Enzymatic Assay of ALLANTOINASE
(EC 3.5.2.5)

PRINCIPLE:
Allantoin + H₂O  Allantoinase → Allantoate
Allantoate + K₃Fe(CN)₆ + Phenylhydrazine HCl → Glyoxylate Colored Product

CONDITIONS:  T = 25°C, pH = 7.0, A₅₄₀nm, Light path = 1 cm

METHOD:  Colorimetric

REAGENTS:

A. 100 mM Tris HCl Buffer, pH 7.0 at 25°C
   (Prepare 100 ml in deionized water using Trizma Base, Adjust to pH 7.0 at 25°C with 1 M HCl.)

B. 33 mM Allantoin Solution, pH 7.0 at 25°C (Allantoin)
   (Prepare 50 ml in Reagent A using Allantoin, Adjust to pH 7.0 at 25°C with either 1 M HCl or 1 M NaOH.)

C. 69 mM Phenylhydrazine HCl Solution (PH)
   (Prepare 10 ml in deionized water using Phenylhydrazine Hydrochloride)

D. 25% (v/v) Hydrochloric Acid Solution (HCl)
   (Prepare 25 ml in deionized water using Hydrochloric Acid)

E. 152 mM Potassium Ferricyanide Solution (K₃Fe(CN)₆)
   (Prepare 10 ml in deionized water using Potassium Ferricyanide)

F. 0.1 mM Glyoxylic Acid Standard Solution (Std)
   (Prepare 5 ml in deionized water using Glyoxylic Acid, Sodium Salt, Monohydrate)

G. Allantoinase Enzyme Solution
   (Immediately before use, prepare a solution containing
0.05 – 0.20 unit/ml of Allantoinase in cold Reagent A.)
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PROCEDURE:

Step 1:

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

<table>
<thead>
<tr>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent B (Allantoin)</td>
<td>4.00</td>
</tr>
</tbody>
</table>

Equilibrate to 25°C. Then add:

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

Immediately mix by swirling and incubate at 25°C for exactly 5 minutes. Remove aliquots from the Test and Blank at 5, 10, and 15 minutes.

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

<table>
<thead>
<tr>
<th>Test</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Test 4</th>
<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 D (HCl)</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Reagent C (Ph)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
</tr>
<tr>
<td>Test Mixture</td>
<td>1.00</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td></td>
</tr>
<tr>
<td>Test Blank Mixture</td>
<td>----</td>
<td>1.00</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td></td>
</tr>
<tr>
<td>Reagent F (Std)</td>
<td>----</td>
<td>----</td>
<td>0.20</td>
<td>0.40</td>
<td>0.60</td>
<td>0.80</td>
<td>----</td>
<td>----</td>
<td></td>
</tr>
<tr>
<td>Deionized Water</td>
<td>----</td>
<td>----</td>
<td>0.80</td>
<td>0.60</td>
<td>0.40</td>
<td>0.20</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mix by swirling and boil for 2 minutes. Place on ice. Then add:

<table>
<thead>
<tr>
<th>Test</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Test 4</th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent D (HCl)</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>
<td>0.50</td>
<td></td>
</tr>
<tr>
<td>Reagent E (K₃Fe(CN)₆)</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Allow to incubate on ice for 20 minutes. Then add:

<table>
<thead>
<tr>
<th>Test</th>
<th>Test 1</th>
<th>Test 2</th>
<th>Test 3</th>
<th>Test 4</th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deionized Water</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td></td>
</tr>
</tbody>
</table>

Mix by swirling and transfer the solutions to suitable cuvettes. Record the A₅₄₀nm for the Tests, Test Blank, Standards, and Standard Blank using a suitable spectrophotometer.
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CALCULATIONS:  

Standard Curve: 

\[ r_{A_{540nm} \text{ Standard}} = A_{540nm} \text{ Standard} - A_{540nm} \text{ Standard Blank} \]  

Prepare a standard curve by plotting \( r_{A_{540nm} \text{ Standard}} \) vs µmoles of glyoxylic acid.  

Sample Determination: 

\[ r_{A_{540nm} \text{ Test}} = A_{540nm} \text{ Test} - A_{540nm} \text{ Test Blank} \]  

Determine the µmoles of glyoxylic acid liberated using the standard curve.  

\[
\text{Units/ml enzyme} = \frac{(\mu\text{moles of glyoxylic acid released})(5)(df)}{(1)(T)}
\]

\[ 5 = \text{Total volume (in milliliters) of reaction mix} \]  
\[ \text{df} = \text{Dilution factor} \]  
\[ 1 = \text{Volume (in milliliter) of enzyme used} \]  
\[ T = \text{Time (in minutes) of assay as per the Unit Definition} \]  

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

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

\[
\text{Units/g protein} = \text{units/mg protein} \times 1000
\]

UNIT DEFINITION:  

One unit will hydrolyze 1.0 µmole of allantoin to allantoate (measured as glyoxylate) per minute at pH 7.0 at 25°C.  

FINAL ASSAY CONCENTRATIONS:  

In a 5.00 ml reaction mix, the final concentrations are 27 mM allantoin, 100 mM Tris, and 0.05 - 0.20 unit allantoinase.
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REFERENCE:


NOTES:

1. Aliquots from the Test and Blank are to be removed at 5, 10, and 15 minutes time points. A standard curve of glyoxylic acid must be prepared for each time point, as the color intensity may vary with time.

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.