

Megazyme

**ASSAY OF
endo-1,4- β -Glucanase
(Cellulase)
using**

AZO-CM-CELLULOSE

S-ACMC 09/2010



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PRINCIPLE:

This assay procedure is specific for the *endo*-1,4- β -D-glucanase activity (*endo*-Cellulase) present in cellulase preparations. On incubation of dyed CM-cellulose with cellulase, the substrate is depolymerised by an *endo*-mechanism to produce low-molecular weight dyed fragments which remain in solution on addition of a precipitant solution to the reaction mixture. High-molecular weight material is removed by centrifugation and the colour of the supernatant is measured. *endo*-Cellulase in the assay solution is determined by reference to a standard curve.

SUBSTRATE:

The substrate is partially depolymerised and dyed CM-Cellulose 4M. The polysaccharide is dyed with Remazolbrilliant Blue R to an extent of approx. one dye molecule per 20 sugar residues.

DISSOLUTION:

Add 2 g of powdered substrate to 80 mL of boiling and vigorously stirring water on a hot-plate, magnetic stirrer. Turn the heat off and continue stirring until the solution/slurry is homogeneous (approx. 20 min). Add 5 mL of sodium acetate buffer (2 M, pH 4.5) and cool the solution to room temperature. Adjust the pH to 4.5 and the volume to 100 mL. Store this solution at 4°C between use. Under these conditions and if the solution is not accidentally contaminated with *endo*-cellulase, the substrate is stable for at least 12 months.

Before use, mix the substrate solution vigorously by shaking the storage bottle. As the substrate solution is viscous, it should preferably be dispensed with a positive displacement dispenser (eg. Eppendorf Multipipette® with a 5.0 mL Combitip).

BUFFER SOLUTION:

(Sodium Acetate buffer, 100 mM, pH 4.6)

Add 6.0 g of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water. Adjust the pH to 4.6 by the addition of 5 M (20 g/100 mL) sodium hydroxide solution. Approximately 50 mL is required. Adjust the volume to 1 L.

PRECIPITANT SOLUTION:

Dissolve 40 g of sodium acetate trihydrate and 4 g of zinc acetate in 150 mL of demineralised water. Adjust the pH to 5.0 with 5 M HCl and the volume to 200 mL with demineralised water. Add 200 mL of this solution to 800 mL of industrial methylated spirits (IMS, 95 %) or ethanol (95 %), mix well and store at room

temperature in a well sealed bottle.

ENZYME EXTRACTION AND DILUTION:

Add 1 mL of liquid enzyme sample to 49 mL of extraction/dilution buffer (pH 4.5) using a positive displacement dispenser (these solutions can be very viscous), and mix thoroughly. This is termed the **Original Extract**. Add 1.0 mL of this solution to 9.0 mL of extraction/dilution buffer (10-fold dilution). Repeat this process of dilution until a concentration suitable for assay is achieved. For example, a dilution of the Original Extract of approx. 100-fold is required for the industrial enzyme preparation, Laminex BG (from *Trichoderma* sp.; Genencor International).

With powdered samples, add 1.0 g of the preparation to 50 mL of extraction/dilution buffer (pH 4.5) and gently mix the slurry over 15 min or until the sample is completely dispersed or dissolved. This is termed the **Original Extract**, and is clarified by centrifugation (1,000 g, 10 min) or filtration through Whatman No. 1 (9 cm) filter circles. Further dilution of this extract with buffer is performed as for the liquid enzyme samples.

ASSAY PROCEDURE:

Add 0.5 mL of enzyme solution (pre-equilibrated at 40°C) to 0.5 mL of pre-equilibrated substrate solution and stir on a vortex mixer. Incubate at 40°C for exactly 10 min and terminate the reaction (with precipitation of high-molecular weight unhydrolysed substrate) by the addition of 2.5 mL of Precipitant Solution with vigorous stirring for 10 sec on a vortex mixer. Allow the reaction tubes to equilibrate to room temperature for 10 min. Stir the tube contents again and centrifuge the tubes at 1,000 g for 10 min. Pour the supernatant solution directly into a spectrophotometer cuvette and measure the absorbance of the reaction solutions at 590 nm against the reaction blank. Determine enzyme activity by reference to a standard curve.

Prepare the reaction blank by adding precipitant solution to substrate solution and mixing, then add the enzyme solution and mix well. Usually, a single blank is required with each set of determinations, and this is used to zero the spectrophotometer.

STANDARD CURVE:

A typical standard curve is shown below. This curve is for pure *Trichoderma* sp. cellulase from Laminex preparation (Genencor International) diluted in 100 mM sodium acetate buffer (pH 4.5). A suitable dilution for Original Extract of Laminex preparation is approx. 200-fold.

CALCULATION OF ACTIVITY:

endo-Cellulase activity is determined by reference to the standard curve to convert absorbance to milliUnits of activity per assay (i.e. per 0.5 mL) on CMC-4M, and then calculated as follows:

Units/mL or gram of Original Preparation:

$$= \text{milliUnits (per assay i.e. per 0.5mL)} \times 2 \times 50 \times \frac{1}{1000} \times \text{Dilution}$$

where:

2 = conversion from 0.5 mL to 1.0 mL.

50 = the volume of buffer used to extract the original preparation (i.e. 1.0 g/50 mL or 1.0 mL of enzyme added to 49 mL of buffer).

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

Dilution = further dilution of the **Original Extract**.

$$\text{milli U/assay} = 412.5 \times \text{Abs} + 7.2; R^2 = 0.99$$

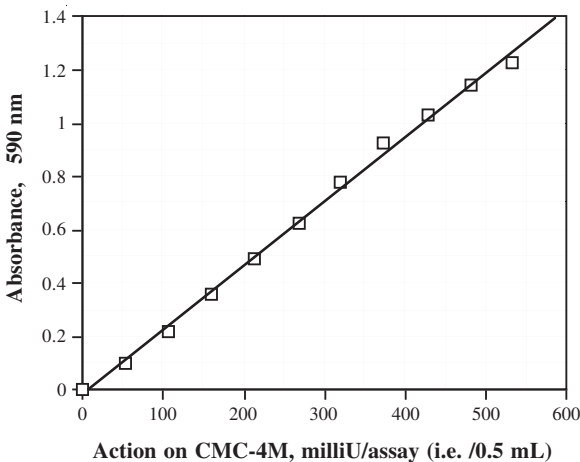


Figure. 1. *Trichoderma* sp. endo-Cellulase standard curve on Azo-CM-Cellulose (Lot 90504).



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