Megazyme

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ASSAY OF

endo-FRUCTANASE

using

AZO-FRUCTAN

S-AZFR5 11/17

PRINCIPLE:

The substrate is the high molecular weight fraction of chicory fructan (DP ~ 20-60) dyed with an azo-dye content of about one dye molecule per 15 sugar molecules.

In the standard assay procedure, Azo-Fructan solution is incubated with preparations containing *endo*-inulinase under defined assay conditions. The reaction is terminated and unhydrolysed Azo-Fructan is precipitated by the addition of an alkaline ethanolic solution. The solution is stirred vigorously, centrifuged and the absorbance of the supernatant solution measured at 590 nm. The activity of *endo*-inulinase is determined by reference to a standard curve or by use of the equation provided.

ACCURACY:

Standard errors of less than 5% are achieved routinely.

SPECIFICITY:

The assay procedure is specific for *endo*-inulinase however, it is evident that the presence of *exo*-inulinase affects the sensitivity of the assay (refer to Figure 1, page 4). As the *endo*-inulinase hydrolyses the fructan molecule, it exposes regions of fructan which are susceptible to *exo*-inulinase. On action of *exo*-inulinase, smaller dyed fragments are produced and these become soluble in the precipitant solution. If measurement of *endo*-inulinase in the presence of *exo*-inulinase is required, we recommend the use of **S-AZFRXO1**, in which *exo*-inulinase is provided and should be included in the assay in excess.

SAFETY

The general safety measures that apply to all chemical substances should be adhered to. For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

EQUIPMENT (RECOMMENDED):

1. Volumetric flasks/beakers.
2. Glass test-tubes (16 x 120 mm).
3. Analytical balance.
4. Vortex mixer (e.g. Vortex-Genie® 2 mixer).
5. Thermostated water bath (set at 40°C).
6. Centrifuge (required speed 3,000 rpm).

7. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 200 µL and 1 mL).
8. Positive displacement pipettor, e.g. Eppendorf Multipette®.
9. Spectrophotometer set at 590 nm.
10. Stop clock.

CONTROLS AND PRECAUTIONS:

1. The time of incubation of the assay must be carefully controlled (i.e. exactly 10 min).
2. Incubation temperatures must be accurately controlled (i.e. exactly 40°C).
3. After addition of Precipitant Solution to the reaction tube, the tubes must be stirred vigorously to ensure thorough mixing.
4. If the absorbance values for a particular assay are greater than 1.0, the enzyme extract should be further diluted in buffer and re-assayed.

REACTION BLANK:
The reaction blank is prepared by using 0.1 M sodium acetate buffer (0.1 mL, pH 4.5) instead of *endo*-inulinase preparation in the standard assay procedure and incubating this under the same conditions as the reaction mixture. Reaction blank absorbance values are approximately 0.25 absorbance units.

PREPARATION OF REAGENTS (NOT SUPPLIED):

1. Sodium acetate buffer (0.1 M, pH 4.5):

Add glacial acetic acid (5.8 g, 1.05 g/mL) to 900 mL of distilled water. Adjust to pH 4.5 using 1 M (4 g/100 mL) sodium hydroxide (approx. 25 mL is required). Adjust the volume 1000 mL. Stable for > 2 years at room temperature.

2. Sodium acetate buffer (0.1 M pH 4.5) containing potassium chloride (0.5 M):

Add potassium chloride (18.6 g) to 500 mL of sodium acetate buffer pH 4.5. Adjust to pH 4.5. using 1 M (4 g/100 mL) sodium hydroxide (approx. 4 drops required). Use this solution to dissolve Azo-Fructan substrate as described below.

3. Potassium hydroxide (2 M):

Add potassium hydroxide (56.1 g) to 500 mL of distilled water. Use this solution to formulate Precipitant Solution as described below. Stable for > 2 years at room temperature.

4. Precipitant Solution:

Add potassium hydroxide (100 mL, 2 M) to 1000 mL of 95% ethanol. Mix thoroughly before use. Stable for > 2 years at room temperature.

PREPARATION OF SUBSTRATE:

Powdered substrate (2.0 g) is added to 100 mL of 0.1 M sodium acetate buffer (pH 4.5) containing 0.5 M potassium chloride, and stirred vigorously at room temperature until it completely dissolves. The solution is stored in an air-tight glass storage bottle (e.g. Duran) and overlain with 2 drops of toluene to prevent microbial contamination. This substrate is stable at room temperature for several days but we recommend storage at 4°C between uses. For long term storage, the solution can be frozen.

ENDO-INULINASE ASSAY

ENZYME EXTRACTION:

Liquid Preparations

1. Add 1 mL of enzyme preparation to 25 mL of 0.1 M sodium acetate buffer (pH 4.5) and gently stir over a period of about 15 min or until the sample is completely dispersed or dissolved.
2. Clarify this solution by centrifugation (3,000 rpm, 10 min) or filtration through Whatman No. 1 (9 cm) filter circles if required.
3. Further dilute in 0.1 M sodium acetate buffer (pH 4.5), as required to obtain the correct range of activity for assay.

Powder Preparations

1. Add 1 g of enzyme powder preparation to 25 mL of 0.1 M sodium acetate buffer (pH 4.5) and gently stir the slurry over a period of about 15 min or until the sample is completely dispersed or dissolved.
2. Clarify this solution by centrifugation (1,000 *g*, 10 min) or filtration through Whatman No. 1 (9 cm) filter circles if required.
3. Further dilute in 0.1 M sodium acetate buffer (pH 4.5), as required to obtain the correct range of activity for assay.

ASSAY PROCEDURE:

1. Dispense 0.5 mL aliquots of Azo-Fructan solution into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate *endo*-inulinase extract at 40°C for 5 min.
3. To each tube containing Azo-Fructan solution (0.5 mL), add 0.1 mL of pre-equilibrated *endo*-inulinase directly to the bottom of the tube and stir contents vigorously on a vortex mixer. Incubate at 40°C for exactly 10 min (from time of addition).
4. At the end of the 10 min incubation period, add exactly 2.5 mL of Precipitant Solution and stir the tube contents vigorously for 10 sec or more.
5. Allow the reaction tubes to equilibrate to room temperature over a 5 min period and then centrifuge at 3,000 rpm for 10 min.
6. Read the absorbance of the solutions and the reaction blank at 590 nm against distilled water. Activity is determined by reference to a standard curve or using **Equation 2** as provided below.

STANDARD CURVE:

Equation 1 (excess *exo*-inulinase):

milli-Units / assay (i.e. 0.1 mL) = 15.1 + 496.8 x Abs + 211 x Abs^2

Equation 2 (no *exo*-inulinase):

milli-Units / assay (i.e. 0.1 mL) = 19.52 + 1416 x Abs + 1877 x Abs^2

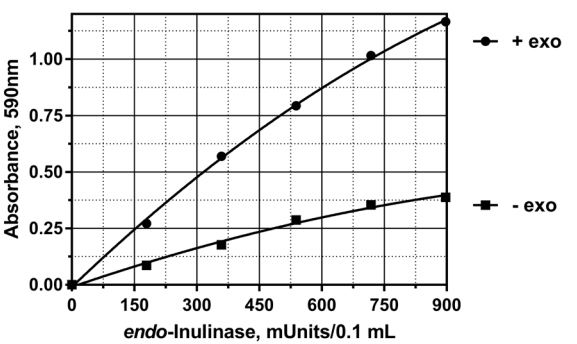

Figure 1. Standard curve relating activity of *endo*-inulinase on Azo-Fructan (Lot 30102) at 40°C and pH 4.5 to activity on inulin (10 mg/mL) at 40°C and pH 4.5 in the presence and absence of added *exo*-inulinase.

One Unit of *endo*-inulinase activity is defined as the amount of enzyme required to release one micromole of fructose reducing-sugar equivalents per minute from inulin (10 mg/mL) at pH 4.5 and 40°C.

CALCULATIONS:

Units/mL of original solution

$$= \text{milliUnits per assay (ie. per 0.1 mL)} \times 10 \times \frac{1}{1000} \times \text{Dilution}$$

where:

mU/assay	= calculated using Equation 1 or 2.
10	= adjustment from 0.1 mL to 1.0 mL.
1/1000	= conversion from milliUnits to Units.
Dilution	= total dilution of enzyme.

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