Creative Enzymes

Enzymatic Assay of TRYPSIN INSOLUBLE
(E.C. 3.4.21.4)

PRINCIPLE:

BAEE + H_2O $\xrightarrow{\text{Trypsin}}$ Nα-Benzoyl-L-Arginine + Ethanol

Abbreviation used:
BAEE = Nα-Benzoyl-L-Arginine Ethyl Ester

CONDITIONS: T = 30°C, pH = 8.0

METHOD: Titrimetric

REAGENTS:

A. 50 mM Nα-Benzoyl-L-Arginine Ethyl Ester and 100 mM Calcium Chloride Substrate Solution, pH 8.0 at 30°C (Substrate)
(Prepare 125 ml in deionized water using Nα-Benzoyl-L-Arginine Ethyl Ester, Hydrochloride, and Calcium Chloride, Dihydrate. Adjust to
pH 8.0 at 30°C with 0.1 M NaOH.)

B. 20 mM Standardized Sodium Hydroxide Solution (NaOH)
(Prepare 100 ml in deionized water using Sodium Hydroxide, Anhydrous,
Standardize according to the ACS Reagent Procedure.[1])

C. Trypsin Insoluble Enzyme Solution
Prepare the trypsin insoluble enzyme solution as follows:

1. Add appropriate amount (ml) of suspension to a graduated (0.1 ml increment) micro-column or Buchner Funnel.

2. Wash sample with Milli-8 water under pressure. The amount of milli-q water used should be 50 X the volume of suspension used.

3. Resuspend the gel in an appropriate amount of water. If using a Buchner Funnel, transfer the suspension to a graduated micro-column. Once the gel is suspended in the micro-column, allow the excess water to drain from the column.

4. Determine the amount of packed gel present from the graduations on the column.

5. Resuspend the packed gel (in micro-column) with the appropriate enzyme diluent to the units required to perform the assay. If the volume required does not fit into the micro-column, perform subsequent dilutions by making the first dilution in the micro column, then transferring the suspension to a plastic container. Continue dilutions in plastic container until the appropriate units are reached. Results shall be reported as unit/ml packaged gel.

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PROCEDURE:

Using a suitable pH meter in conjunction with a magnetic stirrer, pipette (in milliliters) the following reagents into a suitable titration vessel:

	Test
Reagent A (Substrate)	20.00

Equilibrate to 30°C. Adjust to pH 8.0 at 30°C with 20 mM NaOH (do not allow pH to exceed 8.35). Readjust pH to 8.35. When pH reaches 8.0 begin timing. Run blank by adding 0.5 X u equivalents NaOH (X = estimated units of activity in test aliquot, Ideally 1). Time the minutes required to return to pH 8.0. Repeat until time is relatively constant between injections (<10% deviation)

Reagent C (Insol Enz)	0.100

(Aliquot should be adjusted to produce 1-2 units of activity.)

Readjust pH to approximately 8.35. When the pH reaches 8.0 begin timing. Maintain the pH of the reaction mix at 8.0 by the addition of small volumes of NaOH (0.5 equivalents NaOH per unit of activity in aliquot). Record the volumes of NaOH used to maintain the pH at 8.0 and the time required for the consumption of NaOH. Ten injections of NaOH should not exceed a total time of 5 minutes, and pH should not exceed 8.35 at anytime during the titration.[2]

CALCULATION:

$$\text{Units/ml enzyme solution} = \frac{(\text{NaOH})(\text{Molarity of NaOH})(1000)}{(T)(0.1)}$$

NaOH = Volume (in milliliters) of NaOH used
1000 = Conversion factor from millimoles to μmoles as per the Unit Definition
T = Time (in minutes) required for the pH to reach 8.0
0.1 = Volume (in milliliter) of insoluble enzyme added

Units/ml of Packed Gel = Units/ml enzyme solution x 2

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UNIT DEFINITION:

One unit will hydrolyze 1.0 μmole of BAEE per minute at pH 8.0 at 30°C.

INITIAL ASSAY CONCENTRATION:

In a 20.10 ml reaction mix, the initial concentrations are 50 mM Nα-benzoyl-L-arginine ethyl ester, 100 mM calcium chloride and 2-4 units trypsin insoluble enzyme.

REFERENCES:

(1993) *Reagent Chemicals ACS Specifications*, 8th ed., 95

Laskowski, M. (1955) *Methods in Enzymology*, Vol II, 26-36

NOTES:

1. The standardization of NaOH is described in (1993) Reagent Chemicals ACS Specifications.

2. Aliquots of Trypsin and normality of NaOH solution need to be adjusted to insure a pH of <8.35 at all times during titration. Total time of titration should be less than 5 minutes. If these conditions are not met pH induced hydrolysis will effect the rate determination.

   For Trypsin, agarose (T1763) having an activity of approximately 20 units/ml packed gel a 100 μl aliquot (of a 1 to 2 dilution of packed gel) requires 25 μl injections of 20 mM NaOH to maintain the above conditions. This translates into injections equaling 0.5 u equivalents of NaOH for every 1 unit of activity in the aliquot.

   The blank determination should be run using the same size injection and same normality of NaOH as used in test. The blank will not null out pH induced hydrolysis correctly unless this condition is met.

3. This assay is based on Laskowski, M. (1955).

4. Where our Product or Stock numbers are specified, equivalent reagents may be substituted.

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

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