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PROTEASE ANALYTICAL METHOD (PC)

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A. Purpose: This procedure is used to determine protease activity, expressed as PC units, of preparations derived from *Bacillus subtilis* var. and *Basillus licheniformis* var, and it may be used to determine the activity of other proteases at pH 7.0. The assay is based on a 30-min proteolytic hydrolysis of casein at 37° C. and pH 7.0. Unhydrolyzed casein is removed by filtration, and the solubilized casein is determined spectrophotometrically.

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. Spectrophotometer set to read at 275 nm
9. pH Meter
10. Analytical Balance
11. Automatic Pipetters
12. Timer

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 Daily
   a. Place a 2000 to 4000 ml beaker on the heat/stir plate.
   b. Add water (enough to cover approximately 2/3 of the substrate beaker) and bring to a boil.
   c. Dissolve and dilute 1.51g Tris and 2.0 ml of 1N HCL in about 125ml of distilled water in an appropriate size beaker (250ml or 400ml recommended).
   d. Dispense 1.75 g (dry weight) of Hammarsten grade Casein (as determined by moisture determinations for each lot of reagent) into the above solution using a bar and stir plate.
   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. pH the Casein substrate solution to 7.0 with the 0.2N HCL slowly to avoid destruction of the protein matrix. (Approximately 35 ml of the acid will be needed.)
   i. Dilute the Casein solution with distilled water to 250 ml in a volumetric flask.
2. Tris Buffer: Prepare Daily
   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 24.2 g Tris
   d. Stir until dissolved.
   e. pH the Buffer solution to 7.0 with the 1 N HCL (approximately 180 ml will be needed).
   f. Dilute the Buffer with distilled water to 2000 ml in a volumetric flask.

g. Repeat above if necessary.

3. Stopping Solution: (PC-TCA Stock Reagent)
   a. Dissolve 18.0 g trichloroacetic acid and 19.0 g sodium acetate ($3H_20$) in water.
   b. Add 20 g (20 ml) of glacial acetic acid.
   c. Dilute to one liter with distilled water.
   d. Stopping solution is stable at room temperature.

E. Procedure:

1. Enzyme Preparation:
   a. Accurately weigh a sample of the enzyme to be analyzed and dissolve it quantitatively in the Buffer solution. The final dilution concentration should correspond to an Absorbance of approximately 0.3800.
   b. Calculating Enzyme Preparation:

$$\text{Final concentration} = \frac{0.3800 \times \text{PC factor*}}{\text{Target PC/g} \times 2}$$

Example: final concentration of enzyme = 0.0000558
Target = 227,000/g PC
Factor* = 66.7

$$0.0000558 = \frac{0.3800 \times 66.7*}{227{,}000 \times 2}$$

*Previously determined by tyrosine curve

2. Enzyme Evaluation:
   a. Pipette 10 ml of the Casein Substrate solution into 3-labeled 25ml screw cap test tubes. (1A, 1B and 1C... for each sample number is recommended) Additionally prepare 2 tubes to be used as a Substrate blank.
   b. Place the tubes in the 37° C water bath for a minimum of 5 minutes.
   c. Prepare Enzyme solutions to be measured: **Enzyme solutions are stable for 30 minutes.**
   d. Add 2.0 ml of the Enzyme Solution to each of the first two 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 30 minutes.
   f. At 30 minutes, add 10 ml of the Trichloroacetic Acid stopping reagent to each timed tube (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 Trichloroacetic Acid stopping reagent immediately followed by 2 ml of the original enzyme solution to act as the sample blank.
   i. To the Substrate Blank tubes add 10 mL Trichloroacetic Acid followed by 2 mL of buffer and vortex.
   j. Allow all tubes to remain in the 37° C water bath for an additional 30 minutes.
   k. Remove tubes and allow them to cool to room temperature.
   l. Filter the contents of each tube twice through the same Whatman #1 Filter Paper.
   m. Use air to set the spectrophotometer to zero, record and print the absorbance of the clear filtrate from all three tubes.
3. Preparation of Standard Curve: (Performed approximately every 6 months)
   a. Dissolve 0.100 g of Tyrosine in 60 ml of 0.10 N Hydrochloric Acid (HCL) and dilute to volume with distilled water in a 1L volumetric flask. This stock solution contains 100 μg of tyrosine per ml.
   b. Prepare three more dilutions from the above stock solution using distilled water to contain 25.0, 40.0 and 75 μg of tyrosine per ml.
   c. Determine the absorbance of the Tyrosine solutions at 25, 40, and 75 micrograms in a Spectrophotometer at 275 nm in a 1-cm cell using 0.006 N HCl as a blank.
   d. Prepare a plot of absorbance versus tyrosine concentration.
   e. Calculate the slope from the data generated.
   f. Determine the value for 60 μg/ml from the graph and divide it by 40 to obtain the absorbance for 1.50 μg/ml.
   g. The value will be close to 0.0114.

h. Calculation of Factor:

$$\text{Factor} = \frac{22}{\text{A of 1.50 } \mu\text{g/ml tyr x 30}}$$

A = Absorbance
22 = Volume (ml)
30 = Time (minutes)

F. Calculations:

1. Units of activity: One unit of activity is the amount of enzyme which produces in one minute under the conditions of the test a hydrolyzate whose absorbance at 275 mμ is the same as the one of a tyrosine solution containing 1.5 μg tyrosine per 1 ml.

$$\text{PC/g} = \frac{\text{A x Factor*}}{\text{enzyme conc. (g/ml) x 2}}$$

A = Absorbance of test minus the absorbance of the Blank tube

*For ease of calculation the **Factor** is calculated incorporating constant volumes and the slope obtained from the tyrosine curve. Refer to the Tyrosine curve.

Example:
A = 0.3800
Factor = 66.7
Enzyme conc. = 0.00005580 g/ml

PC = 0.3800 x 66.7 / 0.00005580 x 2* = PC
*2.0 ml aliquot

2. 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 test absorbance. The substrate blank (average) would be subtracted from both the enzyme tests 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.m.

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: Absorbance reading after correction for the blank between 0.200 and 0.500 may be used.
2. Duplicate assays should not vary by more than 3%.
3. The Substrate blank will be used as a reference to the Enzyme blank to reflect the integrity of the assay.

H. References:

1. Current Revision of Food Chemical Codex.