**Enzymatic Assay of $_D$-RIBULOSE 1,5-DIPHOSPHATE CARBOXYLASE (EC 4.1.1.39)**

**PRINCIPLE:**

$$RuDP + CO_2 \xrightarrow[Mg^{+2}]{RUBISCO} 2 \text{ moles of 3-phosphoglycerate}$$

$$3\text{-Phosphoglycerate} + ATP \xrightarrow{PGK} \text{Glycerate 1,3-Diphosphate} + ADP$$

$$\text{Glycerate 1,3-Diphosphate} + \text{ß-NADH} \xrightarrow{GAPDH} \text{Glyceraldehyde 3-Phosphate} + NAD + P_i$$

$$\text{Glyceraldehyde 3-Phosphate} \xrightarrow{TPI} DHAP$$

$$DHAP + \text{ß-NADH} \xrightarrow{a\text{-GDH}} \text{a-Glycerophosphate} + \text{ß-NAD}$$

Abbreviations used:
RUDP = $_D$-Ribulose 1,5-Diphosphate
RUBISCO = $_D$-Ribulose 1,5-Diphosphate Carboxylase
ATP = Adenosine 5'-Triphosphate
PGK = 3-Phosphoglyceric Phosphokinase
ADP = Adenosine 5'-Diphosphate
ß-NADH = ß-Nicotinamide Adenine Dinucleotide, Reduced Form
GAPDH = Glyceraldehyde 3-Phosphate Dehydrogenase
$P_i$ = Inorganic Phosphate
TPI = Triosephosphate Isomerase
DHAP = Dihydroxyacetone Phosphate
ß-NAD = ß-Nicotinamide Adenine Dinucleotide, Oxidized Form
a-GDH = a-Glycerophosphate Dehydrogenase

**CONDITIONS:** T = 25°C, pH = 7.8, $A_{340nm}$, Light path = 1 cm

**METHOD:** Continuous Spectrophotometric Rate Determination

**REAGENTS:**

A. 1 M Tris HCl Buffer, pH 7.8 at 25°C
(Prepare 25 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 7.8 at 25°C with 10 M HCl.)

B. 100 mM Magnesium Chloride Solution ($MgCl_2$)
(Prepare 5 ml in deionized water using Magnesium Chloride, Hexahydrate, Sigma Prod. No. M-0250.)

**REAGENTS:** (continued)

C. 2 M Potassium Bicarbonate Solution ($KHCO_3$)
(Prepare 5 ml in deionized water using Potassium Bicarbonate, Sigma Prod. No. P-9144.)

D. 2.5 mM ß-Nicotinamide Adenine Dinucleotide, Reduced Form Solution (ß-NADH)
(Prepare 5 ml in Reagent A using ß-Nicotinamide Adenine Dinucleotide, Reduced Form, Disodium Salt, Sigma Prod. No. N-8129 or dissolve the contents of one 1 mg vial of ß-Nicotinamide Dinucleotide, Reduced Form, Disodium Salt, Sigma Prod. No. 340-101 in the appropriate volume of Reagent A.)

E. 100 mM Adenosine 5'-Triphosphate Solution (ATP)
(Prepare 5 ml in deionized water using Adenosine 5'-Triphosphate, Disodium Salt, Sigma Prod. No. A-5394.)

F. 100 mM Glutathione, Reduced Form, Solution (GSH)
(Prepare 5 ml in deionized water using Glutathione, Reduced Form, Free Acid, Sigma Prod. No. G-4251.)

G. 25 mM D-Ribulose 1,5-Diphosphate Solution (RuDP)
(Prepare 2 ml in deionized water using D-Ribulose 1,5-Diphosphate, Sodium Salt, Hydrate, Sigma Prod. No. R-0878.)

H. a-Glycerophosphate Dehydrogenase-Triosephosphate Isomerase Enzyme Suspension (a-GDH/TPI)
(Immediately before use prepare a solution containing 50 units/ml of a-Glycerophosphate Dehydrogenase-Triosephosphate Isomerase, Sigma Prod. No. G-1881, in cold deionized water.)

I. Glyceraldehyde-3-Phosphate Dehydrogenase/3-Phosphoglyceric Phosphokinase Enzyme Solution (GAPDH/3-PGK)
(Immediately before use prepare a solution containing 50 units/ml of Glyceraldehyde-3-Phosphate Dehydrogenase/3-Phosphoglyceric Phosphokinase, Sigma Prod. No. G-8505, in cold deionized water.)

J. D-Ribulose 1,5-Diphosphate Carboxylase (RUBISCO)
(Immediately before use, prepare a solution containing 0.07 - 0.15 unit/ml of D-Ribulose 1,5-Diphosphate Carboxylase in cold Reagent A.)

## Enzymatic Assay of D-RIBULOSE 1,5-DIPHOSPHATE CARBOXYLASE (EC 4.1.1.39)

**PROCEDURE:**

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

| | |
|---|---|
| Reagent A (Buffer) | 4.40 |
| Reagent B ($MgCl_2$) | 1.50 |
| Reagent C ($KHCO_3$) | 1.00 |
| Reagent D (ß-NADH) | 2.40 |
| Reagent E (ATP) | 1.50 |
| Reagent F (GSH) | 1.50 |
| Reagent G (RuDP) | 0.60 |

Mix by swirling. Adjust to pH 7.8 at 25°C, if necessary, with either 0.1 M NaOH or 0.1 M HCl.

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

| | Test | Blank |
|---|---|---|
| Reaction Cocktail | 1.29 | 1.29 |
| Reagent H (a-GDH/TPI) | 0.10 | 0.10 |
| Reagent I (GAPDH/3-PGK) | | 0.10 |
| | | 0.10 |
| Deionized Water | 1.41 | 1.41 |

Mix by inversion and equilibrate to 25°C. Monitor the $A_{340nm}$ until constant, using a suitably thermostatted spectrophotometer. Then add:

| | | |
|---|---|---|
| Reagent J (RUBISCO) | 0.10 | ------ |
| Reagent A (Buffer) | ------ | 0.10 |

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

**CALCULATIONS:**

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340nm}/\text{min Test} - \Delta A_{340nm}/\text{min Blank})(3)(df)}{(4)(6.22)(0.1)}$$

3 = Total volume (in milliliters) of assay
df = Dilution factor
4 = 4 µmoles of ß-NADH are oxidized for each µmole of D-Ribulose 1,5-diphosphate utilized

6.22 = Millimolar extinction coefficient for ß-NADH at 340 nm
0.1 = Volume (in milliliter) of enzyme used

**CALCULATIONS:** (continued)

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

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

**UNIT DEFINITION:**

One unit will convert 1.0 µmole of D-RuDP and $CO_2$ to 2.0 µmoles of D-3-phosphoglycerate per minute at pH 7.8 at 25°C.

**FINAL ASSAY CONCENTRATIONS:**

In a 3.00 ml reaction mix, the final concentrations are 259 mM Tris, 5 mM magnesium chloride, 67 mM potassium bicarbonate, 0.2 mM ß-nicotinamide adenine dinucleotide, reduced form, 5 mM adenosine 5'-triphosphate, 5 mM glutathione, reduced form, 0.5 mM D-ribulose 1,5-diphosphate, 5 units a-glycerophosphate dehydrogenase-triosephosphate isomerase, 5 units glyceraldehyde-3-phosphate dehydrogenase/3-phosphoglyceric phosphokinase, and 0.007 - 0.015 unit D-ribulose 1,5-diphosphate carboxylase.

**REFERENCES:**

Racker, E. (1962) *Methods in Enzymology* V, 266-270

Racker, E. (1957) *Archives of Biochemistry and Biophysics* **69**, 300-310

**NOTES:**

1. 3-Phosphoglyceric Phosphokinase Unit Definition: One unit will convert 1.0 µmole of 3-phosphoglycerate to 1,3-diphosphoglycerate per minute at pH 7.5 at 25°C.

2. Glyceraldehyde-3-Phosphate Dehydrogenase Unit Definition: One unit will reduce 1.0 µmole of 3-phosphoglycerate to D-glyceraldehyde 3-phosphate per minute in a coupled system with 3-phosphoglyceric phosphokinase at pH 7.6 at 25°C.

**NOTES:** (continued)

3. 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 and 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.