Enzymatic Assay of PEPTIDASE¹

PRINCIPLE:

L-Leucine-β-Naphthylamide + H₂O → L-Leucine + β-Naphthylamine

β-Naphthylamine + NaNO₂ → Diazo Reagent

Diazo Reagent + N-(1-Naphthyl)ethylenediamine → Blue Azo complex

CONDITIONS:  T = 37°C, pH = 7.1, A₅₈₀nm, Light path = 1 cm

METHOD:  Colorimetric

REAGENTS:

A.  100 mM Potassium Phosphate Buffer, pH 7.1 at 37°C
    (Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sigma Prod. No. P-5379. Adjust to pH 7.1 at 37°C with 1 M KOH.)

B.  0.02% (w/v) L-Leucine β-Naphthylamide Solution (Leu-Nap)
    (Prepare 50 ml in Reagent A using L-Leucine β-Naphthylamide Hydrochloride, Sigma Prod. No. L-0376, or use Leucine Aminopeptidase Substrate, Sigma Stock No. 251-1.)

C.  2000 mM Hydrochloric Acid Solution (HCl)
    (Prepare 100 ml in deionized water using Hydrochloric Acid, Sigma Prod. No. H-7020, or Sigma Stock No. 251-2.)

D.  0.5% (w/v) Ammonium Sulfamate Solution (AS)
    (Prepare 150 ml in deionized water using Sigma Prod. No. A-4630 or use Ammonium Sulfamate, 0.5% (w/v) Solution, Sigma Stock No. 251-3.)

E.  0.2% (w/v) Sodium Nitrite Solution (Nit)
    (Prepare 10 ml in deionized water using Sodium Nitrite, Sigma Prod. No. S-2252, or use Sodium Nitrite Tablets, Sigma Stock No. 251-4. PREPARE FRESH.)
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REAGENTS: (continued)

F. 0.05% (w/v) N-(1-Naphthyl)ethylenediamine Solution (CLR)
(Prepare 100 ml in Reagent H using N-(1-Naphthyl)ethylenediamine Dihydrochloride, Sigma Prod. No. N-9125, or dissolve the contents of one bottle of N-(1-Naphthyl)ethylenediamine Dihydrochloride, Sigma Stock No. 251-5, in 110 ml of Ethanol (Reagent H).)

G. 0.1257 mM β-Naphthylamine Solution (Std)
(Prepare 10 ml in deionized water using β-Naphthylamine, Sigma Prod. No. N-8381 or use Leucine Aminopeptidase (LAP) Calibration Solution, Sigma Stock No. 251-10.)

H. 95% Ethanol (v/v)
(Use Ethanol, Nondenatured)

I. Peptidase Enzyme Solution
(Immediately before use, prepare a solution containing 1 - 3 units/ml in cold Reagent A.)

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 A (Buffer)</td>
<td>-----</td>
<td>0.50</td>
</tr>
<tr>
<td>Reagent B (Leu-Nap)</td>
<td>0.50</td>
<td>0.50</td>
</tr>
</tbody>
</table>

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

| Reagent I (Enzyme Solution) | 0.50 | ----- |

Incubate for exactly 60 minutes. Then add:

| Reagent C (HCl) | 0.50 | 0.50 |

COLORIMETRIC ASSAY:

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

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std 5</th>
<th>Std</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Soln</td>
<td>1.50</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
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<td></td>
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<tr>
<td>Blank Soln</td>
<td>----</td>
<td>1.50</td>
<td>-----</td>
<td>-----</td>
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<td></td>
<td>-----</td>
</tr>
<tr>
<td>Reagent C (HCl)</td>
<td>----</td>
<td>-----</td>
<td>1.40</td>
<td>1.30</td>
<td>1.10</td>
<td>0.90</td>
<td>0.70</td>
<td>1.50</td>
</tr>
<tr>
<td>Reagent G (Std)</td>
<td>----</td>
<td>-----</td>
<td>0.10</td>
<td>0.20</td>
<td>0.40</td>
<td>0.60</td>
<td>0.80</td>
<td>-----</td>
</tr>
</tbody>
</table>
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COLORIMETRIC ASSAY: (continued)

Mix by swirling. Then add:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
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<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std 5</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent E (Nit)</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>0.50</td>
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</tbody>
</table>

Mix by swirling and incubate at 25°C for 3 minutes. Then add:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std 5</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent D (AS)</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>
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<th>Std 4</th>
<th>Std 5</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent F (CLR)</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>2.00</td>
</tr>
</tbody>
</table>

Mix by swirling and incubate at 25°C for 45 minutes. Transfer solutions to suitable cuvettes and record the $A_{580\text{nm}}$ for the Test, Blanks and Standards.

CALCULATIONS:

Standard Curve:

$$\Delta A_{580\text{nm}} \text{ Standard} = A_{580\text{nm}} \text{ Std} - A_{580\text{nm}} \text{ Std Blank}$$

Prepare a standard curve by plotting $\Delta A_{580\text{nm}}$ standard vs $\mu$moles of $\beta$-Naphthylamine.

Sample Determination:

$$\Delta A_{580\text{nm}} \text{ Sample} = A_{580\text{nm}} \text{ Test} - A_{580\text{nm}} \text{ Blank}$$

Determine the $\mu$moles of $\beta$-Naphthylamine liberated using the standard curve.

$$\frac{\mu \text{moles of } \beta\text{-Naphthylamine liberated} \times (\text{df})}{(60) (0.5)}$$

$\text{df} = \text{Dilution factor}$

$60 = \text{Time of assay (in minutes) as per the unit definition.}$

$0.5 = \text{Volume (in milliliters) of enzyme used}$
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CALCULATIONS:  (continued)

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

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

UNIT DEFINITION:

One unit will liberate 1.0 µmole of β-naphthylamine from L-leucine β-naphthylamide per minute at pH 7.1 at 37°C.

FINAL ASSAY CONCENTRATIONS:

In a 1.00 ml reaction mix, the final concentrations are 100 mM potassium phosphate, 0.01% (w/v) L-leucine β-naphthylamide and 0.5 - 1.5 units peptidase.

REFERENCE:


NOTES:

1. Procedure can be replaced by using Leucine Aminopeptidase Kit, Sigma Stock No. 251-AW.

2. This assay is based on the cited reference.

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

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