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Glucosidase from *Aspergillus niger* expressed in *Trichoderma reesei* exhibiting α-glucosidase and transglucosidase activity

(JECFA99-4a, JECFA99-4b)

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Glucosidase from *Aspergillus niger* expressed in *Trichoderma reesei* exhibiting α-glucosidase and transglucosidase activity (JECFA99-4a, JECFA99-4b)

New specifications prepared at the 99th JECFA (2024), published in FAO JECFA Monographs 34 (2025). An ADI "not specified" was established at the 99th JECFA (2024).

SOURCES

Produced by a submerged fed-batch pure culture fermentation of a non-pathogenic and non-toxigenic strain of *Trichoderma reesei* for the expression of an enzyme-encoding gene from *Aspergillus niger*. The enzyme that is produced and secreted into the fermentation broth exhibits α-glucosidase or transglucosidase activity. It is separated from the biomass using multiple filtration steps to obtain the enzyme concentrate and is formulated and standardized as a liquid enzyme preparation.

The enzyme preparation is used for its α-glucosidase (JECFA99-4a) or transglucosidase (JECFA99-4b) activity. Transglucosidase activity is assayed and expressed in Transglucosidase Units (TGU). α-Glucosidase activity in the article of commerce is standardized based on the transglucosidase activity units (TGU).

The respective specifications are presented below in Annex 1 (JECFA99-4a) and Annex 2 (JECFA99-4b).

Annex 1: α-glucosidase (JECFA99-4a) from *Aspergillus niger* expressed in *Trichoderma reesei*

SYNONYMS	α-Glucosidase; Maltase; Glucoinvertase; Glucosidosucrase; Maltase-Glucoamylase; α-Glucopyranosidase;
Active principles	α-Glucosidase
Systematic names and numbers	α-D-Glucoside glucohydrolase; EC 3.2.1.20
Reaction catalysed	Hydrolysis of terminal, non-reducing (1→4)-linked α-D-glucose residues with release of α-D-glucose.
Secondary enzyme activities	Transglucosidase
DESCRIPTION	Brown liquid.
FUNCTIONAL USES	Enzyme preparation Used as a processing aid in the manufacture of potable alcohol, organic acids, and monosodium glutamate.
GENERAL SPECIFICATIONS	Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

α-Glucosidase activity	The sample shows α-glucosidase activity. See description under TESTS.
Transglucosidase activity	The sample shows transglucosidase activity. See description under TESTS in Annex 2.

TESTS

IDENTIFICATION TEST

α-Glucosidase activity

PRINCIPLE

The activity of α-glucosidase is determined spectrophotometrically by measuring the formation of yellow colour of *p*-nitrophenol released by the hydrolysis of *p*-nitrophenyl α-D-glucopyranoside (PNPG) substrate at 420 nm, at pH 10.0. This activity, under this assay condition, is expressed in α-Glucosidase Units (AGDU)/g or AGDU/ml.

One AGDU is defined as the amount of enzyme required to generate 1.0 µmole of *p*-nitrophenol per minute at pH 4.8 and 30 °C.

MATERIALS AND EQUIPMENT

- UV-Vis Spectrophotometer
- Magnetic stirrer
- Thermometer
- pH Meter
- Cuvettes, 10 mm path length

REAGENTS AND SOLUTIONS

Deionized water

Sodium acetate trihydrate ($CH_3COONa \cdot 3H_2O$)

Glacial acetic acid (CH_3COOH)

Sodium borate decahydrate ($Na_2B_4O_7 \cdot 10H_2O$)

Sodium hydroxide (NaOH)

Substrate: *p*-nitrophenyl α-D-glucopyranoside (PNPG: $C_{12}H_{15}NO_8$; CAS No. 3767-28-0; N-4351 or N-4350 from Biosynth, or equivalent)

Standard: *p*-nitrophenol ($C_6H_5NO_3$; CAS No. 100-02-7; ≥ 99.0 %)

Assay buffer (Sodium acetate buffer (pH 4.8)): Weigh and dissolve 2.72 g of sodium acetate trihydrate in approximately 800 ml of deionized water with stirring. Adjust the pH to 4.8 with glacial acetic acid at room temperature with stirring. Make up to volume with deionized water in a 1 000-ml volumetric flask and mix well. The solution can be stored in a refrigerator in a closed container.

Stop Solution (0.2 M Borax solution): Weigh and dissolve 76.20 g of sodium borate decahydrate in approximately 800 ml of deionized water with stirring for about 30 minutes. Adjust the pH to 10.0 at room temperature with sodium hydroxide, then make up to volume with deionized water in a 1 000–ml volumetric flask. The solution can be stored at room temperature in a closed container.

Standard dilution buffer: Mix 500 ml of Assay buffer and 250 ml of Stop solution.

SUBSTRATE SOLUTION

Weigh 50.0 mg of PNPG in a 50-ml amber volumetric flask. Add approximately 40 ml of the Assay buffer to the flask and mix on a magnetic stirrer for 20-30 minutes until completely dissolved (Periodic heating in a warm water bath can also aid the dissolution). Make up to volume with the Assay buffer. The solution can be stored for up to 2 weeks in refrigerator.

STANDARD SOLUTIONS

- Standard stock solution (0.001 M *p*-nitrophenol solution): Dissolve 139.11 mg of *p*-nitrophenol previously dried (60 °C, maximum 4 h) in water in a 1-L volumetric flask and dilute to volume with water.
- Standard working solutions (0.005, 0.02 and 0.05 mM *p*-nitrophenol solutions):
  - Standard 0.005 mM: Add 1 ml of the Standard stock solution in a 200-ml volumetric flask, and dilute to volume with Standard dilution buffer
  - Standard 0.02 mM: Add 2 ml of the Standard stock solution in a 100-ml volumetric flask, and dilute to volume with Standard dilution buffer
  - Standard 0.05 mM: Add 5 ml of the Standard stock solution in a 100-ml volumetric flask and dilute to volume with Standard dilution buffer

SAMPLE SOLUTIONS

Work in duplicate. Accurately weigh a sample and dissolve with the Assay buffer in a volumetric flask to obtain a final net absorbance within the range of the Standard solutions prepared for the calibration curve (approximately 0.04 AGDU/ml). The sample solutions are typically stable for 6 hours at room temperature, but storage on ice is recommended.

ASSAY TEST SOLUTIONS AND ASSAY BLANK

Prepare all Assay test solutions, and the Assay blank in duplicate.

- Pipette 500 µl of each Sample solution, or Assay buffer (as Assay blank) to the corresponding tube.
- Pre-heat the tubes in a 30 °C water bath for approximately 5 minutes and allow to equilibrate.
- Add 500 µl of Substrate solution to each tube at timed intervals, vortex, and replace the tube in the water bath.
- After exactly 5 minutes from the addition of Substrate solution, add 500 µl of Stop solution to each tube and mix well by vortex to terminate the reaction.

Measurements

Measure the absorbance of each of the Standard working solutions, Assay test solutions and Assay blanks at 420 nm using deionized water to zero the spectrophotometer in a 10-mm cell.

STANDARD CURVE AND CALCULATION

One AGDU is defined as the amount of α-glucosidase that will liberate 1.0 µmole of *p*-nitrophenol per minute from the Substrate solution under the conditions of the assay.

Standard Curve:

Plot the net absorbance as y-values against the concentration of the three Standard working solutions in µmole/ml as the x values and prepare a standard curve using linear regression. The correlation coefficient must be ≥0.999.

Calculation:

1. Calculate the net average absorbance of the Assay test solutions by subtracting the average absorbance of the two replicates of Assay blanks from the average absorbance of the two replicates of the Assay test solutions. Determine the average concentration of *p*-nitrophenol in the Assay test solutions in µmole of *p*-nitrophenol /ml using the standard curve.
2. Calculate the activity of the Sample solutions

$$\frac{\text{AGDU}}{\text{ml of sample solution}} = \frac{\text{M} \times 1.5}{0.5 \times 5}$$

Where:

M is the average concentration of *p*-nitrophenol in the Assay test solutions in µmole of *p*-nitrophenol/ml using Standard curve;

1.5 is the final volume of the test solutions, in ml;

5 is the reaction time, in min;

0.5 is the sample aliquot, in ml

Calculate the activity of the sample in AGDU per gram (g) using the following equation:

$$\frac{\text{AGDU}}{\text{g}} = \text{S} \times \frac{1}{\text{C}}$$

Where:

S = Activity of the Sample solution in AGDU/ml

C= Concentration of the Sample solution in g/ml

If needed, convert from AGDU/g to AGDU/ml based on the relative density of the sample.

An enzyme preparation with an activity of 2000 TGU/g (or TGU/ml) will have an activity corresponding to approximately 30 AGDU/g (or AGDU/ml).

Annex 2: Transglucosidase (JECFA99-4b) from *Aspergillus niger* expressed in *Trichoderma reesei*

SYNONYMS	Transglucosidase; 1,4-D-glucan 6-D-glucosyltransferase; oligoglucan-branching glycosyltransferase; D-glucosyltransferase
Active principles	Transglucosidase
Systematic names and numbers	1,4-D-glucan 6-D-glucosyltransferase; EC 2.4.1.24
Reaction catalysed	Transfers an α-D-glucosyl residue in a (1→4)- α-D-glucan to the primary hydroxy group of glucose, free or combined in a (1→4)- α-D-glucan.
Secondary enzyme activities	α-Glucosidase
DESCRIPTION	Brown liquid.
FUNCTIONAL USES	Enzyme preparation Used as a processing aid in the production of isomalto oligosaccharide syrups from a variety of sources.
GENERAL SPECIFICATIONS	Must conform to the latest edition of the JECFA General Specifications and Considerations for Enzyme Preparations used in Food Processing.

CHARACTERISTICS

IDENTIFICATION

Transglucosidase activity	The sample shows transglucosidase activity. See description under TESTS.

TESTS

IDENTIFICATION TEST

Transglucosidase activity

PRINCIPLE

The activity of transglucosidase is determined by incubating a diluted enzyme solution with a 10% (w/v) maltose solution in 0.02 M acetate buffer and measuring, using HPLC with RI detector, the amount of trisaccharide produced. This activity is expressed in Transglucosidase Units (TGU)/g or TGU/ml.

One TGU is defined as the amount of enzyme that produces one μmol trisaccharide per minute at pH 4.0 and 50 °C.

MATERIALS AND EQUIPMENT

- HPLC with RI detector
- Magnetic stirrer
- Thermometer
- pH Meter

REAGENTS AND SOLUTIONS

Acarbose ($C_{25}H_{43}NO_{18}$; CAS No. 56180-94-0;

BAY g 5421 from Bayer AG, or equivalent)

Concentrated sulfuric acid (≥ 96%)

Glacial acetic acid (CH_3COOH)

HPLC grade water

Sodium hydroxide (NaOH) AR or ACS Grade.

D-Glucose (anhydrous) ($C_6H_{12}O_6$; CAS No. 50-99-7) AR Grade

Substrate: D-Maltose monohydrate ($C12H22O11 \cdot H_2O$; CAS No. 6363-53-7) ≥ 99% (by HPLC) (Recommend purity test every six months)

D-Panose (anhydrous) (as qualitative standard) ($C_{18}H_{32}O_{16}$; CAS No. 33401-87-5)

Qualitative reference solution: Weigh 0.1 g of D-panose (anhydrous) in a 100-ml volumetric flask and dilute to volume with 0.01 N H_2SO_4.

2 N NaOH: Weigh 80.0 g NaOH and add it carefully with mixing to 500 ml of water in a 1-L beaker. Cool to room temperature, transfer to a 1-L volumetric flask and make up to volume with water.

Acetate buffer (0.02 M, pH 4.0): Add 2.30 ml glacial acetic acid to 1800 ml of water in a 2-L beaker. Adjust the pH to 4.00 ± 0.01 using 2 N NaOH, under stirring. Transfer to a 2 L volumetric flask and make up to volume with water. This solution can be stored in refrigerator.

Acarbose buffer: Weigh 258 mg of acarbose in a 100-ml volumetric flask. Make up to volume with Acetate buffer (0.02 M, pH 4.0) (Stock solution). This can be stored frozen in a suitable closed container. Pipette 10 ml of the Stock solution into a 1-L volumetric flask and make up to volume with Acetate buffer (0.02 M, pH 4.0) (Working solution). This solution can be stored in refrigerator.

0.01 N H_2SO_4 (for HPLC Mobile Phase): Weigh 2.05 g of conc. H_2SO_4, add it to 4.0 L of HPLC grade water and mix.

0.011 N H_2SO_4 (for sample diluent): Weigh 1.13 g of conc. H_2SO_4, add it to 2.0 L of HPLC grade water and mix.

SUBSTRATE SOLUTION (20 % (w/v) D-Maltose monohydrate in Acetate buffer)

Weigh 5.00 g of D-maltose monohydrate in a small beaker, add 20 ml of Acetate buffer (0.02 M, pH 4.0) and mix well. Quantitatively transfer to a 25-ml volumetric flask and make up to volume with acetate buffer (0.02 M, pH 4.0). Solution must be made fresh daily.

STANDARD SOLUTION (GLUCOSE SOLUTION (1 MG/ML))

Weigh 1.000 g of D-Glucose (anhydrous) in a 100 ml volumetric flask. Make up to volume with 0.01 N H_2SO_4. Pipette 10 ml of the solution in a 100-ml volumetric flask and make up to volume with 0.01 N H_2SO_4. This solution can be stored frozen in screw cap tubes.

SAMPLE SOLUTION

Accurately weigh at least 1.0 g (W1) of enzyme in a 100 ml volumetric flask (V1) and make up to volume with Acetate buffer (0.02 M, pH 4.0). Accurately dilute the solution 10 - 100 times with Acetate buffer (0.02 M, pH 4.0) to produce a dilution containing 0.2-1.0 TGU/ml (target 0.5 TGU/ml). (Vs/V2)

In the case that the product of the enzyme is considered insufficiently purified and may contain impurities such as glucoamylase, the following operations should be performed, instead of the above sample solution.

Weigh at least 1.0 g of enzyme in a 100-ml volumetric flask and make up to volume with Acarbose buffer working solution. Accurately dilute the solution 10-100 times with Acarbose buffer working solution to produce a dilution containing 0.2 1.0 TGU/ml (target 0.5 TGU/ml). Incubate the diluted enzyme at room temperature for at least 10 min before assay. Then use it as Sample solution. (Acarbose is added as an inhibitor to prevent interference by the other activity such as glucoamylase.

PROCEDURE

Perform the following procedure on all Sample solutions.

FOR ASSAY TEST SOLUTION

1. Prepare 3 tubes for each Sample solution. Pipette 500 µl of Substrate solution to the tubes. Pre-heat the tubes in a water bath at 50 °C for 10 minutes.
2. Add 500 µl of Sample solution to each tube at timed intervals, seal, vortex, and replace the tube in the water bath at 50 °C.
3. After exactly 60 minutes from the addition of Sample solution, terminate the reaction by removing the tube and immediately placing in boiling water bath for exactly 10 minutes. Cool to room temperature, add 9.0 ml of 0.011 N H_2SO_4 to each tube and mix by inversion.

FOR ASSAY BLANK

1. Prepare 2 tubes for assay blank of each Sample. Pipette 500 µl of Substrate solution to the tubes. Incubate in a water bath at 50 °C for exactly 60 minutes of incubation.
2. Remove the tubes from the water bath, add 500 µl of Sample solution to each test tube, seal, mix, and immediately place in boiling water bath for exactly 10 minutes. Cool to room temperature, add 9.0 ml of 0.011 N H_2SO_4 to each tube and mix by inversion.

HPLC PROCEDURE

- Column: Cation exchange column with guard column (H type) (300 mm x 7.8 mm, 9 µm) with guard column (H type), for example Bio-Rad HPX-87H (cat. no. 125-0140) with Cation H guard cartridge (cat. no. 125-0129), or equivalent.
- Column temperature: 60 °C
- Mobile Phase 0.01N H_2SO_4
- Detector: Refractive Index
- Flow rate: 0.7 ml/min
- Injection volume: 20 µl
- Run Time: 15 min

The retention time of D-panose in the chromatogram of the Sample solution should be determined using the Qualitative reference solution.

(Approximate retention times are as follows: D-Panose 5.7 min, D-Glucose 7.7 min)

CALCULATION

Calculate the activity of the sample in TGU per gram (g) using the following equation:

$$\frac{\text{TGU}}{\text{g}} = \frac{(G_3^{spl} - G_3^{blk}) \times 1\,000 \times 2 \times 10 \times V_1 \times V_2}{G_1^{std} \times 504 \times 60 \times W_1 \times V_s \times 1.30}$$

Where:

G_3^{spl} = Area of trisaccharide peak (average) in Assay test solution

G_3^{blk} = Area of trisaccharide peak (average) in Assay blank

G_1^{std} = Area of glucose standard (1 mg/ml) peak (average of duplicate injection)

1 000 = Conversion of gram to mg

504 = Mol. wt. of trisaccharide (D-panose) (conversion to m moles)

2 = Conversion to 1 ml enzyme (0.5 ml used in assay)

10 = HPLC dilution factor

V_1 = Primary dilution total volume (ml)

V_2 = Secondary dilution total volume (ml)

W_1 = Primary dilution weight of sample (g)

V_s = Secondary dilution sample volume (ml)

1.30 = Calculation factor related to the use of glucose standard

60 = Minutes of assay

One TGU is defined as the amount of enzyme that produces one μmol trisaccharide per minute under the assay condition.

If needed, convert from TGU/g to TGU/ml based on the relative density of the sample.