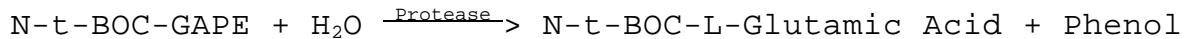


Enzymatic Assay of PROTEASE INSOLUBLE

PRINCIPLE:



Abbreviations used:

BOC = N-tert-Butoxy-Carbonyl

N-t-BOC-GAPE = N-t-BOC-L-Glutamic Acid α -Phenyl Ester

CONDITIONS: T = 37°C, pH = 7.8, A_{270nm}, Light path = 1 cm

METHOD: Spectrophotometric Determination

REAGENT:

- A. 200 mM Tris-Phosphate Buffer, pH 7.8 at 37°C
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 7.8 at 37°C with Phosphoric Acid, Sigma Prod. No. P-6560.)
- B. 60 mM N-t-BOC-L-Glutamic Acid α -Phenyl Ester Substrate Solution (N-t-BOC-GAPE)
(Prepare by dissolving 97 mg of N-t-BOC-L-Glutamic Acid α -Phenyl Ester, Sigma Prod. No. B-3016, in 5 ml of 1,4-Dioxane, Sigma Prod. No. D-9553. Make sure that the solid is at room temperature before opening. Any moisture in this substrate will cause immediate hydrolysis of the substrate. **PREPARE FRESH.**)
- C. Protease Insoluble Enzyme Suspension (Insol Enz)¹
Accurately weigh approximately 0.5 g of insoluble protease and suspend in 10 ml of deionized water in a conical centrifuge tube. Mix by swirling gently and allow to swell for 1 hour. Then centrifuge at 500 - 1000 g for 5 minutes. Measure the packed gel volume. Resuspend the gel and place 2 - 4 ml of suspension on a Whatman #50 filter paper and remove the suspension medium and soluble protease by vacuum filtration. Stop the vacuum filtration before the gel becomes dry or begins to crack. It should be moist. Wash the gel cake with approximately 10 volumes of deionized water, and again stop the vacuum filtration before the gel becomes dry or begins to crack. Resuspend 0.9 g of the washed gel cake with 1 ml of deionized water. Mix by swirling to form a homogenous slurry. Then dilute to 3 - 5 units/ml of protease in cold deionized water.

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PROCEDURE:

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

	<u>Test</u>	<u>Blank</u>
Reagent A (Buffer)	4.87	4.87
Reagent C (Insoluble Enzyme)	0.05	-----
Deionized Water	-----	0.05

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

Reagent B (N-t-BOC-GAPE)	0.083	0.083
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Immediately mix by stirring and incubate at 37°C for exactly 5 minutes. Stir continuously with a magnetic stirrer. Remove 1 ml from the reaction mix, and filter through a 0.45 µm syringe filter. Immediately transfer the supernatant into a suitable cuvette and record the absorbance at $A_{270\text{nm}}$ versus Reagent A using a suitable spectrophotometer.

CALCULATIONS:

$$\text{Units/ml suspension} = \frac{(A_{270\text{nm}} \text{ Test} - A_{270\text{nm}} \text{ Blank})(5.003)(\text{df})}{(5)(1)(1.5)(0.05)}$$

5.003 = Total volume (in milliliters) of assay

df = Dilution factor

5 = Time (in minutes) of assay

1 = Volume (in milliliters) removed from the reaction mixture

for absorbance measurement

1.5 = Millimolar extinction coefficient of phenol at 270 nm

0.05 = Volume (in milliliter) of enzyme used

$$\text{Units/gram solid} = \frac{(\text{units/ml suspension})(1000)}{(15.7)}$$

1000 = Conversion factor from mg to g

15.7 = mg dry agarose/ml suspension

$$\text{Units/ml packed gel} = \text{Units/ml suspension} \times 2$$

UNIT DEFINITION:

One unit will hydrolyze 1.0 μ mole of N-t-BOC-L-glutamic acid α -phenyl ester per minute at pH 7.8 at 37°C.

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FINAL ASSAY CONCENTRATION:

In a 5.003 ml reaction mix, the final concentrations are 195 mM Tris, 1 mM N-t-BOC-L-glutamic acid a-phenyl ester, and 0.15 - 0.25 units of protease insoluble.

REFERENCE:

Drapeau, G.R. (1976) *Methods in Enzymology* **45**, 469-475

NOTES:

1. Mix the enzyme suspension gently. Elevating the suspension surface along the sides of the vessel may result in the attachment of agarose beads to the walls of the vessel effectively reducing the concentration of the enzyme in the suspension. Whenever an insoluble enzyme suspension is sampled, it must be done while the enzyme suspension is stirring.
2. This assay is based on the cited reference.
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.