HEMICELLULASE from *ASPERGILLUS NIGER*, var.

Prepared at the 55th JECFA (2000) and published in FNP 52 Add 8 (2000), superseding tentative specifications prepared at the 31st JECFA (1987) and published in FNP 38 (1988) and in FNP 52 (1992). An ADI "Not specified" was established at the 35th JECFA (1989).

SOURCES

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

Active principles

Endo-1,4-beta-xylanase
Xylan 1,4-beta-xylosidase
alpha-L-Arabinofuranosidase
Cellulase
Galactomannanase

Systematic names and numbers

1,4-beta-D-Xylan xylanohydrolase (EC 3.2.1.8; C.A.S. No. 9025-57-4)
1,4-beta-D-Xylan xylohydrolase (EC 3.2.1.37; C.A.S. No. 9025-53-0)
alpha-L-Arabinofuranoside arabino-furanohydrolase (EC 3.2.1.55; C.A.S. No. 9067-74-7)
1,4-[1,3; 1,4]-beta-D-Glucan 4-glucano-hydrolase (EC 3.2.1.4; C.A.S. No. 9012-54-8)
1,4-beta-D-galactan-4-mannano-hydrolase (C.A.S. No. 50812-17-4)

Secondary Enzyme activities

1) Glucoamylase (1,4-alpha-D-Glucan glucohydrase; EC 3.2.1.3; C.A.S. No. 9032-08-0)
2) Maltase (alpha-D-Glucosidase glucohydrolase; EC 3.2.1.20; C.A.S. No. 9001-42-7)
3) Lactase (beta-galactosidase, beta-D-Galactoside galactohydrolase, EC 3.2.1.23, C.A.S. No. 9031-11-2)
4) Invertase (Saccharase, beta-Fructofuranosidase, beta-D-Fructofuranoside fructohydrolase; EC 3.2.1.26)

Reactions catalyzed

Hydrolyzes the linkages between the various sugar groups of the polysaccharide chains.

DESCRIPTION

Typically off-white to tan amorphous powders or tan to dark-brown liquids. The unformulated product is usually diluted and standardised with food grade lactose, maize starch or maltodextrin powders to obtain commercial preparations. Soluble in water and practically insoluble in ethanol and ether

FUNCTIONAL USES

Enzyme preparation
Used in the manufacture of instant coffee and bread making process

GENERAL SPECIFICATIONS

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

CHARACTERISTICS

IDENTIFICATION

Cellulase activity

The sample shows cellulase activity
See description under TESTS

Galactomannanase activity

The sample shows galactomannanase activity
See description under TESTS

Xylanase activity

The sample shows xylanase activity
See description under TESTS

Cellulase activity

Principle

The assay is based on the ability of the enzyme to hydrolyze carboxymethyl cellulose (CMC) to reducing sugars. The reaction products are determined photometrically at 540 nm by measuring the resulting increase in reducing groups using 3,5-dinitrosalicylic acid. One cellulase unit is defined as the amount of enzyme that liberates reducing sugar at the rate of 1 µmol/min under the conditions of the assay.

Apparatus

Spectrophotometer set at 540 nm.
Water-bath set at 40.0 ± 0.1°

Reagents

1. CMC substrate solution (1.0%): Accurately weigh 0.500 g of CMC (SIGMA C5678-7 or equivalent) and sprinkle on to warm 40 ml of water in a beaker. Place beaker on a hot-plate equipped with a magnetic stirrer, apply heat and stir vigorously. When the liquid has started to boil, cover the beaker with a watch glass, turn off the hot plate and continue stirring until the solution is cool. Quantitatively transfer the solution into a 50 ml volumetric flask, add 5 ml acetate buffer, adjust the pH to 5.0 and make up to volume.

2. 3,5-Dinitrosalicylic acid (DNS) solution: Accurately weigh 10 g of DNS into a 2000-ml beaker. Add 16 g of sodium hydroxide pellets, 300 g of potassium sodium (+)-tartrate and 500 ml of water. Place the beaker on a heater/stirrer and warm gently, whilst stirring, to dissolve. Cool to ambient temperature and transfer the contents of the beaker into a 1000-ml volumetric flask. Rinse the beaker with water, add to volumetric flask and make up to volume with water. Store the solution at ambient temperature for up to 10 weeks. It is possible that DNS reagent get overheated during the preparation making the solution quite dark. The maximum absorbance at 540 nm for a blank (without glucose standard) measured against water shall not be more than 0.050 absorbance units.

3. DNS-lactose solution: Dissolve lactose monohydrate with water to obtain 0.120 g/l solution. Mix l50 ml of DNS solution and 50 ml of Lactose solution. Use freshly prepared mixture.

4. Samples preparation: Dissolve known quantity of sample in distilled water. Make serial dilutions to get a working solution in the absorbance range of
0.150 - 0.400

5. Glucose standard solution: Accurately weigh 0.5g of anhydrous glucose and make up to volume in a 100 ml volumetric flask. Dilute the solution with water to get 5, 10 and 15 µmoles/l of glucose.

Procedure

Measurement of enzyme activity

Add 1 ml of substrate solution (pre-warmed to 40.0± 0.1° for 5 min) to an equal volume of sample solution also pre-warmed to 40.0 ± 0.1°. Mix the resulting solution thoroughly and transfer to a water-bath maintained at 40.0 ± 0.1 °. After 10 minutes (reaction step) remove the test tube from the water bath, and add 4 ml of DNS-Lactose solution and mix to stop the enzymatic reaction. Cover tubes and place in a boiling water bath for 15 min. and then cooled to room temperature with a cooling water bath. Remove insoluble substances by centrifugation (3000 rpm, 10 min). Determine the absorbance at 540 nm against water blank. Prepare a reaction blank in a similar manner but without a reaction step. Prepare a reagent blank omitting substrate and read absorbance against water.

Standard curve

Prepare the glucose standard curve by adding 1 ml glucose standard solution (5, 10 and 15 µmoles/l) instead of CMC substrate solution in the procedure described above. Draw the standard curve in a coordinate system using glucose concentration (µmol/l) as the abscissa and absorbance as the ordinate. The standard curve is a straight line passing through the origin and linear regression can therefore be applied. Calculate the glucose concentration in the sample from the standard curve and calculate the enzyme activity as follows.

Calculation

Calculate the sample enzyme activity (U/g) by reading the equivalent glucose concentration on the standard curve for the sample and the reaction blank and inserting them in the following formula:

$$\text{Cellulase Activity, U/g} = \frac{(C_G - C_{RB}) \times D}{W \times 10 \times V}$$

Where

C_G : Reading from the standard curve for sample enzyme, µmol/l

C_{RB} : Reading from the standard curve for reagent blank, µmol/l

D : Dilution factor of the sample

W : Weight of sample taken, g

10 : Incubation time, min

V : Volume of sample solution taken, 1 ml

Galactomannanase activity

Principle

This assay method is based on the enzymatic hydrolysis of galactomannan. The velocity of the decrease of the viscosity of the substrate under the enzymatic action is measured in a capillary viscometer. One unit of galactomannanase activity (GMA) is defined as that quantity of enzyme that decreases to half the initial viscosity per minute of substrate under the conditions of the assay.

Apparatus

Capillary viscometer such as Cannon-Fenske viscometer, Type #200.
Two digital watches, calibrated in 1/10 sec.
Water-bath set at 40.0 ± 0.1 °

Reagents and solutions

1. Substrate solution: Weigh 0.73 g dry base (loss on drying value: 1g, 105°, 3 hr) of substrate Locust Bean Gum (such as SIGMA G-0735) in 500 ml beaker. Add about 400 ml of water and stir gently by a magnetic stirrer for 10 min and heat with boiling water for more than 3 min. Quantitatively transfer the solution to 500 ml volumetric flask and fill with water and then filter this solution before use.

2. Acetate buffer pH 4.5: Add 30 g of glacial acetic acid in about 800 ml of water.
Adjust pH 4.5 ± 0.02 with 1N sodium hydroxide by pH meter. Quantitatively transfer the solution to 1000 ml volumetric flask, dilute to volume with water, and mix.

3. Sample solution: Dissolve in water to obtain about 0.015-0.040 U/ml enzyme activities that allows the end point of the reactions to be between 4 to 5min.

4. Enzyme standard solutions: Dilute with water Galactomannanase standard (~50,000 U/g, such as Shin Nihon Chemical Co., Ltd) to contain 0.03 U/ml.

Procedure

Allow the temperature of the viscometer to reach equilibrium with the bath thermostatically controlled at 40.0 ± 0.1 ° at least 5 minutes. Place in the bath test tubes containing 10 ml of the substrate solution and 1 ml of buffer, mix and allow it to reach equilibrium with the temperature (~5 min). At zero time measured on watch no. 1, add 1 ml of the sample or the enzyme standard solution to the test tube, mix and pour the quantity needed for the measurements into the bulb of the viscometer. Wait at least two minutes and then suck the reactive mixture into the upper measuring bulb and allow it to flow out; as soon as the meniscus of the liquid becomes tangent to the upper mark, start stop-watch No. 2 and simultaneously record watch No. 1 (time t_1); as soon as the meniscus becomes tangent to the lower mark, stop-watch No. 2 (efflux time t_2). Calculate the reaction time (R) as follows:
The enzyme reaction time, which the efflux of the reaction mixture has been measured, is defined as a half point from the beginning time to the end time of the efflux measurement.

$$R = t_1 + \frac{t_2}{2}$$

Repeat at different reaction times (such as 4, 6, 8 minutes) and calculate the reaction time in the same way described above.

Efflux time of the substrate blank solution

Mix l0 ml of substrate solution, 1 ml of buffer and 1 ml of water into a test tube and equilibrate at 40.0 ± 0.1 °C for about 5 min. Measure the efflux

time of the substrate blank solution in the same way described above. Repeat 3 times each measurement of the efflux time, and average them (t_{2sb}).

Efflux time of the water blank
Pipet 12 ml of water in to a test tube and equilibrate at 40.0 ± 0.1°C for about 5 min. Measure the efflux time of water in the same way described above. Repeat 3 times each measurement of the efflux time, and average them (t_{2w}).

Calculation
Calculate the efflux time when the substrate blank viscosity is reduced to half of the initial viscosity value ($t_{1/2}$) as follows:

$$t_{1/2} = \frac{t_{2sb} + t_{2w}}{2}$$

Plot the efflux time (t_2) as the ordinate against the reaction time (R) as the abscissa and draw the curve. For the sample and for the standard read the end point of the reaction time (R) corresponding to 1/2 efflux time of the substrate blank ($t_{1/2}$) from the curve.
Galactomannanase activity units of the unknown sample are calculated in relation with a known standard.

$$\text{Galactomannanase, U/g} = St \times \frac{Rs \times Cs}{Rx \times Cx}$$

St: galactomannanase activity of the enzyme standard (50000 U/g)
Rs: the reaction time of the enzyme standard solution at the t 1/2 (sec)
Cs: concentration of the enzyme standard solution (g/ml)
Rx: the reaction time of sample solution at the t 1/2 (sec)
Cx: concentration of sample solution (g/ml)

Xylanase activity

Principle
This assay is based on the enzymatic hydrolysis of sodium arabinoxylan. The resulting reducing sugar is allowed to react with 3,5-dinitrosalicylic acid and is determined photometrically at 540 nm. One xylanase unit is defined as that quantity of enzyme that liberates reducing sugar at a rate of 1 µmol /min under the conditions of the assay.

Apparatus
Spectrophotometer set at 540 nm.
Water bath set at 40.0 ± 0.1°

Reagents and solutions
1. Xylan substrate solution (1.0%): Accurately weigh 1.0 g xylan (dry base, from oat spelts; such as SIGMA X-0627), transfer to a beaker with 60 ml of 0.2 M acetate buffer (pH 4.5). Stir for 30 min and incubate at 60 ° for I hr with gradually stirring and check pH (4.50 ± 0.05). Transfer the solution into a 100 ml volumetric flask and make up to volume with water.

2. 3,5-Dinitrosalicylic acid (DNS) solution: Accurately weigh 10 g of DNS into a 2000-ml beaker. Add 16 g of sodium hydroxide pellets, 300 g of potassium sodium (+)-tartrate and 500 ml of water. Place the beaker on a heater/stirrer and warm gently, whilst stirring, to dissolve. Cool to ambient temperature and transfer the contents of the beaker into a 1000-ml volumetric flask. Rinse the beaker with water, add rinsings to the volumetric flask and make up to volume with water. Store the solution at ambient temperature for up to 10 weeks. It is possible that DNS reagent get overheated during the preparation making the solution quite dark. The maximum absorbance at 540 nm for a blank (without xylose standard) measured against water shall not be more than 0.050 absorbance units.

3. DNS-lactose solution: Dissolve lactose monohydrate with water to obtain 0.120 g/l solution. Mix l50 ml of DNS solution and 50 ml of Lactose solution. Use freshly prepared mixture.

4. Samples preparation: Dissolve known quantity of sample in distilled water. Make serial dilutions to get a working solution in the absorbance range of 0.150 - 0.400

5. Xylose standard dilutions: Accurately weigh 0.5g of anhydrous xylose with distilled water and make up to 100 ml in a volumetric flask. Dilute with water to get working standard solutions containing 250, 500 and 750 µmoles/l of xylose.

Procedure

Measurement of enzyme activity

Add 0.1 ml of sample solution to 1.9 ml of substrate solution pre-warmed to 40.0 ± 0.1° for 5 min. Mix the resulting solution thoroughly and transfer to a water-bath maintained at 40 ± 0.1°. After 10 minutes (reaction step) remove the test tube from the water bath, and add 4 ml of DNS-Lactose solution and mix to stop the enzymatic reaction. Cover tubes and place in a boiling water bath for 15 min. and then cooled to room temperature with a cooling water bath. Remove insoluble substances by a centrifuge (3000 rpm, 10 min). Determine the absorbance at 540 nm against water blank. Prepare a reagent blank in a similar manner but without a reaction step.

Standard curve

Prepare the xylose standard curve by adding 0.1 ml xylose standard solution (250, 500 and 750 µmoles/l) instead off xylan substrate solution in the procedure described above. Draw the standard curve in a coordinate system using glucose concentration (µmol/l) as the abscissa and absorbance as the ordinate The standard curve is a straight line passing through the origin and linear regression can therefore be applied. Calculate the xylose concentration in the sample from the standard curve and calculate the enzyme activity as follows.

Calculation

Calculate the sample enzyme activity by reading the equivalent xylose concentration on the standard curve for the sample and the reaction blank and inserting them in the following formula:

$$\text{Xylanase Activity, U/g} = \frac{(C_x - C_{RB}) \times D}{W \times 10 \times V}$$

Where
C_x : Reading from the standard curve for sample enzyme, µmol/l
C_{RB}: Reading from the standard curve for reagent blank, µmol/l
D : Dilution factor of the sample
W : Weight of sample taken, g
10 : Incubation time, min
V : Volume of sample taken, 0.1ml