Enzymatic Assay of PROTEASE[1]
Casein as a Substrate

PRINCIPLE:

Casein + H_2O $\xrightarrow{\text{Protease}}$ Amino Acids

CONDITIONS: T = 37°C, pH = 7.5, A_{660nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

A. 50 mM Potassium Phosphate buffer, pH 7.5 at 37EC.
(Prepare 200 ml in deionized water using Potassium Phosphate, Dibasic, Trihydrate, Sigma Prod. No. P-5504. Adjust to pH 7.5 at 37 °C with 1 M HCl.)

B. 0.65% (w/v) Casein Solution (Casein)
(Prepare 125 ml in Reagent A using Casein, Sigma Prod. No. C-7078. Heat gently (do not boil) to 80-90 °C for 10 minutes with stirring. Adjust the pH to 7.5 at 37 °C, if necessary, with either 1 M NaOH or 1 M HCl.)

C. 110 mM Trichloroacetic Acid Reagent (TCA)
(Dilute 9 ml of Trichloroacetic Acid, 6.1 N, 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 (Na_2CO_3)
(Prepare 500 ml in deionized water using Sodium Carbonate Anhydrous, Sigma Prod. No. S-2127.)

F. 10 mM Sodium Acetate Buffer with 5 mM Calcium Acetate, pH 7.5 at 37 °C (Enzyme Diluent)
(Prepare 100 ml in deionized water using Sodium Acetate, Trihydrate, Sigma Prod. No. S!8625, and Calcium Acetate, Sigma Prod. No. C-1000. Adjust the pH to 7.5 at 37 °C with 0.1 M Acetic acid or 0.1 M NaOH.)

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REAGENTS: (continued)

G. 1.1 mM L-Tyrosine Standard (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.)

H. Protease Enzyme Solution
(Immediately before use, prepare a solution containing 0.1 - 0.2 unit/ml of Protease in cold Reagent F.)

PROCEDURE:

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

	Test	Blank
Reagent B (Casein)	5.00	5.00

Equilibrate to 37 °C. Then add:

Reagent H (Enzyme Solution)	1.00	------

Mix by swirling and incubate at 37 °C for exactly 10 minutes. Then add:

Reagent C (TCA)	5.00	5.00
Reagent H (Enzyme Solution)	------	1.00

Mix by swirling and incubate at 37 °7C for about 30 minutes. Filter through Whatman #50 filter paper or a 0.45 µm filter and use the filtrate in color development.

COLOR DEVELOPMENT:

Standard Curve:

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

	Std 1	Std 2	Std 3	Std 4	Std Blank
Reagent G(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_2CO_3)	5.00	5.00	5.00	5.00	5.00
Reagent D (F-C)	1.00	1.00	1.00	1.00	1.00

COLOR DEVELOPMENT: (continued)

Sample:

Pipette the following reagents into 4 dram vials (in milliliters):

	Test	Blank
Test Filtrate	2.00	------
Blank Filtrate	------	2.00
Reagent E (Na_2CO_3)	5.00	5.00
Reagent D (F-C)	1.00	1.00

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

CALCULATIONS:

Standard Curve:

ΔA_{660nm} Standard = A_{660nm} Standard - A_{660nm} Standard Blank

Plot the ΔA_{660nm} Standard vs µmoles of Tyrosine.

Sample Determination:

ΔA_{660nm} Sample = A_{660nm} Test - A_{660nm} Sample Blank

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

$$\text{Units/ml enzyme} = \frac{(\mu\text{mole Tyrosine equivalents released})\ (11)}{(1)\ (10)\ (2)}$$

11 = Total volume (in milliliters) of assay
10 = Time of assay (in minutes) as per the Unit Definition
1 = Volume of enzyme (in milliliter) of enzyme used
2 = Volume (in milliliters) used in Colorimetric Determination

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

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CALCULATIONS: (continued)

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

UNIT DEFINITION:

One unit will hydrolyze casein to produce color equivalent to 1.0 µmole (181 µg) of tyrosine per minute at pH 7.5 at 37 °C (color by Folin & Ciocalteu's reagent).

FINAL ASSAY CONCENTRATION:

In a 6.00 ml reaction mix, the final concentrations are 42 mM potassium phosphate, 0.54% (w/v) casein, 1.7 mM sodium acetate, 0.8 mM calcium acetate, and 0.1 - 0.2 unit protease.

REFERENCES:

Anson, M.L., (1938) *J. Gen. Physiol.* **22**, 79-89

Folin, O., and Ciocalteu, V., (1929) *J. Biol. Chem.* **73**, 627

NOTES:

1. This assay procedure is to be used to assay Protease, Sigma Prod. Nos.: P-4630, P-4755, P-0384, P-5380, P-7431, P-6141, P-1512, P-9040, P-5147, P-5647, P-8775, P-7026, P-4032, P-8038, P-8298, P-2789, P-4789, P-6670, P-3910, and P-4806.

2. This assay is based on the cited references.

3. 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.