

Megazyme

ASSAY OF *endo*- β -GLUCANASES USING BETA-GLUCAZYME TABLETS

T-BGZ 12/12

Note: Changed assay format for malt β -glucanase



SUBSTRATE:

The substrate employed is Azurine-crosslinked barley β -glucan (AZCL-Beta-Glucan). The substrate is prepared by dyeing and cross linking highly purified β -glucan to produce a material which hydrates in water but is water insoluble. Hydrolysis by cellulases (*endo*-1,4- β -glucanase), malt β -glucanase or bacterial 1,3:1,4- β -glucanase (lichenase), 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 a ready-to-use tablet form as Beta-Glucazyme Tablets.

APPLICATIONS:

This substrate can be used to assay any *endo*-acting enzyme which is active on barley β -glucan. Such enzymes include cellulases (*endo*-1,4- β -glucanase), malt β -glucanase, bacterial 1,3:1,4- β -glucanase (lichenase), and non-specific *endo*-1,3- β -glucanases (i.e. enzymes which can act on 1,3- β -glucans and can also hydrolyse 1,4- β -linkages next to a 1,3- β -linkage as in barley and oat β -glucans).

STOCK BUFFER: (Sodium acetate buffer, 1 M, pH 4.2).

Add glacial acetic acid (60.0 g, 1.05 g/mL) to 800 mL of distilled water. Adjust the pH to 4.2 by the addition of 4 M (16 g/100 mL) sodium hydroxide solution. Adjust the volume to 1 litre.

EXTRACTION/DILUTION BUFFER:

[Sodium acetate, 25 mM, pH 4.2 or pH 4.5, containing sodium azide (0.02 %)].

Add 25 mL of stock buffer solution to 950 mL of distilled water and adjust the pH to pH 4.2 (or pH 4.5) by dropwise addition of 1 M hydrochloric acid solution. Adjust the volume to 1 litre and add 0.2 g of sodium azide.

NOTE: Do not add the sodium azide until the pH is adjusted. Acidification of sodium azide releases a poisonous gas.

Sodium azide is added solely as an anti-microbial agent. This chemical can be deleted from the mixture, but the buffer should then be stored at 4°C.

A. ASSAY OF MALT FLOUR β -GLUCANASE

ENZYME EXTRACTION AND DILUTION:

- 1 Accurately weigh 0.5 g of milled malt (milled to pass a 0.5 mm screen) into a glass centrifuge tube (16 x 120 mm).
- 2 Add 5.0 mL of extraction/dilution buffer (25 mM, pH 4.2) to the tube and stir the contents thoroughly on a vortex mixer.
- 3 Allow the enzyme to extract over a 15 min period at room temperature (less than 30°C), with occasional mixing.
- 4 Centrifuge the tube and contents at 1,000 g (3,000 rpm) for 10 min, or filter the slurry through glass fibre filter paper (Whatman GF/C).

NOTE: The final pH of the extract is pH 4.5-4.6, which is optimal for malt β -glucanase.

ASSAY PROCEDURE:

- 1 Equilibrate 0.5 mL aliquots of suitably diluted and buffered enzyme in glass test tubes (16 x 120 mm) to 30°C for 5 min.
- 2 Initiate the reaction by adding a Beta-Glucazyme Tablet (60 mg). The tablet hydrates rapidly. Do not stir the suspension.
- 3 Exactly **10.0 min** after addition of the tablet, add **10.0 mL** of Trizma Base solution (2 % w/v, Sigma Cat. No. T-1503) to terminate the reaction. Stir the tube contents vigorously on a vortex mixer.
- 4 Allow the tubes to stand at room temperature for approx. 5 min, and once again stir the tube contents. 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 blank.

NOTE:

- 1 Prepare a substrate blank** by adding a Beta-Glucazyme tablet to 0.5 mL of extraction/dilution buffer, incubate at 30°C for 10 min, add 10.0 mL of Trizma Base (2 % w/v) and filter after 5 min.
- 2** A single blank is required for each set of determinations and this is used to zero the spectrophotometer. Measure the absorbance of the reaction solutions at 590 nm against the blank. If the absorbance of the reaction solution is above **1.2**, dilute an aliquot of the enzyme extract with an equal volume of extraction/dilution buffer and **repeat the assay**.

STANDARDISATION:

A standard curve relating the activity of purified malt β -glucanase on Beta-Glucazyme (Lot 110901) is shown in Figure 1. This curve is relatively linear over the absorbance range 0.05 to 1.2 absorbance units (after subtraction of the blank).

Activity on barley β -glucan (Lot 60501) was determined at a substrate concentration of 10 mg/mL in 100 mM sodium acetate buffer (pH 4.6) at 30°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 reducing-sugar equivalents per minute under the defined assay conditions.

To determine Malt β -Glucanase activity, refer to Figure 1 (or to the regression equation) which converts absorbance values to Units of activity / Kg of malt.

The effects of extraction time, pH and the concentration of the extraction buffer on apparent enzyme activity are shown in Figs. 2-4.

$$U/\text{Kg malt} = 94 \times \text{Abs}^2 + 381 \times \text{Abs} + 28$$

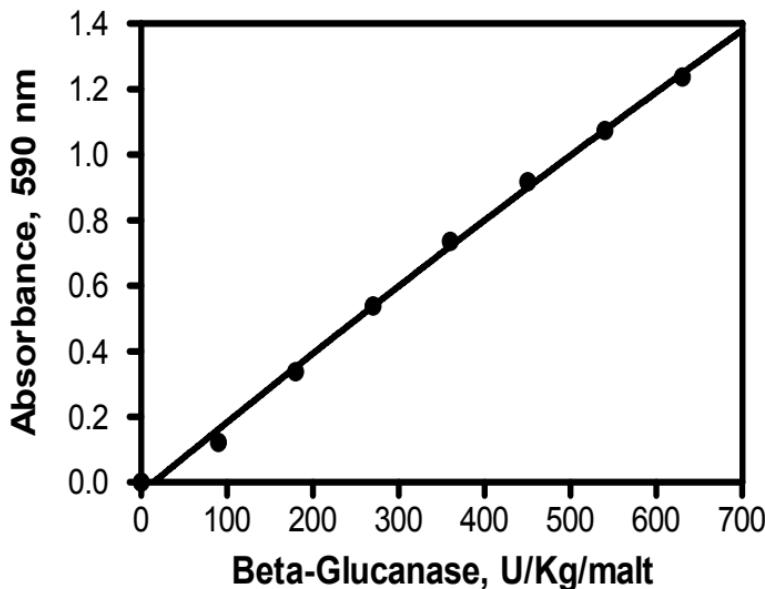


Figure 1. Malt β -glucanase standard curve on Beta-Glucazyme tablets (Lot 110901).

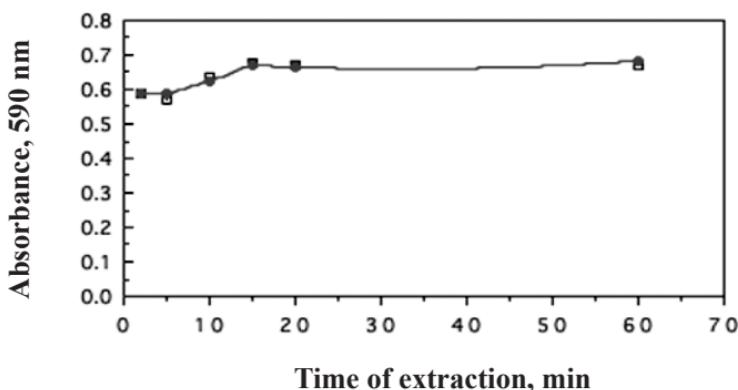


Figure 2. Effect of extraction time on the measured level of β -glucanase (as absorbance values) in malt flour.

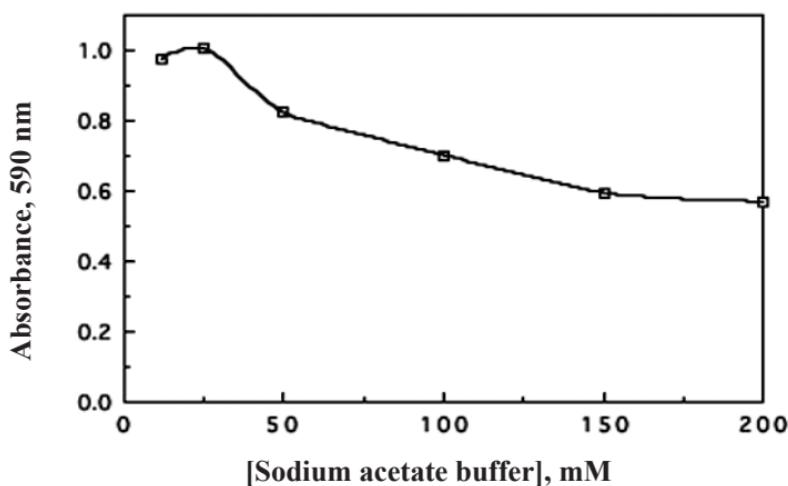


Figure 3. Effect of buffer salt concentration on the sensitivity of the malt β -glucanase, Beta-Glucazyme test procedure.

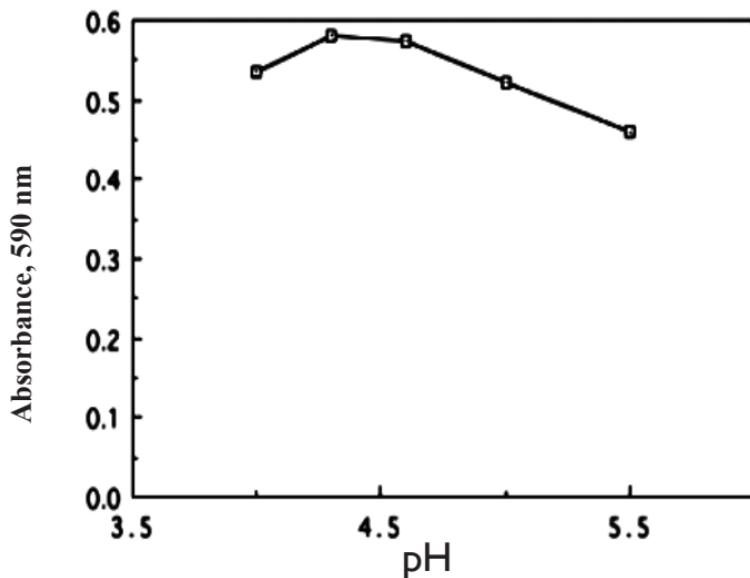


Figure 4. Effect of the pH of the extraction buffer (at 25 mM) on the pH of the extract and the apparent level of β -glucanase (as absorbance values) in malt flour extracts.

B. ASSAY OF MICROBIAL CELLULASES (*endo*-1,4- β -D-GLUCANASE)

EXTRACTION/DILUTION BUFFER:

Sodium acetate (25 mM, pH 4.5) plus sodium azide (0.02 %)

See page 1.

NOTE: Do not add the sodium azide until the pH is adjusted.
Acidification of Na azide releases a poisonous gas.

Sodium azide is added solely as an anti-microbial agent. This chemical can be deleted from the mixture, but the buffer should then be stored at 4°C.

ENZYME EXTRACTION AND DILUTION:

- 1 **With liquid samples**, using a positive displacement, add 1.0 mL of enzyme preparation (these solutions can be very viscous) to 49 mL of dilution buffer (pH 4.5) and mix thoroughly. This is termed the **Original Extract**.
- 2 Dilute an aliquot (1.0 mL) of this solution 10-fold by adding it to 9.0 mL of dilution buffer. Repeat this process of dilution until a concentration of the enzyme suitable for assay is achieved.
- 3 **With powder samples**, add 1.0 g of the preparation to 50 mL of extraction buffer (pH 4.5) and mix the slurry by inversion over a period of 5 min, or until the sample is completely dispersed or dissolved. Clarify this solution (**the Original Extract**) by centrifugation (1,000 g, 10 min), or filtration through Whatman No. 1 (9 cm) filter circles. Dilute this extract further with dilution buffer, as for the liquid samples.

This procedure can be used to extract *endo*-glucanase from industrial enzyme preparations, bread improver blends, enzyme supplemented poultry diets and cereal flours.

ASSAY PROCEDURE:

- 1 Pre-equilibrate 0.5 mL aliquots of suitably diluted enzyme in sodium acetate buffer (25 mM, pH 4.5) at 40°C for 5 min.
- 2 Initiate the reaction by adding a Beta-Glucazyme tablet. The tablet hydrates and swells rapidly.
- 3 Terminate the reaction exactly 10 min after addition of the tablet by adding 10.0 mL of Trizma Base solution (2 % w/v, pH ~ 8.5; Sigma Cat. No. T1503) with vigorous stirring on a vortex mixer.
- 4 Leave the tubes at room temperature for about 5 min, and stir the slurry again. Filter the slurry through a Whatman No. 1 (9 cm) filter circle. Measure the absorbance of the filtrate at 590 nm.
- 5 Prepare a **substrate/enzyme blank** by adding 10.0 mL of Trizma Base solution to the enzyme solution before the addition of the Beta-Glucazyme tablet.

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 pure *Trichoderma* sp. endo-cellulase from pure endo-1,4- β -D-glucanase (EGII) on barley β -glucan and **Beta-Glucazyme** tablets is shown in Figure 5.

Activity on barley β -glucan (Lot 60501) was determined at a substrate concentration of 10 mg/mL in 50 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 barley β -glucan (10 mg/mL) at pH 4.5 and 40°C.

A Beta-Glucazyme tablet (I10901) was added to pure Trichoderma sp. endo-1,4- β -D-glucanase (0.5 mL, 0-33 milliUnits on barley β -glucan) in 25 mM sodium acetate buffer (at 40°C) without stirring. The reaction was terminated after exactly 10 min by adding 10 mL of Trizma base solution (2 % w/v) with vigorous stirring. The tubes were stirred again after approximately 5 min, and the slurries filtered through Whatman No. 1 (9 cm) filter circles. The absorbance of the reaction solutions were read against an enzyme/substrate blank solution at 590 nm.

CALCULATION OF ACTIVITY:

endo-1,4- β -D-glucanase (*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. 1.0 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.

$$\text{mU/assay} = 23.86 \times \text{Abs} + 0.856 - 2.6 \times \text{Abs}^2$$

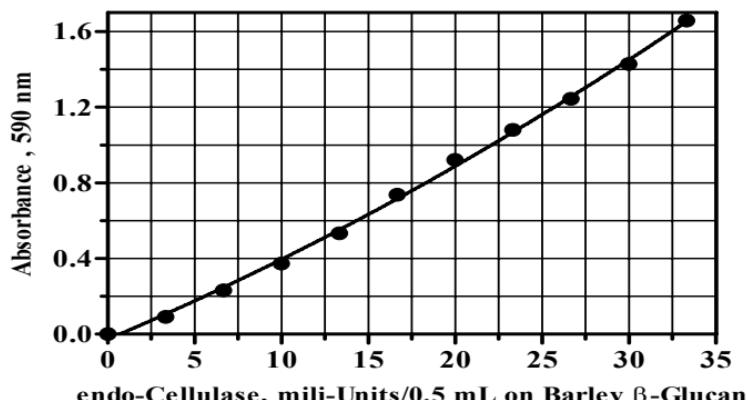


Figure 5. Trichoderma sp. endo-cellulase standard curve on Beta-Glucazyme (Lot I10901).

NOTES:



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