Enzyme Development Corporation
505 8th Avenue, Suite 1500, New York, NY 10018
(212) 736-1580
E-mail: info@EnzymeDevelopment.com

Casein Digestion Unit Analytical method (CDU)

A. Purpose: This method is used to determine the amount of bromelain that will liberate one μg of tyrosine after one minute of digestion at 37°C from a standard casein substrate solution at pH 7.0

B. Equipment:

1. Volumetric Pipettes
2. Volumetric Flasks
3. 25 ml screw capped test tubes
4. Constant temperature water bath at 37° C ± 0.1 C
5. Disposable culture tubes
6. Long stemmed funnels
7. Whatman #1 Filter paper
8. Analytical Balance
9. Spectrophotometer set to read at 275 nm
10. pH Meter
11. Timer
12. Automatic Pipetters

C. Safety Precautions:

1. Utilize standard laboratory safety practices.
2. Acetic acid: Pipetting should be preformed in a fume hood.
3. Trichloroacetic acid: Gloves should be worn to avoid acid burns.

D. Reagent and Reagent preparation: Volumes may be adjusted depending on requirements.

1. Substrate: Prepare fresh daily.
   a. Dissolve and dilute 1.775 g of anhydrous disodium phosphate (Na_2HPO_4) with distilled water to 250 ml in a volumetric flask.
   b. Dispense 0.6 g (adjusted for dry weight by lot of reagent) of Hammarsten grade Casein into 80 ml of the above solution using a stir bar and stir plate.
   c. Cover the solution with tinfoil.
   d. Place the Casein substrate into a constant temperature water bath set to 55°C (or any constant temperature water bath with a range of 50° to 60°C such as a 4000mL beaker on a heated stir plate) using a submersible stir plate with constant gentle stirring of the Casein substrate for 15 minutes.
   e. Remove the beaker from the water bath and cool to room temperature in a cool water bath with constant gentle stirring.
   f. pH the Casein substrate solution to 7.0 with the 0.1 N HCL slowly to avoid destruction of the protein matrix.
   g. Dilute the Casein solution with distilled water to 100 ml in a volumetric flask.
2. Protein Precipitant (TCA) Stock solution:
   a. Prepare a solution containing 0.11 M CCl_3COOH, 0.22 M $CH_3COONa \bullet 3\ H_2O$, and 0.33 M CH_3COOH.
   b. Dissolve the following in distilled water and dilute to volume in a 500 ml volumetric Flask.
      9 g CCl_3COOH (Trichloracetic acid)
      14.95 g $CH_3COONa \bullet 3\ H_2O$ (Sodium Acetate)
      9.9 g CH_3COOH (Acetic Acid)
3. Enzyme Diluent: Prepare fresh daily
   a. Prepare a solution containing 0.03 M L-Cysteine HCl $\bullet$ H_2O and 0.006 M EDTA disodium $\bullet 2\ H_2O$ as follows:
   b. Dissolve the following in approximately 1800 ml of distilled water

10.6 g L-Cysteine HCl • H_2O
4.4 g EDTA

c. pH the solution to 4.5 with 1 N NaOH.
d. Dilute to 2000 ml with distilled water in a volumetric flask.

E. Procedure:

1. Enzyme solution:
   a. Weigh 500 mg of bromelain powder dilute to 100 ml in enzyme diluent
   b. Dilute the solution B fold to the proper concentration (40-50 units/ml).
   c. The solution must be tested within 30 minutes of preparation.
   Calculation of Dilution Volumes:
   Target CDU ÷ 55 = B

   Calculation of pipetting volume:
   100/500 x 100/10 x 100/B = ml in last dilution (round to nearest ml)

2. Enzyme Evaluation:
   a. Pipette 5 ml of the Casein Substrate solution into 3 labeled screw cap test tubes. (1A, 1B and 1C... for each sample number is recommended)
   b. Place the tubes in the 37° C water bath for a approximately of 10 minutes.
   c. Prepare Enzyme solutions to be measured: **Enzyme solutions are stable for 30 minutes.**
   d. At zero time, add 1.0 ml of the Enzyme Solution to each of the tubes (1A and 1B...etc.), vortex the tubes and immediately return them to the bath. Allow sufficient time between injections (One minute between injections is recommended.)
   e. Allow the tubes to incubate in the 37° C water bath for exactly 10 minutes.
   f. At exactly 10 minutes, add 5 ml of the TCA stopping reagent to the tubes (1A and 1B...etc)
   g. Vortex the tubes vigorously and return them to the water bath.
   h. To the third tube (1C...etc) add the TCA stopping reagent immediately followed by 1 ml of the original Enzyme solution to act as the sample blank.
   i. Allow all tubes to remain in the 37° C water bath for an additional 30 minutes.
   j. Remove tubes and allow them to cool to room temperature.
   k. Filter the contents of each tube twice through the same Whatman #1 Filter Paper.
   l. Use air to set the spectrophotometer to zero, record and print the absorbance of the clear filtrate from all three tubes.
3. Standard Tyrosine:
   a. Prepare a standard solution containing exactly 50µg/ml of L-Tyrosine in 0.1 N HCl. Read and record the absorbance of the standard at 275 nm (E_s) using distilled water for the blank.

F. **Calculation:**

1. Definition: A Casein Digestion Unit (CDU) is the amount of enzyme that will liberate one µg of tyrosine after one minute of digestion at 37°C from a standard casein substrate solution at pH 7.0.

$$\text{CDU/mg} = \frac{E_t - E_b}{E_s} \times \frac{50}{10} \times \frac{11}{} \times DF$$

E_t = Absorbance of enzyme sample tube
E_b = Absorbance of enzyme blank tube
E_s = Absorbance of standard Tyrosine
DF = Dilution Factor of enzyme solution in mg

2. Example Calculation
   a. Bromelain (target CDU=1200/mg) was diluted as follows:
      1) B = 1200/55 = 21.8
      ml in last dilution= 100/500 x 100/10 x 100/21.8 = 9.17 rounded to 9 mls
      2) 500 mg of Bromelain diluted as follows:
      500.00 mg/100 ml x 10ml/100ml x 9ml/100ml= 0.045mg/ml

Dilution Factor = 22.22

3) Final Calculation

$$CDU/mg = \frac{0.5073 - 0.1101}{0.377} \times \frac{50}{10} \times \frac{11}{10} \times 22.22 = 1288 \text{ CDU/mg}$$

E_t = 0.5073 (Absorbance of enzyme sample tube)
E_b = 0.1101 (Absorbance of enzyme blank tube)
E_s = 0.377 (Absorbance of standard Tyrosine)
DF = 22.22 (Dilution Factor of enzyme solution in mg)

G. Testing Parameters:

1. Enzyme preparations are diluted to a concentration of 40-50 units/ml.
2. A Reference Material is assayed with each run to ensure method accuracy.

H. References:

1. Dapeau, G.R.: *Methods in Enzymology* Vol XLV (Lorand, L., ed.) pg. 471. Academic Press, NewYork (1976)