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PRINCIPLE:

p-Nitrophenyl Phosphate + H2O $\xrightarrow{\text{Phosphatase, Acid}}$ p-Nitrophenol + Pi

Abbreviation:

Pi = Inorganic phosphate

CONDITIONS: T = 37°C, pH = 4.8, A410nm, Light path = 1 cm

METHOD:

Spectrophotometric Stop Rate Determination

REAGENTS:

A. 90 mM Citrate Buffer, pH 4.8 at 37°C

(Prepare 100 ml in deionized water using Citric Acid, Trisodium, Dihydrate,, or Citrate Buffer Solution. Adjust to pH 4.8 at 37°C with1 M NaOH or 1 M HCl.)

B. 15.2 mM p-Nitrophenyl Phosphate (PNPP)

(Prepare 5 ml in deionized water using 104 Phosphatase Substrate.)

C. 100 mM Sodium Hydroxide Solution (NaOH)

(Prepare 50 ml in deionized water using Sodium Hydroxide, Anhydrous.)

D. Acid Phosphatase Enzyme Solution

(Immediately before use, prepare a solution containing 0.15 - 0.25 unit/ml of Phosphatase, Acid in cold deionized water.)

PROCEDURE:

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

	Test	Blank
Reagent A (Buffer)	0.50	0.50
Reagent B (PNPP)	0.50	0.50

PROCEDURE: (continued)

Mix by inversion and equilibrate to 37°C. Then add:

	Test	Blank
Reagent D (Enzyme Solution)	0.10	------

Immediately mix by inversion and incubate at 37℃ for exactly 10 minutes. Then add:

Reagent C (NaOH)	4.00	4.00
Reagent D (Enzyme Solution)	------	0.10

Mix by inversion and record the A410nm for both the Test and Blank in a suitable spectrophotometer.

CALCULATIONS:

$$\text{Units/ml enzyme}=\frac{(\text{A410nmTest - A410nm Blank})(5.1)(\text{df})}{(10)(18.3)(0.1)}$$

5.1 = Total volume (in milliliters) of solution

df = Dilution factor

10 = Time of assay (in minutes) as per the Unit Definition

18.3 = Millimolar extinction coefficient of p-Nitrophenol at 410 nm

0.1 = Volume (in milliliter) of enzyme used

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

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

UNIT DEFINITION:

One unit will hydrolyze 1.0 µmole of p-nitrophenyl phosphate per minute at pH 4.8 at 37℃

FINAL ASSAY CONCENTRATION:

In a 1.10 ml reaction mix, the final concentrations are 41 mM citric acid, 6.9 mM p-nitrophenyl phosphate and 0.015 - 0.025 unit phosphatase, acid.

REFERENCE:

Bergmeyer, H.U. Gawehn, K., and Grassl, M. (1974) in Methods of Enzymatic Analysis (Bergmeyer H.U.) Volume I, 2nd ed., 495-496, Academic Press, Inc. New York, NY