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Novel substrates for the measurement of *endo*-1,4-β-glucanase (*endo*-cellulase)

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ABSTRACT

A specific and sensitive substrate for the assay of *endo*-1,4-β-glucanase (cellulase) has been prepared. The substrate mixture comprises benzylidene end-blocked 2-chloro-4-nitrophenyl-β-cellotrioside (BzCNPG3) in the presence of thermostable β-glucosidase. Hydrolysis by exo-acting enzymes such as β-glucosidase and *exo*-β-glucanase is prevented by the presence of the benzylidene group on the non-reducing end D-glucosyl residue. On hydrolysis by cellulase, the 2-chloro-4-nitrophenyl-β-glycoside is immediately hydrolysed to 2-chloro-4-nitrophenol and free D-glucose by the β-glucosidase in the substrate mixture. The reaction is terminated and colour developed by the addition of a weak alkaline solution. The assay procedure is simple to use, specific, accurate, robust and readily adapted to automation. This procedure should find widespread applications in biomass enzymology and in the specific assay of *endo*-1,4-β-glucanase in general.

1. Introduction

endo-1,4-β-Glucanase (EC 3.2.1.4) (cellulase) is widely used in the poultry industry to degrade β-glucan and thus increase the digestibility of feeds rich in barley.[1] In the malt industry it is used to hydrolyse β-glucan, increase the rate of wort filtration and reduce the possibility of β-glucan precipitation in beer. In the textile industry *endo*-1,4-β-glucanase is used for denim finishing and cotton softening, in the detergent industry it is used for cleaning and colour care and in the paper industry it modifies fibre and improves drainage.[2] It is generally accepted that the market for this enzyme will increase dramatically when the second generation biofuel industry becomes economic.[3]

endo-1,4-β-Glucanase in biological materials and microbial fermentation broths can be specifically measured by the decrease in the viscosity of water soluble, chemically modified cellulose derivatives such as CM-cellulose 7 M.[4] Cellulose derivatives either dyed or dyed and crosslinked also give specific measurement of *endo*-1,4-β-glucanase. With soluble dyed substrates, hydrolysis by *endo*-1,4-β-glucanase gives an increase in dyed fragments soluble in an aqueous–organic solvent, whereas with insoluble, dyed and crosslinked cellulose substrates, cellulase hydrolysis gives an increase in water soluble, dyed fragments.[5] Pure *endo*-1,4-β-glucanase enzymes can be characterised by studying the rates of hydrolysis of cello-oligosaccharides, borohydride reduced cello-oligosaccharides[4] or nitrophenyl-cello-oligosaccharides.[6–9] However, these substrates cannot be used to measure *endo*-1,4-β-glucanase in the presence of other cellulose degrading enzymes such as β-glucosidase and *exo*-1,4-β-glucanases which occur in fermentation broths and industrial enzyme preparations.

The aim of this study was to develop a specific substrate and a robust assay procedure for the measurement of *endo*-1,4-β-glucanase activity in complex biological materials.

2. Results and discussion

4,6-*O*-Benzylidene end-blocked 4-nitrophenyl-α-maltoheptaoside[10] has been commercially available for 25 years (from Megazyme) and is used, in combination with the ancillary enzymes α-glucosidase and/or amyloglucosidase (AMG), for the measurement of α-amylase.[10–12] The length of the maltodextrin chain ensures maximal rate of hydrolysis by fungal and pancreatic α-amylase, and near maximal rate of hydrolysis by bacterial and cereal α-amylase (which require a longer maltodextrin chain length for binding and hydrolysis). Synthesis of 4,6-*O*-benzylidene end-blocked 2-chloro-4-nitrophenyl cello-oligosaccharides poses a number of challenges. Because cellulose is insoluble, production in good yield of oligosaccharides of the required degree of polymerisation (DP) of 3–6 is challenging. Cello-oligosaccharides of DP 6

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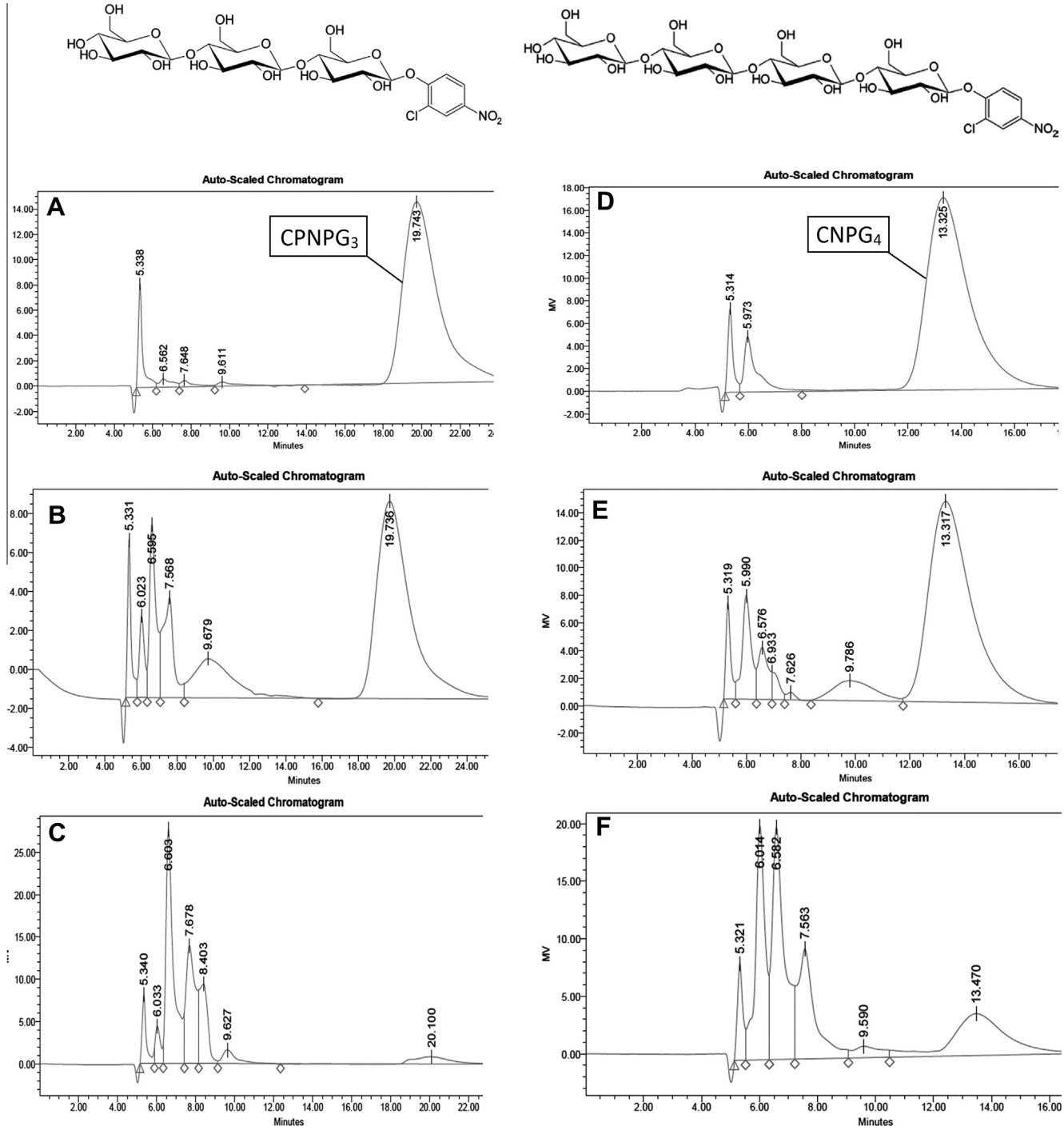

Figure 1. $CNPG_3$ hydrolysis by *A. niger endo*-1,4-β-glucanase (*A* = 0 U, *B* = 0.64 U and *C* = 6.4 U) and $CNPG_4$ hydrolysis by *A. niger endo*-1,4-β-glucanase (*D* = 0 U, *E* = 0.064 U and *F* = 0.64 U). All incubations were carried out at 40 °C, pH 4.5 for 10 min with 6.66 mg/mL substrate concentration.

and greater are sparingly soluble in water and thus are very difficult to purify in quantity. The current work has thus focused on the modification of cello-oligosaccharides of DP 3–5, which could be obtained in quantity from Megazyme.

The synthesis of colourimetric cello-oligosaccharide substrates has been described by a number of groups using a variety of glycosylation methods. Initial approaches employed peracetylated α-bromo glycosyl donors in mono-phasic acetone/aq. NaOH glycosylation reactions with a variety of phenol acceptors.[13,14] This reaction was subsequently improved by replacing the aqueous base with 2,6-lutidine and carrying out the transformation under strictly anhydrous conditions.[15] It was later discovered that the use of a biphasic $CHCl_3$/NaOH solvent system for the same glycosylation reactions afforded higher yields.[16,17] This methodology has been successfully employed here to synthesise a series (DP 2–DP 4) of colourimetric cello-oligosaccharides. Extensive spectroscopic data on these compounds not previously reported are also included here in the Supporting information.

These colourimetric substrates have been further functionalised with the incorporation of a 4,6-*O*-benzylidene group on the non-reducing terminus. Benzylidene acetals have been routinely used for the selective protection of the 4 and 6 positions of glucosyl

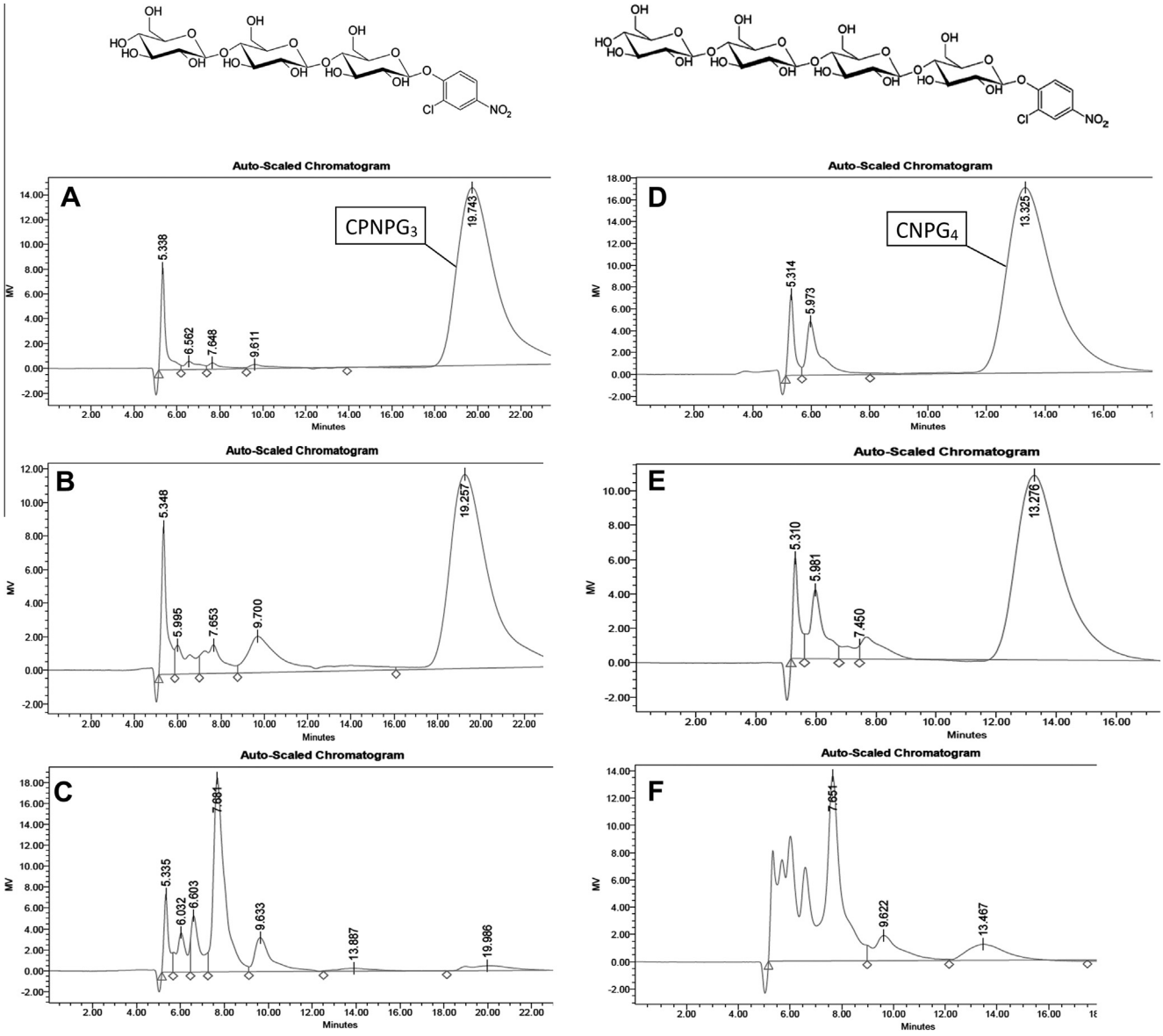

Figure 2. $CNPG_3$ hydrolysis by *T. longibrachiatum endo*-1,4-β-glucanase (*A* = 0 U, *B* = 0.027 U and *C* = 0.27 U) and $CNPG_4$ hydrolysis by *T. longibrachiatum endo*-1,4-β-glucanase (*D* = 0 U, *E* = 0.027 U and *F* = 0.27 U). All incubations were carried out at 40 °C, pH 4.5 for 10 min with 6.66 mg/mL substrate concentration.

residues in a wide range of applications.[18–20] This synthetic modification has been shown to block the action of *exo*-acting glucosidase enzymes in the analogous malto-oligosaccharide series.[9]

Difficulty in the chemical modification of the cello-oligomers increased in moving from cellotriose to cellopentaose, and this was reflected in lower final yields of the modified tetramer (10%), and particularly the modified pentamer (not isolated, estimated 5%). This can be attributed to the fact that a greater number of undesired benzylidene acetal derivatives are obtained during the benzylidene acetal formation step with the higher DP oligosaccharides. In addition, the higher DP oligosaccharides are obviously less amenable to purification and isolation by crystallisation/precipitation methods. Consequently, the work described here has focused on the production and evaluation of 4,6-*O*-benzylidene end-blocked 2-chloro-4-nitrophenyl cellotrioside ($BzCNPG_3$) and 4-6-*O*-benzylidene end-blocked 2-chloro-4-nitrophenyl cellotetraoside ($BzCNPG_4$). 4-6-*O*-Benzylidene end-blocked 2-chloro-4-nitrophenyl cellobioside ($BzCNPG_2$) was also prepared for comparative studies.

It is well known that the rates of hydrolysis of cello-oligosaccharides by *endo*-1,4-β-glucanase is dependent both on the DP of the cello-oligosaccharide (or reduced cello-oligosaccharide) and on the specific *endo*-1,4-β-glucanase being studied. In a comparison of the rates of hydrolysis of borohydride reduced cello-oligomers of DP 2–6 by *endo*-1,4-β-glucanase,[4] it was shown that *Trichoderma longibrachiatum endo*-1,4-β-glucanase hydrolysed cellotriitol at a rate similar to that for cellohexaitol, whereas *Aspergillus niger endo*-1,4-β-glucanase hydrolysed cellotetraitol at just 24% the rate of cellohexaitol, and the hydrolysis of cellotriitol was just 6% the rate for cellohexaitol. The susceptibility to the hydrolysis of 2-chloro-4-nitrophenyl-cellotrioside ($CNPG_3$) and 2-chloro-4-nitrophenyl-cellotetraoside ($CNPG_4$) by *A. niger* and *T. longibrachiatum endo*-1,4-β-glucanase is shown in Figures 1 and 2. Complete hydrolysis of both $CNPG_3$ and $CNPG_4$ is obtained with just 0.27 U of *T. longibrachiatum endo*-1,4-β-glucanase in 10 min at 40 °C. Near complete hydrolysis of $CNPG_4$ by *A. niger endo*-1,4-β-glucanase requires 0.64 U/assay, and of $CNPG_3$ requires 6.4 U/assay. Of the enzymes studied by McCleary et al.[4] these two *endo*-1,4-β-glucanases

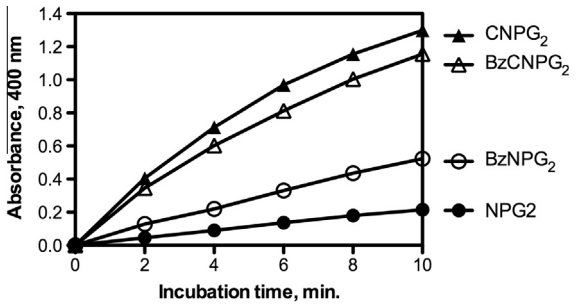

Figure 3. Relative rates of hydrolysis of NPG_2, $CNPG_2$, $BzNPG_2$ and $BzCNPG_2$ by *Trichoderma endo*-1,4-β-glucanase. Incubation of each substrate (0.1 mL, 3 mM in 33% DMSO/0.1 M KCl) in the presence of β-glucosidase (0.82 U) with *endo*-1,4-β-glucanase (1.42 U, 0.1 mL, pH 4.5 100 mM sodium acetate) at 40 °C.

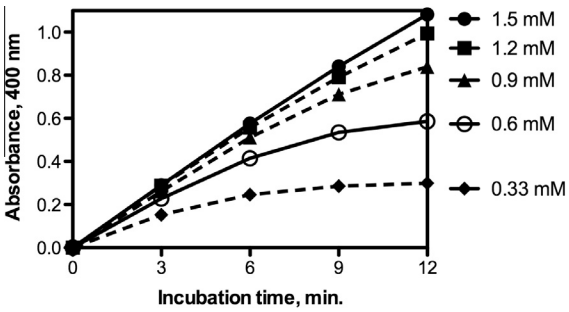

Figure 5. Effect of $BzCNPG_3$ concentration on reaction kinetics over 12 min in the presence of *T. longibrachiatum endo*-1,4-β-glucanase (170 mU/assay) and β-glucosidase (0.82 U/assay) at 40 °C in pH 4.5 100 mM sodium acetate at 40 °C.

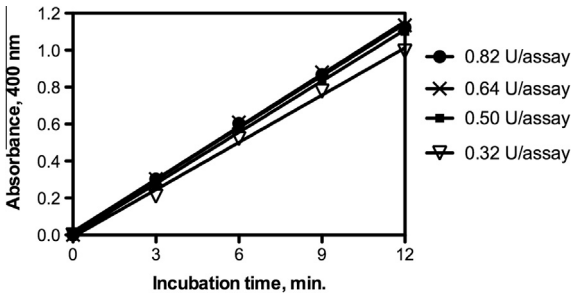

Figure 4. Effect of β-glucosidase concentration on reaction kinetics over 12 min in the presence of *T. longibrachiatum endo*-1,4-β-glucanase (170 mU/assay) and $BzCNPG_3$ (1.5 mM) in pH 4.5 100 mM sodium acetate at 40 °C.

occur at either end of a spectrum in terms of their ability to hydrolyse low DP cello-oligosaccharides as well as their ability to hydrolyse tamarind seed xyloglucan (containing a highly substituted 1,4-β-D-glucan backbone) or konjac glucomannan in which just 40% of the 1,4-β-linked hexoses in the linear polysaccharide chain are D-glucosyl residues (the remainder being D-mannosyl residues).

$BzNPG_2$, $BzCNPG_2$, $BzCNPG_3$ and $BzCNPG_4$ are sparingly soluble in water. They are best handled by dissolving in dimethyl sulphoxide (DMSO) at a concentration of 9.15 mM. Before use, 3 mL of this substrate solution is diluted with 6 mL of 100 mM KCl solution, mixed, and 50 μL of thermostable β-glucosidase is added to a 3 mL aliquot of the result 33% DMSO solution to make the assay reagent mixture.

The relative rates of hydrolysis of NPG_2, $CNPG_2$, $BzNPG_2$ and $BzCNPG_2$ by *T. longibrachiatum endo*-1,4-β-glucanase is shown in Figure 3. These incubations were performed in the absence of β-glucosidase and the release of 4-nitrophenol (NP) or 2-chloro-4-nitrophenol (CNP) was measured. β-Glucosidase was not required in these incubations because with the modified disaccharide, the cellulase cleaves between the terminal D-glucosyl unit and NP or CNP. The CNP derivatives are hydrolysed at 2–3 times the rate for the NP derivatives. This effect was to be expected given the difference in leaving group ability between CNP ($pK_a = 5.45$) and NP ($pK_a = 7.18$) and has been studied in great detail by Tull and Withers.[14] All subsequent research has focused on the CNP derivatives.

The concentration of β-glucosidase required to optimise the sensitivity of hydrolysis of $BzCNPG_3$ by *T. longibrachiatum endo*-1,4-β-glucanase was determined using substrate solutions containing 3 mM $BzCNPG_3$ plus 3.2–8.2 U/mL of β-glucosidase. An aliquot of these solutions (0.1 mL) was incubated with 0.1 mL of *Trichoderma endo*-1,4-β-glucanase (170 mU on this substrate) at 40 °C and the reaction terminated at 0–12 min by the addition of 3 mL of 2% Tris solution (pH 9). β-Glucosidase at a level of 0.5 U/assay (5 U/mL of substrate) was sufficient to give maximal colour release on hydrolysis of the substrate (Fig. 4), but a level of 0.66 U/assay was routinely used to allow for the possibility of loss of enzyme activity on longer term storage of the substrate.

Under the assay conditions employed, the effect of substrate concentration was determined by incubating 0.1 mL of substrate mixture containing from 0.66 to 3.0 mM $BzCNPG_3$ plus β-glucosidase (at 0.82 U/assay) with 0.1 mL of buffered solution containing *T. longibrachiatum endo*-1,4-β-glucanase (170 mU on this substrate) and the reaction was terminated at 0, 3, 6, 9 and 12 min by the addition of 3 mL of 2% Tris solution (pH 9) (Fig. 5). Near maximum rate of hydrolysis was obtained at a final substrate concentration of 1.2 mM in the reaction mixture, so a concentration of 1.5 mM was routinely used. At this substrate concentration, the reaction was linear up to an absorbance value of 1.0 (corresponds to <65% substrate hydrolysis). If absorbance values above 1.0 are obtained, the enzyme should be diluted and the assay re-run.

In the hydrolysis of BzCNP-cello-oligosaccharides, the *endo*-1,4-β-glucanase can potentially cleave within the oligosaccharide chain or between the terminal D-glucosyl residue and the CNP group (Fig. 6). The effect of saturating levels of β-glucosidase on the rates of hydrolysis of $BzCNPG_2$ and $BzCNPG_3$ by *T. longibrachiatum* and *A. niger endo*-1,4-β-glucanase is shown in Figure 7. With *A. niger endo*-1,4-β-glucanase, addition of β-glucosidase has no effect on the rate of hydrolysis of either $BzCNPG_2$ or $BzCNPG_3$, showing that hydrolysis occurs exclusively between the terminal D-glucosyl residue and the CNP group. The same is true for the hydrolysis of $BzCNPG_2$ by *T. longibrachiatum endo*-1,4-β-glucanase, but with $BzCNPG_3$, the rate of release of CNP in the presence of β-glucosidase is approximately fivefold higher than without added β-glucosidase. This shows that the preferred site of hydrolysis is within the oligosaccharide chain, most probably between the reducing-end terminal and the penultimate-D-glucosyl residues.

The relative rates of hydrolysis of $BzCNPG_2$, $BzCNPG_3$ and $BzCNPG_4$ by *A. niger* and *T. longibrachiatum endo*-1,4-β-glucanase are shown in Figure 8. With *T. longibrachiatum endo*-1,4-β-glucanase, $BzCNPG_3$ is hydrolysed at the same rate as $BzCNPG_4$. This is consistent with the relative rates of hydrolysis of cellotetraitol and cellopentaitol by this enzyme.[4] $BzCNPG_2$ is hydrolysed, but very slowly. With *A. niger endo*-1,4-β-glucanase, $BzCNPG_4$ is hydrolysed approximately five times faster than $BzCNPG_3$, again consistent with the rate of hydrolysis of cellopentaitol and cellotetraitol by this enzyme.

The determined activity of different *endo*-1,4-β-glucanase on $BzCNPG_3$ relative to CM-cellulose 4 M varies significantly as shown in Table 1. This can be used to characterise the particular *endo*-1,4-β-glucanase. From a quality control standpoint, this is of no consequence as protocols and activity relationships can be established

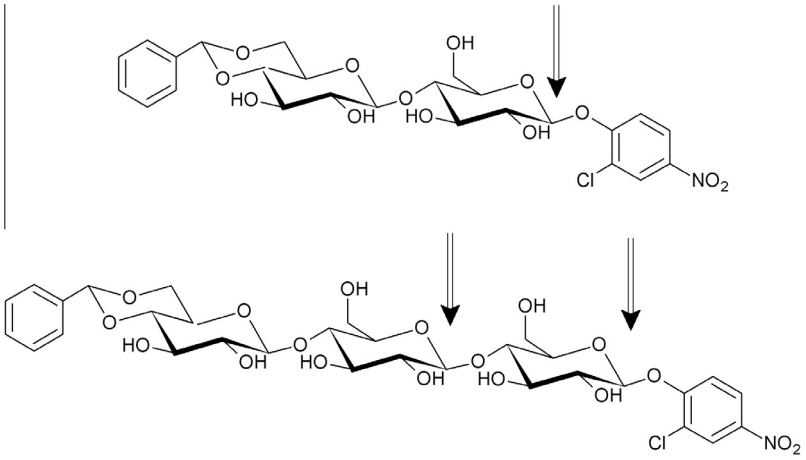

Figure 6. Observed sites for hydrolysis of $BzCNPG_2$ and $BzCNPG_3$ by *endo*-1,4-β-glucanase.

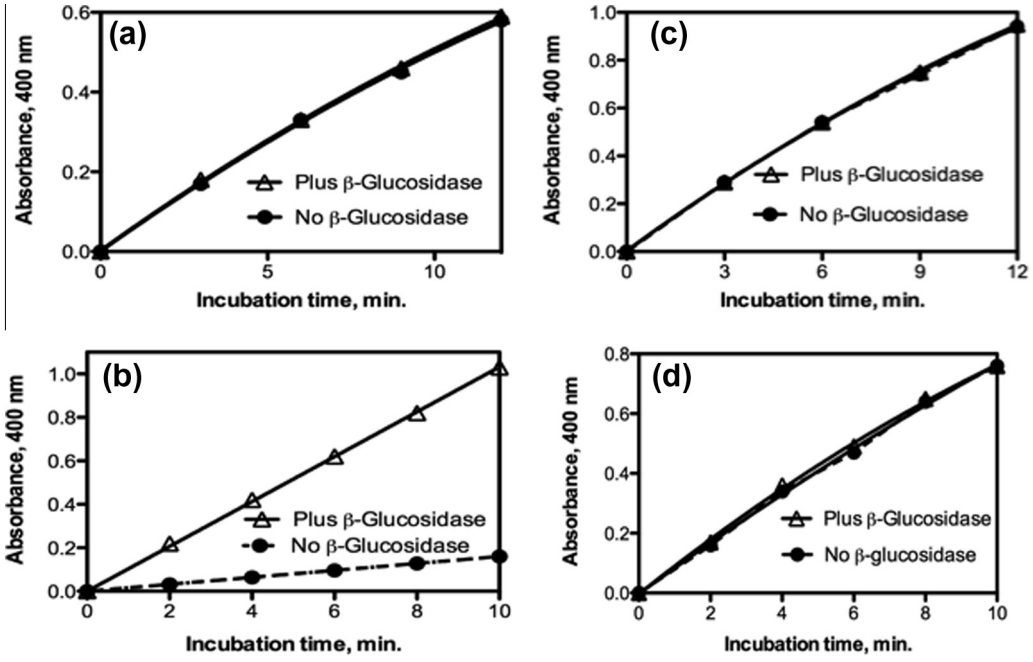

Figure 7. Hydrolysis of BzCNPG2 and BzCNPG3 by *endo*-1,4-*b*-glucanase in the presence and absence of saturating levels of *b*-glucosidase (0.82 U/assay); (a) BzCNPG2 with *T. longibrachiatum endo*-1,4-*b*-glucanase (600 mU/assay), (b) BzCNPG3 with *T. longibrachiatum endo*-1,4-*b*-glucanase (30 mU/assay), (c) BzCNPG2 with *A. niger endo*-1,4-*b*-glucanase (17.5 U/assay), (d) BzCNPG3 with *A. niger endo*-1,4-*b*-glucanase (700 mU/assay). All incubations were carried out at 40 °C in pH 4.5 100 mM sodium acetate.

for the particular *endo*-1,4-β-glucanase and then the determined activity value is the sole consideration.

The thermostable β-glucosidase used in combination with $BzCNPG_3$ is stable up to 90 °C and has activity over the pH range of 4.5–7.5, meaning that the reagent can be used over these ranges of temperature and pH. A standard curve relating absorbance increase at 400 nm to level of *Thermotoga maritima endo*-1,4-β-glucanase at 80 °C is shown in Figure 9. $BzCNPG_3$ substrate mixture is stable at 80 °C for up to 30 min.

The stability of the reagent mixture containing $BzCNPG_3$ and thermostable β-glucosidase (400 U/mL) at −20, 4 and 20 °C is shown in Figure 10. On storage of the reagent for up to 100 days, 0.1 mL aliquots were removed and the blank absorbance was determined as well as the absorbance on incubation of the substrate with a set amount of *T. longibrachiatum endo*-1,4-β-glucanase (20 mU on $BzCNPG_3$) at 40 °C for 10 min. With reagent stored at 20 °C there is a significant increase in the blank absorbance value, but only after extended periods. The increase in the reaction absorbance value parallels the increase in the blank value, such that on subtracting the blank value from the reaction value, the determined absorbance value is very similar to those obtained on storage of the reagent at −20 and 4 °C. This demonstrates that the β-glucosidase in the reagent mixture is stable, even on storage at 20 °C for 100 days. Since on storage of the substrate at 4 or −20 °C there is essentially no increase in blank absorbance value and the determined activity of the cellulase control is the same, these are the recommended storage temperatures. These studies would indicate that the prepared substrate should be stable for >2–3 years at −20 °C.

It can thus be concluded that $BzCNPG_3$ is a useful and versatile substrate for the measurement of *endo*-1,4-β-glucanase from microbial sources. The assay procedure employing this substrate

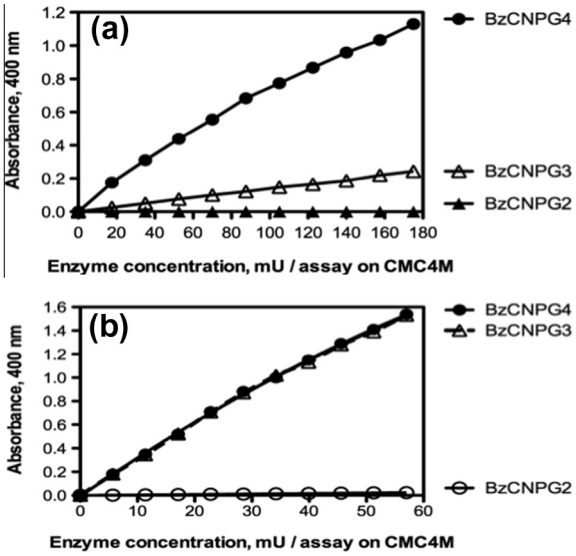

Figure 8. Relative rates of hydrolysis of $BzCNPG_2$, $BzCNPG_3$ and $BzCNPG_4$ by (a) *A. niger endo*-1,4-β-glucanase and (b) *T. longibrachiatum endo*-1,4-β-glucanase at 40 °C in pH 4.5 100 mM sodium acetate.

Table 1
Relative rates of hydrolysis of CellG3 mixture [containing 1.5 mM $BzCNPG_3$ (concentration in the assay) and β-glucosidase] and CM-cellulose 4 M (10 mg/mL) by several *endo*-1,4-β-glucanases at 40 °C and optimal pH

Source of *endo*-cellulase	pH of reaction mixture	CMC-4 M		CellG3	
		Specific activity (U/mg)	Relative activity (%)	Specific activity (U/mg)	Relative activity (%)
T. longibrachiatum	4.5	68	100	47.6	70.0
B. amyloliquefaciens	6.0	82	100	52.2	63.6
T. maritima	6.0	53	100	27.3	51.6
T. emersonii	4.5	64	100	2.6	4.0
A. niger	4.5	82	100	2.1	2.5

is versatile, accurate and very reproducible. Activity on $BzCNPG_3$ compared to that on CM-cellulose 4 M varies from one *endo*-cellulase to the next, but this is of no concern if the aim is to monitor and control a particular fermentation broth when the nature of the major *endo*-cellulase component is known. The principle of the assay procedure is summarised in Figure 11. On hydrolysis of $BzCNPG_3$ by *endo*-1,4-β-glucanase, the β-glucosidase in the substrate mixture immediately hydrolyses the non-blocked CNP-gluco-oligosaccharides releasing D-glucose and free 2-chloro-4-nitrophenol. The reaction is terminated and colour developed by the addition of an alkaline solution. The substrate is absolutely specific for *endo*-1,4-β-glucanase. The benzylidene blocking group prevents hydrolysis by β-glucosidase, cellobiohydrolase or *exo*-glucanases.

3. Experimental

3.1. Materials

Cello-oligosaccharides, thermostable β-glucosidase (*T. maritima*; accession number Q08638, Cat. No. E-BGOSTM) and highly purified *endo*-1,4-β-glucanases[4] from *T. longibrachiatum* (accession number Q12714, Cat. No. E-CELTR), *Bacillus amyloliquefaciens* (accession number Q8RPQ6, Cat. No. E-CELBA), *Thermatoga maritima* (accession number Q9X273, Cat. No. E-CELTM), *Talaromyces emersonii* (accession number Q8NIB5, Cat. No. E-CELTE) and *A. niger* (O74705, Cat. No. E-CELAN), were obtained from Megazyme International, Ireland. All other chemicals used in organic synthesis

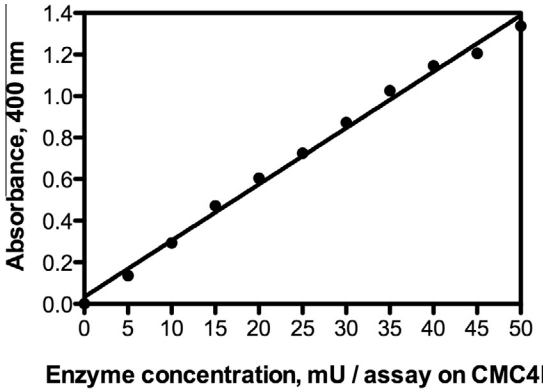

Figure 9. Standard curve relating absorbance increase at 400 nm observed with $BzCNPG_3$ to level of *T. maritima endo*-1,4-β-glucanase activity on CM-cellulose-4 M at 80 °C in pH 6 100 mM sodium phosphate.

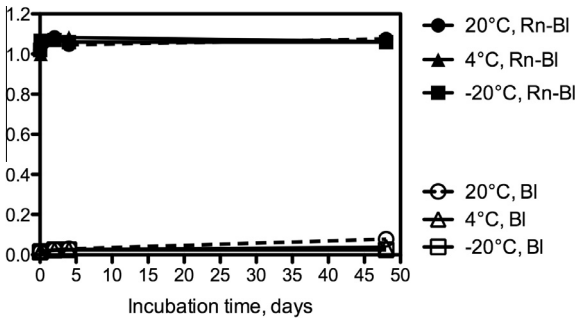

Figure 10. Stability study on $BzCNPG_3$/β-glucosidase assay reagent in 33% DMSO/100 mM KCl at −20, 4 and 20 °C over 50 days.

were purchased from Sigma Aldrich, Lennox Laboratory Supplies or Lab unlimited (Carl Stuart Group) and were analytical reagent grade. A Brüker Avance 400 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 ($\delta_H = 2.50$). 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.

3.2. Colourimetric substrate synthesis

3.2.1. Nitrophenyl-cello-oligosaccharide synthesis

NPG_2, $CNPG_2$, $CNPG_3$ and $CNPG_4$ were synthesised without difficulty according to the general methods employed by Planas et al.[16] The compounds reported here have been previously synthesised by Claeyssens and Henrissat.[9]

3.2.1.1. 4-Nitrophenyl-cellobioside (NPG_2). Yield: 41%; mp (H_2O) 244–247 °C (dec); ^1H NMR (400 MHz C_2D_6SO) δ 2.94–3.57 (m, 8H), 3.58–3.79 (m, 4H), 4.30 (d, $J = 7.53$ Hz, 1H(anom)), 4.62–4.76 (m, 2H), 4.87 (br s, 1H), 4.99–5.12 (m, 2H), 5.18 (d, $J = 7.53$ Hz, 1H(anom)), 5.28 (br s, 1H), 5.65 (br s, 1H), 7.24 (d, $J = 7.53$ Hz, 2H), 8.22, (d, $J = 8.03$ Hz, 2H); ^{13}C NMR (100 MHz, C_2D_6SO) δ 60.3, 61.5, 70.5, 73.3, 73.8, 75.2, 75.6, 76.9, 77.3, 80.0, 99.8, 103.6, 117.0, 117.0, 126.2, 126.2, 142.2, 162.7; HRMS ES^- $[M-H]^-$ Calcd 462.1248, Found 462.1250.

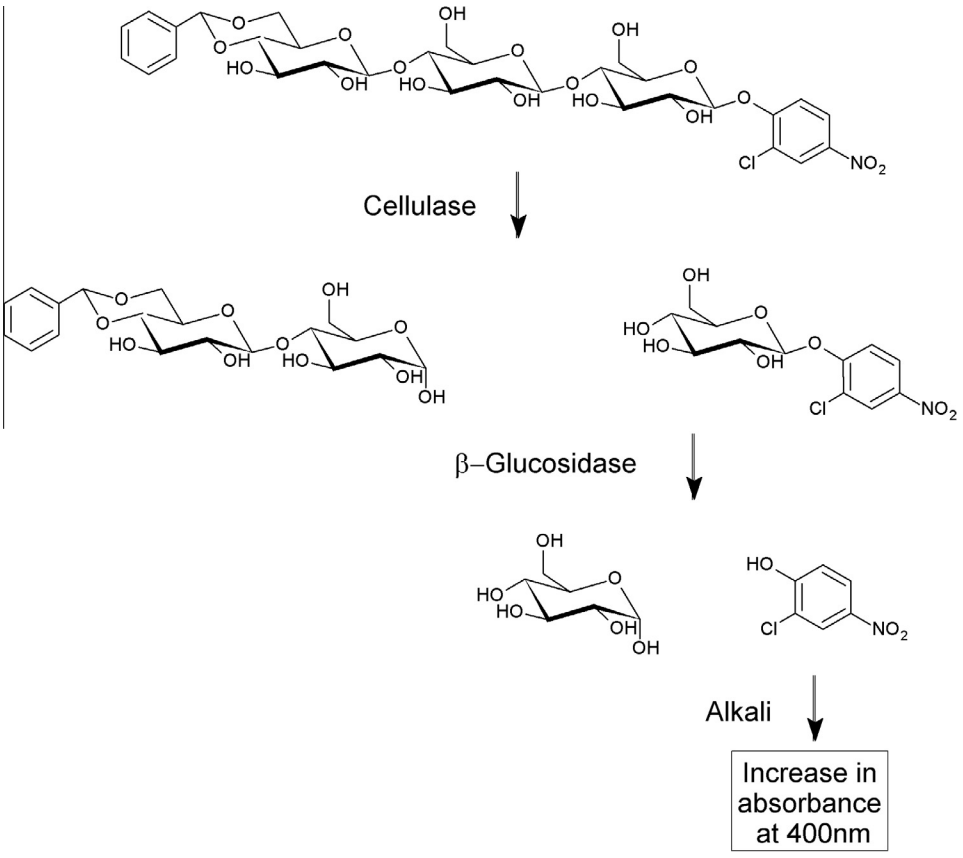

Figure 11. Overview of *endo*-1,4-β-glucanase assay format.

3.2.1.2. 2-Chloro-4-nitrophenyl-cellobioside ($CNPG_2$). Yield: 37%; mp (H_2O) 200–203 °C (dec); ^{1}H NMR (400 MHz C_2D_6SO) δ 2.94–3.08 (m, 2H), 3.09–3.24 (m, 2H), 3.24–3.51 (m, 4H), 3.58–3.78 (m, 4H), 4.28 (d, J = 7.78 Hz, 1H (anom)), 4.62 (t, J = 5.14 Hz, 1H), 4.66 (t, J = 5.53 Hz, 1H), 4.85 (d, J = 2.01 Hz, 1H), 5.00 (d, J = 5.53 Hz, 1H), 5.04 (d, J = 4.77 Hz, 1H), 5.25 (d, J = 5.02 Hz, 1H), 5.32 (d, J = 7.78 Hz, 1H (anom)), 5.64 (d, J = 5.14 Hz, 1H), 7.47 (d, J = 9.09 Hz, 1H), 8.20 (dd, J = 9.09, 2.79 Hz, 1H), 8.32 (d, J = 2.79 Hz, 1H); ^{13}C NMR (100 MHz, C_2D_6SO) δ 60.2, 61.6, 70.5, 73.2, 73.8, 75.4, 75.7, 76.9, 77.3, 79.9, 99.9, 103.6, 116.1, 122.7, 124.7, 126.1, 142.0, 158.1; HRMS ES$^-$ [M−H]$^-$ Calcd 496.0858, Found 496.0858.

3.2.1.3. 2-Chloro-4-nitrophenyl-cellotrioside ($CNPG_3$). Yield: 35%; mp (H_2O) 228–231 °C (dec); ^{1}H NMR (400 MHz C_2D_6SO) δ 2.92–3.87 (m, 18H), 4.23 (d, J = 7.78 Hz, 1H (anom)), 4.35 (d, J = 7.78 Hz, 1H (anom)), 4.43–4.88 (m, 6H), 4.99 (br s, 1H), 5.23 (br s, 1H), 5.31 (d, J = 7.78 Hz, 1H (anom)), 5.41 (br s, 1H), 5.64 (br s, 1H), 7.46 (d, J = 9.23 Hz, 1H), 8.19 (dd, J = 9.23, 2.64 Hz, 1H), 8.32 (d, J = 2.64 Hz, 1H); ^{13}C NMR (100 MHz, C_2D_6SO) δ 60.1, 60.8, 61.5, 70.5, 73.2, 73.5, 73.7, 75.3, 75.3, 75.4, 75.8, 76.9, 77.3, 79.8, 80.9, 99.9, 103.2, 103.8, 116.1, 122.7, 124.7, 126.1, 142.1, 158.1; HRMS ES$^-$ [M−H]$^-$ Calcd 658.1386, Found 658.1392.

3.2.1.4. 2-Chloro-4-nitrophenyl-cellotetraoside ($CNPG_4$). Yield: 32%; mp (H_2O) 243–245 °C (dec); ^{1}H NMR (400 MHz C_2D_6SO) δ 2.89–3.33 (m 6H), 3.24–13.86 (m, 18H), 4.22 (d, J = 7.78 Hz, 1H(anom)), 4.31 (d, J = 8.03 Hz, 1H(anom)), 4.36 (d, J = 7.78 Hz, 1H(anom)), 4.59 (t, J = 5.27 Hz, 1H), 4.62–4.71 (m, 4H), 4.73 (br s, 1H), 4.78 (br s, 1H), 4.98 (d, J = 5.02 Hz, 1H), 5.02 (d, J = 4.77 Hz, 1H), 5.22 (d, J = 4.77 Hz, 1H), 5.31 (d, J = 7.53 Hz, 1H(anom)), 5.41 (d, J = 3.77 Hz, 1H), 5.42 (d, J = 3.76 Hz, 1H), 5.64 (d, J = 5.27 Hz, 1H), 7.46 (d, J = 9.17 Hz, 1H), 8.19 (dd, J = 9.17, 2.38 Hz, 1H), 8.32 (d, J = 2.38 Hz, 1H); ^{13}C NMR (100 MHz, C_2D_6SO) δ 60.2, 60.8, 60.8, 61.5, 70.5, 73.2, 73.5, 73.5, 73.7, 75.2, 75.3, 75.3, 75.3, 75.4, 75.7, 76.9, 77.3, 79.9, 80.9, 80.9, 99.9, 103.2, 103.3, 103.7, 116.1, 122.7, 124.7, 126.1, 142.1, 158.1; HRMS ES$^-$ [M−H]$^-$ Calcd 820.1914, Found 820.1906.

3.2.2. Benzylidene blocked nitrophenyl-cello-oligosaccharide synthesis

$BzNPG_2$, $BzCNPG_2$, $BzCNPG_3$ and $BzCNPG_4$ were synthesised from the corresponding unprotected substrates according to the following general procedure. To a solution of 2-chloro-4-nitrophenyl-cellotrioside (1 g, 1.515 mmol) and *p*-toluenesulfonic acid monohydrate (86 mg, 0.45 mmol) under an argon atmosphere in anhydrous dimethylformamide (10 mL) containing activated 4A molecular sieves (200 mg) was added benzaldehyde dimethylacetal (684 μL, 4.545 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 ACN/H_2O.

3.2.2.1. 4,6-*O*-Benzylidene-4-nitrophenyl-cellobioside ($BzNPG_2$). Yield: 58%; mp (H_2O) 266–267 °C (dec); ^{1}H NMR (400 MHz C_2D_6SO) δ 3.07–3.23 (m, 1H), 3.27–3.84 (m, 10H), 4.13–4.28 (m, 1H), 4.55 (d, J = 6.53 Hz, 1H(anom)), 4.67 (br s, 1H), 4.76 (br s, 1H), 5.17 (d, J = 7.03 Hz, 1H(anom)), 5.44 (br s, 1H), 5.60 (app br s, 2H), 5.67 (br s, 1H), 7.24 (d, J = 7.53 Hz, 2H), 7.31-7.52 (m, 5H), 8.23 (d, J = 7.53 Hz, 2H); ^{13}C NMR (100 MHz, C_2D_6SO) δ 60.0, 66.5, 68.2, 73.3, 73.5, 74.8, 74.8, 75.7, 78.6, 80.8, 99.9, 101.2, 103.5,

117.0, 117.0, 126.2, 126.2, 126.8, 126.8, 128.5, 128.5, 129.4, 138.1, 142.2, 162.7.; HRMS ES$^-$ [M−H]$^-$ Calcd 550.1561, Found 550.1556.

3.2.2.2. 4,6-*O*-Benzylidene-2-chloro-4-nitrophenyl-cellobioside (BzCNPG$_2$). Yield: 57%; mp (H_2O) 236–237 °C (dec); ^{1}H NMR (400 MHz C_2D_6SO) δ 3.07–3.24 (m, 1H), 3.25–3.85 (m, 10H), 4.11–4.30 (m, 1H), 4.56 (d, J = 7.78 Hz, 1H (anom)), 4.71 (d, J = 3.01 Hz, 1H), 4.79 (t, J = 5.51 Hz, 1H), 5.32 (d, J = 7.52 Hz, 1H (anom)), 5.46 (d, J = 3.95 Hz, 1H), 5.53–5.65, (m, 2H), 5.71 (d, J = 4.96 Hz, 1H), 7.27–7.60 (m, 6H), 8.22 (dd, J = 9.31, 2.78 Hz, 1H), 8.35 (d, J = 2.78 Hz, 1H); ^{13}C NMR (100 MHz, C_2D_6SO) δ 59.6, 66.1, 67.9, 73.0, 73.1, 74.5, 74.6, 75.5, 78.1, 80.5, 99.7, 100.8, 103.2, 115.8, 122.4, 124.4, 125.7, 126.5, 126.5, 128.2, 128.2, 129.1, 137.8, 141.7, 157.8; HRMS ES$^-$ [M−H]$^-$ Calcd 584.1171, Found 584.1168.

3.2.2.3. 4,6-*O*-Benzylidene-2-chloro-4-nitrophenyl-cellotrioside (BzCNPG$_3$). Yield: 49%; mp (H_2O) 230–232 °C (dec); ^{1}H NMR (400 MHz C_2D_6SO) δ 3.00–3.18 (m, 2H), 3.21–3.87 (m, 15H), 4.09–4.25 (m, 1H), 4.36 (d, J = 7.78 Hz, 1H(anom)), 4.49 (d, J = 7.78 Hz, 1H (anom)), 4.51 (d, J = 1.75 Hz, 1H), 4.67 (t, J = 5.35 Hz, 1H), 4.73 (t, J = 5.32 Hz, 1H), 4.80 (br s, 1H), 5.32 (d, J = 7.53 Hz, 1H(anom)), 5.35–5.48 (m, 2H), 5.53 (d, J = 4.52 Hz, 1H), 5.57 (s, 1H), 5.64 (d, J = 4.52 Hz, 1H), 7.29–7.53 (m, 6H), 8.20 (dd, J = 9.16, 2.64 Hz, 1H), 8.32 (d, J = 2.51 Hz, 1H); ^{13}C NMR (100 MHz, C_2D_6SO) δ 60.2, 60.6, 66.4, 68.1, 73.2, 73.3, 73.6, 74.7, 74.7, 75.4, 75.5, 75.8, 79.4, 79.8, 80.8, 99.9, 101.1, 103.3, 103.6, 116.1, 122.7, 124.7, 126.1, 126.8, 126.8, 128.5, 128.5, 129.4, 138.1, 142.0, 158.1; HRMS ES$^+$ [M+Na]$^+$ Calcd 770.1675, Found 770.1679.

3.2.2.4. 4,6-*O*-Benzylidene-2-chloro-4-nitrophenyl-cellotetraoside (BzCNPG$_4$). Yield: 31%; mp (H_2O) 235–238 °C (dec); ^{1}H NMR (400 MHz C_2D_6SO) δ 3.00–3.17 (m, 3H), 3.20–3.87 (m, 20H), 4.12–4.21 (m, 1H), 4.31 (d, J = 7.38 Hz, 1H (anom)), 4.36 (d, J = 7.01 Hz, 1H (anom)), 4.48 (d, J = 7.47 Hz, 1H (anom)), 4.52 (br s, 1H), 4.63–4.78 (m, 4H), 4.81 (br s, 1H), 5.31 (d, J = 7.46 Hz, 1H (anom)), 5.37–5.49 (m, 3H), 5.51–5.56, (m, 2H), 5.66 (br s, 1H), 7.23–7.54, (m, 6H), 8.20, (d, J = 8.84 Hz, 1H), 8.32 (br s, 1H); ^{13}C NMR (100 MHz, C_2D_6SO) δ 60.2, 60.5, 60.8, 66.4, 68.1, 73.2, 73.3, 73.5, 73.6, 74.7, 74.7, 75.2, 75.3, 75.4, 75.4, 75.7, 79.4, 79.8, 80.7, 80.7, 99.9, 101.1, 103.2, 103.4, 103.5, 115.7, 122.2, 124.2, 125.6, 126.3, 126.3, 128.0, 128.0, 128.8, 137.7, 141.6, 157.6; HRMS ES$^-$ [M−H]$^-$ Calcd 908.2227, Found 908.2227.

3.3. Dissolution of oligosaccharide substrates

NPG2, CNPG2, CNPG$_3$ and CNPG$_4$ were dissolved directly in 100 mM KCl solution to give a concentration of 3 mM. BzNPG$_2$, BzCNPG$_2$, BzCNPG$_3$ and BzCNPG$_4$ were dissolved at a concentration of 9.15 mM in dimethylsulfoxide (DMSO) and stored at −20 °C between use. In this form they are stable for >4 years. For use as substrates, 3 mL of these solutions were added to 6 mL of 100 mM KCl and mixed thoroughly. Thermostable β-glucosidase (50 μL, 400 U/mL) was then added to 3 mL aliquots of the resulting 33% DMSO solution, mixed by swirling and stored at −20 °C between use. In this form, they were stable for >2 years.

3.4. Standard assay of *endo*-1,4-β-glucanase using 3 mM BzCNPG$_3$ in the presence of β-glucosidase (8.2 U/mL) (CellG3 assay)

To a pre-equilibrated aliquot of substrate solution (CellG3) (0.1 mL, 3 mM) containing thermostable β-glucosidase (0.66 U), a pre-equilibrated aliquot of *endo*-1,4-β-glucanase in 100 mM sodium acetate buffer, pH 4.5 (0.1 mL) or sodium phosphate, pH 6.0 was added and the mixture incubated at 40 °C for exactly 10 min. The reaction was terminated by adding 3 mL of 2% trisodium phosphate (pH 11.0) or 3 mL of 2% Tris (pH 9). The tube contents were well mixed and the absorbance measured at 400 nm against a reagent blank. One Unit of activity is the amount of enzyme required to release one micromole of 2-chloro-4-nitrophenolate per minute at 40 °C and the pH used in the particular assay.

3.5. Standard assay for *endo*-1,4-β-glucananse using CM-cellulose 4 M (10 mg/mL) by reducing sugar determination

To a pre-equilibrated aliquot of CM-cellulose 4 M (0.5 mL, 10 mg/mL) in 100 mM sodium acetate buffer (pH 4.5) or sodium phosphate, pH 6.0, a pre-equilibrated aliquot of *endo*-1,4-β-glucanase was added. The resulting mixture was agitated on a vortex stirrer and incubated at 40 °C. Incubations were terminated by the addition of Nelson–Somogyi solution C[4,21] (0.5 mL) at 0, 3, 6, 9 and 12 min. The colour was developed according to the Nelson–Somogyi procedure[4,21] and incubation tubes centrifuged at 1500 g for 10 min to remove insoluble CM-cellulose 4 M. The absorbance was measured at 520 nm against a substrate/reagent blank and a glucose standard solution (50 μg) was included. One unit of activity is the amount of enzyme required to release one micromole of glucose reducing sugar equivalents per minute at 40 °C and the pH used in the particular assay.

3.6. Assay of *endo*-1,4-β-glucananse on CNP-cello-oligomers and BzCNP-cello-oligomers

The mode of action of *A. niger* and *T. longibrachiatum endo*-1,4-β-glucanase on CNPG$_3$ and CNPG$_4$ was studied by incubating 0.1 mL of the *endo*-1,4-β-glucanase (0–6.4 mU; *A. niger*; Fig. 1) (0–0.27 U; *T. longibrachiatum*; Fig. 2) with 0.2 mL of CNPG$_3$ or CNPG$_4$ (10 mg/mL) in 10 mM sodium acetate buffer (pH 4.5) for 10 min at 40 °C. The reaction was terminated by heating the reaction tubes at ~100 °C for 3 min and the tube contents were transferred to a microfuge tube and centrifuged at 12,000 rpm for 6 min. An aliquot (50 μL) was analysed directly by HPLC using a Waters Sugar-Pac Column (6 × 300 mM), 90 °C, with distilled water containing dicalcium EDTA as mobile phase at 0.5 mL/min. A Breeze HPLC system was used incorporating a Waters 2410 RI detector and Empower 2 software.

The relative rates of hydrolysis of NPG$_2$, CNPG$_2$, BzNPG$_2$ and BzCNPG$_2$ by *T. longibrachiatum endo*-1,4-β-glucanase (Fig. 3) involved the incubation of 0.1 mL of the substrates at 3 mM in the presence of β-glucosidase (0.66 U) with 0.1 mL of *endo*-1,4-β-glucanase at 40 °C. Reaction was terminated at 0, 2, 4, 6, 8 and 10 min by adding 3 mL of 2% Tris solution (pH 10) with mixing. The absorbance values were read at 400 nm against the zero time reading for the respective substrate. Activity was calculated using the extinction coefficient of CNP of 16,600 M^{-1} cm^{-1}.

The effect of the presence of saturating levels of β-glucosidase, and its absence, on the rate of increase in absorbance at 400 nm on hydrolysis of BzCNPG$_2$ and BzCNPG$_3$ by *endo*-1,4-β-glucanase was studied by incubating 0.1 mL of oligosaccharide substrate (3 mM; containing 0 or 0.66 U β-glucosidase per 0.1 mL substrate) with 0.1 mL of *T. longibrachiatum* (Fig. 7a and b) or *A. niger* (Fig. 7c and d) *endo*-1,4-β-glucanase in 100 mM sodium acetate buffer (pH 4.5) at 40 °C. Reaction was terminated at 0, 3, 6, 9 and 12 min by adding 3 mL of 2% Tris solution (pH 9).

To determine the effect of substrate concentration (Fig. 6) on the rate of hydrolysis of BzCNPG$_3$ by *T. longibrachiatum endo*-1,4-β-glucanase, 0.1 mL of BzCNPG$_3$ (0.66–3.0 mM) in the presence of β-glucosidase (0.66 U) was incubated with 0.1 mL of *T. longibrachiatum endo*-1,4-β-glucanase (170 mU on this substrate) in 100 mM sodium acetate buffer (pH 4.5) at 40 °C. Reaction was terminated at 0, 3, 6, 9 and 12 min by adding 3 mL of 2% Tris solution (pH

10) and absorbance measured at 400 nm. The effect of β-glucosidase concentration on the rate of increase in absorbance at 400 nm was determined by preparing solutions containing 3 mM $BzCNPG_3$, plus β-glucosidase (3.2–8.2 U/mL) and was determined as follows (Fig. 4): aliquots (0.1 mL) of the solutions were incubated with 0.1 mL of *T. longibrachiatum endo*-1,4-β-glucanase (170 mU/mL on Cell G3) at 40 °C and reaction terminated at 0, 3, 6, 9 and 12 min by adding 3 mL of 2% Tris solution (pH 9). The relative rates of hydrolysis of $BzCNPG_2$, $BzCNPG_3$ and $BzCNPG_4$ by *A. niger* and *T. longibrachiatum endo*-1,4-β-glucanase was determined by incubating 0.1 mL of substrate (3 mM, containing β-glucosidase at 6.6 U/mL) with 0.1 mL of *A. niger endo*-1,4-β-glucanase (0–180 mU/assay on CM-cellulose 4 M) or *T. longibrachiatum endo*-1,4-β-glucanase (0–57 mU/assay on CM-cellulose 4 M) (Fig. 8).

Action of *T. maritima* thermostable *endo*-1,4-β-glucanase on $BzCNPG_3$ was determined by incubating 0.1 mL of $BzCPNG_3$ (3 mM, containing β-glucosidase at 6.6 U/mL) with 0.1 mL of *T. maritima endo*-1,4-β-glucanase (0–50 mU/assay on CMC4 M in pH 6 100 mM sodium phosphate) at 80 °C for 10 min. The reaction was terminated by the addition of 3 mL of tri-sodium phosphate solution (pH 11.0) and the tubes immediately placed into an ice water bath. They were warmed to ~20 °C before measuring absorbance at 400 nm (Fig. 9).

Longer term stability of $BzCNPG_3$ in the presence of β-glucosidase (CellG3 substrate mixture) was determined by storing the substrate mixture at −20, 4 and 20 °C for up to 100 days. After the various time intervals, stability was determined by running a standard assay by mixing 0.1 mL of the substrate mixture with 0.1 mL of *T. longibrachiatum endo*-1,4-β-glucanase (20.8 mU on CellG3) for 10 min at 40 °C and stopping the reaction with 3 mL of 2% Tris solution (pH 10). Changes in blank absorbance values were determined by adding 3 mL of 2% Tris solution (pH 9) to 0.1 mL of the substrate solution and 0.1 mL of the relevant buffer solution, mixing well and measuring the absorbance at 400 nm (Fig. 10). Clearly, the substrate mixture is very stable under each of the storage conditions. On storage at 20 °C, the absorbance of the blank solution in the assay (no enzyme) increased from 0.013 to 0.078 over 45 days (i.e., sixfold), but the assay absorbance increased by the same amount such that the determined enzyme activity was the same. This increase in absorbance value represents a degradation of approximately 4% of the substrate in the assay mixture. At −20 °C and 4 °C, the absorbance of the blank solution in the assay increased by just 0.013 and 0.025, respectively over the same period. In the assay, the substrate behaved exactly as in the freshly prepared reagent solution.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2013.12.001.

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