Megazyme

ASSAY OF *endo*-FRUCTANASE using AZO-FRUCTAN

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PRINCIPLE:

The assay procedure is specific for *endo*-inulinase in the presence of *exo*-inulinase. The substrate is the high molecular weight fraction of chicory inulin dyed with an azo-dye. *exo*-Inulinase hydrolyses this substrate in an *exo*-fashion, and cannot hydrolyse a fructose molecule containing a dye molecule. However, because the fructan molecules are quite small (20-40 sugar units), the release of fructose from the non-reducing end of some molecules renders these soluble in the presence of the precipitant solution. Thus blank absorbance values in the absence of *exo*-inulinase are about 0.05 absorbance units, and in the presence of excess quantities of *exo*-inulinase (e.g. 20 Units per assay) the blank absorbance value increases to about 0.23 absorbance units.
In the standard assay procedure, Azo-fructan solution is incubated (in the presence of excess *exo*-inulinase) 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. It is evident that the presence of *exo*-inulinase affects the sensitivity of the assay (refer to Figure 1). Basically, 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.

SUBSTRATE:

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. 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 quite stable at room temperature for several days, but, we recommend storage at 4°C between use.
For long term storage, the solution can be frozen.
The *exo*-inulinase, as supplied, is diluted in sodium acetate buffer (0.1 M. pH 4.5) to give a final concentration of 200 U/ml.
This is stored frozen in polypropylene tubes.

BUFFER: SODIUM ACETATE (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. (Approximately 25 ml is required). Adjust the volume 1 litre. Store at 4°C.

PRECIPITANT SOLUTION:

The precipitant solution is prepared by adding 100 ml of 2M potassium hydroxide to 1 litre of 95% ethanol, with thorough mixing.

ENZYME PREPARATION:

Enzyme solution is diluted in 0.1 M sodium acetate buffer (pH 4.5). This solution is filtered if necessary, and further diluted in 0.1 M sodium acetate buffer (pH 4.5), as required to obtain the correct range of activity for assay.

ASSAY PROCEDURE:

To glass test-tubes (16 x 120 mm) add 0.5 ml of Azo-fructan solution (2%w/v) and 0.1 ml of *exo*-inulinase (20 U). Pre-equilibrate the tubes at 40°C for 2 min and then initiate the reaction by the addition of 0.1 ml of *endo*-inulinase enzyme preparation with vigorous stirring on a vortex mixer. This mixture is stirred and incubated at 40°C for exactly 10 min. The reaction is terminated and high-molecular weight substrate is precipitated by the addition of 2.5 ml of precipitant solution with vigorous stirring for 10 sec on a vortex mixer. The reaction tubes are allowed to equilibrate to room temperature for 5 min and are then centrifuged at 3,000 rpm (1,000g) for 10 min. The supernatant solution is poured directly from the centrifuge tube into a spectrophotometer cuvette and the absorbance of blank and reaction solutions are measured at 590nm against the reaction blank. Activity is determined by reference to a Standard Curve.

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 mixture and incubating this under the same conditions as the reaction mixture. Reaction blank absorbance values are approximately 0.230 absorbance units. This solution is used to zero the spectrophotometer.

STANDARD CURVE:

A typical standard curve is shown in Figure 1. This curve is for pure *Aspergillus niger endo*-inulinase diluted in 0.1 M sodium acetate buffer (pH 4.5) on Azo-Fructan Lot 80201. Enzyme activity is standardised using dahlia fructan (10 mg/ml) as substrate in 100 mM sodium acetate buffer (pH 4.5) at 40°C using the Nelson/Somogyi reducing sugar method.

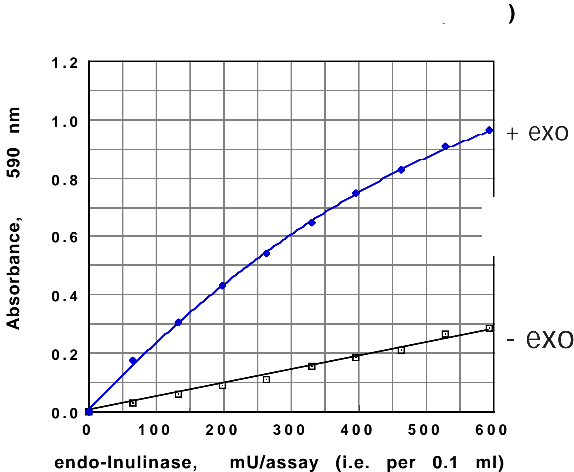

Figure 1. Standard curves for the action of *endo*-inulinase on Azo-Fructan (Lot 80201) in the presence and absence of added *exo*-inulinase.

One Unit of activity is defined as the amount of enzyme required to release one micromole of fructose reducing-sugar equivalents per minute under the defined assay conditions.

CALCULATIONS:

Units/ml of original soln

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

WHERE:

milliUnits per assay is determined by reference to the Standard Curve

10 = adjustment from 0.1 ml to 1.0 ml.

$\frac{1}{1000}$ = conversion from milliUnits to Units.

Dilution = further dilution of the original enzyme extract.

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