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Characterization of a biologically active arabinogalactan from the leaves of *Plantago major* L.

Anne Berit Samuelsen[a,*], Berit Smestad Paulsen[a], Jens Kristian Wold[a], Svein H. Knutsen[b] and Haruki Yamada[c]

[a]*Institute of Pharmacy, Department of Pharmacognosy, P.O. Box 1068, Blindern, N-0316 Oslo, Norway*
[b]*Agricultural University of Norway, Department of Biotechnological Sciences, Chemistry, P.O. Box 5040, N-1432 Ås, Norway*
[c]*Oriental Medicine Research Center of the Kitasato Institute, 5-9-1, Shirokane, Minatoku 108, Tokyo, Japan*

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PMIa is a Type II arabinogalactan with anti-complementary activity isolated from the leaves of *Plantago major* L. It has a molecular weight of 77 000–80 000 Da and consists of arabinose (38%), galactose (49%), rhamnose (6%), galacturonic acid (7%) and 1.5% protein with hydroxyproline, alanine and serine as the main amino acids. Characterization of PMIa by methylation and GC–MS, methanolysis and GC, Smith degradation, weak acid hydrolysis, ^{13}C-NMR, ^{1}H-NMR, two-dimensional heteronuclear NMR and DEPT show that it consists of 1,3-linked galactan chains with 1,6-linked galactan side chains attached to position 6. The side chains are further branched in position 3 with 1,3-linked galactose residues which have 1,6-linked galactose attached to position 6; these 1,3- and 1,6-linked galactose chains altogether probably form a network. Terminal and 1,5-linked arabinose in furanose form are attached to the galactan mainly through position 3 of the 1,6-linked galactose side chains.

INTRODUCTION

The leaves of *Plantago major* L. are used in wound healing in traditional medicine. Either whole or crushed leaves are used directly on burns, wasp stings and wounds of all kinds to stop bleeding, keep the wound clean and to enhance the healing process. The reason for this effect remains unknown. However, several polysaccharides from plants have effects on the human immune system. Examples of such are arabinogalactans from the berries of *Viscum album* (Wagner and Jordan, 1988), *Echinacea purpurea* (Wagner et al., 1988) and pectins from *Angelica acutiloba* (Kiyohara et al., 1988). Biologically active polysaccharides might at least partly explain the wound healing effect of *Plantago major* L.

Polysaccharide fractions have been isolated from the leaves of *Plantago major* L. and tested for so called anti-complementary activity (Samuelsen et al., 1995). The complement system is part of the innate immune system and consists of a group of serum proteins which are activated in a cascade mechanism. The activation might be initiated at the level of C3 by LPS and micro-organisms (alternative pathway) or at the level of C1q by immune complexes containing antigen and IgM or IgG antibodies (classical pathway). There is also a third activation pathway were the C1 complex is substituted by a mannose binding lectin (lectin pathway) (Janeway and Travers, 1996).

The complement system is important in initiating inflammation, and its activation might result in opsonization, activation of leukocytes, mast cell degranulation or lysis of target cells by the endproduct C5b-9 of the cascade. The acquired immune system is also stimulated by complement activation, thus bridging the innate and acquired immune system. There are also interactions between complement and other cascade systems such as coagulation and fibrinolysis (Roitt et al., 1993).

*Corresponding author.

In the test for anti-complementary activity the polysaccharide sample is incubated with human serum which contains intact complement proteins. The serum used is diluted to a concentration giving 50% lysis of the indicator cells, which are antibody-sensitized sheep erythrocytes. The polysaccharide either activates the complement cascade, "burns it" completely or partly out, or the polysaccharides can inhibit the cascade by binding to one or more of the complement proteins. After adding the indicator cells haemolysis will occur to a degree depending on the amount of complement proteins left in the solution after incubation with polysaccharide.

The term 'anti-complementary activity' refers to the observed inhibition of haemolysis due to the interaction between complement proteins and polysaccharide. The test does not differentiate between activation and inhibition of the complement system, but it shows that the complement system is affected by the presence of certain polysaccharides and is used for preliminary testing in the search for biologically active polysaccharides.

Two of the acidic polysaccharide fractions isolated from the leaves of *Plantago major* L. had relatively high anti-complementary activity, PMI and PMII. PMII is a pectin and has been characterized previously (Samuelsen *et al.*, 1996). We now report on the further purification and characterization of PMI.

METHODS

Material

The polysaccharide fraction PMI was isolated from *Plantago major* L. by extraction and ion exchange chromatography as described previously (Samuelsen et al., 1995). A solution of PMI (3 mg/ml) was filtered (0.45 μm) and applied to a Sephacryl S-400 HR (Pharmacia) column (2.6 × 100 cm) which was coupled to a Peristaltic pump P-1 (Pharmacia) and a LKB Super Frac fraction collector and eluted with 0.15 M NaCl at 1 ml/min. The carbohydrate profile obtained was determined using the phenol-sulphuric acid reaction (Dubois et al., 1956), and PMIa and PMIb were obtained as subfractions of PMI (Fig. 1).

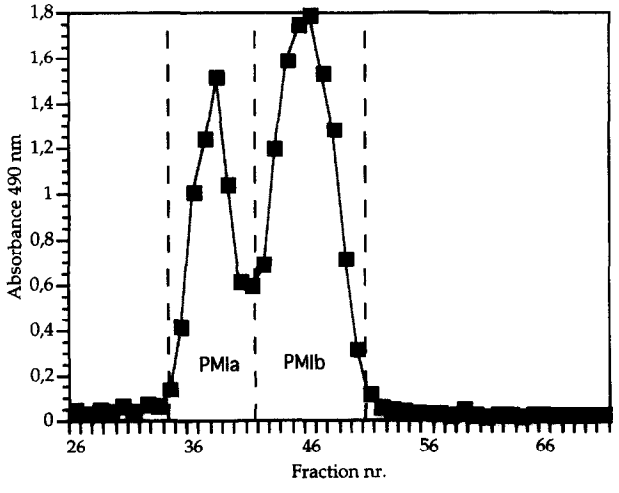

Fig. 1. Separation of PMI on a Sephacryl S-400 HR column. Fractions (10 ml) 34–41 and 42–51 were pooled and designated PMIa and PMIb, respectively.

Monosaccharide compositional analysis by methanolysis and GC

The methods used for methanolysis and GC analysis are described by Samuelsen et al. (1995).

Reduction of uronic acids

For linkage analysis by methylation and GC–MS, it is necessary to reduce the carboxylic acid groups of the uronic acids to the corresponding primary alcohols prior to methylation. To be able to differentiate between reduced galacturonic acid and galactose in GC–MS, sodium borodeuteride is used giving a deviation in molecular weight by 2 of the reduced galacturonic acid and galactose. The reduction of galacturonic acid was performed after activation with carbodiimide as described by Kim and Carpita (1992).

Linkage analysis by methylation and GC–MS

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 Hewlett-Packard methyl silicone capillary column (12 m × 0.22 mm i.d.) with film thickness 0.33 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 and was then 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. Helium was carrier gas with a flow rate of 0.9 ml/min. E.I. mass spectra were obtained using Hewlett-Packard Mass Selective Detector 5970 with a Hewlett-Packard 5890 GC.

Determination of amino acid composition

PMIa was subjected to hydrolysis under vacuum in 6 M HCl at 110°C for 24 h. After removal of HCl under reduced pressure, the amino acid composition was determined using a Biocal JC 5000 automatic amino acid analyser.

M_w determination

The molecular weight was determined by size exclusion chromatography coupled to LALLS (Low Angle Laser Light Scattering) with a B-44 column and pullulan standards

as described by Christensen et al. (1993). This was performed at Department of Biotechnology, Norwegian University of Science and Technology, Trondheim, Norway.

Degree of acetylation

The degree of acetylation was determined as described by Tomoda et al. (1974). The polysaccharide sample was dried over P_2O_5 and hydrolysed with 1 M HCl at 100°C for 2 h. Propionic acid was added as an internal standard. The hydrolysate was applied directly to analysis on a Carlo Erba 6000 Vega series gas chromatograph with control module ICU 600. The chromatograph had a split–splitless injector, a flame ionization detector and was coupled to a Hewlett-Packard HP 3396A integrator. The column was a glass column (2 m × 3 mm i.d.) packed with 10% FFAP (Supelco) on 80/100 Chromosorb WAW. Nitrogen was used as the carrier gas at a flow rate of 50 ml/min. The column temperature was 150°C and the injector and the detector temperature was 200°C.

Weak acid hydrolysis

Arabinose was hydrolysed off the polysaccharide with 50 mM oxalic acid at 100°C for 2 h followed by dialysis and freeze drying (Cartier *et al.*, 1987).

Smith degradation

PMIa (240 μmol) was subjected to oxidation with 0.01 M $NaIO_4$ in 0.1 M NaOAc buffer pH 4 at 4°C in the dark. The reaction was terminated after 80 h with ethylene glycol and the solution was neutralized with $NaHCO_3$ prior to reduction overnight with $NaBH_4$. After neutralizing with dilute acetic acid and desalting by dialysis (cut off 1 kDa), the remaining material was submitted to hydrolysis with 1 M TFA at 40°C for 30 min. Under these conditions only the newly formed acetal bonds but not the glycosidic linkages will be hydrolysed (Percival and McDowell, 1967).

The mixture was concentrated to dryness at 40°C under vacuum and washed with water several times. The remaining glycan was dissolved in water and precipitated with two volumes of ethanol. The precipitate was isolated by centrifugation at 5000 rpm for 10 min. After repeated periodate oxidation and hydrolysis the remaining material was isolated by fractionation on a BioGel P10 (Bio Rad) column (2.6 × 100 cm). The column was coupled to a Peristaltic pump P-1 (Pharmacia) and a LKB-Super Frac fraction collector (Pharmacia) and a Shimadzu RID-6A refractive index detector. The column was eluted with water at 12 ml/h. The high molecular weight fraction which was eluted in the void volume of the column was submitted to methanolysis and methylation/GC–MS analysis.

Precipitation with Yariv β-glucosyl reagent

The precipitation with Yariv β-glucosyl reagent by crossed electrophoresis was performed according to the method by van Holst and Clarke (1986). The first dimension gel (10 × 10 cm) was made of 15.5 ml 1% agarose in Tris–glycine buffer (0.025 M Tris and 0.2 M glycine pH 8.3). Wells of 4 mm in diameter were made, and to each well 30 μl polysaccharide sample (3 mg/ml in 50 mM Tris–HCl pH 8.0) and 0.8 μl bromophenol blue (3 mg/ml in 50 mM Tris–HCl pH 8.0) were added. Using Tris–glycine buffer as a running buffer, the gel was subjected to electrophoresis in a 2117 multiphor LKB electrophoresis chamber coupled to a 2197 Power supply LKB at 5 V/cm until the dyefront had moved about 4 cm. Then a 0.8 × 5 cm slice of gel around the sample lane was removed and transferred to a Gel Bond film (5 × 7 cm), and a second gel consisting of 3 ml 1% agarose in Tris–glycine buffer and 100 μl Yariv β-glucosyl reagent (0.9 mg/ml in Tris–glycine buffer) was made on top of the first gel for second dimension electrophoresis. Electrophoresis was run at 10 V/cm with Tris–glycine buffer as running buffer. Finally, the gels were washed with 1% NaCl to remove excess reagent.

^{1}H-NMR

The sample was dissolved in D_2O (10 mg/ml) and the ^{1}H-NMR spectrum was acquired on Jeol DX270 with a 0.5 s pulse delay and 1.4 s acquisition time, using 3 K sweep width and 8 K data points. Chemical shifts were set relative to residual water; 4.2 ppm at 90°C and 4.8 ppm at ambient temperature.

^{13}C-NMR

The sample was dissolved in D_2O (25–30 mg/ml) and the ^{13}C-NMR spectra were recorded on Jeol DX270 at 27 or 90°C with a 1.2 s pulse delay, 0.3 s acquisition time and a 90° pulse angle, using 13.4 sweep width and 8 K data points. Chemical shifts were set relative to DMSO (39.6 ppm). DEPT were performed at ambient temperature in full automatic mode based on 5000 scans.

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

^{1}H–^{13}C shift correlated two-dimensional NMR were performed at 25°C, and data were processed by DELTA-NMR software.

Anti-complementary activity

Samples were subjected to a test for anti-complementary activity as previously described by Yamada *et al.* (1985) using a mixture of anti-complementary pectic polysaccharides from *Angelica acutiloba* as positive control.

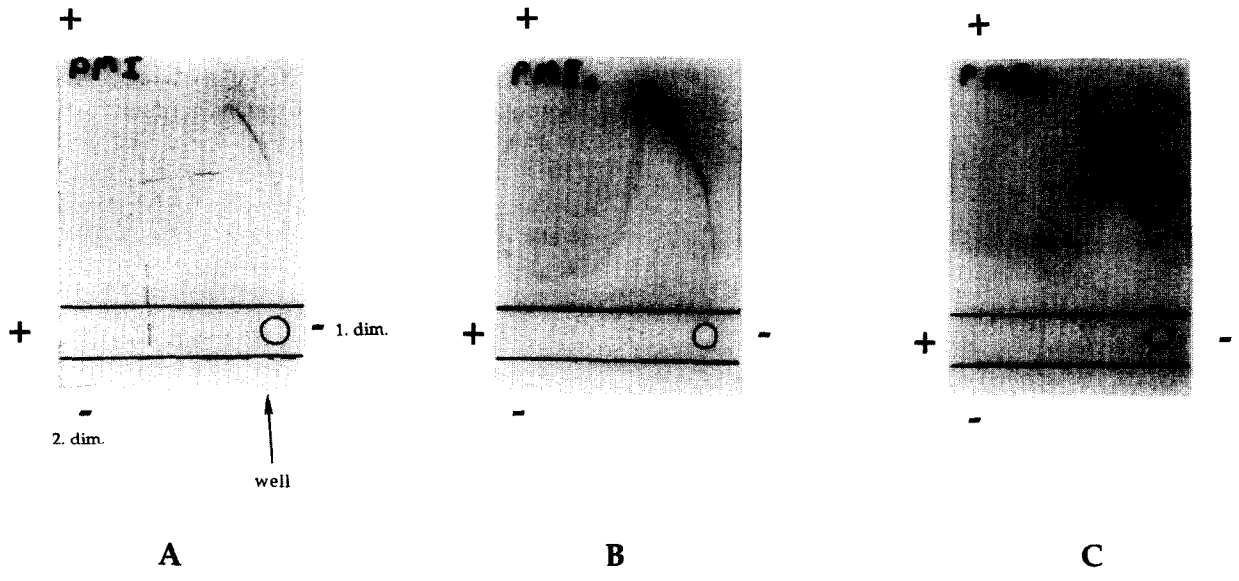

Fig. 2. Precipitation of PMI, PMIa and PMIb with Yariv β-glucosyl reagent by crossed immuno electrophoresis. The first dimension gel was run at 5 V/cm for 1.5 h using 0.025 M Tris and 0.2 M glycine pH 8.3 as running buffer. In the second dimension Yariv β-glucosyl reagent was included in the gel, and electrophoresis was run at 10 V/cm for 3.5 h to achieve a precipitation reaction between polysaccharide and the Yariv reagent. (A) PMI (90 μg); (B) PMIa (110 μg); (C) PMIb (98 μg).

RESULTS AND DISCUSSION

Fig. 2, gel A, shows the precipitation line obtained by crossed immuno-electrophoresis with PMI and Yariv β-glucosyl reagent. The Yariv β-glucosyl reagent is known to precipitate certain types of arabinogalactan proteins, especially those with a high proportion of 1,3, 6-linked galactose residues (Jermyn and Yeow, 1975). A similar but more intense precipitation line was obtained from sub-fraction PMIa (gel B), which contains the major part of the arabinogalactan seen in PMI. PMIa is composed of arabinose (38%), galactose (49%), rhamnose (6%) and galacturonic acid (7%).

The other sub-fraction, PMIb consists of arabinose (31%), galactose (32%), rhamnose (5%), galacturonic acid (8%) in addition to xylose (18%) and glucose (7%). Previously a similar fraction containing 50% xylose was isolated by the same method, but using an analytic Superose 6HR 10/30 column (Samuelsen *et al.*, 1995). According to methylation analysis it consisted of a 1,4-linked xylan. Isolating PMIb using a preparative Sephacryl S-400 HR column gave a similar but not completely identical fraction due to the somewhat different columns used and also due to the heterogeneity of plant polysaccharides in general. The newly isolated PMIb contained an arabinogalactan as well as a xylan as indicated by the positive reaction with Yariv β-glucosyl reagent (Fig. 2, gel C). As seen in Fig. 1, the chromatographic conditions might not have been optimal since the separation of PMIa and PMIb was not at the baseline. Finally, covalent linkages between the different types of polysaccharides might exist and make separation difficult. Solo Kwan and Morvan (1995) have demonstrated the existence of an ether linkage between arabinogalactan type II and a 1,4-linked xylan backbone from a *Silene alba* cell culture.

Anti-complementary activity

PMIa has a higher anti-complementary activity than PMIb (Fig. 3), and the activity increases with increasing polysaccharide concentrations. Since PMIa is the fraction with highest anti-complementary activity, it was submitted to further characterization.

The structure of PMIa

According to the isolation procedure used, PMIa constitutes 2% (w/w) of the carbohydrates in the crude extract. It consists of 93% carbohydrate and 1.5% (w/w) protein. The protein content was determined from the amino acid analysis. According to amino acid analysis the protein consists of 28.7% (mol%) hydroxyproline, 14.9% alanine and 10.9% serine as the main amino acids (Table 1). The amino acid analysis also revealed the presence of 1.9% glucosamine.

The molecular weight of PMIa was estimated to 77–80 kDa, and the material appears quite monodisperse with $M_w/M_n = 1{,}1-1{,}2$.

Only traces of *O*-acetyl groups were detected by GC analysis of the hydrolysate.

According to the methylation results of native PMIa (Table 2, PMIa), it contains a galactan part which is heavily branched: there are 25% 1,3,6-linked galactose residues present. Some 1,3-linked (6%) and 1,6-linked (7%) residues are also found. PMIa contains 38% arabinose which is in

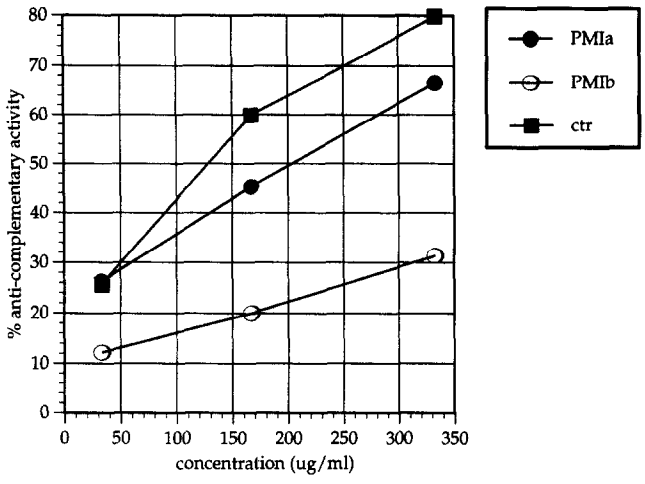

Fig. 3. The anti-complementary activity (%) of PMIa and PMIb. A mixture of anti-complementary pectin polysaccharides from *Angelica acutiloba* were used as positive control.

furanose form. Almost 50% of the arabinose residues are 1,5-linked and the rest are linked terminally. Thus, the average arabinose chains are dimers, but the chains can vary from a single arabinose residue to two or more. A small amount of 1,2 and 1,3-linked arabinose was also detected.

Finally, the galacturonic acid residues are mainly 1, 4-linked and most of the rhamnose are terminal residues. This can be part of a pectin which is either associated with or might be covalently linked to the arabinogalactan.

Table 1. Amino acid composition (mol%) of PMIa. Amino sugars found are included at the bottom

Amino acid	%
Hydroxy proline	28.7
Alanine	14.9
Serine	10.9
Glutamic acid	7.5
Glycine	5.8
Proline	4.7
Valine	4.1
Threonine	4.0
Cysteine	3.7
Leucine	2.9
Arginine	2.8
Methionine	2.0
Isoleucine	1.7
Tryptophan	1.1
Lysine	1.1
Phenylalanine	0.9
Aspartic acid	0.2

Amino sugar	%
Glucosamine	1.9

Table 2. The linkages (mol%) of PMIa and derivatives determined by reduction, methylation and GC–MS. The total mol% of each monosaccharide was determined by methanolysis and GC

Carbohydrate	Linkage	PMIa	H	SD
Ara	T*f*	19.5	3.8	—
	1, 2 + 1, 3	2.6	—	—
	1, 5	15.7	—	—
Total		37.8	3. 8	—
Gal	T	1.7	6.0	2.3
	1, 3	5.6	6.2	21.0
	1, 4	3.9	—	—
	1, 6	7.1	28.9	2.9
	1, 2, 4	0.6	—	—
	1, 3, 6	24.6	8.1	8.0
	1, 4, 6	1.5	—	—
	1, 3, 4	4.4	—	0.5
Total		49.2	49.2	34.7
GalA**	T	0.1	n.d.*	n.d.*
	1, 4	6.0	n.d.*	n.d.*
	1, 2, 4	0.6	n.d.*	n.d.*
Total		6.7	9.8	5.2
Rha	T	5.2	2.0	—
	1, 3	0.9	—	0.5
Total		6.1	2.0	0.5

H = PMIa subjected to weak acid hydrolysis with oxalic acid, the results are correlated to the total amount of galactose found in PMIa.
SD = PMIa subjected to two consecutive Smith degradations, the results are correlated to the amount of galactose in PMIa which theoretically is not degradated by periodate oxidation.
*n.d. = not determined.
**GalA = GalA was reduced with sodium borodeuteride prior to methylation and GC–MS.

Weak acid hydrolysis and Smith degradation

For more detailed structure elucidation, weak acid hydrolysis and Smith degradation were performed.

Weak acid hydrolysis with oxalic acid hydrolyses sugars in furanose form, i.e. arabinose in this case, and the hydrolysis was performed to determine the mode of linkage of arabinose in PMIa. About 10% of the arabinose was left after this hydrolysis. Linkage analyses (Table 2, H) show that there was a relative increase of 1,6-linked galactose residues whereas the amount of 1,3,6-linked residues decreased after hydrolysis. This shows that arabinose was mainly attached to position 3 of 1,6-linked galactose. The amount of 1,3-linked galactose residues remained unchanged, and for that reason arabinose is not attached to those residues in native PMIa.

The 1,3,6-linked galactose rescues left after hydrolysis must be due to branches in the 1,3-linked galactan chains. An increase in terminally linked galactose was seen, indicating that some arabinose residues were attached to the non-reducing end of the 1,6-linked galactan chains. According to these results, PMIa consists of 1,3-linked galactan

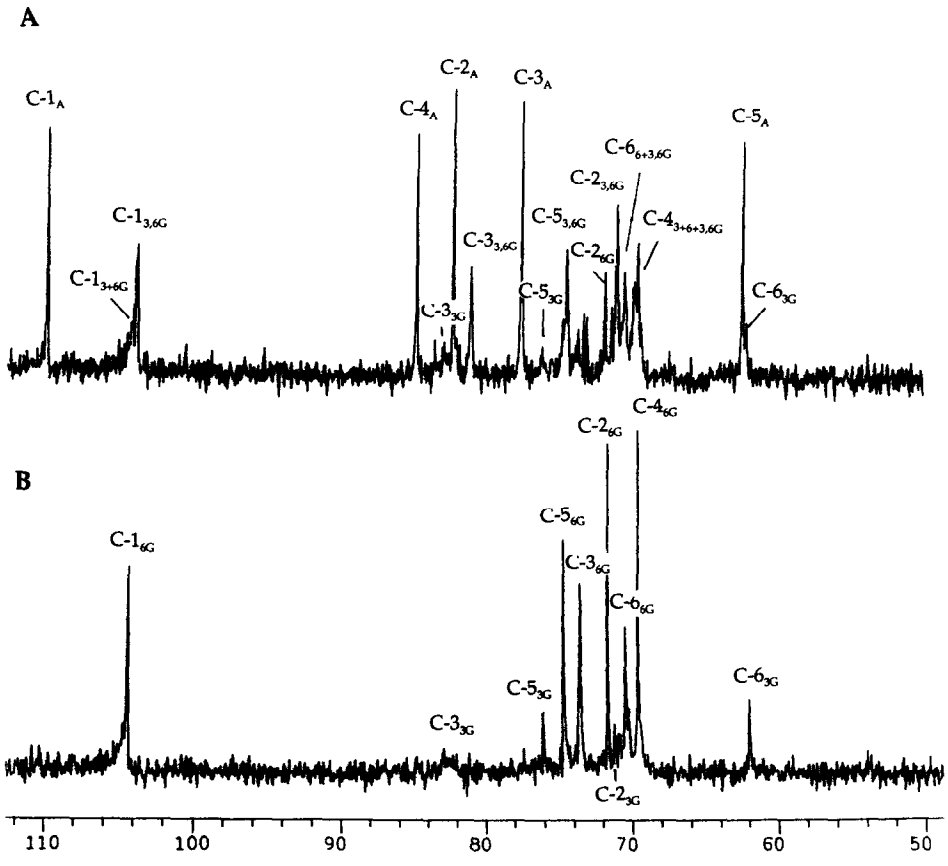

Fig. 4. ^{13}C-NMR spectra of PMIa, an arabinogalactan fraction obtained from *Plantago major* L. where (A) is PMIa and (B) is PMIa after weak acid hydrolysis. C-n refers to the different carbons. Lowercase A, 3,6G and 6G refer to arabinose, 1,3-linked, 1,3,6-linked and 1,6-linked galactose, respectively.

chains with 1,6-linked galactan side chains attached to position 6. Arabinose is linked to position 3 of the 1,6-linked galactan side chains.

In Smith degradation the carbon–carbon linkage between vicinal hydroxyl groups is broken and the hydroxyl groups are oxidized. During the following hydrolysis step the newly formed acetal bonds are hydrolysed. Glycosidic linkages are not hydrolysed except for sugars in furanose form which are hydrolysed under mild conditions. No arabinose was therefore detected after Smith degradation of PMIa; terminally and 1,5-linked arabinose was oxidized, 1,2- and 1,3-linked arabinose was lost by hydrolysis.

The results after two consecutive Smith degradations are shown in Table 2, SD. The 1,3 6-linked galactose residues correspond to the 1,3-linked galactose residues in the back bone with 1,6-linked galactan side chains attached in position 6. In contrast, the 1,3,6-linked galactose residues belonging to the 1,6-linked side chains were oxidized during Smith degradation and are therefore not seen. These results confirm the results from weak acid hydrolysis.

A significant increase in 1,3-linked galactose residues was seen after Smith degradation. This must be due to some galactan residues attached to position 3 of the 1,6-linked galactan side chains. In the original PMIa, these galactose residues are 1,3-linked with 1,6-linked galactose attached to position 6. Summarizing these results, the structure of PMIa seems to be a network formed of 1,3- and 1,6-linked galactan chains with arabinose attached to position 3 of 1,6-linked galactose.

Because little branching in galacturonic acid was observed, no attempts were made to detect it by GC–MS after hydrolysis with oxalic acid. Besides, detection by GC–MS requires reduction prior to methylation, and only a limited amount of material was available.

According to its composition, PMIa is an arabinogalactan–protein (AGP). AGPs are proteoglycans which contain a high proportion of galactose and arabinose residues with $<10\%$ protein which typically consists of high proportions of hydroxyproline, serine and alanine. Stephen (1983) divides arabinogalactans in Type I and Type II arabinogalactans. Type I consists of a 1,4-linked galactose backbone substituted by arabinose. Type II arabinogalactans are composed of a 1,3-linked galactan backbone heavily branched in position 6 with 1,6-linked galactan side chains and arabinose substituted terminally. In contrast to Type I arabinogalactans Type II arabinogalactans are associated with protein, thus the AGPs belong to the latter type.

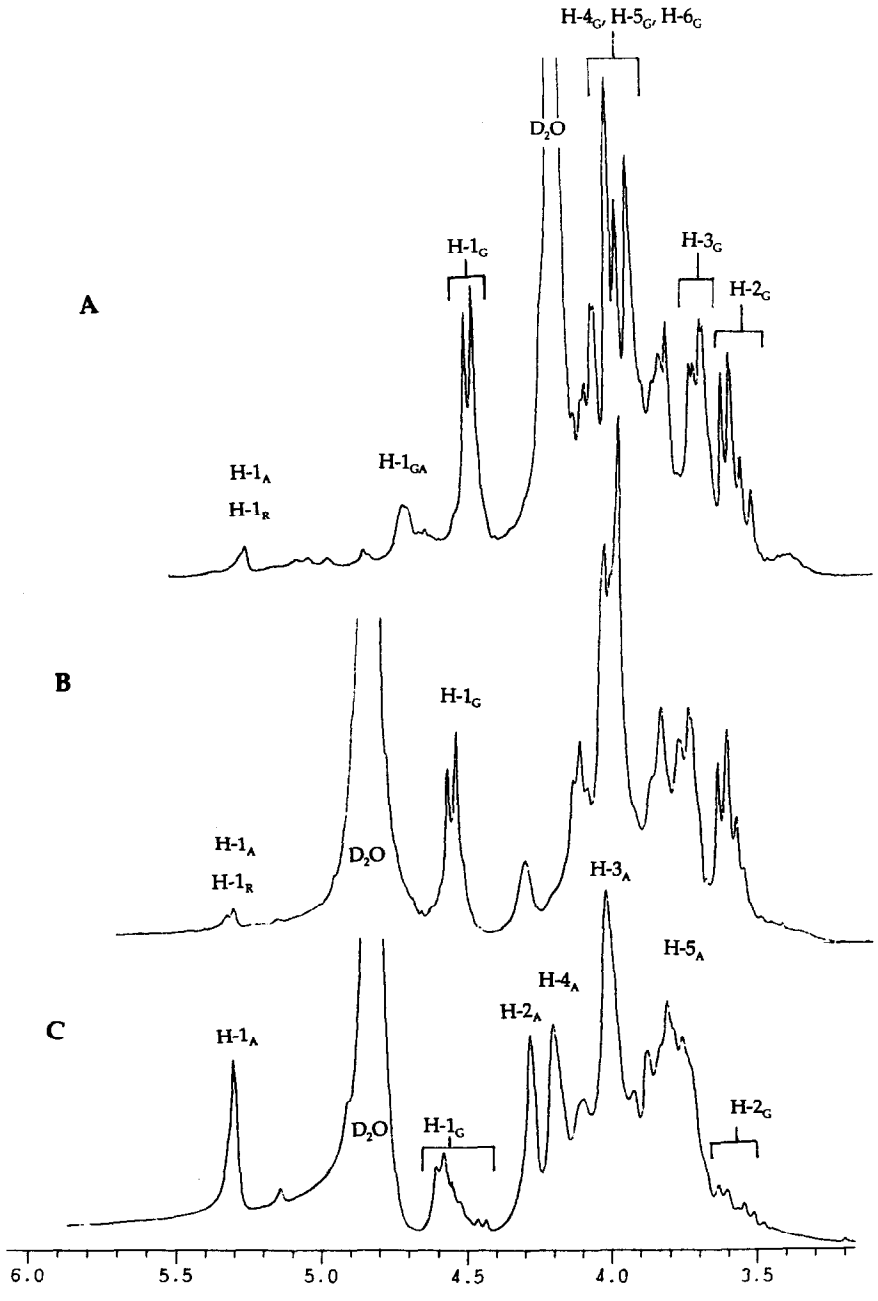

Fig. 5. ^{1}H-NMR spectra of PMIa, an arabinogalactan fraction obtained from *Plantago major* L., where (A) is PMIa after weak acid hydrolysis recorded at 90 °C, (B) is PMIa after weak acid hydrolysis recorded at 27 °C and (C) is the original PMIa recorded at 27 °C. H-n refers to the different proton resonances. Lowercase A, R, GA and G refer to arabinose, rhamnose, galacturonic acid and galactose, respectively.

NMR-spectroscopy

A characteristic region of the ^{13}C-NMR spectrum obtained from PMIa is shown in Fig. 4A. The dominating, well-separated resonances in the spectrum are from terminal arabinose (A) units. The resonances at 110.1, 82.2, 77.4, 84.7 and 62.1 ppm, are from C-1, C-2, C-3, C-4 and C-5, respectively (Cartier et al., 1987). Resonances from corresponding C-1 of 2 and 5-linked arabinose were not detected.

In general resonances originating from galactose (G) are less well defined, due to the occurrence of 1,3-(termed 3G), 1,6-(termed 6G)and 1,3,6-(termed 3,6G) linked units. Other sugar linkages, as detected by methylation analysis (Table 2), may partly overlap or give rise to very low intensity resonances in the spectra which, at present, have not been assigned. The resonances from the anomer carbons of galactose at about 104 ppm are dominated by 3,6G at 104.0 ppm. Partly resolved, less intense anomer signals from 6G and 3G, are observed as a shoulder towards lower field. The C-6 resonances from the different galactose units are split due to the occurrence of some linkages through position 6. The low intensity signal, only partly

resolved from arabinose C-5 at about 61.9 ppm, are from galactose residues with a free C-6, i.e. mostly 3G-type units and terminally linked units. The corresponding C-6 resonances from 1,6-linked galactose, i.e. 6G and 3,6G units are co-occurring at about 70.7 ppm, and are of higher intensities. This confirms the methylation results (Table 2) showing that the major part of the galactose units in PMIa are branched in position 6. According to the methylation analysis the major part of the galactose occurs as 1,3, 6-linked units (3,6G). Resolved signals from these units occur at 80.9 (C-3) and 74.2 ppm (C-5), and in the more crowded regions at 70.8 (C-2) and 69.3 (C-4). Finally, low intensity signals from 3G occurring at about 83.0 ppm and 76.0 ppm are assigned to C-3 and C-5, respectively.

Additional resonances were found at about 18 ppm, typical from CH_3 of rhamnose units (data not shown), but signals from the remaining carbons of rhamnose were not detected by ^{13}C-NMR. Galacturonic acid was not detected by ^{13}C-NMR due to its low content (Table 2) and/or spectral conditions unsuited for quantitative determination of uronic acids.

A modified polysaccharide was obtained after weak acid hydrolysis, giving rise to a much simpler ^{13}C-NMR spectrum (Fig. 4B). The dominating resonances are from 1,6-linked galactose (6G-type units), whereas typical resonances from arabinose are lacking. This confirms the results from the methylation analysis that 1,6-linked galactose is the most abundant linkage found in PMIa after removal of most of the arabinose, and that the latter was linked to position 3 of 1,6-linked galactose. The broad signal at about 82.5 ppm as well as low intensity signals at 76, 71.2 and 61.9 ppm are assigned to C-3, C-5, C-2 and C-6 of 3G-units, respectively.

To confirm some assignments two-dimensional heteronuclear ^{1}H–^{13}C-experiments and DEPT (for C-6) were performed (spectra not shown).

The galactose (measured as C-6) and galactose with a free C-6 ratio determined by ^{13}C-NMR is in quite good agreement with the ratio obtained by methylation and methanolysis–GC analysis (3.7 and 4.0, respectively).

^{1}H-NMR was also performed for the two samples (Fig. 5). Chemical shift values were obtained indirectly via residual water only, which may give rise to deviations from ppm-values published for similar polysaccharides (Gane et al., 1995). Signals from the CH_3 group of rhamnose appeared at about 1.3 ppm (spectral region not shown). A double doublet indicated the occurance of this residue in at least two different linkages/positions.

In Fig. 5 only the typical carbohydrate spectral region is shown. Characteristic features for all spectra are the occurrence of a resonance at about 5.3 ppm, assigned mainly to H-1 of terminal α L-arabinose (A). The occurrence of a low field shoulder is due to H-1 of rhamnose (R) (Colquhoun *et al.*, 1990). This is confirmed by observing the resonances after hydrolysis in spectrum 5B where the rhamnose and arabinose resonances are somewhat better resolved.

H-1 of β D-galactose occur around 4.5 ppm, with a characteristic coupling $J_{1,2b} = 7.3$ Hz (Izumi, 1971). Tentative assignments for H-1 in different liked β D-galactose units, appearing in the spectrum of the original PMIa sample, are marked in Fig. 5C.

Although not confirmed by ^{13}C-NMR, the signal at about 4.65 ppm (also appearing in the spectra of the original sample when run at 90°C) may be attributed to galacturonic acid (CA). This resonance gave a cross peak in the two-dimensional heteronuclear spectrum corresponding to a ^{13}C-NMR resonance at about 100 ppm. Due to the relatively low S/N ratio obtained by limited amount of sample, this spectrum is not included. The galacturonic acid resonance and the anomer signal of galactose ratio is the same as the molar ratio found after methanolysis and GC (0.2).

Other weak signals appearing in the anomeric region, in spectra recorded at 90°C, are not assigned.

Due to the relative low field used (270 MHz for ^{1}H), the remaining signals in the spectra, occurring in the 3.5–4.3 ppm region, are not well resolved. In the 3.85–4.0 ppm region are the partly overlapping unresolved signals from H-4, H-5 and H-6 of galactose. However, some assignments are made based on characteristic splitting, i.e. coupling constants (Knutsen and Grasdalen, 1992; Izumi, 1971). In the spectrum in Fig. 5A, the signal centred at about 3.6 ppm is from H-2 of β D-galactose (6G-units) ($J_{1,2} = 8.1$ Hz and $J_{2,3} = 10$ Hz) and the following at about 3.7 ppm from H-3 of β D-galactose (6G-units) ($J_{2,3} = 10$ Hz and $J_{2,3} = 3.2$ Hz). These assignments were also confirmed by the two-dimensional heteronuclear spectrum.

CONCLUSIONS

Two biologically active polysaccharides have been isolated from the leaves of *Plantago major* L. One has been described earlier as a pectin type polysaccharide (PMII). The second is a Type II arabinogalactan (PMIa) with molecular weight 77–80 kDa. PMIa precipitates with Yariv β-glucosyl reagent and the carbohydrate part consists of arabinose (38%), galactose (49%), rhamnose (6%) and galacturonic acid (7%). PMIa contains 1.5% protein with hydroxyproline, alanine and serine as the main amino acids.

The polysaccharide part of PMIa consists of a network of 1,3-linked galactan chains and 1,6-linked galactan chains: there is a 1,3-linked galactan backbone with 1,6-linked galactan side chains attached to position 6. The side chains are further branched by attachment of 1,3-linked galactose residues which have 1,6-linked galactose residues linked to position 6. Arabinose in furanose form is attached to the galactan mainly through position 3 of the 1,6-linked galactan chains.

PMIa can influence the immune system by anti-complementary activity. For further investigations this arabinogalactan and the pectin PMII should be subjected to immunological testing such as induction of cytokine

production and macrophage activation in addition to complementary activating/inhibitory testing in order to achieve a more definite answer to whether they actually can stimulate wound healing.

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