Victoria Mullen

BIOL 309

10/29/15

Purification of Cellobiase using Portobello Mushrooms

**Introduction:**

Cellobiase is an enzyme with bacteria and protozoa that catalyzes the hydrolysis of β 1-4 glycosidic bonds which is the last step of the process of breaking down cellulose. Cellulose is a monosaccharide made up of long chains of glucose that are found in plant cell walls. This happens by a natural process and is used by fungi and bacteria to obtain glucose as a source of food. Cellobiase also has a white powder appearance and a pKa of 12.39.

Enzymes are proteins that speed up reactions by lowering the activation energy. These are capable of speeding up most chemical reactions, which wouldn’t otherwise occur without the help of an enzyme. Enzymes also bind to the substrate at the enzyme’s active site. If the concentration of the enzyme and substrate are increased, there will be a time when the solution will get saturated with either enzymes or substrates and won’t speed up the reaction any more. When studying enzymes, you will find the substrate that the reaction will use up or the products that created from it.



In this experiment, our substrate will be cellulose and the product will be glucose, which are both colorless. We will use p-nitrophenyl glucopyranoside, which is an artificial substrate, to bind to the enzyme and act as cellulose. When p-nitrophenyl glucopyranoside is broken down by the cellobiase, it will produce the products glucose and p-nitrophenol. In basic solutions, p- nitrophenol will act as a stop solution and the solution will turn yellow. We will want to use a basic solution because pH can cause denaturation of the protein, and will help stop the reaction. In order to get measure absorbance we will need to add stop solution at times such as 0, 15, 30, 45 and 60 seconds after the reaction has taken place. Then we will use multiplication to obtain the value of change in absorbance per minute.

Overall, experiment will take place over three consecutive weeks with each week leading us to further purification of our enzyme. In week one, we homogenize and centrifuge the enzyme. We will then add ammonium sulfate in order to precipitate proteins with lower solubility first and then the protein that we want to purify. In week two, we will use gel filtration chromatography, which separates molecules based on size. In week three, we will use ion exchange chromatography to separate molecules based on charge.

During gel filtration chromatography, we will use the S-300 HR Sephacryl resin. It has a fractional range of 5000-250000 Da and cellobiase has a molecular weight of 93000 Da. Cellobiase would therefore fall within the range and elute in the middle. The buffer we can use is a sodium citrate buffer at a pH of 4.1. This buffer will be used in ion exchange chromatography because we will need to protonate cellobiase in order to make the charge positive. This way we will be able to use a cation exchange. The sodium citrate buffer has an isoelectric point at 6.95. The resin that will be used is SE-Sephadex C-50 because is has an operating pH of 6-10 and exclusion limit of 200,000 daltons. When we take the absorbance of p-nitrophenol we will use a wavelength of 405 nm on the spectrophotometer. We can use Beer’s law of absorbance= extinction coefficient x path length x concentration. The extinction coefficient of p-nitrophenol is 18.1 mM-1 cm-1. The path length of the cuvette will be 1 cm and we can find the absorbance at 405 to find the concentration.

**Hypothesis:**

Week 1: When we add ammonium sulfate, the Cellobiase pellet will precipitate out in 80% saturation. Cellobiase activity and fold purification will be the largest when they are compared to other fractions.

Week 2: Using gel filtration chromatography, the Cellobiase will elute out of the middle. There will be higher fold purification and enzyme activity than the ammonium sulfate precipitation.

Week 3: Using ion exchange chromatography, the buffer and a salt concentration are added together and the cellobiase will elute from the ion exchange column. Ion exchange chromatography will have higher fold purification than the gel filtration chromatography and the highest of all the experiments.

**Materials:**

Chemicals:

Homogenization buffer

Bradford Reagent

Ammonium Sulfate

BSA standard

Blue Dextran

1.5 mM *p*-nitrophenyl glucopyranoside

Sodium Hydroxide at pH 12 (stop buffer)

Sodium Citrate buffer at pH 4.2

Sodium Citrate buffer pH 4.2 with NaCl concentration

Equipment:

Spectrophotometer

1.5 mL microtubes

15mL centrifuge tubes

Ion Exchange column

Gel Filtration Column

Sharpie pen

Centrifuge

Cuvettes

Pipets (p1000, p200, p20)

Pipette tips

S-300 HR Seacryl

SE-Sephadex C-50 resin

**Protocol:**

Week 1: *Ammonium Sulfate Precipitation*

* We will use portabello mushrooms and homogenization buffer to homogenize the fungus by blending until the mushrooms become liquid. Record the volume and save 1 mL to do assays.
* Make sure to balance!
* Put protein into a centrifuge tube and centrifuge at 20,000x g for 20 minutes using a blank with water at the same weight.
* Decant S/N into the 50ml tube. Record volume and again keep 1ml for assays.
* Gradually add 0.313 g of groundammonium sulfate for every 1 ml of S/N to reach 50% saturation.
* Keep in ice bath for 10 minutes.
* Make sure to balance!
* Put into centrifuge tube and centrifuge at 15,000 x g for 15 minutes.
* Decant S/N back into the 50ml tube. Record volume and save 1ml for assays.
* Gradually add 0.186 g of groundammonium sulfate for every 1 ml of S/N in order to reach 80% saturation.
* Keep in ice bath for 10 minutes.
* Make sure to balance!
* Put protein into centrifuge tube and centrifuge at 15,000 x g for 15 minutes.
* Decant S/N back into the 50ml tube. Record volume and keep 1ml for assays.
* Put pellet back in 3 ml of homogenization buffer. Record the volume and keep 0.5ml for assays.
* Place the pellet in the freezer in a 15ml tube for GFC in Week 2. Also, put the remaining samples in the freezer for Week 2’s protocol.
* Make Bradford assay standards by using the concentrations of 0.065, 0.125, 0.25, 0.5 and 1.
* Use a 1 in 2 dilution for your BSA standard.
* Let samples sit for 10 minutes and measure absorbance at 595nm.
* Make a standard curve graph in Excel and use the equation of the line to determine concentration of the protein.
* Find the specific activity and fold purification for each fraction.
* Run an activity assay by labeling one tube Enzyme the other Control.
* Measure out 4 ml of 1.5 mM *p*-nitrophenyl--D glucopyranoside substrate and add to each labeled tube.
* Pipet 1 ml of the sodium hydroxide into 5 cuvettes.
* Put 2ml of the enzyme into the tube labeled enzyme.
* Quickly mix and promptly remove 0.5 ml and add to a cuvette labeled 0. After 15 seconds add 0.5ml to the enzyme reaction to a clean cuvette with 1 ml of the sodium hydroxide. Do this for 15, 30, 45 and 60 seconds.
* Put 1 ml of the control into a cuvette with 1 ml of sodium hydroxide. Label this cuvette blank.
* Measure the absorbance of the yellow p-nitrophenol at 405 nm using a spectrophotometer.

Week 2: *Gel Filtration Chromatography (GFC)*

* Allow the pellet to thaw then divide into two 1.5 ml microtubes.
* Centrifuge protein at 13,000rpm for 5 min then put S/N and 100 μl of Blue Dextran into a 15 ml tube.
* Make sure the buffer Sodium Citrate is at pH 4.2.
* Put your sample at the sample valve and run the GFC-Cellobiase protocol.
* As your sample is being taken up into the machine, make sure to keep the sample inlet tube at the bottom of the sample.
* When peaks appear on the chromatograph, run a quick check prod uction of p-nitrophenol.
* During the quick check put the enzyme in a reaction cocktail and then add the stop solution. If an absorbance appears, there is enzymatic activity.
* Once the run is finished, find the fractions that contain cellobiase.
* Perform assays for cellobiase activity.
* Keep the fraction in the freezer for ion exchange chromatography is week 3.

Week 3: *Ion Exchange Chromotography (IEX)*

* Thaw your GFC fraction from last week.
* Using a minimum of 6 ml of sample, divide the sample into four 1.5ml tubes for centrifugation.
* Combine all 4 supernatants into one 15ml tube. Put the inlet tube of the sample valve in the sample.
* Load 1.5ml tubes into the fraction collector.
* As your sample is being taken up into the machine, make sure to keep the sample inlet tube at the bottom of the sample.
* Run a quick check by putting the enzyme in a reaction cocktail and then adding the stop solution. If an absorbance appears, there is enzymatic activity.
* Once you find the peak that has cellobiase, pool all fractions for that peak.
* Perform assays for cellobiase activity.

**Predicted Outcomes:**

* In the range of 50% and 80% saturation, the cellobiase will precipitate.
* After the ammonium sulfate precipitation, the pellet at 80% saturation will have the highest activity.
* The cellobiase will elute out of the middle during gel filtration chromatography.
* There will be higher fold purification after the gel filtration chromatography than after the ammonium sulfate precipitation.
* After the buffer and a salt concentration are added together, the cellobiase will elute from the ion exchange column.
* Ion exchange chromatography will have higher fold purification than the gel filtration chromatography.
* Fold purification will increase with each experiment, which will result in IEX producing the highest fold purification of all experiments.