SIGMA QUALITY CONTROL TEST

Enzymatic Assay of TRYPsin INHIBITOR

PRINCIPLE:

Trypsin
BAEE + H₂O → Nα-Benzoyl-L-Arginine + Ethanol

Abbreviation used:
BAEE = Nα-Benzoyl-L-Arginine Ethyl Ester

CONDITIONS:  T = 25°C, pH = 7.6, A₂₅₃nm, Light path = 1 cm

METHOD:  Continuous Spectrophotometric Rate Determination

REAGENTS:

A.  67 mM Sodium Phosphate buffer, pH 7.6 at 25°C
(Prepare 100 ml in deionized water using Sodium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. S-0751. Adjust to pH 7.6 at 25°C with 1 M NaOH.)

B.  0.25 mM Nα-Benzoyl-L-Arginine Ethyl Ester Solution (BAEE)
(Prepare 50 ml in Reagent A using Nα-Benzoyl-L-Arginine Ethyl Ester, Hydrochloride, Sigma Prod. No. B-4500.)

C.  1 mM Hydrochloric Acid Solution (HCl)
(Prepare 50 ml in deionized water using concentrated Hydrochloric Acid, Sigma Prod. No. H-7020.)

D.  Trypsin Enzyme Solution (Trypsin)
(Immediately before use, prepare a solution containing 1 mg protein/ml of Trypsin, Prod. No. T-8003, in cold Reagent C.)

E.  Trypsin Inhibitor Solution (Inhib)
(Immediately before use, prepare a solution containing 1.0 mg/ml of Trypsin Inhibitor in cold Reagent A.²³)}
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PROCEDURE:

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

Part A:

<table>
<thead>
<tr>
<th></th>
<th>Inh</th>
<th>Test1</th>
<th>Test2</th>
<th>Test3</th>
<th>Test4</th>
<th>Test5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent E (Inhib)</td>
<td>---</td>
<td>0.10</td>
<td>0.15</td>
<td>0.20</td>
<td>0.25</td>
<td>0.30</td>
</tr>
<tr>
<td>Reagent D (Trypsin)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Allow to stand at 25°C for a minimum of five minutes and no longer than six minutes.

Mix by inversion and pipette (in milliliters) the following reagents into suitable cuvettes:

Part B:

<table>
<thead>
<tr>
<th></th>
<th>Inh</th>
<th>Test1</th>
<th>Test2</th>
<th>Test3</th>
<th>Test4</th>
<th>Test5</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent B (BAEE)</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
<td>3.00</td>
</tr>
<tr>
<td>Reagent C (HCl)</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.10</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 25°C. Monitor the $A_{253\text{nm}}$ until constant, using a suitably thermostatted spectrophotometer. Then add:

Uninh (Part A) 0.10 --- --- --- --- --- ---
Test 1 (Part A) --- 0.10 --- --- --- --- ---
Test 2 (Part A) --- --- 0.10 --- --- --- ---
Test 3 (Part A) --- --- --- 0.10 --- --- ---
Test 4 (Part A) --- --- --- --- 0.10 --- ---
Test 5 (Part A) --- --- --- --- --- 0.10 ---

Immediately mix by inversion and record the increase in $A_{253\text{nm}}$ for approximately 5 minutes. Obtain the $\Delta A_{253\text{nm}}$/minute using the maximum linear rate for the Tests, Blank, and Uninhibited Solution.

CALCULATIONS:

Trypsin Activity in BAEE units/ml enzyme =

$$\frac{(\Delta A_{253\text{nm}}/\text{min Test} - \Delta A_{253\text{nm}}/\text{min Blank})(df)(10.0)}{(0.001)(0.10)(0.5)}$$

$df$ = Dilution factor

0.001 = The change in $A_{253\text{nm}}$/minute per unit of Trypsin at pH 7.6 at 25°C in a 3.2 ml reaction mix
0.10 = Volume (in milliliters) enzyme used (Part B)
10.0 = Total volume in milliliters of assay (Part A)
0.5 = Volume (in milliliters) of enzyme used (Part A)
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CALCULATIONS: (continued)

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

Plot the Trypsin activity (BAEE units/mg protein) vs ml of Trypsin Inhibitor/RM

\[
\text{Mg Trypsin Inhibitor} = (\text{ml of Trypsin Inhibitor})(\text{Conc. of Trypsin Inhibitor, mg/ml})
\]

\[
\text{Mg Trypsin Inhibited by 1 mg Trypsin Inhibitor} = \frac{\text{mg Trypsin/RM (normalizing factor)}}{\text{mg Trypsin Inhibitor (from plot)}}
\]

Normalizing Factor = (BAEE Units of Uninhibited Trypsin per mg solid/10,000 BAEE units of Trypsin per specification.)

UNIT DEFINITION:

One trypsin unit will produce a $\Delta A_{253\text{nm}}$ of 0.001 per minute with BAEE as substrate at pH 7.6 at 25°C in a reaction volume of 3.2 ml.

FINAL ASSAY CONCENTRATION:

In a 3.2 ml reaction mix, the final concentrations are 63 mM sodium phosphate, 0.23 mM BAEE, 0.002 mM HCl, 0.005 mg trypsin, and 0.0003 - 0.001 mg trypsin inhibitor.

NOTES:


2. When assaying Trypsin Inhibitor, Ovoinhibitor, Prod. No. T-1886, the diluent used is 200 mM sodium phosphate, monobasic, pH 7.6 at 25°C.

3. When assaying Trypsin Inhibitor, Type II-S, Sigma Prod. No. T-9128, prepare a solution containing 0.60 mg/ml of Trypsin Inhibitor in cold Reagent A.

4. The uninhibited Trypsin activity should be within 85% of the release value for activity. With a 11,700 to 13,005 Trypsin units/mg solid per label, the acceptable range for activity of the uninhibited Trypsin reaction should be 10,000 to 15,300 Trypsin units/mg solid. This range should also correspond to a corrected $\Delta A_{253\text{nm}}$/minute of 0.0545 to 0.0835. With this rate and an inhibition of 20% to 80% the $\Delta A_{253\text{nm}}$/minute should be above the spectrophotometric rate detection limit of 0.0020.
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NOTES: (continued)

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

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