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Structural characterization of water soluble β-glucan of oat bran

L. Johansson[a,*], L. Virkki[a], S. Maunu[b], M. Lehto[a], P. Ekholm[a], P. Varo[a]

[a]*Department of Applied Chemistry and Microbiology, University of Helsinki, P.O. Box 27, FIN-00014, Helsinki, Finland*
[b]*Department of Chemistry, University of Helsinki, P.O. Box 55, FIN-00014, Helsinki, Finland*

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Abstract

β-glucan was isolated from oat bran in a highly purified form. The bran was characterized for its contents of dietary fiber, β-glucan, fat and protein. The isolated β-glucan was free of protein and contained only glucose in GC sugar analysis. Two types of β-glucan were obtained with different solubilities. Their molar masses were 1.6 million for the less soluble and 1.1 million for the more readily soluble type. No structural differences were found. The two-dimensional correlation NMR spectrum of the isolated β-glucan showed that the glucose units are joined with 1,3- and 1,4-linkages only. The oligosaccharides produced by the action of a specific enzyme, lichenase, were analyzed by HPLC and capillary zone electrophoresis. The major products are 3^2-β-D-glucosyl cellobiose (trisaccharide) and 3^3-β-D-glucosyl cellotriose (tetrasaccharide), which account for 95% of the whole. Also, 3^4-β-D-glucosyl cellotetraose (pentasaccharide) and other oligosaccharides with degree of polymerization (DP) higher than 5 were detected as minor components. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: β-glucan; Oat bran; Lichenase

1. Introduction

Oats are suggested to have positive effects on human health: they may lower serum cholesterol and glucose levels (Anderson, Jones & Riddell-Manson, 1994; Wood, 1994). Janatuinen et al. (1995) have also shown that adult celiac patients can use oats as part of a gluten-free diet. These effects are related to soluble dietary fiber. The main component of soluble fiber in oats is the mixed-linkage polysaccharide $(1 \rightarrow 3),(1 \rightarrow 4)$-β-D-glucan, referred to as β-D-glucan. Most of the molecule consists of cellotriose and cellotetraose blocks separated by $(1 \rightarrow 3)$-linkages, but there is a minor amount of sequences of $(1 \rightarrow 4)$-linkages longer than the tetraose type (Wood, Weisz & Blackwell, 1994). Cellulose is also a β-D-glucan, but it consists only of $(1 \rightarrow 4)$-β-D-linkages and is therefore stiff, highly crystalline and non-soluble. The $(1 \rightarrow 3)$-linkages break up the uniform structure of the β-D-glucan molecule and make it soluble and flexible (Anderson & Bridges, 1993). The amount of the long sequences and their distribution in the polymer backbone effect the properties of the molecule, but they have not been studied thoroughly so far. Also the rheological properties of oat β-glucan and the relation of these properties to its structure need to be studied. The molar mass of β-glucan has been widely studied, but the results are controversial and strongly depend both on the conditions of isolation and the method used for analysis (Autio, 1996; Beer, Arrigoni & Amado, 1996; Beer, Wood & Weisz, 1997a). The molar mass of β-glucan may also vary between cultivars (Beer et al., 1997a; Jaskari, Henriksson, Neiminen, Suortti, Salovaara & Poutanen, 1995) and be effected by heat treatments of the starting material (Beer, Wood, Weisz & Fillion, 1997b; Zhang, Doehlert & Moore, 1998).

The building blocks of β-glucan have been studied by degradation of the polysaccharide to oligosaccharides with a specific β-D-glucanase enzyme i.e. lichenase (Bock, Duus, Norman & Pedersen, 1991; Wood, Weisz & Blackwell, 1991; Wood et al., 1994). The released oligosaccharides have then been analyzed by gel chromatography (Bock et al., 1991) and by HPLC methods (Izydorczyk, Biliaderis, Marci & MacGregor, 1998; Wood et al., 1994). Rydlund (1995) has used capillary electrophoresis (CE) for analyzing mono- and oligo-saccharides from wood samples. CE provides a sensitive and selective analysis method also for the structural components of β-glucan.

NMR spectroscopy has proved to be an excellent method to get structural information about β-glucan and its building blocks (Bock et al., 1991). One-dimensional ^{1}H- and ^{13}C NMR spectra have been used for investigation of anomeric protons (Westerlund, Andersson & Åman, 1993) and carbons of β-glucan and for comparison of β-glucans of different origins (Dais & Perlin, 1982; Wood et al., 1991). With two-dimensional NMR experiments more detailed

* Corresponding author. Tel.: +358-91-9158406; fax: +358-91-9158475.

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information of the structural features of β-glucan can be obtained (Dawkins, 1994; Ensley et al., 1994).

The main aim of this work was to study the details of the structure of highly purified water soluble oat β-glucan. Such a study is needed to reveal the structure–properties relationship of the polymer, which can help to understand the behavior of oat β-glucan in the digestive track and in baking and other processes of industrial importance.

2. Experimental

2.1. Materials

Oat bran was a commercial product of Melia, Finland. Thermamyl 300 L DX was from Novo Nordisk, Denmark, pancreatin 8 × U.S.P. was from Sigma and lichenase (EC 3.2.1.73), a (1 → 3), (1 → 4)-β-D-glucan-4-glucanohydrolase, from Megazyme, $(NH_4)_2SO_4$ and $CaCl_2$ from Merck, NaN_3 and isopropanol from J.T. Baker. Starch, glucose and arabinose were from Merck. Malto-oligosaccharides and myo-inositol were from Sigma. Cello-oligosaccharides were from Merck. 6-Amino quinoline and sodium cyanoborohydride were from Aldrich. Other chemicals were: petroleum ether from Fisher, acetonitrile (HPLC grade) from Rathburn, trifluoro acetic acid (TFA) and acetic acid anhydride from Merck and 1-methyl imidazole from Fluka.

2.2. Isolation and treatment of mixed-linked β-glucans

β-glucan was isolated by the method of Westerlund et al. (1993) with minor modifications. Milled bran was defatted by Soxhlet extraction with hot isopropanol and petroleum ether. Polysaccharides were solubilized in water at 96°C and starch was hydrolyzed by Thermamyl. The insoluble fraction was separated by centrifugation. Proteins in the supernatant were degraded by pancreatin. The polysaccharides were precipitated by 60% ethanol at 4°C. The precipitate was removed by centrifugation and dissolved in water at 70–80°C. β-glucan was precipitated by 30% $(NH_4)_2SO_4$ and separated by centrifugation. The solution containing arabinoxylan was concentrated, centrifuged, dissolved in water and dialyzed against water for 3 × 24 h. The β-glucan precipitate was dissolved in water at 80°C in the dialysis tube. Part of the β-glucan dissolved (S-type) and the rest remained as lumps of gel (G-type). The mixtures were dialyzed against water at room temperature for 3 × 24 h. The two types of β-glucan were separated. The resulting arabinoxylan and β-glucan solutions were freeze dried.

β-glucan and starch samples (2–20 mg) were hydrolyzed to monosaccharides with trifluoroacetic acid (TFA) as described by Olson, Gray, Chiu, Betschart and Turnland (1988) with minor modifications. Starch was used for checking the validity of the method. TFA was added followed by myo-inositol as internal standard. The sample was heated at 120°C for 1 h. The solution was neutralized with conc. ammonia. Standard solutions of glucose were prepared in the same way at three concentrations (2.5, 5 and 10 mg/ml).

The method used for lichenase treatment of oat β-glucan was modified from the McCleary method (AOAC method 995.16) (McCleary & Codd, 1991). Isolated β-glucan samples of oat bran were dried at 70°C for 3 h. The samples (60–110 mg) were weighed accurately and pretreated with aqueous ethanol (50%), dissolved in phosphate buffer (4.0 ml, 20 mM, pH 6.5) in a boiling water bath and stirred on a vortex mixer. The samples were then incubated with lichenase at 60°C for 2 h. The resulting solution was diluted with water and filtered with a membrane filter Whatman Puradisc 25AS 0.45 μm. The decomposition products (oligosaccharides) were analyzed with HPLC and capillary electrophoresis.

2.3. Analytical methods

The β-glucan content of oat bran was determined according to AOAC 995.16. The crude protein was analyzed by the Kjeldahl method. Total dietary fiber was determined by the method of Asp, Johansson, Hallmer and Siljeström (1983).

Gas chromatography (GC) was used to identify monosaccharides obtained from the hydrolysis of β-glucan. GC analysis was performed with a Micromat HRGC 412 (Orion Analytica, Finland) chromatograph equipped with a flame ionization detector. The column was a NB-17 fused silica capillary column (25 m × 0.32 mm i.d., film thickness 0.25 μm, Nordion, Finland). Helium was used as carrier gas (pressure 0.7 bar). Splitless injection was done at 225°C. The detector was at 280°C. The column oven was programmed from 190°C (4 min hold) to 230°C (6 min hold) at a rate of 4°C/min.

The monosaccharides obtained from the hydrolysis step of β-glucan and starch and the glucose standards were acetylated with acetic anhydride as described by Blakeney, Harris, Henry and Stone (1993). They were identified according to their retention times and quantified using an internal standard method involving myo-inositol. A standard curve (concentration ratio of monosaccharide/internal standard vs. peak area ratio of monosaccharide/internal standard) was produced for glucose at three concentrations between 2.5 and 10 mg/ml. All analyses were made in duplicate.

HPLC analysis were carried out with a Varian Vista 5500 chromatograph equipped with a Rheodyne injector, a Hewlett-Packard HP 1047A RI-detector and a Merck Hitachi D2500 Chromato-Integrator. The analytical column used was Spherisorb $S5NH_2$ (15 cm × 4.6 mm i.d., stainless, homemade). A prefilter (Uptight 2 μm) was used to protect the analytical column. All analyses were run with isocratic elution. The mobile phase consisted of acetonitrile and water (70:30). The flow rate was 1.0 ml/min and temperature 30°C. The injection volume for each sample was 20 μl.

Oligosaccharides produced by lichenase treatment of β-glucan were identified according to their relative retention

Table 1
The recoveries of β-glucan and starch as glucose by GC

Sample	*n*	Range (%)	Mean	Standard deviation
β-glucan	24	74–95	86	5.2
Starch	6	84–94	87	3.9

times. The retention time of each peak was confirmed by spiking with the corresponding standard oligomer. The quantitative analyses were made with the external standard method. Standard curves (concentration of each oligomer plotted against peak area) were produced for glucose, maltotriose and maltotetraose. The concentration of standards varied from 0.5 to 4 mg/ml and were in the same range as the concentrations of the samples. All analyses were made in triplicate. The detection limit for the method was 50 μg/ml. Glucose was used to evaluate the accuracy of the method. Glucose was treated in the same way as the samples and the recovery calculated from chromatograms. A standard mixture of oligosaccharides was used to evaluate the day-to-day repeatability of the chromatographic method.

The capillary electrophoresis (CE) analyses were performed with a Hewlett Packard^{3D} capillary electrophoresis system with UV-detection at 245 nm. An uncoated fused silica capillary column was used (total length 60 cm, effective length 51 cm, 50 μm i.d.) at 25°C. Injections were performed hydrodynamically (50 mbar, 2 s). The applied voltage was 21 kV. The running electrolyte was an alkaline borate buffer (pH 9.2, 420 mM H_3BO_3/220 mM NaOH). Samples and buffer were filtrated through a 0.2 μm filter. The standards were aqueous solutions of glucose and cello-oligosaccharides with DP 2-5. The oligosaccharides were derivatized through reductive amination using sodium cyanoborohydride (0.5 M solution in water) and 6-aminoquinoline (0.5 M solution in 5 M aqueous acetic acid). The reagents were added and the mixtures were heated at 40°C for 2 h.

Six β-glucan samples were analyzed for their oligosaccharide contents. The oligosaccharides were identified according to their migration times. Quantification was done for triose, tetraose and pentaose with the internal standard method involving arabinose. Standard curves (concentration ratio of oligosaccharide/internal standard vs. peak area ratio of oligosaccharide /internal standard) were produced for the three standards at three concentration levels varying between 0.07–0.72 mg/ml. The correlation coefficients varied between 0.997–0.999. Linearity was checked with a five point standard curve with concentrations between 0.15–7.2 mg/ml. Glucose was treated with lichenase in the same way as the samples and derivatized, and the recovery calculated from chromatograms. The detection limit for the method was 7.5 μg/ml. All analyses were made in triplicate.

Nuclear magnetic resonance (NMR) spectra were obtained using an Varian ^{UNITY}INOVA spectrometer operating at 300 MHz for ^{1}H and 75 MHz for ^{13}C. β-glucan samples were dissolved in DMSO-d_6 by heating and stirring at 80°C for several hours. The samples dissolved totally and there was no need for filtration. Sample concentrations were 0.8–1.0% (w/w). All measurements were done at 80°C. Chemical shifts were referenced to solvent signal 2.5 ppm for ^{1}H and 39.9 ppm for ^{13}C relative to TMS. Quantitative one-dimensional ^{13}C NMR measurements were done using pulse lengths of 6.5 μs (60°) for ^{13}C with 2 s delays, respectively. The inverse detected H,C-correlation spectra (HSQC) were measured using 256 increments of 80 scans.

3. Results and discussion

3.1. Characterization of bran

The dietary fiber content of the bran measured by the method of Asp et al. (1983) showed that it contained 14% of soluble fiber and 11% of insoluble fiber. The β-glucan content of the bran by the AOAC method 995.16 was 9.5%. The yield of high purity β-glucan by the method used for isolation (Westerlund et al., 1993) was 4.3% (s.d. 0.84, $N = 22$). The fat content of the bran was 8% and the protein content determined by the Kjeldahl method was 17% ($N \times 6.25$).

3.2. Purity and characterization of isolated β-glucan

The purity of β-glucan was checked by hydrolyzing the isolated polysaccharide to monomeric units. The monomers were then analyzed as alditol acetates with gas chromatography. The starch samples were used for checking the validity of the derivatization. The results are given in Table 1.

All β-glucan samples contained only glucose, which indicates that the isolation produces highly purified β-glucan free of other polysaccharides. The protein content of the isolated β-glucan was negligible when determined by the Kjeldahl method.

The isolation procedure produced two types of β-glucan (S and G) differing in their solubility. The molar mass of the isolated β-glucan was measured for both types by the method of Suortti (1993). In this method size exclusion chromatography with fluorescence detection and postcolumn dyeing of β-glucan with calcofluor was used. The results of the molar mass measurements are shown in Table 2. They show that the more easily soluble type S had a lower molar mass than the G-type.

Table 2
The molar masses of the isolated β-glucan samples (M_w is the weight-average molecular weight, M_n is the number-average molecular weight)

Sample	M_w (kD)	M_n (kD)	M_w/M_n
β-glucan, S-type	1100	300	3.6
β-glucan, G-type	1000	630	2.5

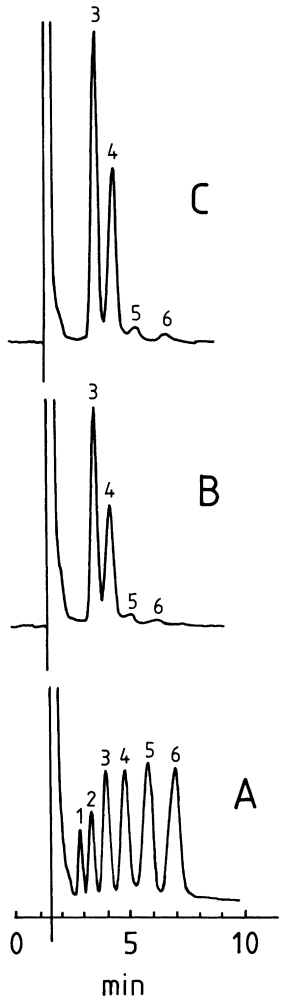

Fig. 1. HPLC chromatogram with refractive index detection of oligosaccharides released from oat β-glucan by lichenase digestion: (a) standard oligomers with DP 1–6; (b) β-glucan G-type; (c) β-glucan S-type. Peaks 1–6 are mono-, di-, tri-, tetra-, penta- and hexa-oligomers, respectively.

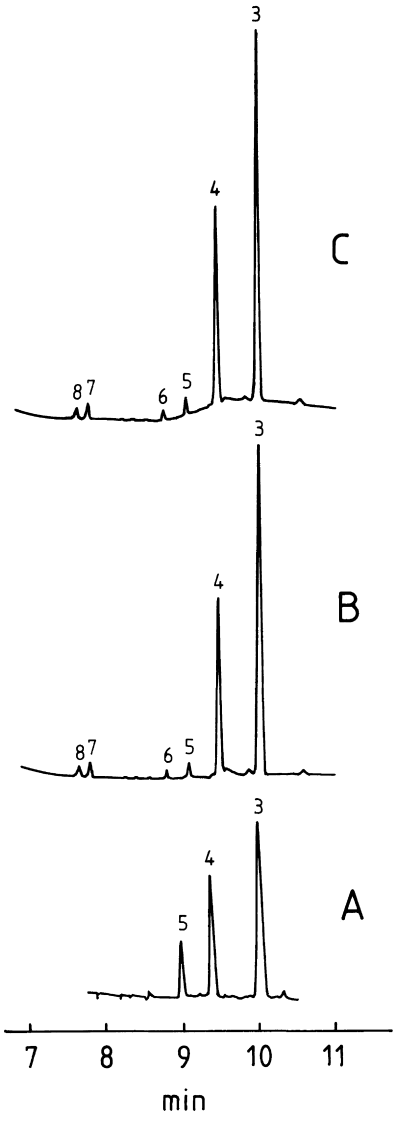

Fig. 2. Capillary zone electropherograms with UV detection of oligosaccharides released from oat β-glucan by lichenase digestion: (a) standard oligomers with DP 3–5; (b) β-glucan G-type; (c) β-glucan S-type. Peaks 3–5 are tri-, tetra- and penta-oligomers, respectively.

3.3. Analyses of oligosaccharides

Lichenase cleaves the β-(1 → 4)-linkages next to a 1,3-linkage. The oligosaccharides thus obtained are the 1,4-linked building blocks of β-glucan with a 1,3-linked end group (Åman & Westerlund, 1996; Wood et al., 1991, 1994). Six isolated β-glucan samples, three totally soluble in water (S-type) and three partially gelled (G-type) samples were treated with lichenase as described above.

Table 3
The relative amounts of the lichenase digestion products of oat β-glucan by capillary electrophoresis (CE) and liquid chromatography (LC)

	Triose (wt%)[a]	Tetraose (wt%)	Pentaose (wt%)	Tri/tetra
G-type CE	53.0 ± 2.9	42.2 ± 0.7	3.1 ± 0.4	1.3
LC	53.4 ± 2.2	41.4 ± 1.0		1.3
S-type CE	52.1 ± 2.3	44.7 ± 2.8	3.1 ± 0.4	1.2
LC	54.8 ± 1.7	40.4 ± 1.8		1.4

[a] For both methods and all samples $n = 3$.

3.4. Liquid chromatography

Fig. 1 shows chromatograms of a standard oligomer mixture and two β-glucan samples (S-type and G-type). The HPLC chromatograms of the different β-glucan samples were similar and showed only minor differences in the contents of oligomers with degree of polymerization (DP) from 3 to 6. The major products were triose and tetraose (peaks 3 and 4, respectively). Pentaose and hexaose were also identified in the chromatograms of all samples (peaks 5 and 6, respectively). No traces of glucose or cellobiose were detected. Table 3 shows the calculated contents of triose and tetraose in two samples. The weight

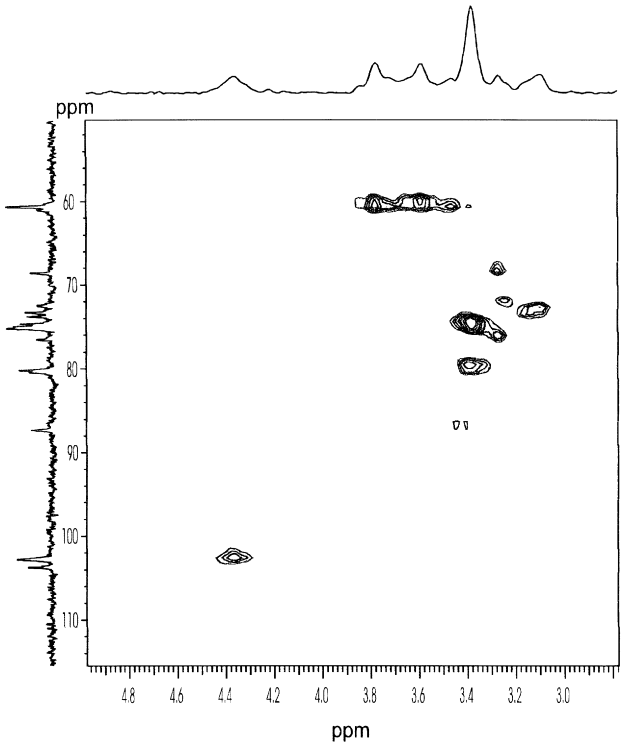

Fig. 3. $^{1}H-^{13}C$ correlation spectrum (HSQC) of β-glucan measured in DMSO-d_6 at 80°C. The measured ^{13}C spectrum is shown on the vertical axis and the ^{1}H projection from the two-dimensional-spectrum is shown on the top.

percentages of the peaks were calculated on the basis of standard curves made for triose and tetraose. Results are corrected for the recovery of glucose obtained from the lichenase treatment procedure. The tri- and tetrasaccharide components account for 95% of the total measured, which is slightly higher value than obtained for oat β-glucan by Izydorczyk et al. (1998) and Wood et al. (1994). The ratio of 1.3 for tri/tetra indicates higher amount of tetraose than found in earlier results of Izydorczyk et al. (1998). Amounts of oligomers higher than DP 3 were not measured.

3.5. Capillary electrophoresis

Fig. 2 shows the electropherograms of isolated β-glucan and a mixture of standard oligosaccharides. The components identified in all samples were triose, tetraose and pentaose (peaks 3, 4 and 5, respectively). No traces of glucose or cellobiose were detected. Peak 6 is most likely hexaose, as identified by HPLC. There were two unidentified components (peaks 7 and 8) in all samples eluting before the hexaose, whose DP must therefore be higher than 6.

The amounts of triose, tetraose and pentaose were calculated as weight percentages from their corrected areas on the basis of standard curves made for triose, tetraose and pentaose and taking the correction factor from glucose treatment into account. The means and the standard deviations are summarized in Table 3. Triose and tetraose account for 95% of the whole. The amount of pentaose is also in good agreement with that reported by Wood et al. (1994) (3.3%), by Doublier and Wood (1995) (3.0%) and by Izydorczyk et al. (1998) (3.6%). In this study the area of the hexaose peak was half of that of the pentaose. The area of the unidentified peak 7 was of the order of pentaose, and the area of the unidentified peak 8 was about half of that of pentaose. Wood et al. (1994) have reported the amount of DP 9 to be 1.6%, Doublier and Wood (1995) (1.4%) and by Izydorczyk (1998) (1.1%).

The results of this study with HPLC and CE methods are in good agreement with each other (see Table 3) and also with the results reported for the amounts of triose and tetraose by Wood et al. (1994) (55 and 36%, respectively), Doublier and Wood (1995) (58.1 and 34.2%), respectively, and by Izydorczyk et al. (1998) (57.6 and 34.1%) respectively, for a commercial β-glucan.

The amounts of triose, tetraose and pentaose were the same for the two types of β-glucan. The ratio of the amount of triose to tetraose was 1.2–1.4 for all samples. This indicates that there is no difference between the structures of the two types. They differ only in their molar masses (1.1 vs. 1.6 million Daltons).

3.6. NMR spectroscopy of β-glucan

Two-dimensional inverse detected H,C-correlation spectroscopy (HSQC) was used to obtain structural information about the polymeric backbone in β-glucan isolated from oat bran. Polysaccharides of high molecular weight are poorly soluble in normal NMR solvents. They yield solutions of high viscosity which cause low resolution especially to the ^{1}H spectra. The major limitation of ^{13}C spectroscopy of polysaccharides is its low sensitivity. In the HSQC measurement the data is obtained in a relatively short experimental time even for low concentration samples.

Fig. 3 shows the HSQC spectrum of oat β-glucan. Correlations with carbon chemical shifts in 87.3, 76.5, 72.5 and 68.6 ppm provide evidence for the 3-O- substituted residues and can be assigned as C-3, C-5, C-2 and C-4 in 3-O-linked glucose, respectively (Dawkins, 1994; Ensley et al., 1994). Other signals originate from 4-O-linked residues. The HSQC NMR spectrum thus confirms the structure of a mixed linked β-glucan earlier assigned by Dais and Perlin (1982). It can also be concluded that the (1→3)-linkages are separate, since no evidence for consecutive (1→3)-linkages were seen in the spectrum.

The ^{13}C spectrum obtained from oat bran β-glucan isolated in this study contained 15 resonances suggesting also a structure of highly pure mixed linked β-glucan with 3-O- and 4-O-substitued residues.

4. Conclusions

The results of the above studies show that β-glucan can

be isolated from oat in a very pure form by the method used in this study. Thus, structural analysis gives reliable results. The structure of β-glucan can be investigated by two-dimensional HSQC NMR spectroscopy, which provides an efficient method for structural analysis with a short analysis time for samples with low concentrations. The results indicate that cello-oligosaccharides are joined by (1 → 3)- and (1 → 4)-linkages only. No evidence was found for linkages of any other type. It was also shown that the (1 → 3)-linkages are separate.

Another way to study the structure of β-glucan is to cleave it to oligosaccharides by a specific enzyme, lichenase. The oligosaccharides thus released were analyzed by both HPLC and CE methods. The results were in good agreement. These studies show that the main structural components of β-glucan are the cellotri- and cellotetrasaccharides. These account for 95% of the whole. The rest consists of oligosaccharides with a DP higher than 4.

Capillary electrophoresis provides a more sensitive method compared to HPLC with RI detection. Ease of use, high sensitivity and higher resolution make it a good choice for the analysis of cereal oligosaccharides in spite of the need for derivatization.

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