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Pullulanase

(*Amylopectin-6-gluconohydrolase*) CAS RN[®]: 9075-68-7.—An enzyme obtained from *Klebsiella pneumoniae*. It contains not less than 30 units per mg of protein. One unit defined for enzymatic activity will liberate 1.0 μmol of maltotriose (measured as glucose) from pullulan per minute at pH 5.0 at 30°. It can be suspended in 3.2 M ammonium sulfate solution, pH 6.2.

• MEASUREMENT OF RELATIVE PULLULANASE ACTIVITY

Determination of pullulanase activity

Substrate: Dissolve pullulan¹ in water to make a 1.25% (w/v) solution.

[NOTE—Add pullulan to the water. Clumping will occur if water is added to pullulan.]

Buffer solution A, pH 5.0: Add 0.1 M disodium phosphate (27 g of dibasic sodium phosphate in each L of the solution) to 0.1 M citric acid (21 g of citric acid in each L of the solution) to adjust pH to 5.0.

Buffer solution B, pH 6.0: Add diluted acetic acid to 1 M sodium acetate (136 g of sodium acetate in each L of solution) to adjust the pH to 6.0. Dilute with water to prepare the final buffer solution as 0.01 M acetic acid buffer, pH 6.0.

Somogyi reagent: Add 54 g of disodium phosphate heptahydrate or 28 g of anhydrous disodium hydrogen phosphate and 40 g of potassium sodium tartrate to about 650 mL of water or about 700 mL for anhydrous disodium hydrogen phosphate. Add 100 mL of 1 N sodium hydroxide to this solution and mix. Add 80 mL of 10% cupric sulfate to the solution, and mix. Heat until any solid is completely dissolved. Add 180 g of anhydrous sodium sulfate to the solution and adjust the volume to 1 L. Allow the solution to stand at room temperature for 1 or 2 days to let insoluble matter precipitate. Filter the solution with standard filter paper, and keep the solution in a brown bottle with a ground-glass stopper.

Nelson reagent: Dissolve 50 g of ammonium molybdate in 900 mL of water. Add 42 g of sulfuric acid, and mix. Dissolve 6 g of sodium arsenate or 3.6 g of monobasic potassium arsenate in 50 g of water. Allow the solution to stand in a brown bottle with a ground-glass stopper at 37° for 1 or 2 days.

Glucose standard solution: Dry anhydrous glucose crystals under less than 50-mm Hg at 60° for 5 hours, and calculate the water content. Transfer 10.00 g of dried glucose to a 1-L volumetric flask, dissolve in and dilute with water to volume, and mix. Transfer 10.0 mL to a 1-L volumetric flask and dilute to volume with water. Each mL contains 100 μg of glucose.

Pullulanase diluent: Dilute pullulanase with *Buffer solution B, pH 6.0* to prepare a solution having the enzyme activity of about 0.2 units per mL.

[NOTE—The measurement range is between 0.1 and 0.4 units per mL.]

Record the dilution factor (F_{PD}). This diluent is used as a diluted enzyme solution.

Procedure: Transfer 4 mL of *Substrate* to a test tube and add 0.5 mL of *Buffer solution A, pH 5.0*, mix, and incubate at 30°. Add 0.5 mL of *Pullulanase diluent*, and mix thoroughly. After 30 seconds, transfer 1 mL of this solution to a test tube labeled as "Pullulan test solution 1", and add 2 mL of *Somogyi reagent*, and mix. After 30 minutes and 30 seconds, transfer 1 mL of the mixture of *Substrate* and *Pullulanase diluent* to a second test tube labeled as "Pullulan test solution 2", add 2 mL of *Somogyi reagent*, and mix. In a third test tube labeled as "Standard blank", mix 2 mL of *Somogyi reagent* and 1 mL of water. In a fourth test tube labeled as "Glucose standard solution", mix 2 mL of *Somogyi reagent* and 1 mL of *Glucose standard solution*, and add 1 mL of water. Incubate the fourth test tube in a boiling water bath for exactly 10 minutes. Remove the tube and allow it to cool in cold running water. Add 2 mL of *Nelson reagent*, mix well, and allow the solution to stand for at least 15 minutes. Add 5 mL of water to each of the four test tubes, and mix thoroughly. Determine the absorbance at 520 nm of the Standard blank, A_{blank} , of the *Glucose standard solution*, A_{Std} , of the Pullulan test solution 1, $A_{0'}$, and of the Pullulan test solution 2, $A_{30'}$, using water as the blank. One unit is defined as the enzymatic activity that produces 1 μmol of maltotriose (measured as glucose) from pullulan per minute. Calculate the pullulanase activity, PA, in units per mL, using the formula:

$$PA = [(A_{30'} - A_{0'}) / (A_{\text{Std}} - A_{\text{blank}})] \times 0.185 \times F_{PD}$$

• MEASUREMENT OF PROTEIN AMOUNT (MEASURED AS ALBUMINOID AMOUNT) FOR THE CALCULATION OF SPECIFIC ACTIVITY

Reagent A: Prepare a solution having known concentrations of about 0.1 N sodium hydroxide and about 0.2 M sodium carbonate.

Reagent B: Transfer 0.5 g of cupric sulfate and 1.0 g of sodium citrate dihydrate to a 100-mL volumetric flask, dissolve in and dilute with water to volume, and mix.

Lowry solution: Mix *Reagent A* and *Reagent B* at the proportion of 50:1.

Diluted Folin-Ciocalteu's phenol reagent (for albuminoid quantification): Prepare a two-fold dilution of 2 N Folin-Ciocalteu's phenol reagent commercially available or prepare a solution by making an appropriate dilution from Folin-Ciocalteu Phenol TS (see [Biotechnology-Derived Articles—Total Protein Assay \(1057\), Method 2](#)).

Bovine serum albumin standard stock solution: Transfer 0.05 g of bovine serum albumin to a 500-mL volumetric flask, dissolve in and dilute with water to volume, and mix. It contains 100 µg of bovine serum albumin per mL.

Standard solutions: Using appropriate dilutions of *Bovine serum albumin standard stock solution* in water, prepare five *Standard solutions* having concentration equally spaced between 5 and 100 µg of bovine serum albumin per mL.

Test solution: Dilute pullulanase with *Buffer solution B, pH 6.0* in order to obtain a solution having a concentration between 60 and 70 µg of albuminoid per mL.

[NOTE—Water can be used as diluent.]

Record the dilution factor, F_{TS} .

Blank solution: Use water.

Procedure: To 0.3 mL in separate tubes of the *Standard solutions*, the *Test solution*, and the *Blank solution*, add 3 mL of *Lowry solution*, and mix. Allow to incubate at room temperature for 10 minutes. Add 0.3 mL of *Diluted Folin-Ciocalteu's phenol reagent* to each tube, mix immediately, and allow to stand at room temperature for 60 minutes. Determine the absorbances of the *Standard solutions* and the *Test solution* at the wavelength of maximum absorbance at about 750 nm, using the *Blank solution* as the blank.

Calculation: [NOTE—The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.]

Using linear regression method, plot the absorbances of the *Standard solutions* versus the protein (bovine serum albumin) concentrations, in µg per mL, and determine the best fit curve. Using the plot, determine the concentration, $C_{\text{albuminoid}}$, in µg per mL, of protein (albuminoid amount) in the *Test solution*. Calculate the albuminoid concentration, in mg per mL, in the pullulanase taken by the formula:

$$C_{\text{protein}} = (C_{\text{albuminoid}} \times F_{TS}) / 1000$$

Calculate the specific activity, SA, in units per mg, of pullulanase using the formula:

$$SA = PA / C_{\text{protein}}$$

¹ A suitable supplier for pullulan is www.intl.hayashibara.co.jp.

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|----------------|--|------------------|
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