

Enzymatic Assay of CATHEPSIN B
(EC 3.4.22.1)

PRINCIPLE:

Na-CBZ-Arg-Arg-7-Amido-4-Methylcoumarin + H₂O $\xrightarrow{\text{Cathepsin B}}$ 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:

	<u>Test</u>	<u>Blank</u>
Reagent B (L-Cys)	0.75	0.75
Reagent C (Brij 35)	0.90	1.00
Reagent F (Enzyme Solution)	0.10	-----

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:

Reagent D (Arg-Arg-7-AMC)	0.75	0.75
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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:

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

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.

$$\text{nanmoles liberated} = \frac{r \text{ Intensity}_{\text{Sample}/5 \text{ min}} - r \text{ Intensity}_{\text{Blank}/5 \text{ min}}}{\text{Intensity/nanomoles of 7-AMC}}$$

$$\text{Units/ml enzyme} = \frac{(\text{nanomoles of 7-Amino-4-Methylcoumarin liberated})(df)}{(5)(1)}$$

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

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

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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:

Barrett, A. J. and Kirschke, H. (1981) *Methods in 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.
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.