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

PRINCIPLE:

\[ \text{PP}_i\text{-PFK} \]
\[ \text{PP}_i + \text{F-6-P} \xrightarrow{\text{Aldolase}} \text{F-1,6-DP} + \text{P}_i \]
\[ \text{F-1,6-DP} \xrightarrow{TPI} \text{GAP} + \text{DHAP} \]
\[ 2\text{DHAP} + 2 \text{ß-NADH} \xrightarrow{\text{GDH}} 2 \text{Glycerol-3-Phosphate} + 2 \text{ß-NAD} \]

Abbreviations used:

- \text{PP}_i = \text{Pyrophosphate}
- \text{F-6-P} = \text{D-Fructose-6-Phosphate}
- \text{F-2,6-DP} = \text{Fructose 2,6-Diphosphate}
- \text{PP}_i\text{-PFK} = \text{Fructose-6-Phosphate Kinase, Pyrophosphate Dependent}
- \text{F-1,6-DP} = \text{D-Fructose-1,6-Diphosphate}
- \text{P}_i = \text{Inorganic Phosphate}
- \text{GAP} = \text{D-Glyceraldehyde-3-Phosphate}
- \text{TPI} = \text{Triosephosphate Isomerase}
- \text{DHAP} = \text{Dihydroxyacetone Phosphate}
- \text{ß-NADH} = \text{ß-Nicotinamide Adenine Dinucleotide, Reduced Form}
- \text{GDH} = \text{a-Glycerophosphate Dehydrogenase}
- \text{ß-NAD} = \text{ß-Nicotinamide Adenine Dinucleotide, Oxidized Form}

CONDITIONS:  \( T = 30^\circ\text{C}, \text{pH 7.6, } A_{340\text{nm}}, \text{Light path} = 1 \text{ cm} \)

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, Magnesium Chloride, Hexahydrate and Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate. 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.)

C. 5.0 mM \(\beta\)-Nicotinamide Adenine Dinucleotide, Reduced 
Form Solution (\(\beta\)-NADH) 
(Dissolve the contents of one 5 mg vial of 
\(\beta\)-Nicotinamide Adenine Dinucleotide, Reduced Form, 
Disodium Salt, 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, in cold Reagent A.)

E. \(\alpha\)-Glycerophosphate Dehydrogenase/Triosephosphate 
Isomerase Enzyme Solution\(^1\) (\(\alpha\)-GDH/TPI) 
(Immediately before use, prepare a solution containing 
50 \(\alpha\)-GDH units/ml of \(\alpha\)-Glycerophosphate Dehydrogenase- 
Triosephosphate Isomerase Type X from Rabbit Muscle, 
in cold Reagent A.)

F. 30 mM Pyrophosphate Solution (PP\(_i\)) 
(Prepare 10 ml in Reagent A using Pyrophosphate, 
Disodium.)

G. 30 \(\mu\)M Fructose 2,6-Diphosphate Solution\(^2\) (F-2,6-DP) 
(Prepare 1 ml in Reagent A using \(d\)-Fructose 
2,6-Diphosphate, Sodium Salt.)

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:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
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</thead>
<tbody>
<tr>
<td>Reagent A (Buffer)</td>
<td>1.45</td>
<td>1.45</td>
</tr>
<tr>
<td>Reagent B (F-6-P)</td>
<td>1.00</td>
<td>1.00</td>
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</tbody>
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PROCEDURE: (continued)

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
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</thead>
<tbody>
<tr>
<td>Reagent C (β-NADH)</td>
<td>0.10</td>
<td>0.10</td>
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<tr>
<td>Reagent D (Aldolase)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent E (a-GDH/TPI)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent G (F-2,6-DP)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent H (PPi-PFK)</td>
<td>0.10</td>
<td>------</td>
</tr>
<tr>
<td>Deionized Water</td>
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<td>0.10</td>
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</table>

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

<p>| | | |</p>
<table>
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<tbody>
<tr>
<td>Reagent F (PPi)</td>
<td>0.10</td>
<td>0.10</td>
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</table>

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

CALCULATIONS:

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

3.05 = Total volume (in milliliters) of assay  
df = Dilution factor  
6.22 = Millimolar extinction coefficient of β-NADH at 340 nm  
2 = Factor accounting for 2 moles of β-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, α-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 β-nicotinamide adenine dinucleotide, reduced form, 0.98 mM pyrophosphate, 5 units aldolase, 5 units α-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:


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 α-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. α-Glycerophosphate Dehydrogenase Unit Definition: One unit will convert 1.0 µmole of dihydroxyacetone phosphate to α-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.
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NOTES: (continued)

6. This assay is based on the cited reference.

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

This procedure is for informational purposes. For a current copy of our quality control procedure contact our Technical Service Department.