CHEMISTRY 365 BIOCHEMISTRY
Team Project #1

ISOLATION AND PURIFICATION OF YEAST β-FRUCTOFURANOSIDASE

References:

1) Melius, Paul *J. Chem. Educ.*, **1971**, *48(11)*, pp 765-766.
2) Bollag, D. M.; Edelstein, S.J. *Protein Methods* Wiley-Liss: New York, 1991.
3) Whitaker, J. R. *Anal. Chem.* **1963**, 35, pp 1950- 1953.

This project involves the analysis of a yeast extract for β-fructofuranosidase specific activity, and then purification of the extract by organic solvent precipitation and by gel permeation chromatography[1]. β-fructofuranosidase (EC 3.2.1.26) , (aka invertase, saccharase or sucrase), has a molecular weight of 270 kD and catalyzes the hydrolysis of the $\alpha 1 \rightarrow \beta 2$ linkage of sucrose yielding an equimolar mixture of glucose and fructose. Protein precipitation as a purification technique has already been discussed. Gel permeation chromatography refers to a technique which separates molecules based on their molecular size and shape, and therefore their molecular weight. Under appropriate conditions, this technique may be used to separate mixtures of large biomolecules or to remove low molecular weight components from solutions of large molecules. The technique may also be used to estimate the molecular weights of unknown macromolecule fractions from plots of log molecular weight versus distribution coefficient[3]. Large molecules will be excluded from the porous gel beads, travel through the channels between beads, and be eluted first. Small molecules are able to penetrate the gel beads, have a much larger volume in which to travel, and are eluted later.

The degree of purification is found from comparing the specific activity of the redissolved precipitate, and the specific activity of the chromatography fractions, to the specific activity of the initial extract. **Specific activity** is a measure of how much desired enzyme is present in a mixture of proteins (μmoles sucrose hydrolyzed per minute divided by milligrams of total protein for invertase). Higher specific activities indicate higher purities of the desired enzyme.

The enzyme activity units (μmoles sucrose hydrolyzed per minute) are determined by incubation of enzyme with sucrose in 0.05 M acetate buffer at pH 4.8 and stopping the reaction at exactly 3 minutes by the addition of alkaline 3,5-dinitrosalicylate (DNS) reagent. The amount of reducing sugar formed is then determined spectrophotometrically from the absorbance at 540 nm. The Bradford assay is used for total protein determination.

First, the invertase specific activity of a protein extract from yeast will be determined. The protein extract will then be subjected to precipitation in a 50% (v/v) aqueous solution of ethanol[2]. The precipitate is collected by centrifugation (10 min at 10000xg) and redissolved in pH 4.8 acetate buffer. The invertase specific activity of the redissolved precipitate is then determined. Also, the protein extract containing a blue marker will be passed through a Sephadex G-150 column, and three fractions collected from the column. The invertase specific activity of each column fraction is then determined.

Solutions:

1) 0.1 M sodium bicarbonate.
2) 0.1 M acetate buffer, pH 4.8. 2.3 mL glacial acetic acid and 4.92 grams of sodium acetate in 1 L.
3) 34.2 g/L sucrose solution.
4) DNS reagent. Dissolve, with warming, 5 g 3,5-dinitrosalicylic acid in 100 mL of 2M NaOH (8 g NaOH/100 mL). Add 150 g sodium potassium tartrate to 250 mL water and warm to dissolve. Mix the two solutions and dilute to 500 mL with water.
5) Sephadex G-150 hydrated in buffer (2).
6) Solution of Blue Dextran (5 mg/mL).
7) Bradford reagent.
8) 95% ethanol solution (ice cold).

Laboratory:

A) Preparation of yeast extract (Bulk preparation done by Instructor.)

1) Dried baker's yeast is dissolved in a 0.1 M $NaHCO_3$ solution at a rate of ≈4 mL solution per gram of dry yeast.
2) Incubate for a day at 35-40°C.
3) Centrifuge at 15000xg for 15 minutes and save the supernatant as the extract.

B) Analysis of Organic Solvent Precipitate

Obtain 9.0 mL of the yeast extract in a centrifuge tube. Put on ice. While on ice, slowly dropwise add 10.0 mL of cold 95% ethanol solution (Take around 10 minutes). Centrifuge at 10000xg for 10 minutes. Decant the supernatant, then redissolve the precipitate in 2.0 mL of the acetate buffer. Determine the specific activity of this solution using results from Parts D and E.

C) Fractionation by Gel Permeation Chromatography

You will be applying the yeast extract to a column of Sephadex G-150, that will fractionate proteins in the molecular weight range of 5 kD to 400 kD. A blue marker will be used to indicate the excluded volume, V_0, for your column (where the 400 kD proteins elute).

Obtain 3.5 mL of the yeast extract and mix with 0.5 mL of the Blue Dextran marker solution. After flowing the solution from the top of the column to the top of the gel bed, carefully apply the extract/marker solution to the top of the column bed with a Pasteur pipet. (**Your instructor wants to do this step for you.**) Allow this solution to slowly flow to the top of the gel bed and then replace the buffer solution on top of the column slowly with a Pasteur pipet. Resume column flow and collect 3, 2.0 mL fractions beginning with the elution of the blue color and analyze for invertase specific activity using results from Parts D and E.

D) Total protein determination by Bradford Method

Prepare one blank and five more tubes for the Bradford protein assay. The crude extract and redissolved precipitate (**both 1:5 diluted**), and three column fractions (**no dilution**) are the five protein solution samples.

Tube	Bradford Reagent	Protein Soln.	Water
Blank	4.0 mL	0.00 mL	0.08 mL
Five Samples	4.0 mL	0.02 mL	0.06 mL

Mix well and read the absorbance of each solution after 15 minutes at 595 nm. Calculate the protein concentration of the extract using data from your standard bovine serum albumin.

E) Determination of Specific Activity

Three test tubes are needed for each assay (a control and 5 assays will be done). See the table below for the contents of each tube. When ready to do the activity assay, pour the sucrose solution from the second test tube into the first tube (mix well) and begin timing **using a timer capable of measuring seconds.** After **exactly** 3 minutes, pour the third tube (DNS! solution) into the reaction test tube (mix well). Put the tube in a boiling water bath for 10 minutes, then cool. **After cooling, dilute by adding 5.0 mL of DW and 5.0 mL of buffer; read the absorbance at 540 nm.**

Solutions to Assay	**First Test Tube**			**Second Test Tube**	**Third Test Tube**
	Acetate Buffer	Distilled Water	Undiluted Enzyme Solution	Sucrose Solution	DNS Reagent
Control (Blank)	3.0 mL	2.0 mL	0.00 mL	1.0 mL	3.0 mL
Crude Extract	3.0 mL	2.0 mL	0.02 mL	1.0 mL	3.0 mL
Rediss. Ppt.	3.0 mL	2.0 mL	0.02 mL	1.0 mL	3.0 mL
Each of the 3 Column Fractions	3.0 mL	2.0 mL	0.02 mL	1.0 mL	3.0 mL

Report: Hand in an Abstract and a completed Purification Chart as shown on reverse side of this page.

Table 1. Summary of Yeast Invertase Specific Activity Results for Crude and Purified Extracts							
Bradford Total Protein Assay			DNS Kinetic Assay				
slope=	35.00		slope=	1330			
intercept=	0.00		intercept=	-0.012			
	Final Volume (mL)		Concentration (mg/mL)		Enzyme Units (micromol/min) {per mL}	Specific Activity (Units/mg)	Purification (fold)
	This is the total volume of the protein solution.	Bradford Assay Abs. at 595 nm	This is the concentration of all proteins in solution.	Kinetic Assay by DNS method; Abs. after 3 min; at 540 nm	This indicates how much of the wanted protein is present.	This indicates how many of all proteins present are the ones wanted.	This is specific activity after the current step divided by the initial specific activity.
Crude Extract	9.0	0.120	3.50	0.300	74.29	21.24	1.0
Alcohol Precipitate	2.0	0.300	8.74	1.500	360.00	41.18	1.9
Column Fraction 1	2.0	0.275	1.60	0.400	98.10	61.20	2.9
Column Fraction 2	2.0	0.250	1.46	0.500	121.90	83.66	3.9
Column Fraction 3	2.0	0.180	1.05	0.300	74.29	70.81	3.3