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TYROSINE UNIT (TU) ANALYTICAL METHOD

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A. **Principle:** Proteolytic enzymes will hydrolyze a protein substrate with the formation of various degradation products that are amino acids using casein as a substrate, one of the amino acids found upon hydrolysis is L-Tyrosine. L-Tyrosine absorbs strongly at 280 nm and the concentration of Tyrosine as a function of optical density follows Beers' Law.

B. Equipment:

1. pH Meter
2. Constant Temperature Water Bath at 40.0° ± 0.1°C
3. Analytical Balance
4. Spectrophotometer with wavelength setting at 280nm
5. Volumetric Flasks
6. Volumetric Pipettes
7. Long stemmed funnels
8. 25 ml screw cap tubes
9. Disposable culture tubes
10. Timer
11. Whatman #1 filter paper
12. Automatic Pipetters

C. **Safety precautions:**

1. Utilize standard laboratory safety practices.
2. Trichloroacetic acid: Gloves should be worn to avoid acid burns

D. Reagents and Reagent Preparation: Substrate, Buffer and Stopping reagent must be prepared daily. (Volumes prepared may be adjusted as needed for testing.)

1. Casein Substrate:
   a. Place a 2000 to 4000 ml beaker on the heat/stir plate.
   b. Add water and bring to a boil.
   c. Dissolve and dilute 1.775 g of anhydrous disodium phosphate with distilled water to 250 ml in a volumetric flask.
   d. Dispense 2.5 g of Calbiochem Casein (or dry weight as determined by moisture determinations for each lot of reagent utilizing the general procedure for Loss on Drying) into 125 ml of the phosphate solution made above in an appropriate size beaker (400 ml suggested) using a stir bar and mixer.
   e. Cover the solution with tinfoil.

f. Place the Casein substrate into the Boiling water bath with constant gentle stirring for 30 minutes, making sure the water level does not reach the top of the beaker. (Weigh the Casein beaker down with another beaker filled with water to minimize movement caused by boiling.)
g. Remove the beaker from the boiling water bath and cool to room temperature in a cool water bath with constant gentle stirring.
h. Dissolve 1.05 g of Citric Acid Monohydrate in distilled water and dilute to 100 ml in a volumetric flask.
i. pH the Casein substrate (original pH should be about 7.2) solution to 6.0 with the Citric Acid Solution slowly to avoid destruction of the protein matrix. (Approximately 35 ml of the acid will be needed.)
j. Quantitatively transfer the casein to a 250 ml volumetric flask and dilute to volume with distilled water.

2. Cysteine-Versene Buffer - 2 or 4 L of the buffer may be needed based on the number of samples and the need for dilutions: To make 2 Liters:
   a. Place a 2000-ml or greater beaker with a stir bar on the magnetic mixer.
   b. Add approximately 1600-1700 ml of distilled water to the beaker.
   c. Quantitatively add:
      14.20 g Anhydrous Sodium Phosphate Dibasic
      12.20 g L-Cysteine
      28.00g EDTA
   d. Allow all ingredients to stir until dissolved.
   e. pH the Buffer solution (original pH should be about 5.7) to 6.0 with the 1 N NaOH (approximately 20 ml will be needed.)
   f. Quantitatively transfer the buffer to a 2000 ml volumetric flask and dilute to volume with distilled water.
   g. Repeat above if more buffer is necessary.
3. Stopping Solution: (TCA): Trichloroacetic acid (30%) - 18 ml is needed for each sample to be tested.
   a. Dissolve 30 g of Trichloroacetic Acid in distilled water, quantitatively transfer to a 100 ml volumetric flask and dilute to volume with distilled water.

E. **Procedure**
1. Enzyme Preparation:
   a. Dissolve an appropriate amount of enzyme preparation in Cysteine-Versene Buffer. Use the same buffer if serial dilutions are required. The dilution of the enzyme should be used within 30 minutes. The final dilution concentration should correspond to an absorbance of approximately 0.2600 and a concentration between 2.5 - 6.5 TU/ml.
   b. Calculating Enzyme preparation:

$$\text{gram weight of sample} = \frac{0.2600 \times (\text{tyrosine curve factor})}{\text{TU/g (Target)}}$$

2. Enzyme Evaluation: Each set of tests comprises 2 enzyme tests and 1 enzyme blank. Proceed as follows:

    a. Pipette 10.0 ml of the Casein substrate solution into 3 labeled 25 ml screw cap tubes, two for each enzyme test and one for the enzyme blank. (1A, 1B, and 1C... for each sample number is recommended)
    b. Equilibrate the tubes for about 10 minutes at 40° C.
    c. At zero time start a timer and add 4.0 ml enzyme solution to the first tube for the enzyme test. Close the tube and invert gently several times. Place in water bath at 40° C. Continue the enzyme addition at a sufficient interval (1 minute is recommended) to each tube except the enzyme blanks.
    d. After exactly 60 minutes rapidly pipette 6.0 ml of TCA solution to each enzyme preparation tube. Shake vigorously and return the tubes to the water bath for 30 minutes at 40°C to complete the coagulation of precipitated casein.
    e. To prepare enzyme blanks add 6.0 ml TCA solution to the Casein substrate solution followed by 4.0 ml of enzyme solution. Shake vigorously and return the tubes to the water bath for 30 minutes at 40°C to complete the coagulation of precipitated casein.
    f. At the end of the 30 minute period remove each tube from the water bath and allow to cool to room temperature.
    g. After cooling, filter through a Whatman #1 filter paper. (Tubes may be shaken prior to filtering to dislodge protein precipitant.) Refilter the filtrate through the same filter paper.
    h. Read the absorbencies of the filtrates in a 1- cm cuvette at 280 nm using air to set the spectrophotometer to zero. Correct the A_{280} value of each enzyme test by subtracting the reading of the respective enzyme blank.

3. Tyrosine curve (performed approximately every 3 months)
    a. Dissolve 100 mg of L-Tyrosine in 0.10 N hydrochloric acid (HCL) and dilute to 1 L in a volumetric flask.
    b. Prepare the following solutions using 0.10 N HCL as a diluent:

Final concentration	Dilution from Stock Tyrosine Solution
25.0µg/ml	25ml/100ml
50.0µg/ml	50ml/100ml
75.0µg/ml	75ml/100ml

    c. Determine the absorbance of the tyrosine solutions at 25.0, 50.0, 75.0 and 100.0 µg of tyrosine per ml spectrophotometrically at 280 nm in a 1-cm cell using 0.1N HCL to zero the instrument.
    d. Prepare a plot of absorbance versus tyrosine concentration.
    e. Determine the slope of the curve in terms of absorbance per µg of tyrosine. (The slope must be between 0.0064 and 0.0076)

F. Calculations:

1. Definition of Units: One unit of potency may be defined as that unit which, while acting on the specified casein substrate at the specified conditions, will produce one microgram of Tyrosine per minute.

2. The number of TU/g in a digestion mixture or in the amount of enzyme preparation contained therein is:

   O.D. x D.F./**4 x 20/60 x 1/slope** = TU/g

O.D.	=	Optical Density of test minus the Optical Density of the Blank tube
D.F.	=	Dilution factor of Enzyme solution (1/final concentration of enzyme)
4	=	Volume of Enzyme solution injected
20	=	Total Volume of Substrate, Enzyme and Stopping reagent
60	=	Hydrolysis duration in minutes
Slope	=	Slope obtained by tyrosine curve (see above)

   For ease of calculation a **Factor** (calculation of bolded formula above) can be calculated incorporating constant volumes and the slope obtained from the tyrosine curve. (Refer to the Tyrosine curve.)

3. The number of TU per gram of an enzyme preparation is the TU activity of the preparation.

   Thus: TU Activity = $\frac{A_{280} \text{ of filtrate x Tyrosine curve factor}}{\text{enzyme conc. (g/ml)}}$

Example:	O.D.	=	0.2600
	Factor	=	13.0
	Final conc.	=	0.00003600 (1/Dilution Factor)

   TU = 0.2600 x 13.0 / 0.00003600 = 94,000 TU/G

G. Testing Accuracy Parameters:

1. Range: Absorbency readings after correction for the blank between 0.150 and 0.700 may be used to find the approximate activity of unknown preparations. Final test, however, should be made in the range of 0.200 to 0.500.
2. Duplicate tests at different levels of enzyme should not vary by more than 3%.

H. Reference:

1. *A.S.B.C. PROCEEDINGS* p. 225-228, Some Physical and Chemical Properties of Commercial Chillproofing Compounds, Harold E. Weissler and Adan C. Garza

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