Enzymatic Assay of LACTATE OXIDASE

PRINCIPLE:

\[ \text{L-Lactate} + \text{O}_2 \overset{\text{LOX}}{\longrightarrow} \text{Pyruvate} + \text{H}_2\text{O}_2 \]

\[ 2\text{H}_2\text{O}_2 + 4\text{-AAP} + \text{DMA} \overset{\text{POD}}{\longrightarrow} \text{Quinonediimine dye} + \text{H}_2\text{O} \]

Abbreviations used:
LOX = L-Lactate Oxidase
4-AAP = Aminoantipyrine
DMA = N,N-Dimethylaniline
POD = Peroxidase

CONDITIONS:  \( T = 37^\circ\text{C}, \text{pH} = 6.5, A_{565\text{nm}}, \text{Light path} = 1 \text{ cm} \)

METHOD:  Spectrophotometric Stop Rate Determination

REAGENTS:

A. 200 mM 3,3-Dimethylglutaric Acid-NaOH Buffer, pH 6.5 at 37\text{EC} (DMGA)
(Prepare 5 ml in deionized water using 3,3-Dimethylglutaric Acid, Sigma Prod. No. D-4379.
Adjust to pH 6.5 at 37\text{EC} with 1 M NaOH.)

B. 15 mM 4-Aminoantipyrine Solution (4-AAP)
(Prepare 1 ml in deionized water using 4-Aminoantipyrine, Free Base, Sigma Prod.
No. A-4382.)

C. 500 mM \( \text{L}(+)\text{Lactic Acid} \), pH 6.5 at 37\text{EC} (Lactic Acid)
(Prepare 1 ml in deionized water using \( \text{L}(+)\text{Lactic Acid} \), Free Acid, Sigma Prod. No. L-1750.
Adjust to pH 6.5 with 1 M NaOH.)

D. Peroxidase Enzyme Solution (POD)
(Immediately before use, prepare a solution containing 50 Purpurogallin units/ml of Peroxidase
Type II from Horseradish, Sigma Prod. No. P-8250, in cold deionized water.)
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REAGENTS: (continued)

E. 10 mM Potassium Phosphate Buffer with 0.010 mM Flavin Adenine Dinucleotide (FAD), pH 7.0 at 37°C (Enzyme Diluent)
   (Prepare 50 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379, and Flavin Adenine Dinucleotide, Disodium Salt, Sigma Prod. No. F-6625. Adjust to pH 7.0 at 37°C with 1 M NaOH. PREPARE FRESH.)

G. 0.2% (v/v) N,N-Dimethylaniline Solution (DMA)
   (Prepare 10 ml in deionized water using N,N-Dimethylaniline, Sigma Prod. No. D-8509.)

H. 0.25% (w/v) Dodecylbenzenesulfonic Acid Solution (DBS)
   (Prepare 5 ml in deionized water using Dodecylbenzenesulfonic Acid, Sodium Salt, Sigma Prod. No. D-2525.)

I. Lactate Oxidase Enzyme Solution (LOX)
   (Immediately before use, prepare a solution containing 0.1 - 0.2 units/ml of Lactate Oxidase in cold Reagent E.)

PROCEDURE:

Prepare a reaction cocktail by pipetting (in milliliters) the following reagents into a suitable container.

| Reagent A (DMGA) | 2.00 |
| Reagent D (POD) | 1.00 |
| Reagent B (4-AAP) | 1.00 |
| Reagent C (Lactic Acid) | 1.00 |
| Deionized Water | 3.00 |

Mix by inversion and equilibrate to 37°C.

Pipette (in milliliters) the following reagents into a suitable cuvette:

<table>
<thead>
<tr>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction Cocktail</td>
<td>0.80</td>
</tr>
<tr>
<td>Reagent G (DMA)</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 37°C. Then add:

<table>
<thead>
<tr>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent I (LOX)</td>
<td>0.020</td>
</tr>
<tr>
<td>Reagent E (Enzyme Diluent)</td>
<td>-----</td>
</tr>
</tbody>
</table>
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PROCEDURE: (continued)

Immediately mix by inversion and incubate at 37°C for exactly 10 minutes. Then add:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent H (DBS)</td>
<td>2.00</td>
<td>2.00</td>
</tr>
</tbody>
</table>

Mix by inversion and record the $A_{565\text{nm}}$ for both the Test and Blank using a suitable spectrophotometer.

CALCULATIONS:

\[
\text{Units/mg enzyme} = \frac{(A_{565\text{nm Test}} - A_{565\text{nm Blank}})(3.02)(df)}{(35.33)(0.5)(10)(0.02)}
\]

3.02 = Total volume of assay
df = Dilution factor
35.33 = Millimolar extinction coefficient of Quinonedimine dye at 565 nm.
0.5 = Conversion factor based on one mole of H$_2$O$_2$ produces half a mole of Quinonedimine dye
10 = Time of assay (in minutes) as per unit definition
0.02 = Volume (in milliliter) of enzyme used

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

FINAL ASSAY CONCENTRATIONS:

In a 1.02 ml reaction mix, the final concentrations are 39 mM 3,3 dimethylglutaric acid, 5 units peroxidase, 1.5 mM 4-aminoantipyrine, 49 mM L(+)-lactic acid, 0.04% (v/v) N,N-dimethylaniline, 0.20 mM potassium phosphate, 0.20 µm FAD and 0.002 - 0.004 unit lactate oxidase.

UNIT DEFINITION:

One unit will oxidize 1.0 µmole of L-lactate to pyruvate and H$_2$O$_2$ per minute at pH 6.5 at 37°C.

REFERENCES:

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NOTES:

1. Unit Definition for Peroxidase: One unit will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C. This purpurogallin (20 seconds) unit is equivalent to approximately 18 μM units per minute at 25°C.

2. All products and stock numbers, unless otherwise indicated, are Sigma product and stock numbers.

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