AMYLOGLUCOSIDASE from *ASPERGILLUS NIGER*, var.

Prepared at the 59th JECFA (2002) and published in FNP 52 Add 10 (2002), superseding tentative specifications prepared at the 55th JECFA (2000) and published in FNP 52 Add 8 (2000). An ADI "Not specified" was established at the 35th JECFA (1989).

SYNONYMS INS No. 1100

SOURCES Produced by the controlled fermentation of non-pathogenic and non-toxicogenic strains of *Aspergillus niger*, isolated from the growth medium.

Active principles: Glucan 1,4-α-glucosidase (synonyms: glucoamylase, acid maltase, lysosomal α-glucosidase, exo-1,4-α-glucosidase)

Systematic names and numbers: 1,4-α-D-Glucan glucohydrolase (EC 3.2.1.3; C.A.S. No. 9032-08-0)

Reactions catalysed: Hydrolyzes 1,4-α, 1,6-α and 1,3-α glucosidic linkages to yield glucose.

Secondary enzyme activities: α-Amylase (1,4-α-D-Glucan-glucanohydrolase; EC 3.2.1.1; C.A.S. No. 9000-90-2)
Cellulase (1,4-[1,3; 1,4]-β-D-Glucan 4-glucano-hydrolase; EC 3.2.1.4; C.A.S. No. 9012-54-8)

DESCRIPTION Typically off-white to tan amorphous powders or tan to dark-brown liquids. The unformulated products are concentrated and standardised with food-grade diluents or carriers and may contain stabilizers as glycerol and preservatives as sodium benzoate or as the dried powder form with food grade sucrose or glucose. Soluble in water and practically insoluble in ethanol and ether.

FUNCTIONAL USES Enzyme preparation
Used to convert starches to dextrins and glucose in the preparation of starch syrups, dextrose, fruit juices and low-calorie beer.

GENERAL SPECIFICATIONS Must conform to the General Specifications for Enzyme Preparations Used in Food Processing (See Volume Introduction)

CHARACTERISTICS

IDENTIFICATION: The sample shows amyloglucosidase activity.
See description under TESTS

TESTS

Amyloglucosidase activity

METHOD I

This method applies to products not containing glucose.

Principle

The method is based on the ability of the enzyme to hydrolyze maltose to glucose. The formed glucose is converted to gluconolactone by glucose

dehydrogenase in the presence of NAD+ that is converted to NADH. The change in the concentration of NAD^+ is measured photometrically at 340 nm. One amyloglucosidase unit (AGU) is defined as the amount of enzyme that cleaves 1 µmol of maltose per minute under standard assay conditions (substrate concentration 10 mg/ml; temperature 37.0 o; pH 5.0; incubation time 30 min).

Apparatus
Spectrophotometer set at 340 nm
Vortex mixer
Water bath set at 37.0 ± 0.1º

Reagents and solutions
Maltose substrate: Dissolve 1000 mg maltose (Merck No. M5912 or equivalent) in 0.1 M citrate buffer, pH 5.0, making a total volume of 50 ml (to be prepared each day of analysis).

Glucose dehydrogenase (GluDH) reagent: Add buffer solution (Merck No. M14051 or equivalent) to the enzyme mixture (Merck No. 14055, bottles '1' and '2', or equivalent). Allow the mixture to stand for 15 min. Transfer the solution to a 500 ml volumetric flask, containing 200 ml of 0.1 M citrate buffer pH 5.0. Fill the flask to the mark with the same buffer. This solution can be stored for two weeks at room temperature (not in a refrigerator).

Preparation of standard enzyme solutions: Dilute an amyloglucosidase standard (Sigma A-7420 or equivalent) with known activity in 10 mM NaCl to give the following enzyme units per ml: 0.015, 0.030, 0.045, 0.060 and 0.075.

Procedure
Measurement of enzyme activity: Add 500 µl of substrate solution (20 mg/ml maltose, prepared in 0.1 M citrate buffer, pH 5.0, pre-warmed to 37 °) to an equal volume of enzyme solution also pre-warmed to 37 °. Mix the resulting solution thoroughly and transfer to a water bath maintained at 37 °. After 30 minutes remove the test tube from the water bath, and add 1000 µl of the stop reagent (0.06 N NaOH) and shake vigorously. Add 3 ml of GluDH reagent and mix again. Leave the test tube at room temperature for exactly 30 min and measure absorbance (A) at 340 nm. Prepare a blank in a similar manner except that the stop reagent is added before the substrate.
Calculation of enzyme activity: Draw the enzyme standard curve in a coordinate system using enzyme activity (AGU/ml) as the abscissa and ΔA (sample-blank) as the ordinate. Calculate the activity of the sample by reading ΔA of sample dilutions on the standard curve and then multiplying by the corresponding dilution factors. The standard curve is a straight line passing through the origin and linear regression can therefore be applied.

Basic standardization
Glucose stock solution: Accurately weigh 1.60 g of glucose, analytical grade, in a 1000 ml volumetric flask and make up to volume with water.
Standard solutions: Dilute 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 ml of stock solution to volume with water in 100-ml volumetric flask to give standard solutions of 88.8, 178, 266, 355, 444 and 533 µmol.

Standard curve: Prepare the glucose standard curve by mixing 2.0 ml glucose

solution with 3.0 ml GluDH reagent and incubating at room temperature for 30 min. Read A at 340 nm. Prepare a common blank using water instead of glucose solution. Plot glucose concentration (µmol/l) versus ΔA.

Calculation

Calculate the enzyme activity (AGU/g) by reading the equivalent glucose concentration on the standard curve and inserting it in the following formula:

$$\text{AGU/g} = \frac{A \times 4 \times F}{30 \times 1000}$$

Where:
A = reading on the standard curve, µmol/l
F = dilution factor of the enzyme solution, ml/g
4 = the ratio between amount of enzyme dilution in sample and amount of glucose solution standard
30 = incubation time, 30 min
1000 = conversion from litre to ml

METHOD II

This method is designed for an autoanalyzer system and applies to products including those containing glucose.

Principle

Amyloglucosidase hydrolyzes maltose to form beta-D-glucose and mutarotase isomerizes alpha-D-glucose to form beta-D-glucose. The produced sugar is changed to gluconolactone in the presence of glucose dehydrogenase reagent. Glucose dehydrogenase requires NAD^+ which is converted in the course of the reaction to NADH. The disappearance of glucose is monitored by measuring the change in optical density at 340 nm.

Apparatus

Autoanalyzer, e.g. Technicon Autoanalyzer II system
Thermostable water bath (37 °)
Automatic diluter
Analytical balance
pH meter
Dialysis membrane, e.g. Technicon dialysis membrane, Type C.

Reagents and substrates

Acetate buffer 1.0 M: Dissolve 88.8 g of sodium acetate trihydrate (Merck No. 6267 or equivalent) in approximately 1.8 l of water and add 77 ml of glacial acetic acid (Merck No. 63 or equivalent). Transfer the solution to 2 l volumetric flask and fill to the mark with demineralized water.

Acetate buffer 0.1 M, pH 4.30: Transfer 100 ml of acetate buffer 1.0 M to a 1 l volumetric flask and add 20 drops of Triton X-100 or Triton X-405 (Sigma or equivalent). Add 700 ml of water, adjust the pH to 4.3 ± 0.5 using NaOH or HCl and make up to 1 litre with demineralized water.

Maltose substrate 10 g/l: Dissolve 10 g of maltose monohydrate (Merck No. 5912 or equivalent) in 100 ml of 1.0 M acetate buffer. Add 10 drops of Triton X-405 or Triton X-100 (Sigma or equivalent), and adjust the pH to 4.30 ± 0.05

using NaOH or HCl as appropriate. Make up to 1 l in a volumetric flask with demineralized water. During analysis, keep the solution on ice in a brown bottle. (Storability: 3 days in refrigerator).

Glucose dehydrogenase reagent (GlucDH-R) stock solution: Pour the contents of the enzyme mix vial (Merck No. 12194 Granutest 250 glucose or equivalent) into a 300 ml volumetric flask and fill to the mark with buffer pH 7.6 (Merck No. 14051 or equivalent). Stir for at least 15 minutes. (Storability: 5 days at 4 °.)

GlucDH-R working solution: Transfer 100 ml of GlucDH-R stock solution to a 1 l volumetric flask and add 10 drops of surfactant (FC 135 or equivalent). Make up to volume with buffer pH 7.6 (Merck No. 14051 or equivalent). The working solution can be stored for 5 days at 4°.

Saturated benzoic acid: Weigh 5.40 g benzoic acid (Merck No. 136 or equivalent) into a 2 l volumetric flask and add 1.8 l of demineralized water. Stir the flask until all the benzoic acid dissolves (can be heated) and fill to the mark with demineralized water. (Storability: 5 days at 4°).

Glucose 20 g/l: Weigh 4.00 g D(+)-glucose anhydrous (Fluka 49139, or equivalent) into a beaker and add 150 ml of saturated benzoic acid. Stir the solution in the beaker until the sugar has dissolved completely. Transfer quantitatively to a 200 ml volumetric flask and fill to the mark with saturated benzoic acid solution. The solution is stable for 1 month at room temperature.

Samples and standards

Amyloglucosidase standard: Prepare a stock solution having 12 AGU/ml by dissolving the appropriate weight of the enzyme standard in a 25 ml volumetric flask using demineralized water. Dilute proper volumes of the stock solution in demineralized water to obtain the following enzyme activity (AGU/ml): 0.5, 1.0, 2.0, 3.0, and 4.0. The amyloglucosidase standard solutions are prepared daily prior to assay.

Controls: The analysis is performed relative to a known amyloglucosidase serving as a control for the level of the run (level control). The 3.0 AGU/ml standard and 20 g/l glucose are used as an AutoAnalyzer drift control. Unknown samples: Dilute samples to an estimated enzyme activity of 2.0 AGU/ml with demineralized water and stir for about 15 minutes to ensure complete dissolution. The normal working range is 0.5 - 4.0 AGU/ml. If the diluted samples are cloudy, centrifuge at approximately 3000 rpm (or about 1100 × g) for 10 minutes and analyze the supernatant.

Procedure

Prior to analysis, pump demineralized water containing 10 drops of Triton X-405 or Triton X-100 per litre through all the tubing for at least 10 minutes. Place the diluted unknown samples in the sampler, 3 cups per sample.

Calibrate the blank channel relative to the sample channel with a solution of 20 g/l glucose. The standard calibration for the blank channel is such that it gives the same response as the sample channel. Carry out analysis in both channels at the same time. These two physically independent channels are identical except that the blank channel uses 0.1 M acetate buffer pH 4.30

while the sample channel uses maltose reagent.

Incubate the sample to be tested with the substrate for 5 minutes at 37°. Stop the reaction by dialysis (approx. 3 seconds). Determine the concentration of glucose in the dialyzer's recipient stream using the glucose dehydrogenase reaction. Translate the OD values obtained for the standards or samples to amyloglucosidase activity units using appropriate software.

Calculation

The AutoAnalyzer method is a relative method in that the regression for the standards is calculated and the OD values for the samples correlated to the regression line. All values for optical density measurements must be within the range of the standard curve. If readings are above the standard curve, samples must be diluted. Samples that give values below 600 AGU/g or ml can be considered acceptable. If results are below the lowest standard, they may be reported as follows:

$$\text{Result} < \frac{\text{Lowest Standard (0.5 AGU/ml)} \times \text{dilution factor}}{\text{Weight (g)}}$$