Enzymatic Assay of PROTEASE
Casein as a Substrate

**PRINCIPLE:**

Casein + H₂O Æ Amino Acids

**CONDITIONS:** T = 37°C, pH = 7.5, A₆₆₀nm, 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) 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 (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.)
Enzymatic Assay of PROTEASE
Casein as a Substrate

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):

<table>
<thead>
<tr>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent B (Casein)</td>
<td>5.00</td>
</tr>
</tbody>
</table>

Equilibrate to 37°C. Then add:

<table>
<thead>
<tr>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent H (Enzyme Solution)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent C (TCA)</td>
<td>5.00</td>
</tr>
<tr>
<td>Reagent H (Enzyme Solution)</td>
<td>------</td>
</tr>
</tbody>
</table>

Mix by swirling and incubate at 37°C 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):

<table>
<thead>
<tr>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent G (Std Soln)</td>
<td>0.05</td>
<td>0.10</td>
<td>0.20</td>
<td>0.40</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>1.95</td>
<td>1.90</td>
<td>1.80</td>
<td>1.60</td>
</tr>
<tr>
<td>Reagent E (Na2CO3)</td>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Reagent D (F-C)</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Enzymatic Assay of PROTEASE
Casein as a Substrate

COLOR DEVELOPMENT: (continued)

Sample:

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

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Filtrate</td>
<td>2.00</td>
<td>------</td>
</tr>
<tr>
<td>Blank Filtrate</td>
<td>------</td>
<td>2.00</td>
</tr>
<tr>
<td>Reagent E (Na₂CO₃)</td>
<td>5.00</td>
<td>5.00</td>
</tr>
<tr>
<td>Reagent D (F-C)</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

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:

\[ \Delta A_{660nm} \text{ Standard} = A_{660nm} \text{ Standard} - A_{660nm} \text{ Standard Blank} \]

Plot the \( \Delta A_{660nm} \) Standard vs µmoles of Tyrosine.

Sample Determination:

\[ \Delta A_{660nm} \text{ Sample} = A_{660nm} \text{ Test} - A_{660nm} \text{ Sample Blank} \]

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

\[ \text{Units/ml enzyme} = \frac{\text{µmole Tyrosine equivalents released}}{(1) \times (10) \times (2)} \]

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

\[ \text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}} \]
Enzymatic Assay of PROTEASE
Casein as a Substrate

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 \( \mu \)mole (181 \( \mu \)g) 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:


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, P 5459 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.

Sigma warrants that the above procedure information is currently utilized at Sigma and that Sigma products conform to the information in Sigma publications. Purchaser must determine the suitability of the information and products for its particular use. Upon purchase of Sigma products, see reverse side of invoice or packing slip for additional terms and conditions of sale.