each of maltose and cellobiose:
120 μL of 0.0100 M sugar solution
427 μL of buffer
53 μL of enzyme (using less invertase since it is more efficient than lactase)

Produce additional identical samples, if possible.

Prepare a control with sucrose to verify that the invertase works:
120 μL of 0.0100 M sucrose solution
427 μL of buffer
53 μL of enzyme

After adding the enzyme to a tube, immediately move the tube to the warm water bath. Note the starting time. Let the reaction progress for 5 minutes.

Add 400 μL of 3,5-DNS solution, which contains NaOH that will denature the enzyme and stop the reaction. Place in a boiling water bath for 5 minutes to develop the color. Let cool.

Add 3000 μL of water.

Take absorbance readings of all samples and blanks. If, as hypothesized, that invertase does not hydrolyze maltose and cellobiose, then the absorbance readings should not be significantly higher than that of their blanks.