

**Enzymatic Assay of PROTEASE
(EC 3.4.23.6)**

PRINCIPLE:

Hemoglobin + H₂O $\xrightarrow{\text{Protease}}$ Amino Acids

CONDITIONS: T = 37°C, pH = 2.8, A_{660nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

- A. 50 mM Potassium Phthalate Buffer, pH 2.8 at 37°C.
(Prepare by dissolving 4.03 g of Phthalic Acid, Monopotassium Salt, Sigma Prod. No. P-6758 in 100 ml of deionized water. Then add 53 ml of 200 mM HCl. Adjust to pH 2.8 at 37°C with 1 M HCl. Dilute to 400 ml with deionized H₂O.)
- B. 2.5% (w/v) Hemoglobin Solution
(Prepare by dissolving 2.5 g of Hemoglobin, Bovine Sigma Prod. No. H-2625, in 70 ml of deionized water. To this solution add 20 ml of 0.5 M HCl. Adjust the pH to 2.8 at 37°C with 1 M NaOH. Dilute to 100 ml with deionized water. Note: Hemoglobin slowly dissolves and clumps if it is all added at one time.)
- C. 110 mM Trichloroacetic Acid Reagent (TCA)
(Dilute 9 ml of Trichloroacetic Acid, 6.1 N Solution, approximately 100% (w/v), Sigma Stock No. 490-10, to 500 ml with deionized water.)
- D. Folin & Ciocalteu's Phenol Reagent (F-C)
(Dilute 10 ml of Folin & Ciocalteu's Phenol Reagent, Sigma Prod. No. F-9252, to 40 ml with deionized water.)
- E. 500 mM Sodium Carbonate Solution (Na₂CO₃ Soln)
(Prepare 500 ml in deionized water using Sodium Carbonate Anhydrous, Sigma Prod. No. S-2127.)

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REAGENTS: (continued)

- F. 1.1 mM L-Tyrosine Standard Solution (Std Soln)
(Prepare 100 ml in deionized water using L-Tyrosine, Free Base, Sigma Prod. No. T-3754. Heat gently (do not boil) until tyrosine dissolves and cool to room temperature.)
- G. Protease Enzyme Solution
(Immediately before use, prepare a solution containing 0.5 - 1.0 unit/ml of Protease in cold Reagent A.)

PROCEDURE:

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

	<u>Test</u>	<u>Blank</u>
Reagent B (Hemoglobin Solution)	5.00	5.00

Incubate the vials at 37°C until equilibrated. Then add:

Reagent G (Enzyme Solution)	1.00	-----
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Mix by swirling and incubate at 37°C for exactly 10 minutes. Then add:

Reagent C (TCA)	10.00	10.00
Reagent G (Enzyme Solution)	-----	1.00

Mix by swirling and incubate at 37°C for approximately 30 minutes. Filter through a Whatman #50 filter paper and use the filtrate in the color development.

COLOR DEVELOPMENT:

Standard Curve:

Prepare a standard curve by pipetting (in milliliters) the following reagents into suitable vials:

	<u>Std 1</u>	<u>Std 2</u>	<u>Std 3</u>	<u>Std 4</u>	<u>Std Blank</u>
Reagent F (Std Soln)	0.05	0.10	0.20	0.40	0.00
Deionized Water	1.95	1.90	1.80	1.60	2.00
Reagent E (Na ₂ CO ₃ Soln)	5.00	5.00	5.00	5.00	5.00

Reagent D (F-C)	1.00	1.00	1.00	1.00	1.00
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COLOR DEVELOPMENT: (continued)

Sample:

Pipette (in milliliters) the following reagents into a suitable vial:

	<u>Test or Blank</u>
Filtrate	2.00
Reagent E (Na ₂ CO ₃ Soln)	5.00
Reagent D (F-C)	1.00

Mix by swirling and incubate at 37°C for 30 minutes. Remove the vials and allow them to cool to room temperature. If the solutions are hazy, filter through a 0.45 µm filter prior to reading. Read the absorbance at 660nm for each of the vials in suitable cuvettes.

CALCULATIONS:

Standard Curve:

$$r A_{660\text{nm}} \text{ Standard} = A_{660\text{nm}} \text{ Standard} - A_{660\text{nm}} \text{ Standard Blank}$$

Plot the $r A_{660\text{nm}}$ Standard vs µmoles Tyrosine.

Sample Determination:

$$r A_{660\text{nm}} \text{ Sample} = A_{660\text{nm}} \text{ Test} - A_{660\text{nm}} \text{ Test Blank}$$

Determine the µmoles of Tyrosine equivalents liberated using the Standard curve.

$$(\mu\text{mole Tyrosine equivalents released}) (16)$$

$$\text{Units/ml enzyme} = \frac{\text{---}}{(1)(10)(2)}$$

16 = Total volume (in milliliters) of stopped reaction

2 = Volume (in milliliters) used in
Colorimetric Determination

1 = Volume (in milliliters) of enzyme used

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

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

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UNIT DEFINITION:

One unit will hydrolyze hemoglobin to produce color equivalent to 1.0 μ mole (181 μ g) of tyrosine per minute at pH 2.8 at 37°C (color by Folin & Ciocalteu's reagent).

FINAL ASSAY CONCENTRATION:

In a 6.00 ml reaction mix, the final concentrations are 8.3 mM potassium phthalate, 2.1% (w/v) hemoglobin, and 0.5 - 1.0 unit protease.

REFERENCE:

Yoshida, F. (1956) *Bull. Agr. Chem. Soc. Japan* **20**, 252-256.

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.