

**Enzymatic Assay of PROTEASE INHIBITOR, of
Calcium Activated Neutral Protease**

PRINCIPLE:

N,N-Dimethylated Casein + H₂O ~~Protease, Calcium Activated Neutral~~ → Amino Acids

This reaction is inhibited by the protease inhibitor.

CONDITIONS: T = 30°C, pH = 7.5, A_{293nm}, Light path = 1 cm

METHOD: Stopped Spectrophotometric Rate Determination

REAGENTS:

- A. 200 mM Tris HCl Buffer with 1.5 mM β-Mercaptoethanol and 2.0 mM Calcium Chloride, pH 7.5 at 30°C (Substrate Buffer)
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503, 2-Mercaptoethanol, Sigma Prod. No. M-6250, and Calcium Chloride, Dihydrate, Sigma Prod. No. C-3881. Adjust to pH 7.5 at 30°C with 1 M HCl.)
- B. 0.5% (w/v) Casein, N,N-Dimethylated Solution (Casein)
(Prepare 15 ml in Reagent A using Casein, N,N-Dimethylated, Sigma Prod. No. C-9801.)
- C. 200 mM Tris HCl Buffer, pH 7.5 at 30°C (Protease Buffer)
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 7.5 at 30°C with 1 M HCl.)
- D. 100 mM Ethylenediaminetetraacetic Acid Solution (EDTA)
(Prepare 2 ml in deionized water using Ethylenediaminetetraacetic Acid, Tetrasodium Salt, Hydrate, Sigma Prod. No. ED4SS.)
- E. 6.1 N Trichloroacetic Acid Solution (TCA)
(Use Trichloroacetic Acid, 6.1 N Solution, approximately 100% (w/v), Sigma Stock No. 490-10.)

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REAGENTS: (continued)

- F. Protease Solution (Protease)
(Immediately before use, prepare a solution containing 2.5 units/ml of Protease, Calcium Activated Neutral, Sigma Prod. No. P-4533, in cold Reagent C.)
- G. Protease Inhibitor Solution (Inhibitor)
(Immediately before use, prepare a solution containing 20 mg/ml of Protease Inhibitor in cold Reagent B.)

PROCEDURE:

Pipette (in milliliters) the following reagents into suitable containers, with the exception of Reagent F (Protease):

Tube Number	Reagent B (Casein)	Reagent G (Inhibitor)	Reagent D (EDTA)	Deionized Water	Reagent F (Protease)
1 (Blank)	1.00	-----	0.10	-----	0.50
1 (Control)	1.00	-----	-----	0.10	0.50
3 (Blank)	0.80	0.20	0.10	-----	0.50
4 (Test)	0.80	0.20	-----	0.10	0.50
5 (Blank)	0.60	0.40	0.10	-----	0.50
6 (Test)	0.60	0.40	-----	0.10	0.50
7 (Blank)	0.40	0.60	0.10	-----	0.50
8 (Test)	0.40	0.60	-----	0.10	0.50
9 (Blank)	0.20	0.80	0.10	-----	0.50
10 (Test)	0.20	0.80	-----	0.10	0.50
11 (Blank)	-----	1.00	0.10	-----	0.50
12 (Test)	-----	1.00	-----	0.10	0.50

Mix by inversion and then add 0.50 ml of Reagent F (Protease) to each tube. Mix by inversion and incubate at 30°C for exactly 30 minutes. Then add 0.20 ml of Reagent E (TCA) to each tube. Mix by inversion and allow the tubes to stand at room temperature for 10 minutes.

Centrifuge in a clinical centrifuge at maximum speed in order to clarify the samples. Remove the clear

supernatant from tubes 1 and 2 and record the absorbance at 280 nm using water as the blank. Save these supernatants.

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PROCEDURE: (continued)

Pipette 1.0 ml of each of the 12 clear, acidic supernatant into 0.4 ml of 5 N NaOH. Allow to stand for 5 minutes and clarify by centrifugation. Record the absorbance at 293 nm for each of the supernatants, using water as the Blank.

CALCULATIONS:

$$\text{Units of protease per assay} = \frac{(A_{280\text{nm}} \text{ Control} - A_{280\text{nm}} \text{ Blank No. 1})}{(0.5)(0.5)}$$

0.5 = Change in absorbance at $A_{280\text{nm}}$ per unit as per the Unit Definition

0.5 = Volume (in milliliter) of protease used

$$\% \text{ Inhibition} = \left[1 - \frac{(\Delta A_{293\text{nm}} \text{ Test})}{(\Delta A_{293\text{nm}} \text{ Control})} \right] \times 100$$

$$r \quad A_{293\text{nm}} \text{ Test} = A_{293\text{nm}} \text{ Test} - A_{293\text{nm}} \text{ Blank}$$

$$r \quad A_{293\text{nm}} \text{ Control} = A_{293\text{nm}} \text{ Control} - A_{293\text{nm}} \text{ Blank}$$

Plot the % inhibition versus mg of protease inhibitor per assay. Calculate from the graph the mg of protease inhibitor required for 50% inhibition.

Divide the mg of inhibitor required for 50% inhibition by the units of protease per assay. This is equivalent to the mg of inhibitor required to inhibit 1 unit of protease.

$$\text{Units/mg solid} = \frac{1}{(\text{mg inhibitor required to reduce the activity of 1 unit of protease by 50\%})}$$

UNIT DEFINITION:

One unit will reduce the activity of one unit of calcium-activated neutral protease (P4533) by 50% at pH 7.5 at 30°C. (Final volume = 1.8 ml, Light path = 1 cm)

FINAL ASSAY CONCENTRATION:

In a 1.60 ml reaction mix, the final concentrations are 188 mM Tris, 0.9 mM 2-mercaptoethanol, 1.3 mM calcium chloride, 0.3% (w/v) casein, N,N-dimethylated, 1.25 units protease and 4 - 20 mg of protease inhibitor.

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REFERENCE:

Goodwin, T.W., and Morton, R.A. (1946) *Biochemical Journal* **40**, 628-632

Melloni, E., Salamino F., Sparatore, B., Michetti, M., Pontremoli, S., and Horecker, B.L., (1984) *Archives of Biochemistry and Biophysics* **232**, 513-519

NOTES:

1. This assay is based on the cited references.
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.