Enzymatic Assay of PHOSPHORIBULOKINASE
(EC 2.7.1.19)

PRINCIPLE:
D-Ribulose 5-Phosphate + ATP \( \overset{PRK}{\rightarrow} \) D-Ribulose 1,5-Bisphosphate + ADP
ADP + PEP \( \overset{PK}{\rightarrow} \) Pyruvate + ATP
Pyruvate + \( \beta \)-NADH \( \overset{LDH}{\rightarrow} \) Lactate + \( \beta \)-NAD

Abbreviations used:
ATP = Adenosine 5'-Triphosphate
PRK = Phosphoribulokinase
ADP = Adenosine 5'-Diphosphate
PEP = Phospho(enol)pyruvate
PK = Pyruvate Kinase
\( \beta \)-NADH = \( \beta \)-Nicotinamide Adenine Dinucleotide, Reduced Form
LDH = Lactic Dehydrogenase
\( \beta \)-NAD = \( \beta \)-Nicotinamide Adenine Dinucleotide, Oxidized Form

CONDITIONS:  T = 37°C, pH = 7.9, \( A_{340nm} \), Light path = 1 cm

METHOD:  Continuous Spectrophotometric Rate Determination

REAGENTS:
A. 67 mM Tris HCl Buffer, pH 7.9 at 37°C
   (Prepare 100 ml in deionized water using Trizma Base,
   Adjust to pH 7.9 at 37°C with
   1 M HCl.)

B. 50 mM Potassium Chloride Solution (KCl)
   (Prepare 10 ml in Reagent A using Potassium Chloride.)

C. 6 mM Magnesium Sulfate Solution (MgSO\(_4\))
   (Prepare 10 ml in Reagent A using Magnesium Sulfate,
   Heptahydrate.)

D. 0.54 mM Phospho(enol)pyruvate Solution (PEP)
   (Prepare 10 ml in Reagent A using
   Phospho(enol)pyruvate, Monopotassium Salt.)
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REAGENTS:  (continued)

E.  0.36 mM Ethylenediaminetetraacetic Acid Solution (EDTA)
    (Prepare 10 ml in Reagent A using Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate.)

F. β-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (β-NADH)
    (Use β-Nicotinamide Adenine Dinucleotide, Disodium, 1 mg vial.)

G. 50 mM Tris HCl Buffer, pH 7.1 at 37°C (Enz Dil)
    (Prepare 25 ml in deionized water using Trizma Base, Adjust to pH 7.1 at 37°C with 1 M HCl.)

H. 20 mM D-Ribulose 5-Phosphate Solution (RU-5-P)
    (Prepare 1 ml in deionized water using D-Ribulose 5-Phosphate, Sodium Salt.)

I. 9.1 mM Adenosine 5'-Triphosphate Solution (ATP)
    (Prepare 1 ml in deionized water using Adenosine 5'-Triphosphate Disodium Salt.)

J. 82 mM Glutathione, Reduced Form Solution (GSH)
    (Prepare 1 ml in deionized water using Glutathione, Reduced Form, Free Acid.)

K. PK/LDH Enzymes Suspension
    (Use PK/LDH Enzymes Suspension.)

L. Phosphoribulokinase Enzyme Solution (PRK)
    (Immediately before use, prepare a solution containing 0.3 - 0.6 unit/ml of Phosphoribulokinase in cold Reagent G.)

PROCEDURE:

Prepare an initial reaction cocktail by pipetting (in milliliters) the following reagents into a suitable container:

- Reagent A (Buffer) 60.00
- Reagent B (KCl) 10.00
- Reagent C (MgSO₄) 10.00
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
<th>Price</th>
</tr>
</thead>
<tbody>
<tr>
<td>D (PEP)</td>
<td></td>
<td>10.00</td>
</tr>
<tr>
<td>E (EDTA)</td>
<td></td>
<td>10.00</td>
</tr>
</tbody>
</table>
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PROCEDURE:  (continued)

Mix by swirling and adjust to pH 7.9 at 37°C with either 1 M HCl or 1 M NaOH, if necessary.

Prepare a final reaction cocktail by pipetting 10 ml of the initial reaction cocktail into Reagent F (β-NADH).

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

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final Reaction Cocktail</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Reagent I (ATP)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent J (GSH)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Reagent K (PK/LDH)</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Reagent L (PRK)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 37°C. Monitor the \(rA_{340nm}\) until constant, using a suitably thermostatted spectrophotometer. Then add:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent H (RU-5-P)</td>
<td>0.10</td>
<td>------</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>------</td>
<td>0.10</td>
</tr>
</tbody>
</table>

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

CALCULATIONS:

\[
\text{Units/ml enzyme} = \frac{(rA_{340nm}/\text{min Test} - rA_{340nm}/\text{Blank})(2.93)(\text{df})}{(6.22)(0.1)}
\]

2.93 = Total volume (in milliliters) of assay
\(\text{df} = \) Dilution factor
6.22 = Millimolar extinction coefficient of β-NADH at 340 nm
0.1 = Volume (in milliliter) of enzyme used

\[
\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}
\]
Units/mg protein = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}
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UNIT DEFINITION:

One unit will transfer 1.0 µmole of phosphate from ATP to D-ribulose 5-phosphate per minute at pH 7.9 at 37°C.

FINAL ASSAY CONCENTRATION:

In a 2.93 ml reaction mix, the final concentrations are 57 mM Tris, 4.3 mM potassium chloride, 0.5 mM magnesium sulfate, 0.046 mM phospho(enol)pyruvate, 0.031 mM ethylenediaminetetraacetic acid, 0.31 mM adenosine 5'-triphosphate, 2.8 mM glutathione, reduced form, 0.12 mM ß-nicotinamide adenine dinucleotide, reduced form, 0.68 mM D-ribulose 5-phosphate, 21 units pyruvate kinase, 30 units lactic dehydrogenase, and 0.03 - 0.06 unit phosphoribulokinase.

REFERENCE:


NOTES:

1. Contains not less than 700 Pyruvate Kinase units and 100 Lactic Dehydrogenase units per ml.

2. Pyruvate Kinase Unit Definition: One unit will convert 1.0 µmole of phospho(enol)pyruvate to pyruvate per minute at pH 7.6 at 37°C.

3. L-Lactic Dehydrogenase Unit Definition: One unit will reduce 1.0 µmole of pyruvate to L-lactic per minute at pH 7.5 at 37°C.

4. This assay is based on the cited reference.