Enzyme Development Corporation
(212) 736-1580 505 8th Avenue, 15th Fl., New York, NY 10018
E-mail: info@EnzymeDevelopment.com

PLANT PROTEOLYTIC ANALYTICAL METHOD (FCC-PU)

UNCONTROLLED COPY

A. Principle: This procedure is used to determine the proteolytic activity of papain, ficin, and bromelain. The assay is based on a 60-minute proteolytic hydrolysis of a casein substrate at pH 6.0 and 40°C. Unhydrolyzed substrate is precipitated with trichloroacetic acid and removed by filtration; solubilized casein is then measured 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 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: Volumes may be adjusted depending on requirements.

1. Casein Substrate: Prepare fresh daily.
   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 2.0 g (adjusted dry weight as determined by moisture determinations for each lot of reagent utilizing the general procedure for Loss on Drying) of Hammarsten-grade casein (United States Biochemical corp., Catalog No. 12840) or equivalent into 100 ml of the phosphate solution made above in an appropriate size beaker (250 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.
j. Quantitatively transfer the casein to a 200 ml volumetric flask and dilute to volume with distilled water.

2. Phosphate-Cysteine-EDTA Buffer Solution: Prepare fresh 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:
      14.20 g Anhydrous Sodium Phosphate Dibasic
      12.20 g L-Cysteine
      28.00 g 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 necessary.

3. Stopping Solution: (TCA): Trichloroacetic acid (30%) Prepare fresh daily. 9 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. Calculating Enzyme preparation:

$$\text{gram weight of sample} = \frac{244}{\text{PU/mg (Target)}} \times \frac{1\text{g}}{1000\text{mg}}$$

   b. Dissolve an appropriate amount of enzyme preparation in Phosphate-Cysteine-EDTA Buffer Solution. 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 between 0.2 and 0.5 for each 2 ml preparation.

2. Enzyme Evaluation: Each set of tests comprises 2 enzyme tests and 1 enzyme blank. Proceed as follows:
   a. Pipette 5.0 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 for each sample to be analyzed and 6 sets of 3 labeled tubes for the standard curve. (1A, 1B, and 1C... for each sample number is recommended).
   b. Prepare Stock Standard solution: Transfer 100.0 mg of USP Papain Reference Standard into a 50 ml beaker, dissolve in Phosphate-Cysteine-EDTA Buffer Solution, quantitatively transfer the solution to a 100 ml volumetric flask and dilute to volume with buffer.
      1) Dilute Stock Standard Solution for standard curve.
      2) Pipet 2, 3, 4, 5, 6 and 7 ml of Stock Standard Solution into a series of 100 ml volumetric flasks and dilute each to volume with Phosphate-Cysteine-EDTA Buffer solution and mix by inversion.
   c. Equilibrate the casein substrate tubes for about 15 minutes at 40° C.
   d. At zero time start a timer and add 2.0 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.
   e. After exactly 60 minutes rapidly pipette 3.0 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.
   f. To prepare enzyme blanks add 3.0 ml TCA solution to the Casein substrate solution followed by 2.0 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.
   g. At the end of the 30 minute period remove each tube from the water bath and allow to cool to room temperature.
   h. After cooling, filter through a medium porosity filter paper (Whatman #1 filter paper), discarding approximately the first 3.0 ml of filtrate. (Tubes may be shaken prior to filtering to dislodge protein precipitant).
   i. 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.

F. Calculations:

1. Definition of Units: papain unit (PU) is defined in this assay as that quantity of enzyme that liberates the equivalent of 1 microgram of tyrosine per hour under the conditions of the assay.

2. Prepare the standard curve by plotting the absorbances of filtrates (minus the blank absorbance) from the Diluted Standard solutions against the corresponding enzyme concentrations, in mg/ml. By interpolation from the standard curve, obtain the equivalent concentration of the filtrate from the Test solution.

$$\mathbf{PU/mg = (A \times C \times 10)/W}$$

A	=	Activity of the USP Papain Reference Standard in PU/mg
C	=	Concentration, in mg/ml, of reference standard extrapolated from the standard curve
W	=	Weight in mg of original enzyme preparation x 2 (2 ml injection)
10	=	Total Volume of Substrate, Enzyme and Stopping reagent

3. Example calculations:
   a. Stock Solution Concentration = 100.00 mg/100 ml (will vary with actual weight used) or 1mg/ml.
   b. Diluted standard calculation: Stock solution Concentration x volume of stock solution ÷ 100 ml dilution in volumetric flask x 2 ml injection volume ÷ 10 ml total volume in final test assay tube
      1) 1mg/ml x 2 ml ÷ 100 ml x 2 ml ÷ 10 ml = 0.004 mg/ml
      2) 1mg/ml x 3 ml ÷ 100 ml x 2 ml ÷ 10 ml = 0.006 mg/ml
      3) 1mg/ml x 4 ml ÷ 100 ml x 2 ml ÷ 10 ml = 0.008 mg/ml
      4) 1mg/ml x 5 ml ÷ 100 ml x 2 ml ÷ 10 ml = 0.010 mg/ml
      5) 1mg/ml x 6 ml ÷ 100 ml x 2 ml ÷ 10 ml = 0.012 mg/ml
      6) 1mg/ml x 7 ml ÷ 100 ml x 2 ml ÷ 10 ml = 0.014 mg/ml
   c. Enzyme activity calculation:
      1) Enzyme dilution in grams
         0.1308g/100ml x 10ml/100ml = 0.0001308g/ml
      2) Convert to mg:
         0.001308g/ml x 1000mg/1g= 0.1308mg/ml

$$\mathbf{PU/mg = (A \times C \times 10)/W}$$

6100	=	Activity of the USP Papain Reference Standard in PU/mg
0.0088	=	Concentration, in mg/ml, of reference standard extrapolated from the standard curve
0.2616	=	Weight in mg of original enzyme preparation x 2 (2 ml injection)
10	=	Total Volume of Substrate, Enzyme and Stopping reagent

$$\mathbf{PU/mg = (6100 \times 0.0088 \times 10)/0.2616 = 2052 PU/mg}$$

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 should not vary by more than 8 %.

H. Reference:

1. Current Revision of Food Chemical Codex

UNCONTROLLED COPY
July 2008