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SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of PROTEASE IMPURITY
using Fluorescein Isothiocyanate-Casein

PRINCIPLE:

FITC-Casein + H_2O $\xrightarrow{\text{Protease}}$ FITC + Casein

Abbreviations:
FITC-Casein = Fluorescein Isothiocyanate-Casein
FITC = Fluorescein Isothiocyanate

CONDITIONS:

Part A: T = 37°C, pH 7.6,
Part B: T = 25°C, pH 8.5, Excitation = 490 nm, Emission = 525 nm, Light path = 1 cm

METHOD: Fluorometric Stopped Reaction

REAGENTS:

A. 20 mM Sodium Phosphate Buffer with 150 mM Sodium Chloride, pH 7.6 at 37°C
(Prepare 200 ml in deionized water using Sodium Phosphate, Dibasic, Anhydrous, Sigma Prod. No. S-0876, and Sodium Chloride, Sigma Prod. No. S-9625. Adjust to pH 7.6 at 37°C with 1 M HCl.)

B. 0.5% (w/v) Fluorescein Isothiocyanate-Casein Solution (FITC-C)
(Prepare 1.0 ml in Reagent A using Casein-Fluorescein Isothiocyanate, Sigma Prod. No. C-3777. **KEEP FROM LIGHT!**)

C. Protease Enzyme Solution
(Immediately before use, prepare a solution containing 10 µg/ml in cold Reagent A using Protease Type VIII, Sigma Prod. No. P-5380.)

D. 10% (v/v) Trichloroacetic Acid Solution (TCA)
(Prepare 10 ml in deionized water using Trichloroacetic Acid, 6.1 N Solution, Sigma Stock No. 490-10.)

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REAGENTS:

E. 500 mM Tris HCl Solution, pH 8.5 at 25°C
(Prepare 500 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 8.5 at 25°C with 5 M HCl.)

F. 0.001% (w/v) Fluorescein Isothiocyanate Standard Solution (FITC)
(Prepare 1 ml in Reagent E using Fluorescein Isothiocyanate, Sigma Prod. No. F-7250. **KEEP FROM LIGHT!**)

G. Sample Solution (Sample)
(Prepare solution in cold deionized water as per the requested assignment.)

PROCEDURE:

Part A:

To prepare Protease Standards (PS), pipette (in milliliters) the following reagents into suitable containers:

	PS 1	PS 2	PS 3	PS 4
Reagent A (Buffer)	1.90	0.90	0.70	0.50
Reagent C (Protease)	0.10	0.10	0.30	0.50

Mix by swirling.

Pipette (in milliliters) the following reagents into suitable microcentrifuge tubes:

	Test		PS				
	Test	Blank	PS 1A	PS 2A	PS 3A	PS 4A	Blank
Reagent A (Buffer)	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Reagent B (FITC-C)	0.02	0.02	0.02	0.02	0.02	0.02	0.02

Equilibrate to 37°C. Then add:

Reagent G (Sample)	0.01	----	----	----	----	----	----
PS 1	----	----	0.01	----	----	----	----
PS 2	----	----	----	0.01	----	----	----
PS 3	----	----	----	----	0.01	----	----
PS 4	----	----	----	----	----	0.01	----

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PROCEDURE: (continued)

Immediately mix by swirling and incubate at 37°C for exactly 60 minutes. Then add:

	Test	PS					
	Test	Blank	PS 1A	PS 2A	PS 3A	PS 4A	Blank
Reagent D (TCA)	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Reagent G (Sample)	0.01	----	----	----	----	----	----
PS 4 (Protease Standard)	----	----	----	----	----	0.01	

Mix by swirling and incubate at 37°C for exactly 60 minutes. Centrifuge all tubes for 15 minutes.[1]

Part B:

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

Reagent E (Tris)	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Supernatant-Test	0.10	-----	-----	-----	-----	-----	-----
Supernatant-Test Blank	-----	0.10	-----	-----	-----	-----	-----
Supernatant-PS-1A	-----	-----	0.10	-----	-----	-----	-----
Supernatant-PS-2A	-----	-----	-----	0.10	-----	-----	-----
Supernatant-PS-3A	-----	-----	-----	-----	0.10	-----	-----
Supernatant-PS-4A	-----	-----	-----	-----	-----	0.10	-----
Supernatant-PS Blank	-----	-----	-----	-----	-----	-----	0.10

Mix by swirling and transfer the solutions to suitable cuvettes. Record the fluorescence intensity at the excitation wavelength of 490 nm and the emission wavelength of 525 nm for all the containers in a suitable fluorometer at 25°C.

Prepare the FITC (FS) standards by pipetting
(in milliliters) the following reagents into suitable containers:

	FS 1	FS 2	FS 3	FS 4	PS Blank
Reagent E (Tris)	10.00	10.00	10.00	10.00	10.00
Reagent F (FITC)	0.01	0.05	0.07	0.10	-----

Mix by swirling and transfer to suitable cuvettes. Record the fluorescence intensity at the excitation wavelength of 490 nm and the emission wavelength of 525 nm for all the containers in a suitable fluorometer at 25°C.

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CALCULATIONS:

F = Fluorescence at the excitation wavelength of 490 nm and the emission wavelength of 525 nm

ΔF_{FS} Standard = F_{FS} Standard - F_{FS} Blank

ΔF_{PS} Standard = F_{PS} Standard - F_{PS} Blank

SPECIFICATIONS:

The change in fluorescence for the sample must be less than the 10 mg fluorescein isothiocyanate (FITC) standard and the 5 ng protease standard. The detection limit for this assay is 2.56×10^{-5} µmoles FITC released per minute.

FINAL ASSAY CONCENTRATIONS:

In a 0.05 ml reaction mix, the final concentrations are 16 mM sodium phosphate, 120 mM sodium chloride and 0.2% (w/v) casein-fluorescein isothiocyanate. The units of protease will vary.

NOTES:

1. At this point, 0.10 ml of the supernatant from all samples and standards (FITC standard must be included) can be pipetted into 4 dram vials, wrapped in foil and placed in the cooler for up to a week before diluting with 10 ml of Reagent E (Tris) and read on the fluorometer.

2. Where Sigma Product or Stock numbers are specified, equivalent reagents may be substituted.