Carbo Assay Spec

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CARBOHYDRATE ASSAYS ON DUAL λ SPECTROPHOTOMETER

Materials:
G6P, Glucose, G1P, F6P, Fructose, Sucrose Assay:
150 mM Hepes Buffer pH 7.2
50 mM NADP (Sigma N-0505)
50 mM ATP (Sigma A-2383)
1 u 4^{-1} µl Glucose-6-phosphate dehydrogenase, G6PDH (Sigma G-8529)
1 u 4^{-1} µl Hexokinase, HEX (Sigma H-4502)
21 u 1^{-1} µl Phosphoglucomutase, PGM (Sigma P-3397)
1 u 1^{-1} µl Phosphogucose isomerase, PGI (Sigma P-9544)
5 u 1^{-1} µl Invertase, INV (Sigma I-4504)

DHAP, GAP Assay:
100 mM Bicine buffer pH 7.6
1 M DTT
1 M KH_2PO_4
50 mM NAD (Sigma N-7004)
50 mM ADP (Sigma A-5285)
6 u 1^{-1} µl PGA kinase, PGK (Sigma P-7634)
1 u 1^{-1} µl Triose-phosphate isomerase, TPI (Sigma T-6258)
1 u 1^{-1} µl Glyceraldehyde-phosphate dehydrogenase, GAPDH (Sigma G-5537)

PGA Assay:
100 mM Bicine Buffer pH 7.6
1 M DTT
50 mM ATP (Sigma A-2383)
10 mM NADH (Sigma N-8129)
6 u 1^{-1} µl PGA kinase, PGK (Sigma P-7634)
1 u 1^{-1} µl Glyceraldehyde-phosphate dehydrogenase, GAPDH (Sigma G-5537)

Procedure:
G6P, Glucose, Sucrose, G1P, F6P, Fructose Assay
1. To help insure consistency and eliminate some pipetting errors pre-mix the phosphate buffer, NADP, ATP, MER and in cases of assays needing only glucose measurements (starch, sucrose, maltose, etc) add G6PDH as well in a falcon tube according to the following formulas
    1 ml Hepes buffer * X (number samples)
    + 10 µl NADP * X (488 µM)
    + 10 µl ATP * X (488 µM)
2. Add 800 µl of the mix to each cuvette
3. Add 5 - 50 µl of sample to cuvette
4. Mix cuvette contents with a stir rod, and allow cuvette to to warm up before placing in spec
5. Place cuvette in spec and shut the cuvette holder door, Do not slam door as shutter mechanism to protect the photomultiplier tube is fragile.
6. Select to display the signal output from light source A
7. Open the shutter from A all the way by moving the shutter on the left all the way down

8. Using the Gain on the photomultiplier tube or Hochspannung set the signal output to be around 900
9. Select to display the signal output from light source B
10. We want the output of light source A and B to match (At a sensitivity of 50 they must match exactly at 10 or 20 you can be off by 0.005)
11. Use the shutter on the right to adjust the output of B to match A, this can be tricky small movements of the shutter make a big difference in signal output, have patience
12. Select to display the difference in signal output from A and B by selecting ΔE, we want this to read somewhere in the neighborhood of 0.5, it doesn't really matter what it reads just so long as your starting base line is sufficiently low on the chart paper so that your curve does not run off the chart paper. (To make this determination see the table below), If you have not used the spec in awhile make sure the chart recorder is properly zeroed and attenuated. i.e. ΔE = 0 should = 0, bottom on the chart paper, and ΔE = -1000 should = 100, top on the chart paper. If the chart recorder is not zeroed use the zero and atten knob to adjust this, try just the zero knob first, if that isn't sufficient then use the atten knob, be careful small movements of the knob make a big difference in the pen position.
13. Depress the chart button to start advancing the chart paper and wait until you have observed a steady base line around 1 to 2 cm long, Be sure to label your graph with the sample, the date and sensitivity setting, and the assay being run.
14 If you did not add G6PDH to your buffer mix proceed to step 15, If you did add G6PDH proceed to step 17
15. Once you have a steady base line add 4 µl of G6PDH to the stir rod, quickly open the cuvette holder and stir in the G6PDH and close the cuvette holder, Do this as quickly as possible, but do not slam the cuvette holder door.
16. Allow reaction to precede. This will tell you how much G6P is in your sample.
17. Add 4 µl Hexokinase by placing on stirring rod and then adding to cuvette making sure to thoroughly mix, again close cuvette holder door and allow reaction to precede. This will tell you how much Glucose is in your sample.
18. To assay G1P add 5 µl PGM by placing on stirring rod and then adding to cuvette making sure to thoroughly mix, again close cuvette holder door and allow reaction to precede. This will tell you how much G1P is in your sample.
19. To assay fructose and F6P add 4 µl of PGI and allow reaction to go to completion.
NOTE: If you with to separate the Fructose and F6P, you will need to perform a second assay adding G6PDH first to get rid of any G6P, then add PGI this will tell you how much F6P you can then subtract this from you combined fructose F6P measurement. If your extra paranoid you can then add hexokinase and this will tell you how much Glucose and Fructose combined you have.
20. To assay sucrose add 5 µl of Invertase and allow the reaction to go to completion.
NOTE: The invertase has a pH optimum ≈ 5 and a temperature optimum of 55°C however the enzyme will work at pH 7 and room temp, just slower so it will work to add it directly to the plate, we just have to add a little more enzyme i.e. 25 units instead of 0.5 untis.
NOTE: Since sucrose breaks down to glucose and fructose if you have previously added PGI you will be measuring both the glucose and the fructose from the sucrose, so make sure to divide your calculated sucrose concentration by two.

DHAP, GAP Assay:
1. To help insure consistency and eliminate some pipetting errors pre-mix the Hepes buffer, DTT, KH_2PO_4, NAD, ADP and PGK in a falcon tube according to the following formulas
    1 ml Bicine buffer * X (number of samples)
    + 10 µl DTT * X (9.7 mM)
    + 10 µl NAD * X (483 µM)
    + 10 µl ADP * X (483 µM)
    + 5 µl PGK * X
    + 1 µl KH_2PO_4 * X (965 µM)
2. Add 800 µl of the mix to each cuvette
3. Add 5 µl of sample to cuvette

4. Mix cuvette contents with a stir rod, and allow cuvette to warm up before placing in spec
5. Place cuvette in spec, balance beams, and obtain a stable baseline. For more detailed instructions as to how to do this see steps 5 – 13 of the glucose assay directions.
6. Add 5 µl of GAPDH to the cuvette and allow reaction to precede, this is how much GAP is is in your sample
7. After the GAP reaction has finished add 5 µl of TPI to the cuvette and allow reaction to precede, this is how much DHAP is in your sample

PGA Assay:
1. To help insure consistency and eliminate some pipetting errors pre-mix the Hepes buffer, DTT, NADH, ATP and GAPDH in a falcon tube according to the following formulas
    1 ml Bicine buffer * X (number of samples)
    + 20 µl NADH * X (191 µM)
    + 10 µl ATP * X (483 µM)
    + 10 µl DTT * X (9.7 mM)
    + 5 µl GAPDH * X
2. Add 800 µl of the mix to each cuvette
3. Add 5 µl of sample to cuvette
4. Mix cuvette contents with a stir rod, and allow cuvette to warm up before placing in spec
5. Place cuvette in spec, balance beams, and obtain a stable baseline. Since we are going be looking at a reduction in ABS (i.e. NADH to NAD) obtain your baseline on the right hand side of the chart paper For more detailed instructions as to how to do this see steps 5 – 13 of the glucose assay directions.
6. Add 5 µl of PGK to the cuvette and allow reaction to precede, this is how much PGA is is in your sample

G1P (*PGM*) → **G6P**

Fructose (*HXK* + *ATP*) → **F6P** (*PGI*) → **G6P**

Sucrose (*INV*) → **Glucose** + Fructose

DHAP (*TPI*) → **GAP** (*GAPDH* + *NAD*) → **1,3-Bisphosphoglycerate** + $NADH^{(absorbs\ at\ 340)}$ (*PGK* + *ADP* + *Pi*) → **PGA** + ATP

For all assays use the following formula to calculate concentration in sample:

1 / Sensitivity / 20 (width of chart paper) * Height of curve / 6180 (extinction coefficient) / 5e-6 (amount of sample added to cuvette) * (805 + (X * 5))e-6 X = number of enzymes added to assay particular carbohydrate, or 855e-6 (total volume of cuvette sucrose assay) = Molarity of sample

These assays were adapted from:

Lowry, Oliver H., and Passonneau, Janet V., (1972) A flexible system of enzymatic analysis. Academic Press Inc.

Shirokane, Y., Ichikawa, K., and Suzuki, M., (2000) A novel enzymic determination of maltose. Carbohydrate Research 329:699-702.

Sensitivity Settings for Various Concentrations				
Sensitivity	**Max Conc in Cuvette**	**Min Conc in Cuvette[1]**	**Max Conc in Sample[2]**	**Min Conc in Sample[2]**

1	161.8 µM	4.0 µM	26.1 mM	651 µM
2	80.9 µM	2.0 µM	13 mM	326 µM
5	32.4 µM	0.81 µM	5.2 mM	130 µM
10	16.2 µM	0.405 µM	2.6 mM	65 µM
20	8.1 µM	0.202 µM	1.3 mM	33 µM
50	3.2 µM	0.081 µM	521 µM	13 µM
100	1.6 µM	0.040 µM	261 µM	7 µM

[1] assuming a minimum of 0.5 cm displacement on the chart recorder
[2] assuming 5 ul of sample in a total cuvette volume of 805 µl

Quick Equations to Determine Sample Concentration		
Sensitivity	**Cuvette Vol**	**Equation (X = height of curve)**
1	805	X * $1.30259e^{-3}$ = Concentration (M)
1	855	X * $1.3835e^{-3}$ = Concentration (M)
2	805	X * $6.51294e^{-4}$ = Concentration (M)
2	855	X * $6.91748e^{-4}$ = Concentration (M)
5	805	X * $2.60518e^{-4}$ = Concentration (M)
5	855	X * $2.76699e^{-4}$ = Concentration (M)
10	805	X * $1.30259e^{-4}$ = Concentration (M)
10	855	X * $1.3835e^{-4}$ = Concentration (M)
20	805	X * $6.51294e^{-5}$ = Concentration (M)
20	855	X * $6.91748e^{-5}$ = Concentration (M)
50	805	X * $2.60518e^{-5}$ = Concentration (M)
50	855	X * $2.76699e^{-5}$ = Concentration (M)

CARBOHYDRATE ASSAY REAGENTS

Hepes buffer pH 7.2:
In 500 ml of distilled H_2O
+ 17.9 G Hepes, 150 mM (fw 238.3)
+ 1.5 g $MgCl_2$, 15 mM (fw 203.3)
+ 0.6 g EDTA, 3 mM (fw 416.2)
pH to 7.2 using KOH or HCl

50 mM ATP:
In 10 ml of distilled H_2O

pH to 7.0 using KOH

50 mM ADP:
In 10 ml of distilled H_2O
+ 0.214 g ADP (fw 427.2; Sigma A-5285)
pH to 7.0 using KOH

50 mM NADP:
In 10 ml distilled H_2O
+ 0.383 g NADP (fw 765.4; Sigma N-0505)

50 mM NAD:
In 10 ml distilled H_2O
+ 0.332 g NAD (fw 663.4; Sigma N-7004)

10 mM NADH:
In 10 ml 100 mM Carbonate buffer pH 10.6
+ 0.071 g NADH (fw 709.4; Sigma N-8129)

1 M DTT:
In 10 ml dH_2O
+ 1.54 g DTT (fw 154.2; Sigma D-5545)
Divide into 1.5 ml microfuge tubes and store in -80° C

30% Glycerol solution for enzymes:
In 7 ml distilled H_2O
+ 3 ml Glycerol
usually X units of enzyme per Y μl of 30% glycerol solution

100 mM Carbonate buffer pH 10.6:
In 100 ml of distilled H_2O
+ 0.848 g Na_2CO_3 (fw 105.99)
+ 0.168 g $NaHCO_3$ (fw 84.01)
pH to 10.6 using NaOH or HCl

Adapted from Sean's protocol
Ziru Li

Perchloric acid extraction of oak leaves followed by DHAP and GAP measurement

Last modified 07/15/10
Part adapted from Sean Weise's carbohydrate assay protocol

1. Measure the leaf area before gas exchange experiment. Make an envelope with aluminum foil and pre-weigh this envelope.
2. At different times during the gas exchange experiment, open up the chamber lid, quickly excise the leaf,

wrap it up in the aluminum envelope and flash-freeze the whole thing in liq. N2.

3. Weigh the envelope with leaf on the balance and put back into liq. N2. (Record minimum weight on balance because condensation will start to occur.) Calculate the weight of leaf.
4. Pre-chill a mortar in liquid nitrogen. Grind up the leaf to a fine powder in perchloric acid, use approx. 1ml per g of tissue. Record the amount used.
5. Place the mortar on ice and let incubate for 3 minutes. Take the supernatant and pipette into new eppendorf tubes.
6. Centrifuge at 15,000g at 4C for 10 min to remove pellet.
7. Transfer the supernatant to a new tube (record the volume transferred). Neutralize supernatant with neutralizing buffer to bring the pH to ~7, if unsure check pH using pH sticks. Record the volume of neutralizing buffer used.
8. Freeze sample in liq. N2 to precipitate salts. Then thaw on ice.
9. Spin for 2 minutes on micro-centrifuge, transfer the supernatant to a new tube. Take a small amount of sample for carbohydrate assay. Lyophilize the rest for NMR studies. Stop point: flash freeze the sample and store in -80, or store the lyophilized powder at -80.
10. Turn on the dual-wavelength spec at least 20 minutes before the experiment to allow warm up. Match light source A and B at 900 if needed. Calibrate (i.e. zero and attenuate) chart recorder if needed.
11. Make up the reaction buffer by mixing up the following in a falcon tube according to the following formulas
    1ml Bicine/HEPES buffer * X (X=number of samples)
    + 10ul DTT * X (9.7mM)
    + 10ul NAD * X (483µM)
    + 10ul ADP * X (483uM)
    + 5ul PGK * X
    + 1ul KH2PO4 * X (965uM)
12. Add 800ul of the mix to each cuvette
13. Add 5 ul of sample to cuvette
14. Mix cuvette contents with a plastic applicator, and allow cuvette to warm up before placing in spec.
15. Place cuvette in spec, obtain a baseline. Add 5ul of GAPDH to the cuvette and allow reaction to proceed, the difference will reflect the amount of GAP is in the sample.
16. After the GAP reaction is complete add 5ul of TPI to the cuvette and allow reaction to proceed, the difference will reflect the amount of GAP in the sample.

Materials needed:
Ice-cold perchloric acid
Neutralizing buffer
100 mM Bicine buffer pH 7.6
1 M DTT
1 M KH_2PO_4
50 mM NAD (Sigma N-7004)
50 mM ADP (Sigma A-5285)
6 u 1^{-1} µl PGA kinase, PGK (Sigma P-7634)
1 u 1^{-1} µl Triose-phosphate isomerase, TPI (Sigma T-6258)
1 u 1^{-1} µl Glyceraldehyde-phosphate dehydrogenase, GAPDH (Sigma G-5537)

3.5% Perchloric Acid:
In 400 ml of distilled H2O
+ 20 ml of 70% perchloric acid
remember acid into water never the reverse

Neutralizing Buffer:
2M KOH, 150 mM Hepes, 10 mM KCl
In 250 ml of distilled H2O
+ 8.936 g Hepes

+ .186 g KCl
+ 28.055 g KOH

Hepes buffer pH 7.2:
In 500 ml of distilled H_2O
+ 17.9 G Hepes, 150 mM (fw 238.3)
+ 1.5 g $MgCl_2$, 15 mM (fw 203.3)
+ 0.6 g EDTA, 3 mM (fw 416.2)
pH to 7.2 using KOH or HCl

50 mM ADP:
In 10 ml of distilled H_2O
+ 0.214 g ADP (fw 427.2; Sigma A-5285)
pH to 7.0 using KOH

50 mM NAD:
In 10 ml distilled H_2O
+ 0.332 g NAD (fw 663.4; Sigma N-7004)

1 M DTT:
In 10 ml dH_2O
+ 1.54 g DTT (fw 154.2; Sigma D-5545)
Divide into 1.5 ml microfuge tubes and store in -80° C

30% Glycerol solution for enzymes:
In 7 ml distilled H_2O
+ 3 ml Glycerol
usually X units of enzyme per Y μl of 30% glycerol solution