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Carbohydrate Polymers 38 (1999) 133–143

Carbohydrate Polymers

Structural features and anti-complementary activity of some heteroxylan polysaccharide fractions from the seeds of *Plantago major* L.

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Received 6 April 1998; received in revised form 13 August 1998; accepted 31 August 1998

Abstract

Polysaccharides were isolated from the seeds of *Plantago major* L. by water extraction and fractionated by ion exchange and size exclusion chromatography. Methods such as methanolysis and GC, methylation analysis and GC-MS, weak acid hydrolysis, enzyme hydrolysis, ^{13}C, APT, ^{1}H and 2D heteronuclear ($^{1}H-^{13}C$) chemical shift correlated NMR spectroscopy have revealed that the polysaccharides isolated are heteroxylans which consist of a 1,4-linked β-D-Xyl*p* backbone with short side chains attached to position 2 in some 1,4-linked β-D-Xyl*p* residues and to position 3 in other 1,4-linked β-D-Xyl*p* residues. The side chains consist of β-D-Xyl*p*, α-L-Ara*f*, α-L-Ara*f* 1 → 3 β-D-Xyl*p* and α-D-Glc*p*A1 → 3 α-L-Ara*f*. 1,4-linked α-D-GalpA residues were also detected in addition to small amounts of 1,2,4-linked Rha*p* and 1,3-, 1,6- and 1,3,6-linked Gal*p*. The crude extract and some of the fractions obtained had potent anti-complementary activity.

Keywords: *Plantago major* L; Polysaccharides; Anti-complementary activity, Heteroxylan

1. Introduction

The seeds of some *Plantago* spp. are used in medicine, mainly as mild laxatives because of the high mucilage content in the seed epidermis. Examples of plants used for seed production are *Plantago psyllium* L. and *Plantago ovata* Forsk., which are cultivated in the south of Europe and in India, respectively.

Sandhu et al. (1981) have characterised a polysaccharide faction from *Plantago ovata* Forsk., which consists of a 1,3- and 1,4-linked β-D-Xyl*p* backbone *O*-2 and/or *O*-3 substituted by α-D-Gal*p*A 1 → 2α-L-Rha*p*, α-L-Ara*f* and β-D-Xyl*p*.

In Japan the seeds of *Plantago asiatica* L. are used as an antiphlogisticum, against diarrhoea and as an antitussivum in the traditional medicine, and the seed polysaccharides have been characterised. One fraction, ''Plantago mucilage A'' consists of a 1,4-linked β-D-Xyl*p* backbone *O*-3 substituted by single β-D-Xyl*p* residues, α-D-Glc*p*A1 → 3 α-L-Ara*f* and α-D-Gal*p*A1 → 3 α-L-Ara*f*. ''Plantago mucilage A'' has potent anti-complementary activity (Yamada et al., 1986).

In Norway *Plantago major* L., is the most common *Plantago* species. The leaves of this plant are well known in traditional medicine as a wound healing remedy, and two biologically active polysaccharide fractions have been isolated and characterised (Samuelsen et al., 1995;Samuelsen et al., 1996;Samuelsen et al., 1998).

We now report on some structural features and the anti-complementary activity of some polysaccharide fractions isolated from *P. major* seeds.

2. Methods

2.1. Preparation of the crude extract

The seeds of *Plantago major* L. were collected in Oslo, Norway, in August 1993. Seeds (100 g) were extracted with distilled water (3000 ml) at 50°C for 2 h under reflux after removal of the seed capsules. The extract formed was a gel of high viscosity, and it was prefiltered through a cotton cloth bag in a herbal press. The extraction was repeated two times. The filtrates from the three extractions were pooled, filtered through gauze, concentrated under a vacuum at 40°C and dialysed (*Mw* cut-off 3500) against distilled water.

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0144-8617/99/$ - see front matter
PII: S0144-8617(98)00115-5

The crude polysaccharide extract was highly viscous, making filtration almost impossible. Therefore, to reduce the viscosity the crude extract (1000 ml) was heated on a boiling water bath for 2 h while depolymerisation was followed by measuring the increase of reducing sugars (method described below). The extract was then filtered through a Gelman Acro 50 A 5 μm filter.

2.2. Determination of reducing sugars

The cleavage of the glycosidic linkages was followed by measuring the increase in reducing sugars in the reaction mixture using dinitrosalicylic acid (DNS) (Miller, 1959, modified by Knutsen, 1991). The DNS reagent was made by adding NaOH (8 g), potassium sodium tartrate (150 g) and dinitrosalicylic acid (5 g) to 400 ml of distilled water at 60°C. Finally the solution was diluted with water to 500ml.

The test was performed by adding 500 μl sample solution to 500 μl DNS reagent. Acidic sample solutions must be neutralised before addition to the DNS reagent, otherwise precipitation will occur. The sample and reagent were mixed and boiled in a water bath for 5 min. Finally 2.5 ml water was added and the absorbance was measured at 540 nm.

2.3. Ion-exchange chromatography

The filtered extract was applied to a DEAE-Sepharose fast flow (Pharmacia) column (500 ml) with chloride as counter-ion. The column was coupled to a Peristaltic pump P-3 (Pharmacia) and 10 ml fractions were collected using a LKB-Super Frac (Pharmacia). The column was first eluted with water at 1 ml/min, followed by a NaCl gradient (0–1 M). The carbohydrate profile was determined using the phenol–sulphuric acid test of Dubois et al. (1956).

2.4. Size exclusion chromatography

Polysaccharide fractions were applied to a Sephacryl S-400 HR (Pharmacia) column (2.6 × 100 cm) which has a molecular weight fractionation range of 10–2000 kDa for dextrans. The column was coupled to a Peristaltic pump P-3 (Pharmacia), a LKB-Super Frac (Pharmacia) fraction collector and a Shimadzu RID-6A refractive index detector. The column was eluted with 15 mM NaCl at 20 ml/h.

2.5. Methanolysis and GC

The polysaccharide samples (1 mg) were subjected to methanolysis with 4 M hydrochloric acid in anhydrous methanol for 24 h at 80°C (Chambers and Clamp, 1971). Mannitol was added as an internal standard. The samples were dried with nitrogen at 40°C, methanol was added and the samples were dried again. This washing was repeated twice. Prior to trimethylsilylation the samples were dried in vacuum over P_2O_5 for 1 h. The samples were subjected to gas chromatographic analysis on a Carlo Erba 4200 instrument with a 430 LT programmer and a flame ionisation detector, a split–splitless injector and a LKB 2220 Recording integrator. The column was a DB-5 fused silica capillary column (30 m × 0.32 mm i.d.) with film thickness 0.25 μm. Helium was used as carrier gas at a flow rate of 3.0 ml/min. Both injection- and detector temperature were 300°C. The column temperature was initially 140°C, then an increase of 1°C/min to 170°C, followed by 6°C/min to 250°C and then 30°C/min to 300°C

2.6. Reduction

The uronic acid residues in polysaccharide samples were reduced with sodium borodeuteride ($NaBD_4$) in two steps. In the first step esterified uronic acids were reduced, in the second step un-esterified uronic acids were activated with carbodiimide before reduction (Kim and Carpita, 1992). The reduction was followed by methylation and GC-MS as described below.

2.7. Methylation analysis

The polysaccharides were methylated by the method described by Harris et al. (1984) modified by Kvernheim (1987) using the lithium salt of methylsulphinyl carbanion (Blakeney and Stone, 1985). The partially methylated alditol acetates were analysed by GC-MS. The gas chromatograph was fitted with a split–splitless injector, used in the split mode and a Supelco Fused silica capillary column (30 m × 0.20 mm i.d.) with film thickness 0.20 μm. The column was inserted directly into the ion source of the mass spectrometer. The injector temperature was 250°C and the detector temperature was 300°C. The column temperature was 80°C at the time of injection, after 5 min temperature was increased with 30°C/min to 170°C, followed by 0.5°C/min to 200°C and then 30°C/min to 300°C in which it was kept for 25 min. Helium was the carrier gas with a flow rate of 0.9 ml/min. E.I. mass spectra were obtained using a Fisons Instruments GC8000 series (8065) gas chromatograph.

2.8. De-esterification

The pH of the polysaccharide solution (2.4 mg/ml) was increased to 12.5 using 0.2 M NaOH. After 30 min at 25°C the pH was lowered to 6.5 with 0.2 M HCl. The solution was then dialysed (*Mw* cut-off 3500) against distilled water and freeze dried.

2.9. Weak acid hydrolysis

Arabinose was partly removed from the polysaccharide fractions by hydrolysis with 50 mM oxalic acid at 100°C for 2 h followed by dialysis (*Mw* cut-off 3 500) and freeze drying (Cartier et al., 1987).

2.10. Formic acid hydrolysis

Polysaccharide samples (10 mg) were hydrolysed with

90% formic acid (2 ml) at 100°C for 6 h. Then water (6 ml) was added and the solution was left at 100°C for 2 h, concentrated at 40°C under vacuum and washed several times with methanol to remove the reagent. Finally the residue was dissolved in water and acidic monosaccharides were separated from the neutral by ion exchange chromatography, using AG® 1 × 8 formate from (Bio Rad). The neutral monosaccharides were eluted with water and the uronic acids were eluted with 1 M formic acid.

2.11. Identification of neutral methylated monosaccharides

The neutral monosaccharides isolated after formic acid hydrolysis were reduced with $NaBD_4$ overnight, neutralised with Dowex H^+form, filtered and borate was removed by washing and subsequent evaporation several times with acetic acid in methanol (1:9) followed by washing with pure methanol. The dried alditols were acetylated using pyridine (1 ml) and acetic acid anhydride (1 ml) at 100°C for 10 min. The samples were dried under vacuum and washed with methanol. Finally the samples were dissolved in methanol (250 μl) and analysed by GC-MS as described under ''methylation analysis''.

2.12. Identification of uronic acids by paper chromatography

The acid monosaccharides isolated after formic acid hydrolysis were dissolved in ethanol–water (1:1) and applicated on a Whatman NV.1 paper (23 × 50 cm). Galacturonic acid and glucuronic acid (Sigma), 2% in ethanol–water (1:1) were used as standard. The chromatogram was eluted overnight using ethylacetate – acetic acid – formic acid – water (18:3:1:4) as mobile phase. The uronic acids were detected by spraying with saturated aniline oxalate and drying at 100°C.

2.13. Enzyme degradation

A purified endoxylanase from *Trichoderma viride* (EC 3.2.1.8) (Sigma prod. No. X 3876) was added to polysaccharide in 30 mM sodium acetate buffer pH 4.5 and incubated at 30°C. Enzyme hydrolysis was followed by measuring the increase in reducing sugars.

2.14. ^1H NMR

Fractions were dissolved in D_2O and NMR spectra were acquired on Jeol DX270 at 80°C with a 0.5 s pulse delay and 1.4 s acquisition time, using 3K sweep width and 8K data points. Chemical shifts were set relative to residual water (4.29 ppm).

2.15. ^{13}C NMR

Fractions were dissolved in D_2O and ^{13}C NMR spectra were recorded on Jeol DX270 at 50°C with a 1.2 s pulse delay, 0.3 s acquisition time and a 90°C pulse angle, using 13.4 sweep width and 8K data points. Chemical shifts were set relative to DMSO (39.6 ppm). APT was performed at ambient temperature in full automatic mode based on 5000 scans.

2.16. 2D heteronuclear ($^1H–^{13}C$) chemical shift correlation spectra

$^1H–^{13}C$ shift correlated 2D NMR were performed at 80°C, and data were processed by DELTA-NMR software.

2.17. IR spectroscopy

Polysaccharide samples (5 mg) were dried at 40°C in vacuum over P_2O_5 for 48 h prior to preparing a tablet with KBr. The IR spectra were recorded with a Beckman Accu-Lab 2 IR spectrophotometer.

2.18. Anti-complementary activity

Sheep erythrocytes were washed twice with 9 mg/ml NaCl and once with veronal buffer pH 7.2 containing 145 m M NaCl, 190 μM Ca^{2+}, 826 μM Mg^{2+}, 2 mg/ml BSA and 0,02% sodium azide (VB/BSA) and sensitised with rabbit anti-sheep erythrocyte antibodies (Virion amboceptor 9020, the National Institute of Public Health). After incubation at 37°C for 30 min on a shaker, the cells were washed as described above, and a 1% cell suspension in VB/BSA was prepared and stored at 4°C until use the same day.

The human serum with intact complement proteins was form a healthy adult, and it was pre-treated for removal of antibodies against sheep erythrocytes as described by Michaelsen et al. (1991). The serum was diluted with VB/BSA (1:40) to a concentration giving about 50% hemolysis.

Samples dissolved in VB/BSA (50 μl) and serum (50 μl) were added in duplicates to wells on a microplate and incubated on a shaker at 37°C. After 30 min, the sensitised sheep erythrocytes (50 μl) were added and the microplate was incubated as earlier. After centrifugation at 1700 rpm for 10 min., 100 μl of the supernatants were transferred to a flat bottom microplate for absorbance reading (A) at 405 nm using a MR 700 Dynatech microplate reader.

100% lysis was obtained with distilled water and sensitised sheep erythrocytes ($=A_{water}$). VB/BSA, serum and sensitised sheep erythrocytes were the control of the medium ($=A_{control}$), and the pectin fraction PMII from the leaves of *Plantago major* L. (Samuelsen et al., 1996) was used as positive control.

The degree of lysis is given by the formula

$$\left[\frac{A_{control}}{A_{water}}\right]\times 100\%$$

and the anti-complementary activity (%) is calculated from the formula

$$\left[\frac{A_{control}-A_{sample}}{A_{control}}\right]\times 100\%$$

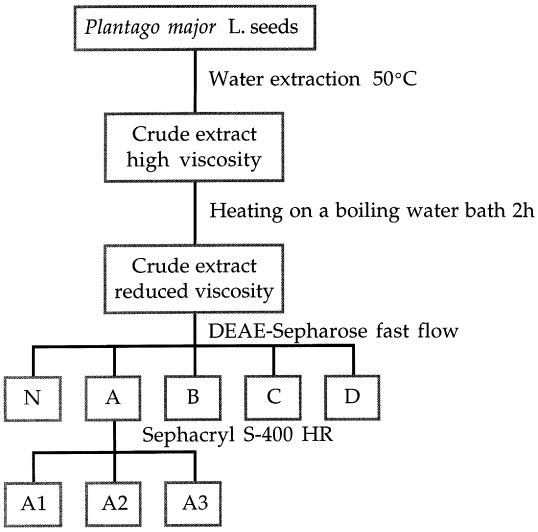

Figure 1. Isolation of polysaccharide fractions from the seeds of *Plantago major* L.

The results were plotted as the initial sample concentrations when added to the wells versus % anti-complementary activity.

2.19. Endotoxin contamination

The *Limulus amebocyte* lysate pyrogen (LAL) test (Bio Whittaker, Inc.), endpoint method was employed for the determination of endotoxin contamination. Lipopolysaccharide (LPS) from *E. coli* was used as positive control.

3. Results and discussion

3.1. The crude extract

The polysaccharids extracted with water at 50°C gave a viscous solution that was impossible to filter. Addition of EDTA did not decrease the viscosity. This problem was however minimised by boiling the extract, i.e. to introduce depolymerisation to some extent. After 2 h of boiling, the concentration of reducing sugars had increased from undetectable amounts to about 40 μg/ml, and the extract could be filtered through a 5 μm filter.

Table 1
Linkage analysis by methylation and GC-MS of polysaccharide fractions from *Plantago major* L. seeds

				Fractions							Fractions after oxalic acid hydrolysis					
	Linkage	Rt[a]	Primary fragments	N	A1	A2	A3	B	C	D	A1	A2	A3	B	C	D
Ara*f*	T-	0.43	45, 118, 161	7.5	7.5	10.4	15.3	8.3	8.4	2.8	3.8	1.0	1.4	1.6	2.8	2.7
	1,3	0.48	45, 118	2.0	20.1	4.6	2.5	4.3	7.2	12.8	16.6	4.3	1.6	1.8	6.2	2.9
	Total[b]			9.5	27.6	15.0	17.8	12.6	15.6	15.9	20.4	5.3	3.0	3.4	9.0	5.6
Xyl*p*	T-	0.45	117, 118, 161, 162	0.5	14.1	6.6	4.8	12.7	12.2	4.0	15.0	11.5	3.9	11.7	10.2	18.5
	1,3	0.50	117, 118	—	8.1	6.0	3.5	13.0	22.4	18.7	4.3	0.8	—	3.3	14.5	7.9
	1,4	0.51	118, 162, 189	3.1	2.4	3.8	11.8	3.7	2.9	1.5	10.7	7.4	18.6	19.3	17.9	22.2
	1,3,4	0.57	118	—	6.8	3.0	1.4	4.9	5.4	—	3.8	1.9	—	2.3	5.6	2.0
	1,2,4	0.58	189, 190	—	6.1	2.3	2.0	4.7	10.5	28.6	3.5	—	0.7	2.4	5.1	2.2
	Total[b]			3.6	37.5	21.7	23.5	39.0	53.3	52.8	37.5	21.7	23.2	39.0	53.3	52.8
Rha*p*	1,2,4	0.59	190, 203	—	—	3.7	—	3.4	—	—	—	3.3	3.1	3.1	3.2	4.6
	Total[b]			1.0	Trace	3.7	3.5	3.4	2.1	2.9	Trace	3.3	3.1	3.1	3.2	4.6
Gal*p*	T-	0.56	45, 118, 161, 162, 205	—	0.9	11.1	7.2	3.0	Trace	—	2.2	5.6	3.0	1.9	—	—
	1,3	0.64	45, 118, 161, 234	—	2.6	5.0	—	2.0	—	—	1.1	10.8	—	1.6	—	—
	1,6	0.67	118, 162, 189, 233	0.7	—	3.4	4.0	1.0	Trace	—	2.8	1.3	4.9	1.5	—	—
	1,3,4	0.70	45, 118	2.8	—	—	—	—	—	—	—	1.8	3.0	—	—	—
	1,2,4	0.73	45, 190, 233	1.1	—	—	—	—	—	—	—	0.3	—	—	—	—
	1,3,6	0.83	118, 189	1.8	3.3	4.2	2.5	—	—	—	1.8	3.3	2.2	0.8	—	—
	Total[b]			6.5	6.8	23.7	13.8	6.0	1.6	1.1	7.9	25.3	13.1	5.8	2.5	1.5
Glc*p*	T-	0.54	45, 118, 161, 162, 205	7.1	1.5	1.2	5.9	—	0.3	—	—	1.1	5.8	1.8	—	—
	1,4	0.63	45, 118, 162, 233	64.4	2.5	3.0	20.5	3.5	0.3	—	—	0.8	19.4	—	—	—
	1,4,6	0.79	118, 189, 234	2.0	—	—	—	—	—	—	—	2.4	1.5	—	—	—
	Total[b]			73.5	4.0	4.2	26.4	3.5	0.6	—	5.7	4.3	26.7	1.8	—	—
Man*p*	Total[b]			1.5	1.6	Trace	2.0	—	1.6	0	Trace	1.4	2.6	0.6	Trace	—
Gal*p*A[c]	T	0.56	47, 118, 162, 163, 207	n.d.[d]	0.1	—	n.d.[d]	6.0	4.2	—	—	—	—	—	Trace	0.3
	1,4	0.62	47, 118, 162, 235	n.d.[d]	17.2	31.7	n.d.[d]	14.1	9.6	13.2	16.1	29.0	10.2	21.5	12.9	10.8
	Total[b]			4.4	17.3	31.7	10.3	20.1	13.8	13.2	16.1	29.0	10.2	21.5	12.9	11.1
Glc*p*A[c]	T-	0.54	47, 118, 162, 163, 207	—	5.2	Trace	n.d.[d]	15.6	13.1	14.1	Trace	Trace	3.2	—	4.5	3.3
	Total[b]			—	5.2	Trace	2.7	15.6	13.1	14.1	Trace	Trace	3.2	—	4.5	3.3

[a] Retention time, relative to myo inositol.
[b] Determined by methanolysis and GC.
[c] Gal*p*A and Glc*p*A were detected by reduction with $NaBD_4$ prior to methylation.
[d] Not detected, failure in reduction procedure, not enough material left for repeating experiment.

The carbohydrates in the crude extract consisted of 39.7% xylose, 13.1% arabinose, 17.2% galacturonic acid, 15.5% glucuronic acid, 2.1% rhamnose, 2.5% galactose and 9.9% glucose. The lyophilised crude extract contained about 76% carbohydrate. Only traces of proteins were detected by the method of Lowry et al., (1951).

The presence of galacturonic acid and glucoronic acid was verified by formic acid hydrolysis of the crude extract followed by paper chromatography of the acidic fraction. No methylated uronic acids were detected by this method. The neutral fraction after hydrolysis was acetylated, and no methylated neutral monosaccharides were detected by GC-MS.

3.2. Fractionation of the crude extract

The crude extract was separated into five fractions by ion-exchange chromatography (Fig. 1.). The neutral fraction, N, constituted only 4% of the total carbohydrate recovery after this separation. Four acidic fractions, A, B, C and D were eluted as follows: A~0.5–0.78 M NaCl, B~0.79–0.83 M NaCl, C~0.84–0.90 M NaCl and D~0.91–1.0M NaCl. C was the major of the acidic fractions (39%), followed by D (34%), A (15%) and B (12%).

Fraction A was applied to size exclusion chromatography using a Sephacryl S-400 HR column and separated into three fractions: A1 (204–278 ml, corresponding to blue dextran, $\overline{Mw}$ 2000 kDa (Pharmacia)), A2 (282–350 ml) and A3 (354–435 ml, corresponding to green dextran, $\overline{Mw}$ 16400, $\overline{Mn}$ 15500 (Pharmacia)). The separation gave a 62% recovery, A3 was the major fraction (54% of the total recovery) and fractions A1 and A2 were obtained in about equal yields.

3.3. Structure analysis

The neutral fraction (N) contained high amounts of glucose (73.5%), arabinose (9.5%), galactose (6.5%) and small amounts of xylose (3.6%), galacturonic acid (4%), rhamnose (1%) and mannose (1.5%). Fraction N gave positive reaction for starch with iodine. This was supported by the methylation data showing that most of the glucose was 1,4-linked (Table 1.).

Fractions A1, A2, A3, B, C and D consisted of higher amounts of xylose (22–53%) in addition to arabinose, rhamnose, galactose, glucose, galacturonic acid, glucuronic acid and small amounts of mannose.

The xylose residues were 1,4- and 1,3-linked in pyranose from (Xyl*p*). *O*-2 or *O*-3 substituted 1, 4-linked Xyl*p* residues were detected by GC-MS, but no completely *O*-substituted Xyl*p* residues were found.

The ratio between the 1,4- and 1,3-linked residues differed among the fractions as well as the degree of branching. All fractions except A3 contained more 1,3-linked than 1,4-linked Xyl*p*. A1 had the highest degree of *O*-3 substitution of Xyl*p*, and fraction D had almost no such branching. Instead, D was heavily *O*-2 substituted while the other fractions contained less of this branching type. C and D contained the highest amount of xylose (ca. 50%).

Arabinose was in furanose form (Ara*f*) and was 1,3- and terminally linked in all fractions. A1 contained the highest amount of arabinose (27.6%).

The total amount of uronic acid was higher in fraction B than in both C and D although these two latter fractions were eluted from the ion exchange column at higher sodium chloride concentrations. This must imply esterification of uronic acid residues in fraction B. Galacturonic acid (Gal*p*A) was 1,4-linked in all fractions, and the glucuronic acid residues (Glc*p*A) were found to occupy only terminal positions. Fractions B, C and D consisted of almost equal amounts of glucuronic acid and galacturonic acid while A1, A2 and A3 contained more galacturonic acid than glucuronic acid. The reduction of the uronic acids by $NaBD_4$ in fraction A3 failed (Table 1.), but after weak acid hydrolysis the reduction was carried out successfully, revealing after methylation that the uronic acids were linked as in the other fractions. Galacturonic acid was therefore assumed to be 1,4-linked and glucuronic acid terminally linked in fraction A3. Galactose, when detected, was 1,3-, 1,6- and 1,3,6-linked.

The glucose found in these fractions were considered to be a contaminant. Mannose was not detected by methylation and GC-MS.

3.4. The position of arabinose

Glycosidic linkages of carbohydrate residues in the furanose form are hydrolysed under mild conditions compared to residues in the pyranose form. By such hydrolysis Ara*f* residues can be removed selectively from the polysaccharide, and the position of Ara*f* in the original polysaccharide can be determined by methylation of the remaining polysaccharide.

In this case the conditions might have been too mild (Table 1,), especially for fraction A1 in which only 26% of the Ara*f* was lost during this treatment. However, if all arabinose residues had been removed, hydrolysis might have occurred also for other glycosidic linkages in addition to those of Ara*f*.

According to the GC-MS analysis after hydrolysis, the remaining arabinose was also in furanose form and no arabinose in pyranose form (Ara*p*) was detected. One large peak in the GC chromatogram had the fragmentation pattern of terminally linked Ara*p* or Xyl*p*. Non-reducing ends of the latter was confirmed by ^{13}C NMR (see below).

After weak acid hydrolysis an increase in the amount of 1,4-linked Xyl*p* and a decrease in the amount of 1,3,4- and 1,2,4-linked Xyl*p* were observed. The decrease in 1,2,4-linked Xyl*p* was most obvious in C and D because of the relatively larger amounts of this linkage type in C and D compared with the other fractions. The original A1, A2, A3 and B contained relatively small amounts of 1,3,4- and 1,2,4-linked Xyl*p*, but decreases in both linkage types

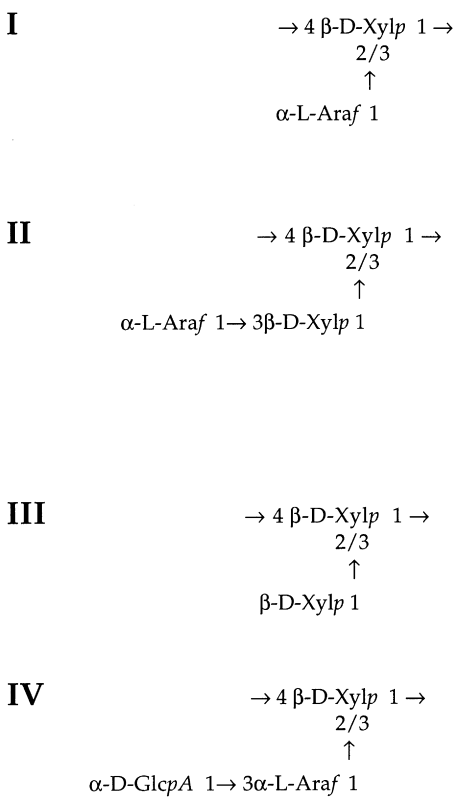

Figure 2. Proposed structural fragments of the heteroxylan polysaccharide from the seeds of *Plantago major* L.

were observed after hydrolysis. This indicates that Ara*f* was linked to *O*-2 and *O*-3 of 1,4-linked Xyl*p* (Fig. 2(I)). A decrease in 1,3-linked Xyl*p* was also observed in all fractions, and an increase in non-reducing Xyl*p* was seen in A2 and D. This indicates that Ara*f* was linked to position 3 of some of the terminal Xyl*p* units which are most likely located in side chains (Fig. 2(II)). The relatively high proportion of non-reducing ends of Xyl*p* before and after hydrolysis indicate that the polysaccharide fractions either are of a relatively low d.p. and/or that small Xyl*p* side chains are present (Fig. 2(III)).

The findings that the content of Glc*p*A decreased in B, C, and D after the mild hydrolysis, favouring splitting of Ara*f* linkages, indicated that Glc*p*A was substituted to *O*-3 of Ara (Fig. 2(IV)).

The 1,4-linked α-D-Gal*p*A residues, small amounts of 1,2,4-linked Rha*p* and 1,3-, 1,6- and 1,3,6-linked Gal*p* might be part of a pectin type polysaccharide consisting of a 1,4-linked α-D-Gal*p*A backbone with some 1,2,4-linked α-L-Rha*p* residues and neutral galactan side chains attached to position 4 of the rhamnose residues.

According to the methylation results of A1, A2, A3, B, C and D (Table 1), these fractions are similar to one another, only differing in the relative amounts of each monosaccharide and linkage type. Therefore, there are reasons to believe that these fractions originate from the same polysaccharide and might have been produced during heating of the crude extract.

3.5. NMR spectroscopy

^{13}C NMR spectra were recorded at 50°C for fractions B and C (Fig. 3). Because of the limited solubility of B in water, this spectrum (Fig. 3(I)) has a low signal/noise ratio making interpretation difficult. The solubility, and hence the quality of the spectra, was not improved by attempts to solubilise in DMSO or 1 M NaOH (data not shown). As seen in Fig. 3(II) and (III), the signal/noise ratios were improved in the spectra of C, although still complex due to different sugar residues with several different substitution patterns and sequences. By comparing the spectra of B and C it is however obvious that the fractions are of a similar polysaccharide type. The solubilised samples had a relatively low viscosity, as indicated by fairly narrow lines, and spectral quality was not improved markedly by increasing the temperature to 80°C.

Some assignments were made by comparing with spectra of a xylan from *Leptusarea simplex* composed of 1,3-linked and 1,4-linked β-D-Xyl*p* residues (Adams et al., 1988), an arabinoxylan from wheat endosperm containing a 1,4-linked β-D-Xyl*p* backbone that is *O*-3 or *O*-2 and *O*-3 substituted by single α-L-Ara*f* residues (Hoffman et al., 1992), a heteroxylan from wheat pericarp consisting of short chains of α-L-Ara*f* residues and Glc*p*A distributed along the 1,4-linked β-D-Xyl*p* backbone (Brillouet and Joseleau, 1987) and the spectrum of a pectin which contains 1,4-linked α-D-Gal*p*A residues (Westerlund et al., 1991).

Multiple resonances from C-1 of the β-D-Xyl*p* residues appear at 102–104.1 ppm (Fig. 3 (II)). The signals at 104.1 ppm are from 1,3-linked β-D-Xyl*p* and terminally linked β-D-Xyl*p* substituted to *O*-3 of β-D-Xyl*p* residues. The C-1 resonance from 1,4-linked β-D-Xyl*p* is detected at 102.6 ppm (Brillouet and Joseleau, 1987; Adams et al., 1988). This latter signal increased after weak acid hydrolysis for removal of α-L-Ara*f* (Fig. 3(III)). The other anomer signals from the xylose residues are due to branching.

In the 2D heteronuclear correlation spectrum (not shown) the C-1 signals at 102–104.1 ppm correspond to partly unresolved signals from H-1 of the β-D-Xyl*p* residues which appear in the 4.50–4.72 ppm region.

The signals corresponding to C-3, C-2, C-4 and C-5 of 1,3-linked β-D-Xyl*p* residues appear at 84.3, 74.0, 68.4 and 65.8 ppm, respectively (Adams et al., 1988). After hydrolysis a significant amount of 1,3-linked β-D-Xyl*p* units was still present (C-3 at 84.3 ppm, Fig. 3(III)). The signals from C-4, C-3, C-2 and C-5 of 1,4-linked β-D-Xyl*p* appear at 77.1, 74.1, 73.5 and 63.6 ppm, respectively (Adams et al., 1988, Brillouet and Joseleau, 1987).

Resonances from C-3, C-2, C-4 and C-5 from the non-reducing ends of β-D-Xyl*p* are at 76.4, 73.5, 70.0 and 65.9 ppm, respectively. These signals have relatively high

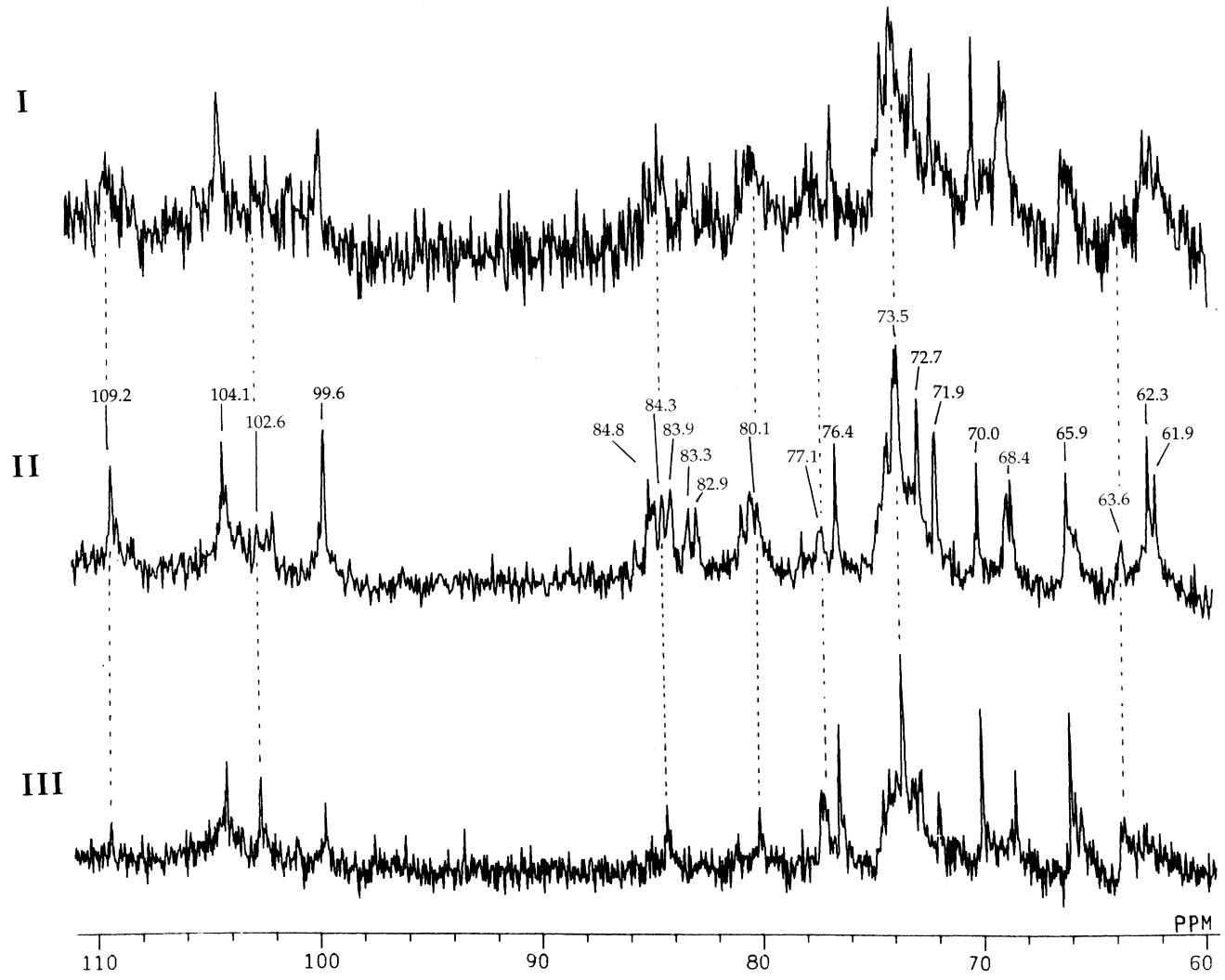

Figure 3. ^{13}C NMR spectra of heteroxylan fractions from the seeds of *Plantago major* L. (I): Fraction B, (II): Fraction C, (III): Fraction C after weak acid hydrolysis for removal of arabinose.

intensities, thus they reflect a high degree of branching and / or a relatively low d.p. Signals for C-1α or C-1β from the reducing end-groups at 92.8 or 97.3 ppm are not detected in the spectra, indicating a high degree of branching rather than a very low d.p. (Brillouet and Joseleau, 1987). The is confirmed by multiple signals in the C-1 and C-5 regions which indicate a high degree of branching.

In the case of short xylose side chains linked to C-2 of the backbone, the signal from C-2 of *O*-2 substituted 1,4-linked β-D-Xyl*p* co-occur with the C-4 signal of 1,4-linked β-D-Xyl*p* at 77.1 ppm (Brillouet and Joseleau, 1987). The resonance at 80.1 ppm might be assigned to C-3 of 1,4-linked β-D-Xyl*p* with a xylose side chain in position 3 (Kovac et al., 1980, measured from a xylan tetrasaccharide), or to the signal from C-4 of 1,4-linked α-D-Gal*p*A (Westerlund et al., 1991).

The signal at 82.9 ppm from C-3 of 1,4-linked β-D-Xyl*p* *O*-3 linked to α-L-Ara*f* (Brillouet and Joseleau, 1987) in the spectrum of the original sample was not detected in the spectrum obtained from the hydrolysed sample. This is however not in agreement with the methylation analysis where no decrease in the 1,3,4-linked β-D-Xyl*p* residues was observed after hydroysis of fraction C.

According to the methylation data (Table 1.), α-L-Ara*f* was linked to position 2 of the 1,4-linked β-D-Xyl*p* residues in fraction C (Fig. 2(I)). At 74.1 ppm the C-2 signal from 1,4-linked β-D-Xyl*p* with α-L-Ara*f* linked to position 2 (tentative assignment by Hoffman et al., 1992) co-occur with the signal from C-3 of 1,4-linked β-D-Xyl*p*. These signals appear in the most crowded region of the spectra, therefore a reduction in the C-2 signal intensity due to removal of the arabinose residues after weak acid hydrolysis could not be observed.

The anomer signals from the α-L-Ara*f* residues are at about 109.2 ppm. In the 2D heteronuclear correlation spectrum these signals correspond to two partly overlapping signals which have different intensities in the ^{1}H NMR spectrum with resonances occurring at 5.29 and 5.37 ppm. Thus,

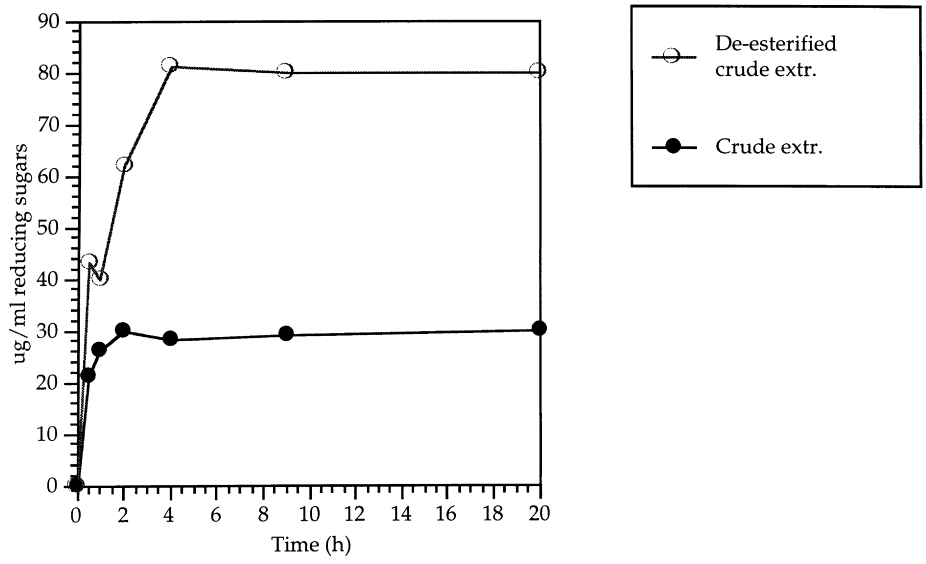

Figure 4. Increase in the amount of reducing sugars measured as μg/ml xylose equivalents during endoxylanase treatment of the high viscosity crude extract and the high viscosity crude extract after alkali de-esterification.

two α-L-Ara*f* linkage types are present (i.e. 1,3- and terminally linked units). The α-L-Ara*f* signals decreased after hydrolysis with oxalic acid as expected (Fig. 3(III)).

According to earlier studies (Brillouet and Joseleau, 1987; Hoffman et al., 1992) the resonance from C-3 of terminally linked α-L-Ara*f* co-occur with the β-D-Xyl*p* signals at about 77.1 ppm. Multiple C-4 signals from α-L-Ara*f* are in the 84.5–85.0 ppm region. The C-5 signals from terminal α-L-Ara*f* residues linked to *O*-2 or *O*-3 of β-D-Xyl*p* co-occur at 62.3 ppm (Hoffman et al., 1992), and the signal from C-5 of 1,3-linked α-L-Ara*f* appear at 61.9 ppm. The identity of the C-5 signals which contain a CH_2 functional group were confirmed by the spectrum obtained by the APT technique.

The signals at 83.3 and 83.9 ppm might be due to the C-3 signals from α-L-Ara*f* *O*-3 linked to α-L-Ara*f* and α-D-Glc*p*A. The signals in the region 80.2–80.8 ppm are tentatively assigned to C-2 of the α-L-Ara*f* residues. These signals were not detected in spectra obtained from the sample which had been subjected to hydrolysis.

The C-1 signals from α-D-Glc*p*A and α-D-Glc*p*A co-occur at 99.7 ppm (Bock et al., 1984). The cross peak in the 2D heteronuclear correlation spectrum correspond to two partly overlapping peaks with different intensities in the ^{1}H NMR spectrum occurring at 5.08 at 5.17 ppm. No signal corresponding to *O*-methyl was detected at 53.5 ppm in the ^{13}C NMR spectrum of C (spectral region omitted in Fig. 3), indicating that the uronic acid residues in this fraction were not methyl-esterified. A signal of relatively high intensity was present at 53.5 ppm in the spectrum of B. This confirms that the uronic acids in fraction B were esterified and thus, despite of the higher total uronic acid content in B than in C, B was eluted from the ion exchange column at a lower NaCl concentration.

The signals at 73.5, 72.7 and 71.9 ppm which decreased after hydrolysis are due to terminally linked α-D-Glc*p*A (Bock et al., 1984), and the signal at 80.1 ppm is from C-4 of 1,4-linked α-D-Gal*p*A (Westerlund et al., 1991).

3.6. Enzyme degradation

The crude extract, without a previous heating was subjected to enzyme degradation by a purified endoxylanase from *Trichoderma viride*, specific for β-1,4-linked Xyl*p* residues in xylans. The hydolysis was followed by measuring the increase in reducing sugars. About 30 μg/ml reducing ends (measured as xylose equivalents) were released after 2 h (Fig. 4). De-esterification of the substrate before enzyme treatment for removal of *O*-acetyl groups on the polysaccharide, increased the release of reducing ends to 80 μg/ml. No further increase was registered after 20 h. Thus, de-esterification increased the enzyme activity.

The presence of *O*-acetyl groups in the original crude extract was confirmed by IR spectroscopy. After de-esterification with alkali, the absorbance bands at 1740 cm^{-1} and 1240 cm^{-1} decreased (spectra not shown). The band at 1740 cm^{-1} is related to the carboxyl groups, and the band at 1240 cm^{-1} is associated with the vibrations involving bonds CC and CO and angles OCO and CCO of the acetyl groups (Filippov, 1992).

3.7. Biological activity

The test used for biological activity was the anti-complementary activity test which is an in vitro test for the ability of the polysaccharides to interact with the complement cascade reaction. The complement system is a part of the innate immune system consisting of a group of serum proteins which are activated in a cascade mechanism.

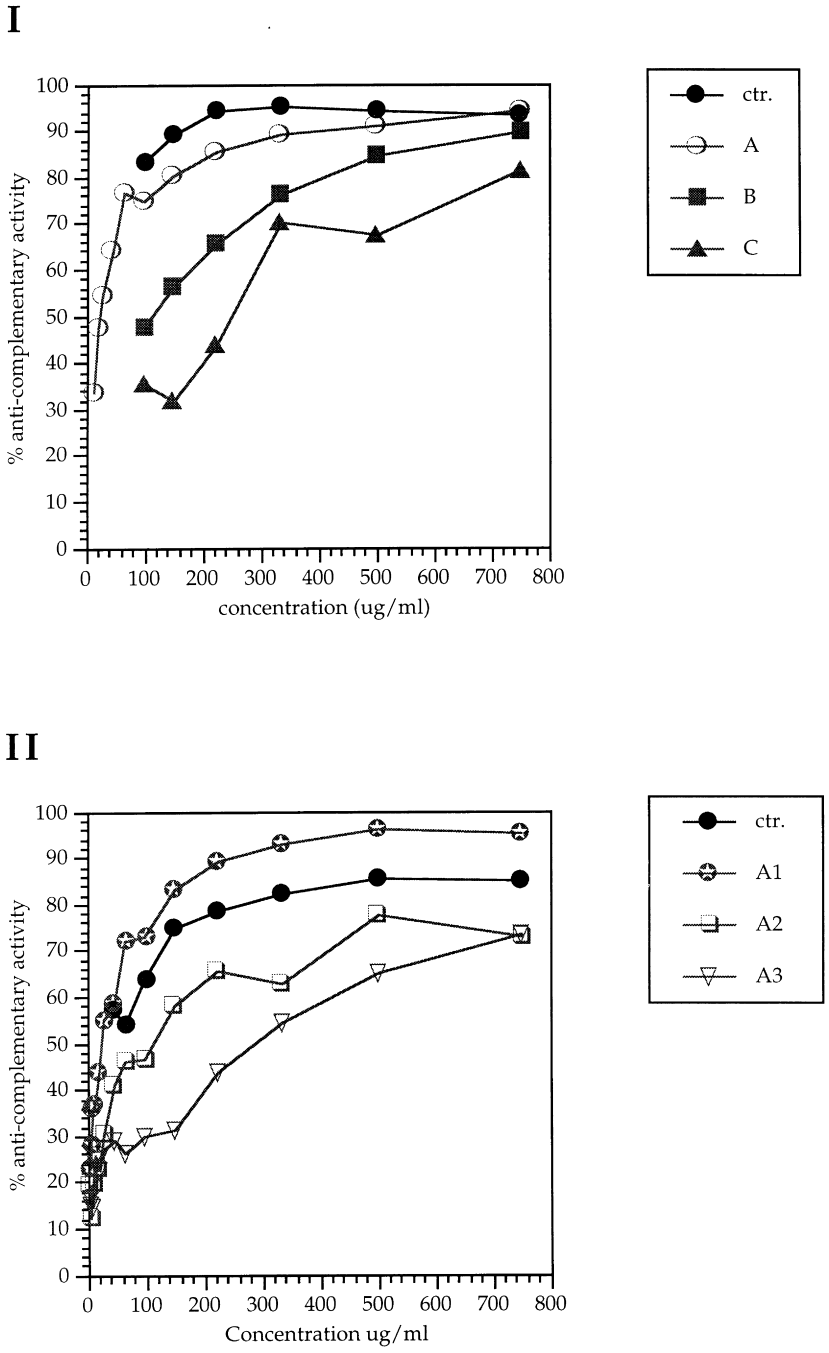

Figure 5. The % anti-complementary activity of heteroxylan polysaccharide fractions from *Plantago major* L. seeds. (I): Fractions A, B and C obtained after ion exchange chromatography, (II): A1, A2 and A3 obtained after size exclusion chromatography of A. The pectin fraction PMII from the leaves of *Plantago major* L. was used as positive control (ctr.).

Activation can be initiated by LPS, micro-organisms (the alternative pathway), by immune complexes containing antigen and IgM or IgG antibodies (classical pathway) or by the binding to a mannose binding lectin present in serum (lectin pathway). The activation of the complement system important in initiating inflammation, and some of the cleavage products formed during activation might induce opsonisation, leukocyte activation and degranulation of basophils and mast cells. The end-product of the cascade, the membrane attack complex, can damage invading cells by entering their lipid membrane. The crude extract from *Plantago major* L. seeds had potent anti-complementary activity, 90% at a concentration of 750 μg/ml. The crude extract was contaminated with 0.02% LPS, measured by the LAL test. False positive results in the LAL test could not be ruled out. LPS from *E. coli* (7603II/09 (MPI) S_3, the National Institute of Public Health) and *N. meningitidis* (MK-88, the National Institute of Public Health) were

subjected to the test of anti-complementary activity but were found to be inactive in the concentrations tested (3–750 μg/ml).

From the dose–response curves in Figs. 5(I) and 5(II), fraction A was the most active fraction obtained after ion exchange chromatography (Fig. 5(I)). Fraction D was not included in Fig. 5(I) since it has very low activity in this test. The activities of fractions A1, A2, and A3 obtained by further fractionation of A by size exclusion chromatography are given in Fig. 5(II). The highest molecular weight fraction, A1 had retained the activity of fraction A, and the lower molecular weight fractions, A2 and A3 were less active.

4. Conclusions

The polysaccharides isolated from the seeds of *Plantago major* L. are mainly heteroxylans which consist of a 1,3- and 1,4-linked β-D-Xyl*p* backbone with short side chains attached to position 2 in some of the 1,4-linked β-D-Xyl*p* residues and to position 3 of other 1,4-linked β-D-Xyl*p* residues. The side chains consist of β-D-Xyl*p* and α-L-Ara*f* residues, α-L-Ara*f*1→3 β-D-Xyl*p* and α-D-Glc*p*A1 →3α-L-Ara*f*. 1,4-linked α-D-GalpA residues were also detected in addition to small amounts of 1,2,4-linked Rha*p* and 1,3- 1,6- and 1,3,6-linked Gal*p*. The linkages of these latter monosaccharide residues to the xylan backbone were not determined, but they might originate from a pectin polysaccharide.

The crude extract and some of the fractions of this heteroxylan had potent anti-complementary activity.

The structure of the polysaccharides from *P. major* seeds have some similarities to the polysaccharides from *P. ovata* Forsk. and *P. asiatica* L. The polysaccharide fractions from *P. major* and P. *ovata* both contain 1,3- and 1,4-linked β–D-Xyl*p* residues in the backbone, but the α-D-Gal*p*A 1→2 α-L-Rha*p* side chains found in *P. ovata* were not detected in *P. major.*

α-D-Glc*p*A 1→3 α-L-Ara*f* side chains were found in *P. major* as well as in *P*. asiatica, but different xylan backbone structures are present in these species. Morphologically, *P. asiatica* and P. *major* are closely related and previously *P. asiatica* was designated ''*Plantago major* var. *asiatica*''. Structure analysis of the seed polysaccharides confirm that the two plants belong to two different *Plantago* species.

The isolation of oligosaccharides for more detailed structure elucidation of the polysaccharides from *P. major* seeds is in progress.

Acknowledgements

This work has been supported by The Norwegian Research Council, project no. 100594/410. The authors are indebted to Finn Tønnesen for recording the GC-MS data, to Astrid Giljie for preparing the serum for anti-complementary activity testing and to Torun Helene Aslaksen and Ellen Hanne Cohen for technical assistance. Prof. Lars Skjeldal is thanked for advices related to processing of NMR data. Thanks are also given to Dr. Haruki Yamada and his co-workers at the Oriental Medicine Research Center of the Kitasato Institute, Tokyo, Japan for preliminary testing of anti-complementary activity, to Dr. Erik Jantzen at the National Institute of Public Health for kindly supplying pure endotoxin and to Karianne Lunde at the National Institute of Public Health for performing the LAL test for endotoxin determination.

References

Adams, N. M., Furneax, R. H., Miller, I. J., & Whitehouse, L. A. (1988). Xylan from Leptosarca simplex and Carrageenas from Iridea, Cenacrum and Nemastoma Species from the Subantarctic Islands of New Zealand. *Botanica Marina*, *31*, 9–14.

Blakeney, A. B., & Stone, B. A. (1985). Methylation of carbohydrates with lithium methylsulphinyl carbanion. *Carbohydr. Res.*, *140*, 319–324.

Bock, K., Pedersen, C., Pedersen, H. (1984) Carbon-13 nuclear magnetic resonance data for oligosaccharides, In Advances in carbohydrate chemistry and biochemistry, Tipsen & Horton (Eds.) 42, pp. 193–225, Academic Press.

Brillouet, J. -M., & Joseleau, J. -P. (1987). Investigation of the structure of a heteroxylan from the outer pericarp (beeswing bran) of wheat kernel. *Carbohydr. Res.*, *159*, 109–126.

Cartier, N., Chambat, G., & Joseleau, J. -P. (1987). An arabinogalactan from the culture medium of Rubus fruticosus cells in suspension, Carbohydr. *Res.*, *168*, 275–283.

Chambers, R. E., & Clamp, J. R. (1971). An assessment of methanolysis and other factors used in the analysis of carbohydrate-containing materials. *Biochem. J.*, *125*, 1009–1018.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, *28*, 350–356.

Filippov, M. P. (1992). Practical infrared spectroscopy of pectic substances. *Food Hydrocolloids*, *6*, 115–142.

Harris, P. J., Henry, R. J., Blakeney, A. B., & Stone, B. A. (1984). An improved procedure for the methylation analysis of oligosaccharides and polysaccharides. *Carbohydr. Res.*, *127*, 59–73.

Hoffman, R. A., Kamerling, J. P., & Vliegenthart, J. F. G. (1992). Structural features of a water-soluble arabinoxylan from the endosperm of wheat. *Carbohydr. Res.*, *226*, 303–311.

Kim, J. -B., & Carpita, N. C. (1992). Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. *Plant Physiol.*, *98*, 646–653.

Knutsen, S.H. (1991) Carrageenase production in a culture of *Pseudomonas carregeenovora* growing on *kappa*-carrageenan. In Proceedings of a COST-48 workshop at Grand Canaria, Spain. R Garcia-Reina and M. Pedersen, (Eds.), ISBN 84-604-1766-7, pp. 277–281.

Kovac, P., Hirsch, J., Shashkov, A. S., Usov, A. I., & Yarotsky, S. V. (1980). 13C –N.M.R. spectra of xylo-oligosaccharides and their application to the elucidation of xylan structures. *Carbohydr. Res.*, *85*, 177–185.

Kvernheim, A. L. (1987). Methylation analysis of polysaccharides with butyllithium in dimethyl sulfoxide. *Acta Chem. Scand.*, *B41*, 150–152.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, *193*, 265–275.

Michaelsen, T. E., Garred, P., & Aase, A. (1991). Human IgG subclass pattern of inducing complement mediated cytolysis depends on antigen

concentration and to a lesser extent on epitope patchiness, antibody affinity and complement concentration. *Eur. J. Immunol.*, *221*, 11–16.

Miller, G. L (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* , *3*, 426–428.

Samuelsen, A. B., Paulsen, B. S., Wold, J. K., Otsuka, H., Yamada, H., & Espevik, T. (1995). Isolation and partial characterization of biologically active polysaccharides from Plantago major L. *Phytotherapy Res.*, *9*, 211–218.

Samuelsen, A. B., Paulsen, B. S., Wold, J. K., Otsuka, H., Kiyohara, H., Yamada, H., & Knutsen, S. H. (1996). Characterization of a biologically active pectin from Plantago major L. *Carbohydr. Polymers*, *30*, 37–44.

Samuelsen, A. B., Paulsen, B. S., Wold, J. K., Knutsen, S. H., & Yamada, H. (1998). Characterisation of a biologically active arabinogalactan from Plantago major L. *Carbohydr. Polymers*, *35*, 145–153.

Sandhu, J. S., Hudson, G. J., & Kennedy, J. F. (1981). The gel nature and structure of the carbohydrate of Ispaghula husk ex Plantao ovata Forsk. *Carbohydr. Res.*, *93*, 247–259.

Westerlund, E., Åman, P., Andersson, R. E., & Andersson, R. (1991). Investigation of the distribution of methyl ester groups in pectin by high-field ^{13}C NMR. *Carbohydr. Polymers*, *14*, 179–187.

Yamada, H., Nagai, T., Cyong, J. C, Otsuka, Y., Tomoda, M., Shimizu, N., & Gonda, R. (1986). Relationship between chemical structure and activating potencies of complement by an acidic polysaccharide, Plantago-Mucilage A, from the seed of Plantago asiatica. *Carbohydr. Res.*, *156*, 137–145.