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Note

Quantitative fluorometric assay for the measurement of *endo*-1,4-β-glucanase

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ABSTRACT

There is a growing demand for research tools to aid the scientific community in the search for improved cellulase enzymes for the biofuel industry. In this work, we describe a novel fluorometric assay for cellulase (*endo*-1,4-β-glucanase) which is based on the use of 4,6-*O*-benzylidene-4-methylumbelliferyl-β-cellotrioside (BzMUG3) in the presence of an ancillary β-glucosidase. This assay can be used quantitatively over a reasonable linear range, or qualitatively as a solution screening tool which may find extensive use in the area of metagenomics.

Cellulose is the most abundant polymer on Earth. The attractiveness of biomass as a renewable energy source has led to increased funding into the research of cellulose degrading enzymes.[1] There currently exists a requirement for a rapid, sensitive and specific method for the assay of these enzymes given that current methods suffer from a number of deficiencies.

Traditional reducing sugar assays[2] on cello-oligosaccharides or borohydride reduced cello-oligosaccharides are time consuming. The use of nitrophenyl cello-oligosaccharides[3–6] allows for rapid assays but can only be used on pure enzymes as they are susceptible to hydrolysis by *exo*-acting hydrolytic enzymes often found in crude preparations containing cellulase. Viscometric methods[7] and dyed or dyed and cross-linked substrates[8] are used for this purpose but they are difficult to use in automated formats.

Mewis et al. have recently reported a high throughput screening protocol for biomining cellulase activity from metagenomic libraries in a 384-well microplate format that is based on the hydrolysis of 2,4-dinitrophenyl-β-cellobioside.[9] While this method demonstrates the power of high throughput screening, the substrate employed could be improved upon as 2,4-dinitrophenyl-β-cellobioside is not selectively hydrolysed by cellulase and can be readily cleaved by *exo*-acting enzymes such as β-glucosidase (EC 3.2.1.21) and cellobiohydrolase (EC 3.2.1.91).[10,11]

We have recently reported a detailed study on the use of 4,6-*O*-benzylidene protected chromogenic cello-oligosaccharides for the measurement of cellulase (*endo*-β-1,4-glucanase, EC 3.2.1.4).[12] This work focused on a series of cello-oligosaccharide substrates with either 4-nitrophenol or 2-chloro-4-nitrophenol employed as the chromogen. We report herein how this methodology has been extended to produce an analogous fluorometric assay that allows for even higher sensitivity. The advantages of an assay based on fluorescence spectroscopy over UV absorption spectroscopy are obvious with the former having a considerably lower limit of detection.

Outlined in Scheme 1 is the proposed format of the enzyme-coupled assay. The target substrate 1 (BzMUG3) is cleaved in the presence of cellulase to generate a protected cello-oligosaccharide fragment **2** (or similar) and a fluorogenic cello-oligosaccharide **3** (or similar) which will then be acted on by excess β-glucosidase (EC 3.2.1.21) present in the reagent mixture to liberate 4-methylumbelliferone. The 4,6-*O*-benzylidene protecting group prevents the action of β-glucosidase on the parent substrate **1**. The rate of formation of 4-methylumbelliferone is directly proportional to the cellulase activity in a given sample.

The synthesis of 4-methylumbelliferyl-β-cellotrioside (**8**) was achieved without difficulty following the synthetic protocol developed by Planas et al.[13] Benzylidene protection of the 4 and 6 positions of the glucosyl residue on the non-reducing terminus to afford **1** was carried out using standard transacetalisation conditions (Scheme 2).

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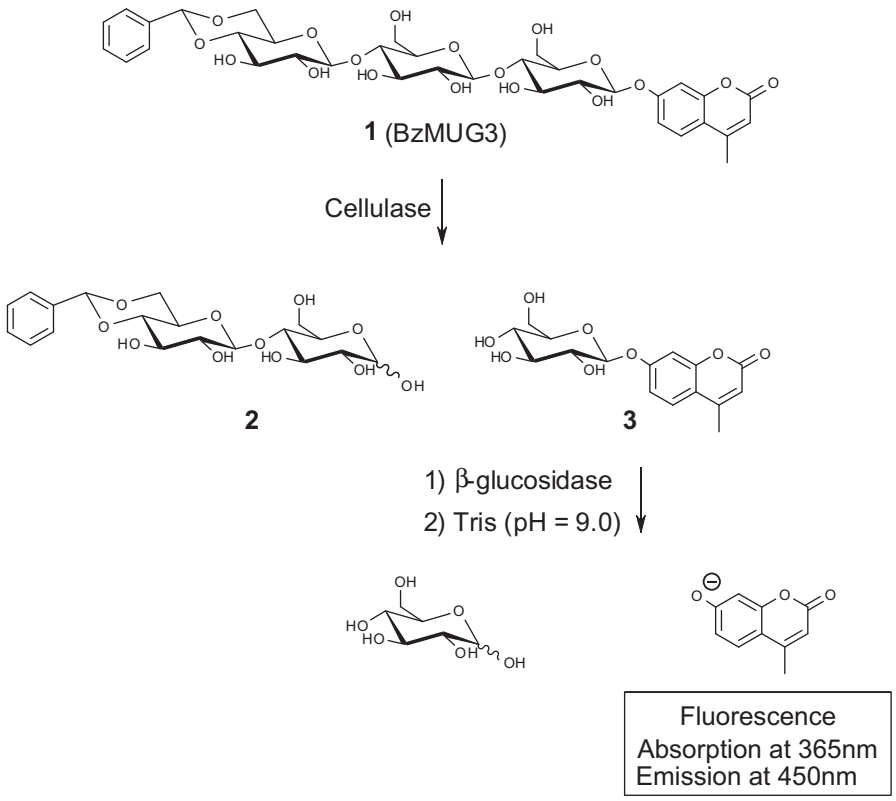
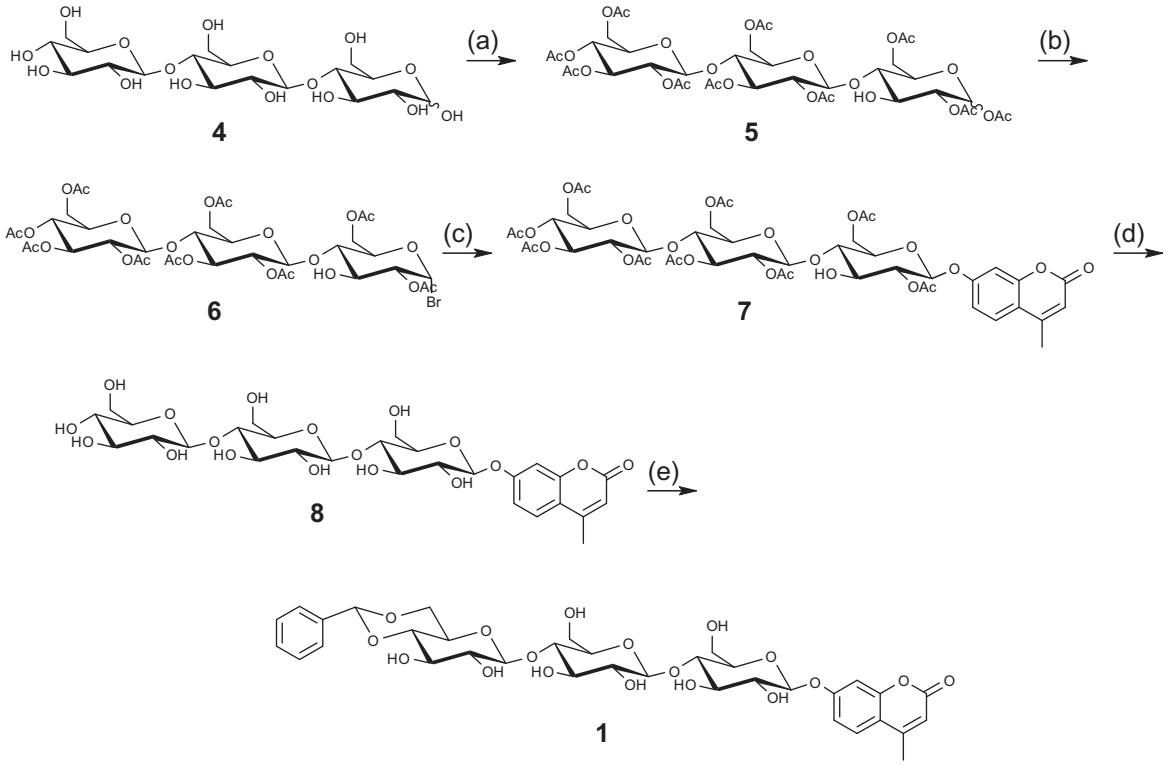


Scheme 1. Proposed enzyme-coupled cellulase assay format.



Scheme 2. Reagents and conditions: (a) Ac_2O, I_2 (cat.); (b) 33% HBr in AcOH, CH_2Cl_2, 0 °C; (c) 4-methylumbelliferone, tetra *N*-butylammoniumhydrogensulfate, CH_2Cl_2/1 M aq. NaOH; (d) $NaOCH_3$ (cat.), CH_3OH; (e) benzaldehyde dimethyl acetal, *p*-toluenesulfonic acid (cat.), DMF, 50 °C.

With the desired substrate in hand, we turned our attention to the development of a simple, easy to use assay format. A standard curve for fluorescence response versus 4-methylumbelliferyl concentration was constructed after performing serial substrate dilutions and this relationship was shown to be linear over at least a range of 1 nM–5 μM (Fig. 1).

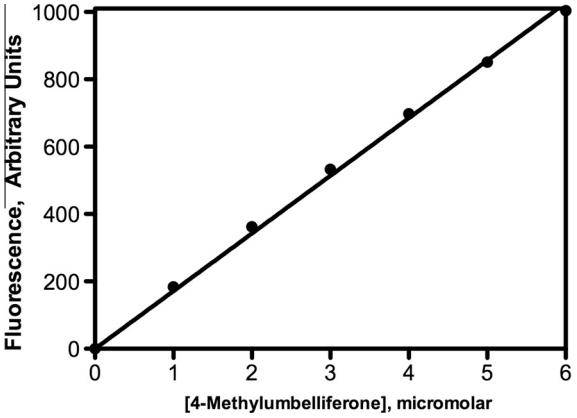


Figure 1. Standard curve relating fluorescence response against concentration of 4-methylumbelliferone in 2% Tris (pH 9.0).

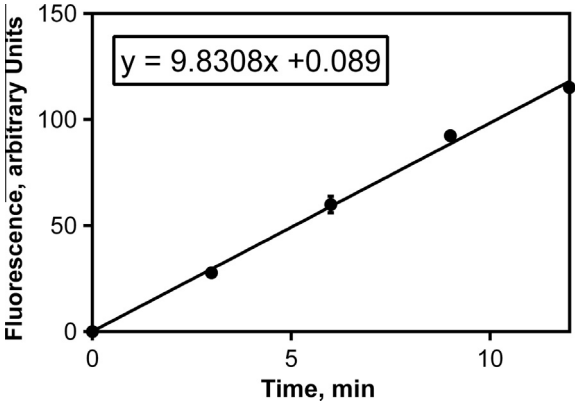


Figure 2. Fluorescent response observed over 12 min with BzMUG3 incubated in the presence of *T. longibrachiatum endo*-1,4-β-glucanase (3.16 mU/assay) and β-glucosidase (0.65 mU/assay) in pH 4.5 100 mM sodium acetate at 40 °C.

It was evident from the outset that compound **1** suffered from low aqueous solubility. Dimethyl sulfoxide (DMSO) was employed as a co-solvent to solubilise this substrate for use in the assay. It was also important to have β-glucosidase present in the substrate mixture in stable, solubilised form. Addition of β-glucosidase (50 μL, 400 U/mL in 3.2 M ammonium sulphate suspension) to a 3 mL aliquot of 50% v/v DMSO/H_2O solution of BzMUG3 (0.51 mM) resulted in a soluble mixture that was completely stable at −20 °C for at least 2 years. This BzMUG3/β-glucosidase mixture, hereafter referred to as Cellafluor, served to produce the expected linear response when incubated with a cellulase enzyme from *Trichoderma longibrachiatum* and sampled at 3 min intervals over 12 min (Fig. 2).

The assay format was further optimised by examining the effect of varying the β-glucosidase level while maintaining constant substrate concentration for a fixed cellulase activity level (Fig. 3). An activity level of 0.65 U/assay of β-glucosidase was found to be sufficient in this case to eliminate any lag phase that may be associated with the use of a coupled enzyme system.

The effect of substrate concentration on the assay was determined by incubating substrate mixtures over a concentration range of 0.1–1.0 mM containing β-glucosidase (0.65 U/assay) with a fixed quantity of *T. longibrachiatum* (31.6 mU/mL on CM-cellulose) and monitoring each reaction over a duration of 6 min (Fig. 4). Michaelis–Menten plots (Fig. 5) revealed a K_m value of 0.43 mM.

To test the sensitivity of the assay, serial dilutions of the cellulase solution were performed to probe the detection limit (Fig. 6). This experiment highlights the potential use of this substrate as a solution screening tool for metagenomic applications where extremely low level cellulase activity can be reliably identified. Cellulase activity levels as low as 1.6×10^{-4} U/assay produce a fluorescent response with a magnitude of double that of the mean blank measurement. This figure compares very favourably with the analogous UV based assay[12] where an absorbance with a magnitude of double that of the mean blank is obtained with 1.5×10^{-3} U/assay.

Stability trials for the key substrate, **1**, are currently underway. We expect this compound to be completely stable when stored at −20 °C as analogous compounds have exhibited long term storage stability (>2 years) in our previous in-house stability trials.

In conclusion, a highly sensitive, specific, fluorometric assay that is completely specific for cellulase activity has been developed. This assay can be used quantitatively over a reasonable linear range, or qualitatively as either a solution-based or agar plate-based screening tool, both of which may find extensive use in the area of metagenomics.

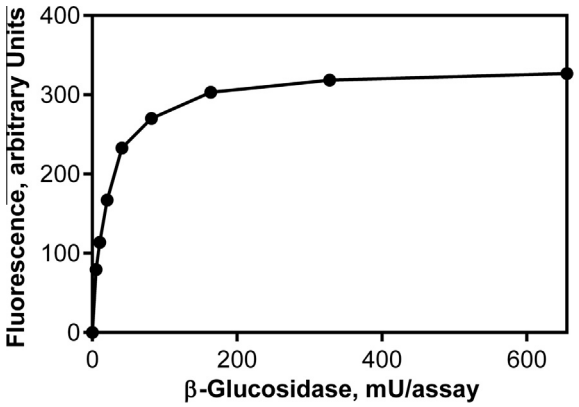


Figure 3. Effect of β-glucosidase concentration on observed fluorescence over 10 min in the presence of *T. longibrachiatum endo*-1,4-β-glucanase (1.58 mU/mL) and Cellafluor reagent mixture in pH 4.5 100 mM sodium acetate at 40 °C.

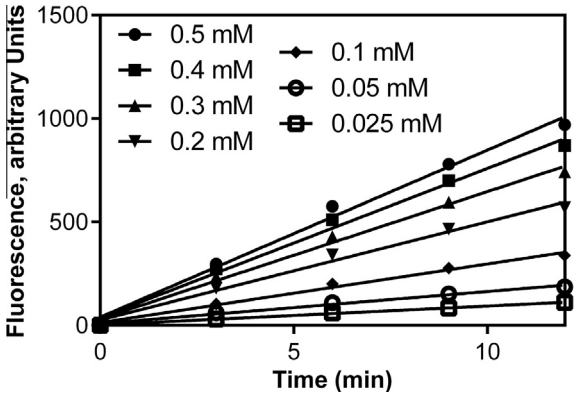


Figure 4. Effect of $BzMUG_3$ concentration on reaction kinetics over 12 min in the presence of *T. longibrachiatum endo*-1,4-β-glucanase (3.16 mU/assay) and β-glucosidase (0.65 U/assay) in pH 4.5 100 mM sodium acetate at 40 °C.

1. Experimental

1.1. General

Cellotriose (Megazyme Cat. No. O-CTR100), 4,6-*O*-benzylidene-4-methylumbelliferyl-β-cellotrioside (BzMUG3) (Megazyme Cat. No O-B4MUG3), Cellafluor (Megazyme Cat. No. R-CELLF), thermostable β-glucosidase (*Thermotoga maritima*; accession number

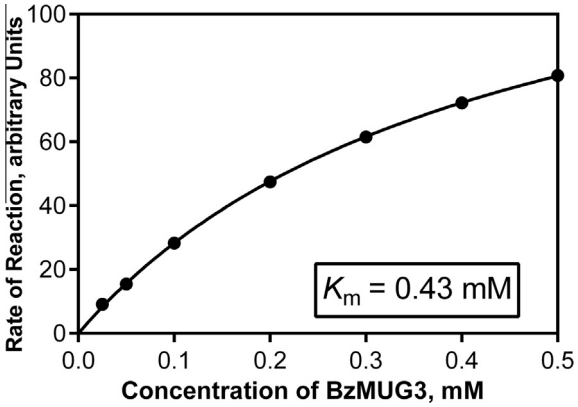


Figure 5. Determination of the Michaelis–Menten K_m constant for *T. longibrachiatum endo*-1,4-β-glucanase on $BzMUG_3$. Initial rates of reaction were determined as described in Figure 4 and Section 3.

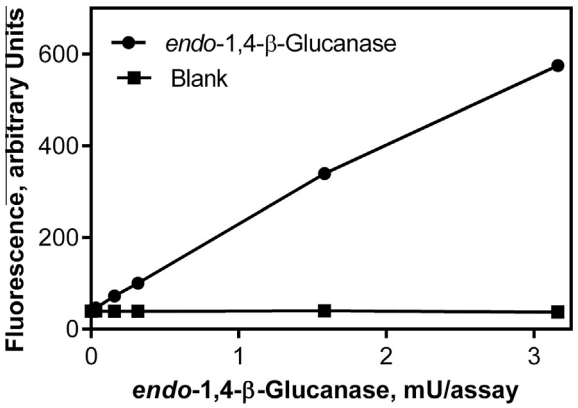


Figure 6. Effect of *T. longibrachiatum endo*-1,4-β-glucanase concentration on increase in relative fluorescence units over 10 min in the presence of Cellafluor in pH 4.5 100 mM sodium acetate at 40 °C.

Q08638, Megazyme Cat. No. E-BGOSTM, 800 U/mL) and *endo*-1,4-β-glucanase from *Trichoderma longibrachiatum* (accession number Q12714, Megazyme Cat. No. KC-CELLG3-4, 1.58 U/mL) were obtained from Megazyme International Ireland. Quoted enzyme activities were calculated as follows: 1 Unit of cellulase activity is defined as the amount of enzyme required to release 1 μmol of reducing sugar equivalents as glucose per minute from CM-Cellulose 4 M (10 mg/mL) in sodium acetate buffer (0.1 M), pH 4.5 at 40 °C. One Unit of β-glucosidase activity is defined as the amount of enzyme required to release 1 μmol of 4-nitrophenol per minute from 4-nitrophenyl-β-D-glucopyranoside (10 mM) in sodium maleate buffer (50 mM), pH 6.5 at 40 °C.

All other chemicals used in organic synthesis were purchased from Sigma Aldrich or Lennox Laboratory Supplies and were of analytical reagent grade. A Bruker Avance 400 MHz was employed for ^1H (400.13 MHz) and ^{13}C (100.61 MHz) NMR spectra. Resonances δ, are in ppm units downfield from an internal reference in C_2D_6SO (δ_H = 2.50). Mass spectrometry analysis was performed with a Q-Tof Premier Waters Maldi-quadrupole time-of-flight (Q-Tof) mass spectrometer equipped with Z-spray electrospray ionisation (ESI) and matrix assisted laser desorption ionisation (MALDI) sources. Silica gel Florisil (200 mesh; Aldrich) was used for column chromatography. Analytical thin-layer chromatography was performed using Merck 60 F_{254} silica gel (pre-coated sheets, 0.2 mm thick, 20 cm × 20 cm) and visualised by UV irradiation or 5% H_2SO_4/EtOH staining. Fluorescence measurements were obtained using a Promega Quantifluor® ST fluorometer. A Tris calibration standard (50 μg/L of 4-methylumbelliferone in 2% Tris (pH 9.0)) was used throughout this work. All biochemical assays were performed in duplicate and the average value taken. Fluorescence values (arbitrary units) for graphs were obtained by subtracting the blank (no reaction) from the averaged values of measured fluorescent response. Relative fluorescent units can be converted to 4-methylumbelliferone concentration using the standard curve (Fig. 1).

1.2. Substrate synthesis

1.2.1. 4-Methylumbelliferyl-β-cellotrioside (8)

4-Methylumbelliferyl-β-cellotrioside was synthesised from cellotriose according to the general method of Planas et al.[13] This compound has been previously synthesised by Claeyssens et al.[6] among others.

Yield: 31% from cellotriose; mp (H_2O) 225–235 °C; ^1H NMR (400 MHz, C_2D_6SO) δ 2.38 (s, 3H), 2.93–3.23 (m, 5H), 3.25–3.52 (m, 7H), 3.52–3.86 (m, 6H), 4.23 (d, J = 8.10 Hz, 1H (anom)), 4.35 (d, J = 7.93 Hz, 1H, (anom)), 4.61 (t, J = 5.29 Hz, 1H), 4.64–4.79 (m, 4H), 5.00 (d, J = 5.47 Hz, 1H), 5.04 (d, J = 5.01 Hz, 1H), 5.12 (d, J = 7.75 Hz, 1H (anom)), 5.24 (d, J = 5.02 Hz, 1H), 5.43 (d, J = 5.02 Hz, 1H), 5.57 (d, J = 5.27 Hz, 1H), 6.23 (d, J = 1.25 Hz, 1H), 6.98–7.07 (m, 2H), 7.69 (d, J = 8.71 Hz, 1H) ^{13}C NMR (100 MHz, C_2D_6SO) δ 18.7, 60.4, 60.8, 61.5, 70.5, 73.3, 73.5, 73.7, 75.2, 75.3, 75.3, 75.5, 77.0, 77.3, 80.2, 80.9, 99.9, 103.2, 103.6, 103.7, 112.2, 113.8, 114.6, 127.0, 153.9, 154.9, 160.5, 160.6; HRMS ESI$^+$ $[M+Na]^+$ Calcd 685.1956, Found 685.1982.

1.2.2. 4,6-*O*-Benzylidene-4-methylumbelliferyl-β-cellotrioside (1, BzMUG3)

To a solution of 4-methylumbelliferyl-β-cellotrioside (1 g, 1.52 mmol) and *p*-toluenesulfonic acid monohydrate (86 mg, 0.45 mmol) under an argon atmosphere in anhydrous dimethylformamide (10 mL) containing activated 4 Å molecular sieves (200 mg) was added benzaldehyde dimethylacetal (684 μL, 4.55 mmol) via syringe. The reaction was heated to 50 °C and stirred for 14 h. Triethylamine (86 μL, 0.62 mmol) was added and the reaction cooled to room temperature. The crude reaction mixture was absorbed onto silica gel and semi-purified by flash chromatography. The fractions obtained containing the desired product were combined and further purified by recrystallisation from CH_3CN/H_2O.

Yield: 30% mp (H_2O) 229–238 °C; ^1H NMR (400 MHz, C_2D_6SO) δ 2.42 (d, J = 1.01 Hz, 3H), 3.06–3.19 (m, 2H), 3.30–3.57 (m, 9H), 3.58–3.88 (m, 6H), 4.15–4.26 (m, 1H), 4.38 (d, J = 8.04 Hz, 1H (anom)), 4.52 (d, J = 7.78 Hz, 1H (anom)), 4.55 (d, J = 2.51 Hz, 1H), 4.72 (t, J = 5.52 Hz, 1H), 4.79 (t, J = 5.27 Hz, 1H), 4.82 (d, J = 2.26 Hz, 1H), 5.15 (d, J = 7.78 Hz, 1H (anom)), 5.43 (d, J = 4.52 Hz, 1H), 5.47 (d, J = 5.02 Hz, 1H), 5.57 (d, J = 5.27 Hz, 1H), 5.60 (s, 1H), 5.62 (d, J = 5.27 Hz, 1H), 6.27 (d, J = 1.25 Hz, 1H), 7.02–7.11 (m, 2H), 7.34–7.43 (m, 3H), 7.43–7.50 (m, 2H), 7.72 (d, J = 9.09 Hz, 1H) ^{13}C NMR (100 MHz, C_2D_6SO) δ 18.6, 60.4, 60.6, 66.4, 68.1, 73.3, 73.3, 73.6, 74.8, 74.8, 75.2, 75.4, 75.5, 79.4, 80.1, 80.8, 99.9, 101.2, 103.3, 103.5, 103.6, 112.2, 113.9, 114.7, 126.8, 126.8, 127.0, 128.5, 128.5, 129.4, 138.1, 153.9, 154.9, 160.5, 160.6; HRMS ESI$^-$ $[M-H]^-$ Calcd 749.2293, Found 749.2299.

1.3. Enzyme assays

1.3.1. Formulation of assay 'substrate/ancillary enzyme' mixture—Cellafluor

Substrate **1** was dissolved at a concentration of 1.02 mM in DMSO and stored at −20 °C between uses. In this form compound **1** is stable for >4 years. For use as a substrate, 1.5 mL of this solution was added to 1.5 mL of deionised water and mixed thoroughly. The solution was allowed to cool to room temperature

and thermostable β-glucosidase (50 μL, 400 U/mL in 3.2 M ammonium sulphate suspension) was then added and mixed thoroughly (final substrate concentration = 0.5 mM). This solution is hereafter referred to as Cellafluor. The substrate is stable for >2 years when stored at −20 °C.

1.3.2. Standard assay of *endo*-1,4-β-glucanase using Cellafluor (Fig. 2)

To a pre-equilibrated aliquot of Cellafluor (0.1 mL, 0.5 mM), a pre-equilibrated aliquot of *T. longibrachiatum endo*-1,4-β-glucanase (0.1 mL, 3.16 mU/mL) (or cellulase aliquot of interest) in 100 mM sodium acetate buffer pH 4.5 was added and the mixture incubated at 40 °C. The reaction was terminated after 0, 3, 6, 9 and 12 min by adding 3 mL of 2% Tris solution (pH 9.0). The tube contents were well mixed and the relative fluorescence units were determined using a Promega Quantifluor®-ST fluorometer after calibration using a 50 μg/L solution of 4-methylumbelliferone in 2% Tris solution (pH 9.0) and using 2% Tris solution as a blank.

1.3.3. Optimisation of β-glucosidase content in standard assay format (Fig. 3)

The effect of β-glucosidase concentration on the rate of hydrolysis was investigated by preparing solutions of β-glucosidase in 3.2 M ammonium sulphate suspension (400–3.125 U/mL) and was determined as follows: to a 3 mL aliquot of substrate **1** (0.51 mM) in 50% v/v DMSO/H_2O, 50 μL of β-glucosidase solution was added (to give β-glucosidase concentrations of 1.31–0.0051 U/assay). Aliquots of the substrate/β-glucosidase mixtures (0.1 mL) were each equilibrated to 40 °C and a pre-equilibrated aliquot of *T. longibrachiatum endo*-1,4-β-glucanase (0.1 mL, 15.8 mU/mL) in 100 mM sodium acetate buffer pH 4.5 was added to each aliquot and mixed thoroughly. The solutions were incubated at 40 °C for 10 min before termination of the reactions using 3 mL of 2% Tris solution (pH 9.0). The tube contents were mixed thoroughly and the relative fluorescence units were determined using a Promega Quantifluor®-ST fluorometer.

1.3.4. Investigation into the enzyme/substrate reaction kinetics—K_m (Figs. 4 and 5)

The effect of substrate concentration under the standard assay format was investigated by preparing solutions of substrate **1** in 50% v/v DMSO/H_2O at varying concentrations (1.02–0.051 mM). β-Glucosidase (50 μL, 400 U/mL) was added to 3 mL aliquots of each substrate solution. Effect of substrate concentration was determined as follows: aliquots (0.1 mL) of the substrate/β-glucosidase mixtures were equilibrated to 40 °C and aliquots of pre-equilibrated *endo*-1,4-β-glucanase (0.1 mL, 0.1 M sodium acetate, pH 4.5, 31.6 mU/mL) were added and mixed thoroughly. The mixtures were incubated at 40 °C for 0, 3, 6, 9 and 12 min and reactions were terminated by addition of 3 mL 2% Tris solution (pH 9.0). The tube contents were mixed thoroughly and the relative fluorescence units determined using a Promega Quantifluor®-ST fluorometer.

1.3.5. Investigation into the detection limit of cellulase activity (Fig. 6)

The detection limit of the assay system was investigated by preparation of solutions of *T. longibrachiatum endo*-1,4-β-glucanase in 0.1 M sodium acetate buffer pH 4.5 at varying concentrations (316–0.016 mU/mL) and was determined as follows: aliquots (0.1 mL) of the *endo*-1,4-β-glucanase solutions were equilibrated to 40 °C. Cellafluor was also pre-equilibrated to 40 °C and aliquots (0.1 mL, 0.5 mM) were added to each *endo*-1,4-β-glucanase solution and the resulting mixtures were incubated at 40 °C for 10 min. The reactions were terminated by addition of 3 mL 2% Tris solution (pH 9.0). The tube contents were mixed well and relative fluorescence units were determined using a Promega Quantifluor®-ST fluorometer.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2014.05.002.

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