Isolation of Homogeneous Fractions from Wheat Water-Soluble Arabinoxylans. Influence of the Structure on Their Macromolecular Characteristics

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Water-soluble arabinoxylans from wheat flour were purified and fractionated by graded ethanol precipitation. Six fractions were obtained at 20% (F20), 30% (F30), 40% (F40), 50% (F50), 60% (F60), and 70% (F70) saturation with ethanol. Neutral sugars and ^{1}H NMR analyses revealed differences in structural characteristics. The Ara/Xyl ratio and the amount of Xyl*p* residues disubstituted increased with ethanol concentration. Ferulic acid content was higher in fractions precipitated at low ethanol percentage. Fractions were refractionated by SEC, leading to 46 subfractions with low polydispersity index. Substitution degree was apparently linearly related to the amount of disubstituted Xyl*p*. Macromolecular characteristics (M_w, [η], R_G, q, ν) determined by multiangle laser light scattering and viscosimetry were similar among all fractions. A rather flexible conformation was determined for the arabinoxylans, in conflict with the admitted rodlike conformation. The substitution degree had no influence on the conformation or on the rigidity of the polymers. Evidence for the presence of ferulic acid dimers in the water-soluble arabinoxylans is provided, which probably explains the unexpected conformation and macromolecular characteristics.

Keywords: *Arabinoxylans; fractionation; structure; macromolecular characteristics*

INTRODUCTION

Endosperm of cereal grains contains between 2 and 7% cell wall material, which consists largely of nonstarch polysaccharides (NSP). In wheat, arabinoxylans are major components of NSP. They have a linear backbone of β-(1→4)-linked D-xylopyranosyl units to which α-L-arabinofuranosyl substituents are attached through O-2 and/or O-3. Some ferulic acids are esterified to arabinose via O-5 (Smith and Hartley, 1983). Despite being relatively minor constituents of the wheat flour (0.3−0.7% w/w), water-soluble arabinoxylans are believed to have an impact on dough rheology and on bread quality parameters such as loaf volume, crumb texture, and staling characteristics (Biliaderis et al., 1995) and are recognized as antinutritive factors in poultry diets (Fincher and Stone, 1986). These effects might be related to the high viscosity they exhibit in aqueous solutions (Fincher and Stone, 1986; Girhammar and Nair, 1992b).

On average, an arabinose-to-xylose ratio of 0.6 is usually found (Hoffmann et al., 1991; Izydorczyk and Biliaderis, 1992; Izydorczyk et al., 1991), but high natural variations with respect to arabinose-to-xylose ratio, substitution pattern of arabinose, content of feruloyl groups, and molar mass is observed in arabinoxylans (Cleemput et al., 1995; Izydorczyk and Biliaderis, 1995; Girhammar and Nair, 1992a; Andrewartha et al., 1979). Andrewartha et al. (1979) suggested that the high viscosity of arabinoxylan solutions is due to a rigid rodlike conformation of the polymers caused by the relatively high ratio of arabinose to xylose. It is assumed that an increase in arabinose content appears to "stiffen" the xylan backbone into a more extended conformation (Dea et al., 1973). A great deal of uncertainty, however, remains as to the relation that might exist between structural characteristics and macromolecular features; research papers in this area are contradictory (Meuser and Suckow, 1986; Hoseney, 1984; Ali and D'Appolonia, 1979). A reason for such conflicting results may lie in differences in the purity and composition of arabinoxylans preparations.

The purpose of this work was first to isolate well-defined arabinoxylan populations with narrow distribution. The structure of the fractions was determined through NMR; macromolecular characteristics were investigated through viscosity and laser light-scattering measurements. These techniques allow parameters such as molar mass (M_w), intrinsic viscosity ([η]), and weight-average radius of gyration (R_G) to be obtained, and then the persistence length (q) and the exponent ν (from $R_G \propto M_w^{\nu}$) can be calculated, which give information on the conformation of the macromolecule. We studied whether the substitution patterns affected the rigidity, the conformation of the polymers, and, consequently, their macromolecular characteristics.

MATERIALS AND METHODS

Isolation of Arabinoxylans. Wheat (variety Soissons, harvest 1993) water-soluble arabinoxylans (crude extract) prepared on a large scale (Faurot et al., 1995) have been purified (Figure 1): 80 g of arabinoxylans was blended with 2 L of distilled water for 2 h at 60 °C. After centrifugation (13000*g*, 20 min), a heat-stable α-amylase (EC 3.2.1.1, Termamyl 120 L Novo 120 Knu/mL) was used to eliminate starch contaminants from the aqueous extract (95 °C, 1 h). The mixture was cooled and incubated with protease (Sigma

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10.1021/jf990222k CCC: $19.00
Published on Web 01/14/2000

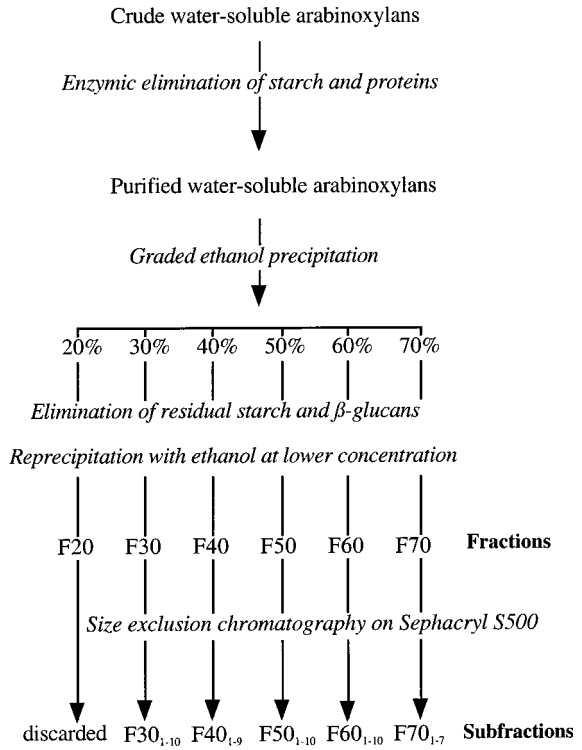

Figure 1. Purification and fractionation of water-soluble arabinoxylans.

Chemicals, A 5380 25 units/g, 200 mg, 1 h, pH 7, 60 °C) to remove residual proteins. The enzyme was inactivated by heat (100 °C, 10 min). The mixture was centrifuged (13000*g*, 20 min), and the supernatant was filtered and then adjusted to 80% (v/v) ethanol and allowed to stand at 4 °C for a night. The precipitate was collected by centrifugation (13000*g*, 20 min), washed twice with ethanol 80%, ground in ethanol 100%, washed with acetone, and then dried in an oven overnight at 40 °C to give purified water-soluble arabinoxylans: 43 g of purified arabinoxylans was obtained from 80 g of crude arabinoxylans.

Purification and Fractionation of Arabinoxylans. The purified arabinoxylans were fractionated (Figure 1) by a graded ethanol precipitation: increasing volumes of ethanol were added to a solution of arabinoxylans (40 g in 2 L of distilled water) to obtain ethanol concentrations ranging from 20 to 70%. Solutions were allowed to stand overnight at 4 °C for each ethanol concentration; after centrifugation, the residues were kept and supernatants were further saturated. Residues were ground in ethanol 100%, washed with acetone, and then dried in an oven overnight at 40 °C.

The fractions were further purified to eliminate residual β-glucans and the remaining starch. Each fraction was solubilized (0.5%) in distilled water and incubated with Lichenase (EC 3.2.1.73, Megazyme, 50 units/mL) for 1 h at 40 °C. The fractions were then incubated (30 min, pH 4.5, 40 °C) with β-glucosidase (EC 3.2.1.21, Megazyme, 40 units/mL) and amyloglucosidase (EC 3.2.1.3, Megazyme, 200 units/mL). The enzymes were inactivated by heat (100 °C, 10 min), and the solutions were centrifuged (13000*g*, 20 min). As the first ethanol precipitations were realized at an arabinoxylan concentration (2%) above the critical concentration (c^* = 0.3−0.4%) (Izydorczyk and Biliaderis, 1995), leading probably to physical entanglement, each of the six supernatants was reprecipitated with ethanol at its initial ethanol percentage and the residues were recovered as described above. In each case supernatants were then discarded. The fractions are designated F20, F30, F40, F50, F60, and F70 (the numbers refer to the saturation level of ethanol at which the material was collected). F20 was not studied.

The fractions obtained by ethanol precipitation, F30−F70, were solubilized (60 mg in 10 mL) in 0.1 M NaCl and eluted on a Sephacryl S500 HR column (100 × 2.6 cm) with 0.1 M NaCl at a flow rate of 100 mL/h at room temperature, and fractions (6.5 mL) were collected and analyzed for neutral sugar content by an automated orcinol method (Tollier and Robin, 1979). To obtain sufficient material, the chromatography was repeated four times for each fraction. Subfractions were obtained by pooling tubes; 46 subfractions were referred to as F30$_{1-10}$, F40$_{1-9}$, F50$_{1-10}$, F60$_{1-10}$, and F70$_{1-7}$.

Chemical Analyses. The amounts of monosaccharides were determined according to the method of Englyst and Cummings (1988): polysaccharides were hydrolyzed with 2 N sulfuric acid at 100 °C for 2 h. Individual sugars were then converted into alditol acetates and analyzed by gas−liquid chromatography. Analyses were made in duplicate, and arabinoxylan content was calculated from the sum of arabinose and xylose [except in purified water-soluble arabinoxylans and F70, in which arabinose arising from arabinogalactans was estimated from galactose content by assuming an arabinose-to-galactose ratio of 0.7 (Loosveld et al., 1998)].

The phenolics contents were determined in fractions F30−F70. Fifteen milligrams was dissolved in 2 mL of 2 N NaOH and incubated for 30 min at 35 °C in the dark. After *o*-coumaric acid (internal standard, 20 μg) had been added, the pH was adjusted to 2 with 2 N HCl. Phenolic acids were extracted twice with 2 mL of ether. The ether phase was transferred to a test tube and evaporated at 40 °C; 0.3 mL of methanol/H_2O (50:50 v/v) was added, and samples were injected (20 μL) on an HPLC system equipped with a C_{18} column (250 × 4 mm). Detection was done by UV absorbance at 320 nm. Gradient elution was performed using methanol/1% acetic acid and H_2O/1% acetic acid solvents at 0.7 mL/min at 25 °C, in linear gradients from 20:80 to 60:40 in 20 min and from 60:40 to 80:20 in 1 min, maintained at 80:20 for 9 min, from 80:20 to 20:80 in 1 min, and finally maintained at 20:80 for 5 min (Saulnier et al., 1999). Results given are means of two measurements; the coefficients of variation were <2% for ferulic acid contents and <6% for ferulic acid dimers contents.

Physicochemical Determinations. The purified polysaccharides were dissolved (5 mg/mL) for 2 h at 40 °C under magnetic stirring, filtered over a 0.45 μm membrane, and injected at 25 °C on a high-performance size exclusion chromatography (HPSEC) system constituted of two Shodex OH-pak SB HQ 804 and 805 columns eluted at 0.7 mL/min with 50 mM $NaNO_3$, containing 0.02% NaN_3. On-line molar mass and intrinsic viscosity determinations were performed at room temperature using a multiangle laser light scattering (MALLS) detector (mini-Dawn, Wyatt, Santa Barbara, CA, operating at three angles: 41°, 90°, and 138°), a differential refractometer (ERC 7517 A) (dn/dc = 0.146 mL/g), a UV detector (λ = 280 nm), and a differential viscometer (T-50A, Viscotek). M_w, R_G, q, and ν were calculated using Astra 1.4 software, and [η] was calculated using Trisec software.

The effect of alkaline treatment on arabinoxylans was studied by solubilizing them (0.5% v/v) in 2 N NaOH during 1 h at room temperature. Fractions were then filtered on 0.45 μm membranes and injected on HPSEC as described above with 0.1 N NaOH as eluant. A prime was added to the names of fractions studied in NaOH (e.g., F30′).

The polydispersity index, I, is defined by the ratio M_w/M_n, where M_w represents the weight-average molar mass and M_n the number-average molar mass. The radius of gyration R_G determined by elastic laser light scattering represents the square root of the weight-average mean square radius.

Persistence lengths (q) representing the chain extension were determined by using the following equations: $q = (C_\infty + 1)I_0/2$ [where C_∞, the characteristic ratio, is obtained from $C_\infty = 6R_G^2M_0/I_0^2M_W$ (Roger and Colonna, 1992), I_0 = 0.54 nm (length of a xylose residue), M_0 = 132 g/mol (molar mass of a xylose residue), R_G = radius of gyration, and M_w = weight-average molar mass]. The persistence lengths were calculated using M_w of the xylan backbone (i.e., molar mass was corrected from arabinose contribution).

NMR Spectroscopy. ^{1}H NMR spectra (400 MHz) were recorded at 60 °C on a Brücker ARX spectrometer. Arabinoxy-

Table 1. Composition of Crude Extract, of Purified AX, and of Fractions Obtained by Ethanol Precipitation before Purification with Lichenase, β-Glucosidase, and Amyloglucosidase

arabino-xylan	sugar content[a] (wt %)						proteins[a] (wt %)	ferulic acid[b] (wt %)
	Glc	Xyl	Ara	Man	Gal	Ara/Xyl		
crude extract	10.4	14.3	15.0	1.3	10.3	0.63[c]	12.1	nd[d]
purified AX	9.3	23.8	23.9	1.6	15.1	0.63[c]	10.5	0.08
20%	27.3	27.6	11.8	3.9	2.0	0.42	nd	nd
30%	16.8	48.0	19.5	2.4	0.7	0.40	nd	nd
40%	9.3	48.6	23.6	1.6	1.3	0.48	nd	nd
50%	4.9	51.1	29.5	1.5	0.8	0.57	nd	nd
60%	5.1	43.6	33.6	1.7	1.2	0.77	nd	nd
70%	4.4	14.5	23.0	2.5	15.8	0.97[c]	nd	nd

[a] Results obtained from duplicates, coefficients of variation <4%. [b] Results obtained from duplicates, coefficients of variation <2%. [c] Corrected from the presence of arabinogalactans. [d] nd, not determined.

lans were dissolved in D_2O (10 mg/mL). Approximately 128 pulses were collected, pulse repetition time was 4 s, and pulse angle was 6 μs.

RESULTS AND DISCUSSION

Compositions of the crude extract and of the purified water-soluble arabinoxylans are given in Table 1. The main neutral sugars of the crude extract were arabinose and xylose, but non-negligible amounts of glucose arising from starch and β-glucans and galactose arising from arabinogalactans were also detected. The purified water-soluble arabinoxylans contained slightly less glucose and proteins than the crude extract. The remains of glucose probably arose from β-glucans and proteins that were certainly bound to arabinogalactans (Fincher et al., 1974). The high degree of substitution (0.63) of the xylan backbone with arabinose residues is characteristic of wheat endosperm arabinoxylans and is in the range of the values usually found (0.53−0.70) (Rattan et al., 1994; Hoffmann et al., 1991; Izydorczyk et al., 1991). The content of ferulic acid is in accordance with that found for water-soluble wheat endosperm arabinoxylans (1.14−0.89 mg/g arabinoxylans) (Izydorczyk et al., 1991). The elution profile (Figure 2) shows the presence, in the purified arabinoxylans, of arabinogalactans eluted after 17 mL. Macromolecular characteristics are presented in Table 2: The value of M_w of 300000 g/mol is higher than that determined by other authors using sedimentation techniques (Andrewartha et al., 1979; Girhammar et al., 1992a) or gel filtration (Cleemput et al., 1995; Rattan et al., 1994) and lower than that determined by gel filtration by Fincher and Stone (1986). These differences probably arise from the fact that M_w values are determined on very polydisperse arabinoxylans that need to be fractionated. The intrinsic viscosity was similar to that found by Rattan et al. (1994).

Ethanol Precipitation. *Chemical Composition and Macromolecular Characteristics.* Fractionation with ethanol of the purified arabinoxylans solubilized (2%) in H_2O above the critical concentration resulted in six fractions (20−70%). The composition of each fraction is presented in Table 1. These fractions were still contaminated with glucose arising from β-glucans and remains of starch and needed to be further purified. The substitution degrees of the 20 and 30% fractions were similar and then increased with ethanol concentration.

Fractions obtained with 20−70% ethanol were therefore treated with Lichenase, β-glucosidase, and amyloglucosidase and were reprecipitated with ethanol to give fractions F20−F70 (a concentration of 0.5% was used to avoid precipitation of material due to entanglement). Yields and compositions of each fraction are presented in Table 3. On the basis of the total amount of material recovered after fractionation, fraction F20 was a minor one. Fractions F30, F40, F50, and F60 were mainly composed of arabinose and xylose and contaminated with small amounts of mannose, glucose, and galactose, whereas F20 was more contaminated with glucose and mannose arising from galactomannanes and F70 with galactose arising from arabinogalactans. Protein contents were low for fractions F30−F60, whereas F70 contained more proteins, suggesting that proteins are bound to arabinogalactans (Fincher et al., 1974).

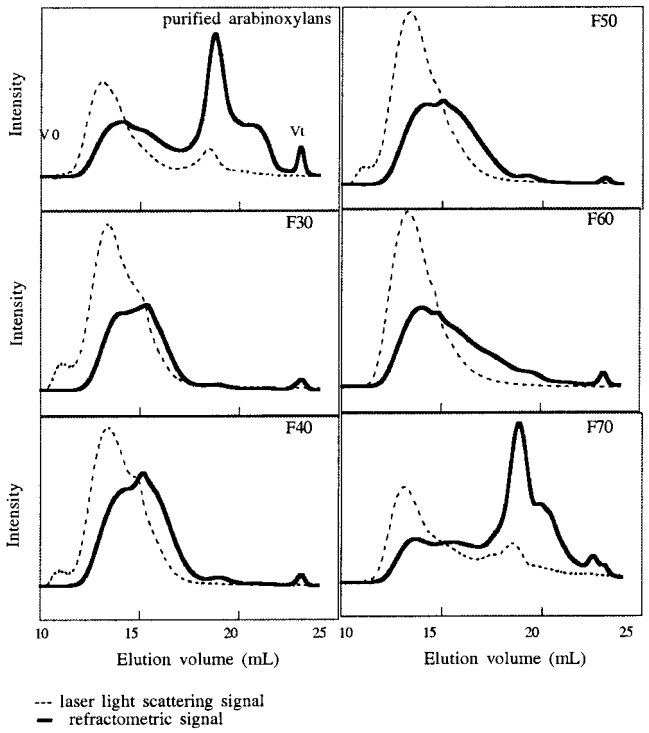

Figure 2. Elution profiles of purified arabinoxylans and of fractions F30, F40, F50, F60, and F70 obtained by chromatography on Shodex OH-pak 804 and 805 columns.

Table 2. Macromolecular Characteristics of Purified Arabinoxylan, Its Fractions, and Subfractions $F30_3$−$F70_3$[a]

arabino-xylan	A/X	$M_w \times 10^{-3}$[b] (g/mol)	R_G[b] (nm)	I	$[\eta]$[b] (mL/g)	q[b,g] (nm)	ν
purified AX	0.63[c]	300[e]	44	1.65	530[f]	7.8	0.58
F30	0.37[c]	408[e]	46	1.45	520	5.4	0.41
F40	0.46[c]	307[e]	44	1.50	497	7.3	0.50
F50	0.57[c]	392[e]	46	1.66	488	6.5	0.37
F60	0.75[c]	375[e]	46	1.67	483	7.5	0.45
F70	0.93[c]	370[e]	45	1.39	320[f]	8.0	0.50
$F30_3$	0.41[d]	490	54	1.14	610	6.4	0.49
$F40_3$	0.55[d]	500	56	1.20	650	7.4	0.46
$F50_3$	0.73[d]	590	59	1.11	690	7.7	0.50
$F60_3$	0.95[d]	590	58	1.12	550	8.4	0.54
$F70_3$	1.06[d]	530	50	1.20	450	7.4	0.50

[a] A/X, arabinose-to-xylose ratio; M_w, weight-average molar mass; R_G, radius of gyration; I, polydispersity index; $[\eta]$, intrinsic viscosity; q, persistence length; ν, exponent ν. [b] Coefficients of variation <5%. [c] Determined by GLC. [d] Determined by NMR. [e] Molar masses for the fractions eluted within 12 and 16.5 mL. [f] Intrinsic viscosity determined without the arabinogalactans. [g] Persistence length calculated on the basis of unbranched arabinoxylans.

Table 3. Yield and Composition of the Fractions after Ethanol Precipitation

arabino-xylan	yield[a]	sugar content[b] (wt %) Glc	Xyl	Ara	Man	Gal	Ara/Xyl[c]	ferulic acid[d] (wt %)	proteins[b] (wt %)
F20	2.4	3.6	26.5	8.5	9.3	0.4	0.31	nd	nd
F30	23.8	0.6	60.2	22.3	2.7	0.2	0.37	0.17	2.7
F40	10.2	0.3	53.6	24.6	2.2	0.2	0.46	0.15	3.5
F50	27.3	0.2	56.6	31.8	1.7	0.2	0.57	0.09	3.1
F60	25.2	0.2	50.0	37.7	2.4	0.6	0.75	0.07	2.7
F70	11.1	0.5	21.9	28.6	3.6	14.1	0.93	0.08	8.8

[a] Based on total amount of material recovered. [b] Results obtained from duplicates, coefficients of variation <4%. [c] Determined by GLC and corrected from the presence of arabinogalactans in F70. [d] Results obtained from duplicates, coefficients of variation <2%.

The arabinose-to-xylose ratio varies in the same way for F20−F70 as for 20−70%. However, substitution degrees of 20 and 30% fractions are higher than those of F20 and F30, respectively, which suggests that above the critical concentration, precipitation of material due to entanglement occurred, especially when the concentration in arabinoxylans is high. This explains why the differences in the substitution degrees among fractions obtained above and under the critical concentration are less marked at high ethanol percentages.

The increasing arabinose-to-xylose ratio with increasing ethanol concentration from F20 to F70 reflects the chemical heterogeneity of the unfractionated arabinoxylans. These results are in agreement with those found by Cleemput et al. (1995), Gruppen et al. (1992), and Hoffmann et al. (1991), who also used ethanol to fractionate arabinoxylans. The content of ferulic acid varied also significantly among the fractions, as there was a shift from F30 to F70 of the ferulic acid amount, indicating that it decreased when the substitution degree of the fractions increased. Izydorczyk and Biliaderis (1992) have also seen the same trend for fractions obtained by stepwise precipitation with ammonium sulfate.

Although the fractions have been obtained with different ethanol concentrations, their macromolecular characteristics were similar (Table 2). Their weight-average molar masses, in the range of 300000−400000 g/mol, are not apparently related to the ethanol concentrations, whereas the intrinsic viscosities decreased from 520 to 320 mL/g when ethanol concentration increased. Elution profiles (Figure 2) show the polydispersity of the fractions. All of the fractions showed a relatively high index of polydispersity ($I \approx 1.4-1.6$) as compared to the value of 1.65 observed for the purified arabinoxylans. Different molar masses (25000 to 1×10^6 g/mol) have been obtained by other authors (Cleemput et al., 1995; Hoffmann et al., 1992) by gel permeation chromatography with pullulans as standards.

The exponent ν of the R_G−molar mass relationship ($R_G \propto M^{\nu}$) gives information about the polymer conformation, typical values of ν being 0.33 for a sphere, 0.5−0.6 for a random coil, and 1 for a monodisperse rigid rodlike conformation. Values of the exponent ν close to 0.5 indicated that the molecule has a random coil conformation (Adolphi and Kulicke, 1997). This fact conflicts with the usually admitted rodlike conformation for arabinoxylans (Andrewartha et al., 1979). Besides, the persistence lengths calculated (Table 2) indicated that the polymers are semiflexible, in comparison with rigid polysaccharides ($q = 310$ nm for xanthan) and with very flexible polysaccharides ($q = 1.2-1.7$ nm for pullulans, $q = 1.7$ nm for amylose) (Roger and Colonna, 1992). The persistence lengths of arabinoxylans in all of the fractions are very similar and do not increase with the ethanol concentration. It can be concluded that the rigidity does not increase with substitution degree in the range (0.37−0.93) of our arabinoxylans.

Structural Determination of Fractions Obtained by Ethanol Precipitation. The anomeric regions of ^{1}H NMR spectra confirmed the large variation of the structure of the arabinoxylans in the fractions F30−F70. Published data (Cleemput et al., 1993; Bengston et al., 1992; Gruppen et al., 1992; Hoffmann et al., 1991) allowed assignments of the peaks. The resonance at 5.38 ppm can be unequivocally assigned to H-1 of Ara*f* linked to O-3 of xylopyranose residue. Anomeric protons of Ara*f* linked to O-3 and O-2 of the same Xyl*p* were responsible for peaks at 5.22 and 5.28 ppm. Ara*f* linked to O-2 of a Xyl*p* residue was not observed: this may be explained by overlapping of the ^{1}H NMR signal of the anomeric proton of this arabinose with the signal originating from the anomeric protons of the arabinoses linked to O-3 and O-2 of the same xylose (Viëtor et al., 1994). However, this pattern of substitution is very slightly present in wheat (Gruppen et al., 1992).

The ratio of integrals of resonances of anomeric protons of Ara*f* to those of anomeric protons of Xyl*p* for fractions F30−F70 allowed calculation of Ara/Xyl ratios that were higly correlated ($R^2 = 0.99$) with those determined by GLC. They increased with ethanol concentration. Relative percentages of disubstituted, monosubstituted, and unsubstituted xyloses were calculated. It appeared that monosubstituted and unsubstituted xylose levels decreased, whereas disubstitution levels increased, as ethanol percentage increased. The ratio of unbranched to branched xyloses decreased from 2.54 to 1.40 as the Ara/Xyl ratio increased, whereas the ratio of double-branched to single-branched xyloses increased from 0.44 to 3.13. These values were higher than those determined by Gruppen et al. (1992) on fractions obtained by ethanol precipitation (respectively, 1.7 to 1.1 and 0.7 to 2.5). The increasing splitting of peaks at 5.23 and 5.29 ppm from F30 to F70 revealed (Vinkx et al., 1993; Hoffmann et al., 1992) that double substitutions were more and more adjacent on the polymer when substitution degree increased.

Gel Filtration Chromatography on Sephacryl S500. *Chemical and Macromolecular Characteristics.* To get populations of low polydispersity index, fractions F30−F70 were chromatographed on a Sephacryl S500 column (Figure 3). In F70, the peak in the high-K_{av} region is due to the arabinogalactans. Forty-six subfractions were pooled as indicated (Table 4), and their yields, based on total amount recovered after fractionation on Sephacryl S500, are given in Table 4. Yields of chromatographies were 80, 81, 88, 92, and 67% for F30, F40, F50, F60, and F70, respectively.

Arabinose-to-xylose ratios of the fractions eluted in the fractionation range of the column are presented in Figure 4. A great chemical heterogeneity within each fraction was observed because the arabinose-to-xylose ratio decreased when elution volume increased. For F70, Ara/Xyl ratios over $K_{av} = 0.6$ were not reported due to the presence of arabinogalactans in the fractions. Within each fraction, high molar mass subfractions had higher substitution degree. Hoffmann et al. (1991) obtained similar trends.

The separation on Sephacryl S500 showed that the fractions isolated by fractionated precipitation with

Table 4. Yields of Each Subfraction Based on Total Amount Recovered after Fractionation

	subfraction									
	1	2	3	4	5	6	7	8	9	10
K_{av}	0.11−0.23	0.25−0.30	0.31−0.36	0.38−0.43	0.45−0.51	0.53−0.55	0.57−0.63	0.65−0.72	0.73−0.80	0.82−0.95
F30	1.5	6.3	9.4	10.0	10.8	21.3	16.9	10.1	8.5	4.7
F40	1.0	4.4	11.1	13.0	12.4	12.9	15.7	18.9	10.2	
F50	1.6	4.9	7.5	8.9	9.9	11.9	16.6	15.9	15.7	6.6
F60	3.0	6.3	9.8	11.2	15.0	6.4	16.5	12.8	10.5	8.0
F70	1.4	3.4	6.2	11.9	14.5	20.5	41.9			

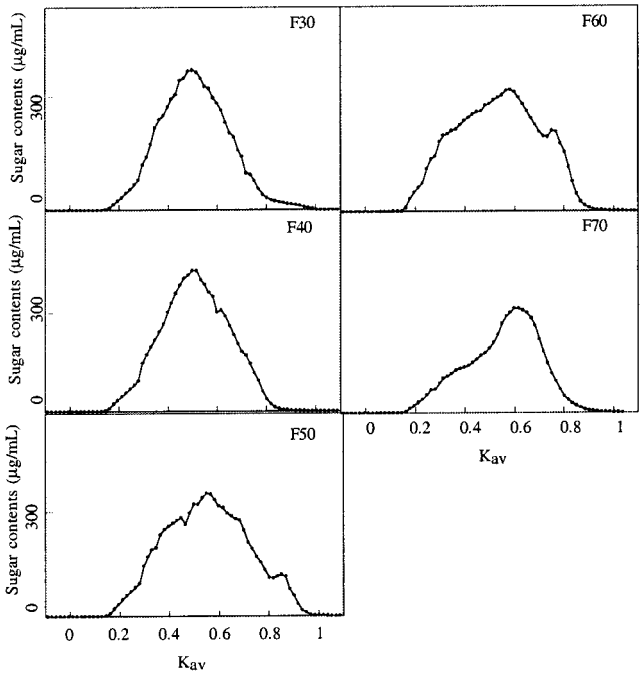

Figure 3. Elution profiles of fractions F30, F40, F50, F60, and F70 obtained by chromatography on a gel permeation Sephacryl S500 column.

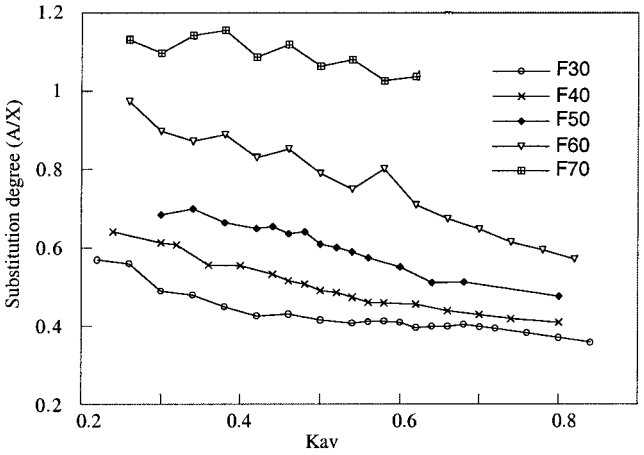

Figure 4. Arabinose-to-xylose ratios determined by NMR of the arabinoxylans isolated by ethanol precipitation, as a function of K_{av} on Sephacryl S500.

ethanol contained a mixture of arabinoxylans differing in their molar masses as well as in their structures. Indeed, for the same ethanol percentage, there is precipitation of high molar mass, highly substituted arabinoxylans, and of low molar mass, lesser substituted arabinoxylans. Differences in precipitation behavior of arabinoxylans with the same substitution degree might be attributed to different molar masses or differences in the pattern of substitution of the arabinose residues on the xylan backbone.

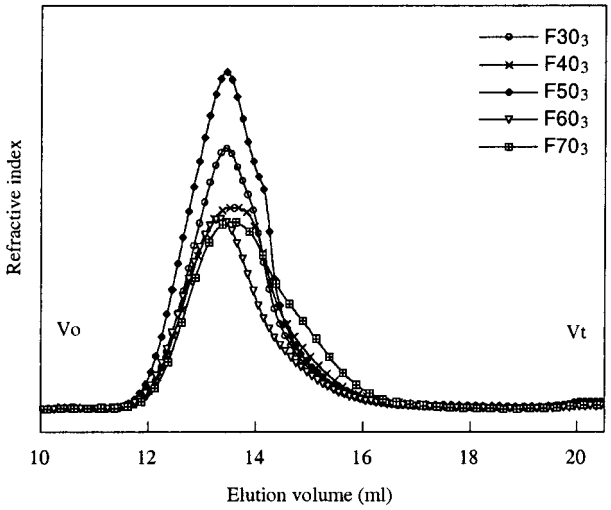

Figure 5. Elution profiles on Shodex OH-pak columns of subfractions $F30_3$, $F40_3$, $F50_3$, $F60_3$, and $F70_3$ obtained by chromatography on Sephacryl S500.

As this heterogeneity might have induced erroneous interpretation of the results, we have studied in detail narrow subfractions ($F30_3$, $F40_3$, $F50_3$, $F60_3$, and $F70_3$) of the same hydrodynamic volume (K_{av} = 0.31−0.36 on Sephacryl S500) (Figure 5). These fractions have similar range of M_w (490000−590000 g/mol) (Table 2) but exhibit increasing Ara/Xyl ratios (Figure 4). The polydispersity index (1.1) of the populations is low, indicating a chemical and structural homogeneity within each subfraction (Table 2). Because they were collected in the low elution volumes, they have high molar masses, radii of gyration, and intrinsic viscosities. Their exponent ν was close to 0.5, indicating a random coil conformation. Again, the same conformation and the same values of persistence lengths were determined whatever the substitution degrees, which suggested that structural characteristics (Ara/Xyl ratios) had no influence on the macromolecular characteristics of the arabinoxylans. The same results were obtained in the other subfractions.

Structural Determination of Subfractions Obtained by SEC. The anomeric regions of ^{1}H NMR spectra of fractions $F30_3$−$F70_3$ are shown in Figure 6. As previously seen, disubstitutions occurred more and more adjacent when substitution degree increased. Each of the 46 subfractions has been studied with NMR, and their relative percentages of disubstituted, monosubstituted, and unsubstituted xyloses are presented on Figure 7. Monosubstitution levels remained rather low (<20%) and constant within the subfractions of the same fraction. Within each fraction, subfractions with higher molar masses were more disubstituted and less unsubstituted than those with lower molar masses. Levels of di- and unsubstituted xyloses seemed to evolve in the opposite direction, and the proportion of xyloses monosubstituted did not apparently vary with the substitu-

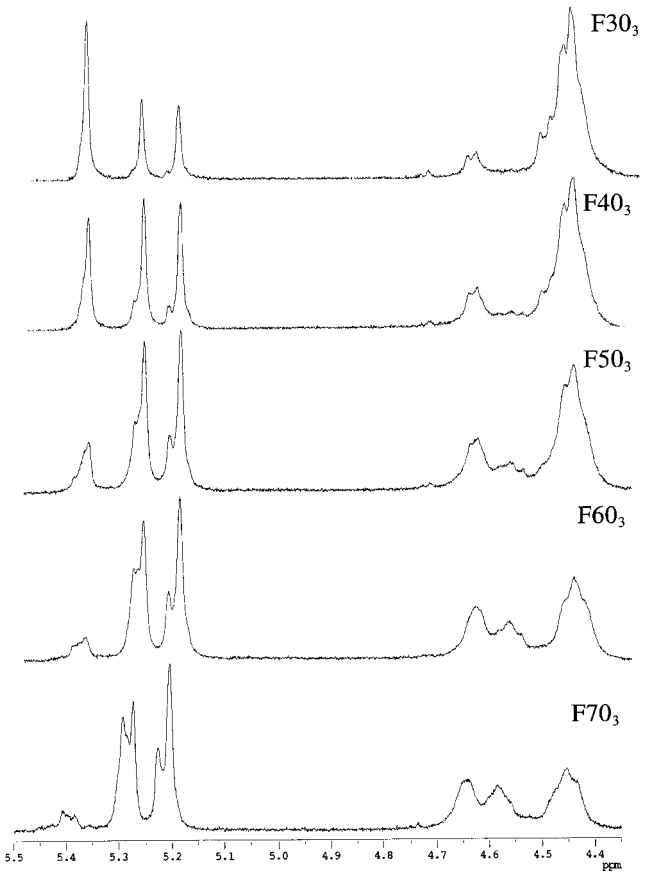

Figure 6. ^{1}H NMR spectra of arabinoxylans from fractions F30$_3$, F40$_3$, F60$_3$, and F70$_3$.

tion degree. To confirm this assumption, we looked at the relations between substitution degree and the different substitution patterns (Figure 8). It clearly appeared that the substitution degree was correlated with the disubstitution level; in other words, the arabinose-to-xylose ratio represented this substitution pattern.

Hoffmann et al. (1991) obtained nine subfractions by ethanol precipitation followed with SEC but observed that substitution degree was correlated with both di- and monosubstitutions. Gruppen et al. (1992) expressed the relative proportions of the partially methylated xylose residues present in five ethanol precipitates versus the Ara/Xyl ratio and obtained the same trends. However, in both cases the fractions were not homogeneous.

Therefore, water-soluble wheat arabinoxylan is composed of a family of polysaccharides varying from a few disubstituted ones to disubstituted ones, the level of monosubstitution remaining constant. Izydorczyk and Biliaderis (1995) isolated two fractions at two different saturation levels of ammonium sulfate and demonstrated that for each fraction different regions existed, varying according to their substitution patterns. However, they used heterogeneous fractions, and the regions they determined could correspond to different polymers.

Effect of Alkaline Treatment on Water-Soluble Arabinoxylans. As the flexibility, the lack of relation between substitution degree, and the rigidity of arabinoxylans were in disagreement with the generally admitted behavior for arabinoxylans, we have explored other chromatographic conditions.

When solubilized in 50 mM $NaNO_3$, arabinoxylans had little propensity to aggregate as seen after HPSEC

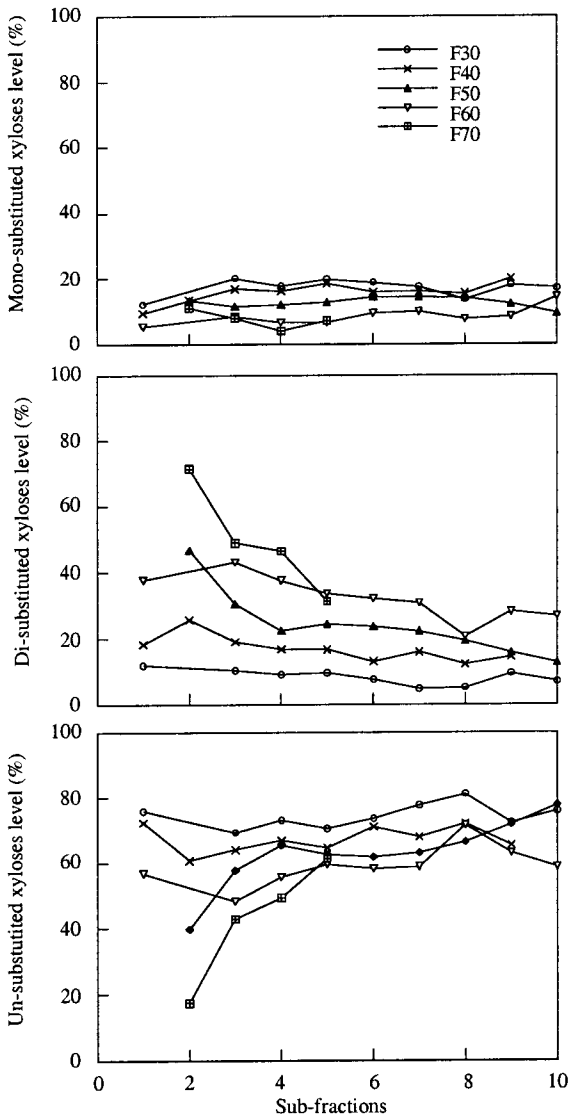

Figure 7. Relative percentage of the different substitution patterns for each subfraction.

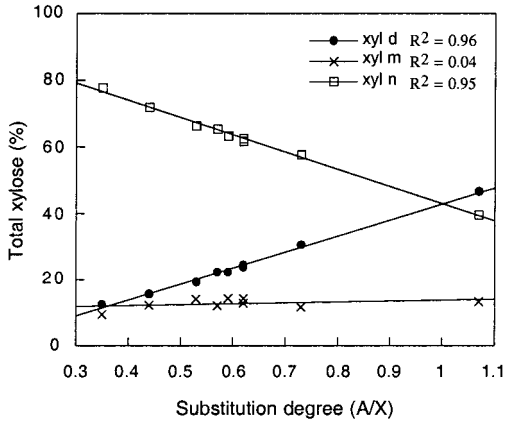

Figure 8. Correlation between proportions of unsubstituted xylose (xyl n), disubstituted xylose (xyl d), monosubstituted xylose (xyl m), and substitution degree (example of subfractions F50$_1$−F50$_{10}$).

by the occurrence of a laser light scattering signal at the void elution volume and no refractometric signal (Figure 2: fractions F30, F40, and F50). NaOH was thought to be a better solvent that eliminates aggregate formation. Arabinoxylans were then dissolved in 2 N

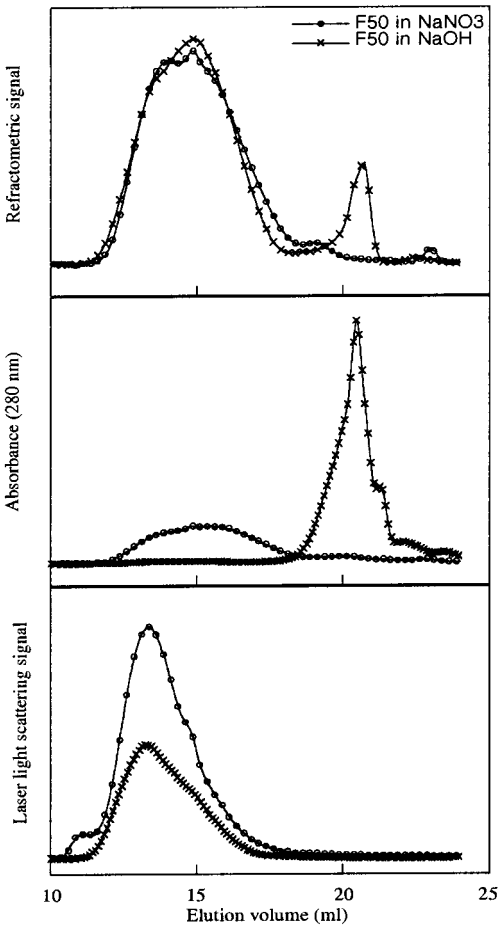

Figure 9. Comparison of refractometric, absorbance at 280 nm, and laser light scattering signals of F50 in $NaNO_3$ or in NaOH on Shodex OH-pak 804 and 805 columns.

NaOH to improve their solubility. Furthermore, NaOH would deesterify the ferulic acid esterified on the arabinose, leading perhaps to modification of the elution profiles.

As seen in Figure 9, the refractometric profiles were similar for fractions dissolved and eluted either in $NaNO_3$ or in NaOH. However, laser light scattering profiles (Figure 9) showed that the presence of aggregated populations had disappeared at the void elution volume. A great change in M_w occurred and the fractions dissolved in NaOH had lower M_w, as if the presence of aggregates in fractions dissolved in $NaNO_3$ had overestimated these M_w (Table 5). The UV profiles (Figure 9) showed besides that material initially bound to arabinoxylans had been deesterified with NaOH. The UV absorbance was clearly due to phenolic acids, and analysis of the phenolic contents showed that ferulic acid dimers, in addition to ferulic acid monomers, were present in the fractions (Table 6). Three types of dimers were essentially found: the 8-O-4′, 8−8′, and 5−5′ forms. Their contents remained constant within the fractions. Figueroa-Espinoza and Rouau (1998) also showed the occurrence of ferulic acid dimers in unfractionated water-extractable arabinoxylans from wheat flour (thesis).

The presence of ferulic acid dimers suggested that some arabinoxylans might be cross-linked. Therefore, the study of fractions non-deesterified gave results (M_w, $[\eta]$, ν, ...) for polymers partially coupled, which could explain their unexpected macromolecular characteristics.

Table 5. Macromolecular Characteristics of Deesterified Arabinoxylan Fractions and Subfractions F30$_3$′−F70$_3$′[a]

arabino-xylan	A/X	$M_w \times 10^{-3}$ [b] (g/mol)	R_G [b] (nm)	I	$[\eta]$ [b] (mL/g)	q [g] (nm)	ν
F30′	0.37[c]	167[e]	35	1.20	617	7.6	0.54
F40′	0.46[c]	191[e]	32	1.45	567	6.0	0.63
F50′	0.57[c]	185[e]	34	1.51	555	7.4	0.65
F60′	0.75[c]	230[e]	37	1.70	576	7.9	0.58
F70′	0.93[c]	290[e]	35	1.52	848[f]	6.3	0.76
F30$_3$′	0.41[d]	190	36	1.20	nd[h]	7.3	0.55
F40$_3$′	0.55[d]	260	44	1.45	nd	8.7	0.58
F50$_3$′	0.73[d]	330	50	1.51	nd	9.8	0.52
F60$_3$′	0.95[d]	220	35	1.70	nd	8.2	0.64
F70$_3$′	1.06[d]	270	40	1.52	nd	9.2	0.58

[a] M_w, weight-average molar mass; R_G, radius of gyration; I, polydispersity index; q, persistence length; ν, exponent ν. [b] Coefficients of variation <5%. [c] Determined by GLC. [d] Determined by NMR. [e] Molar masses for the fractions eluted within 12 and 16.5 mL. [f] Intrinsic viscosity determined without the arabinogalactans. [g] Persistence length calculated on the basis of unbranched arabinoxylans. [h] nd, not determined.

Table 6. Ferulic Acid Dimer Content in Purified Arabinoxylans and in Fractions F30−F70

	ferulic acid dimers[a] (μg/g)			
arabinoxylan	5−5′	8-O-4′	8−5′	8−8′
unfractionated	10	7	0	4
F30	8	6	6	2
F40	10	8	0	2
F50	10	8	0	3
F60	10	9	8	5
F70	10	9	0	4

[a] Results obtained from duplicates. Coefficients of variation <6%.

Table 6 gives the macromolecular characteristics of fractions F30−F70 and F30$_3$−F70$_3$ after deesterification: deesterified arabinoxylans had lower R_G values and lower molar masses but had a more extended conformation as seen by their exponent ν ($\nu = 0.6$). As for the persistence lengths, the evolution toward a more "rigid" polymer was more evident for homogeneous subfractions.

Therefore, arabinoxylans have to be studied both in their native form, that is to say with the occurrence of coupled polymers, and in their deesterified form. Particularly, care must be taken during enzymic purification of the arabinoxylans, as indeed we have observed that some arabinoxylan fractions presented more ferulic acid dimers after they had been purified with Lichenase, amyloglucosidase, and β-glucosidase (data not shown). These enzymes may be contaminated with peroxidases or polyphenol oxidases, promoting the oxidative coupling of ferulic acid. These dimers may exist initially in the arabinoxylans: endogeneous enzymes of wheat, such as peroxidases or polyphenol oxidases, would be likely to induce these couplings. Their activity has been described in wheat (Baik et al., 1994), and we have detected the presence of dimers in native water-soluble arabinoxylans from different wheat varieties (thesis, Rialto) (data not shown).

The use of NaOH as the solvent would allow us to get rid of these dimers that couple the native arabinoxylans.

CONCLUSION

Although the fractionation of arabinoxylans was generally restricted to graded precipitation with ethanol

or ammonium sulfate, here the combination of ethanol precipitation with SEC allowed the isolation of fractions homogeneous in molar mass and substitution pattern. This approach permitted us to show that the substitution degree of arabinoxylans was governed by the level of disubstituted xylose residues and that substitution degree had no influence on the conformation or on the rigidity of water-soluble arabinoxylans; however, the range of variation in substitution degree might be too limited to observe any effect on conformation. From this study, it also appeared that arabinoxylans behaved as random coils. This point particularly conficts with the results of Andrewartha et al. (1979), who postulated an extended rodlike conformation for arabinoxylans. However, these authors have used a sedimentation equilibrium ultracentrifugation technique on very polydisperse samples and obviously have underestimated the molar weight of their arabinoxylan sample ($M_w = 65000$ for an intrinsic viscosity $[\eta]$ of 620 mL/g) so that an inaccurate extended conformation has been deduced. Although arabinoxylans behave as random coils, their rigidity, as shown by their persistence length $q \sim 7-8$ nm, is higher than that of very flexible random coil polysaccharides such as pullulan ($q \sim 2$ nm), which explains their higher intrinsic viscosity as compared with that of pullulans ($[\eta] = 116$ mL/g for an $M_w = 380000$).

Ferulic acid dimers were detected in the arabinoxylans, leading to the conclusion that arabinoxylans might be partially coupled in their native form, and deesterification involved modifications of the macromolecular characteristics.

Although the substitution degree of arabinoxylans was generally believed to affect their physicochemical behavior, our results suggest that the amount of ferulic dimers might be a major parameter to explain variations in the macromolecular characteristics of arabinoxylans. Furthermore, the mode of fractionation seems to be governed not only by the molar mass and the substitution degree of the arabinoxylans but also by their content in ferulic acid dimers.

As the presence of dimers has probably modified the fractionation behavior of the arabinoxylan molecules, further fractionation using ethanol precipitation and gel filtration is in progress starting from deesterified arabinoxylans. The aim of this fractionation is to obtain homogeneous fractions and to establish the Mark−Houwink relations ($[\eta] = KM_w^a$).

ABBREVIATIONS USED

a, Mark−Houwink exponent; AX, arabinoxylans; Ara, arabinose; Ara/Xyl, arabinose-to-xylose ratio; Xyl, xylose; Glc, glucose; Gal, galactose; Man, mannose; Ara*f*, arabinofuranose; Xyl*p*, xylopyranose; NMR, nuclear magnetic resonance; SEC, size exclusion chromatography; HPSEC, high-performance size exclusion chromatography; GLC, gas chromatography; M_w, weight-average molar mass; M_n, number-average molar mass; $[\eta]$, intrinsic viscosity; R_G, radius of gyration; *q*, persistence length; ν, exponent ν; MALLS, multiangle laser light scattering.

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Received for review March 1, 1999. Revised manuscript received July 27, 1999. Accepted November 19, 1999.

JF990222K