Enzymatic Assay of CATHEPSIN B  
(EC 3.4.22.1)

PRINCIPLE:

Na-CBZ-Arg-Arg-7-Amido-4-Methylcoumarin + H_2O \xrightarrow{Cathespin B} \text{Arg-Arg + 7-AMC}

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

CONDITIONS:  T = 40°C, pH = 6.0, Excitation = 348 nm, 
Emission = 440 nm, Light path = 1 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, Sigma Prod. No. P-5379, Sodium Phosphate, Dibasic, Anhydrous, Sigma Prod. No. S-0876, and Ethylenediaminetetraacetic Acid, Disodium salt, Dihydrate, Sigma Stock No. ED2SS. 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, Sigma Prod. No. C-7880. 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, Sigma Stock No. 430AG-6.)
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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, Sigma Prod. No. C- 
5429 in  
0.14 ml of Dimethyl Sulfoxide, Sigma Prod. No. D-5879.  
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, Sigma Prod. No. A-9891 in  
1.0 ml of Dimethyl Sulfoxide,  
Sigma Prod. No. D-5879. 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 r intensity/5 minutes by using the maximum  
linear rate for both the Test and Blank.
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PROCEDURE:  (continued)

Standard Curve:

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

<table>
<thead>
<tr>
<th></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.

\[ \text{niamoles liberated} = \frac{r \text{ Intensity}_{\text{Sample/5 min}} - r \text{ Intensity}_{\text{Blank/5 min}}}{\text{Intensity/\text{nanomoles of 7-AMC}}} \times \frac{\text{(nanomoles of 7-Amino-4-Methylcoumarin liberated)}}{(\text{df})} \]

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

\( df \) = Dilution factor  
5 = Reaction time  
1 = Volume (in milliliter) of enzyme used

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:


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.

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.