CSUDH Biochemistry II Laboratory – CHE 453

Lab 2B – Glucose-1-Phosphate Chemical Characterization

INTRODUCTION

It is necessary to establish the identity of any newly isolated compound. In this experiment, the first step consists of showing that the product presumed to be glucose-1-phosphate is in fact composed of equal quantities of glucose and phosphate. Second, it is also necessary to distinguish between the various isomers of glucose-phosphate. The second step is particularly pertinent to Experiment 2B because we know that glucose-1-phosphate may be converted to glucose-6-phosphate by the enzyme phosphoglucomutase, which is present in the crude potato extracts from Experiment 2A.

The characterization of glucose-1-phosphate is based on the relative stability of various sugar phosphates in acid solution. Sugar phosphor-acetals are nonreducing sugars, and they release equal amounts of reducing sugar (characterizable by chromatography) and phosphate upon 7-min hydrolysis in 1N acid at 100°C. In contrast, glucose-6-phosphate and other phosphate esters require more concentrated acid or more prolonged heating for complete hydrolysis. Therefore, it is possible to characterize glucose-1-phosphate by qualitative (chromatographic) and quantitative analysis of materials present before and after 7-min hydrolysis. It is also possible to estimate the purity of the product by careful evaluation of the results.

Chromatographic characterization of sugar phosphates can be achieved in two different ways. First, you can usually detect the sugar portion of sugar phosphates by using sugar-specific reagents, such as the aniline-acid-oxalate or *p*-anisidine spray reagents. Organic phosphates, including sugars phosphates, can also be detected with phosphate specific methods. The modified Hanes-Ischerwood spray reagent used in this experiment hydrolyzes any organic phosphate so that inorganic phosphate is released. The inorganic phosphate then combines with the molybdic acid and is reduced to yield a blue spot (phosphomolybdous acid). The alternate iron-sulfosalicylic acid complex assay features binding of Fe^{+3} by organic phosphates. The bound Fe^{+3} cannot then form a red-brown complex with sulfosalicylic acid.

It is convenient to begin the experiment by preparing the chromatograms and starting chromatographic development. Then you should perform the phosphate and reducing sugar analyses as described. Finally, spray the developed chromatograms to detect the separated products.

SAFETY CONSIDERATIONS

Substance	GHS	Signal Word
Nelson's reagents		**DANGER** May be corrosive to metals. Causes severe skin burns and eye damage. Toxic to aquatic life with long lasting effects.
Glucose standard	none	none
Sodium Hydroxide, 1N NaOH		**DANGER** May be corrosive to metals. Causes serve skin burns and eye damage.
Iron III Chloride, FeCl3		**DANGER** Harmful if swallowed. Causes skin irritation. Causes serious eye damage. Toxic to aquatic life.
Acetone		**DANGER** Highly flammable liquid and vapour. Causes serious eye irritation. May cause drowsiness or dizziness.
Glucose-1-phosphate	none	none
Glucose-6-phosphate	none	none
Glucose	none	none
Formic Acid		**DANGER** Flammable liquid and vapour. Harmful if swallowed. Causes severe skin burns and eye damage. Toxic is inhaled. Harmful to aquatic life.
Methanol		**DANGER** Highly flammable liquid and vapour. Toxic if swallowed, in contact with skin or if inhaled. Causes damage to organs (Eyes).
p-Anisidine spray reagent		**DANGER** Toxic if swallowed. Fatal in contact with skin or if inhaled. May cause cancer. Very toxic to aquatic life.
Ammonium molybdate	none	none
Hydrochloric Acid, 1N HCl		**WARNING** May be corrosive to metals. Causes skin irritation. Causes serious eye irritation. May cause respiratory irritation.

EXPERIMENTAL PROCEDURE

Materials

Nelson' s reagents A and B
Glucose standard
Sodium hydroxide, 1N NaOH
Reducing reagent
Acid-$FeCl_3$ in Acetone
1 % Glucose-1-phosphate
1 % Glucose
UV lamp
Phosphate (Pi) standard (1 µmole / ml)
Arsenomolybdate reagent
10 % $Mg(NO_3)_2$ in ethanol
88 % Formic acid
Methanol
1 % Glucose-6- phosphate
1 % *p*-Anisidine spray reagent
Whatman No. 1 paper
100°C Oven
Acid molybdate reagent
Hydrochloric acid; 1N HCl
1.25% Sulfosalicylic acid in acetone
Modified Hanes-Ischerwood spray reagent [4%$(NH_4)_6$ Mo_7O_{24}•$4H_2O$:1N HCl:70% $HClO_4$:H_2O (5:2:1:12)]

Characterization of Compounds Present Before and After Hydrolysis

You can check a sample of the suspected glucose-1-phosphate for purity by evaluating the following quantities (tubes with prime values are duplicates; instructions for preparing tubes follow list):

1. reducing equivalents before 7-min hydrolysis (tube l and 1')
2. reducing equivalents after 7-min hydrolysis (tubes 2 and 2')
3. inorganic phosphate present before 7-min hydrolysis (tube 3 and 3')
4. inorganic phosphate present after 7-min hydrolysis (rubes 4 and 4')
5. phosphate present after total hydrolysis (tubes 5 and 5')

To determine these values, prepare a 10 mL solution of the isolated, suspected glucose-1-phosphate (1.60-2.20 mg/mL to two decimal places), and place 0.1 mL aliquots in each of 10 tubes. Number the tubes 1, 1', 2, 2', 3, 3', 4, 4', 5, and 5'. To another tube, Tube 6, add 0.1 ml of a 10 mg/mL solution of isolated glucose-1-phosphate and add 0.1 mL 2N HCl.

7-Min Hydrolysis

Add 1 mL of 1N HCl to tubes 2, 2', 4, and 4'. Heat them for 7-8 min in a boiling water bath; then cool them at once in a beaker of cold water. Finally, neutralize the contents by adding 1 mL of 1N NaOH to each of the tubes. At the same time as the other tubes, heat tube 6 at 100°C, but do not add additional HCl or NaOH to this tube. Cool tube 6 with the other tubes and save the contents for chromatographic analysis.

Total Hydrolysis

Add 1 mL 1N HCl and heat in a boiling water bath for 15 min. Cool the tubes, neutralize with 1 mL 1N NaOH, and analyze for inorganic phosphate as with the other tubes.

Preparation for Assay of Unhydrolyzed Product

Add 2 mL of H_2O to tubes 1, l', 3, and 3' and save for the assay of unhydrolyzed product.

Nelson's Test for Equivalents of Reducing Sugar. Analyze tubes 1 and 1' (untreated) and tubes 2 and 2' (hydrolyzed 7 min) by Nelson's test as follows: Pre-pare a blank sample containing 2 mL H_2O and standards containing 0.1, 0.2, 0.4, 0.5 and 0.8 µmoles of glucose in 2-mL final volume. Mix 0.5 mL Nelson's reagent B with 12.5 mL of Nelson's reagent A. Add 1 mL of the combined reagent to each tube (i.e., the blank, the standards, and 1, 1', 2, and 2'). Place the tubes simultaneously in a vigorously boiling water bath (500 mL beaker or larger), and heat for exactly 20 min. Remove the tubes simultaneously and place them in a beaker of cold water to cool. When the tubes are cool (25°C), add 1 mL of arsenomolybdate reagent to each and shake well occasionally during a 5 min period to dissolve the precipitated Cu_2O and to reduce the arsenomolybdate. Dilute the contents of each tube to 10 mL with H_2O. Read the absorbance at 540 nm and calculate the µmoles of reducing equivalents in tubes 1, 1', 2, and 2' from your glucose standard curve.

Determination of Inorganic Phosphate

Using the modified Fiske-Subbarow method, assay the following for inorganic phosphate: tubes 3 and 3' (no hydrolysis), tubes 4 and 4' (7-min hydrolysis), tubes 5 and 5' (total hydrolysis), five standards (0.1, 0.3, 0.5, 0.7, and 0.9 µmoles of phosphate) and a blank. Add 1 mL of acid molybdate reagent and 1 ml of reducing reagent to all tube, and make up to 10 mL with H_2O. Allow them to stand for 20 min, then read the absorbance of each at 660 nm against the blank. Determine the µmoles of inorganic phosphate in tubes 3, 3', 4, 4', 5, and 5' from your standard curve.

Report of Results

Determine the R_f values for the various standard and experimental spots on both chromatograms and record them in a table. Prepare a table similar to **Table 1**. From the weight of isolated glucose-1-phosphate dissolved in H_2O for analysis, calculate the theoretical values for reducing equivalents and inorganic phosphate in each sample assuming a MW of 372 and 100% purity. Then determine the observed values from your analyses. In all cases, report the values as µmoles/0.1 mL of the original glucose-I-phosphate solution analyzed.

How many µmoles of inorganic phosphate contaminate your sample? How many moles of glucose-6-phosphate contaminate your sample? (You have *two* independent determinations of this value. What are they? What do you assume in using them as assays for glucose-6- phosphate? Do they agree? If not, suggest why not) Do your chromatographic results agree with the conclusions drawn from the chemical analyses?

Determine the extent of hydration of your isolated compound by performing the following operations:

1. Calculate the percentage of 7-min phosphorus (not phosphate) in your sample (e.g., µg P/100 µg sample), assuming that the sample is pure.
2. Calculate the expected percentage of 7-min phosphorus from dipotassium glucose-1-phosphate
3. Calculate the expected percentage of 7-min phosphorus from dipotassium glucose-1-phosphate dihydrate.
4. Compare your value for the percentage of phosphorus (1) with the two theoretical values (2 and 3), and decide which formula best fits your data. Comment on the reliability of your conclusion.

Weigh and label the remaining glucose-1-phosphate, and turn it in to the instructor.

EXERCISES

1. Describe chemical tests for determining whether a given pure sample of unknown is (a) glucose-6-phosphate, (b) glucose-1-phosphate, (c) β-methylglucoside, (d) glucose, (**e)** fructose-1,6-di phosphate, or (f) sorbitol
2. Which of the following compounds yield inorganic phosphate upon 7 min hydrolysis: acetyl phosphate, 3-phosphoglyceraldehyde, ribose-5-phosphate, adenosine-5'-phosphate, ribose-1-phosphate, and pyrophosphate?
3. Point out the chemical similarities between Nelson's test and the Fiske-Subbarow test for phosphate, and between these tests and the Folin-Ciocalteu (Lowry) protein determination.

REFERENCES

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