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Structural features of water soluble (1,3) (1,4)-β-D-glucans from high-β-glucan and traditional oat lines

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Abstract

Information on structural features of β-glucans purified from a traditional (4.4% β-glucan) and two high β-glucan oat lines (6.0 and 7.3%) was obtained by partial and complete hydrolysis with lichenase. The depolymerization process was monitored with disodium 2,2′-bicinchoninate reductometry (BCA), fluorophore-assisted capillary-electrophoresis (FACE) and nuclear-magnetic-resonance spectroscopy (NMR). The average degree of polymerization (DP) of the completely hydrolyzed β-glucans was the same for β-glucans from the three oat lines, as determined from NMR spectra (3.7), by FACE (3.4), and by BCA (3.9). By FACE, the β-glucans from all three lines had the same molar ratio of cellobiosyl-(1,3)-D-glucose/cellotriosyl-(1,3)-D-glucose units of 1.6. By NMR the ratio of β (1,4)/β(1,3) before hydrolysis was 2.4 and after complete hydrolysis 1.7, with no significant differences among oat lines. Identical molar ratios of penultimate oligosaccharides (DP6, DP7 and DP8) released during partial depolymerization suggested the same arrangement of cellotriosyl/cellotetraosyl residues in the β-glucan polymers from normal and high β-glucan oat lines. Differences in lichenase specificity toward the β-glucans from the three sources, however, were observed during partial depolymerization, which were attributed to differences in chain lengths of the three polymers. The preferred substrate was the polymer with the highest initial DP. A more limited specificity was observed for the β-glucans with the lowest initial DP.

Keywords: β-glucan; Oats; Structure; Lichenase; NMR; Capillary electrophoresis; Soluble fiber; Dietary fiber

1. Introduction

Food products derived from oat are known to reduce plasma cholesterol, glucose and insulin concentrations in humans. These effects are attributed to the water-soluble mixed linkage (1,3)(1,4)-β-D-glucans and seem to be a result of different mechanisms, whether working alone or in combination. The physiological effects exerted by soluble fibers, such as β-glucans from oats, are likely related to their gel-forming properties, which increase viscosity of intestinal chyme. Increased viscosity disturbs micelle formation, which may inhibit cholesterol absorption, slow cholesterol transfer across the unstirred layer, and increase bile acid excretion by inhibiting bile acid reabsorption (Anderson, 1995).

The physical properties (viscosity, solubility) of β-glucans depend not only on the degree of polymerization (DP), but also on small differences in the structural features that influence the ability of the molecules and chains to align into relatively stable molecular aggregates (Gómez, Navarro, Garnier, Horta, & Carbonell, 1997; Woodward, Phillips, & Fincher, 1983). The β-glucan structure was established using a sequence-dependent *Bacillus subtilis* endoglucanase (lichenase) that cleaves (1,4)-β-D-glucosyl units only if preceded by (1,3)-β-units and yields primarily a trisaccharide, cellobiosyl-(1,3)-D-glucose, and a tetrasaccharide, cellotriosyl-(1,3)-D-glucose (Anderson & Stone, 1975). Over 90% of the polymer consists of cellotriosyl and cellotetraosyl units, each connected by single (1,3) linkages. The remaining polymer consists of longer runs of cellodextrin interspersed within the polymer and connected by single β-(1,3) linkages (Wood, Weisz, & Blackwell, 1994). Although present in small amounts, the long sequences of adjacent β-(1,4) linkages have been proposed to be responsible for aggregation between chains mediated by hydrogen bonds (Fincher & Stone, 1986). Small

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proportions of longer sequences of β-(1,4) linkages could significantly increase the molecular weight of β-glucans, thus increase the viscosity (Buliga, Brant, & Fincher, 1986). It was suggested that differences in number of consecutive β-(1,4) linkages is responsible for differences in solubility of cereal β-glucan fractions (Izydorczyk, Macri, & Mac Gregor, 1998; Woodward, Phillips, & Fincher, 1988).

This study aimed to enhance knowledge of oat β-glucan structure–function relationships by comparing the chemical structure of β-glucans extracted from oats with high β-glucan quantities (up to 7.3%, dry weight basis-dwb) and from traditional oat lines (4.4% β-glucan, dwb). We previously reported differences in rheological properties of these oat β-glucans, which we related to differences in peak molecular weight and polymer distribution (Colleoni-Sirghie, Kovalenko, Briggs, Fulton, & White, 2003). The (1,3)-(1,4)-β-D-glucan 4-glucanohydrolase (lichenase) was used in this study to partially and totally cleave the polymeric chains to better characterize the sequences present in the β-glucans from three different oat sources. The concomitent changes in the reducing end-group, profile of oligosaccharides released (analyzed by capillary electrophoresis), and chemical structure (by one and two dimensional NMR), were compared among the three β-glucan polymers.

2. Experimental

2.1. Isolation and purification of β-glucans

Oat (*Avena sativa*) grain types were grown in 2001 at the Agronomy and Agricultural Engineering Field Research Center near Ames, IA, on a Nicollet loam soil (fine-loamy, mixed, mesic Aquic Hapludoll). Experimental lines, IA95205 and IA95258, had high β-glucan concentrations (up to 7.3% β-glucan), as previously described (Cervantes-Martinez, Frey, White, Wesenberg, & Holland, 2001). The Paul oat is a naked oat variety (McMullen, Doehlert, & Miller, 1997). Water-soluble β-glucans were extracted, in replicate, as previously described (Colleoni-Sirghie, Kovalenko, Briggs, Fulton, & White, 2003). Pentosan was analyzed by the phloroglucinol colorimetric method (Douglas, 1981). Proteins in extracts were analyzed by the Bradford dye-binding method (Bradford, 1976) with bovine serum albumin as a standard. Starch was analyzed by the enzymatic procedure of McCleary, Gibson, and Meyford (1997). Ash was determined by Method 08-01 (AACC, 1995). The mixed linkage β-glucan content of oats and purified extracts were determined according to the method of McCleary and Glennie-Holmes (1985) using a Mixed-linkage β-glucan assay kit (Megazyme International, Wicklow, Ireland). Purified β-glucans were freeze-dried for further investigation. Solutions were prepared when needed from freeze-dried β-glucans by dissolving them at 80 °C with stirring.

2.2. Partial hydrolysis of β-glucans by lichenase and time course of enzymatic reaction

To determine whether differences in fine structure occurred among the three oat β-glucans, the polymers were partly hydrolyzed by lichenase provided by Megazyme. Substrate specificity of the lichenase towards the three substrates was obtained by monitoring the level of reducing sugars and determining the products profile by fluorophore-assisted capillary-electrophoresis (FACE), during the time course degradation. Enzymatic hydrolysis of β-glucans at 7.5 mg/ml was performed in 50 mM acetate buffer (pH 6.5) at 50 °C, with lichenase at 0.001 U/mg β-glucans. A β-glucan control was prepared by adding buffer instead of enzyme solution. To follow the reducing-end group formation, 2.0 ml aliquots were removed from the reaction mixture at various time intervals and the enzyme inactivated by boiling for 20 min. The concentration of reducing-end groups was determined by the disodium 2,2′-bicinchoninate (BCA) method according to Doner and Irwin (1992), which is most appropriate for the highly sensitive (glucose 3–60 μM) evaluation of oligosaccharide homologues and provides a stoichiometric estimation of hydrolysis products (Garcia, Johnston, Whitaker, & Shoemaker, 1993).

Products released following the time-course incubation were analyzed by FACE, using the following procedure. Aliquots of reaction mixture were centrifuged at 10,000*g* for 5 min and aliquots of supernatant, each containing up to 4 μg glucose equivalents, were evaporated to dryness in a Speed Vac. The reducing ends of the liberated oligosaccharide chains were derivatized with the fluorescent compound, 8-amino-1, 3, 6-pyrenetrisulfonic acid (APTS Catalog No. 09341, Sigma-Aldrich, St Louis, MO), as below. The dried sample was suspended in 2 μl of 1 M sodium cyanoborohydride in tetrahydrofuran (Catalog No 29, 681-3, Sigma-Aldrich) and 2 μl APTS (0.1 mg/μl in 15% acetic acid). The reaction was incubated overnight at 42 ° C, then diluted with 46 μl water, vortexed, and centrifuged briefly in a microfuge. A 5 μl aliquot, containing less or equal to 0.4 μg glucose equivalents, was added to 195 μl purified water, and this sample was applied to a Beckman P/ACE capillary electrophoresis instrument with the cathode on the injection side (reversed polarity) and monitored with a laser-induced fluorescence detector (Beckman Instruments, Fullerton, CA) fitted with an argon-ion laser as the excitation source. The sample injection parameters were 5 s and 0.5 psi. Separation was accomplished at 23.5 kV in an uncoated capillary of 50 μm diameter eCAP™, obtained from the eCAP™ N-linked oligosaccharide profiling kit (Beckman Instruments), by using Carbohydrate Separation Gel Buffer N (Catalog Nos 338451 and 477623, Beckman). The oligosaccharides were detected according their migration times.

2.3. Complete hydrolysis of β-glucans by lichenase

To achieve complete hydrolysis, β-glucans at 2 mg/ml were incubated with lichenase (1 U/mg β-glucan) in 20 mM sodium phosphate buffer, pH 6.5, for 24 h at 50 °C. Reducing end formation was monitored after 1, 4 and 24 h, by using the BCA method. Distribution of oligosaccharides in the final products of hydrolysis were analyzed by FACE.

2.4. NMR spectroscopy

NMR spectroscopy was performed on a Bruker DRX500 spectrometer operating at 60 °C. One dimensional ^{13}C spectra were acquired by using a 5 mm Nalorac BB-{^{1}H} probe. All other spectra were acquired by using a 5 mm Bruker $^{1}H/^{13}C/^{15}N$ TXI probe. Two dimensional $^{1}H-^{13}C$ Heteronuclear Single-Quantum Coherence (HSQC) spectra were acquired with standard experimental protocol (Bodenhausen & Ruben, 1980). β-glucan polymers, and intermediate and final products of enzymatic hydrolysis by lichenase, were deuterium-exchanged by freeze-drying two times from D_2O and then examined as solutions in 99.97% D_2O. Chemical shifts were referenced to internal acetone at a temperature of 60 °C. Laminaribiose and cellobiose were used as controls.

2.5. Statistical analysis

Experiments and individual chemical analyses were conducted in replicate. Results were analyzed by using the Statistical Analysis Computer Program (SAS Institute, Cary, NC). Multiple comparison of means was performed by least significant difference (LSD) at $\alpha = 0.05$.

3. Results and discussion

3.1. Isolation and purification of β-glucans

β-Glucans were purified from three oat lines: Paul, IA95205 and IA95258, that differed by their percentages of β-glucans: 4.4, 6.0 and 7.3% (dwb), respectively. The composition of oats was reported previously by Colleoni-Sirghie et al. (2003). No significant differences ($P < 0.05$) in levels of starch, protein, arabinoxylans, and ash were observed between oat lines, which differed only in β-glucan and lipids levels. For molecular structural characterization high purity of the β-glucans was obtained, with complete removal of starch, and with only traces of protein and arabinoxylans present in the extracts. The extraction yield of β-glucan was about 70% (w/w, dry basis). The β-glucan content in purified β-glucan samples was between 80.2 and 82.3% (w/w, dwb), with 1.5% ash, 0.30–0.33% protein, 0.5–0.7%, arabinoxylans, and with no starch detected. As reported previously (Colleoni-Sirghie et al., 2003), the three β-glucan types exhibited differences in rheological behavior and molecular weight. Viscosity of their solutions (measured at the same concentration) and peak molecular weight (MW) decreased in the order: IA95258 ($\eta =$ 0.528 Pa s at 1% w/w, MW $\cong 2 \times 10^6$ Da), IA95205 ($\eta =$0.177 Pa s at 1% w/w, MW $< 2 \times 10^6$ Da) and Paul ($\eta =$0.042 Pa s at 1% w/w, MW $\ll 2 \times 10^6$ Da).

3.2. Monitoring of reducing ends after hydrolysis of β-glucans by lichenase

3.2.1. Reducing-end formation during partial hydrolysis

Levels of reducing sugars released during the time course degradation of β-glucans at 7.5 mg/ml by lichenase at 0.001 U/mg β-glucan indicated that hydrolysis proceeded linearly in all three samples, up to 90 min of reaction time (Fig. 1A). The three substrates tested were hydrolyzed at similar initial velocities, suggesting the same activity of the enzyme on all three polymers. As the polymerization degree (DP) decreased, the rate of degradation decreased (after 90 min) and differences were observed among the three β-glucan substrates, with the lowest hydrolysis rate for the oat Paul, and the greatest for the oat IA95258.

Equal activity of lichenase on β-glucans differing by their initial MW also was reported by Carbonell, Izquierdo, Rendra, and Manzanares (1998). A decrease in hydrolysis rate as the depolymerization occurred suggested that the substrate-binding cleft in the enzyme accommodated the shorter chains less easily. For endoglucanases with no *exo* activity, low-molecular mass oligosaccharide derivatives are often poor substrates (Chen et al., 1995). Differences in the kinetics observed among the three β-glucans, after the first stage of depolymerization, could be related to a change in enzyme specificity toward fragments released from the three different substrates. The differences in average MW of the initial substrates might be responsible for these patterns. When a β-glucan molecule is enzymatically cleaved, two fragments of lower MW are released, which in turn become substrates. The fragments released from the three polymers with different initial average MW would have different lengths, thus the enzyme might have different specificities toward these molecules. For long-chain substrates, it can be assumed that that all linkages have the same probability of being cut by the endo-enzyme, but for short-chain-length molecules, the linkages occupying different positions have a different probability to be cut, depending on the subsite structure of the endo-enzyme (Sendra & Carbonell, 1999). The β-glucan with the greatest initial DP, oat IA95258, was the best substrate. Lower specificity was observed for the glucans with the lowest initial DP (oat Paul). Differences in enzyme specificity could also be related to differences in the arrangement of β-(1,3) and β-(1,4) linkages in the three polymers. This hypothesis was tested by analysis of oligosaccharides released by FACE (see below).

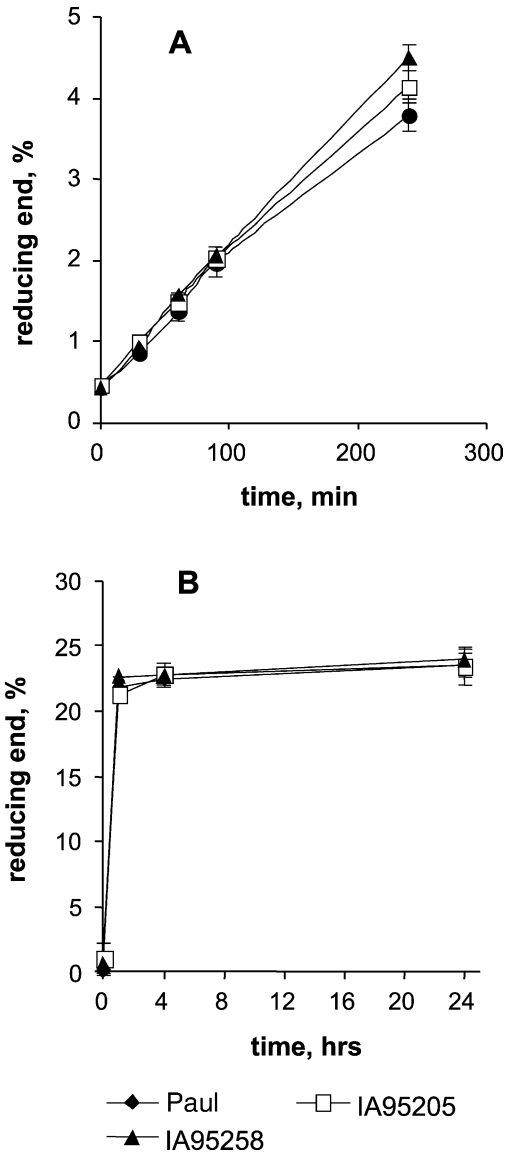

Fig. 1. Time course of enzymatic hydrolysis. Reducing ends were monitored by BCA reductometric method. Results are means from two replicate experiments. (A) Partial hydrolysis of β-glucans at 7.5 mg/ml, with 0.001 U lichenase/mg β-glucan. (B) Complete hydrolysis of β-glucans at 2 mg/ml, with 1 U lichenase/mg β-glucan.

3.2.2. Reducing-end formation during complete hydrolysis

The extent of reducing end formation during hydrolysis in conditions of enzyme saturation (Fig. 1B), showed that the samples were nearly completely hydrolyzed after 1 h of reaction. At complete reaction (after 24 h) the proportion of reducing ends was about 24%, with no significant differences between the three samples. Thus, the three polymers were hydrolyzed approximately to the same extent when lichenase was used in excess. Normally, lichenase can degrade polymers to completion. In contrast, β-1,4-glucan-cellobiohydrolase exhibits product inhibition (Huber & Nevins, 1977). For structural studies, the absence of product inhibition is a distinct advantage, because in exhaustive degradations the terminal products directly reflect the enzyme specificity. An average DP of 3.42 (about 30% reducing ends) is the theoretically lowest value obtainable if the end products are solely 3-*O*-cellobiosyl-glucose and 3-*O*-cellotriosyl-D-glucose with a molar ratio of 1.38 (Roubroeks, Mastromauro, Andersson, Christensen, & Aman, 2000a). For the current work, a reducing end proportion of less than 30% indicated either the presence of products with a DP higher than four or the presence of some polymeric material that was not completely hydrolyzed.

3.3. Analysis of enzymatic products by capillary electrophoresis with fluorescent detection (FACE)

3.3.1. FACE method

In this work, a highly sensitive method was developed for monitoring the enzymatic reaction products released by lichenase from β-glucans. The capillary gel electrophoretic method using laser-induced fluorescence detection (FACE) enabled full quantification at high resolution of the cello-oligosaccharides released during hydrolysis. The method has high sensitivity and resolution, which enabled also separation of cellobiose and laminaribiose (Fig. 2A). The reproducibility of labeling was maintained over the entire chain-length distribution, as demonstrated by comparing separate labeling experiments with different concentrations of β-glucan hydrolysates. The preservation of β-(1,3) linkages during the procedure (these linkages are very fragile and easily hydrolyzed -*Dr P.J. Wood, personal communication*) was tested by labeling and injecting laminaribiose. Only one peak was obtained, demonstrating that molecular integrity was preserved during the analysis (Fig. 2B). Several techniques have been reported in the literature to analyze oligosaccharides released by lichenase from β-glucans. The most used technique is high-performance anion-exchange chromatography (HPAEC-PAD). The quantification of oligosaccharides by this technique is limited, however, by a lack of knowledge of the weight-response factors. The reported accuracy of the molar ratio of tri- to tetrasaccharides, as analyzed by HPAEC-PAD is ±10%, because of different weight response factors of tri- and tetra-saccharides to PAD (Wood et al., 1994). A key advantage of the reductive amination technique is that a single fluorophore is attached per reducing end and the detector response remains constant as the oligomeric chain length increases, thus enabling a molar quantification.

3.3.2. Products released during partial hydrolysis

When β-glucans at 7.5 mg/ml were incubated with lichenase at 0.001 U/mg, depolymerization of substrate produced a complex mixture of oligosaccharides with a wide range of MW, thus confirming that lichenase has a typical endo-action pattern (Fig. 3A–C). The pattern of products released during the enzymatic reaction depends on several factors, including enzyme dosage, initial reaction products acting as substrates for the next phase of

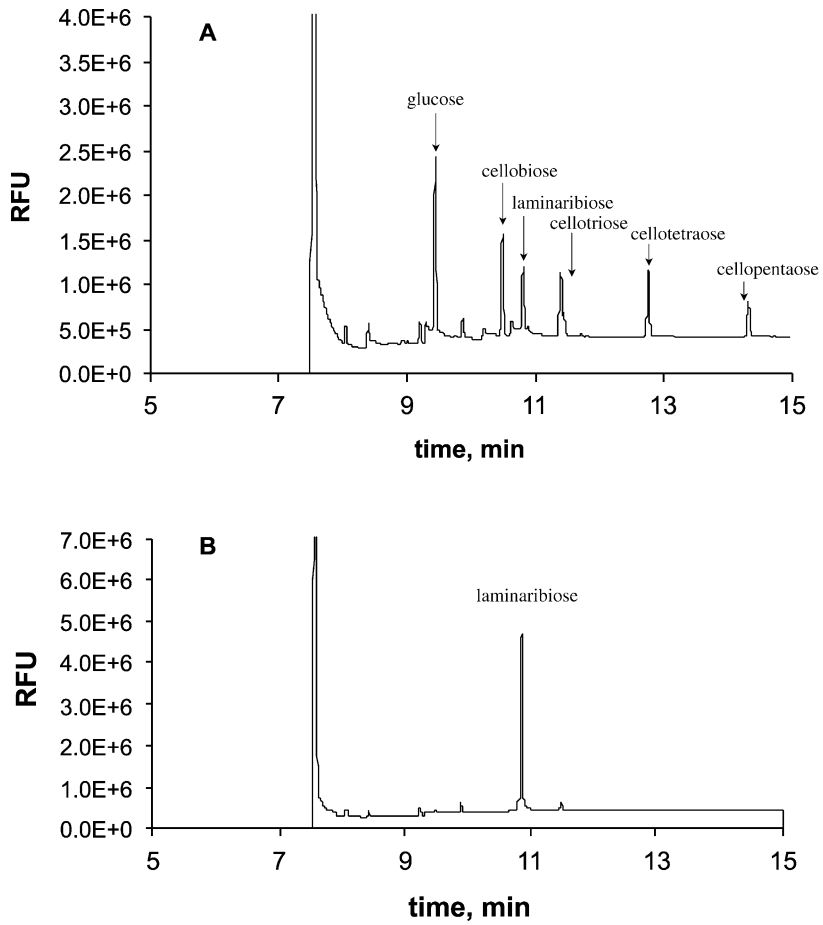

Fig. 2. Analysis of oligosaccharides by fluorophore assisted capillary electrophoresis (FACE). Operating conditions are contained in the experimental section. The peak at 7.5 min corresponds to the APTS. (A) Fractionation of glucose, cellobiose, laminaribiose, cellotriose, cellotetraose and cellopentaose. (B) Analysis of laminaribiose.

the reaction and binding characteristics of the enzyme itself. As noted by Staudte, Woodward et al. (1983), the presence of DP7 (penultimate oligosaccharide, which further generates DP3 and DP4 residues) confirms that β-glucans do not consist of two discrete populations (in this case, only oligosaccharides of DP6 and DP8 would be released), one containing DP3 residues, the other DP4 residues.

As the reaction proceeded (240 min), the relative amount of polymers of DP greater than 16 decreased, with a marked increase in those of DP 3, 4, 6, 7 and 10, suggesting that former oligosaccharides serve as substrates during the later stages of hydrolysis (Fig. 4). The observation that relatively high DP oligosaccharides were released first confirmed that the enzyme has a relatively long substrate-binding domain. For the first 90 min of hydrolysis, β-glucans from the three sources yielded similar results, which confirmed that lichenase had the same activity on the three polymer substrates. After 240 min of hydrolysis, differences were observed in the amounts of products released from the three polymers (Fig. 5): IA 95258 yielded a greater amount of oligosaccharides than did IA95205 and Paul. These results confirmed results obtained by BCA reductometry: there was a greater specificity of lichenase toward the substrate from IA95258 than toward the substrate from IA95205 and Paul oats.

To evaluate whether the differences in specificity observed were related to differences in the arrangement of cellotriosyl and celloteraosyl units in the three polymers, the relative abundance of penultimate oligosacharides, DP6, DP7, and DP8, released during hydrolysis, were evaluated, according to the study of Staudte, Woodward, Fincher and Stone (1983). The time course of release of penultimate oligosacharides, DP6, DP7, and DP8, during enzymatic hydrolysis of the three samples is shown (Fig. 6). For the first 90 min of hydrolysis no differences were observed in the rate of accumulation of these oligosaccharides, among three oat samples. Differences in the rate of accumulation of residues released from three β-glucan samples were observed after 90 min, with the highest rate for IA95258 and the lowest for Paul oat. At 90 min the relative molar abundance of DP6: DP7: DP8 was 0.429: 0.428: 0.143, and was identical for the three oat types. At 240 min the same ratio was observed (0.414:0.446:0.140) with small but not

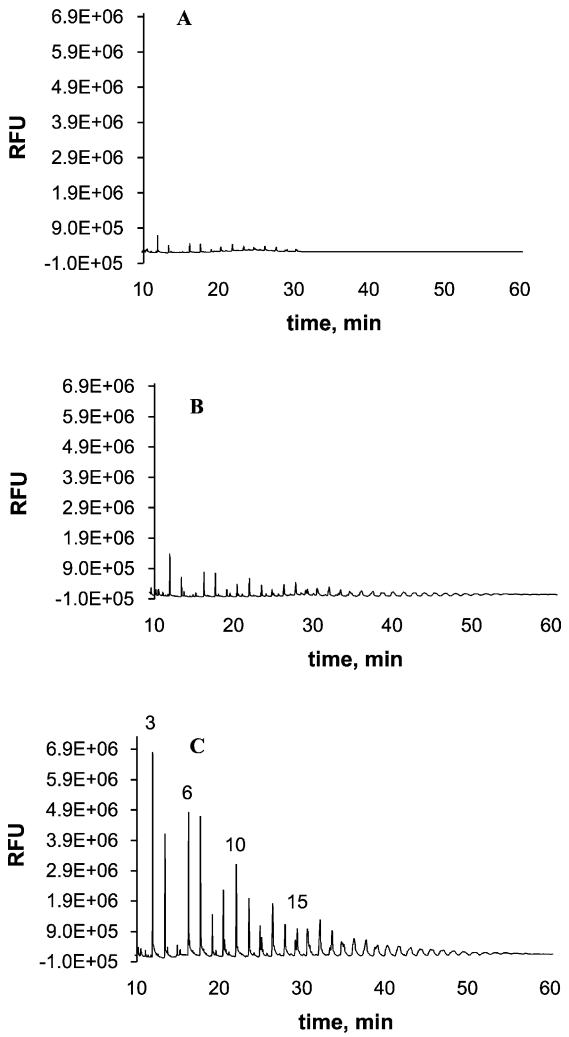

Fig. 3. Time course of release of oligosaccharides from β-glucan (Paul oat line) by lichenase. Electrophoregrams obtained from capillary electrophoresis separation of partially hydrolyzed β-glucan at 7.5 mg/ml with 0.001 U lichenase/mg β-glucan, at different times of reaction: A-60 min, B-90 min, C-240 min. The numbers indicate the polymerization degree of products.

significant differences among oat types. For barley, the reported molar ratio of DP6, DP7 and DP8 released during partial hydrolysis by lichenase was 0.512:0.408:0.08 (Staudte et al., 1983).

The DP6, DP7 and DP8 residues were described as penultimate oligosaccharides because they ultimately hydrolyze to form two cellotriosyl units (2 G4G4G3 from DP6), one cellotriosyl and one cellotetraosyl units (G4G4G3 and G4G4G3G from DP7), and two cellotetraosyl units (2 G4G4G3G from DP8), respectively, which do not degrade further (Staudte et al., 1983). The frequency of two adjacent cellotriosyl units, a cellotriosyl followed by a cellotetrosyl residue, and two adjacent cellotetraosyl units, could be estimated from the relative abundance of DP9, DP7 and DP8. Because the three polymers appeared to have identical relative abundances of penultimate oligosaccharides, observed differences in enzyme specificity toward the three β-glucan polymers could not be explained by differences in polymer sequence.

3.3.3. Products released after complete hydrolysis

Completely hydrolyzed β-glucan polymers contained mainly DP3 and DP4, with smaller amounts of higher MW cello-oligomers of DP up to 10 (Fig. 7). The stoichiometry of the APTS-labelling reaction permitted quantification of substrates on a molar basis (Table 1). No statistically significant differences were observed in the molar distribution of oligosaccharides released from the β-glucans purified from the three oat lines. Complete enzymatic hydrolysis with lichenase confirmed that the backbone structure of β-glucan was similar among the three oat types. In the β-glucans from all oat types, DP3 and DP4 accounted for 89-90% of the total oligosaccharides measured, nearly identical to the value (88–89%) reported previously (Wood et al., 1994). The remaining products consisted of blocks of up to 10 adjacent (1,4)-linkages. The amount of pentaose (2.8–3.1%) was close to that reported elsewhere: 3.3% (Wood et al., 1994) and 3.1% (Johansson et al., 2000).

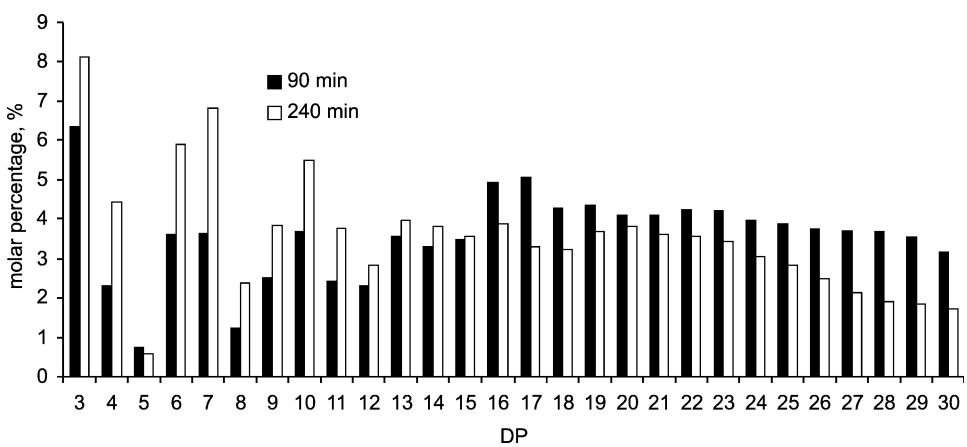

Fig. 4. Molar distribution (relative normalized integration area,%) of oligosaccharides released during partial hydrolysis of oat β-glucans (Paul line) by lichenase.

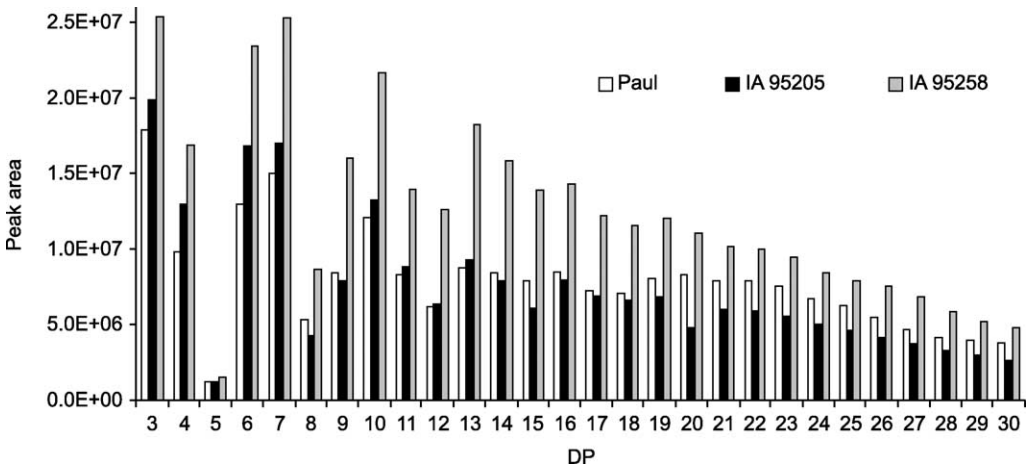

Fig. 5. Products released by partial hydrolysis of β-glucan from Paul, IA95205 and IA95258 oat types, at 7.5 mg/ml with 0.001 U lichenase/mg β-glucan, after 240 min of reaction.

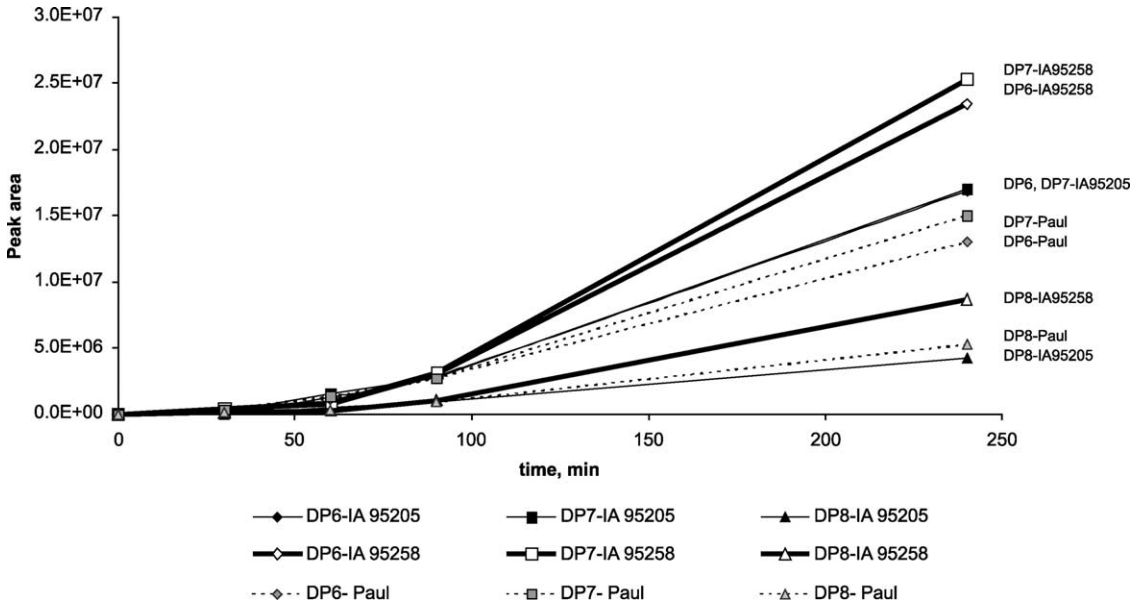

Fig. 6. Time course of release of penultimate oligosaccharides, DP6, DP7 and DP8, from three β-glucan oat lines: A-Paul, B-IA95205 and C-IA95258. The detector response is constant, proportional to the molar amount. The numbers indicate the polymerization degree of products.

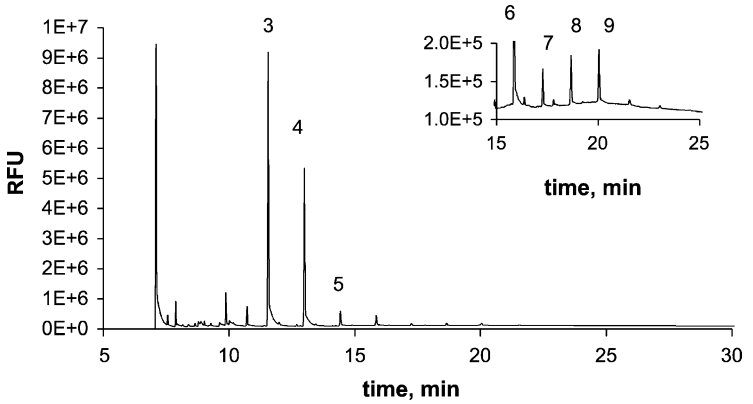

Fig. 7. Electrophoregram obtained from capillary electrophoresis separation of products of completely hydrolyzed β-glucan (Paul oat) at 2 mg/ml with 1 U lichenase/mg β-glucan, after 24 h of reaction.

Table 1
Relative percentages[a] of oligosaccharides released by complete hydrolysis with lichenase of β-glucans from three oat lines, as determined by FACE

Product	Oat line		
	Paul	IA95205	IA95258
Cellobiose	3.68	3.02	2.64
DP3[b]	55.61	55.91	55.80
DP4	33.66	33.62	34.41
DP5	3.15	2.99	2.89
DP6	2.30	2.36	2.16
DP7	0.38	0.43	0.36
DP8	0.51	0.72	0.68
DP9	0.63	0.85	0.91
DP10	0.09	0.11	0.14
Ratio DP3/DP4	1.65	1.66	1.62
Sum DP3 + DP4	89.27	89.52	90.21
Average DP	3.52	3.55	3.55

[a] Values are means from two separate hydrolysis experiments. Samples were analyzed in duplicate. No significant differences in molar percentages ($p > 0.05$) were observed between the three oat lines.
[b] Cellooligosaccharides having 3-*O*-β-substituted terminal glucose residue (DP_n, where n is the number of degrees of polymerization).

A peak corresponding to cellobiose (β-D-glucosyl-1,4-glucose) and an unidentified peak also were observed. No laminaribiose was observed in the final products. The molar ratio of cellobiosyl-(1,3)-D-Glc/cellotriosyl-(1,3)-D-Glc ranged from 1.6 to 1.7, slightly higher than values reported by Johansson et al. (2000) (1.3–1.4) by capillary electrophoresis with UV detection, and lower than the values reported by Wood et al. (1994) (2.1–2.4), obtained by HPAEC-PAD and not corrected for differences in response factors. This ratio depends on the extent of β-glucan degradation and the lowest theoretical value is 1.38, if cellobiosyl-(1,3)-D-Glc and cellotriosyl-(1,3)-D-Glc are the only end products generated (Roubroeks et al., 2000a).

4. NMR spectroscopy

4.1. NMR analysis of polymers before hydrolysis

The ^{13}C NMR and ^{1}H NMR spectra of purified oat β-glucans (Fig. 8) from the Paul oat line confirmed the structure of a mixed β-glucan polymers as assigned by Dais and Perlin (1982), with four types of glucosyl residues: → 4)-β-**Glc***p*(1 → 4) preceded by → 4)-β-Glc*p* (referred as **a** in Fig. 9); → 4)-β-**Glc***p*(1 → 3) (**b**, Fig. 9); → 3)-β-**Glc***p*(1 → 4) (c, Fig. 9); and → 4)-β-**Glc***p*(1 → 4) preceded by → 3)-β-Glc*p*(**d**, Fig. 9). No differences were observed in chemical shifts or the magnitude of signals between β-glucans from the three sources. The ^{13}C NMR and ^{1}H NMR anomeric signals were tentatively assigned by referring to previously reported data (Dais & Perlin, 1982) and data from 2D-HSQC experiments (Fig. 10) which correlate the frequencies of carbon atoms and their directly bonded protons. The 2D-HSQC spectrum for oat β-glucan was similar to those reported for a purified wheat β-glucan (Cui, Wood, Blackwell, & Nikiforuk, 2000) and for a purified rye β-glucan (Roubroeks, Andersson, & Aman, 2000b).

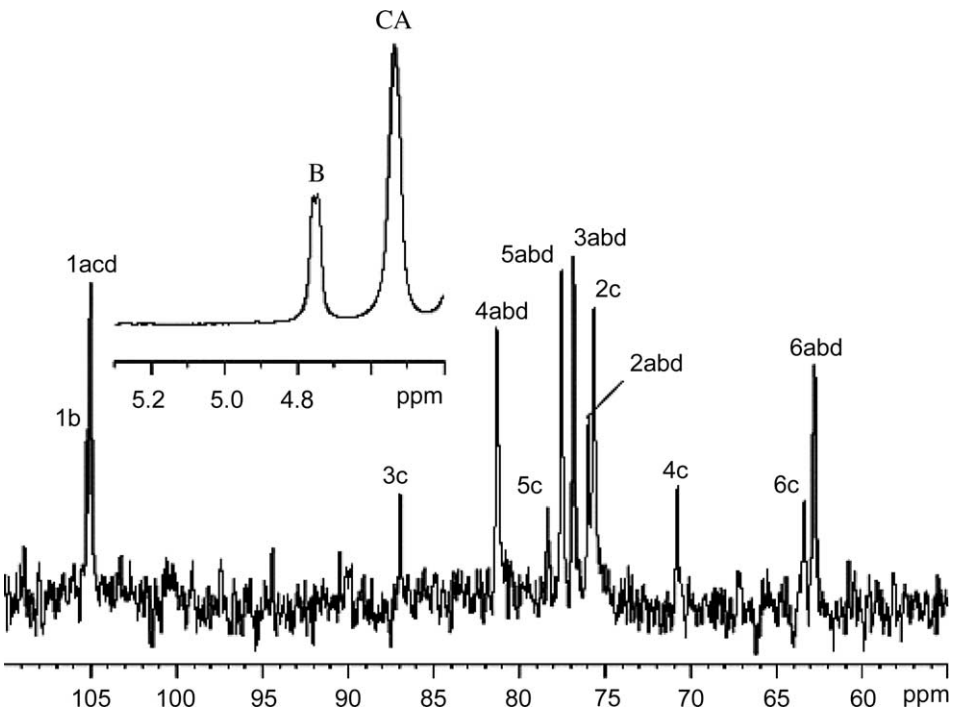

Fig. 8. ^{13}C NMR spectrum (500 MHz, D_2O, 60 °C) of purified β-glucan from Paul oat line. Letters a–d refer to residues shown in Fig. 9. Inset, the ^{1}H NMR spectrum of the anomeric region, showing distinct anomeric protons labeled A–C, with reference to Fig. 9.

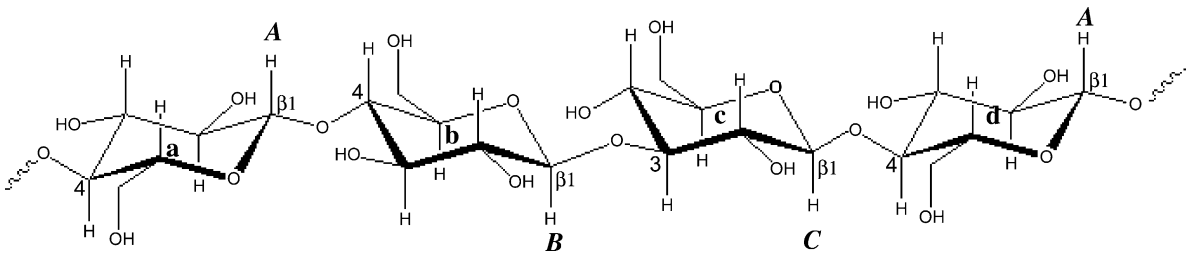

Fig. 9. β-glucan structure indicating four types of residues (a-d). Anomeric protons are indicated with A–C.

The ^{13}C NMR data did not reveal peaks corresponding to the α configuration of the anomeric carbon, meaning the β-glucan extract contained no starch. The α-Glc anomeric carbons would resonate at approximately 100.0 ppm and anomeric β-Glc carbons slightly downfield at about 104 ppm. For C2–C6 of the 3-*O*-substituted residue (residue c in Fig. 9) only sharp single resonances were observed, confirming that 1,3-β-linkages occurred only in isolated locations in the polymer. Cui et al. (2000), Wood et al. (1994) and Woodward et al. (1983) reported similar results. The β-glucan polymers had a molecular mass of 2×10^6 Da, so that no signals from terminal G4 or 4G were visible in the ^1H NMR spectrum, due to extreme line broadening. The absence of signals at 5.4, 5.3 and 5.2 ppm, in ^1H NMR spectrum due to anomeric protons of α-L-arabinofuranosyl residues of arabinoxylan (Bengtsson & Åman, 1991) also confirmed a pure β-glucan extract relatively free from pentosans.

The group of overlapped signals at 4.50 ppm in the ^1H NMR spectrum corresponded to protons **A** or **C** (Fig. 9) of the 1,4-linked-β-D-glucopyranosyl units **a**, **c** or **d** (Fig. 9) substituted at position 3 or 4. The doublet at 4.75 ppm corresponded to proton **B** of 1,3-linked-β-D-glucopyranosyl unit **b** (Fig. 9). The ratio between β-(1,3) and β-(1,4) linkages calculated by the ratio of the integral of the signal at 4.75 ppm to that of the signal at 4.50 ppm, was 1:2.4 with no differences among oat lines. The signals for C-3 (86.8 ppm) and C-4 (81 ppm) from the ^{13}C NMR spectra,

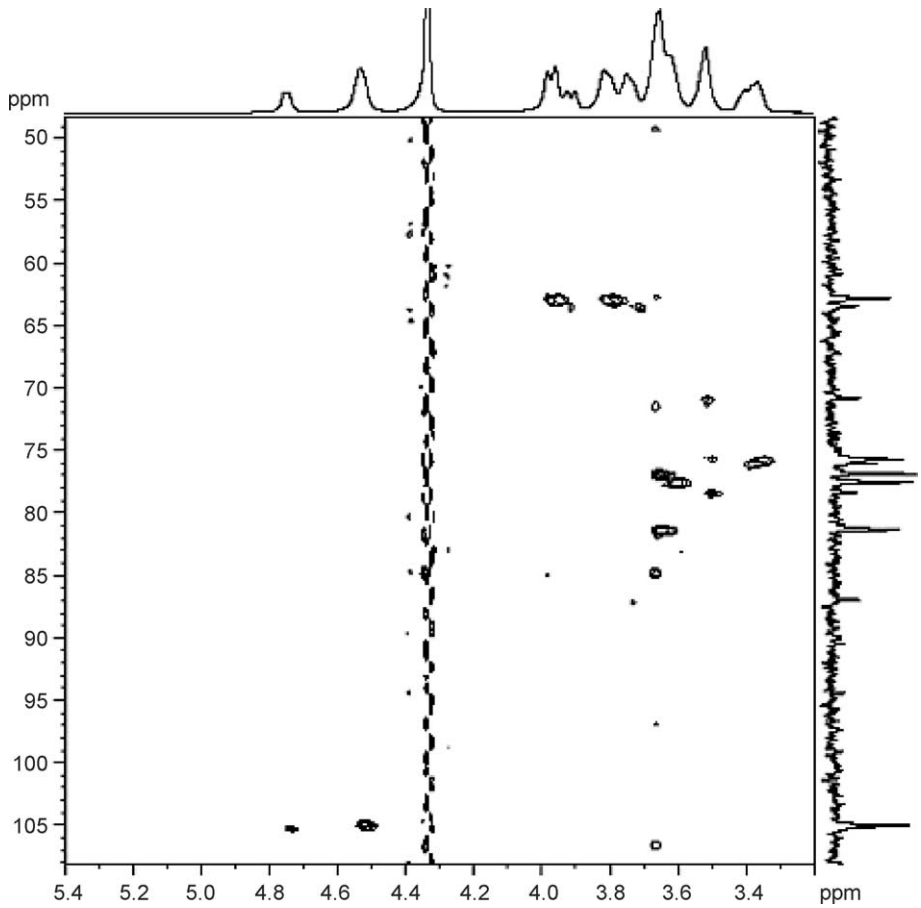

Fig. 10. $^1H-^{13}C$ HSQC spectrum of purified β-glucan from Paul oat line used to map connectivities between carbon atoms and their directly bound protons (500 MHz, D_2O, 60 °C).

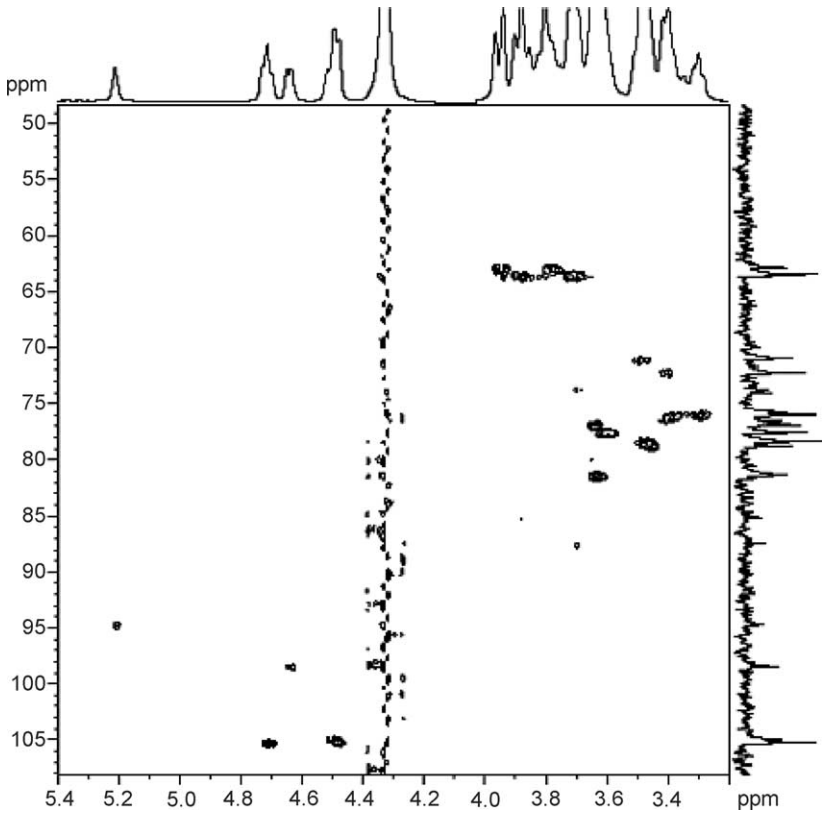

Fig. 11. $^{1}H-^{13}C$ HSQC spectrum of completely hydrolyzed β-glucan from Paul oat line (500 MHz, D_2O, 60 °C).

engaged in the inter-residue linkages were well separated, and their integrals were also used to estimate the ratio of β (1,3) to β (1,4) linkages, which was 1:2.4 for all sources. The ratio of β-(1,4) to β-(1,3) linkages in β-glucans of oats estimated from ^{13}C-signals by Dais and Perlin (1982) was 2.3 and from ^{1}H NMR signals by Roubroeks et al. (2000a) was 2.4.

4.2. NMR analysis of partially and completely hydrolyzed polymers

Similar spectra were obtained for all three β-glucan hydrolyzed polymers, thus only spectra of β-glucans corresponding to the Paul oat line are presented here. Assignments were made by using 2D-HSQC data of completely hydrolyzed β-glucan (Fig. 11) and laminaribiose and cellobiose standard spectra.

The major changes observed in the ^{1}H NMR spectra of partially (Fig. 12A) and completely hydrolyzed (Fig. 12B) β-glucan, when compared to the spectra of the starting polymer (Fig. 8, inset) were the appearance of signals corresponding to the non-reducing ends at 4.65 ppm, characteristic of the anomeric signal of the β-configuration, and at 5.25 ppm, corresponding to the anomeric α-signals. The signal at 5.25 ppm probably resulted from mutarotation of the initially formed β-oligosaccharides, considering that the hydrolysis of β-glucans by lichenase

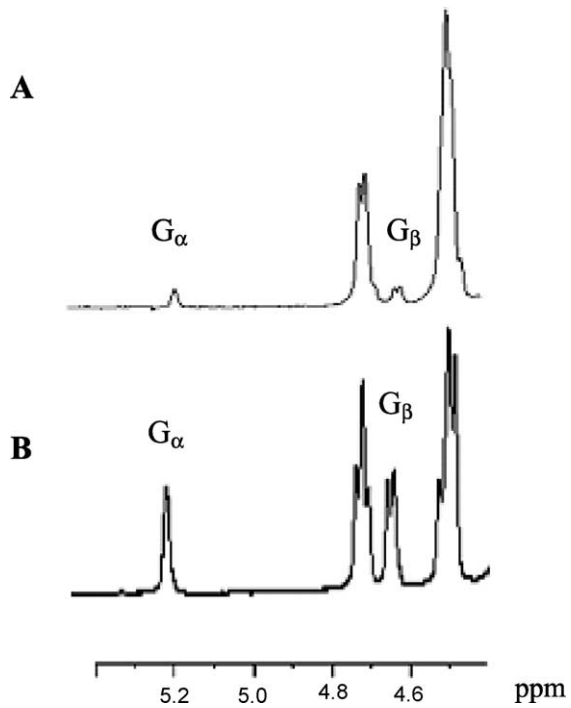

Fig. 12. ^{1}H NMR spectroscopic changes observed during hydrolysis: (A) Partial hydrolyzed β-glucans (Paul line) at 7.5 mg/ml, with 0.001 U lichenase/mg β-glucan after 240 min of reaction. (B) Completely hydrolyzed β-glucans (Paul line) at 2 mg/ml, with 1 U lichenase/mg β-glucan, after 24 h of reaction.

Table 2
Structural parameters obtained by the integration of signals in the anomeric region of ^{1}H NMR spectra

	Oat type	Average DP[a]	Reducing end (%)	Molar ratio β(1 → 4)/β(1 → 3)
Polymer	Paul	ND[b]	ND	2.45
	IA95205	ND	ND	2.37
	IA95258	ND	ND	2.42
Polymer partial hydrolyzed	Paul	18.8	5.3	2.37
	IA95205	20.4	4.9	2.42
	IA95258	17.2	5.8	2.39
Products after complete hydrolysis	Paul	3.8	26.5	1.67
	IA95205	3.7	26.9	1.64
	IA95258	3.7	27.1	1.57

[a] DP is degree of polymerization, determined as the ratio of the sum of all anomeric signals (between 4.5 and 5.2 ppm) to the sum of reducing end signals (at 4.65 and 5.2 ppm). Reducing end percentage was calculated from DP as 100/DP (%).
[b] ND is non determined (non detected reducing ends).

occurs with retention of the β-configuration at the anomeric carbon (Malet, Jimenez-Barbero, Bernabe, Brosa, & Planas, 1993). The anomeric chemical shifts observed for completely hydrolyzed β-glucan (Fig. 12B) correspond to three different types of linkage environments in 1,4-β-linked glucose: at 4.48, 4.50, and 4.52 ppm, corresponding to the G4 non reducing end in the tetrasaccharide (**G4**G4G3G), G4 non- reducing end in the trisaccharide (**G4**G3G) and to the residue 4G4 in the tetrasaccharide (G**4G4**G3), respectively. At least two different environments were also observed for 1,3-β-linked glucose residues, corresponding to the 4G3 residues in the two main products of hydrolysis, the trisaccharide (G**4G3**G) at 4.75 ppm, and the tetrasaccharide (G4G**4G3**G) at 4.76 ppm, respectively. Hydrolysis occurred with an increase of the doublets at 4.65 and 5.2 ppm, signals corresponding to the reducing ends. As a result of the *endo* activity of the enzyme, the doublets at 4.65 and 5.25 ppm were broad signals arising from a complex oligosaccharide mixture. In addition, hydrolysis results in an increase in the amount of non-reducing terminal glucose G4. Other major spectral changes, compared to polymers before hydrolysis, included the sharpening of resonance lines, and the formation of complex multiples, both due to cleavage of relatively large polymeric precursors into more mobile oligoglucosides.

To compare the fine structures of β-glucan polymers from the three sources, the ratio between the β-(1,4) and β-(1,3) linkages, the ratio between β- and α-reducing ends, the average DP and the reducing end percentage, were calculated for partially and completely cleaved polymers (Table 2). The ratios β-(1,4)/β-(1,3) were similar before and after partial hydrolysis, indicating no changes during the first stage of depolymerization. In completely hydrolyzed polymers this ratio decreased to 1.57–1.67, a value close to those reported by Roubroeks et al. (2000a) (1.59, obtained by ^{1}H NMR). No significant differences in the ratio of the β-(1,4) to β-(1,3) linkages occurred between the three β-glucans for either partially hydrolyzed or totally degraded polymers.

The reducing end percentage and average DP, were calculated from the sum of all anomeric signals and the sum of the reducing end signals. In partially hydrolyzed polymers, the reducing end percentage of the IA95258 sample was slightly greater than those calculated for IA95205 and Paul samples. These results agreed with results obtained by the BCA method and capillary electrophoresis, indicating a greater extent of hydrolysis for the IA95258 polymer. The average DP value (3.7) in completely hydrolyzed polymers was similar to those reported by Roubroeks et al. (2000a), obtained by ^{1}H NMR (3.3).

The ratio between α- and β-reducing ends calculated by the integration of the signals at 4.6 and 5.2 ppm was 0.7, indicating the mutarotational equilibrium of α and β anomers. No differences were observed between the three polymer sources.

5. Conclusion

This study characterized structural features of β-glucans purified from three oat lines with different percentages of β-glucans, previously found to differ in viscosity. The lichenase enzyme was used to characterize the fine structure of the three types of β-glucans by both complete and partial hydrolysis. Results obtained by three complementary techniques, BCA reductometry, NMR spectroscopy, and capillary electrophoresis-FACE, were consistent. No differences were observed between products generated by complete hydrolysis of three β-glucans polymers with lichenase in excess. When partially hydrolyzed by lichenase, the three polymer substrates were degraded through a similar process and initial velocity, indicating equal activity of lichenase on the three substrates. Differences were observed, however, in the specificity of lichenase toward the three polymer

substrates during partial degradation. The preferred substrate was the β-glucan with the highest initial DP (IA95258) and the least preferred was that with the lowest initial DP (Paul). Differences among the three oat lines, also were observed in the rate of accumulation of penultimate oligosaccharides (DP6, DP7 and DP8) released during partial depolymerization, but a similar molar ratio suggested the same arrangement of cellotriosyl and cellotetraosyl sequences was present in the three polymers. These results demonstrated that β-glucans from high and normal β-glucan oat lines differ by molecular size, but they have a highly defined structure with the same iteration of cellotriosyl, cellotetraosyl and higher cellodextrins units, each connected by single (1,3)-β-linkages.

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