Structural Studies of (1→3),(1→4)-β-D-Glucans by ^{13}C-Nuclear Magnetic Resonance Spectroscopy and by Rapid Analysis of Cellulose-Like Regions Using High-Performance Anion-Exchange Chromatography of Oligosaccharides Released by Lichenase[1]

PETER J. WOOD,[2] JOHN WEISZ,[2] and BARBARA A. BLACKWELL[3]

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ABSTRACT

The structures of (1→3),(1→4)-β-D-glucans (β-glucans) were evaluated by high-performance anion-exchange chromatography (HPAEC) of the oligosaccharides produced by the action of (1→3),(1→4)-β-D-glucan-4-glucanohydrolase (lichenase; EC 3.2.1.73). The relative amounts of the released oligosaccharides constitute a fingerprint of structure, and the method is particularly useful for identification of minor, cellulose-like features in the polysaccharides. In general, the cereal β-glucans were similar, but the ratio of (1→3)-linked cellotriosyl to (1→3)-linked cellotetraosyl units, which constituted ~90% of the polysaccharides, was lower for oats (2.1–2.4) than it was for barley and rye (2.8–3.3). A portion (3–4%) of oat and barley β-glucan was rendered water-insoluble by lichenase. HPAEC revealed that these products, shown by ^{13}C-nuclear magnetic resonance spectroscopy to be cellulose-like, were composed of oligosaccharides of degree of polymerization 9–15. Lichenan is distinct from cereal β-glucans, being mainly (1→3)-linked cellotriosyl units. No evidence for consecutive (1→3)-linkages was found in any of the β-glucans. The extractability of oat and barley β-glucan was not related to identifiable structural differences. Oat aleurone β-glucan has less of the (1→3)-linked cellotetraosyl units than did endospermic β-glucan.

The physical and physiological properties of (1→3),(1→4)-β-D-glucan (β-glucan) are of commercial and nutritional importance. The viscous nature of barley β-glucan can cause problems in brewing processes (Bamforth 1985), and it limits the value of feed barley (Campbell and Bedford 1992). On the other hand, cereal β-glucan improves glucose and insulin regulation in a viscosity-dependent fashion and reduces serum cholesterol levels in hypercholesterolemic subjects (Braaten et al, *in press*; Wood et al, *in press). Lichenan,* a closely related structure from the lichen *Cetraria islandica*, has antitumor properties (Watanabe et al 1986). Numerous structural studies (Peat et al 1957, Parrish et al 1960, Perlin and Suzuki 1962, Dais and Perlin 1982, Woodward et al 1983, Vaårum and Smidsrφd 1988) have established that (1→3),(1→4)-β-D-glucans are unbranched, that the (1→3) linkages occur singly, and that most of the (1→4) linkages occur in groups of two or three. The resultant structure is a polysaccharide built mainly from β-(1→3)-linked cellotriosyl and cellotetraosyl units. Oat and barley β-glucan have been generally categorized as being structurally similar and containing more of the (1→3)-linked cellotetraosyl units than lichenan does (Parrish et al 1960, Perlin and Suzuki 1962, Fleming and Manners 1966).

Lichenase, a (1→3),(1→4)-β-D-glucan-4-glucanohydrolase (E.C. 3.2.1.73) cleaves the (1→4) linkage of the 3-*O*-substituted glucose units in β-glucan. The released oligosaccharides are the building blocks of the β-(1→4)-linked polysaccharide in the original chain. High-performance liquid chromatography (HPLC) of the major products, 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose (Wood et al 1991), allowed rapid definition of the major structural feature of different β-glucans, without prior purification. The method used automated chemistry (orcinol-sulfuric acid) for postcolumn analysis of the carbohydrate. Peaks from products of degree of polymerization (DP) >4 were not well resolved in this system. Furthermore, it is difficult to dispose of the concentrated acid, which is hazardous.

[1]Contribution 2191 of the Centre for Food and Animal Research, and 1517 of the Plant Research Centre, Agriculture Canada, Ottawa.
[2]Centre for Food and Animal Research, Agriculture Canada, Ottawa.
[3]Plant Research Centre, Agriculture Canada, Ottawa.

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The Dionex HPLC system identifies oligosaccharides of a wide DP range as anions at elevated pH (Koizumi et al 1989); pulsed amperometric detection (PAD) provides sensitive and selective analysis. This article describes rapid assessment of both major and minor structural features of (1→3),(1→4)-β-D-glucans by analyzing oligosaccharides of DP 3–15 released by lichenase.

METHODS

General

Analytical-grade reagents and distilled water were used. Lichenase from *Bacillus subtilis* was supplied by Biocon (Lexington, KY) and can now be obtained from Megazyme (Aust. Pty. Ltd., 6 Altona Place, North Rocks, Sydney, NSW 2151). One unit of enzyme releases 1 μmol/min of reducing sugar, measured as glucose, from an oat β-glucan substrate. The specificity, mode of action, and purity of this enzyme has been well established (McCleary 1988). Lichenan (*C. islandica*) was obtained from the Sigma Chemical Company (L-8378) and purified by precipitation by two freeze-thaw cycles (Peat et al 1957) and a final precipitation by 2-propanol. Barley β-glucan was obtained from Biocon. 3-*O*-β-Cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose were prepared as previously described (Erfle et al 1988) and stored frozen. Cellobiose was purchased from Eastman Kodak (Rochester, NY).

Oat gum (OG, ≈80% β-glucan) and purified oat β-glucan (96% β-glucan) were prepared as described by Wood et al (1989). Partially acid-hydrolyzed gum (91% β-glucan) was prepared by heating OG (1%, w/v) in 0.1*M* HCl at 70°C for 60 min. The mixture was rapidly cooled in an ice bath to ≈30°C. The pH was adjusted to 6.5–7.0 with 1*M* NaOH followed by 0.1*M* NaOH. An equal volume of 95% ethanol was slowly added, with vigorous stirring, to the solution of hydrolyzed gum. The precipitate was recovered, after settling, by syphoning and centrifugation. The precipitate was washed with 47.5% ethanol, recovered, and redispersed in 95% ethanol, then filtered and dried in an oven at 30°C. The yield of hydrolyzed gum was 83%.

β-Glucan was determined by the method of McCleary and Glennie-Holmes (1985) using a Biocon kit. Starch was determined as previously described (Wood et al 1991). Monosaccharide composition was determined by hydrolysis in 1*M* H_2SO_4 at 100°C for 4 hr, followed by appropriate dilution and high-performance anion-exchange chromatography (HPAEC) with PAD. The re-

sponse of each component monosaccharide was determined from a calibration with standards. The water-insoluble oligosaccharides released by lichenase from β-glucan were first hydrolyzed by 1*M* H_2SO_4. The residue was then dissolved in 72% sulfuric acid at room temperature before dilution to 1*M* H_2SO_4 and further hydrolysis. Data reported are the sum of monosaccharides released from the two hydrolyses.

Hull-less or dehulled oat cultivars were provided by V. D. Burrows, Plant Research Centre, Agriculture Canada, Ottawa. Barley cultivars were breeder lines, eight 6-row and eight 2-row; four of each were bred for malting and four of each were bred for feed. These samples were provided by A. W. MacGregor, Grain Research Laboratories, Winnipeg. Rye cultivars and oat pericarp tissue were provided by S. S. Miller of this Research Centre (Ottawa).

HPAEC

A Dionex system (Sunnyvale, CA) using a Carbopac PA1 column (4 × 250 mm) and guard (3 × 25 mm) was used for HPAEC. Gold electrodes were used with PAD. Samples were filtered (0.45 μm) before analysis. Eluent A was 150 m*M* sodium acetate in 150 m*M* sodium hydroxide. Eluent B was 150 m*M* sodium hydroxide. Elution was with 70% A and 30% B for 1 min, then with a gradient to 100% eluent A for 9 min, which was continued for 11 min. The initial conditions were maintained for 10 min between each injection of sample. The flow rate was 1.0 ml/min at ambient temperature. Pulse potentials, *E* (volts), and durations, *t* (milliseconds), initially were: $E_1 = 0.1$, $t_1 = 300$; $E_2 = 0.6$, $t_2 = 120$; $E_3 = -0.8$, $t_3 = 300$. Response time of the detector was 3.0 sec. Following an electrode change, pulse potentials and durations were changed to: $E_1 = 0.05$, $t_1 = 480$; $E_2 = 0.6$, $t_2 = 180$; $E_3 = -0.6$, $t_3 = 60$, with a detector response time of 1.0 sec. The instrument was controlled, and data were processed, using Dionex AI 450 software.

^{13}C-Nuclear Magnetic Resonance Spectroscopy

^{13}C-nuclear magnetic resonance (NMR) spectra were acquired at 90°C on a Bruker AM 500 spectrometer at 125.77 MHz. A sweep width of 11 kHz with a 60° pulse width and recycle time of 1.5 sec was used for 32,000 data points. Spectra were processed with resolution enhancement; chemical shifts were determined relative to deuterated methyl sulfoxide (DMSO-d_6) at 39.5 ppm and reported relative to trimethylsilyl (TMS). Samples were dissolved in the DMSO-d_6 by heating and stirring at 90°C for 1–2 hr; water-insoluble residue was heated for 3 hr. Stirring continued overnight at room temperature; undissolved material was removed by centrifugation. Acid hydrolyzed gum was analyzed at 3% (w/v), unhydrolyzed β-glucan at 1%, and lichenan at 4%. Insoluble residue from lichenase digestion was analyzed at 2% in DMSO-d_6.

Lichenase Treatment of Samples

Isolated β-glucan samples or cereal flours, pretreated with hot aqueous ethanol, were incubated with lichenase (Wood et al 1991). Aliquots of the digest were diluted with water 20-fold for analysis of the tri- and tetrasaccharide products. A solution of increased concentration was required for determination of oligosaccharides of higher DP. To ensure that there was no significant change in detector response, either the two dilutions should be analyzed concurrently or a glucose standard should be used. Initially, undiluted enzyme digest was injected, but in subsequent experiments, following a change in the Carbopac PA1 column, the phosphate buffer (20 m*M*) resulted the appearance of additional peaks and peak shoulders; therefore, samples were diluted 1:1 with water, or incubated in 10 m*M* phosphate.

Isolation of Insoluble Precipitate from Lichenase-Treated Oat and Barley β-Glucan

Oat gum or barley β-glucan (2 g) was dissolved in 0.05*M* phosphate buffer (400 ml, pH 6.9) containing 10 m*M* NaCl by heating (60°C) and stirring for 3 hr. Insoluble material was removed by centrifugation (33,000 × *g* for 30 min). The supernatant was treated with 30 units of hog pancreatic α-amylase (Type 1A, DFP-treated, Sigma, A-6255) for 1 hr at room temperature. The solution was heated (70–75°C) for 45 min to deactivate enzyme, then it was centrifuged at 33,000 × *g* for 1 hr; 2-propanol (400 ml) was added dropwise to the supernatant. The precipitate was collected by centrifugation at 4,100 × *g* for 20 min. It was redispersed using a Virtis homogenizer, recentrifuged in 50% 2-propanol, dispersed in 100% 2-propanol, filtered, and air-dried.

The α-amylase-treated gum was wetted with 80% ethanol (8 ml), dissolved in 20 m*M* phosphate buffer (200 ml, pH 6.5), and treated with lichenase (100 units) for 4 hr at 40°C. The solution was heated to 80°C for 15 min to deactivate enzyme and stored at 5°C overnight. The precipitate was recovered by centrifugation (33,000 × *g*, 30 min). The water-insoluble precipitate was washed twice with water, suspended in 2-propanol, recovered by centrifugation, and air-dried. For HPAEC analysis, the water-insoluble samples were dissolved (≈1 mg/ml) in DMSO by stirring at 90°C for 4 hr and then stirring overnight at room temperature. Residue was removed by centrifugation.

Three volumes of 2-propanol were added to the supernatant of the lichenase digest. The mixture was stored at 5°C for three days; the precipitate was recovered by centrifugation (33,000 × *g* for 30 min), washed with 75% 2-propanol followed by 100% 2-propanol, and air-dried. For HPAEC analysis, the 2-propanol precipitates were dissolved (1 mg/ml) in water (70°C, 3 hr).

Consecutive Aqueous Extraction of Samples

Dried, ethanol-treated flours from two oat cultivars (Donald and Tibor, a naked oat) and from two barleys (one 6-row and one 2-row) were analyzed. Triplicate samples (250 mg) were extracted with 20 ml of water at 40°C for 2 hr. The mixtures were centrifuged at 7,000 × g for 20 min, and the residue was reextracted with 10 ml of water at 65°C for 2 hr. Ethanol (1.5 vol) was added to each of the two aqueous extracts. The resultant precipitates were isolated by centrifugation at 7,000 × g for 20 min and redissolved in 20 m*M* phosphate buffer (pH 6.5, 2.5 ml) before treatment with lichenase (200 μl, 50 units/ml). Residue was similarly treated with lichenase in 5 ml of buffer. Samples were diluted with an equal volume of water for HPAEC analysis of oligosaccharides of DP 5–9; they were further diluted, as necessary, for those of DP 3 and 4. Standards of oat and barley β-glucan were also analyzed.

Analysis of Oat Pericarp Fraction

Oat pericarp tissue, prepared by abrasion in an elbow blender lined with sand paper (Miller 1992), was treated with aqueous ethanol and dried. However, HPAEC of a lichenase digest revealed some unknown peaks and shoulders to peaks. Consequently, the sample was further extracted with ethanol before lichenase treatment. Pericarp tissue (50 mg) was heated for 15 min in a boiling water bath in 50% (v/v) aqueous ethanol (5 ml). The mixture was cooled, and a further 5 ml of 50% ethanol was added. The mixture was centrifuged, and the supernatant was removed carefully by siphoning. The residue was washed twice with 50% ethanol (10 ml), and the supernatant was removed after centrifuging. The sample was treated with lichenase (≈10 units) in 10 m*M* phosphate buffer (2.2 ml) before HPAEC, without dilution. Whole flour (cv. Marion) and oat β-glucan standard were also analyzed.

RESULTS

Oligosaccharide Response Factors in PAD

Weight response factors relative to glucose (RRF) were 0.65 for cellobiose and 0.49 and 0.42 for 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose, respectively, based on concentrations determined by the automated orcinol-sulfuric acid reaction (Wood et al 1991). Purity of the oligosaccharides (>90%) was assessed from the HPAEC peak area and expressed as percent of total peak area. RRF values were then calculated on the basis of orcinol-determined concentration and HPAEC-evaluated purity.

The mean molar ratio of tri- to tetrasaccharide, released by

lichenase from 11 oat cultivars, as determined by the HPLC-orcinol method was 2.1 (Wood et al 1991). Initially, the mean molar ratio of tri- and tetrasaccharide from the same 11 cultivars, as determined by HPAEC-PAD, was similar at 2.1, indicating that the weight response factors for these two oligosaccharides in HPAEC-PAD were identical. However, the ratio increased, subsequently, to 2.3 with PAD after replacement of the electrode. Application of the individual RRFs (0.49 and 0.42), which were determined with this replacement electrode, reduced the ratio from 2.3 to 2.0. Clearly, the RRF of each oligosaccharide may vary with electrode change. The value of the molar ratio of tri- to tetrasaccharide was therefore somewhat uncertain, although the accuracy is probably within ±10%.

Thus, although the relative peak areas of oligosaccharides released by lichenase are reproducible over long periods (months), when an electrode is replaced, or is ready for replacement, some changes may occur. In this instance, change to a third electrode and changes in detector response times and voltages were without effect. By inclusion of external standards of lichenase-treated oat β-glucan and glucose in each analysis, changes in the tri- to tetrasaccharide ratio, or total oligosaccharide response relative to glucose (≈0.4), can be detected. A correction may then be applied, when necessary, for comparison of different sets of data. This was not required for results reported here. The use of β-glucan or glucose as a standard allows an estimation of β-glucan in unknown samples.

The response of PAD to tri- and tetrasaccharide is linear in the range used (5–40 μg/ml), but the regression line may depart slightly from the origin. The lower concentration range should therefore be avoided, and similar concentrations used, to validate small differences between samples.

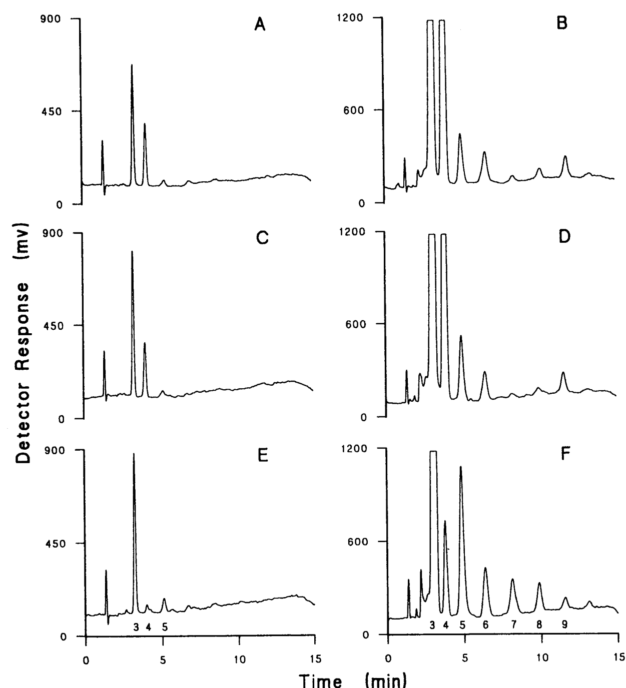

Fig. 1. High-performance anion-exchange chromatograms with pulsed amperometric detection of lichenase-treated oat (**A,B**) and barley (**C,D**) β-glucan and lichenan (**E,F**). **B**, **D** and **F** have increased concentrations to analyze oligosaccharides of DP 5–9. Numbers 3–9 indicate oligosaccharide DP.

Analysis of Oligosaccharides of DP 3–9 in Lichenase Digests of Purified β-Glucans

The chromatography of the soluble oligosaccharides produced by the action of lichenase on purified oat and barley β-glucan and lichenan (Fig. 1) was unchanged when the duration of enzyme treatment (routinely 1.5 hr) was extended to 4.0 hr or reduced to 0.5 hr. The percentage of each peak was calculated on the basis of total peak area (Table I). The tri- and tetrasaccharide components from oat and barley β-glucan accounted for 91–92% of the total analyzed (Fig. 1A–D). Penta- and hexasaccharide each accounted for 2–4%, with the peaks at DP 7 and 8 declining to less than 1% each. There followed an increase in peak size at DP 9 to about 1.2–1.5% of the total.

The different structure of lichenan is apparent in Figure 1E,F and Table I. Tri- and tetrasaccharide accounted for 82% of the total area, 95% of which was the trisaccharide. The pentasaccharide (9.3%) was present in amounts approximately twice that of the amount of the tetrasaccharide (4.2%). Subsequent peaks then declined in area with no increase at DP 9. There were traces (not measured) of peaks of DP > 9 in all three samples.

Analysis of DP 5–9 required injection of concentrated solutions of the enzyme digestion mixture. In phosphate buffer (≥20 m*M*) and 50 m*M* sodium 2-(*N*-morpholino) ethane sulfonate (MES) buffer (pH 6.5), shoulders or secondary peaks appeared with oligosaccharides from β-glucans and with maltodextrins. This was not evident with samples in ≤10 m*M* phosphate.

Analysis of Insoluble Oligosaccharide Products from Lichenase Digestion

Between 27 and 65% of the insoluble products released by lichenase treatment of oat gum and barley β-glucan were from glucan (Table II), although some glucose may be fr̀om glucomannan. Approximately 50% was carbohydrate, as determined by acid hydrolysis and HPAEC. The low carbohydrate yield may be due to small amounts (2–5 mg) of material and the use of 72% sulfuric acid. The starting material from oats was less pure than that from barley, as reflected in the arabinose, xylose, and galactose content of the 75% 2-propanol precipitated fraction.

The oligosaccharide components of the water-insoluble products were similar for oats (Fig. 2A) and barley (Fig. 2B), with some quantitative differences (Table III). The major component (≈40%) of the water-insoluble material was DP 9, but identifiable peaks were analyzed to DP 15.

TABLE I
Analysis of Oligosaccharides Released by Lichenase from Oat and Barley β-Glucan and Lichenan

DP of Oligosaccharide	Proportions					
	Weight, %[a]			Mole, %		
	Oat	Barley	Lichenan	Oat	Barley	Lichenan
3	55.0 (1.8)	62.1 (0.3)	78.1 (0.4)	64	70	86
4	36.0 (1.2)	29.4 (0.4)	4.2 (0.8)	31	25	3.5
5	3.3 (0.1)	3.9 (0.1)	9.3 (0)	2.3	2.6	6.2
6	2.6 (0.2)	2.2 (0.1)	3.4 (0.1)	1.5	1.2	1.9
7	0.6 (0.1)	0.4 (0.1)	2.6 (0.1)	0.3	0.2	1.2
8	0.8 (0.1)	0.4 (0.1)	1.8 (0.1)	0.3	0.2	0.7
9	1.6 (0.2)	1.5 (0.1)	0.7 (0)	0.6	0.6	0.3
3 + 4 total	91.0	91.5	82.3	95	95	90
5 − 9 total	8.9	8.4	17.8	5	5	10

[a] Oat (n = 8), barley (n = 4), lichenan (n = 4). Standard deviation in parenthesis.

TABLE II
Yield and Monosaccharide Composition of Fractions Insoluble in Water and in 75% 2-propanol (IPA), After Treatment of Oat and Barley β-Glucan with Lichenase

	Yield, %[a]	Monosaccharide Composition, %[b]				Total Carbohydrate, %
		Ara	Xyl	Gal	Gluc	
Oat						
75% IPA insoluble	8.8	10	12	1.0	27	51
Water-insoluble	3.9	0.9	1.4	nd[c]	52	54
Barley						
75% IPA insoluble	5.6	1.4	2.7	nd	44	48
Water-insoluble	2.6	nd	nd	nd	65	65

[a] Percent (as is basis) of fraction isolated from oat gum or barley β-glucan.
[b] Percent (as is basis) in fraction.
[c] Not detected.

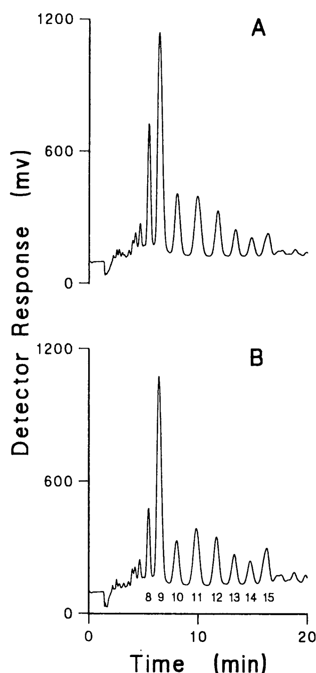

Fig. 2. High-performance anion-exchange chromatograms with pulsed amperometric detection of oligosaccharides in the water-insoluble material released during lichenase digestion from oat (**A**) and barley (**B**) β-glucan.

2-Propanol-insoluble products from oats and barley were also similar in composition; an oligosaccharide of DP 6, 3-*O*-β-cellopentaosyl-D-glucose, was the major component of both (Table IV). Oligosaccharides of DP > 6 did not readily redissolve in water. With DMSO as solvent, 80–85% of the fraction was analyzed as DP 6–9; the oligosaccharide of DP 6 remained the predominant component.

Although a water-insoluble residue was isolated in 3.5% yield from lichenan after treatment with lichenase, this material contained traces of oligosaccharides only in decreasing amounts from DP 3 to 15.

^{13}C-NMR Spectroscopy

The spectrum from oat β-glucan (Fig. 3A) was qualitatively identical to that of barley β-glucan and lichenan as previously reported (Wood et al 1991). The spectrum shows acid-hydrolyzed, low-viscosity oat gum that could be analyzed at 3% without interference from excessive solution viscosity. This improved the quality of the spectrum, which otherwise was identical to that of unhydrolyzed β-glucan. Oligosaccharide products from lichenase digestion of the acid-hydrolyzed glucan were identical to those from unhydrolyzed β-glucan.

The ^{13}C-NMR spectrum (Fig. 3B) of the water-insoluble residue produced by the action of lichenase on oat β-glucan was considerably simplified, with six major resonances (102.1, 79.3, 74.7, 74.3, 72.8, and 60.2 ppm) corresponding to a cellulose-like oligosaccharide (or oligosaccharide mixture). The minor resonance at 79.4 ppm was presumably from C-4 of the unit adjacent to the 3-*O*-linked reducing end or C-4 of the glucose adjacent to the nonreducing end. Additional resonances of low intensity were identified as arising from nonreducing-end glucose and both α- and β-anomers of 3-linked glucose, identifying this unit as the reducing end. There was no evidence for consecutive (1→3) linkages. The spectrum of the insoluble residue from barley β-glucan was similar.

The predominant resonances of the ^{13}C-NMR spectra of the 75% 2-propanol-insoluble residues were similar to those from the water-insoluble material, but the increased influence from the reducing end complicated the spectra and, particularly in the spectrum of the oat sample, there were resonances from contaminating pentosan.

Analysis of Oligosaccharides of DP 3–9 Released by Lichenase from β-Glucan in the Whole Grain of Barley, Oat, and Rye

The data in Table V show that analysis of whole cereal flours gives results similar to those of the purified β-glucans. A selection of 11 oat, 16 barley, and 3 rye cultivars was examined. Barley samples had a higher mean (3.0) and wider range (2.8–3.3) of values for the molar ratio of tri- to tetrasaccharide than did oats (2.3, 2.1–2.4) (Table V). Only three samples of rye were analyzed, but the tri- to tetrasaccharide molar ratio (3.1) was similar to that of barley. The tri- and tetrasaccharide accounted for 91–93% (mean 92%) of the total oligosaccharides from oats and barley. Rye was somewhat different, with tri- and tetrasaccharide accounting for 95% of the total. The DP 9 product was present at 1.2–1.5% in oats and barley, but it was <1% in rye.

Analysis of β-Glucan Extracted by Water at 40 and 65°C

The data in Table VI summarize oligosaccharide analysis of β-glucan fractions extracted by water from oat and barley flours. The proportions of oligosaccharides from β-glucan extracted at 40 or 65°C, and in the final residue of unextracted β-glucan, were similar to the proportions in the original grain, although the molar ratio of tri- to tetrasaccharide was somewhat higher (2.5) for the residue from the oat extractions. The amounts of oligosaccharides of DP 5–9 (7.6–8.6%) showed no obvious trends related to extraction.

Analysis of Oat Pericarp Tissue

Microscopic examination of the pericarp fraction showed that aleurone, but little, if any, endosperm, was present (Miller 1992). The β-glucan content, calculated with glucose standard from total oligosaccharide area, was ≈0.8%. The tri- to tetrasaccharide ratio (2.6) was distinctly higher in the pericarp than it was in the whole flour (2.2) or oat β-glucan standard (2.2) (Table VII). The amount of the oligosaccharide of DP 9 (0.8%) was slightly less than that from flour and standard (1.2%).

DISCUSSION

The results reported here extend previous structural studies of (1→3),(1→4)-β-D-glucans and focus on sequences of the molecule containing more than three consecutive (1→4)-linked units.

TABLE III
Percentage Composition by Weight[a] of Oligosaccharides in Fractions Insoluble in Water After Treatment of Oat and Barley β-Glucan with Lichenase[b]

	DP of Oligosaccharide								
	7	8	9	10	11	12	13	14	15
Oat	2.0	13.2	38.9	10.4	12.8	7.8	4.2	3.2	4.6
	(0.04)	(0.2)	(3.6)	(0.8)	(0.7)	(0.6)	(0.4)	(0.2)	(0.2)
Barley	1.7	7.7	37.0	8.0	12.9	9.3	5.3	4.4	7.9
	(0.05)	(0.3)	(4.2)	(0.6)	(0.5)	(0.5)	(0.2)	(0.2)	(0.4)

[a] Based on normalized area percent.
[b] n = 3. Standard deviation in parenthesis.

Although present in relatively small amounts, these sequences may disproportionately influence chain extension in solution, and hence viscosity (Buliga et al 1986). It is therefore important to know whether these structural characteristics vary between cereals and between different cultivars and their mill fractions.

Quantitation of oligosaccharides by HPAEC required knowledge of response factors. The behavior of the mixed-linkage oligosaccharides was clearly similar to that of cellodextrins (and other gluco-oligosaccharides) as reported by Koizumi et al (1989) and van Riel and Olieman (1991): namely an initial rapid decline in weight response from glucose (1.0) to cellobiose (0.65) but slower further decline above DP 2 (0.4–0.5). Although the response factors of the higher DP oligosaccharides were not known with accuracy, variations were not sufficient to greatly affect structural assessment based on peak area response.

There was a consistently higher ratio of tri- to tetrasaccharide in β-glucan from whole barley (2.8–3.3) and rye (3.0–3.2) compared to that of oats (2.1–2.4), as previously reported (Wood et al 1991). There was no difference in the mean ratio (3.0 ± 0.1) from 6-row and 2-row barley or from those lines bred for feed or for malting. Therefore, oat β-glucan has the greatest number of β-(1→3)-linked cellotetraosyl sequences. These are a minor structural feature of lichenan. However, the β-(1→3)-linked cellopentaosyl sequence is relatively enriched in lichenan (Table I) (Perlin and Suzuki 1962). As a consequence, the ^{13}C-NMR spectra of lichenan and cereal β-glucans were very similar.

The similarity in the amounts of oligosaccharides of DP 5–9 from oat and barley β-glucan is remarkable: the total consistently accounts for 7–9% of the lichenase-solubilized fraction (Tables I and V). In contrast, twice as many (18%) of these oligosaccharides were released from lichenan; somewhat less (5%) were released from rye. As previously reported for barley (Yin and MacGregor 1989), following a decline through DP 8, there is an increase of the DP 9 oligosaccharide in the lichenase-solubilized products from oats, barley, and rye, but not from lichenan.

Lichenase digestion of solutions of purified oat and barley β-glucan forms a water-insoluble product (≈3% yield). This product was also detected in the residue from lichenase digestion of oat bran when the bran residue was extracted with DMSO. Recovery was low (≈0.4% of the original β-glucan), but the characteristic peaks of DP 8–15 were observed.

Methylation analysis previously showed that the water-insoluble material released from cereal β-glucan by lichenase contained 4–14 contiguous β-(1→4)-linked D-glucopyranosyl units terminated at the reducing end by 3-linked glucose (Wood et al 1991, Woodward et al 1983). Analysis of the oligosaccharides in the oat and barley products now show these to be similar; the largest fraction (≈40%) was of DP 9 (Table III). The increase at DP 9 in the water-soluble fraction presumably reflects slight solubility of this cellodextrin-like oligosaccharide. Among the minor products of lichenase digest in both oats and barley, the prominence of this DP oligosaccharide clearly has some physiological significance. Most likely, it is associated with the colligative properties responsible for precipitation after release from the polymer chain. The ability of the polymer to self-associate, possibly through such foci, is evident in partially depolymerized β-glucan that shows weak gel-like characteristics in solution, whereas the unhydrolyzed polymer behaves as a random coil (Doublier and Wood 1993). In the intact cell wall, cellulose-like sequences may assist in cohesion (Fincher and Stone 1986), but they also provide a specific site for cell-wall disruption by enzyme action during germination (Yin and MacGregor 1989).

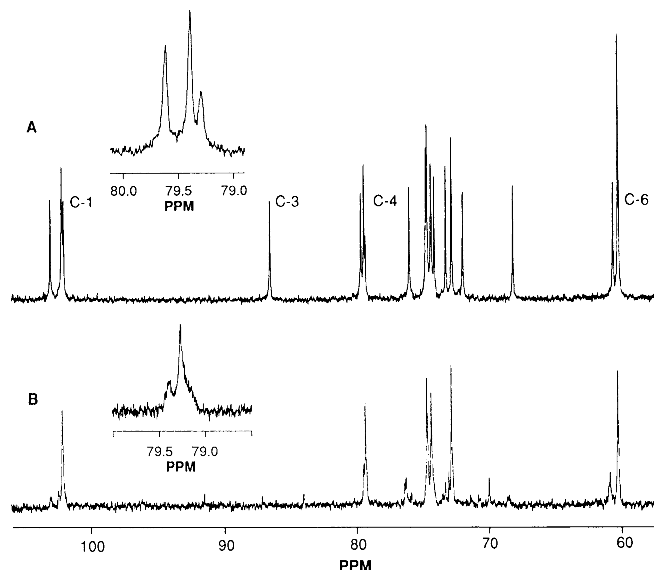

Fig. 3. ^{13}C-nuclear magnetic resonance spectra obtained at 125.77 MHz in deuterated methyl sulfoxide (DMSO-d_6) from oat β-glucan (**A**). **B**, water-insoluble product from lichenase treatment of oat β-glucan. Insets show resonances from glycosidically linked C-4 (C-4). C-3 is resonance from glycosidically linked C-3. C-1 is resonance from anomeric carbons. C-6 is resonance from primary hydroxyl carbons.

TABLE IV
Percentage Composition by Weight[a] of Oligosaccharides in Fractions Insoluble in 75% 2-propanol After Treatment of Oat and Barley β-Glucan with Lichenase[b]

	DP of Oligosaccharide					
	3	4	5	6	7	8
Oat	15.9	11.8	10.9	47.0	10.1	4.4
	(0.7)	(0.7)	(0.3)	(0.2)	(0.1)	(1.2)
Barley	18.0	11.2	12.6	46.6	9.6	2.0
	(1.5)	(0.2)	(0.1)	(1.0)	(0.2)	(0.3)

[a] Based on normalized area percent.
[b] n = 2. Standard deviation in parenthesis.

Identification of consecutive (1→3) linkages in oat β-glucan by Smith degradation (Goldstein et al 1965) was not confirmed by Vaårum and Smidsrød (1988). In the present study, ^{13}C-NMR at higher field and greater sensitivity than that previously reported (Wood et al 1991) was unable to detect consecutive (1→3) linkages. The spectra (Fig. 3A) showed single resonances for each carbon of the 3-linked glucose, indicating a single environment in the polymer for this residue. The resonance at 86.5 ppm, for example, is assigned to glycosidically attached C-3. In exclusively β-(1→3)-linked glucose (curdlan), this resonance occurred at 85.8 ppm (not shown), indicating that additional upfield resonances would be detected for consecutive (1→3)-linked glucose in the mixed linkage β-glucan, similar to those that arise from C-4 of consecutive (1→4)-linked glucose (see below). It seems likely, therefore, that the presence of consecutive (1→3)-linked glucose, de-

tected by the Smith degradation technique (Goldstein et al 1965), was caused by incomplete hydrolysis (Woodward et al 1983).

There were three distinct resonances from glycosidically attached C-4 for both oat β-glucan (Fig. 3A) and lichenan, arising from the three different environments possible for this carbon (Dais and Perlin 1982, Wood et al 1991). The two more intense resonances at 79.4 and 79.6 ppm arise from β-(1→4)-linked glucose flanked on either the reducing or nonreducing end by β-(1→3)-linked glucose. The smaller resonance at 79.3 ppm is from the more cellulose-like environment in the polysaccharides, in which β-(1→4)-linked glucose is flanked on both sides by further β-(1→4)-linked glucose. This feature is not present in the β-(1→3)-linked cellotriosyl residues of the β-glucans. Therefore, the resonance at 79.3 ppm is a rough measure of the proportion of se-

TABLE V
Percentage Composition by Weight[a] of Oligosaccharides Released by Lichenase from Oat, Barley, and Rye Cultivars[b]

	MR[c]	DP of Oligosaccharide 3+4	5	6	7	8	9
Oat[d]	2.3	92.0	3.0	2.3	0.4	0.6	1.4
	(0.1)	(0.4)	(0.2)	(0.1)	(0)	(0)	(0.2)
Barley[e]	3.0	92.2	3.5	2.0	0.4	0.4	1.3
	(0.1)	(0.5)	(0.3)	(0.1)	(0)	(0)	(0.1)
Rye[f]	3.1	94.6	3.0	1.4	tr[g]	0.1	0.9
	(0.1)	(0.3)	(0.3)	(0)	...	(0)	(0.1)

[a] Normalized area percent.
[b] Standard deviation in parenthesis.
[c] Molar ratio of tri- to tetrasaccharide.
[d] n = 11.
[e] n = 16.
[f] n = 3.
[g] trace.

TABLE VI
Percentage Composition by Weight[a] of Oligosaccharides Released by Lichenase from Oats and Barley Consecutively Extracted by Water at 40 and 65°C[b]

	MR[c]	DP of Oligosaccharide 3+4	5	6	7	8	9
Barley							
Whole flour	3.0	92.4	3.4	2.0	0.4	0.4	1.3
	(0.0)	(0.8)	(0.4)	(0.2)	(0)	(0.1)	(0.2)
40°C extract	2.8	91.3	3.8	2.3	0.4	0.5	1.7
	(0.1)	(0.2)	(0)	(0.1)	(0)	(0)	(0.1)
65°C extract	3.0	91.4	4.1	2.2	0.3	0.4	1.5
	(0.1)	(0.1)	(0.5)	(0.1)	(0)	(0.1)	(0.1)
Residue	2.9	91.7	3.7	2.3	0.3	0.4	1.5
	(0.1)	(0.2)	(0)	(0)	(0)	(0)	(0.1)
Oat							
Whole flour	2.3	91.7	3.6	2.3	0.4	0.7	1.2
	(0.0)	(0.8)	(0.5)	(0.3)	(0)	(0.1)	(0.1)
40°C extract	2.2	92.0	3.2	2.3	0.4	0.7	1.3
	(0.1)	(0.1)	(0)	(0)	(0)	(0)	(0.1)
65°C extract	2.3	91.6	3.5	2.4	0.4	0.7	1.3
	(0.1)	(0.2)	(0.2)	(0.1)	(0)	(0)	(0.1)
Residue	2.5	91.9	3.6	2.4	0.3	0.6	1.1
	(0.1)	(0)	(0.1)	(0)	(0)	(0)	(0.1)
Oat β-Glucan[d]	2.2	91.7	3.2	2.4	0.4	0.8	1.5
	(0)	(0.1)	(0)	(0)	(0)	(0)	(0.1)
Barley β-Glucan[d]	3.1	92.1	3.8	2.1	0.4	0.4	1.3
	(0)	(0.2)	(0)	(0)	(0)	(0)	(0.3)

[a] Normalized area percent.
[b] Average of two cultivars.
[c] Molar ratio of tri- to tetrasaccharide.
[d] n = 3.

TABLE VII
Percentage Composition by Weight[a] of Oligosaccharides Released by Lichenase from Whole Oat Flour (cv. Marion) and from Pericarp Fraction[b]

	MR[c]	DP of Oligosaccharide 3+4	5	6	7	8	9
Whole flour	2.2	92.5	2.9	2.3	0.4	0.7	1.2
	(0)	(0.4)	(0.5)	(0.1)	(0)	(0)	(0)
Pericarp	2.6	92.4	3.5	2.4	0.3	0.6	0.8
	(0.01)	(0.1)	(0)	(0)	(0)	(0)	(0)
Oat β-glucan	2.2	92.2	3.1	2.3	0.4	0.7	1.2
	(0.01)	(0.1)	(0)	(0)	(0)	(0)	(0)

[a] Normalized area percent.
[b] n = 2. Standard deviation in parenthesis.
[c] Molar ratio of tri- to tetrasaccharide.

quences with three or more consecutive (1→4) linkages. In the cereal β-glucans, this resonance arises mostly from the β-(1→3)-linked cellotetraosyl residue. In lichenan, where this residue is present in relatively small amounts, the resonance must arise instead from the sequences giving rise to oligosaccharides of DP 5–9.

The relative intensity of the resonance at 79.3 ppm from glycosidically attached C-4 will increase as the number of consecutive (1→4) linkages increase until it is dominant, as in the considerably simplified spectrum of the water-insoluble product formed from lichenase digestion of oat β-glucan (Fig. 3B). Thus, the predominantly β-(1→4)-linked nature of the water-insoluble material was verified by ^{13}C-NMR.

Structural differences might account for differences in aqueous extractability of β-glucan (Woodward et al 1988), but no differences in structure of β-glucan extracted at 40 and 65°C from oats and barley were evident in the present analysis (Table VI). The proportions of the different oligosaccharides released by lichenase were essentially the same in both extracts, and unchanged from the whole flour. The higher tri- to tetrasaccharide ratio in the residual β-glucan left after aqueous extraction of oats probably reflects the anatomical origin of β-glucan in this fraction, namely the outer and aleurone layers. This suggests that differences in extractability are more likely to be consequences of features of microstructure and cell-wall organization than of chemical structure. However, the cellulose-like areas of the molecule that are rendered insoluble by lichenase were not analyzed. Additionally, there may be other features, such as structural regularity, not detected by this method.

Previously, the structure (tri- to tetrasaccharide ratio) of β-glucan from oat brans was shown to be the same as that of β-glucan from the whole flour (Wood et al 1991). However, milling of oats does not generally achieve a clean separation of aleurone and outer layers; bran is significantly contaminated by endosperm. The pericarp tissue prepared by Miller (1992) contained aleurone, but little, if any, endosperm. The present results indicate that oat aleurone cell wall β-glucan has a decreased proportion of β-(1→3)-linked cellotetraosyl units relative to the proportion of the endospermic β-glucan (Table VII).

CONCLUSIONS

HPAEC with PAD, in conjunction with the specific enzyme lichenase, was used to evaluate the fine structure of (1→3),(1→4)-β-D-glucans. The method was particularly useful for detecting minor cellulose-like features that have an important influence on physical properties. In general, the cereal β-glucans were structurally similar, except for variations in the relative amounts of (1→3)-linked cellotriosyl and cellotetraosyl units, which constituted approximately 92% of the polysaccharide as solubilized by lichenase. The remaining structure contained 4–15 consecutive (1→4)-linked D-glucopyranosyl units. No structural differences were detected in fractions extracted from oats or barley by water at 40 and 65°C. β-Glucan from oat aleurone had proportionately less of the (1→3)-linked cellotetraosyl units than did endospermic β-glucan. Lichenan had mainly (1→3)-linked cellotriosyl units. No evidence for consecutive (1→3)-linkages was found.

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