

**Enzymatic Assay of PROTEASE INHIBITOR COCKTAIL
Casein as a Substrate**

PRINCIPLE:

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

This reaction is inhibited by the protease inhibitor cocktail.

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 37°C.
(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) until a homogenous dispersion is obtained. 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 (Na₂CO₃)
(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 10 mg/ml of Pancreatin, Sigma Prod. No. P-1500, in cold Reagent F.)
- I. Protease Inhibitor Cocktail (Inhibitor)
(Prepare a 10 X stock, solution of the Protease Inhibitor Cocktail by adding 10 ml of deionized water to a 100 ml bottle of lyophilized cocktail.)

PROCEDURE:

Protease Inhibitor Complex:

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

	<u>Unhib.</u> <u>Test</u>	<u>Inhib.</u> <u>Test</u>	<u>Blank</u>
Reagent A (Buffer)	2.00	1.80	1.80
Reagent I (Inhibitor)	-----	0.20	0.20
Reagent H (Protease)	0.10	0.10	-----
Reagent F (Enzyme Diluent)	-----	-----	0.10

Mix by inversion and incubate at room temperature for one hour.

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

Reagent B (Casein)	5.00	5.00	5.00
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Equilibrate to 37°C. Then add:

Protease Inhibitor Complex	1.00	1.00	-----
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Mix by swirling and incubate at 37°C for exactly 10 minutes. Then add:

Reagent C (TCA)	5.00	5.00	5.00
Protease Inhibitor Complex	-----	-----	1.00

Mix by swirling and incubate at 37°C for exactly 30 minutes.

Filter through Whatman #50 filter paper and use the filtrate in color development.

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COLOR DEVELOPMENT:

Standard Curve:

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

	<u>Std 1</u>	<u>Std 2</u>	<u>Std 3</u>	<u>Std 4</u>	
	<u>Blank</u>				
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 ₂ CO 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

Sample:

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

	<u>Inhib.</u>	<u>Unhib.</u>	<u>Blank</u>
	<u>Test</u>	<u>Test</u>	
Inhib. Test Filtrate	2.00	-----	-----
Unhib. Test Filtrate	-----	2.00	2.00
Blank Filtrate	-----	-----	2.00
Reagent E (Na ₂ CO ₃)	5.00	5.00	5.00
Reagent D (F-C)	1.00	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. 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:

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

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

Sample Determination:

$$r_{A_{660nm} \text{ Sample}} = A_{660nm} \text{ Uninhib. Test} - A_{660nm} \text{ Sample Blank}$$

$$r_{A_{660nm} \text{ Sample}} = A_{660nm} \text{ Inhib. Test} - A_{660nm} \text{ Sample Blank}$$

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

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CALCULATIONS: (continued)

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

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

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

Inhibition of Pancreatin =

$$\frac{\text{Uninhib.Units/mg S of pancreatin} - \text{Inhib. units/mg S of Pancreatin}}{\text{Uninhibited units/mg S of Pancreatin}}$$

S = Solid

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 and 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 0.48 mg pancreatin.

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 is based on the cited references.

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NOTES: (continued)

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.