Megazyme

ASSAY OF

endo-CELLULASE

using

CELLAZYME T TABLETS

T-CTZ200
T-CTZ1000
03/05

SUBSTRATE:

The substrate employed is azurine-crosslinked Tamarind Xyloglucan (AZCL-Xyloglucan). The substrate is prepared by dyeing and cross-linking highly purified xyloglucan to produce a material which hydrates in water but is water insoluble. Hydrolysis by *endo*-(1-4)-β-D-glucanase (cellulase) produces water soluble dyed fragments and the rate of release of these (increase in absorbance at 590 nm) can be related directly to enzyme activity. The substrate is supplied commercially in ready-to-use tablet form as **Cellazyme T Tablets**.

BUFFER STOCK SOLUTION: (Sodium Acetate buffer, 2 M, pH 4.5)

Add 120.0 g of glacial acetic acid (1.05 g/mL) to 1600 mL of distilled water. Adjust the pH 4.5 by the addition of 5 M (20 g/100 mL) sodium hydroxide solution. Adjust the volume to 1 litre. Stable at room temperature for > 2 years.

DILUTION/ASSAY BUFFER: (Sodium acetate, 100 mM, pH 4.5) containing sodium azide (0.02% w/v)

Add 50 mL of buffer stock solution (1 M) to 850 mL of distilled water. Adjust the pH 4.5 by dropwise addition of 2 M hydrochloric acid solution. Add 0.2 g of sodium azide and dissolve. Adjust the volume to 1 litre. Stable at 4°C for approx 1 month.

NOTES:

1. When preparing the extraction buffer, do not add the **sodium azide** until the pH is adjusted. Acidification of sodium azide releases a poisonous gas.
2. In the assay format described here, **a single blank** is required for each set of determinations and this is used to zero the spectrophotometer. The absorbance of the reaction solutions are read against this blank.
3. Test tubes should not be stirred on addition of the Cellazyme T tablet to the enzyme solution. This gives a slight (about 5%) increase in the absorbance value, but the results are less reproducible. The standard curves given in this booklet were prepared using the standard assay format (tubes not stirred).

ENZYME EXTRACTION AND DILUTION:

With liquid preparations, using a positive displacement dispenser (these solutions can be very viscous) add 1.0 mL of enzyme preparation to 49 mL of extraction/dilution buffer (100 mM, pH 4.5) and mix thoroughly. This is termed the **Original Extract**. Dilute 1.0 mL of this solution 10-fold by addition to 9 mL of extraction/dilution buffer. Repeat this process of dilution until a concentration of enzyme suitable for assay is obtained. For example, with the industrial enzyme preparation Celluclast (from *Trichoderma* sp.; Novozymes, Denmark) a dilution of the original extract of approximately 100-fold is required.

With powder preparations, add 1.0 g of sample to 50 mL of extraction/dilution buffer and gently stir the suspension for approx. 15 min. Clarify this solution, the **Original Extract**, by centrifugation (1,000 *g*, 10 min) or by filtration through Whatman No. 1 (9 cm) filter circles. Dilute 1.0 mL of this solution 10-fold by addition to 9 mL of extraction/dilution buffer. Repeat this process of dilution until a concentration of enzyme suitable for assay is obtained.

ASSAY PROCEDURE:

1. Add 0.5 mL of suitably diluted enzyme preparation in sodium acetate buffer (100 mM, pH 4.5) to a glass test-tube (16 x 120 mm) and pre-equilibrate to 40°C for 5 min.
2. Initiate the reaction by the addition of a Cellazyme T tablet. The tablet hydrates rapidly. Do not stir the tube.
3. Terminate the reaction after exactly 10 min at 40°C by adding 10.0 mL of tri-sodium phosphate solution (2% w/v, pH 11.0) with vigorous stirring on a vortex mixer.
4. Allow the tubes to stand for approx. 4-5 min at room temperature and then stir the contents again. Filter the slurry through a Whatman No. 1 (9 cm) filter circle.
5. Measure the absorbance of the filtrate at 590 nm against a substrate/enzyme blank. Prepare the **substrate/enzyme blank** by adding tri-sodium phosphate to the enzyme solution before the addition of the Cellazyme T tablet. This slurry **must** be left at room temperature.

NOTE: A single blank is required for each set of determinations and this is used to zero the spectrophotometer. The absorbance of the reaction solutions are measured against this blank.

STANDARDISATION:

A standard curve relating the activity of purified *Trichoderma* sp. cellulase (EG II) on CM-cellulose 4M and Cellazyme T tablets (Lot 00601) is shown in Figure 1. Activity on CM-cellulose 4M was determined at a substrate concentration of 10 mg/mL in 100 mM sodium acetate buffer (pH 4.5) at 40°C using the Nelson/Somogyi reducing sugar procedure.

A standard curve relating the activity of purified *Trichoderma* sp. cellulase (EG II) on barley β-glucan and Cellazyme T tablets (Lot 00601) is shown in Figure 2. Activity on barley β-glucan was determined at a substrate concentration of 10 mg/mL in 100 mM sodium acetate buffer (pH 4.5) at 40°C using the Nelson/Somogyi reducing sugar procedure.

One Unit of activity is defined as the amount of enzyme required to release one micromole of glucose reducing-sugar-equivalents per minute from either CM-cellulose 4M (10 mg/mL; Lot 81101) or barley β-glucan (10 mg/mL; Lot 90603) at pH 4.5 and 40°C.

CALCULATION OF ACTIVITY:

***endo*-Cellulase** activity is determined by reference to the standard curve to convert absorbance to milliUnits of activity per assay on barley β-glucan, and then calculated as follows:

Units/mL or gram of Original Preparation:

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

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 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**.

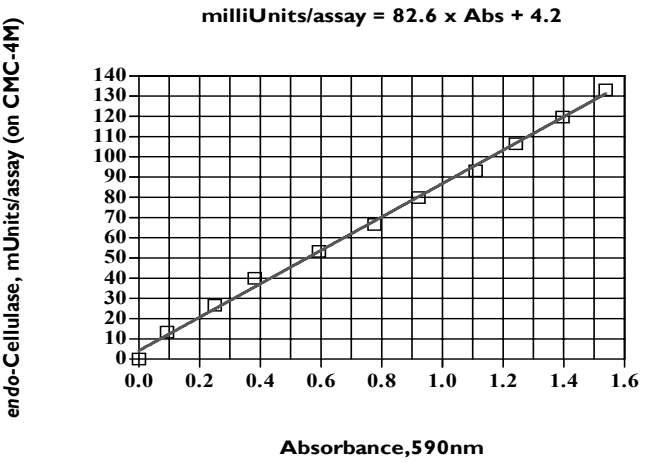

Figure 1. *Trichoderma* sp. *endo*-Cellulase (EG II) standard curve on Cellazyme T (Lot 00601) in 100 mM sodium acetate buffer (pH 4.5). Enzyme standardised on CM-cellulose 4M at pH 4.5.

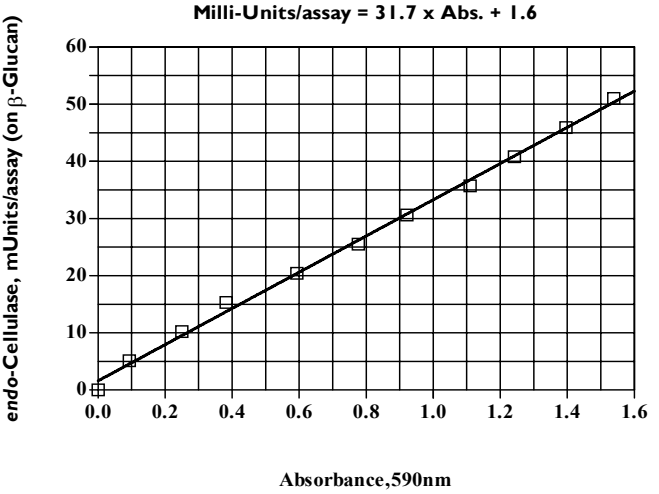

Figure 2. *Trichoderma* sp. *endo*-Cellulase (EG II) standard curve on Cellazyme T (Lot 00601) in 100 mM sodium acetate buffer (pH 4.5). Enzyme standardised on barley β-glucan (at pH 4.5).

BACKGROUND:

Cellazyme T tablets can be used to selectively assay for some *endo*-cellulases in the presence of others. The highly regular nature of the substitution of the "cellulose backbone" of tamarind xyloglucan means that this backbone is susceptible to hydrolysis by some *endo*-cellulases, but not by others. Table 1 shows the relative rates of hydrolysis of tamarind xyloglucan by a highly purified cellulase from *Trichoderma longibrachiatum* (EG II) and by the major *endo*-cellulase in *Aspergillus niger* preparations (MW = 25,769, pI = 4.55).

Table 1. Relative rates of hydrolysis of CM-cellulose 4M, barley β-glucan and tamarind xyloglucan by EG II from *T. longibrachiatum* and *endo*-cellulase (crystalline) from *A. niger*.

Substrate	Relative rates of hydrolysis, %	
	EG II (*Trichoderma*)	*A. niger* cellulase
CM-cellulose 4M	100	100
Barley β-glucan	38	133
Tamarind xyloglucan	63	< 0.00001

Substrate concentration, 10 mg/mL in 100 mM sodium acetate buffer (pH 4.5). Activity measured with the Nelson-Somogyi reducing sugar method with D-glucose as standard. After colour development, tubes were centrifuged (1,000 *g*, 10 min) if necessary, to remove insoluble substrate.

Clearly, tamarind xyloglucan is readily hydrolysed by *T. longibrachiatum* cellulase (EG II), but is highly resistant to hydrolysis by *A. niger* cellulase. Cellazyme T tablets are also very resistant to hydrolysis by *A. niger* cellulase. On incubation of 6,300 mUnits of *A. niger endo*-cellulase with Cellazyme T tablets for 10 min, an absorbance increase of 0.537 was obtained. In a similar incubation with *T. longibrachiatum* EGII, the same colour increase was obtained with just 40 mUnits of enzyme, i.e. a difference in sensitivity of approx. 4000-fold.

NOTES:

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