SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of CASEINASE
(Collagenase Products)

PRINCIPLE:

\[
\text{Casein} + \text{H}_2\text{O} \xrightarrow{\text{Protease}} \text{Amino Acids}
\]

CONDITIONS: \( T = 37^\circ C, \text{pH} = 7.5, A_{660\text{nm}}, \text{Light path} = 1 \text{ cm} \)

METHOD: Colorimetric

REAGENTS:

A. 50 mM Sodium Phosphate Buffer, pH 7.5 at 37°C.
   (Prepare 200 ml in deionized water using Sodium Phosphate, Dibasic, Anhydrous, Sigma Prod. No. S-0876. 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 to 80 - 85°C (do not boil) until a homogenous dispersion is obtained. Allow the solution to cool to 37°C. Adjust the pH to 7.5 at 37°C with 0.1 M HCl or 0.1 M NaOH, if necessary.)

C. 6.1 N Trichloroacetic Acid Reagent (TCA)
   (Use Trichloroacetic Acid, 6.1 N Solution, approximately 100% (w/v), Sigma Stock No. 490-10.)

D. Folin & Ciocalteu's Phenol Reagent (F-C)
   (Dilute 10 ml of Folin & Ciocalteu's Phenol Reagent, 2.0 N, Sigma Prod. No. F-9252, to 40 ml with deionized water.)

E. 500 mM Sodium Carbonate Solution (Na$_2$CO$_3$)
   (Prepare 500 ml in deionized water using Sodium Carbonate, Anhydrous, Sigma Prod. No. S-2127.)

F. 50 mM TES Buffer with 0.36 mM Calcium Chloride, pH 7.4 at 37°C (Enzyme Diluent)
   (Prepare 100 ml in deionized water using TES Free Acid, Sigma Prod. No. T-1375, and Calcium Chloride, Dihydrate, Sigma Prod. No. C-3881. Adjust the pH to 7.4 at 37°C with 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, Sigma Prod. No. T-3754. Heat gently until tyrosine dissolves and cool to room temperature.)

H.  Collagenase Enzyme Solution
(Immediately before use, prepare a solution containing 0.05 - 0.10 mg/ml of Collagenase in cold Reagent F.)

PROCEDURE:

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

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

Equilibrate to 37°C. Then add:

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

Mix by inversion and incubate at 37°C for exactly 30 minutes. Then add:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent C (TCA)</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Reagent H (Enzyme Solution)</td>
<td>-----</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Filter through Whatman #50 filter paper or 0.8 µm syringe filters and use the filtrate in the color development.

COLOR DEVELOPMENT:

Standard Curve:

Prepare a standard curve by pipetting (in milliliters) the following reagents into suitable vials:

<table>
<thead>
<tr>
<th></th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std 5</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>
<td>0.50</td>
<td>-----</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>1.95</td>
<td>1.90</td>
<td>1.80</td>
<td>1.60</td>
<td>1.50</td>
<td>2.00</td>
</tr>
<tr>
<td>Reagent E (Na₂CO₃)</td>
<td>5.00</td>
<td>5.00</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>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Mix vigorously by inversion.
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COLOR DEVELOPMENT: (continued)

Sample:

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

<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 vigorously by inversion. Incubate sample and standard vials at 37°C for 30 minutes. Remove and allow the vials to cool to room temperature. Transfer to suitable cuvettes. (If the solutions are hazy either centrifuge or filter through a 0.45 µm filter prior to determining the A₆₆₀nm.) Determine the A₆₆₀nm for the Test, Test Blank, Standards, and Standard Blank.

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 Tyrosine.

Sample Determination:

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

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

Units/ml enzyme = \[
\frac{(\mu\text{mole Tyrosine equivalents released}) \times 10 \times 6.5 \times \text{df}}{(1 \times 2)}
\]

10 = Time conversion from 30 minutes to 5 hours (Unit Definition)
6.5 = Total volume (in milliliters) of stopped reaction
df = Dilution factor
2 = Volume (in milliliters) of sample used in Colorimetric Assay
1 = Volume (in milliliters) of enzyme used
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CALCULATIONS: (continued)

\[
\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}
\]

UNIT DEFINITION:

One unit will hydrolyze casein to produce color equivalent to 1.0 \(\mu\)mole (181 \(\mu\)g) of tyrosine per 5 hours at pH 7.5 at 37°C (color by Folin & Ciocalteu reagent).

FINAL ASSAY CONCENTRATION:

In a 6.00 ml reaction mix, the final concentrations are 42 mM potassium phosphate, 0.54% (w/v) casein, 8.3 mM TES, 0.06 mM calcium chloride and 0.05 mg - 0.10 mg collagenase.

REFERENCES:


NOTES:

1. This assay is based on the cited references.

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

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