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The hydrolysis of barley β-glucan by the cellulase EC 3.2.1.4 under dilute conditions is identical to that of barley solubilase

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Abstract

Barley β-glucan solubilase is an enzyme that degrades barley β-glucan in extracts obtained from barley flour. The solubilase preferentially attacks the longer blocks of β-(1→4) linkages, i.e., those containing at least nine glucosyl residues. There is strong evidence to suggest that the solubilase derives from fungi associated with the husk of the grain. It was found that cellulase (EC 3.2.1.4) from *Trichoderma* sp. shows similar activity under dilute conditions. Since fungi associated with the husk of the grain are known to produce these types of cellulases, there is no need, based on current evidence, to propose the existence of a unique enzyme, i.e., solubilase, for the solubilising behaviour of enzymes in the barley grain.

Keywords: β-Glucan; Solubilase; Cellulase

1. Introduction

Glucagel™ is a mixed-linkage β-(1→3), (1→4)-glucan (β-glucan in this paper) isolated from barley using a hot water extraction followed by a freeze and thaw of the extract.[1] The process produces a gelatinous precipitate that can be dried. A significant part of the process is the partial hydrolysis of the β-glucan by an enzyme present in the grain. The enzyme helps, not only to release the β-glucan from the grain, which increases the yield of product, but it is also essential for the freeze and thaw step. A high-molecular-weight β-glucan obtained from an enzyme-deactivated flour will not form a precipitate during the freeze–thaw step. The enzyme can also be used to vary the molecular weight of the product. Longer extraction times lead to increased hydrolysis and decreased molecular weight.

The enzyme is present in the grain after the grain has matured and leads to the solubilisation of β-glucan. It has therefore been called a solubilase.[2] Other β-glucan degrading enzymes appear to be expressed only after germination. Preece and Aitken[3] in 1953 found that a cellulase associated with the barley appeared to be responsible for solubilising β-glucan. Martin et al.[2,4,5] and Martin and Bamforth and[6] concluded that the solubilase was an acidic carboxypeptidase which hydrolysed ester linkages from carboxyl groups on peptides to β-glucan. However, this conclusion was later contradicted by the work of Yin and MacGregor[7,8] and Yin et al.[9]

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In a series of papers, Yin and MacGregor[7,8] and Yin et al.[9] reported on β-glucan solubilase. They purified the solubilase, examined the release of β-glucan from barley grain, determined substrate specificity, and identified the distribution of the enzyme in the grain. They found that a crude solubilase preparation made from the whole barley flour did have carboxypeptidase activity. In contrast, the partially purified solubilase enzyme was found to have no carboxypeptidase activity, although it did solubilise β-glucan. Yin and MacGregor[7] found that the carboxypeptidase activity was present within the barley kernel. In contrast, the solubilase activity was associated with the husks of the barley grain, since extracts of the dehusked barley flour (containing the carboxypeptidase) had little solubilising activity. Yin and MacGregor[7] also discovered that the enzyme extracted from the whole-kernel flour, that is the flour including the husk, was stable for 40 min at 62 °C, but the enzyme extracted from the unmilled whole-barley kernels was inactivated within 5 min at 62 °C. It therefore appeared that the enzyme was stabilised by other material extracted from the flour.

Yin et al.[9] concluded that field fungi associated with the husks of the grain are the probable source of the solubilase activity, since extracts of fungi grown on barley husks showed similar activity to that of the solubilase. Also, solubilase activity in the grain increased with increasing number of days of rain prior to the harvest, presumably due to increased fungal growth on wet days.

The solubilase has unusual specificity. β-Glucans contain mostly cellotriosyl and cellotetraosyl residues joined by single β-(1→3) linkages, with about 10% of the polymer formed from residues containing higher cello-oligomer residues. Although these can have a degree of polymerisation (DP) as high as 19, most have a DP ≤ 9.[10,11] It is those cello-oligomers residues of DP 9 and above that the solubilase appears to specifically cleave.[8] This results in relatively high-molecular-weight fragments (20,000 amu). As noted by Yin and MacGregor,[7] other enzymes known to hydrolyse barley β-glucan, e.g., *endo*-β-(1→3)-D-glucanase (EC 3.2.1.39), *endo*-β-(1→3), (1→4)-D-glucanase (EC 3.2.1.73) and *endo*-β-(1→4)-D-glucanase (EC 3.2.1.4) produce low- as well as high-molecular-weight fragments. Because of its specificity for the longer cello-oligomers, MacGregor came to the conclusion that the solubilase was a type of '*endo*-β-(1→4)-glucanase that attacks long blocks of glucosyl residues between two single (1→3)-β-linkages in barley β-glucan'.[8] So although the action of the enzyme, as well as its occurrence and origin are known, the solubilase has not been fully characterised to date. In this paper we show that, contrary to what MacGregor suggested,[7] cellulases (EC 3.2.1.4) can have similar activity to that of the solubilase under dilute conditions.

2. Results

The molecular structures of the extracted β-glucans were analysed by lichenase hydrolysis.[8,10–14] Lichenase specifically degrades β-(1→4) linkages on the reducing end of a 3-linked β-D-glucopyranosyl residue in a β-(1→3), (1→4) linkage sequence. This forms a series of oligomers with a β-(1→3)-D-glucopyranosyl residue at the reducing terminus, which we have called licheno-oligomers. The licheno-oligomers can be quantitatively determined by chromatography.[8,10–14] Since each of the licheno-oligomers has the same DP to that of the corresponding cello-oligomer residues in the β-glucan, the determination gives a measure of the quantity of each cello-oligomer residue.

MacGregor showed that the solubilase activity is associated with the husk of the barley grain, and this activity decreases the molecular weight of the β-glucan. This is consistent with what we found. Hulled varieties tend to produce low-molecular-weight β-glucans even with short extraction times. Therefore to minimise enzyme hydrolysis and produce a high-molecular-weight β-glucan, a hull-less barley variety was chosen for extraction.

Two samples of Glucagel were prepared from the hull-less variety by a freeze–thaw process.[1] The first, H-Glucagel, prepared from an extraction at 45 °C that lasted for 0.5 h, produced a sample that had a relatively high-

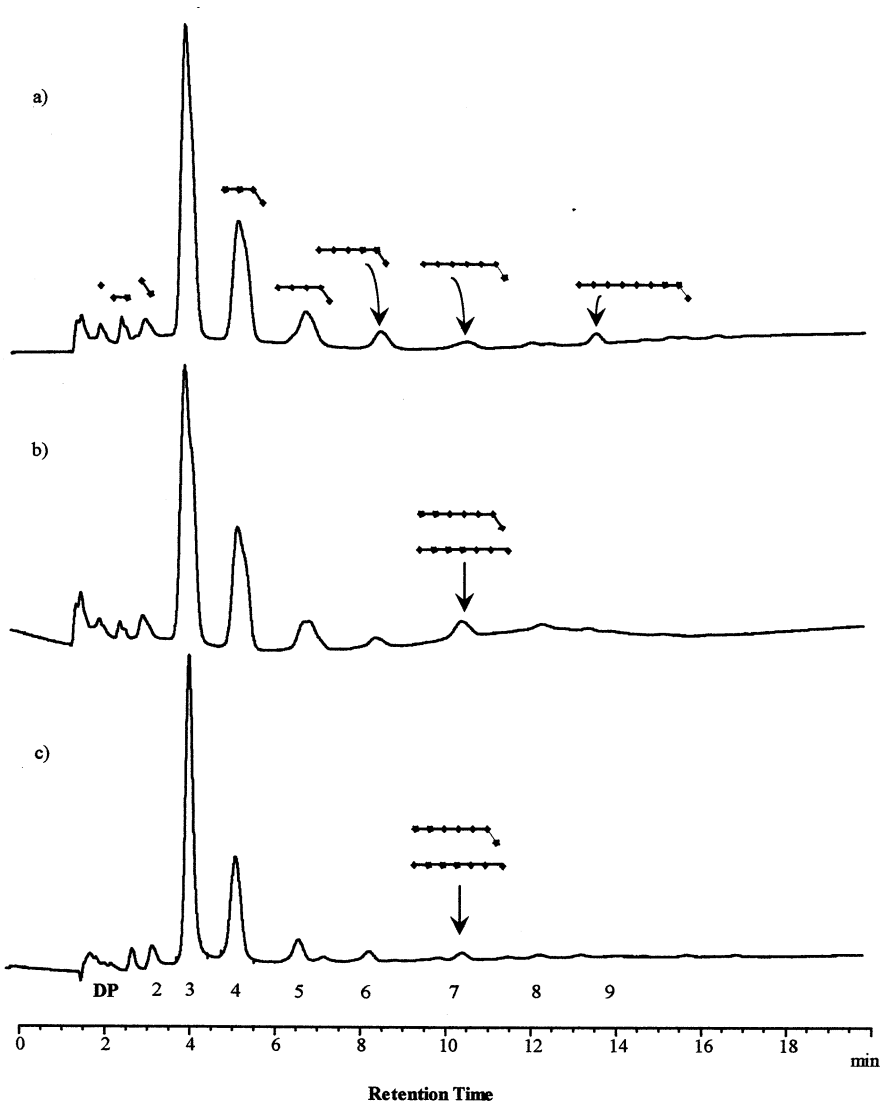


Fig. 1. Dionex chromatogram of lichenase-treated (a) H-Glucagel; (b) L-Glucagel and (c) H-Glucagel that had been partially hydrolysed with cellulase (2 U/g). Key: ●, glucose unit; —, β-(1→4)-link; \, β-(1→3)-link.

molecular-weight. The second, L-Glucagel, of low-molecular-weight was prepared from an extraction at 45 °C that lasted for 6 h. Even though hull-less varieties had decreased solubilase activities, this length of time still resulted in considerable hydrolysis of the β-glucan by the solubilase.

Results obtained for lichenase digestion of the H- and L-Glucagel samples are shown in Fig. 1. As expected, both samples contained predominately cellotriosyl (DP 3) and cellotetraosyl (DP 4) units with smaller amounts of higher DP cello-oligomers up to about DP 9. The ratio of the cellotrisoyl to cellotetraosyl units (2.04) was consistent with that obtained by others[8,10–14] for β-glucan extracted from barley. There are, however, important differences found in their structure (Table 1). The high-molecular-weight material, H-Glucagel, contained significant quantities of the DP 9 cello-oligomer, whereas in the low-molecular-weight material, L-Glucagel, there were only small amounts of the DP 9 cello-oligomer. The L-Glucagel sample also had a significant increase in the height of the peak eluting at the position expected for a DP 7 licheno-oligomer. These results are similar to those obtained by MacGregor and Yin[8] for the hydrolysis of a barley β-glucan by a partially purified solubilase extracted from barley and confirm that the enzyme responsible for the hydrolysis of the β-glucan in the Glucagel

Table 1
Retention times and % peak areas for Dionex chromatograms of lichenase-treated Glucagel samples [a]

Sample	Retention time (min)	% peak areas				
		H-Glucagel	L-Glucagel	H-Glucagel 2 U/g cellulase [c]	H-Glucagel 10 U/g cellulase [c]	H-Glucagel 50 U/g cellulase [c]
Glucose	2.1	1.2	1.3	[b]	[b]	7.6
Cellobiose	2.5	2.0	1.5	3	7.5	18.9
Laminariboise	3.1	2.0	3.1	5.5	13.3	20.1
DP 3	4.1	52.5	52.5	57.3	45.9	26.5
DP 4	5.3	25.8	26.0	24.7	18.0	4.7
DP 5	6.9	8.0	8.3	5.0	8.2	7.4
DP 6	8.6	3.9	2.1	2.2	2.7	2.7
DP 7	10.7	2.1	3.6	1.3	4.2	8.1
DP 8	12.2					
DP 9	13.7	1.5	<0.5	<0.5	<0.5	0

[a] Samples that were treated with cellulase before lichenase treatment are indicated with the U quantity of cellulase added per gram of Glucagel.
[b] Peak obscured by Me_2SO.
[c] Hydrolysis occurring over 1 h (see Section 4 for details).

process has equivalent activity to that of the solubilase.

The action of the cellulase (EC 3.2.1.4) at various concentrations on H-Glucagel was also examined using lichenase hydrolysis and ion chromatography. Results for several different cellulase concentrations are shown in Figs. 1(c) and 2, and % peak intensities are recorded in Table 1. At low cellulase concentrations, the DP 9 cello-oligomeric residues were preferentially hydrolysed, and the chromatograph (Fig. 1(c)) is similar to that obtained on lichenase hydrolysis of L-Glucagel. Since L-Glucagel was formed by degradation of the β-glucan by solubilases in the grain, this would indicate that cellulase (EC 3.2.1.4.) has equivalent activity to solubilase.

In all the lichenase digests of the β-glucan, there are three peaks that elute prior to the DP 3 oligomer (lichenotrisaccharide) but after the 'solvent' peak, and these are particularly prominent in the chromatograms of the Glucagel that has been treated with large amounts of cellulase (Fig. 2(a,b)). MacGregor and Yin[8] did note the presence of two of these peaks that they identified as glucose and an unspecified disaccharide. By comparison to reference samples, we found that the peak having the shortest retention time was due to glucose, the peak having the next shortest retention time was due to cellobiose, and that of the third peak was due to laminarabiose. Chromatograms of a cellulase-treated sample that had not been further treated with lichenase show that both glucose and cellobiose are being produced during cellulase hydrolysis, but not significant quantities of laminarabiose. Most of the laminarabiose appears during lichenase hydrolysis of the cellulase-treated samples.

By examining the hydrolysis of a series of cello-oligomers, Vincken et al.[15] found that the active site for cellulase EC 3.2.1.4. hydrolysis was at least one or two units removed from the reducing end at least for those cello-oligomers having DP > 3. Similarly it was expected that the active site for cellulase hydrolysis in a β-glucan chain would be towards the reducing end of the DP 9 cello-oligomer. We assumed, based on the studies of Vincken et al., that cellulase hydrolysis of the β-glucan could produce a DP 7 cello-oligomer and laminarabiose after lichenase treatment (see Scheme 1). Further hydrolysis of the same chain could lead to production of cellobiose and a DP 5 cello-oligomer, or glucose and a DP 6 cello-oligomer. In fact significant increases in the intensity of the DP 5 and 7 peaks were observed. The increase in the intensity of the DP 6 peak was less matching

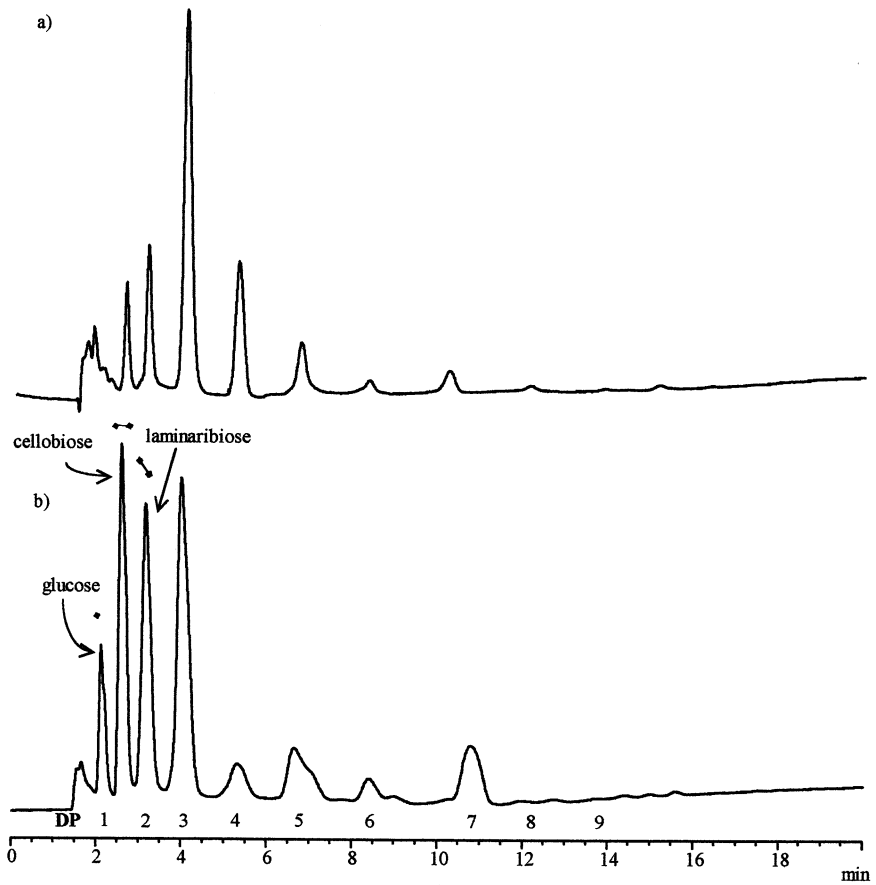


Fig. 2. Dionex chromatogram of lichenase-treated H-Glucagel that had been partially hydrolysed with cellulase (a) 10 U/g; (b) 50 U/g.

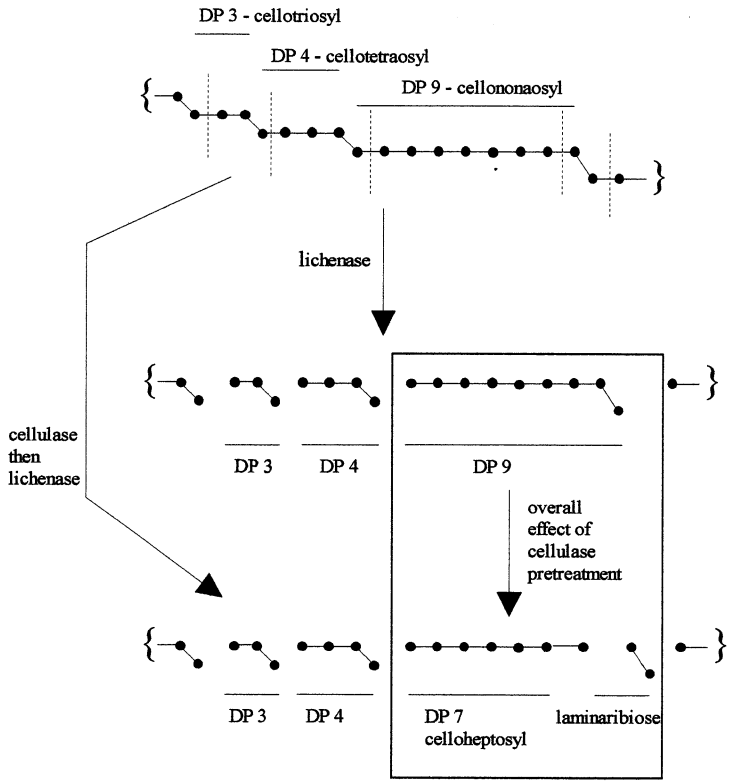
the smaller increase observed in glucose intensity at least at low cellulase levels.

3. Discussion

That cellulase should preferentially cleave the DP 9 cello-oligomer is probably related to the shape of the enzyme. For cellulase, the active cleft of the enzyme, including binding and catalytic sites, is probably sufficiently large that only cello-oligomeric sequences with DP ≥ 9 in the β-glucan chain can be readily accommodated within the active cleft. Smaller cello-oligomeric residues probably fit less readily because the cleft then has to accommodate at least one β-(1 → 3) linkage. Thus the cello-oligomeric residues with DP ≥ 9 hydrolyse faster.

That cellulase (EC 3.2.1.4) preferentially hydrolyses cello-oligomers having DP ≥ 9 is perhaps not surprising, given that cellulases are optimised for binding to and degrading cellulose. However, cellulase (EC 3.2.1.4) does degrade mixed-linkage cereal β-glucans to low-molecular-weight products. Results using higher concentrations of enzyme do show (Fig. 2(a,b)), as expected, that the enzyme can degrade regions that include smaller cello-oligomeric residues, i.e., those less than DP 9. This indicates that the active cleft of the enzyme can accommodate β-(1 → 3)-linked glucose units even though the hydrolysis always occurs at a β-(1 → 4) linkage. The binding to sites including β-(1 → 3) linkages, however, must be significantly weaker, since at low enzyme concentration there is not significant release of low-molecular-weight products.

Under dilute conditions cellulase behaves like solubilase. Cellulase, when more concentrated, however, will also cleave smaller cello-oligomer residues in the β-glucan chain. This type of behaviour has not been reported for solubilase, probably because solubilase has



Scheme 1. Model of β-glucan showing a region containing cellotriosyl, cellotetraosyl and cellononaosyl residues and the effect of either lichenase or cellulase then lichenase treatment of the β-glucan. Key: ●, glucose unit; — β-(1→4)-link; \, β-(1→3)-link.

only been isolated as a very dilute solution. If solubilase were to cleave smaller oligosaccharides, then increased extraction times should lead to decreased yields of β-glucan. This is precisely what is observed for β-glucans extracted by traditional methods[3] and those extracted by the Glucagel process.[1]

The quantity of cellulase (EC 3.2.1.4) required to cause significant reduction in the molecular weight of the β-glucan is small. This may explain why solubilase has not been identified previously as a cellulase of type EC 3.2.1.4. Certainly cellulases of this type are known to occur in barley grain, and like the solubilases, they appear to be associated with the husk of the grain.[16] The family of cellulases of type EC 3.2.1.4 is quite large, and a wide variety of fungi produce them. Similarly the fungi associated with the cereal grain are reasonably numerous.[9] Different agronomic factors could, therefore, lead to colonisation of the grain by different fungi and the release of different cellulases including cellulases of type EC 3.2.1.4 in the husk of the grain. However, the fact that the cellulase from *Trichoderma* sp. used in this study showed similar behaviour under dilute conditions to that of solibulase suggests strongly that solubilase is a cellulase of type EC 3.2.1.4. Until contrary evidence is available, there is, therefore, no reason to propose the existence of a unique enzyme solubilase for solubilising barley β-glucan.

4. Experimental

β-Glucan extraction.—Two types of Glucagel were prepared by altering the extraction times. With a short extraction time, there was not much hydrolysis of the β-glucan by the solubilase, and the molecular weight was comparatively high. With long extraction times, there was more hydrolysis of the β-glucan by the solubilase and the molecular weight was comparatively low. The high-molecular-weight sample (H-Glucagel™) was formed by extracting a hull-less barley flour for a short time period.[1] Thus, pollard flour (30 g) from a hull-less breeders selection was mixed with

distilled water (150 mL) at 45 °C. The mixture was heated at 45 °C for 30 min. The solids were removed by centrifugation at 3000 rpm for 5 min. The supernatant was filtered through glass fibre and frozen for 14 h. The frozen solution was thawed, and the precipitate in the thawed solution was recovered by filtration, washed with EtOH and dried at 60 °C. The material was purified by forming a 3% solution at 75 °C and repeating the freeze–thaw step. Yield was approximately 1.2 g.

A low-molecular-weight sample (L-Glucagel) was formed by extracting the barley flour for a longer period of time.[1] Thus pollard flour (40 g) from the same breeders selection was mixed with water (300 mL) at 45 °C. The mixture was heated for 6 h at 45 °C. The Glucagel was then isolated by a procedure similar to that used for the high-molecular-weight sample. Yield was approximately 1.3 g.

Three samples of H-Glucagel were partially hydrolysed with different quantities of cellulase. Each sample of H-Glucagel (100 mg) was mixed in a sodium phosphate buffer (20 mL, pH 6.5) in a tube and dissolved by heating on a boiling water bath for 5 min. The solution was cooled to 40 °C in a heating block, and aliquots (4, 20 and 100 μL) of a solution of cellulase (Megazyme EC 3.2.1.4 (EG II)) from *Trichoderma* sp., ~50 U/mL] in water were added. The cellulase concentrations correspond to approximately 2, 10 and 50 U of cellulase per gram of Glucagel.[†] After 1 h of hydrolysis the tube was placed in a boiling water bath for 15 min to destroy cellulase activity. The solutions were assayed for cello-oligomers as described below.

Determination of the cello-oligomeric residues in Glucagel and cellulase-treated Glucagel.—The β-glucan of the Glucagel and the cellulase-treated Glucagel solutions were characterised by a lichenase hydrolysis using a procedure similar to that of Yin and MacGregor.[8] Solutions of Glucagel (100 mg) or cellulase-treated Glucagel in sodium phosphate buffer (20 mL, 20 mM, pH 6.5) were placed in a sealed tube on a heating block at 50 °C. An aliquot of a solution of Lichenase (0.4 mL) in sodium phosphate buffer (20 mM, pH 6.5) (Megazyme EC 3.2.1.73, ~50 U/mL) was added. After 1 h reaction, the tube was heated in a boiling water bath for 15 min to deactivate the enzyme. The oligomers were isolated by lyophilisation. Sizes of the oligomers were examined on a Dionex ion-chromatography instrument using a CarboPac PA1 column, eluting with a binary gradient system (NaOH (150 mmol) and NaOAc (0–300 mmol over 30 min)). Detection was with an integrated amperometer. Samples for injection were prepared by dissolving the lyophilised oligomers in Me_2SO and diluting with water to form a 20% v/v solution of Me_2SO. Concentrations of oligomers were about 0.5 mg/mL.

Chromatograms of glucose, cellobiose and laminarabiose under similar conditions were also obtained.

[†] 'Cellulase (*endo*-1,4-β-glucanase) from *Trichoderma* sp.' Data sheet, Megazyme International Ireland Limited, Bray Business Park, Bray, Wicklow, Ireland.

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