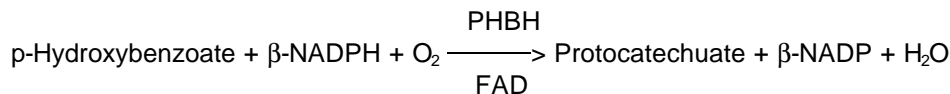


Enzymatic Assay of p-HYDROXYBENZOATE HYDROXYLASE (EC 1.14.13.2)

PRINCIPLE:



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, $A_{340\text{nm}}$, 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:

	<u>Test</u>	<u>Blank</u>
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 $\Delta A_{340nm}/\text{minute}$ 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 μ mole 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.