CSUDH Biochemistry II Laboratory – CHE 453

Lab 2A – Glucose-1-Phosphate: Enzymatic Formation from Starch

INTRODUCTION

Starch, a carbohydrate found in most plants, is a mixture of two polysaccharides: amylose, a straight-chain polymer of glucose units joined by *a*-1,4 linkages, and amylopectin, a branched-chain glucose polymer that differs from glycogen primarily in its larger number of *a*-1,4- linked glucose units between the *a*-1,6 branch points. Both glycogen and starch are acted upon by the enzyme phosphorylase, which catalyzes the phosphorolysis of glycogen or starch to glucose-1-phosphate (see **Figure 1**).

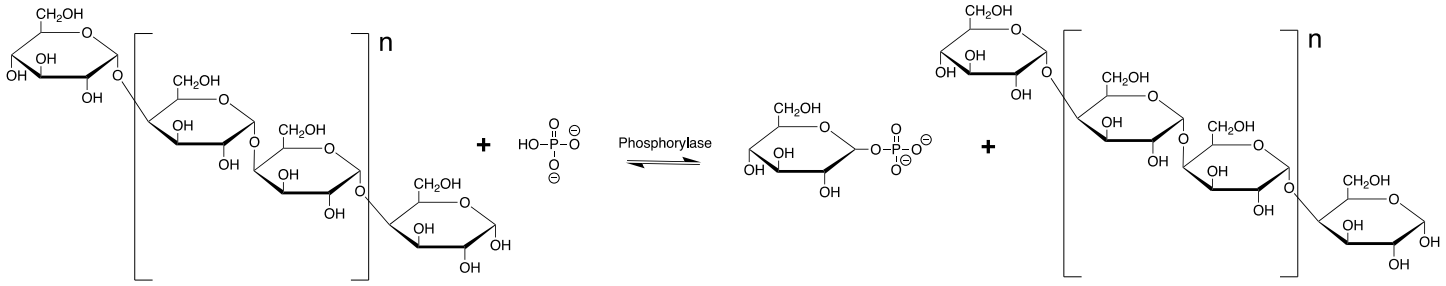

Figure 1. Phosphorylase-catalyzed phosphorolysis of glycogen or starch to glucose-1-phosphate

The primary function of starch is to serve as a storage form for carbohydrate. Mobilization of starch for metabolism or synthesis of other polysaccharides requires cleavage of monosaccharide units from the polymer and formation of phosphorylated sugar derivatives, which are the substrates for glycolysis or for formation of nucleoside diphosphosugars. Phosphorolysis of starch fulfills both requirements in a single step. If starch were cleaved by hydrolytic reactions (e.g., by amylases), subsequent formation of the sugar phosphate from the glucose released would require the expenditure of metabolic energy in the form of ATP. Thus, the phosphorylase reaction increases the efficiency of starch utilization by conserving the energy of the acetal links between glucose units through formation of a phosphoacetal linkage.

The regulation of glycogen phosphorylase in mammalian muscle and liver has been intensively studied. As might be expected for a pathway governing use of a major energy reserve, this enzyme (and the corresponding synthetic enzyme, glycogen synthetase) is under complex metabolic and hormonal control. Students should consult a textbook for details.

In this experiment, soluble starch is incubated with phosphate and a phosphorylase preparation from potatoes. After 24-48 hrs, the reaction is stopped by heating of the mixture to destroy the enzyme. After removal of the denatured enzyme, the unreacted inorganic phosphate is removed from the filtrate by precipitation as magnesium ammonium phosphate. The glucose-I-phosphate is then isolated by ion exchange chromatography and is subsequently crystallized.

Glucose-I-phosphate is determined by measurement of the inorganic phosphate released by acid hydrolysis. Phosphoacetals of all sugars can be hydrolyzed by treatment with lN acid for 7 min at 100°C, but the various sugar phosphate esters (i.e., glucose-6-phosphate) ordinarily require longer heating times or more concentrated acid for complete hydrolysis.

Inorganic phosphate is measured by the colorimetric method of Fiske and Subbarow (1925), which is specific for inorganic phosphate; i.e., inorganic phosphate can be analyzed in the presence of organic phosphates such as phosphate esters or phosphor-acetals. Hence, the increase in inorganic phosphate after 7-min hydrolysis of a sample (over an unhydrolyzed blank) is a measure of the phosphor-acetal content of the sample. The color developed in the Fiske-Subbarow reaction is dependent on the formation of a phosphomolybdic acid complex, which forms an intense blue color when reduced by a mixture of bisulfite and *p*-methylamino-phenol.

SAFETY CONSIDERATIONS

Substance	GHS Pictogram	Signal Word
Phenylmercury nitrate		**DANGER** Toxic is swallowed. Causes severe skin burns and eye damage. Causes damage to organs through prolonged or repeated exposure. Very toxic to aquatic life with long lasting effects.
Potassium phosphate, KH_2PO_4, pH 6.7	none	none
Soluble starch	None	none
Ammonium hydroxide, 14% NH_4OH		**DANGER** May be corrosive to metals. Harmful is swallowed. Causes serve skin burns and eye damage. Very toxic to aquatic life.
Sodium hydroxide, 2N NaOH		**DANGER** May be corrosive to metals. Causes serve skin burns and eye damage.
Magnesium acetate tetrahydrate, $Mg(OAc)_2 \cdot 4H_2O$		**DANGER** May be corrosive to metals. Harmful is swallowed. Causes serve skin burns and eye damage. Very toxic to aquatic life
Amberlite Ion Exchange Resin IR-45, stored in H_2O at pH 10	none	none
Acid molybdate reagent	none	none
Phosphate standard solution, KH_2PO_4 in H_2O	none	none
Hydrochloric acid, 2N HCl		**WARNING** May be corrosive to metals. Causes skin irritation. Causes serious eye irritation. May cause respiratory irritation.
Dowex 50 Resin, stored in H2O at pH 2		**WARNING** May be corrosive to metals. Causes skin irritation. Causes serious eye irritation. May cause respiratory irritation
Potassium hydroxide, 5% KOH		**DANGER** May be corrosive to metals. Harmful if swallowed. Causes severe skin burns and eye damage. Harmful to aquatic life.
Charcoal	none	none
Sodium bisulfate, 3% $NaHSO_3$		**WARNING** Harmful if swallowed.
1% methylaminophenol		**WARNING** Harmful is swallowed. May cause an allergic skin reaction. May cause damage to organs through prolonged or repeated exposure. Very toxic to aquatic life with long lasting effects.
Potassium iodine, 0.01 N KI		**DANGER** Causes damage to organs (Thyroid) through prolonged or repeated exposure if swallowed/
Iodine, 0.01 N I_2		**DANGER** Harmful in contact with skin or if inhaled. Causes skin irritation. Causes serious eye irritation. May cause respiratory irritation. Causes damage to organs (Thyroid) through prolonged or repeated exposure if swallowed. Very toxic to aquatic life.
Phosphorus pentoxide, P_2O_5	none	none

EXPERIMENTAL PRODECURES

Materials

Phenylmercuric nitrate slurry
0.8 M Potassium phosphate buffer pH 6.7
Soluble Starch
Filter aid
Potassium phosphate, 14% NH_4OH
Sodium hydroxide, 2 N NaOH
Magnesium acetate tetrahydrate, $Mg(OAc)_2 \cdot 4H_2O$
Amberlite Ion Exchange Resin IR-45, stored in H_2O at pH 10
Methanol
Cheesecloth
Acid molybdate reagent
Reducing reagent (3% NaHSO3, 1% methylaminophenol)
Potassium iodine, 0.01 N KI; Iodine, 0.01 N I_2
2 N Hydrochloric acid, HCl
Potatoes
Dowex 50 Resin, stored in H_2O at pH 2
Potassium hydroxide, 5% KOH
Blender
Glass wool
Charcoal
Centrifuges
300 mL bottles
Vacuum desiccator
Phosphorus pentoxide, P_2O_5
Phosphate standard solution

General

The preparation and isolation of glucose-1-phosphate can be completed within three laboratory periods. The enzyme incubation can be started in the first period. The removal of cations with Dowex 50 should be completed in the second period and the product stored at 0°-5°C. The ion exchange can then be competed and the crystallization begun in the third period. Experiment 2B may be started in the fourth period, after the collecting and drying of the crystals of dipotassium glucose-phosphate dihydrate. Deionized or distilled water should be used throughout, and no solutions should be discarded until a product is obtained. Store all solutions at 0°-5°C between laboratory periods to a void bacterial or chemical degradation.

Preparation of Starch

Using a minimum amount of H_2O, make a smooth slurry of 5 g of soluble starch. Add this to 35 mL of vigorously boiling H_2O**,** and stir until the solution is nearly clear. The solution may be cloudy, but should not be milky. Further heating may be required to dissolve the starch, but avoid prolonged heating. Add 90 mL of cold H_2O to help cool the solution to room temperature. Do not add the enzyme until the solution has cooled to room temperature (heat inactivates the enzyme).

Preparation of Enzyme

Cut a medium-sized potato (precooled for 4 hrs at 1°-5°C; you need not peel the potato) into half-inch cubes. Blend 75 g of these cube, added over a 30-sec period, with 75 mL of H_2O for 2 min in a blender. Then quickly pour the resultant slurry onto a Buchner funnel lined with 2-4 layers of cheesecloth, and filter with vacuum, washing the crude pulp with 15 ml of H_2O to insure thorough enzyme extraction. Failure to complete these operations within 2 min of blending may result in loss of enzyme activity. After filtration, stir in 50 mg of phenylmercuric nitrate, as a powder or slurry, to inhibit the action of other enzymes and bacterial growth. Allow the preparation to stand for 1 min; then decant the solution and any floating phenyl-mercuric nitrate from the accumulated precipitate. Adjust the extract to a volume of 125 mL with H_2O. The cooled starch solution should be mixed with the potato enzyme extract ***immediately*** after the extract is prepared.

Incubation of the Enzyme with Starch

Add the 125 mL of enzyme solution to 125 mL of starch solution in a labeled clean 500 mL Erlenmeyer flask. Then add 125 mL of 0.8 M phosphate buffer solution. record the total volume ~375 mL), and store the solution in a stoppered Erlenmeyer flask in your locker. During the incubation period (24-48 hrs) the reaction mixture will turn red and then dark blue or purple because of the action of other enzymes present in the crude extract. The colored materials will be removed during later procedures.

Removal of Inorganic Phosphate

After 24-48 hrs of incubation, stop the enzymatic reaction by rapidly heating the solution to 95°C and then slowly cooling it over a 30-min period. Remove the coagulated protein by careful filtration using

cheesecloth and a Buchner funnel. Then remove the excess phosphate by dissolving 0.1 mole of magnesium acetate (22 g of $Mg(OAc)_2 \cdot 4H_2O$) in the solution and adjusting the pH to 8.5 with 14% NH_4OH (use pH paper first, then a pH meter; about 15 ml of 14% NH_4OH will be required, but avoid adding excess NH_4OH). Cool the solution in a salted ice bath for 10 min, and remove the precipitated magnesium ammonium phosphate by suction filtration. Use filter paper covered with a thin layer of celite filter aid. If filtration is very slow, use more than one Buchner funnel or change the filter. Record the volume of the filtrate. Remove duplicate 0.05, 0.1, 0.2, and 0.5 ml aliquots of the filtered solution for the inorganic phosphate and 7- min phosphate assays described later. If the phosphate assay reveals that an excess of inorganic phosphate is still present in the incubation filtrate (i.e., if an intense blue color forms in the unhydrolyzed 0.1 mL aliquot), add l g of $Mg(OAc)_2 \cdot 4H_2O$, adjust to pH 8.5 with 14% NH_4OH, cool the solution, and filter it again before repeating the phosphate assays. Calculate the number of µmoles of inorganic phosphate and 7-min phosphate in the entire volume of filtered solution. If the inorganic phosphate in the filtrate is less than 15% of the phosphate found after 7 min hydrolysis, you may proceed with the rest of the experiment.

Assay of Inorganic Phosphate and Glucose-1-Phosphate. Range Finding for Phosphate Assay

In the Fiske-Subbarow colorimetric method for the determination of phosphate, the color yield is directly proportional to the amount of inorganic phosphate only when the aliquot taken for analysis contains between 0.1 and 1.0 µmole of phosphate. The efficiency of the enzymatic formation of glucose-1-phosphate will vary somewhat depending upon the sources of the enzyme and the starch as well as the length of the incubation period. Accordingly, the aliquot sizes suggested may not lie within the range in which the assay is valid. In this event, analyze several different aliquot sizes until you find one that falls within the accurate range of the assay. In this experiment, as in all isolation procedures, you must obtain an accurate measurement of the amount of the desired compound in each fraction. Therefore. you will need to determine and use appropriate aliquot ranges for analysis before going to the next steps in the experiment. Further, you must keep accurate records of aliquot sizes and protocols in order to evaluate your data correctly. Most laboratory detergents contain large amounts of phosphate, which may contaminate your glassware. All tubes used in the phosphate analyses should be thoroughly cleaned and rinsed with deionized water.

7-Min Hydrolysis

Set aside one of the duplicates of each pair of samples to be assayed for inorganic phosphate. To the other sample add an equal volume of 2N HCl; 0.5 ml of 1N HCl is preferable with aliquots of less than 0.2 mL. Now place the acidified samples in a boiling water bath for 7 min. Remove the samples, cool and neutralize them to pH 6.5-7.5 by adding (pipette) a stoichiometric amount of 2.0N NaOH. It is important that the solutions be nearly neutral; use pH paper to check them. Dilute all the tubes (including the unhydrolyzed aliquots) to 1.5 mL with H_2O.

Inorganic Phosphate Determination (Modified Fiske-Subbarow Method)

For each phosphate analysis prepare tubes containing a water blank, unhydrolyzed aliquots, hydrolyzed aliquots, and phosphate standards (0.1, 0.2, 0.4, 0.6, 0.8, and 1 µmoles of inorganic phosphate). Adjust the volume of all tubes to 1.5 mL with water, and add, in order, 0.5 mL of acid molybdate reagent, 0.5 mL of reducing reagent (3% $NaHSO_3$, 1% *p*-methylaminophenol), 2.5 mL of H_2O. Mix the solutions by inverting the tubes and allow the color to develop for 20 min before reading the absorbance at 660 nm. Calculate the quantity of inorganic phosphate and glucose-1-phosphate in the aliquot and in the entire reaction mixture**.** In subsequent phosphate analyses you may use the standard curve obtained during your first analysis, ***but only if*** you include one or two standards (0.4 and 0.8 µmoles) to ensure that the color yield is constant.

Use of Cation-Exchange Resin

Decolorize the solution of glucose-1-phosphate (freed of inorganic phosphate) by stirring with 1 g of charcoal and then removing the charcoal by vacuum filtration using filter aid. This procedure yields a clear or yellowish solution containing glucose-1-phosphate, unreacted starch, and many salts that were present either in the original potato extract or in the reagents added during the course of the experiment

Remove the cations by treatment with Dowex 50 in the following manner: Add 175 mL of moist Dowex 50 in H^+ form to the decolorized solution and stir gently for 5 min before separating by vacuum filtration. Do not use filter aid when removing the Dowex. Return the Dowex at the end of the period. **(Note: Do NOT allow the Dowex to become mixed with the Amberlite resin used in the next step!)** If the pH of the filtrate is not acidic (pH 1.0-3.0), add 50 mL of moist Dowex 50 in H^+ form,

then stir and filter the solution as before. Repeat this procedure until the pH of the solution is 1.0-3.0. Record the volume of the resultant solution, and remove duplicate aliquots identical to those previously used in the assay of the $MgNH_4PO_4$ supernate. Assay these for inorganic phosphate and 7-min phosphate. If necessary, store the remaining solution at 0°-5°C for several days. There may be small losses of glucose-1-phosphate during storage in acidic solution. These are fewest at 0-5°C. Alternatively, if time is sufficient, proceed directly to the next step.

Use of Anion-Exchange Resin

The next step in the purification procedure is the column chromatography of the Dowex 50 filtrate on Amberlite IR-45 in OH^- form. This involves removing the anions in the acid solution from all other contaminating materials. Thus, when the acidic filtrate (pH 1-3) contacts the IR-45 in OH^- form, the glucose-1-phosphate is ionically adsorbed on the resin while unionized materials in the acid solution, such as acetic acid and unreacted starch, pass through the column. When the resin is eluted with strong alkali (5% KOH), the adhering anions are displaced by the OH^- ions and are obtained in the eluate from the column.

Prepare a column about 4 cm in diameter and 20-30 cm long using a rubber stopper, screw clamp, and glass wool plug (**Figure 2**). Mix 125 mL of H_2O with 125 mL of moist IR-45 (OH^-), and pour the resultant slurry into the column so that there are no air pockets in the settled resin. Then wash the resin with H_2O until the effluent is about pH 9.0 or lower (pH meter). Drain or pipette off the excess fluid until the fluid surface just covers the top of the resin bed. Cover the surface of the resin with a layer of glass wool.

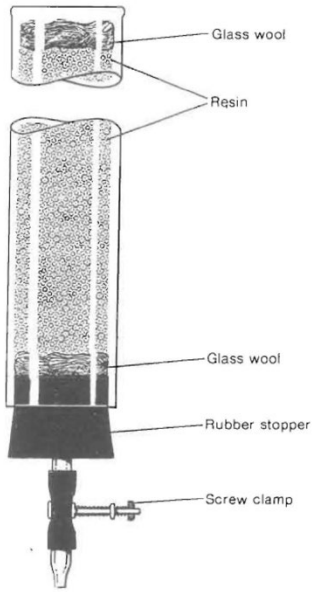

Figure 2. Ion-exchange column

To cause adsorption of the glucose-1-phosphate by the IR-45, gently pour the acidic solution treated with Dowex 50 into the column, avoiding the introduction of air bubbles into the resin bed. Open the screw clamp, and adjust the flow rate to about 15 mL/min. Pass the entire solution through the IR-45 resin without permitting air to enter the column. Collect the entire effluent, determine the volume, and assay duplicate aliquots for inorganic phosphate and 7-min phosphate.
After the original solution has passed into the column and the effluent has been satisfactorily freed of the 7-min phosphate, wash the column with deionized H_2O until the effluent no longer gives a positive test for starch (i.e., a blue color upon mixing a drop of effluent with a drop of 0.01 N I_2 in 0.01 N KI). About 500 ml of water will be required to remove all of the starch from the column.

To elute the glucose-1-phosphate from the resin, pass 5% KOH through the column, adjusting the flow rate to 20 mL/min. Collect separate, successive 50 mL fractions in small Erlenmeyer flasks. Continue collecting fractions for about 250 mL after the pH of the effluent becomes markedly alkaline (pH 11.0- 13.0) as observed with pH paper. (The usual total volume of all fractions equals 150 mL) Then test 0.1 ml aliquots from each fraction for 7-min phosphate. Some fractions require smaller aliquots to fall within the linear range of the standard curve, but 0.1 mL aliquots will serve to find the "peak" for glucose-1-phosphate. Combine the fractions containing 80-90% of the recovered glucose-1-phosphate and adjust the solution to pH 8.0 or higher by adding a few drops of 5% KOH, if necessary. Determine the volume of the combined KOH effluent (glucose-1-phosphate "peak") fractions. Save duplicate 0.05, 0.1, 0.2, and 0.5 mL aliquots for inorganic phosphate and 7-min phosphate analysis.

Add 3 vol of absolute methanol, 450 mL, to the combined fractions, and leave them at 0°-5°C for at least 12 hrs for crystallization of dipotassium glucose-1-phosphate dihydrate. In this temperature range this compound will remain stable indefinitely. Finally, collect the crystals by centrifugation, after pouring off the bulk of the clear supernatant fluid. Wash the crystals with 5 mL of absolute methanol, centrifuge again, then dry them in a tared container in a vacuum desiccator over P_2O_5 at 5°C. Weigh the crystals to determine the final yield of glucose-I phosphate and save them for analysis in Experiment 2B.

Report of Results

1. Prepare a flow sheet of the steps in the isolation procedure, indicating the purpose of each step.
2. Prepare a table (see **Table 1**) showing the percentage of the glucose-1-phosphate recovered at each step in the procedure. Account for any poor recoveries.

Table 1. Percentage of Glucose-1-Phosphate Recovered During Purification

Step	Vol of Solution	µmoles of 7-min phosphate/mL	Total µmoles of 7-min phosphate	Percent recovered
$MgNH_4PO_4$ Supernatant				100
Dowex 50 Supernatant				
Original IR-45 (OH^-) washes (before eluting with H_2O or KOH)				
Combined KOH effluent fractions				
Glucose-1-phosphate crystals*				

* Assume that the crystals are dipotassium glucose-1-phosphate dihydrate (MW = 372)

3. Prepare a graph of the elution pattern of the glucose-1-phosphate, plotting µmoles of glucose-1-phosphate/100 mL on the Y-axis and the fraction numbers of mL of effluent on the X-axis.
4. Calculate the maximum yield of glucose-1-phosphate that would be expected if the reaction had reached equilibrium. The quantity of starch is altered only slightly during the incubation; therefore, the starch concentrations in the numerator and denominator are roughly equivalent and cancel out. Use as your equilibrium constant for calculations:

$$K_{eg} = \frac{\text{Glucose-1-phosphate}}{\text{Inorganic phosphate}} = 0.088$$

Remember that the initial concentration of inorganic phosphate [(0.8M x 250 mL) / 750 mL = 0.267 M] has been decreased at equilibrium by the amount of glucose-1-phosphate formed. Compare your yield (glucose-1-phosphate found in the $MgNH_4PO_4$ supernatant fluid) with the theoretical yield expected. Suggest reasons for any discrepancy between your results and the theoretical value calculated form the phosphorylase constant. Comment of the recovery of the various steps.

EXERCISE

1. Consider the following set of data for inorganic phosphate determinations on unhydrolyzed and 7-min hydrolyzed aliquots taken from a 700 mL volume of the $MgNH_4PO_4$ supernatant obtained in the experiment.

Volume of aliquot	Absorbance at 660 nm	
	Unhydrolyzed	7-min Hydrolyzed
0.1 mL	0.004	0.225
0.2 mL	0.010	0.435
0.5 mL	0.022	0.860
Standards		
0.4 µmole inorganic phosphate		0.150
0.8 µmole inorganic phosphate		0.300

   a. Which absorbance values can be used for further calculations?
   b. Assume 100% recovery of the glucose-1-phosphate and inorganic phosphate at the beginning of the anion exchange step. How many milliequivalents of anion exchange resin must be used to handle a pH 3.0 solution of these anions ($pKa_1 = 1$ and $pKa_2 = 6$, or glucose-1-phosphate)?
   c. Assume 80% recovery of the glucose-1-phosphate as crystals. How many grams of dipotassium glucose-1-phosphate dihydrate would be isolated?
2. Propose a series of steps using ion exchange resins for the isolation of glucose-6-phosphate from a solution containing methylamine, sodium acetate, glucose and glucose-6-phosphate.
3. What steps would you perform to convert the rather insoluble barium salt of glucose-6-phosphate to the dipotassium salt?

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