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# HEMOGLOBIN UNITS ON THE TYROSINE BASIS (HUT) ANALYTICAL METHOD

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**A. Principle:** This procedure is used to determine the proteolytic activity, expressed as hemoglobin units on the tyrosine basis (HUT), of preparations derived from *Aspergillus oryzae* var. and *Aspergillus niger* var., and it may be used to determine the activity of other proteases at pH 4.7. The test is based on the 30 minute enzymatic hydrolysis of a hemoglobin substrate at pH 4.7 and 40°C. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration. The quantity of solubilized hemoglobin in the filtrate is determined spectrophotometrically.

**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 275nm
5. Volumetric Flasks
6. Volumetric Pipettes
7. Long stemmed funnel
8. Disposable culture tubes
9. 25 ml screw cap tubes
10. Timer
11. Whatman #1 filter paper
12. Automatic Pipetters

**C. Safety Precautions:**

1. Utilize standard laboratory safety practices.
2. Acetic Acid – Pipetting should be performed in the Fume hood.
3. Trichloroacetic Acid – Gloves should be worn to avoid acid burns

**D. Reagents and Reagent Preparation: (Volumes prepared may be adjusted as needed for testing.)**

1. Sodium Acetate (2M) - Dissolve 54.4 g sodium acetate - $3H_2O$, reagent grade in approximately 150 ml of distilled water. Transfer the solution quantitatively to a 200 ml volumetric flask and dilute to volume with distilled water.
2. Acetic acid (1M) - Pipette 11.6 ml of glacial acetic acid into a 200 ml volumetric flask containing approximately150 ml of distilled water and dilute to volume with distilled water.
3. Acetate buffer (0.1M), pH 4.7 - Add 50.0 ml 2M Sodium Acetate and 100.0 ml 1M Acetic acid to a 2000 ml beaker containing approximately 1800 ml of distilled water and a stir bar. Using a pH meter, adjust the pH to 4.7 with 2M Sodium Acetate or 1M Acetic Acid. Transfer the solution quantitatively to a 2000 ml volumetric flask and dilute to volume with distilled water.
4. Sodium acetate (0.5M) – Pipette 25.0 ml 2M Sodium acetate into a 100 ml volumetric flask and dilute to volume with distilled water.
5. Hydrochloric Acid (HCL) (0.3N) – Pipette 30.0 ml 1.0 N HCL into a 100 ml volumetric flask and dilute to volume with distilled water.
6. Trichloroacetic acid solution (TCA) - Dissolve 35.0 g TCA in approximately 125 ml of distilled water. Transfer the solution quantitatively to a 250 ml volumetric flask and dilute to volume with distilled water.
7. Substrate: Hemoglobin solution
   a. Weight 5.0 g Hemoglobin substrate powder (SIGMA H-2625) into a 250 ml beaker containing a stir bar.
   b. Add 125 ml distilled water and mix with continuous stirring for 10 minutes.
   c. With continuous stirring and a pH meter, titrate the hemoglobin solution to pH 1.7 with 0.3 N HCL (consumption will be between 24 and 32 ml).

d. After 10 minutes titrate with 0.5 M sodium acetate to pH 4.7 (consumption will be between 24 and 32 ml).
e. Transfer the solution quantitatively to a 250 ml volumetric flask carefully to minimize foaming and dilute to volume with distilled water.
f. Prepare the substrate fresh daily, if necessary substrate may be stored refrigerated overnight.

**E. Procedure:**

1. Enzyme Preparation:
   a. Dissolve an appropriate amount of enzyme preparation, stirring for approximately 10 minutes, with 0.1M sodium acetate buffer, pH 4.7. Use the same buffer if serial dilutions are required. The final dilution of the enzyme should be used within 10 minutes. Solutions containing between 9 and 22 HUT/ml will give results within the preferred range of the method, i.e., from $A_{275}$ 0.200 to 0.500.
   b. Calculating Enzyme preparation:

$$\text{gram weight of sample} = \frac{0.38 \times (\text{tyrosine curve factor})}{\text{HUT/g (Target)}} \div 2$$

   c. Weigh an appropriate amount of enzyme in grams, and dilute to the desired concentration using serial dilutions. Generally powders should be weighed > 0.1g, and liquids should be weighed>1.0g for greater accuracy.
   Example: The sample has a theoretical activity of 222,000 HUT/g

$$\text{Sample concentration} = \frac{0.38 \times 88.4}{222{,}000\text{HUT/g}} \div 2 = 0.000076\text{g/ml (approximate)}$$

   Therefore, the following serial dilutions would be made,
   Dilution 1: ≈0.76g enzyme to 100 ml total volume (≈0.0076g/ml)
   Dilution 2: 10 ml of dilution to 100 ml total volume (0.1)
   Dilution 3: 10 ml of dilution to 100 ml total volume (0.1)
   The concentration is calculated (use exact recorded weight) as 0.0076g/ml (dil1) x 0.1 x 0.1 = 0.000076g/ml
   Perform the assay procedure within 30 minutes of dissolution of the Enzyme Preparation
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 hemoglobin 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) Additionally prepare 2 tubes to act as a substrate blank.
   b. Equilibrate the tubes for about 10 minutes at 40° C.
   c. Add 2.0 ml enzyme solution to the tube and start a timer (zero time) for the enzyme test. Close the tube and tap gently for 30 seconds against palm of hand to mix. 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 blank.
   d. After exactly 30 minutes, rapidly pipette 10 ml of TCA solution to each enzyme preparation tube. Shake vigorously for about 40 seconds and keep the mixture for 1 hour at room temperature. During this period, shake the tubes at least 5 times.
   e. To prepare the enzyme blanks add 10.0 ml TCA solution to the hemoglobin solution, followed by 2.0 ml of enzyme solution. Shake vigorously for about 40 seconds and keep the mixture for 1 hour at room temperature. To prepare the substrate blanks add 10.0 mL of TCA solution followed by 2.0 mL buffer and shake vigorously. Keep the tubes at room temperature for 1 hour. During this period, shake the tubes at least 5 times.
   f. At the end of the hour shake each tube vigorously and filter through a Whatman #1 filter paper. Re-filter again through the same filter paper.
   g. Read the absorbencies of the filtrates in a 1 cm cuvette at 275 nm using air to set the spectrophotometer to zero. Correct the $A_{275}$ value of each enzyme test by subtracting the reading of the respective enzyme blank.
3. Tyrosine curve (performed approximately every 6 months)
   a. Stock Tyrosine Solution 100μg/ml: Dissolve 100 mg of L-Tyrosine in 60 mL 0.10 N HCL and dilute with distilled water to 1 L in a volumetric flask.
   b. Prepare the following solutions using distilled water 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 |
| 100.0μg/ml | Stock Solution |

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 275 nm in a 1-cm cell using 0.006 N 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.
f. Multiply this value by 1.10 and record it as $A_{275}$ of 1.10 μg ty/ml. (A value of approximately 0.0084 should be obtained.)

**F. Calculations:**

1. Definition of Units: One HUT unit of proteolytic (protease) activity is defined as that amount of enzyme that produces, in 1 minute under the specified conditions, a hydrolysate whose absorbance at 275 nm is the same as that of a solution containing 1.10 μg/ml of tyrosine in 0.006 N HCL.

2. Calculation of the Tyrosine curve factor.

$$\textbf{Tyrosine curve factor} = \frac{\textbf{Total volume in ml}}{\textbf{A}_{275}\textbf{ of 1.10 μg ty/ml x Time in minutes}}$$

3. Calculate the activity (HUT/g) of the enzyme preparation as follows:

$$\text{HUT/g Activity} = \frac{A_{275}\text{ of filtrate x Tyrosine curve factor}}{\text{enzyme conc. (g/ml)}} \div 2$$

4. The substrate blank absorbance will be used as a reference to reflect the integrity of the assay and aid in evaluating reagent variability. Food Chemical Codex method uses the substrate blank to set the spectrophotometer to zero (blank) effectively subtracting its value from both the enzyme test absorbance and the enzyme blank absorbance automatically. EDC will set the spectrophotometer to zero (blank) with air and then determine the substrate blanks and enzyme blanks and enzyme tests absorbance. The substrate blank (average) would be subtracted from both the enzyme test prior to calculating the enzyme activity. Mathematically this step is equivalent to the evaluation of the enzyme activity as listed in Section E. Procedure: 2. g.

Proof:

a= enzyme reaction absorbance (Example: 0.5000)
b=enzyme blank absorbance (Example: 0.1400)
c=substrate blank absorbance (Example: 0.1385)
d=final calculated absorbance

Therefore:

(a-c) – (b-c) = d is equivalent to a-b = c
(0.5000 – 0.1385) – (0.1400 – 0.1385) =0.3600
0.5000– 0.1400=0.3600

**G. Testing Accuracy Parameters:**

1. Range: Absorbency readings 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 should not vary by more than 6%.
3. A standard enzyme preparation should be evaluated simultaneously with all other samples. The standard should not deviate from its predetermined value by more than ± 3.0%.

**H. Reference:** Current Revision of: Food Chemical Codex