Enzymatic Assay of PROTEASE
Casein as a Substrate

PRINCIPLE:

Casein + H₂O $\xrightarrow{\text{Protease}}$ 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. 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. 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), to 500 ml with deionized water.)

D. Folin & Ciocalteu's Phenol Reagent (F-C)
   (Dilute 10 ml of Folin & Ciocalteu's Phenol Reagent, to 40 ml with deionized water.)

E. 500 mM Sodium Carbonate Solution (Na₂CO₃)
   (Prepare 500 ml in deionized water using Sodium Carbonate Anhydrous.)

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, and Calcium Acetate. 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.
Heating 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>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>
**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 (Na$_2$CO$_3$)</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 \( \Delta A_{660nm} \text{ Standard} \) 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.

\[ \text{Units/ml enzyme} = \frac{(\text{µmole Tyrosine equivalents released}) \ (11)}{(1) \ (10) \ (2)} \]

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

\[ \text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}} \]
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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 µmole (181 µg) 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.

2. This assay is based on the cited references.

3. Where our Product or Stock numbers are specified, equivalent reagents may be substituted.

This procedure is for informational purposes. For a current copy of our quality control procedure contact our Technical Service Department.