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Carbohydrate Polymers 46 (2001) 275–285

Carbohydrate Polymers

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Molecular weight, structure and shape of oat (1 → 3),(1 → 4)-β-D-glucan fractions obtained by enzymatic degradation with (1 → 4)-β-D-glucan 4-glucanohydrolase from *Trichoderma reesei*

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Received 14 September 2000; accepted 2 October 2000

Abstract

Oat β-glucan was partially degraded with (1 → 4)-β-D-glucan 4-glucanohydrolase for different periods of time. Weight average molecular weight and weight average intrinsic viscosity were obtained from SEC-RI-RALLS-Visc and ranged from 2200 to 213,900 g/mol and 7 to 316 ml/g, respectively. The viscosity equation determined $[\bar{\eta}]_w = 1.06 \times 10^{-2} \bar{M}_w^{0.86}$ indicated an extended random coil conformation. When the coil was modelled as a worm-like chain a persistent length of 3.65 nm was obtained. The hydrolysis products identified after extensive degradation were glucose, cellobiose, laminaribiose, 4-*O*-β-laminaribiosyl D-glucose, 4-*O*-β-laminaribiosyl D-cellobiose and 3-*O*-β-cellobiosyl D-cellobiose showing that other enzyme activities were present. The depolymerization kinetics suggested that longer sequences of consecutive (1 → 4)-linkages represent fast hydrolysable sites and the presence of higher relative proportions of (1 → 3)-linkages restricts the affinity of the enzyme.

Keywords: Mixed-linkage (1 → 3),(1 → 4)-β-D-glucan; Oats; Cellulase; Molecular weight; Shape; Structure; Kinetics

1. Introduction

The content of (1 → 3),(1 → 4)-β-D-glucan (hereafter referred to as β-glucan) in oats is 2.0–6.2% (Åman & Graham, 1987; Beer, Wood & Weisz, 1997; Wood & Weisz, 1984). It has been shown to be an unbranched polysaccharide composed of (1 → 4)-linked β-D-glucopyranosyl units (~70%) substituted at position 3 or 4, hereafter referred to as 3G4 and 4G4, respectively, and (1 → 3)-linked β-D-glucopyranosyl units (~30%) substituted at position 4, hereafter referred to as 4G3 (Aspinall & Carpenter, 1984; Parrish, Perlin & Reese, 1960). While consecutive (1 → 3)-linkages have been identified in the β-glucan from *Zea mays* stems (Kato & Nevins, 1986) and germinated barley (Bathgate, Palmer & Wilson, 1974), most of the evidence suggests that (1 → 3)-linkages occur singly (Dais & Perlin, 1982; Vårum & Smidsrød, 1988; Woodward, Fincher & Stone, 1983). The (1 → 4)-linkages occur in groups of two or three (Perlin & Suzuki, 1962; Woodward et al., 1983), although longer sequences have been reported (Wood, Weisz & Mahn, 1991a; Woodward et al., 1983). Approximately 90% (w/w) of the main chain has been reported to consist of the oligosaccharides 3-*O*-β-cellobiosyl D-glucose and 3-*O*-β-cellotriosyl D-glucose (Woodward et al., 1983) and their molar ratio has been determined to 2.0–2.4 (Erfle, Teather, Wood & Irvin, 1988; Wood, Weisz & Blackwell, 1994a). These oligosaccharides are also the major products after complete enzymatic hydrolysis with lichenase (Huber & Nevins, 1977; Wood, Weisz & Blackwell, 1991b).

Partial hydrolysis with lichenase and its effect on molecular weight, structure and shape has been described previously (Roubroeks, Mastromauro, Andersson, Christensen and Åman, 2000b). Another enzyme that can be used for controlled depolymerization is *endo*-1,4-β-glucanase (E.C. 3.2.1.4). These enzymes have been claimed to hydrolyse at random (Nakayama, Tomita, Suzuki & Nisizawa, 1976) the internal (1 → 4)-linkages next to 4-substituted glucose residues (Anderson & Stone, 1975; Parrish et al., 1960). The major end products after hydrolysis of barley β-glucan are glucose, cellobiose, 4-*O*-β-laminaribiosyl D-glucose and 4-*O*-β-laminaribiosyl D-cellobiose (Igarashi, Noguchi & Fujimaki, 1968). The specificity of *endo*-(1 → 4)-β-glucanases might be determined by the sequence of glucose residues for which the enzyme has affinity, rather than by the linkage being hydrolysed (Bhat, Hay, Claeyssens & Wood, 1990;

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0144-8617/01/$ - see front matter
PII: S0144-8617(00)00329-5

Christensen & Smidsrød, 1996; Shiroishi, Amano, Hoshino, Nisizawa & Kanda, 1997).

The fundamental molecular characteristics of structure and molecular weight are directly related to the viscosity of polysaccharide solutions. Reports on the relationship between β-glucan structure and conformation (Buliga, Brant & Fincher, 1986; Forrest & Wainwright, 1977; Gómez, Navarro, Manzanares, Horta & Carbonell, 1997a; Gómez et al., 1997b; Vårum, Martinsen and Smidsrød, 1991; Wood et al., 1994a; Woodward et al., 1983) and on behaviour in solution (Buliga et al., 1986; Vårum and Smidsrød, 1988; Vårum et al., 1991; Woodward et al., 1983) have appeared previously. The exponent *a* in the Mark–Houwink–Sakurada (MHS) viscosity equation for aqueous β-glucan has been determined to be 0.75 (Vårum & Smidsrød, 1988; Vårum et al., 1991) and 0.81 (Roubroeks et al. 2000b) which was in good agreement with a theoretical extended random coil conformation (Buliga et al., 1986). In the literature only three studies were found which used light scattering experiments to determine the random coil conformation of β-glucans (Gómez et al., 1997b; Roubroeks et al., 2000b; Vårum, Smidsrød & Brant, 1992).

Reports on fractionation of partially degraded β-glucan are scarce in the literature. Ultrasonic irradiation (Vårum & Smidsrød, 1988), acid hydrolysis (Doublier & Wood, 1995), lichenase hydrolysis (Roubroeks et al., 2000b) and lichenase hydrolysis in combination with ammonium sulphate precipitation (Izawa, Kano & Koshino, 1993) were used in previous studies.

In the present work, the partial depolymerization studies were continued with a cellulase preparation from a *Trichoderma* species. It was expected that this enzyme would have a different effect on the stiffness of the polymer in solution compared to the effects obtained in a previous study with lichenase (Roubroeks et al., 2000b). Data on molecular weight, viscosity and ^{1}H-NMR of the different fractions were used to analyse depolymerization kinetics and possible changes in shape during the degradation.

2. Experimental

2.1. Oat β-glucan solution

Oat β-glucan (Lot.30101) was obtained from Megazyme (Bray, Ireland). It had been extracted from the cell walls of oat endosperm, with a yield of >95%. The molecular size of the β-glucan was approximately 200,000 g/mol according to the supplier. The polysaccharide (277.75 mg) was heated in 20 ml sodium acetate buffer (0.05 M, pH 4.7) until total solubilization. The solution was allowed to cool down to room temperature and adjusted to 25 ml.

2.2. Cellulase digestion

The assay mixture consisted of 0.9 ml β-glucan solution (10 mg/ml) and 0.1 ml (0.01 U/ml) cellulase (Lot.50201, isolated from *Trichoderma reesei* and obtained from Megazyme, Bray, Ireland). The enzyme preparation contains low side activities e.g. *endo*-(1 → 3)-β-glucanase (specific activity <0.001 U/mg; specific activity of cellulase is 76.5 U/mg) according to the supplier. Samples used for conventional size-exclusion chromatography were incubated for 1, 2, 6, 12, 18, 24 and 48 h in 0.05 M sodium acetate buffer pH 4.7 in a shaking water bath at 40°C. The enzyme was inactivated by a 30 min treatment in a boiling water bath, since cellulase is known to have a high thermostability. In this respect the enzyme might be similar to *cellulase II B* (Okada, 1975) which retained approximately 41% of the activity after 10 min. boiling. Samples for light scattering and viscometry experiments were incubated for 1, 2, 4, 8, 18, 24, 72, 96, 168, 240 and 314 h using the same conditions as described above. All samples, including a 0 h sample, were cooled, centrifuged and stored at 4°C prior to analysis. Control samples, which consisted of inactivated enzyme and substrate, were used to ensure that the hydrolytic activity was completely related to the cellulase.

2.3. Conventional size-exclusion chromatography (SEC) of cellulase hydrolysates

Hydrolysates were applied on a Sephacryl S-500 (40 × 2.6 cm) or Sephacryl S-300 (35 × 1.1 cm) column. Void volume (V_0) and included volume (V_t) were determined with dextran T2000 and glucose, respectively, and the column was calibrated with dextran standards T-500, T-150, T-70, T-40 and T-10 (Amersham Pharmacia Biotech, Uppsala, Sweden). Elutions were performed with 0.05 M sodium acetate buffer pH 4.7 and 0.01% NaN_3 at room temperature with a constant flow rate of 0.4 ml/min. This separation was performed using a FPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden) equipped with a differential refractometer (R401, Waters Associates, Milford, MA, USA). Fractions of 1 ml were collected. The injected sample amount was 10 mg on S-500 and 5 mg on S-300.

2.4. High performance size-exclusion chromatography combined with multiple angle laser light scattering and viscometry detection

The HPSEC-RI-MALLS-Visc system consisted of an online degasser (Shimadzu DGU-4A), a pump (Shimadzu LC-10AD) and 3 serially connected columns (OHpak SB-806M HQ, OHpak SB-804 HQ and OHpak SB-803 HQ, Shodex, Showa Denko KK, Miniato, Japan). The eluent was 0.1 M $NaNO_3$ containing 0.02% NaN_3 at 0.5 ml/min. Detectors were refractive index (RI) and viscometer (Dual detector model 250, Viscotek Corp, Houston, TX, USA), UV monitor (Pharmacia LKB UV-M II, Amersham Pharmacia Biotech, Uppsala, Sweden) and multiple angle laser light scattering (MALLS; Dawn DSP equipped with a He–Ne laser 632.8 nm, Wyatt Technology Corp., Santa Barbara,

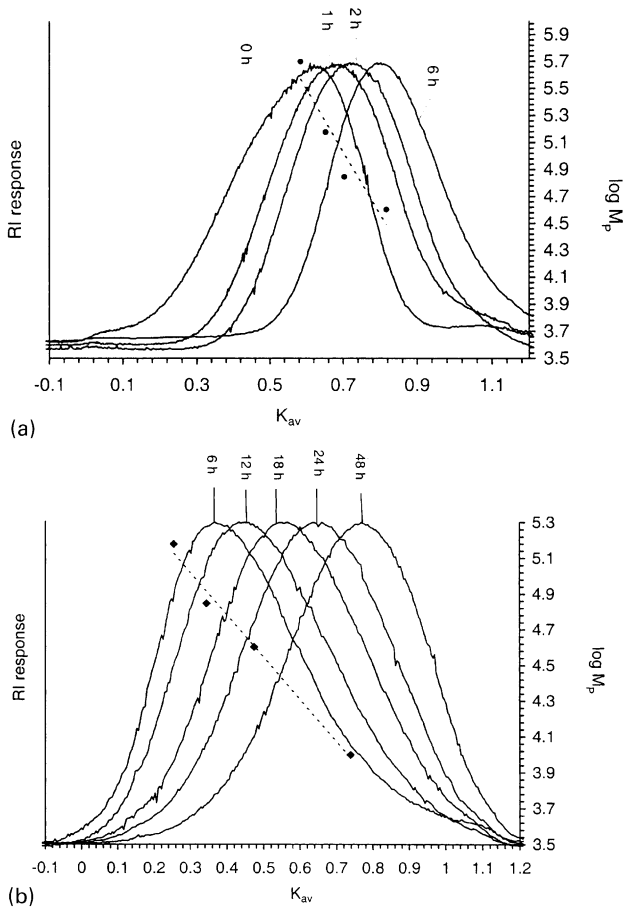

Fig. 1. (A) Distribution of partially hydrolysed β-glucan (0-6 h) on Sephacryl S-500. Peak molecular weights (M_p) are expressed as dextran equivalents (●). (B) Distribution of partially hydrolysed β-glucan (6-48 h) on Sephacryl S-300. Peak molecular weights (M_p) are expressed as dextran equivalents(◆).

CA, USA). Columns and RI-Visc detector were controlled at 35°C.

Samples were centrifuged and filtered (0.45 μm) before injection of 107 μl. Data for molecular weight determinations and conformation were analysed using ASTRA software (Version 4.70.07, Wyatt Technology Corp., Santa Barbara, CA, USA) based on a dn/dc of 0.147 (Vårum et al., 1992). The angular fit was based on the Debye procedure, weight average molecular weight $\bar{M}_w$ and number average molecular weight $\bar{M}_n$were obtained following a 1st order polynomial curvefitting of log *M* (molecular weight) versus elution volume. Calculations based on viscometry data were performed using TriSEC software (Version 3.0, Viscotek Corp., Houston, TX, USA). $\bar{M}_w$, $\bar{M}_n$and weight average radius of gyration $\langle s \rangle_w$ were obtained from unfitted data and no additional data smoothing was applied according to the manual.

2.5. Structural analysis of cellulase hydrolysates

NMR spectroscopy was performed on a Bruker DRX-400 spectrometer (Bruker Spectrospin, Milton, ON, Canada) operating at 60°C. An aliquot of the hydrolysate (5.5 mg) was freeze-dried and dissolved in 0.9 ml D_2O. The sample was centrifuged (a small pellet was obtained) before transferring to the NMR-tube. ^1H, ^{13}C and COSY spectra were recorded and chemical shifts were determined relative to the internal standard acetone-d_6 (δ_H 2.225; δ_C 31.00). The heteronuclear single quantum coherence-distortionless enhancement by polarization transfer (HSQC-DEPT) used an echo-antiecho sequence and a CNST2 of 145 Hz (Willker, Leibfritz, Kerssebaum & Bermel, 1993). Laminaribiose (Sigma, St. Louis, MO, USA), cellobiose (Kebo-Lab, Stockholm, Sweden) cellotriose, cellotetraose and cellopentaose (V-Labs, Covington, LA, USA) were used as reference substances.

The presence of specific di- and oligosaccharides was determined by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex DX 500 instrument (Sunnyvale, CA, USA) as described by Roubroeks, Andersson and Åman (2000a).

3. Results and discussion

3.1. Conventional size-exclusion chromatography of cellulase hydrolysates

Oat β-glucan was treated with cellulase for different periods of time (0–48 h) and hydrolysates were analysed by size-exclusion chromatography (SEC). A concentration of 0.001 U/ml enzyme resulted in a very slow degradation of the polymer. This concentration had been used in a previously published experiment with lichenase, but the substrate affinity was apparently higher for lichenase than for cellulase. Therefore, an enzyme concentration of 0.01 U/ml was used. The results of this pre-experiment are presented in Fig. 1A and B (0–6 h hydrolysates and 6–48 h hydrolysates, respectively). No significant accumulation of low molecular weight fragments was observed and the depolymerization occurred gradually. Similar results were obtained when β-glucan was degraded with lichenase (Roubroeks et al., 2000b). The columns were calibrated with dextrans in sodium-acetate buffer since the elution volume of the β-glucan, when dissolved in sodium-acetate buffer, was more comparable to dextrans than the elution volumes of pullulan standards in sodium-acetate buffer. This might be explained by differences in column interaction at different pH. The peak molecular sizes, expressed as dextran equivalents (Vårum et al., 1991), varied between 211,600 (0 h) and 7800 (48 h).

3.2. High performance size-exclusion chromatography combined with refractive index, light scattering and viscometry detection

Size-exclusion chromatography combined with refractive index-multiple (right) angle laser light scattering and

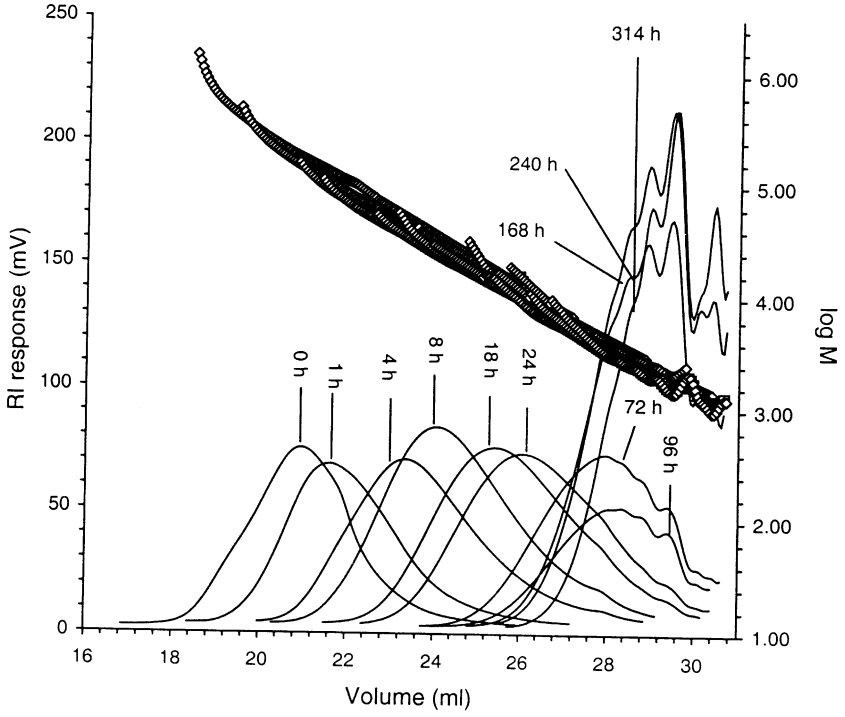

Fig. 2. RI-elution profiles of partially hydrolysed β-glucan (0-314 h) as determined with SEC-RI-RALLS-Visc. Insertion of molecular weight variation for each fraction with elution volume as overlay.

viscometry detection (SEC-RI-M(R)ALLS-Visc) was conducted to obtain more detailed information on molecular weight (M) and weight average intrinsic viscosity $[\bar{\eta}]_w$. The elution profiles of SEC-RI-RALLS-Visc are depicted in Fig. 2. The decrease in weight average molecular weight ($\bar{M}_w$) is reflected in a progressive shift towards higher elution volumes with increasing hydrolysis time (Table 1). These results were comparable to the peak molecular weights obtained with conventional chromatography for 1–48 h. The insertion of log M in Fig. 2 gave an indication of the molecular weight distribution over the whole elution profile. Since the peaks of the longer hydrolysis times (168, 240 and 314 h) were truncated due to partial overlap with the included volume, it can be expected that correct values for $\bar{M}_n$could not be obtained (Table 1). Lower calculated values (1.7–1.2) for the polydispersity index ($\bar{M}_w/\bar{M}_n$) than the theoretical 2.0 (Kuhn distribution) (Tanford, 1961) can possibly be ascribed to band broadening (van Dijk, Varkevisser & Smit, 1987), but Fig. 2 does not suggest a pronounced effect of band broadening since the linear parts of log M overlap. However, it should be noted that at least for longer hydrolysis times, the polydispersity index is largely affected by the unreliable $\bar{M}_n$.

Information about the depolymerization kinetics can be

Table 1
Data from SEC-RI-RALLS-Visc; weight average molecular weight ($\bar{M}_w$), number average molecular weight ($\bar{M}_n$), polydispersity index (($\bar{M}_w/\bar{M}_n$), radius of gyration ($\langle s \rangle$) and weight average intrinsic viscosity ($[\bar{\eta}]_w$) of fractions with increasing hydrolysis time. Values within parentheses are calculated from SEC-RI-MALLS

Hydrolysis time (h)	$\bar{M}_w \times 10^{-3}$(g/mol)		$\bar{M}_n \times 10^{-3}$(g/mol)		$\bar{M}_w/\bar{M}_n$		$\langle s \rangle$ (nm)[a]	$[\bar{\eta}]_w$ (ml/g)
0	213.9	(208.1)	133.7	(135.6)	1.60	(1.53)	30.0	316.3
1	100.6	(105.8)	66.1	(67.5)	1.52	(1.57)	19.5	191.1
4	37.4	(39.3)	22.5	(21.0)	1.66	(1.87)	10.9	91.5
8	24.7	(26.8)	14.9	(15.2)	1.66	(1.76)	8.5	66.7
18	12.9	(13.6)	8.0	(7.1)	1.61	(1.91)	5.6	36.8
24	10.0	(10.2)	6.4	(6.0)	1.58	(1.70)	4.8	28.5
72	4.9	(4.8)	3.6	(3.3)	1.38	(1.45)	3.0	13.9
96	3.8	(4.1)	2.8	(3.1)	1.35	(1.32)	2.5	12.0
168	2.7	(3.2)	2.2	(2.3)	1.28	(1.42)	2.1	8.4
240	2.5	(3.0)	2.0	(2.1)	1.25	(1.47)	1.9	7.7
314	2.2	(2.1)	1.8	(1.4)	1.22	(1.55)	1.7	6.6

[a] Radius of molecules with molecular weight equal to $\bar{M}_w$.

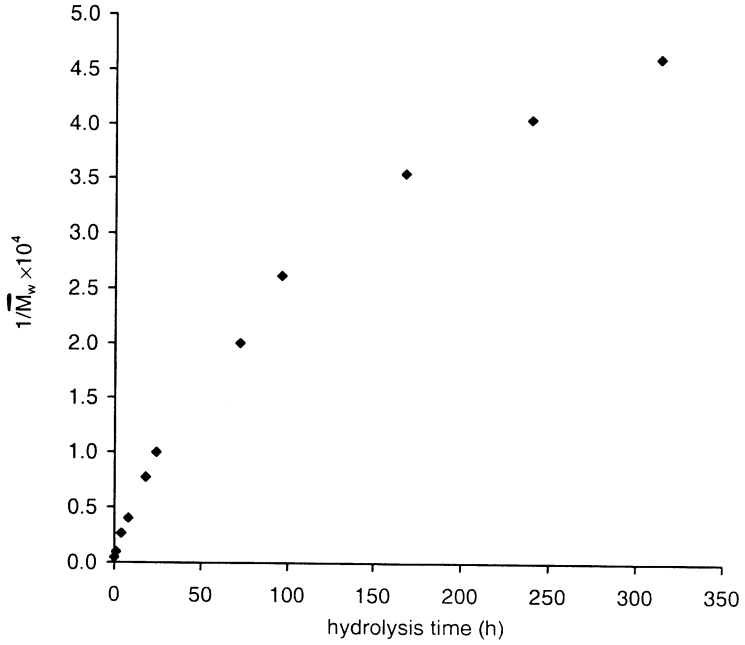

Fig. 3. $1/\bar{M}_w$ of partially hydrolysed β-glucan as a function of hydrolysis time.

obtained from a plot of $1/\bar{M}_w$ versus the hydrolysis time (Fig. 3). For a truly random depolymerization, $\bar{M}_w$ should decrease with time according to the equation (Tanford, 1961) $\bar{M}_w^{-1}(t) = \bar{M}_w^{-1}\ (t=0) + 1/2\ kt\ M_o^{-1}$, where k is the pseudo first-order rate constant for breaking a glycosidic linkage and M_o is the equivalent weight of the monosaccharide. An apparent decreasing degradation rate with increasing hydrolysis time was observed. The same tendency was reported for the enzymatic hydrolysis of CMC and xanthan with cellulases (Christensen & Smidsrød, 1996; Wirick, 1968). This is consistent with an enzyme which has a different affinity for different sites of the substrate and it might therefore be suggested that longer sequences of consecutive (1 → 4)-linkages are attacked more rapidly, and therefore consumed earlier, than chains with a higher relative proportion of (1 → 3)-linkages. The latter may eventually become resistant to degradation by cellulase. The latter effect might also be reflected by the polymodal peak shape from the RI detector for hydrolysates 72, 96, 168, 240 and 314 h. This was confirmed by analysis with HPAEC-PAD clearly showing the presence of distinct populations at retention times 10, 15 and 18 min. (Fig. 4). This finding further indicates that the enzyme depolymerization is indeed non-random. A similar pattern was previously reported by Wood, Erfle, Teather, Weisz and Miller (1994b). Five oligosaccharides were grouped together, but the relative proportions of the oligosaccharides differed from our results.

Information about chain shape and extension may be obtained by analyzing the dependence of $\langle s \rangle$ and $[\eta]$ on M. This can either be performed on the basis of individual chromatographic slices, in each fraction, or by computing and comparing proper averages. An advantage of the former is that slices with poor or noisy data may be excluded from the analysis. The weight average intrinsic viscosity ($[\bar{\eta}]_w$) values, determined by SEC-RI-Visc, ranged from 7 to 316 ml/g (Table 1). The dependency of $[\eta]$ upon M can be expressed with the MHS equation $[\eta] = KM^a$ (Fig. 5). The calculated values for each fraction from SEC-RI-RALLS-Visc could be fitted to $[\bar{\eta}]_w = 1.06 \times 10^{-2}\ \bar{M}_w^{0.86}$. In contrast, when $[\eta]$ and M were calculated for each elution slice and analysed independently for each fraction, it was observed that the exponent a increased from 0.64 for the unhydrolysed material to 1.05 for the 314 h hydrolysate suggesting a gradual shape change towards a stiffer coil. This may simply be ascribed to the fact that the number of statistical segments becomes too low to fulfil the criterion for a statistical chain (where $a = 0.5$–0.6). A similar value for a was obtained when $\bar{M}_w$ from SEC-RI-MALLS was used. In comparison, the degradation of oat β-glucan with lichenase resulted in $a = 0.82$ (Fig. 5) (Roubroeks et al., 2000b). The cellulase hydrolysates had a consistently higher $[\bar{\eta}]_w$ than the lichenase hydrolysates (at the same $\bar{M}_w$) which normally suggests that the hydrodynamic volume is larger in the former case. The structural basis for this observation remains unclear. Other researchers who determined viscosity equations showed a values close to the a value obtained in this study (Böhm & Kulicke, 1999; Gómez et al., 1997b; Grimm, Krüger & Burchard, 1995; Vårum et al., 1992). As in previously obtained results for a β-glucan which was partially degraded with lichenase (Roubroeks et al., 2000b), the coil could be modelled as a worm-like chain. The theory behind this model has been extensively described (Bohdanecký, 1983; Gómez et al., 1997b; Roubroeks et al., 2000b; Yamakawa & Fujii, 1973).

Values for the intercept A_η (44.2), slope B_η (1.04), Kuhn length l_k (7.3 nm), persistent length l_p (3.65 nm) and the

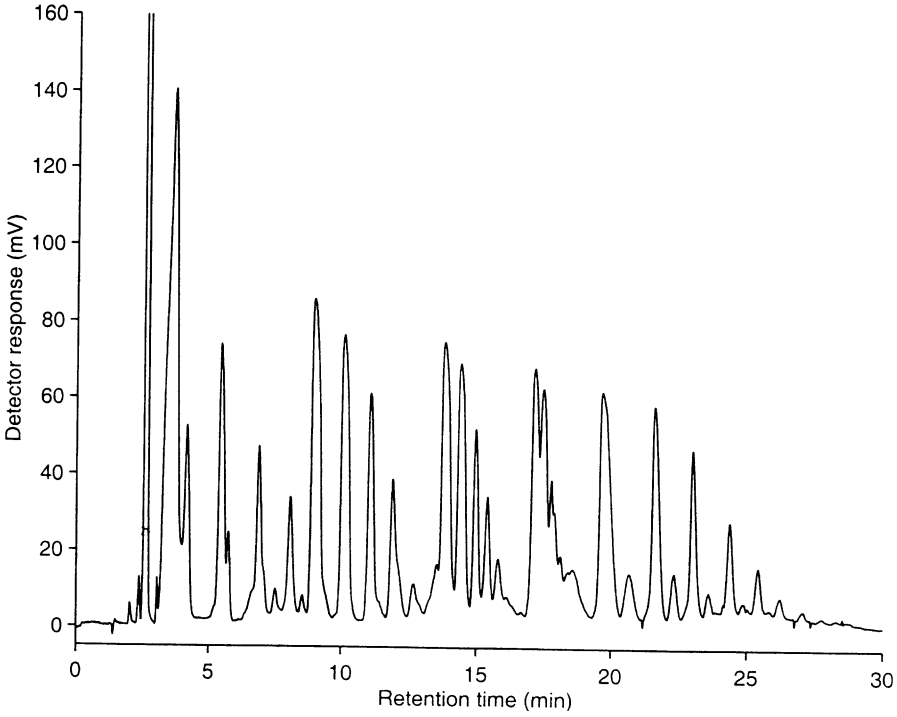

Fig. 4. Fractionation by HPAEC-PAD of oligosaccharides after 314 h of hydrolysis.

thickness d (0.96 nm) were obtained. A value of 7.3 nm for the statistical segment length corresponds to about 14 residues or approximately three to four (1 → 3)-linkages per statistical segment. The value of l_p (3.65 nm) and the thickness (0.96 nm) were in the same range as the values 3.47 and 0.45 nm, respectively, obtained by Gómez et al. (1997b).

The characteristic ratio (C_∞), which is defined as the ratio of the unperturbed value to the value it would have assuming freely jointed units of the chain (N/b^2), is totally consistent with l_k/b in the case of a worm-like chain. A value of 13.7 corresponds to a rigid chain and is in good agreement with the theoretical calculations (Buliga et al., 1986).

3.3. NMR

Structural information and characteristics concerning the

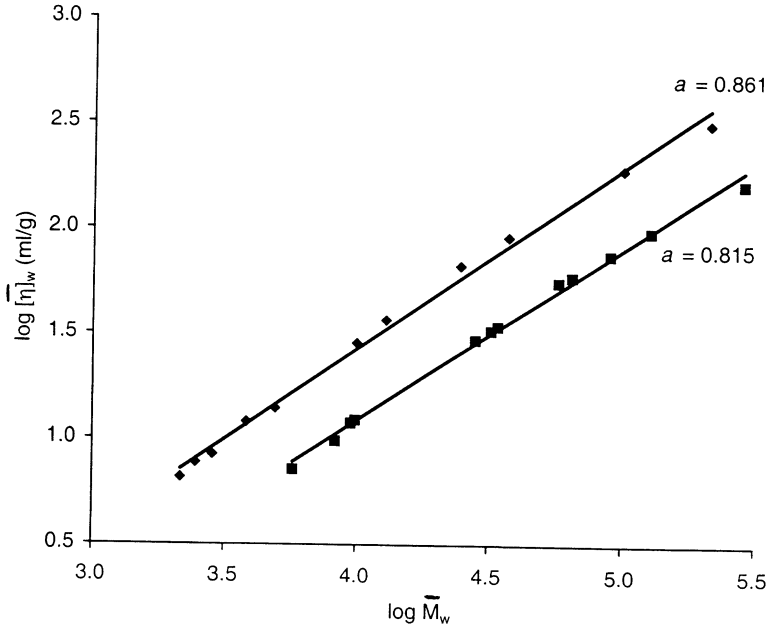

Fig. 5. Function of log $[\bar{\eta}]_w$ versus log $\bar{M}_w$ as determined with SEC-RI-RALLS-Visc (◆) cellulase (■) lichenase (adapted from Roubroeks et al., 2000b).

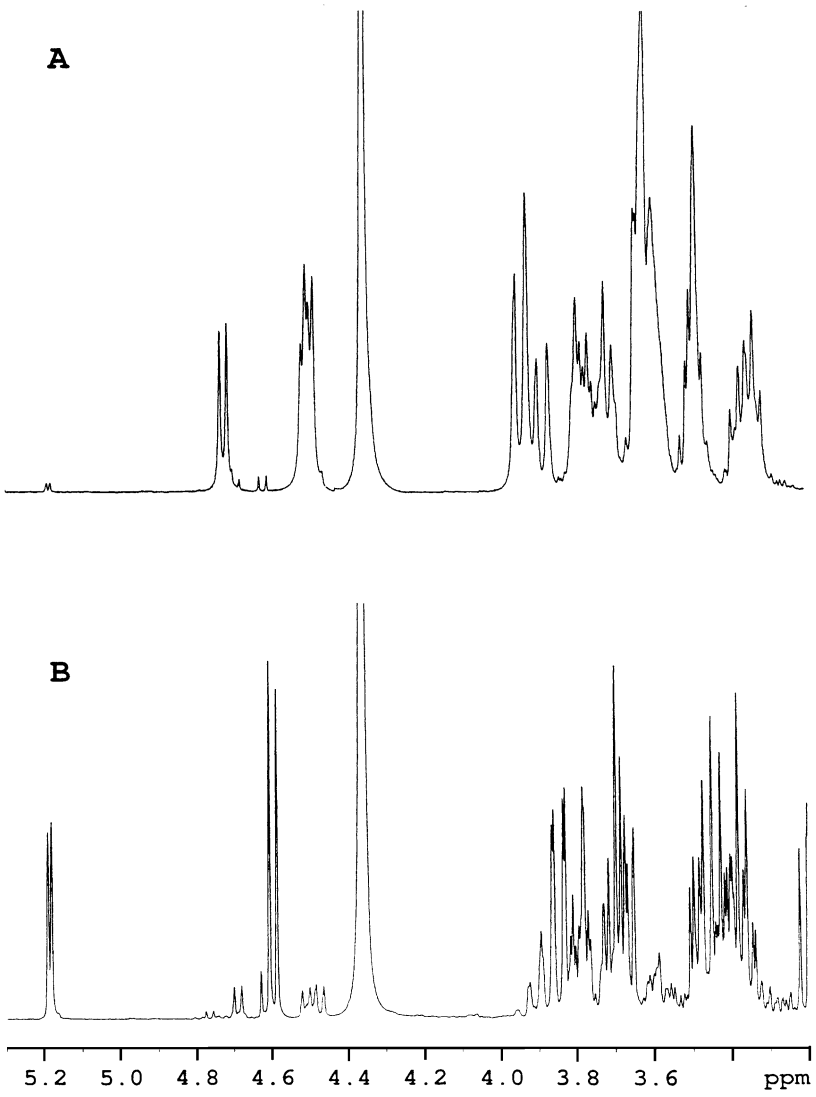

Fig. 6. ^{1}H-spectrum of β-glucan after 240 h of hydrolysis (A), and exhaustive hydrolysis (10 U/ml, 20 h) (B).

enzymatic attack on the polysaccharide can be obtained from NMR. A typical ^{1}H-NMR spectrum from a cellulase hydrolysate is shown in Fig. 6A and the chemical shifts of the signals in the anomeric region were assigned from those reported by Bock, Duus, Norman and Pedersen (1991) (Table 2). H-2 signals were assigned from COSY and ^{13}C-signals by using HSQC-DEPT on the 240 h sample. There was still polymeric material present after 240 h of hydrolysis since the proportion of reducing end and non-reducing end signals was relatively low. Although the spectrum resembled that of the starting material, some new signals were observed. The doublet at 4.722 ppm could be assigned to a polymeric 4G3 and the doublet upfield of this signal at 4.689 was undoubtedly a G3 residue (confirmed with laminaribiose reference). From the specificity of the enzyme it can be expected that the polymeric 4G3 will decrease while the G3 will increase. This was confirmed with the ^{1}H-spectrum of the completely degraded β-glucan (Fig. 6B). The hydrolysis products which could be derived from this spectrum are presented in Fig. 7. Product A was 4-*O*-β-laminaribiosyl D-glucose and product B 4-*O*-β-laminaribiosyl D-cellobiose in accordance with previous results (Bock et al., 1991; Henriksson, Teleman, Suortti, Reinikainen, Jaskari, Teleman & Poutanen, 1995; Igarashi et al., 1968; Parrish et al., 1960; Shiroishi et al., 1997). Product C, 3-*O*-β-cellobiosyl-cellobiose (Bock et al., 1991; Henriksson et al., 1995; Parrish et al., 1960), could be present in minor amounts. However, our findings suggest that product C is degraded by *endo*-(1 → 3)-β-glucanase activity and produces cellobiose (D). The high amount of glucose could not be explained by the degradation of released cellobiose alone. The fast hydrolysis of longer sequences of consecutive (1 → 4)-linkages and the apparent presence of concomitant enzyme activities might partly explain the

Table 2
Chemical shifts (measured at 400 MHz (^{1}H) and 100 MHz (^{13}C) in D_2O at 60°C (internal acetone δ_H 2.225; δ_C 31.000)) of the ^{1}H- and ^{13}C- resonances of the sugar residues from β-glucan after 240 h with 0.001 U/ml cellulase

Sugar residue	H1	H2	C1	C2	C3	C4	C5	C6
→ 4)-β-Glc*p*[a]	5.186 (α)	3.537 (α)	96.560 (β)					
	4.617 (β)	3.251 (β)						
→ 4)-β-Glc*p*(1 → 3)[b]	4.722	3.370	103.327	74.110	74.976	79.457	75.656	61.527
β-Glc*p*(1 → 3)[c]	4.689	3.313	103.366	74.110	74.976	79.457	75.656	61.527
→ 3)-β-Glc*p*(1 → 4)[d]	4.508	3.491	103.106	73.719	85.141	68.910	76.444	60.980
→ 4)-β-Glc*p*(1 → 4)[e]	4.497	3.333	103.067	73.812	74.976	79.374	76.813	60.940
→ 4)-β-Glc*p*(1 → 4)[f]	4.497	3.333	103.067	73.812	74.976	79.432	76.813	60.940
β-Glc*p*(1 → 4)[g]	4.469	3.273	103.067		76.444	70.382	76.813	61.092

[a] Reducing end substituted at 4 position.
[b] Polymeric oat β-glucan.
[c] Non-reducing end residue of the laminaribiosyl part of hydrolysis products A and B (Fig. 7).
[d] Polymeric oat β-glucan.
[e] Adjacent to 3G4 in polymer
[f] Polymeric oat β-glucan and oligosaccharides
[g] Non-reducing end in oligosaccharides.

relatively high proportion of non-reducing end signals at 4.619 and 4.595 ppm of 4-substituted oligosaccharides and glucose, respectively. Moreover, the HPAEC-PAD chromatogram shows glucose and cellobiose as the two main products (Fig. 8). Besides these, a peak co-eluting with laminaribiose and six unidentified peaks were observed. The two main unidentified peaks of these six (denoted as peaks 1 and 2) were probably the hydrolysis products A and

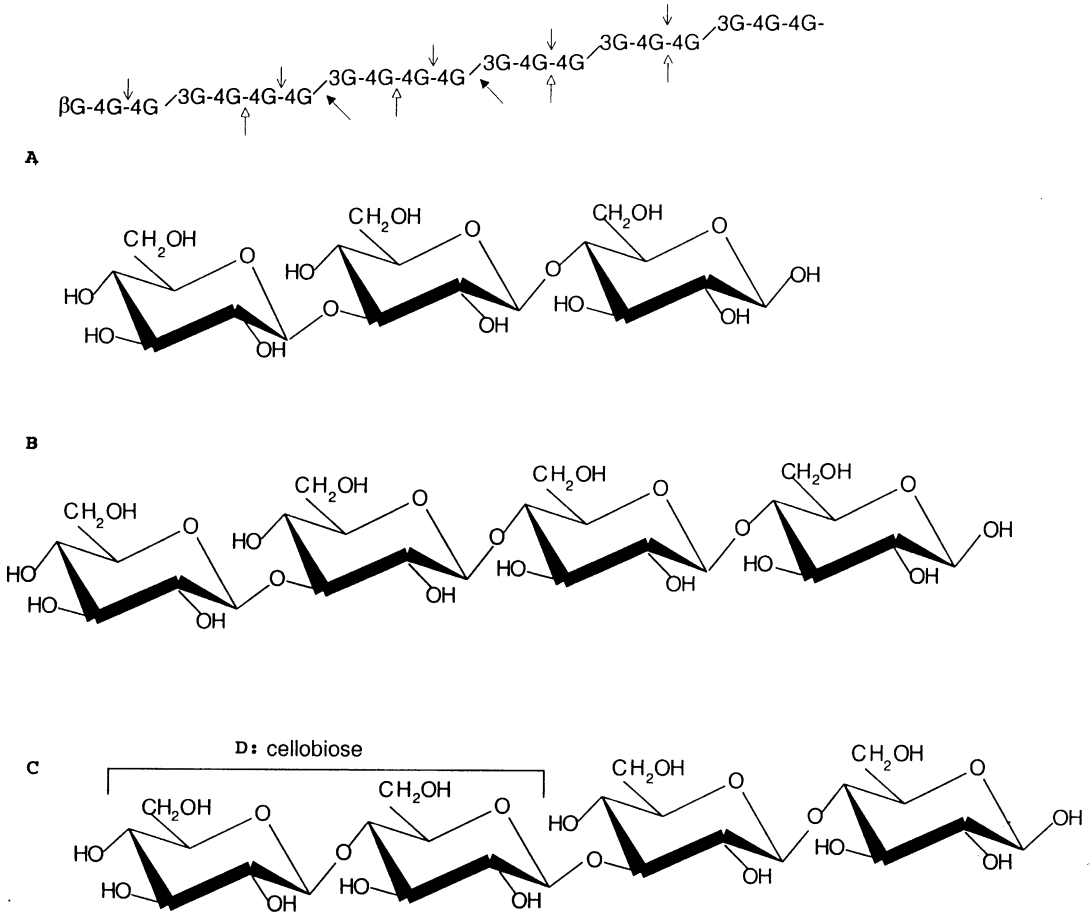

Fig. 7. Degradation sites of β-glucan with cellulase. (↓) results in hydrolysis products A and B; (↑) products A and C; (↖ *endo*-(1 → 3)-β-glucanase) product D (see text).

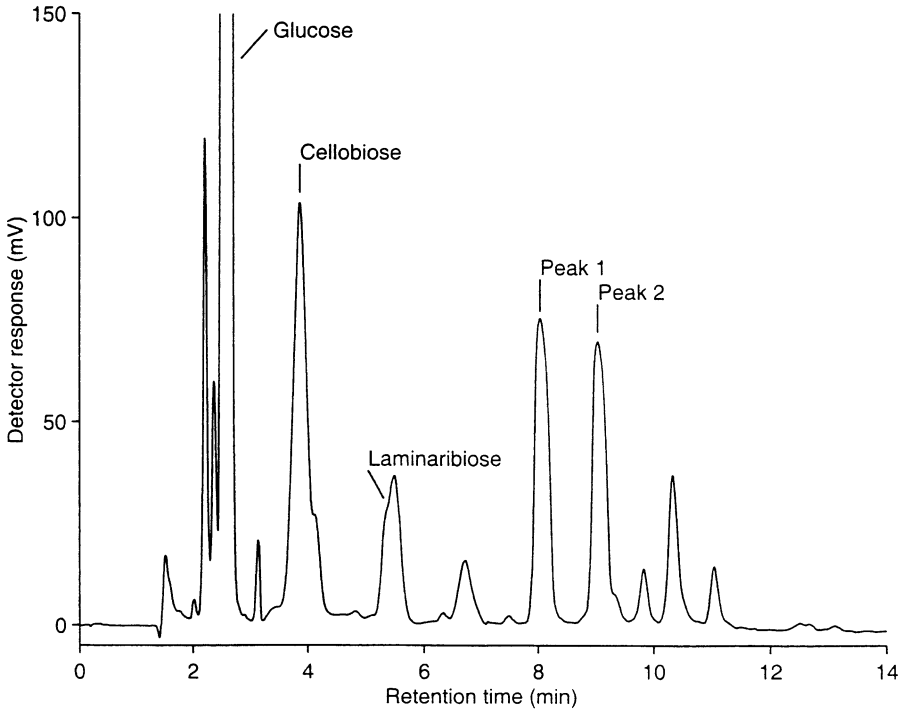

Fig. 8. Fractionation by HPAEC-PAD of oligosaccharides released by exhaustive hydrolysis (10 U/ml, 20 h) with *endo*-(1 → 4)-β-glucanase from *Trichoderma reesei*. Peak 1 and Peak 2 are probably 4-*O*-β-laminaribiosyl D-glucose and 4-*O*-β-laminaribiosyl D-cellobiose (see text).

B. It could also be observed that these peaks were rather broad, which might indicate that other peaks overlap. The low side activity of *endo*-(1 → 3)-β-glucanase of an *endo*-(1 → 4)-β-glucanase from *Trichoderma reesei* has been described previously (Shiroishi et al., 1997). These authors reported only an effect on product C, which might indicate that it is unlikely that products A and B have been attacked by *endo*-(1 → 3)-β-glucanase enzyme. Whether product B is degraded to product A was pursued through a second addition of 10 U/ml enzyme, and the ^{1}H-spectrum (not shown) revealed an increase in the reducing end, non-reducing end and free glucose signals. All other signals decreased proportionally, which might be interpreted as a product transformation (B → A).

There was an indication of small amounts of laminaribiose in the HPAEC-PAD chromatogram (Fig. 8). This can only be explained when lichenase activity is present in the enzyme preparation. However, no signals in the ^{1}H-spectrum (Fig. 6A) could be identified as being derived from a 3-substituted reducing end, but these could be overlapped by the strong reducing end signals of glucose. The presence of laminaribiose and the occurrence of higher undegradable oligosaccharides might be related to the presence of an alternating structure of (1 → 3)- and (1 → 4)-linkages as has been suggested previously (Roubroeks et al., 2000b).

The polysaccharide behaviour in solution might be related to the enzyme hydrolysis. The attack on longer sequences of consecutive (1 → 4)-linkages and blocks of cellotetraosyl units seems to be favourable, which results in an enrichment of oligosaccharides with a higher relative proportion of (1 → 3)-linkages which in turn could result in a larger hydrodynamic volume. This might also be reflected in Fig. 3. A fast decrease in $\bar{M}_w$ over 48 h resulted in a lower proportion of fast hydrolysable linkages. The availability of shorter, less favourable, chains and the presence of different enzymatic side activities, with different kinetics, resulted in a decreasing degradation rate.

4. Conclusions

Partially hydrolysed oat β-glucan fractions were obtained by *endo*-(1 → 4)-β-glucanase digestions. The weight average molecular weight ($\bar{M}_w$) of the fractions ranged between 2.2×10^3 and 2.1×10^5 and the weight average intrinsic viscosity $[\bar{\eta}]_w$ values between 7 and 316 ml/g which resulted in a viscosity equation that indicated an extended coil conformation. These results do not differ from results obtained of the hydrolysis of β-glucan with lichenase (Roubroeks et al., 2000b). However, the depolymerization kinetics changed notably, since longer sequences of consecutive (1 → 4)-linkages appeared to be the primary target, indicating that the *endo*-(1 → 4)-β-glucanase was site specific. The viscosity decreased significantly during this phase, indicating that these sequences are located within the β-glucan chain, since a localization at the ends would not show this effect. Sequences with a higher relative proportion of (1 → 3)-linkages (blocks of cellotetraosyl < blocks of

cellotriosyl units) were degraded at a lower rate, which was reflected by the curvature in the depolymerization kinetics. Moreover, it was shown that distinct populations of hydrolysis products were produced which were refractory towards further depolymerization by cellulase. ^{1}H-NMR of exhaustively hydrolysed β-glucan (10 U/ml, 20 h) showed the presence of four main hydrolysis products, namely glucose, cellobiose, 4-*O*-β-laminaribiosyl D-glucose and 4-*O*-β-laminaribiosyl D-cellobiose and to a lesser extent 3-*O*-β-cellobiosyl D-cellobiose. This shows that the protein band ascribed to *endo*-(1 → 4)-β-glucanase contains several side activities. Although laminaribiose could not be detected with ^{1}H-NMR due to overlapping of the broad reducing end signals of the free glucose, its presence was indicated by HPAEC-PAD, which also suggests lichenase activity.

Acknowledgements

An ERASMUS scholarship for Domenico I. Mastromauro is gratefully acknowledged.

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