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Originals

Structural features of water-soluble arabinogalactans from Norway spruce and Scots pine heartwood

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Abstract Isolated water-soluble acidic arabinogalactans from Norway spruce and Scots pine heartwood were analysed and compared to Siberian larch heartwood arabinogalactans. The carbohydrate monomer composition was determined by acid methanolysis and gas chromatography, while structural studies were performed by ^{13}C NMR spectroscopy and methylation analysis. The main structural features were found to be the same in the three types of arabinogalactans. However, the structure of the arabinogalactans from spruce and pine were found slightly different from the structure of larch arabinogalactans. The amount of single unit side-chains, consisting of arabinose and glucuronic acid units, was higher in the spruce and pine arabinogalactans than in the larch arabinogalactans. The amount of glucuronic acid was higher in the spruce arabinogalactans than in the pine arabinogalactans. The pine arabinogalactans had a higher amount of side chains with more than two sugar units than the spruce arabinogalactans.

Introduction

Water-soluble polysaccharides are released and accumulated into process waters in the production of mechanical pulp and wood-containing paper. Especially detrimental in papermaking are anionic polysaccharides, so-called "anionic trash", which can form complexes with various cationic polymers used by the

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paper industry. Acidic arabinogalactans (AG) constitute the main part of the anionic trash released from spruce and pine heartwood in mechanical pulping (Willför, Holmbom 1999; Willför et al. 1999; Thornton et al. 1993; 1994). The molecular structures of water-soluble arabinogalactans from different Larix species have been investigated by e.g. Ponder and Richards (1997; 1997a, b), Karácsonyi et al. (1984), Simionescu et al. (1976) and Odonmazig et al. (1994). Larch arabinogalactans consist of a main chain of β-D-(1→3)-galactopyranose units (β-D-(1→3)-Gal*p*) where most of the main-chain units carry a side chain on C-6 [→3,6)-Gal*p*-(1→]. Almost half of these side chains are β-D-(1→6)-Gal*p* dimers, and about a quarter are single Gal*p* units. The rest contain three or more units. Arabinose is present both in the pyranose (Ara*p*) and furanose (Ara*f*) forms, attached to the side chains as arabinobiosyl groups [β-L-Ara*p*-(1→3)-L-Ara*f*-(1→] or as terminal α-L-Ara*f*. Laine et al. (1999) found 1,3-linked and partly 6-substituted galactans in samples derived from unbleached spruce kraft pulp. The presence of glucuronic acid units in the side chains of larch arabinogalactans has not been properly addressed due to the lack of suitable analytical techniques.

In this study, the structural features of isolated water-soluble acidic arabinogalactans from the heartwood of Norway spruce and Scots pine were investigated and compared to the known structure of Siberian larch heartwood arabinogalactans. The presence of glucuronic acid units was of particular interest.

Materials and methods

Isolation of water-soluble arabinogalactans from heartwood

Stem cross sections of fresh and healthy Norway spruce, Scots pine and Siberian larch trees, grown in southern Finland, were cut and stored at −24 °C. Knot-free parts of the heartwood, with no visible compression wood, were splintered, freeze-dried and ground in a Cyclo-Tec mill (Tecator Inc.). The wood powders were extracted in a Soxhlet apparatus with methyl *tert*-butyl ether (MTBE), to remove lipophilic extractives. The dry matter content of the air-dried wood powders was determined.

Portions of MTBE-extracted wood powder (50 g o.d.) were suspended in 2 l distilled water and the pH was adjusted to about 7 with 0.1 M NaOH (Willför, Holmbom 1999). The suspensions were stirred vigorously at room temperature for 1.5 h with a Vibro-mixer (Chemap AG). These mild extraction conditions were used in order to obtain a selective dissolution of the easily soluble arabinogalactans. The suspensions were vacuum-filtered on a GF 50 glass fiber filter (Schleicher & Schuell). The supernatants were concentrated by vacuum evaporation using a water bath at 40 °C. The concentrated supernatants were then added to technical grade ethanol, the volume percentage ethanol being at least 90, and the polysaccharides were allowed to precipitate overnight. The samples were centrifuged and the supernatants were carefully pipetted off. The precipitates (Spruce AG, Pine AG, Larch AG) were washed with ethanol and MTBE before they were air-, and vacuum-dried.

Isolation of galactoglucomannans from TMP

Extracted TMP (hexane:acetone 9:1) from Norway spruce was suspended in distilled water at 2% consistency (Sundberg et al. 1999). The suspension was stirred with a blade propeller at about 200 min^{-1} and 60 °C for 3 h. The suspension was then filtered on a paper machine wire. The TMP was again suspended in distilled water, stirred as above and filtered. The filtrates from the

first and second stirrings were mixed and centrifuged at 500 g for 30 min. The supernatant was collected and concentrated by vacuum evaporation using a water bath at 40 °C. The concentrate was filtered on a Polycap^{TM} 75AS filter obtained from Whatman with a 0.2 μm pore size to remove colloidal substances. Ethanol was added to the filtrate, the volume percentage of ethanol being at least 80. The polysaccharides were allowed to precipitate. The precipitated polysaccharides were collected and washed twice with ethanol, twice with methanol and once with MTBE. The precipitate, mainly composed of crude acetyl-galactoglucomannans (GM), was finally dried in a vacuum drier.

Analyses

Carbohydrates were analysed by acid methanolysis followed by gas chromatography (GC), according to Sundberg et al. (1996), to determine the amount and sugar composition of hemicelluloses.

^{13}C NMR spectroscopic analysis. The dried precipitates were dissolved in D_2O, using a short ultrasonic treatment. Acetone was added as an internal standard and given a shift of 31.5 ppm (van Hazendonk et al. 1996). ^{13}C NMR spectra were recorded at 323 K with a JEOL JNM-A500 NMR spectrometer, operating at 125 MHz. An inverse gated pulse sequence was used to suppress the nOe enhancement. The pulse angle was 45° and the pulse interval ca. 6 s. These parameters facilitated the use of the signal intensities for the determination of the relative amounts of different carbon atoms in the samples, and a rough estimation of the relative amounts of the units building up the polysaccharides could be made.

Methylation analysis was performed using a modification of the method of Ciucane and Kerek (Ciucane and Kerek 1984, Laine et al. in preparation). About 20 mg ground sodium hydroxide and 100 μl methyl iodide were added to 3–5 mg sample in 500 μl dimethylsulfoxide (DMSO). The sample was kept for 30 min in an ultrasonic bath at room temperature. Distilled water was added to the sample and the water phase was extracted with dichloromethane. The organic phase was extracted three times with distilled water, dried and evaporated. After the methylation, acid methanolysis was performed as described by Sundberg et al. (1996). After that the samples were silylated with 250 μl N,O-bis(trimethylsilyl)trifluoroacetamide containing 5% trimethylchlorosilane and the samples were analysed by GC/MS. About 1 μl of silylated sample was injected via a split injector (HP7863 Series Injector/Autosampler, 260 °C, split ratio 1:50) into a 30 m/0.25 mm HP-5 column (film thickness 0.25 μm) in an HP6890 Series GC System with an HP5973 Series Mass Selective Detector. The temperature program was 100 °C (2 min) – 4 °C/min – 220 °C (2 min) – 15 °C/min – 300 °C (2 min). The carrier gas was helium (1 ml/min, constant flow). The detector conditions were 70 eV, 40–600 amu. The retention times and mass spectra of the methylated and/or silylated monosaccharides were confirmed by comparison with earlier results of model compounds (Laine et al., in preparation).

Response factors of the permethylated and partly silylated monosaccharides were calculated based on molar responses for flame ionisation detection (Verhaar and Wilt 1969). The ratios of molar responses for mass detector and flame ionisation detector were determined experimentally and mean values of 1.40 for permethylated monosaccharides and 1.1 for partly silylated monosaccharides related to sorbitol were applied (Laine et al., in preparation).

Table 1. Weight-% of carbohydrate sugar units in the four studied samples as determined by acid methanolysis and GC

	Gal	Ara	GlcA	Man	Glc	Other
Spruce AG	35	11	8	30	8	8
Pine AG	69	14	4	7	3	3
Larch AG	79	16	1	1	1	1
GM	16	4	2	54	20	3

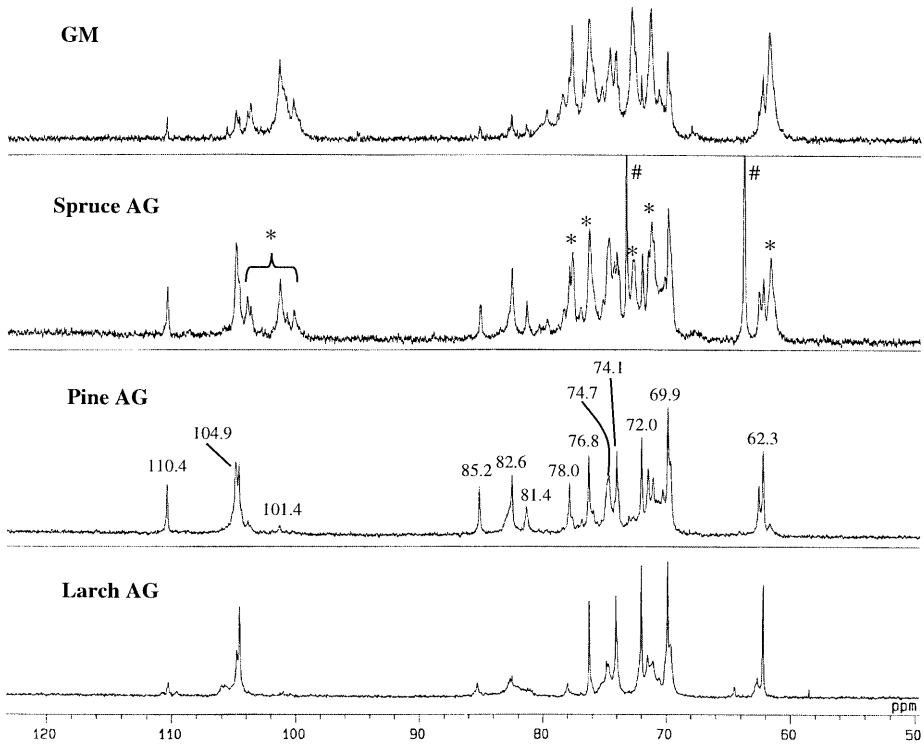

Fig. 1. Quantitative mode ^{13}C NMR spectra of spruce acetyl-galactoglucomannans (GM), spruce (Spruce AG), pine (Pine AG) and larch (Larch AG) arabinogalactans at 50 °C. D_2O was used as solvent and acetone as an internal standard ($\delta = 31.5$ ppm). In the spectrum of Spruce AG, the signals, which are mainly due to glucomannans, are marked with an asterisk *. Unidentified impurities in the spectrum of Spruce AG are marked with #

Results and discussion

Acid methanolysis and GC showed that the Spruce AG sample contained both acidic arabinogalactans and neutral galactoglucomannans, while the Pine AG sample and the Larch AG sample contained mainly acidic arabinogalactans (Table 1). This was also found in an earlier study (Willför and Holmbom 1999). The GM sample isolated from spruce TMP was analysed and found to contain galactoglucomannans and a small amount of arabinogalactans.

^{13}C NMR spectra are shown in Fig. 1. The signals supposed to be derived mainly from GM are marked with an asterisk in the spectrum of Spruce AG. The chemical shifts and signal assignments for the arabinogalactans are given in

Table 2. ^{13}C chemical shifts of the most significant signals in the spectra of the arabinogalactans

Glycosidic linkage	Chemical shifts in ppm[a]					
	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Galp-(1→	104.6	72.0	74.1	69.9	76.4	62.3
→6)-β-D-Galp-(1→	104.6	72.0	73.9	69.9	74.7	70.3
→3, 6)-β-D-Galp-(1→	104.9	71.6	82.6	69.9	74.7	71.1
α-L-Araf-(1→	110.4	81.4	78.0	85.2	62.6	
β-L-Arap-(1→	101.4	69.7	–[b]	–[b]	63.9[c], 64.6[d]	
→3)-α-L-Araf-(1→	108.6[d]	–[b]	–[b]	–[b]	–[b]	

[a] Relative to internal acetone ($\delta = 31.5$ ppm);
[b] Not detected;
[c] In the spectrum of Pine AG;
[d] In the spectrum of Larch AG

Table 3. Mole-% of carbohydrate monomers as determined by methylation analysis; T = terminal non-reducing end unit

	Gal				Ara	Man			Glc
	T	1,3	1,6	1,3,6	T	T	1,4	1,4,6	1,4
Spruce AG	7.1	1.4	1.2	3.7	3.1	2.0	61.5	4.6	15.3
Pine AG	26.0	8.3	6.9	23.9	17.3		13.2	1.0	3.5
Larch AG	36.8	2.3	14.6	26.6	19.7				

Table 2. The assignments were based on the data published by Ponder et al. (1997a) on arabinogalactans isolated from Western larch (*Larix occidentalis*).

The three arabinogalactan samples were also analysed by methylation analysis and the results are given in mole percent in Table 3. The quantification gives approximate ratios between the structural elements in one sample. We experienced some solubility problems of the arabinogalactans in the alkaline solvent (DMSO) during the methylation. Consequently, the results may not be fully representative for the sample as a whole. We cannot exclude that the structure of the DMSO-soluble fraction of the arabinogalactans differs from the D_2O-soluble fraction analysed by ^{13}C NMR spectroscopy. The methylation analysis did not give information about acidic groups, which were not identified in the gas chromatograms.

Galactose units

The ^{13}C NMR results showed that the water-soluble arabinogalactans all have a main chain consisting of β-D-(1→3)-Gal*p*-units. The main-chain units are mainly (1→6)-linked to side chains of galactose, arabinose or glucuronic acid units. The Gal*p* monomer unit ratios, based on the ^{13}C NMR analysis, in the three AG-samples are given in Table 4. The ratios of the signals at $\delta = 82.6$ ppm [→3,6)-Gal*p*-(1→, C-3] and $\delta = 62.3$ ppm [β-D-Gal*p*-(1→, C-6] were used for the determination of the monomer unit ratio →3,6)-Gal*p*-(1→/Gal*p*-(1→. From the signals at $\delta = 102.6$–106.1 ppm the monomer unit ratio →3,6)-Gal*p*-(1→/Gal*p*-(1→+→6)-Gal*p*-(1→ could be determined. Combining the information from these signals facilitated the determination of the ratios →3,6)-Gal*p*-(1→/→6)-Gal*p*-(1→, Gal*p*-(1→/→6)-Gal*p*-(1→ and Gal*p*-(1→/→3,6)-Gal*p*-(1→+→6)-Gal*p*-(1→.

Table 4. Monomer unit ratios in the samples, based on ^{13}C NMR

Ratios	Monomer unit ratios		
	Spruce AG	Pine AG	Larch AG
→3, 6)-Gal*p*-(1→/Gal*p*-(1→	2.5/1	2.1/1	2.0/1
→3, 6)-Gal*p*-(1→/Gal*p*-(1→+→6)-Gal*p*-(1→	2.25/1	1.7/1	1.4/1
→3, 6)-Gal*p*-(1→/→6)-Gal*p*-(1→	31.1/1	2.8/1	20.6/1
Gal*p*-(1→/→6)-Gal*p*-(1→	12.7/1	0.9/1	3.9/1
Gal*p*-(1→/→3,6)-Gal*p*-(1→+→6)-Gal*p*-(1→	0.4/1	0.3/1	0.4/1

The monomer unit ratios suggest that almost 70% of the galactose in the Spruce AG sample is present in a main chain as [→3,6)-Gal*p*-(1→]-linked units. For the Pine AG sample this number is about 55% and for the Larch AG sample about 65%. The percentage of terminal [Gal*p*-(1→]-units is for the Spruce AG, Pine AG and Larch AG samples 28%, 22% and 28%, respectively. The percentage of [→6)-Gal*p*-(1→]-linked non-terminal side chain units is as much as 23% for the Pine AG sample, while for Spruce AG it is only 2% and for Larch AG 7%. It is therefore likely that the Pine AG sample has longer side chains, with at least two [→6)-Gal*p*-(1→]-linked non-terminal side chain units.

We were not able to show the presence of any unsubstituted (1→3)-linked Gal*p*-units by ^{13}C NMR analysis, based on comparison with NMR data published by Usov et al. (1997). However, the methylation analysis (Table 3) clearly showed the presence of such units. Slightly more than 10% of the Spruce AG and Pine AG and under 3% of the Larch AG galactose was unsubstituted (1→3)-linked Gal*p*-units. However, these numbers are approximate, since the yield of the methylation analysis was low, as mentioned earlier. For larch arabinogalactans, Ponder and Richards (1997b) suggested the presence of about 4% of unsubstituted (1→3)-linked Gal*p*-units.

Arabinose units

In all samples, arabinose was present as terminal α-L-Ara*f* end groups attached to the galactopyranose-backbone. Earlier it has been shown by Ponder and Richards (1997b) that arabinose is present as terminal Ara*f* or as arabinobiosylgroups [β-L-Ara*p*-(1→3)-α-L-Ara*f*-(1→]. According to Karácsonyi et al. (1984), Odonmazig et al. (1994) and Ponder and Richards (1997a), C-5 in Ara*p* units absorbs at $\delta = 63.8$, 64.5 or 64.4 ppm, respectively. There was no signal detected for the Spruce AG sample, which could be assigned to Ara*p* units. For the Pine AG sample there was a weak signal at $\delta = 64.1$ ppm that indicated the presence of Ara*p* units. The intensity of the signal as compared with the total of the anomeric carbons (excluding Ara*p*) was Ara*p* C-5/-O-*C*-O- ≈ 1/93.6, corresponding to an amount of ca. 1% Ara*p* units. For the Larch AG sample there was a weak signal at $\delta = 64.6$ ppm that indicated the presence of Ara*p* units. The intensity of the signal as compared with the total of the anomeric carbons (excluding Ara*p*) was Ara*p* C-5/-O-*C*-O- ≈ 1/18.0, corresponding to an amount of ca. 5% Ara*p* units. The methylation analysis showed the presence of terminal Ara*f* but also weak signals that could be assigned to Ara*p* units.

The intensity ratios of the signals at $\delta = 110.3$ ppm (Ara*f*, C-1) and $\delta = 102.6$–106.1 ppm (Gal*p*, C-1) were used to determine the Ara*f*/Gal*p* ratio. The contribution of the GM signals was subtracted from the Gal*p* signals in the Spruce and

Pine AG samples. The calculated ratio Ara*f*/Gal*p* for the Spruce AG sample was found to be ≈0.21/1. Ca. 6% of the signals from anomeric carbons remained unidentified (including Ara*p*, C-1). The calculated ratio Ara*f*/Gal*p* for the Pine AG sample was found to be ≈0.26/1. Ca. 3% of the signals from anomeric carbons remained unidentified (including Ara*p*, C-1). The calculated ratio Ara*f*/Gal*p* for the Larch AG sample was found to be ≈0.14/1. Ca. 3% of the signals from anomeric carbons remained unidentified. These numbers are well in agreement with the ones obtained from the methylation analysis, except for the Larch AG sample. The calculated ratio Ara*f*/Gal*p* for the Larch AG sample was ≈0.25/1 according to the methylation analysis.

Glucuronic acid units

The amount of acidic groups (GlcA) was determined as the relative intensities of the signals of *C*=O (δ = 173.6–175.1 ppm) and the anomeric carbon (C-1). The sample of Spruce AG contained some GM and the spectrum contained strong signals, which could be assigned to acetyl carbons in spruce glucomannans. The intensity of the signal of *C*=O was determined by manual subtraction of the intensity of the corresponding GM signal. The contribution of GM to the carbonyl signal was estimated by first determining the intensity ratio of the signals at δ = 97.6–102.6 ppm and the carbonyl signal in the spectrum of GM. The ratio was found to be 2.53/1. Then the intensities of the corresponding signals in the spectrum of Spruce AG were determined. In this way the ratio *C*=O/C-1 was found to be ≈0.08, corresponding to an amount of ca. 7–8% uronic acid units. As the Pine AG sample also contained small amounts of GM, the contribution of GM to the carbonyl signal was first determined. Determined in the same way as described for the Spruce AG sample the ratio *C*=O/C-1 was found to be ≈0.02, corresponding to an amount of ca. 2% uronic acid units. No signals from carboxyl carbons could be detected in the Larch AG sample. This was probably due to the low amount of GlcA (Table 1).

Suggested major structural features for spruce and pine arabinogalactans

The major structural conclusions in this study are drawn from the ^{13}C NMR analysis. In addition, the methylation analysis and the carbohydrate monomer composition provided some important structural features: e.g the presence of unsubstituted (1→3)-bonded Gal*p*-units was shown by the methylation analysis and the presence of GlcA in the Larch AG sample was seen from the carbohydrate monomer composition. The amounts of these units were probably under the limit of detection for the NMR analysis.

Figure 2 shows a suggested average structure for water-soluble Norway spruce arabinogalactans. The structure is probably slightly different for different molar masses and that the mass range of these samples was quite broad (Willför and Holmbom 1999). An average molar mass of about 22 kdaltons corresponds to a degree of polymerisation (DP) of 130–140. It is therefore unlikely that the different types of side chains will all be present in one single molecule. However, a typical spruce arabinogalactan molecule has a shorter main chain than a typical larch arabinogalactan molecule. It has also more single unit side chains than larch arabinogalactan and a higher amount of these are acidic. The low amount of [→6)-Gal*p*-(1→]-units suggests that the probability of side chains with more than two units is very low.

Figure 3 shows a suggested average structure for water-soluble Scots pine arabinogalactans. As mentioned above, the DP (190–200) and broad mass range

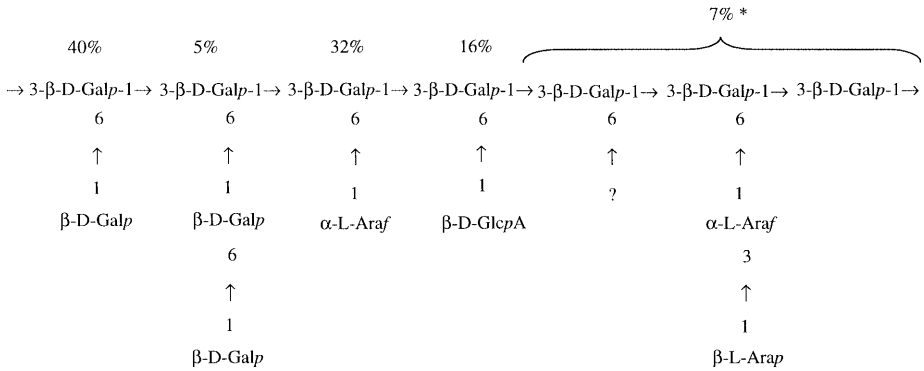

Fig. 2. Suggested major structural features of a typical spruce arabinogalactan molecule. The probability and ratio of the different side chains probably varies with the molar mass and between different molecules. *The ^{13}C NMR analysis did not show the presence of any arabinobiosyl groups or unsubstituted galactose units in the main chain. The presence of these groups was shown by the methylation analysis. About 7% of the sugar units remain undefined

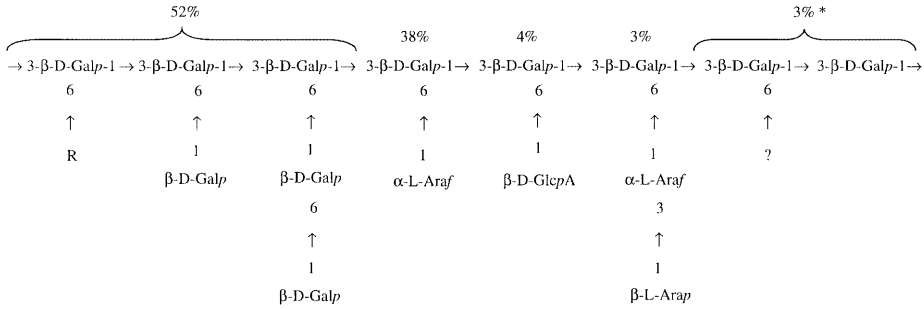

Fig. 3. Suggested major structural features of a typical pine arabinogalactan molecule. The probability and ratio of the different side chains probably varies with the molar mass and between different molecules. *The ^{13}C NMR analysis did not show the presence of any unsubstituted galactose units in the main chain. The presence of these groups was shown by the methylation analysis. About 3% of the sugar units remain undefined. R = side-chains containing three or more β-D-Gal*p* unit

Table 5. Molar sugar unit ratios of the isolated AG samples determined with the different analytical techniques

	Acid methanolysis and GC Gal:Ara:GlcA	Methylation analysis Gal:Ara	^{13}C NMR Gal:Ara:GlcA
Spruce AG	2.6:1:0.6	2.8:1	4.8:1:0.5
Pine AG	3.9:1:0.2	3.7:1	3.7:1:0.1
Larch AG	4.1:1:0.04	4.1:1	5.0:1:–

suggest a variable side chain structure for single molecules. The typical pine arabinogalactan molecule is probably slightly shorter than the spruce molecule. There are less acidic side chains and the relatively high amount of [→6)-Gal*p*-

(1→]-units suggests that the probability of side chains with more than two units is high. It was not possible to suggest the ratio of the three Gal*p*-type side chains.

Sugar unit ratios

The sugar unit ratios of the isolated AG samples determined by acid methanolysis and GC, methylation analysis, and ^{13}C NMR spectroscopy were in good agreement (Table 5). The Gal:Ara ratio for Spruce AG, which was determined by ^{13}C NMR, was high. This may be explained by the unidentified signals from the anomeric carbons. Some arabinogalactans were lost during the isolation procedure. Therefore, it is likely that the previously reported ratios of 3.6:1:0.8 for spruce, 3.8:1:0.2 for pine, and 5.6:1:0.08 for larch are more representative for the total water-soluble arabinogalactans (Willför, Holmbom 1999).

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