Enzymatic Assay of p-HYDROXYBENZOATE HYDROXYLASE (EC 1.14.13.2)

PRINCIPLE:

$$\text{p-Hydroxybenzoate} + \beta\text{-NADPH} + O_2 \xrightarrow[\text{FAD}]{\text{PHBH}} \text{Protocatechuate} + \beta\text{-NADP} + H_2O$$

Abbreviations used:
β-NADPH = β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form
PHBH = p-Hydroxybenzoate Hydroxylase
FAD = Flavin Adenine Dinucleotide
β-NADP = β-Nicotinamide Adenine Dinucleotide Phosphate, Oxidized Form

METHOD: T = 37°C, pH = 8.2, A_{340nm}, Light path = 1 cm

CONDITIONS: Continuous Spectrophotometric Rate Determination

REAGENTS:

A. 50 mM Tris Maleate Buffer, pH 8.2 at 37°C
(Prepare 100 ml in deionized water using Trizma Maleate, Sigma Prod. No. T-3128. Adjust to pH 8.2 at 37°C with 1 M NaOH.)

B. 5.0 mM p-Hydroxybenzoate Solution (p-Hydroxybenz)
(Prepare 10 ml in Reagent A using p-Hydroxybenzoic Acid, Sigma Prod. No. H-5376. **PREPARE FRESH**.)

C. 0.20 mM Flavin Adenine Dinucleotide Solution (FAD)
(Prepare 10 ml in Reagent A using Flavin Adenine Dinucleotide, Disodium Salt, Sigma Prod. No. F-6625. **PREPARE FRESH**.)

D. 3.0 mM β-Nicotinamide Adenine Dinucleotide Phosphate Reduced Form Solution (β-NADPH)
(Dissolve the contents of one 5 mg vial of β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form, Tetrasodium Salt, Sigma Stock No. 201-205, in the appropriate volume of Reagent A or dissolve the appropriate amount of β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form, Tetrasodium Salt, Sigma Prod. No. N-6505 in the appropriate volume of Reagent A. **PREPARE FRESH**.)

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REAGENTS: (continued)

E. 50 mM Potassium Phosphate Buffer, pH 6.0 at 37°C (Enz Dil)
(Prepare 50 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 6.0 at 37°C with 1 M KOH.)

F. p-Hydroxybenzoate Hydroxylase Enzyme Solution
(Immediately before use, prepare a solution containing 0.20 - 0.50 unit/ml of p-Hydroxybenzoate Hydroxylase in cold Reagent E.)

PROCEDURE:

Prepare a reaction cocktail in an amber bottle by pipetting (in milliliters) the following reagents into a suitable container (**PREPARE FRESH.**):

Reagent A (Buffer)	21.00
Reagent B (p-Hydroxybenz)	3.00
Reagent C (FAD)	3.00
Reagent D (β-NADPH)	3.00

Mix by stirring and adjust to pH 8.2 at 37°C with either 1 M HCl or 1 M NaOH.

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

	Test	Blank
Reaction Cocktail	3.00	3.00

Equilibrate to 37°C. Monitor the A_{340nm} until constant, using a suitably thermostatted spectrophotometer. Then add:

Reagent F (Enzyme Solution)	0.05	------
Reagent E (Enz Dil)	------	0.05

Immediately mix by inversion and record the decrease in A_{340nm} for approximately 5-10 minutes. Obtain the ΔA_{340nm}/minute using the maximum linear rate for both the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(\Delta A_{340nm}/\text{min Test} - \Delta A_{340nm}/\text{min Blank})(3.05)(df)}{(6.22)(0.05)}$$

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CALCULATIONS: (continued)

3.05 = Total volume (in milliliters) of assay
df = Dilution factor
6.22 = Millimolar extinction coefficient of β-NADPH at 340 nm
0.05 = Volume (in milliliter) of enzyme used in assay

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

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

UNIT DEFINITION:

One unit will hydroxylate 1.0 μmole of p-hydroxybenzoate to protocatechuate per minute at pH 8.2 at 37°C in the presence of β-NADPH.

FINAL CONCENTRATION:

In a 3.05 ml reaction mix, the final concentrations are 49 mM Tris maleate, 0.49 mM p-hydroxybenzoate, 0.02 mM flavin adenine dinucleotide, 0.3 mM β-nicotinamide adenine dinucleotide phosphate reduced form, 0.8 mM potassium phosphate, and 0.01 - 0.025 unit p-hydroxybenzoate hydroxylase.

REFERENCES:

Hosokawa, K. and Stanier, R.Y. (1966) *Journal of Biological Chemistry* **241**, 2453-2460

NOTES:

1. This assay is based on the cited reference.

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

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