Enzymatic Assay of Cathepsin B (EC 3.4.22.1)

PRINCIPLE:

\[ \text{Na-CBZ-Arg-Arg-7-Amido-4-Methylcoumarin} + \text{H}_2\text{O} \xrightarrow{\text{Cathepsin B}} \text{Arg-Arg} + 7\text{-AMC} \]

Abbreviations:

CBZ = Carbobenzoxy
Arg-Arg = Arginylarginine
7-AMC = 7-Amino-4-Methylcoumarin

CONDITIONS:  
\[ T = 40^\circ\text{C}, \quad \text{pH} = 6.0, \quad \text{Excitation} = 348 \text{ nm}, \]
\[ \text{Emission} = 440 \text{ nm}, \quad \text{Light path} = 1 \text{ cm} \]

METHOD:  
Fluorometric Rate Determination

REAGENTS:

A. 352 mM Potassium Phosphate Buffer with 48 mM Sodium Phosphate and 4.0 mM Ethylenediaminetetraacetic Acid, pH 6.0 at 40°C  
(Prepare 100 ml in deionized water using Potassium Phosphate, Monobasic, Anhydrous, Sodium Phosphate, Dibasic, Anhydrous, and Ethylenediaminetetraacetic Acid, Disodium salt, Dihydrate.  
Adjust to pH 6.0 at 40°C with 1 M HCl.)

B. 8.0 mM L-Cysteine HCl Solution, pH 6.0 at 40°C (L-Cys)  
(Prepare 50 ml in Reagent A using L-Cysteine Hydrochloride, Monohydrate.  
Adjust to pH 6.0 at 40°C with 1 M NaOH.  
PREPARE FRESH.)

C. 0.1% (v/v) Brij 35 Solution (Brij 35)  
(Prepare 100 ml in deionized water using Brij 35 Solution, 30% (w/v) solution.)
Reagents: (continued)

D. 0.02 mM Na-CBZ-Arg-Arg-7-Amido-4-Methylcoumarin
(Arg-Arg-7-AMC)
(Prepare by dissolving 1 mg of Na-CBZ-Arg-Arg-7-Amido-
4-Methylcoumarin, Hydrochloride, in 0.14 ml of Dimethyl
Sulfoxide Dilute to 70 ml with Reagent C.)

E. 5.0 µM 7-Amino-4-Methylcoumarin (7-AMC)
(Prepare by dissolving 1 mg of 7-Amino-4-Methylcoumarin,
in 1.0 ml of Dimethyl Sulfoxide.
Dilute to 5.0 µM
(approximately 1125 fold dilution) with Reagent C.)

F. Cathepsin B Enzyme Solution
(Immediately before use, prepare a solution containing
5 - 10 units Cathepsin B in cold Reagent C.)

Procedure:

Pipette (in milliliters) the following reagents into
suitable fluorometric cuvettes:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent B (L-Cys)</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Reagent C (Brij 35)</td>
<td>0.90</td>
<td>1.00</td>
</tr>
<tr>
<td>Reagent F (Enzyme Solution)</td>
<td>0.10</td>
<td>------</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 40°C. Monitor the
fluorescence intensity at the excitation wavelength of 348
nm and the emission wavelength of 440 nm until constant
using a suitably thermostatted fluorometer. Then add:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent D (Arg-Arg-7-AMC)</td>
<td>0.75</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Immediately mix by inversion and record the increase in
fluorescence intensity at the excitation wavelength of 348
nm and the emission wavelength of 440 nm for 5 minutes.
Obtain the intensity/5 minutes by using the maximum
linear rate for both the Test and Blank.
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(EC 3.4.22.1)

**PROCEDURE:** (continued)

Standard Curve:

Pipette (in milliliters) the following reagents into suitable quartz fluorometric cuvettes:

<table>
<thead>
<tr>
<th>Std</th>
<th>Std1</th>
<th>Std2</th>
<th>Std3</th>
<th>Std4</th>
<th>Std5</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent B (L-Cys)</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Reagent C (Brij 35)</td>
<td>1.55</td>
<td>1.35</td>
<td>1.15</td>
<td>0.95</td>
<td>0.75</td>
<td>1.75</td>
</tr>
<tr>
<td>Reagent E (7-AMC)</td>
<td>0.20</td>
<td>0.40</td>
<td>0.60</td>
<td>0.80</td>
<td>1.00</td>
<td>----</td>
</tr>
</tbody>
</table>

Mix by inversion and equilibrate to 40°C. Obtain the fluorescence intensity for each Standard and the Standard Blank at the excitation wavelength of 348 nm and the emission wavelength of 440 nm.

**CALCULATIONS:**

Standard Curve:

\[ r \text{ Intensity Standard} = \text{Intensity}_{\text{Std}} - \text{Intensity}_{\text{Std Blk}} \]

Construct a Standard curve by plotting the \( r \) Intensity of the Standards versus nanomoles of 7-Amino-4-Methylcoumarin.

Sample Determination:

Determine the nanomoles of 7-Amino-4-Methylcoumarin liberated using the standard curve.

\[ \text{nаномолей либерировано} = \frac{r \text{ Intensity}_{\text{Sample/5 min}} - r \text{ Intensity}_{\text{Blank/5 min}}}{\text{Intensity/nаномолей of 7-AMC}} \]

\[ (\text{nаномолей of 7-Amino-4-Methylcoumarin либерировано})(\text{df}) \]

Units/ml enzyme = \[ \frac{\text{nаномолей либерировано}}{(5)(1)} \]

\[ \text{df} = \text{Dilution factor} \]
\[ 5 = \text{Reaction time} \]
\[ l = \text{Volume (in milliliter) of enzyme used} \]

units/ml enzyme

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

One unit will liberate 1.0 nanomole of 7-amino-4-
methylcoumarin from Na-CBZ-L-arginyl-L-arginine 7-amido-4-
methylcoumarin per minute at pH 6.0 at 40°C.

FINAL ASSAY CONCENTRATION:

In a 2.50 ml reaction mix, the final concentrations are  
106 mM potassium phosphate, 14 mM sodium phosphate,  
1.2 mM ethylenediaminetetraacetic acid, 2.4 mM L-cysteine,  
0.07% (v/v) Brij 35, 0.006 mM Na-CBZ-Arg-Arg-7-amino-4-
methylcoumarin, 0.06% (v/v) dimethyl sulfoxide, and 0.05 -  
0.1 mg cathepsin B.

REFERENCES:

Enzymology, Volume 80, 535-538

NOTES:

1. This solution must be made fresh and used within 3  
hours of preparation.

2. This assay is a modification of the procedure cited in  
the reference.

This procedure is for informational purposes.