Assay method for Protease activity (Folin method pH 8.0)

Solutions

1) 0.2 mol/L Hydrochloric acid solution

Dissolve 18 mL of Hydrochloric acid in about 500 mL of water and dilute to 1000 mL with water.

2) 6 mol/L Acetic acid solution

Dilute 180.0 g of Acetic acid to 500 mL with water.

3) Sodium hydroxide solution

Dissolve 43.0 g of Sodium hydroxide in about 400 mL of water and cool. Dilute to 1000 mL with water.

4) 0.55 mol/L Sodium carbonate solution

Dissolve 116.58 g of Anhydrous sodium carbonate in about 1500 mL of water and dilute to 2000 mL with water.

5) 1 mg/mL Tyrosine stock solution

Dissolve 0.1000 g of L-Tyrosine in 0.2 mol/L Hydrochloric acid solution and dilute to 100 mL with 0.2 mol/L Hydrochloric acid solution.

6) Trichloroacetic acid solution

Dissolve 36.00 g of Trichloroacetic acid and Anhydrous sodium acetate in about 1600 mL of water. Add to 110 mL of 6 mol/L Acetic acid solution and dilute to 2000 mL with water.

7) 0.1 mol/L Potassium dihydrogen phosphate solution

Dissolve 27.21 g of Potassium dihydrogen phosphate in about 1000 mL of water and dilute to 2000 mL with water.

8) 0.1 mol/L Disodium hydrogen phosphate solution

Dissolve 28.39 g of Anhydrous disodium hydrogen phosphate about 1000 mL of water and dilute to 2000 mL with water.

9) 0.1 mol/L Phosphate buffer pH 8.0

Add 0.1 mol/L Potassium dihydrogen phosphate solution to 0.1 mol/L Disodium hydrogen phosphate solution until the pH stabilizes at 8.00

10) 0.02 mol/L Phosphate buffer pH 8.0

Mix 2 L of 0.1 mol/L Phosphate buffer pH 8.0 and 8 L of water.

11) 3 fold Folin & Ciocalteu's phenol reagent

Dilute 50 mL of Folin & Ciocalteu's phenol reagent to 150 mL with water.

12) 0.05 mol/L Disodium hydrogen phosphate solution

Dissolve 14.2 g of Anhydrous disodium hydrogen phosphate in about 1000 mL of water and dilute to 2000 mL with water.

13) Substrate solution

Dissolve 1.20 g of Hammarsten casein in 160 mL of 0.05 mol/L Disodium hydrogen phosphate solution while heating at 60 ℃ and stirring occasionally. After cooling, add Sodium hydroxide solution until the pH stabilizes at 8.00. Dilute to 200 mL with water.

Preparation of the sample solution

Dissolve the sample in an appropriate amount of water or a buffer solution.

PROCEDURE

Tyrosine Standard Curve

Dilute 1 mL, 2 mL, 3 mL and 4 mL of 1 mg/mL Tyrosine stock solution to 100 mL with 0.2 mol/L Hydrochloric acid solution. Pipet 2 mL of each these solutions in a test tube. Add 5 mL of 0.55 mol/L Sodium carbonate solution and 1 mL of 3 fold Folin＆Ciocalteu's phenol reagent, and mix. Place the tubes in water bath maintained at 37±0.5 ℃ for 30 minutes. Measure the absorbance at 660 nm.

As the blank, use 0.2 mol/L Hydrochloric acid solution instead of diluted Tyrosine stock solution in the same manner.

Plot the absorbance against the quantity of Tyrosine (μ g) to determine the slop of the standard curve.

$$F = \{20/(A_{s20}-A_{sBL})+40/(A_{s40}-A_{sBL})+60/(A_{s60}-A_{sBL})+80/(A_{s80}-A_{sBL})\}/4$$

Sample Assay

Pipet 5 mL of Substrate solution into test tubes and place in a water bath maintained at 37±0.5 ℃ for 10 minutes. Add 1mL of sample solution and mix. Place the tubes in the water bath at 37±0.5℃ for 10 minutes. Add 5mL of Trichloroacetic acid solution and mix. Place the tubes in the water bath at 37±0.5 ℃ for 30 minutes and filtrate with filter paper (Toyo: No.131).

Pipet 5 mL of 0.55mol/L Sodium carbonate solution in test tubes, add 2 mL of above filtrate and 1 mL of 3 fold Folin&Ciocalteu's phenol reagent. Mix and place in the water bath at 37±0.5 ℃ for 30 minutes. Measure the absorbance at 660nm.

As the blank, pipet 1 mL of Sample solution in test tubes, add 5 mL of Trichloroacetic acid solution and mix. Add 5 mL of Substrate solution and mix. Place the tubes in the water bath at 37±0.5 ℃ for 30 minutes and filtrate with filter paper (Toyo: No.131).

Pipet 5m L of 0.55 mol/L Sodium carbonate solution in test tubes, add 2 mL of above filtrate and 1 mL of 3 fold Folin&Ciocalteu's phenol reagent. Mix and place in the water bath at 37±0.5 ℃ for 30 minutes. Measure the absorbance at 660 nm.

Definition of Activity Unit

One unit is defined as the quantity of enzyme that liberates the coloring substance equivalent of 1 μg of Tyrosine for 1 minute under the conditions of the assay.

CALCULATION

Protease activity unit/g,mL = $(A_{10} - A_{0BL}) \times F \times (11/2) \times (1/10) \times n$

A_{10}	: Absorbance of the Sample.
A_{0BL}	: Absorbance of the Blank.
F	: Quantity of Tyrosine from the calibration curve (μg)
11/2	: Conversion factor for the total volume
1/10	: Conversion factor for 1 minute
n	: Dilution factor of the enzyme