Enzymatic Assay of FRUCTOSE-6-PHOSPHATE KINASE, PYROPHOSPHATE DEPENDENT (EC 2.7.1.90) from Mung Bean

PRINCIPLE:

$$PP_i + \text{F-6-P} \xrightarrow[\text{F-2,6-DP}^1]{PP_i\text{-PFK}} \text{F-1,6-DP} + P_i$$

$$\text{F-1,6-DP} \xrightarrow{\text{Aldolase}} \text{GAP} + \text{DHAP}$$

$$\text{GAP} \xrightarrow{\text{TPI}} \text{DHAP}$$

$$2\text{DHAP} + 2\ \text{ß-NADH} \xrightarrow{\text{GDH}} 2\ \text{Glycerol-3-Phosphate} + 2\ \text{ß-NAD}$$

Abbreviations used:
PP_i = Pyrophosphate
F-6-P = D-Fructose-6-Phosphate
F-2,6-DP = Fructose 2,6-Diphosphate
PP_i-PFK = Fructose-6-Phosphate Kinase, Pyrophosphate Dependent
F-1,6-DP = D-Fructose-1,6-Diphosphate
P_i = Inorganic Phosphate
GAP = D-Glyceraldehyde-3-Phosphate
TPI = Triosephosphate Isomerase
DHAP = Dihydroxyacetone Phosphate
ß-NADH = ß-Nicotinamide Adenine Dinucleotide, Reduced Form
GDH = a-Glycerophosphate Dehydrogenase
ß-NAD = ß-Nicotinamide Adenine Dinucleotide, Oxidized Form

CONDITIONS: T = 30°C, pH 7.6, A_{340nm}, Light path = 1 cm

METHOD: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 100 mM Imidazole HCl Buffer with 1 mM Magnesium Chloride and 0.2 mM Ethylenediaminetetraacetic Acid, pH 7.6 at 30°C
(Prepare 100 ml in deionized water using Imidazole, Sigma Prod. No. I-0250, Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250, and Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate, Sigma Stock No. ED2SS. Adjust to pH 7.6 at 30°C with 1 M HCl.)

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REAGENT: (continued)

B. 100 mM D-Fructose-6-Phosphate Solution (F-6-P)
(Prepare 10 ml in Reagent A using D-Fructose 6-Phosphate, Dipotassium Salt, Sigma Prod. No. F-1502.)

C. 5.0 mM ß-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (ß-NADH)
(Dissolve the contents of one 5 mg vial of ß-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Stock No. 340-110, in the appropriate volume of Reagent A. **PREPARE FRESH.**)

D. Aldolase Enzyme Solution
(Immediately before use, prepare a solution containing 50 units/ml of Aldolase, Sigma Prod. No. A-7145 in cold Reagent A.)

E. a-Glycerophosphate Dehydrogenase/Triosephosphate Isomerase Enzyme Solution[1] (a-GDH/TPI)
(Immediately before use, prepare a solution containing 50 a-GDH units/ml of a-Glycerophosphate Dehydrogenase-Triosephosphate Isomerase Type X from Rabbit Muscle, Sigma Prod. No. G-6755 in cold Reagent A.)

F. 30 mM Pyrophosphate Solution (PP_i)
(Prepare 10 ml in Reagent A using Pyrophosphate, Disodium, Sigma Prod. No. P-8135.)

G. 30 µM Fructose 2,6-Diphosphate Solution[2] (F-2,6-DP)
(Prepare 1 ml in Reagent A using D-Fructose 2,6-Diphosphate, Sodium Salt, Sigma Prod. No. F-7006.)

H. Fructose-6-Phosphate Kinase, Pyrophosphate Dependent Enzyme Solution (PP_i-PFK)
(Immediately before use, prepare a solution containing 0.02 - 0.07 unit/ml in deionized water.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable cuvettes:

	Test	Blank
Reagent A (Buffer)	1.45	1.45
Reagent B (F-6-P)	1.00	1.00

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PROCEDURE: (continued)

	Test	Blank
Reagent C (ß-NADH)	0.10	0.10
Reagent D (Aldolase)	0.10	0.10
Reagent E (a-GDH/TPI)	0.10	0.10
Reagent G (F-2,6-DP)	0.10	0.10
Reagent H (PP_i-PFK)	0.10	------
Deionized Water	------	0.10

Mix by inversion and equilibrate to 30°C. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent F (PP_i)	0.10	0.10

Immediately mix by inversion and record the decrease in A_{340nm} for approximately 5 minutes. Obtain the ΔA_{340nm} using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340nm}/\text{min Test} - \Delta A_{340nm}/\text{min Blank})(3.05)(df)}{(6.22)(2)(0.1)}$$

3.05 = Total volume (in milliliters) of assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of ß-NADH at 340 nm
2 = Factor accounting for 2 moles of ß-NADH oxidized per mole of pyrophosphate converted
0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

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UNIT DEFINITION:

One unit will convert 1.0 µmole of pyrophosphate and fructose 6-phosphate to fructose 1,6-diphosphate and inorganic phosphate per minute at pH 7.6 at 30°C in a coupled system with aldolase, a-glycerophosphate dehydrogenase, triosephosphate isomerase, and 1 µmole fructose 2,6-diphosphate.

FINAL ASSAY CONCENTRATION:

In a 3.05 ml reaction mix, the final concentrations are 97 mM imidazole buffer, 1 mM magnesium chloride, 0.2 mM ethylenediaminetetraacetic acid, 33 mM D-fructose-6-phosphate, 0.16 mM ß-nicotinamide adenine dinucleotide, reduced form, 0.98 mM pyrophosphate, 5 units aldolase, 5 units a-glycerophosphate dehydrogenase, 40 units of triosephosphate isomerase, 0.002 - 0.007 unit fructose-6-phosphate kinase, pyrophosphate dependent, and 0.98 µM fructose 2,6-diphosphate.

REFERENCE:

Sabularse, D.C. and Anderson, R.L. (1981) *Biochemical and Biophysical Research Communications* **103**, 848-855

NOTES:

1. Fructose 2,6-Diphosphate is an activator of Fructose-6-Phosphate Kinase, Pyrophosphate Dependent from Mung Bean.

2. The Triosephosphate Isomerase activity is approximately 8 fold that of the a-Glycerophosphate Dehydrogenase.

3. Aldolase Unit Definition: One unit will convert 1.0 µmole of fructose 1,6-diphosphate to dihydroxyacetone phosphate and glyceraldehyde 3-phosphate per minute at pH 7.4 at 25°C.

4. a-Glycerophosphate Dehydrogenase Unit Definition: One unit will convert 1.0 µmole of dihydroxyacetone phosphate to a-glycerophosphate per minute at pH 7.4 at 25°C.

5. Triosephosphate Isomerase Unit Definition: One unit will convert 1.0 µmole of D-glyceraldehyde 3-phosphate

to dihydroxyacetone phosphate per minute at pH 7.6 at 25°C.

NOTES: (continued)

6. This assay is based on the cited reference.

7. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.