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Isolation of cellotriosyl blocks from barley β-glucan with *endo*-1,4-β-glucanase from *Trichoderma reesei*

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Abstract

Mixed-linkage β-glucan from barley, with a cellotriosyl to cellotetraosyl ratio of 2.9, was hydrolysed with two *endo*-1,4-β-glucanases (cellulases) and one non-cellulolytic β-glucanase isolated from *Trichoderma reesei*. The hydrolysates were precipitated in 90% ethanol and the fragments obtained were further treated with lichenase, followed by analysis of the oligosaccharides released. One of the *endo*-1,4-β glucanases, Cel 5A (EG II), selectively degraded cellotetraosyl units in the polymer and left the cellotriosyl units unhydrolysed. Blocks of cellotriosyl units were isolated on a larger scale using this enzyme. The isolated blocks were fractionated into four fractions using gel permeation chromatography on Biogel P-6 and the structures of the blocks were analysed by ^{1}H NMR spectroscopy. The fractions essentially contained cellotriosyl units of different sizes with a 3-linked glucose residue at the non-reducing end and a reducing end linked to two 4-linked glucose residues. The results thus indicated that the enzyme could hydrolyse a 4-linked glucose residue next to the 3-linked residue at the non-reducing terminal but left two 4-linked glucose residues at the reducing end. The isolated blocks of cellotriosyl units had a molecular weight distribution that fits a theoretical model based on random blocks of triosyl units in the mixed-linkage β-glucan.

Keywords: Barley β-glucan; Cellotriosyl blocks; *Endo*-glucanase

1. Introduction

Mixed-linkage (1→3), (1→4)-β-D-glucan (hereafter referred to as β-glucan) is a major component of endosperm cell walls of cereals, especially in oats and barley (Åman & Westerlund, 1996). β-Glucan has gained a lot of interest over recent years, since it has been shown to improve glucose and insulin regulation and to lower blood cholesterol levels (Braaten et al., 1994; Duss & Nyberg, 2004; Wood, 2004; Wood, Beer, & Butler, 2000). The physiological functions of β-glucan are primarily related to its concentration, structure, molecular weight and interaction with other components in the diet (Tosh, Wood, Wang, & Weisz, 2004). Hence, studies of β-glucan from different sources have gained importance over the years.

β-Glucans are shown to be linear polysaccharides composed of (1→4)-linked β-D-glucopyranosyl units (~70%) substituted at positions 3 or 4, hereafter referred to as 3G4 and 4G4, respectively, and (1→3)-linked β-D-glucopyranosyl units (~30%) substituted at position 4, hereafter referred to as 4G3 (Roubroeks, Andersson, & Åman, 2000a; Wood, Weisz, & Blackwell, 1991). Most of the (1→4)-linkages occur in groups of two (cellotriosyl) or three (cellotetraosyl) separated by isolated (1→3) linkages. Longer blocks of 5–11 contiguous (1→4)-linkages in small but significant proportions are also present (Woodward, Fincher, & Stone, 1983). The cellotriosyl and cellotetraosyl residues have been reported to be arranged in a random fashion (Staudte, Woodward, Fincher, & Stone, 1983).

The most frequently used enzyme in the analysis of the structure of β-glucan is lichenase (Wood, 2004). Lichenase (licheninase) is a β-(1→3), (1→4)-D-glucan 4-glucanohydrolase, i.e. *endo*-β-1,3-1,4-glucanase (EC 3.2.1.73) which specifically cleaves the (1→4)-linkage of the 3G4 units in β-glucan, yielding oligosaccharides containing a single (1→3)-linkage adjacent to the reducing end (Wood et al., 1991). Controlled depolymerisation is also obtained by cellulase, a β-(1→4)-D-glucan 4-glucanohydrolase, i.e. *endo*-1,4-β-glucanase (EC. 3.2.1.4) which hydrolyses the internal (1→4)-

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linkages next to 4-substituted glucose residues (Roubroeks, Andersson, Mastromauro, Christensen, & Åman, 2001; Tosh, Wood, et al., 2004). β-Glucan may be also hydrolysed by a specific type of laminarinase, a β-(1→3)-D-glucan 3/4-glucanohydrolase (EC. 3.2.1.6), which acts on the next (1→3) or (1→4)-linkage after 3-substituted glucose residues. Thus, its action on β-glucan is similar to that of lichenase but in addition to this it is able to hydrolyse laminarin. All these endoglucanases act in a random mode making internal cuts in β-glucan backbone. In addition β-glucan is hydolyzed by some (1→4)-β-glucan glucohydrolases (EC 3.2.1.74) and (1→4)-β-cellobiohydrolases (EC 3.2.1.91), which are exoglucanases and act from the chain end (Henriksson et al., 1995; Hrmova & Fincher, 2001).

Cellulase (endoglucanase) isolated from *Trichoderma reesei* hydrolyses β-glucan from barley and oats to glucose, cellobiose, 4-*O*-β-laminaribiosyl D-glucose, 4-*O*-β-laminaribiosyl D-cellobiose and 3-*O*-β-cellobiosyl-D-cellobiose (Parrish, Perlin, & Reese, 1960; Roubroeks et al., 2001). Partial hydrolysates produced with a *T. reesei* cellulase, tend to produce more elastic gels with stronger junction zones than partial hydrolysates produced with lichenase (Tosh, Wood, et al., 2004). It was suggested that β-(1→3)-linked cellotriose sections of β-glucan were the segments that formed the junction zones in the gel network. The elasticity and the peak temperature in the melting endotherm of gels formed from solutions of cereal β-glucans and lichenan increased linearly with cellotriol content (Tosh, Brummer, Wood, Wang, & Weisz, 2004). This behaviour of the gels was consistent with the above suggestion.

The aim of the present work, was to identify an enzyme that, could specifically cleave cellotetraosyl units and higher homologues, and thus could be used for the isolation of cellotriosyl blocks. Isolated blocks of cellotriosyl units were characterised by lichenase degradation, gel filtration and ^{1}H NMR studies in order to establish the action of the enzyme.

2. Materials and methods

2.1. General

Barley β-glucan (lot 60501) was obtained from Megazyme (Bray, Ireland). The molecular weight of the β-glucan was approximately 216,000 g mol^{-1} according to the supplier. All experiments were performed in duplicate.

2.2. Enzymes

Cel 5A (EG II; 1.4 g l^{-1}) and Cel 7B (EG I; 5.74 g l^{-1}) were purified from *T. reesei* as described by Rahkamo et al. (1996). A β-glucanase (BG I) was purified from a genetically modified strain of *T. reesei* lacking *egl2* and *cbh2* genes, using the general methods described in Rahkamo et al. (1996). The purification procedure consisted of bentonite treatment, anion exchange chromatography on DEAE Sepharose FF, hydrophobic interaction chromatography on Phenyl Sepharose FF, and two successive cation exchange chromatography steps on CM Sepharose FF. The purified protein was homogeneous in SDS-PAGE (molar mass of 31 kDa), and it possessed high activity against barley β-glucan but no detectable cellulase (endoglucanase) activity, as assayed on hydroxyethyl cellulose (HEC) according to IUPAC (1987). The gene or peptide sequences of the purified BG I are not known and thus it has not yet been classified to any glycoside hydrolase family. Therefore, the enzyme was simply named BG I. The other enzymes used are named according to the new nomenclature (Henrissat, Teeri, & Warren, 1998).

2.3. Determination of working enzyme concentration

A solution of barley β-glucan (0.75 mg ml^{-1}) in sodium acetate buffer (0.1 M, pH 4.6) was made by heating in a boiling water bath for 20 min. One millilitre samples of the solution were incubated separately with 50 μl each of BG I (0.029 and 0.0029 g l^{-1}), Cel 5A (0.14 and 0.014 g l^{-1}) and Cel 7B (0.57, 0.057 and 0.0057 g l^{-1}) at room temperature. The time-dependent degradation of β-glucan molecular weight was monitored every 45 min over a period of 28 h by a high-performance size-exclusion chromatography system with specific fluorescence detection (HPSEC-FD) using calcofluor (Rimsten, Stenberg, Andersson, Andersson, & Åman, 2003).

2.4. Preparation of degraded β-glucan

Samples (1 ml) of barley β-glucan solution (3 mg ml^{-1}) were incubated separately with 200 μl each of BG I (0.0029 g l^{-1}), Cel 5A (0.014 g l^{-1}) and Cel 7B (0.057 g l^{-1}) at room temperature. The degradation of β-glucan molecular weight was monitored by HPSEC-FD. The enzyme was inactivated after 90 min of reaction by treatment in a boiling water bath for 15 min. The samples were cooled in an ice bath and the degraded β-glucan was recovered by precipitation in 90% ethanol for 2 h, after which the samples were centrifuged (1000*g*, 10 min) and the supernatants removed.

2.5. Determination of oligosaccharides after lichenase degradation of the degraded β-glucan

The pellets of degraded β-glucan were dissolved in 0.9 ml of sodium phosphate buffer (20 mM, pH 6.5) by heating in a boiling water bath for 20 min. After cooling to room temperature, 100 μl of lichenase solution (EC. 3.2.1.73, Megazyme, 1000 U ml^{-1}) was added and samples were incubated for 2 h at 40 °C. The enzyme was inactivated by treatment in a boiling water bath for 15 min, samples were filtered (0.45 μm) and oligosaccharides analysed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex DX 500 instrument (Sunnyvale, CA, USA) as described by Roubroeks et al. (2000a).

2.6. Scaled-up preparation of β-glucan degraded with Cel 5A

Barley β-glucan (500 mg) suspended in 10 ml of sodium acetate buffer (0.1 M, pH 4.6) was incubated with 2 ml of Cel 5A (0.014 g l^{-1}) at 35 °C for 24 h. The partially degraded β-glucan was dissolved by heating the mixture to 100 °C. After cooling to room temperature, the hydrolysis was continued at 35 °C for 24 h after a fresh addition of 2 ml of Cel 5A (0.014 g l^{-1}). The enzyme was inactivated by treatment in a boiling water bath for 15 min, and the degraded β-glucan was precipitated in 90% ethanol and centrifuged (1000*g*, 10 min). The pellet was dissolved in 10 ml sodium acetate buffer (0.1 M, pH 4.6) and hydrolysis was repeated (2 ml Cel 5A, 0.014 g l^{-1}, 35 °C, 24 h). The final β-glucan fragments were obtained by precipitation in 90% ethanol, centrifugation (1000*g*, 10 min) and freeze-drying the pellet. The molecular weight distribution was analysed on HPSEC-FD.

2.7. Fractionation of isolated fragments from the scaled-up Cel 5A hydrolysis

The freeze-dried fragments from above were fractionated by gel permeation chromatography on Bio-Gel P-6 column (75 × 1.6 cm) (Bio-Rad, Richmond, CA, USA) using water as eluent at a flow-rate of 0.4 ml min^{-1}. The sample (two runs of 20 mg) was monitored by a refractive index (RI) detector (Model R-403, Waters, Milford, MA, USA) and 4 ml fractions were collected. They were pooled into four fractions (F1, 170–269 min; F2, 270–339 min; F3, 340–389 min; F4, 390–480 min) on the basis of the elution profile and freeze-dried.

2.8. NMR spectroscopy

^{1}H NMR spectroscopy was performed on a Bruker DRX-400 spectrometer (Bruker Spectrospin Canada, Milton, Ont., Canada) operating at 80 °C. The samples (F1, 2.5 mg; F2, 5.3 mg; F3, 9 mg; F4, 11.2 mg) were dissolved in 0.7 ml D_2O.

2.9. Theoretical model of triosyl and tetraosyl distribution

A β-glucan polymer with randomly distributed triosyl and tetraosyl units was simulated in Matlab (The MathWorks Inc., Natick, MA, USA). The polymer had a triosyl to tetraosyl ratio of 2.9 and a degree of polymerisation of 3.26×10^7 glucose residues. The number of sequential triosyl blocks with lengths up to 40 units (120 glucose residues) was counted and the proportion of glucose residues in each block calculated.

3. Results and discussion

3.1. Determination of working enzyme concentration

Two major *endo*-1,4-β-glucanases (cellulases) from *T. reesei* belonging to two different glycosyl hydrolase families (Cel 5A and Cel 7B) were chosen for the study. These enzymes are known to possess different hydrolytic properties. Cel 5A has much higher specific activity towards hydroxyethyl cellulose (HEC) and barley β-glucan than Cel 7B. On the other hand, Cel 7B acts very efficiently on xylan too, which Cel 5A does not (Tenkanen, Niku-Paavola, Linder, & Viikari, 2003). In addition, one non-cellulolytic unclassified β-glucanase (BG I) from *T. reesei* was included in the study.

Barley β-glucan was first hydrolysed with BG I, Cel 5A and Cel 7B at different concentrations and the hydrolysates were monitored on HPSEC-FD every 45 min in order to find appropriate conditions for hydrolysis (results not shown). At higher concentrations, BG I and Cel 7B degraded the β-glucan to fragments of molecular weight less than 10^4 g mol^{-1}, which is below the limit of molecular weight detection by HPSEC-FD (Munck, 1989). Even at high concentrations and longer reaction times, Cel 5A left an end product that was detectable by HPSEC-FD. This indicated selectivity in the mode of hydrolysis with Cel 5A.

3.2. Determination of oligosaccharides after lichenase degradation of the hydrolysates

The dominant oligosaccharides obtained by lichenase degradation of the starting material were 3-*O*-β-cellobiosyl D-glucose and 3-*O*-β-cellotriosyl D-glucose, as previously shown in literature (Wood et al., 1991). The molar ratio of cellotriosyl to cellotetraosyl was 2.9. Small amounts of other oligosaccharides of higher DP (5–9) were also detected.

Isolated fragments after extensive hydrolysis of β-glucan with BG I, Cel 5A and Cel 7B, respectively, were precipitated in 90% ethanol and subjected to further lichenase digestion. Oligosaccharides and polysaccharides precipitated from the BG I hydrolysis showed a similar composition to the starting material after lichenase hydrolysis. Analysis of the supernatant after ethanol precipitation from BG I hydrolysis also revealed a very similar pattern, indicating a lichenase activity in BG I. Thus, BG I could be classified as a lichenase.

Lichenase degradation of the precipitates obtained after Cel 7B and Cel 5A hydrolysis gave chromatograms where the weight ratio of trisaccharide to tetrasaccharide was 10.4 and 11.4, respectively, showing an accumulation of triosyl residues. However, the yield of larger hydrolysis products after Cel 7B treatment was very low, as mentioned above.

3.3. Isolated fragments from Cel 5A hydrolysis

The isolated fragments after the scaled-up Cel 5A hydrolysis were analysed by ^{1}H NMR spectroscopy (Fig. 1). The assignment of signals in the anomeric region was carried out in comparison with results from the study by Roubroeks et al. (2000b). The anomeric signals assigned to 3G4 and 4G4 residues indicated that their amounts were almost equal, which is true for fragments containing only cellotriosyl units. This confirmed the inference that Cel 5A preferentially degraded cellotetraosyl units as well as the higher homologues and the main constituent of the residual fragments after Cel 5A treatment was cellotriosyls. Judging by the intensity of the 4G3 signal, the cellotriosyls did not appear to be isolated. The signal due to a G4 non-reducing end (δ 4.51) is insignificant when

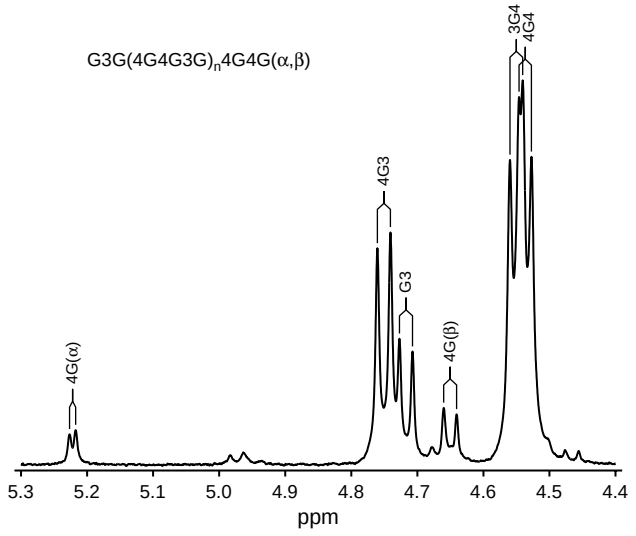

Fig. 1. ^{1}H NMR spectrum (anomeric region) of isolated blocks of cellotriosyl units after hydrolysis of barley β-glucan with *endo*−1,4-β-glucanase (Cel 5A) from *Trichoderma reesei*.

compared to the G3 non-reducing end signal, which indicates that the enzyme selectively hydrolysed the linkage between the 4G4 residue and its adjacent 4G3 residue of cellotetraosyl units. Hence, the isolated fragments from Cel 5A hydrolysis should consist of contiguous cellotriosyl units (blocks) with a G3 non-reducing end and a reducing end linked to two 4-linked glucose residues.

Using gel filtration, it was possible to separate the isolated fragments from Cel 5A hydrolysate into four fractions (F1, F2, F3, and F4) in the order of decreasing molecular weight (Fig. 2). In the ^{1}H NMR spectra (Fig. 3), the relative proportion of signals due to α and β reducing ends increased from F1 to F4. The amount of G3 residues as non-reducing ends also increased from F1 to F4, which showed an increase in the

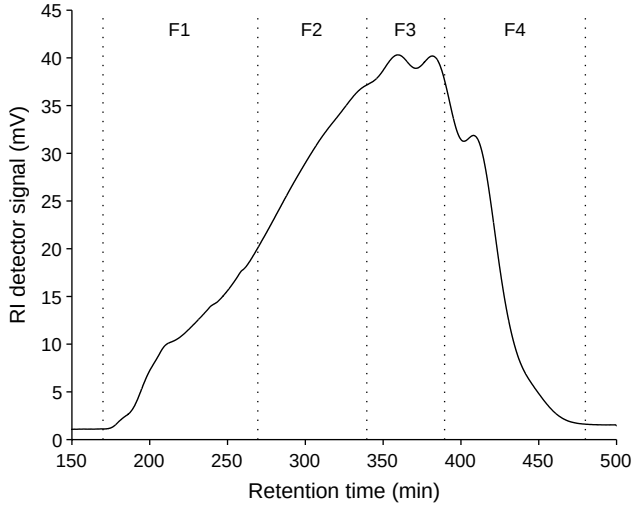

Fig. 2. Fractionation of the isolated blocks of cellotriosyl units from the scaled-up hydrolysis of barley β-glucan with *endo*-1,4-β-glucanase (Cel 5A) from *Trichoderma reesei* on Biogel P-6 using a refractive index detector.

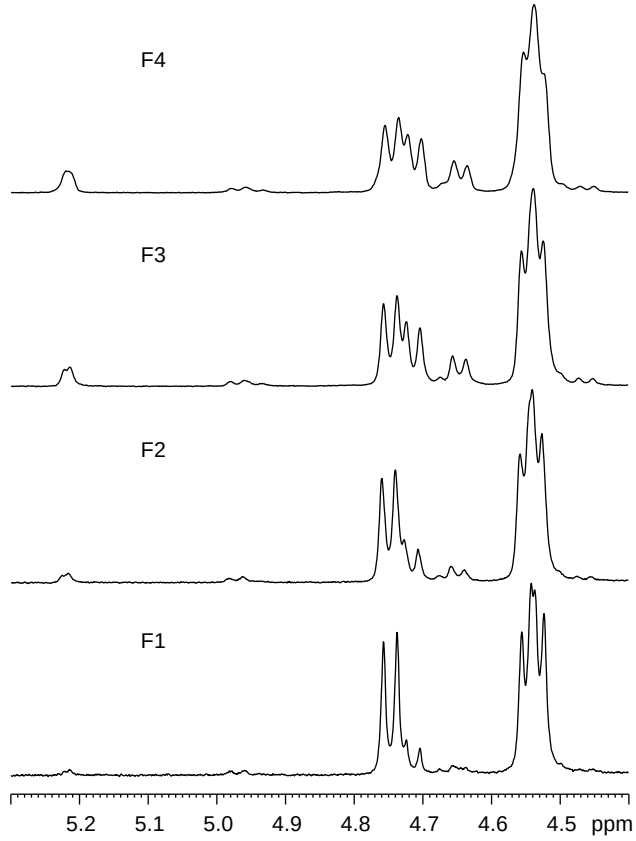

Fig. 3. ^{1}H NMR spectrum (anomeric region) of fractions F1, F2, F3 and F4 (in the order of decreasing molecular weight) after fractionation of blocks of cellotriosyl units on Biogel P6. The blocks were obtained by hydrolysis of barley β-glucan with *endo*-1,4-β-glucanase (Cel 5A) from *Trichoderma reesei.* Signal assignments are given in Fig. 1.

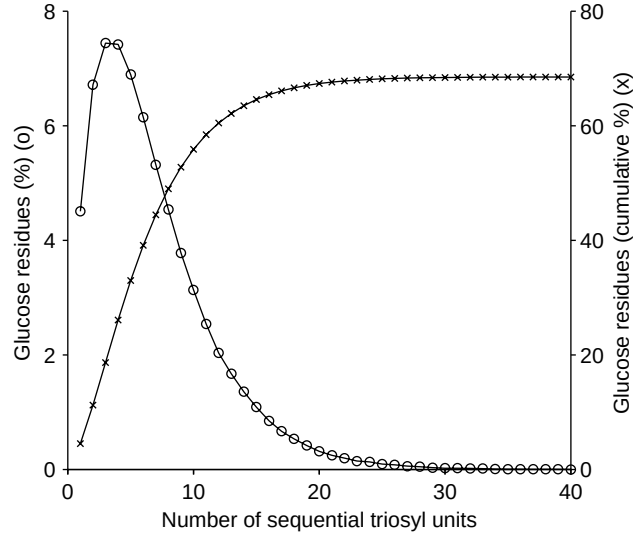

Fig. 4. Theoretical percentage of glucose residues (-○-) present in blocks of sequential cellotriosyl units in a theoretically simulated barley β-glucan (molar ratio of cellotriosyl to cellotetraosyl 2.9). The cumulative percentage of these glucose residues is also shown (-×-).

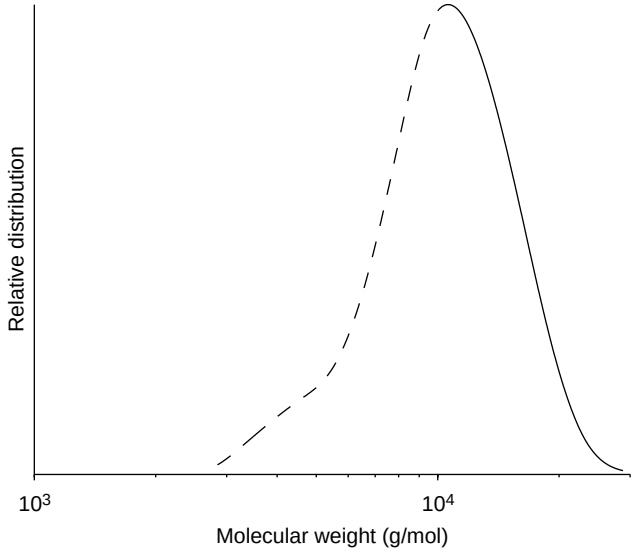

Fig. 5. Molecular weight distribution of isolated blocks of cellotriosyl units from the scaled-up hydrolysis with *endo*-1,4-β-glucanase (Cel 5A) from *Trichoderma reesei*. The blocks were fractionated on HPSEC and detected with specific fluorescence detection using calcofluor. The quantitatively valid part of the distribution is shown as a solid line.

number of short-chain fragments. This was also shown by the amount of 4G3 residues in polymer, which decreased from F1 to F4. The symmetry of the 3G4 and 4G4 overlapped signals in F1 indicated the presence of cellotriosyl units. This and the amount of 4G3 residues in polymer confirmed that cellotriosyls blocks of significant length were present in the F1 fraction. Visual inspection of the samples showed that fractions F1 and F2 formed gels after refrigeration. This confirms the hypotheses by Tosh, Wood, et al. (2004) and Tosh, Brummer, et al. (2004) that β-(1→3)-linked cellotriose sections of β-glucan were the segments that formed the junction zones in the gel network. Tosh, Wood, et al. (2004) also used endoglucanase (cellulase) from *T. reesei* but the paper does not describe in more detail which one.

The number of sequential cellotriosyl units in β-glucan with a molar ratio of cellotriosyl to cellotetraosyl of 2.9 (Erfle, Teather, Wood, & Irvin, 1988; Wood, Weisz, & Blackwell, 1994) was theoretically calculated assuming a random distribution of cellotriosyl and cellotetraosyl units (Fig. 4). The theoretical percentage of glucose residues in triosyl blocks of more than 20 units (molecular weight $>10^4$ g mol^{-1}) is 1.1% and the cumulative percentage of glucose residues present in triosyl units is 68.5%, assuming a molar ratio of 3.0. This corresponds to the triosyl blocks of molecular weight greater than 10^4 g mol^{-1} present in ethanol-precipitated fragments from the scaled-up Cel 5A hydrolysis (Fig. 5). The experimentally determined distribution shown in Fig. 5 is not complete since the calcofluor response is only quantitatively valid above 10^4 g mol^{-1} corresponding to blocks of 20 triosyl units. The experimentally determined yield of these blocks, calculated from the HPSEC-FD analysis, was 1.2% of the starting material, which is in excellent agreement with the theoretically calculated value. In the theoretical model the percentage of glucose residues in triosyl blocks of 30–40 units is approaching zero, which also fits well with the experimental distribution.

4. Conclusions

A method for isolation of blocks of cellotriosyl units from β-glucan using a specific *endo*-1,4-β-glucanase Cel 5A from *T. reesei* was developed. The presence of cellotriosyl blocks in β-glucan in small but significant amounts was established. The number of cellotriosyl units present in these blocks can reach at least 40 units, which could be involved in the formation of junction zones in β-glucan gel networks. More knowledge about the distribution of cellotriosyl residues in mixed-linkage β-glucan is of great importance for understanding the biological, technological and nutritional role of this polymer.

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