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Structural studies of the pectic polysaccharide from Siberian fir (*Abies sibirica* Ledeb.)

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ABSTRACT

The pectic polysaccharide named abienan AS-A was isolated from the wood greenery of *Abies sibirica* using dilute hydrochloric acid (pH 4.0) at 70 °C. The structure of abienan AS-A was elucidated using sugar composition analysis, ion-exchange chromatography and partial acid hydrolysis followed by NMR spectroscopy. The linear region of abienan AS-A was shown to contain linear 1,4-α-D-galactopyranosyluronan partially substituted with methyl esters or 3-*O*-acetyl groups and rhamnogalacturonan blocks consisting of 1,4-α-D-galacturonan partially substituted with methyl ester groups and connected by 2-*O*-substituted α-rhamnopyranose residues. The branched region of abienan AS-A was found to be made of RG-I. The side chains of RG-I were shown to contain 1,4-β-galactan and branched arabinan. Some 4-*O*-substituted β-galactopyranose residues were shown to be attached to the 4-position of the 2-*O*-substituted α-rhamnopyranose residues of the RG-I backbone. The arabinan groups were made up of a 1,5-linked α-L-arabinofuranan backbone that was 3-*O*-, 2-*O*-, and 2,3-di-*O*-substituted with the terminal and 1,3-linked α-L-arabinofuranose residues.

1. Introduction

Pectins belong to a class of high molecular weight polysaccharides that are a part of the intercellular space and the cell walls of plants along with cellulose and hemicellulose (Caffall & Mohnen, 2009; Ovodov, 2009; Ridley, O'Neill, & Mohnen, 2001).

Wood has been found to contain only small amounts of pectic substances (*ca.* 0.5–1.5%) (Thornber & Northcote, 1961a). The amounts of water-soluble polysaccharides and their sugar compositions have been estimated for the heartwood and sapwood of *Abies balsamea*, *Abies sibirica*, *Larix sibirica*, *Larix lariciana*, *Pinus banksiana*, *Pinus resinosa*, *Pinus silvestris*, and *Picea abies*. The largest quantities of pectic polysaccharides have been discovered in the heartwood of *A. balsamea*, *A. sibirica* and *Thuja occidentalis*. Acidic arabinogalactans containing various amounts of galactose, arabinose and glucuronic acid residues have been detected as constituents of the heartwood of larch (*L. sibirica* and *L. lariciana*), fir (*P. abies*), and pine (*P. banksiana*, *P. resinosa*, and *P. silvestris*) (Willfor, Sundberg, Hemming, & Holmbom, 2005). The carbohydrate composition of phloem, cambium, heartwood and sapwood from *Pinus ponderosa* has also been estimated. The highest quantities of pectic polysaccharides have been discovered in the phloem and cambium of *P. ponderosa* (8–10%) (Thornber & Northcote, 1961a, 1961b).

Biological processes are most active in the wood greenery of conifers. The largest amounts of metabolites used to build plant mass over long-term cycles are shuttled predominantly to these plant tissues (Little, 1970; Robakidze & Bobkova, 2003; Robakidze & Patov, 2000). Thus, wood greenery is a raw material rich in energy and biologically active substances as well as of strong interest for scientific research. The structural studies of pectins and other related polysaccharides of conifer storage organs have been limited to the elucidation of their sugar compositions (Ovodova, Kushnikova, Popov, & Bushneva, 1997; Robakidze & Patov, 2000). The main neutral sugar constituents of pectins from the needles of *Picea obovata*, *A. sibirica*, *Pinus sibirica* and *L. sibirica* have been shown to be arabinose and galactose residues, which have been confirmed by paper chromatography (Ovodova et al., 1997).

The Siberian fir *A. sibirica* Ledeb. is widespread in the northeast regions of European Russia and in West and East Siberia. Siberian fir extracts have been used for many years in medicine as a therapeutic for diseases (Koctesha, Luk'yanenok, & Strelis, 1997). The pectic polysaccharide termed abienan was isolated from Siberian fir wood greenery using consecutive extraction with water and dilute hydrochloric acid. Abienan was found to exert a significant influence on the germination and speed of seed sprouting as well as the growth of roots and plantlets of soft wheat *Triticum aestivum* L. (Makarova, Patova, Mikhailova, & Demin, 2011). The present paper

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presents structural studies of abienan AS-A extracted from Siberian fir wood greenery using dilute hydrochloric acid.

2. Materials and methods

2.1. Processing of plant raw material and isolation of pectic polysaccharides

Wood greenery from Siberian firs was collected in October 2010 near Syktyvkar (Komi Republik, Russia). The isolation and purification of abienan AS-A were performed according to a procedure described previously (Makarova et al., 2011). The fresh plant material was homogenised using a knife mill RM-120 (particle size 0.5–15 mm, Russia) and successively extracted with ethyl acetate and chloroform to remove low molecular weight contaminants. The residual raw material (100 g) was treated with distilled water at 70 °C for 2 h to afford abienan AS-W. The residual material was treated with dilute hydrochloric acid at pH 3.8–4.2 with rigorous stirring and heated to 70 °C for 2 h. The extract was filtered, concentrated and centrifuged. The supernatant was collected and precipitated with four volumes of 96% ethanol. The precipitate was separated by centrifugation and redissolved in water. Dialysis and lyophilisation furnished abienan AS-A (3.5 g).

The residual supernatant was concentrated and precipitated with fifteen volumes of 96% ethanol. The precipitate was separated by centrifugation and redissolved in water. Dialysis and lyophilisation provided the polysaccharide fraction (130 mg). A small amount of this fraction (40 mg) was subjected to further purification using gel filtration chromatography on a Sephacryl S-300 to afford two peaks. Fractions corresponding to those peaks were collected and lyophilised to furnish the polysaccharide AS-A-S (31.2 mg) and a minor polysaccharide fraction (3.3 mg).

2.2. General analytical methods

The uronic acid content was determined by reacting the material with 3,5-dimethylphenol in concentrated sulphuric acid (Usov, Bilan, & Klochkova, 1995) and measuring absorbance at 400 and 450 nm using D-galacturonic acid (Sigma–Aldrich) as a standard. Protein concentration was calculated using the Bradford procedure (Bradford, 1976) with bovine serum albumin (BSA) as a standard. The amount of methyl ester groups was determined as previously described (Wood & Siddiqui, 1971) at 412 nm using methanol as a standard. Spectra were measured on an Ultrospec 3000 spectrophotometer (UK). The specific optical rotations were determined on a Polatronic MHZ polarimeter (Germany).

The solutions were concentrated on a rotary evaporator under reduced pressure at 40–45 °C and centrifuged at 5000–6000 rpm for 10–20 min. Samples were lyophilised.

Molecular weights and polydispersities of the polysaccharide fractions were determined by high pressure liquid chromatography (HPLC) as previously described (Khramova et al., 2011). The chromatographic system (Shimadzu, Japan) used for analysis consisted of an LC-20AD pump, a DGU-20A3 degasser, a CTO-10AS thermostat, an RID-10A refractometer as the detector, and a Shodex OH-pak SB-804 HQ column (Shimadzu, Japan). Pullulans from Fluka, Germany (1.3, 6, 12, 22, 50, 110, 200, 400, 800 kDa) were used as standards. Gel filtration chromatography was carried out on a Sephacryl S-300 column (1.3 cm × 37 cm, a void volume of 12 ml, Sigma, USA). Distilled water was used as the eluent at a flow rate of 0.25 ml/min and 3 ml fractions. The presence of carbohydrates in the fractions was determined using the phenol–sulphuric acid procedure (Smith's procedure) with photocolorimetry carried out at 412 nm (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Fractions corresponding to separate peaks were combined, concentrated, dialysed and lyophilised.

NMR spectra were obtained on a Bruker Avance II-300 MHz (Bruker, Germany) using 3–5% solutions of polysaccharides in D_2O (99.9 atom% D, Sigma–Aldrich) containing 0.75% 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid, sodium salt (TMSP, Sigma–Aldrich) or acetone (Sigma–Aldrich) at 303 and 328 K. The samples were lyophilised twice from a D_2O (99.9 atom% D, Sigma–Aldrich) solution before each experiment. Chemical shifts are expressed in parts per million (ppm) relative to internal TMSP at 0.00 ppm or acetone (δC 30.89 ppm, δH 2.225 ppm). NMR signals were assigned using two-dimensional (2D) experiments (TOCSY, COSY, ROESY, and ^{1}H and ^{13}C HSQC). Two-dimensional spectra were recorded using the manufacturer's procedures. The ROESY experiments were conducted using a mixing time of 60 ms. A 90 ms MLEV17 spin-lock time for the TOCSY experiments was used.

2.3. Monosaccharide analysis

The samples (3–5 mg) were hydrolysed with 2 M TFA (1 ml) containing *myo*-inositol (1 mg/ml) at 100 °C for 5 h. The mixture of neutral monosaccharides was converted to alditol acetates (York, Darvill, McNeil, Stevenson, & Albersheim, 1985) and identified by gas–liquid chromatography (GLC) on a Varian 450-GC chromatograph (United States) equipped with a flame ionisation detector using a capillary column RTX-1 as previously described (Khramova et al., 2011). The absolute configuration of arabinose was determined by GLC of the acetylated (S)-2-octyl glycoside (Leontein & Lonngren, 1993).

2.4. Ion-exchange chromatography of abienan AS-A on DEAE-cellulose

Abienan AS-A (200 mg) was dissolved in 0.01 M NaCl (3 ml) and fractionated on a column of DEAE-cellulose OH^{-}-form (34.5 cm × 2.2 cm). Fractions were eluted consecutively with 0.01, 0.1, 0.2, 0.3, 0.4 and 0.5 M NaCl at a flow rate of 1 ml/min. Fractions were collected and analysed by Smith's procedure (Dubois et al., 1956). Fractions corresponding to separate peaks were combined, concentrated, dialysed and lyophilised. The five polysaccharide fractions were as follows: AS-A-D-0 (eluted with 0.01 M NaCl, 11.9 mg), AS-A-D-1 (eluted with 0.1 M NaCl, 12.7 mg), AS-A-D-2 (eluted with 0.2 M NaCl, 82.8 mg), AS-A-D-3 (eluted with 0.3 M NaCl, 63.6 mg), and AS-A-D-4 (eluted with 0.4 M NaCl, 7.2 mg).

2.5. Partial acid hydrolysis of abienan AS-A

Abienan AS-A (700 mg) was heated with 0.05 M TFA (130 ml) at 70 °C for 4 h (Fig. 1). Excess acid was removed from the reaction mixture by evaporation, and the polysaccharides were precipitated with four volumes of 96% ethanol. The precipitate was separated by centrifugation and washed with ethanol. The purified precipitate was dissolved and lyophilised to furnish the polysaccharide fragment AS-A-H (560 mg). The mixed aqueous-alcoholic supernatant was concentrated and precipitated with fifteen volumes of 96% ethanol. The precipitate was separated by centrifugation and washed with ethanol, dissolved and lyophilised to furnish the carbohydrate fraction (46 mg). The carbohydrate fraction was subjected to a further purification using gel filtration chromatography on a Sephacryl S-300 column to afford two peaks (Fig. 1). Fractions corresponding to those peaks were collected and lyophilised to furnish the homogeneous polysaccharide fraction AS-A-H-1 (25.7 mg) and the minor polysaccharide fraction (4.6 mg). Alcoholic supernatants were mixed and concentrated to furnish the mono/oligosaccharide fraction (67.0 mg). The monosaccharide composition of the fractions was determined using GLC analysis.

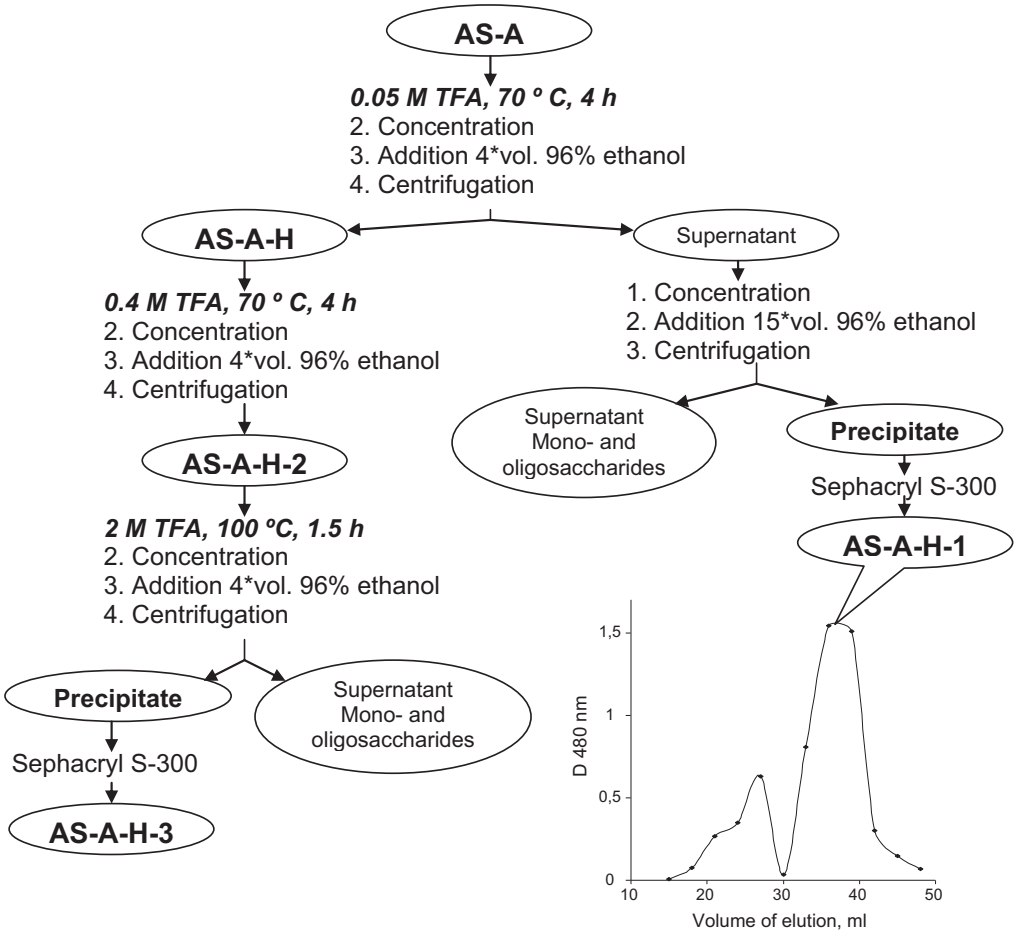

Fig. 1. Schema of partial acid degradation of abienan AS-A.

The polysaccharide fragment AS-A-H (540 mg) was heated with 0.4 M TFA (100 ml) at 70 °C for 4 h (Fig. 1). The mixture was processed as described earlier for AS-A-H to obtain the polysaccharide fragment AS-A-H-2 (430 mg). AS-A-H-2 (400 mg) was heated with 2 M TFA (70 ml) at 100 °C for 1.5 h. The mixture was treated as described earlier for AS-A-H to obtain the galacturonan AS-A-H-3 (150 mg). Alcoholic supernatants were mixed and concentrated to furnish the mono/oligosaccharide fraction (230 mg). The monosaccharide composition of the fractions was determined using GLC analysis.

2.6. Identification of galacturonic acid

Abienan AS-A (50 mg) was dissolved in water (18 ml), and the aqueous solution (0.2 ml) of exo- and endo-1,4-α-D-polygalacturonase (2 mg, activity 500 U/mg, EC 3.2.1.15; Fluka, Germany) was added. The mixture was incubated at 37 °C for 4 h. The following treatment was carried out as described earlier (Makarova et al., 2011). The 1,4-α-D-polygalacturonase was inactivated by boiling at 100 °C and removed by centrifugation. The supernatant was concentrated and precipitated with four volumes of 96% ethanol to obtain the polysaccharide fragment (24.8 mg). Alcoholic supernatant was concentrated to furnish the mono/oligosaccharide fraction. The monosaccharide composition of the fraction was determined using paper chromatography (Filtrak FN-12, butanol–pyridine–water 6:4:3). The sugars were detected by spraying with a solution of aniline hydrogen phtalate following heating at 105 °C.

2.7. Partial acid hydrolysis of polysaccharide AS-A-S

The polysaccharide AS-A-S (90 mg) was heated with 0.01 M TFA (13 ml) at 50 °C for 1 h. Excess acid was removed from the reaction mixture by evaporation, and the polysaccharides were precipitated with fifteen volumes of 96% ethanol. The precipitate was separated by centrifugation and washed with ethanol. The purified precipitate was dissolved and lyophilised to furnish polysaccharide fragment AS-A-S-H-1 (25 mg). The mixed aqueous-alcoholic supernatant was concentrated and precipitated with fifteen volumes of 96% ethanol. The precipitate was separated by centrifugation and washed with ethanol. The residue was then redissolved and lyophilised to furnish polysaccharide fragment AS-A-S-H-2 (34 mg). The fragment AS-A-S-H-2 (30 mg) was heated with 2 M TFA (5 ml) at 100 °C for 1 h. The hydrolysed product was evaporated to dryness, and the carbohydrates were isolated using preparative paper chromatography (Whatman no. 3, butanol–pyridine–water 6:4:3).

3. Results and discussion

3.1. Isolation of abienan AS-A

Crude abienan AS-A was obtained with a yield of 3.5% from air-dried plant material, whereas the pectic substances from *A. sibirica* heartwood have been isolated with a yield of 0.3% (Willfor et al., 2005).

Complete acid hydrolysis of abienan AS-A with 2 M TFA gave the following monosaccharides in the hydrolysate: galacturonic

Table 1
Yields and composition (%, w/w) of abienan fractions.

Fraction	Yield, % of AS-A	$[\alpha]_D^{20}$	Mw, kDa	Mw/Mn	Protein	GalpA	OMe	Neutral monosaccharides					
								Rha	Ara	Xyl	Man	Glc	Gal
AS-A	3.5[a]	n.d.	n.d.	n.d.	3.0	67.6	4.0	2.4	10.0	0.4	2.4	2.2	7.0
AS-A-S	0.1[a]	−65	12.0	1.8	1.8	9.7	0.5	1.6	48.0	0.7	6.8	3.7	3.6
AS-A-S-H-1	27.7[b]	−68	n.d.	n.d.	1.2	10.0	n.d.	1.3	51.4	0.8	11.8	5.3	4.2
AS-A-S-H-2	37.8[b]	−61	n.d.	n.d.	1.4	6.4	n.d.	1.8	61.3	1.0	6.0	3.3	4.7
AS-A-D-0	5.9	+4	20.0	3.1	3.4	5.9	n.d.	2.3	45.8	0.9	12.0	8.5	11.4
AS-A-D-1	6.4	n.d.	21.0	2.9	5.1	21.0	n.d.	3.1	14.5	0.8	2.4	5.8	22.2
AS-A-D-2	41.4	+196	111.0	5.6	3.5	60.0	n.d.	4.4	7.4	0.4	1.7	1.7	9.0
AS-A-D-3	31.8	+296	140.0	8.6	3.3	87.0	n.d.	3.5	1.9	0.2	0.2	0.4	1.6
AS-A-D-4	3.6	n.d.	n.d.	n.d.	6.1	74.0	n.d.	5.3	2.5	0.2	0.8	3.7	3.0
AS-A-H	80.0	n.d.	n.d.	n.d.	3.0	68.3	n.d.	2.4	3.5	0.2	2.6	2.2	7.0
AS-A-H-1	3.7	+51	13.0	1.7	4.3	29.0	1.4	5.2	15.2	1.4	3.9	5.9	18.6
AS-A-H-2	63.0	n.d.	n.d.	n.d.	2.7	74.5	1.5	2.0	0.6	0.2	2.3	2.5	8.3
AS-A-H-3	24.0	+336	54.0	2.7	3.1	95.0	1.2	–	–	–	0.2	0.5	–

[a] Yields of the abienan AS-A and polysaccharide AS-A-S are calculated from the air-dried plant material.
[b] Yields are calculated from the polysaccharide AS-A-S; n.d., not determined.

acid (GalA), galactose (Gal), arabinose (Ara), and rhamnose (Rha) (Table 1). These sugars are the main constituents of the macromolecule carbohydrate chains and are typical components of pectins.

Identification of the galacturonic acid residues was observed during treatment of abienan with 1,4-α-D-polygalacturonase, which possesses both endo- and exo-activities. Digestion of AS-A with enzyme yielded in quantity of free galacturonic acid and oligogalacturonides that were detected by paper chromatography in the supernatant after digestion. This should be assigned to the D-galacturonic acid residues of the 1,4-α-linked backbone of abienan AS-A.

3.2. Ion-exchange chromatography of abienan AS-A on DEAE-cellulose

Abienan AS-A was separated by ion-exchange chromatography on a DEAE-cellulose column into five fractions (Table 1). The major fractions AS-A-D-2 and AS-A-D-3 represented approximately 70% of abienan AS-A and consisted mainly of GalA residues. The highly positive specific rotations of these fractions indicated an α-configuration of the glycosidic linkages in the galacturonan backbone of abienan AS-A. The fractions AS-A-D-2 and AS-A-D-3 were distinguishable by the ratio of the GalA backbone residues to the neutral sugar side chain residues of abienan (Table 1). These differences are probably due to the irregular block structure of pectins (Caffall & Mohnen, 2009; Ovodov, 2009; Ridley et al., 2001).

The weight-average molecular weights of these fractions ranged from 100 to 140 kDa. Thus, abienan AS-A may exist as a mixture of pectic polysaccharides that differ in backbone length.

The fraction AS-A-D-0 (6%) was distinguishable by its high content of neutral sugar residues and its low content of GalA residues (Table 1). AS-A-D-0 appeared to contain hemicellulose along with pectin found in Siberian fir wood greenery. Hardwood is known to contain water- and alkali-soluble galactomannans (Sharkov & Kuibina, 1972).

3.3. Partial acidic hydrolysis of abienan AS-A

Abienan AS-A was subjected to sequential hydrolysis with increasing acid concentration to partially fragment the macromolecule (Fig. 1). Hydrolysis with 0.05 M TFA to furnish two polysaccharide fragments, AS-A-H and AS-A-H-1, and a mono/oligosaccharide fraction (Table 1). A complete acidic hydrolysis of AS-A-H with 2 M TFA led to GalA and Gal in the hydrolysate as the main constituents of the macromolecule. The amount of Ara in the hydrolysate of AS-A-H was significantly less than that in the hydrolysate of abienan AS-A (Table 1). The sugar chains obtained from AS-A-H-1 were shown to contain considerable amounts of GalA, Ara and Gal residues in the ratio of 1.9:1.0:1.2 (Table 1). AS-A-H-1 appeared to contain the sugar chain fragments of linear and branched regions of abienan AS-A.

The sugar chains of the mono/oligosaccharide fraction (9.5%) obtained from the alcoholic supernatant were shown to contain considerable amounts of Ara (47%). This finding suggests that having labile glycoside linkages the Ara residues are in a furanose form. The Ara residues appeared to also be terminal residues in the pectin side chains consisting of the same residues.

AS-A-H was subjected to hydrolysis with 0.4 M TFA to furnish the polysaccharide fragment AS-A-H-2 (Table 1). AS-A-H-2 was subjected to hydrolysis with 2 M TFA to afford galacturonan with a high positive specific rotation (Table 1). These data imply that α-galacturonan is the backbone of abienan AS-A. The sugar chains of the mono/oligosaccharide fraction (57.5%) obtained from the alcoholic supernatant were shown to contain considerable amounts of GalA (60%) and Gal (17%). Ara residues were also detected in trace amounts. Thus, the acid hydrolysis of abienan AS-A resulted in the polysaccharide fragments of the backbone (galacturonan AS-A-H-3) and the branched region (polysaccharide fragment AS-A-H-1).

3.4. Isolation of polysaccharide AS-A-S

Pectic substances are known to exist in plant cell walls as independent constituents, such as arabinogalactans, galacturonan and rhamnogalacturonan, and as the macromolecules with completed biosynthesis, such as heteroglycanogalacturonans (Ridley et al., 2001). Therefore, pectin extraction can result in the isolation of pectin components generated during different stages of the pectin macromolecule biosynthesis. The structures of neutral polysaccharides usually correlate with the structures of the side chains of the branched pectic regions (Albersheim, Darvill, O'Neill, Schols, & Voragen, 1996; Ovodov, 1998). Thus, the structure of pectin may be supplemented by the more simple constituents with the lower molecular weight.

The polysaccharide AS-A-S was isolated from the aqueous-alcoholic supernatant obtained after the precipitation of abienan AS-A (Table 1). The sugar chains of the polysaccharide consisted of Ara, GalA, Rha, and Gal in the ratio of 30:6:1:2. Thus, AS-A-S seemed to contain the highly branched side chains and to represent the neutral component of pectin AS-A made by the biosynthesis of abienan.

Polysaccharide AS-A-S was subjected to acid hydrolysis with 0.01 M TFA to furnish two polysaccharide fragments: AS-A-S-H-1 and AS-A-S-H-2 (Table 1). The sugar chains of the polysaccharide

fragments were shown to contain considerable amounts of Ara. The negative specific rotation and easy cleavage of the arabinosyl linkages upon hydrolysis indicated the presence of predominantly α-L-arabinofuranosyl residues (Joseleau, Chambat, Vignon, & Barnoud, 1977). The L-configuration of Ara was confirmed by GLC of the corresponding acetylated (S)-2-octyl glycosides (Leontein & Lonngren, 1993). In addition, the sugars of AS-A-S-H-2 hydrolysed with 2 M TFA were isolated by preparative paper chromatography. Elution of the appropriate fractions gave mainly L-Ara, $[\alpha]_D^{20}$ +106.0° (*c* 0.5; H_2O).

3.5. NMR spectroscopy of galacturonan AS-A-H-3

The ^{13}C NMR spectrum of the polysaccharide fragment AS-A-H-3 (Fig. 2) showed that the resonance from C1 of GalA at 99.6 ppm was the most intense peak in the high-field anomeric area. This signal belongs to the GalA residues of the α-1,4-linked backbone. The resonances from the other atoms of 1,4-linked GalA residues (C2 68.9, C3 69.5, C4 78.5, C5 71.9, and C6 175.9 ppm) corresponded to the peaks in the ^{13}C NMR spectrum of 1,4-α-D-galacturonan (Petersen, Meier, Duus, & Clausen, 2008; Taboada et al., 2010). The signal at C 22.9 ppm in the ^{13}C NMR spectrum (Fig. 2) revealed the presence of *O*-acetyl groups on the GalA residues of the abienan AS-A backbone (Caffall & Mohnen, 2009; Ridley et al., 2001).

3.6. NMR spectroscopy of polysaccharide AS-A-S

A group of intense signals in the anomeric region of the ^{13}C NMR spectrum of polysaccharide AS-A-S (106.9–108.1 ppm) was assigned to C1 of Ara residues. The correlation signal at C 21.4/H 2.15; 2.18 ppm in the two-dimensional NMR-spectra revealed the presence of *O*-acetyl groups on the GalA residues of AS-A-S (Fig. 3). The ^{13}C- and ^{1}H NMR spectra of the polysaccharide AS-A-S were interpreted using two-dimensional spectroscopic experiments such as TOCSY, COSY, HSQC and ROESY (Table 2).

Analysis of the ROESY spectrum of AS-A-S (Fig. 4) confirmed the presence of the 1,4-linked GalA residues and showed that the polysaccharide was pectin. The correlation peaks of H1/H2, H1/H3 and H1/H4 of the 4-*O*-substituted GalA residues (at 5.04/3.84, 5.04/4.00, and 5.04/4.46 ppm) and the 4-*O*-substituted GalA methyl ester residues (H1/H2 at 4.96/3.75 and H1/H4 at 4.96/4.46 ppm) were also observed.

The presence of the corresponding correlation peaks in the $^{1}H/^{13}C$ HSQC spectrum (Fig. 3 and Table 2) confirmed the occurrence of a considerable amount of terminal, 5-*O*-, 2,5-di-*O*, 3,5-di-*O*-, or 2,3,5-tri-*O*-substituted Ara residues in AS-A-S (Capek, Matulová, Navarini, & Suggi-Liverani, 2010; Cardoso, Silva, & Coimbra, 2002; Dourado, Cardoso, Silva, Gama, & Coimbra, 2006; Habibi, Heyraud, Mahrouz, & Vignon, 2004; Khramova et al., 2011; Nunes et al., 2008; Westphal et al., 2010).

The ROESY spectrum revealed the following correlation peaks: H1/H2, H1/H3, H1/H4, and H1/H5, H5′ of 5-*O*-substituted Ara residues at 5.08/4.13, 5.08/4.01, 5.08/4.19, and 5.08/3.87, 3.81 ppm; H1/H2 and H1/H4 of 3-*O*-substituted Ara residues at 5.10/4.28 and 5.10/4.21 ppm; and H1/H3 of terminal and 3-*O*-substituted Ara residues at 5.14/4.03 ppm (Fig. 4). These data demonstrated the occurrence of these fragments: …→5)-α-L-Ara*f*-(1→5)-α-L-Ara*f*-(1→…; …→3)-α-L-Ara*f*-(1→3)-α-L-Ara*f*-(1→…; and α-L-Ara*f*-(1→3)-α-L-Ara*f*-(1→….

The ROESY spectrum also showed the following correlation peaks: H1/H2, H1/H3, and H1/H4 of 3,5-di-*O*-substituted Ara residues at 5.11/4.28, 5.11/4.07, and 5.11/4.29 ppm and H1/H2, H1/H3, H1/H4, and H1/H5, H5′ of 2,3,5-tri-*O*-substituted Ara residues at 5.23/4.30, 5.23/4.08, 5.23/4.24, and 5.23/3.93, 3.83 ppm (Fig. 4). These data demonstrated the occurrence of the branching point of AS-A-S as …→3,5)-α-L-Ara*f*-(1→… and …→2,3,5)-α-L-Ara*f*-(1→…. Previously, similar branching points were detected in pectins from sugar beets and almonds (Dourado et al., 2006; Westphal et al., 2010). In addition, the ROESY spectrum showed the correlation peak of H1 of the terminal Ara residues with H2 of the 2,3,5-tri-*O*-substituted Ara residues at 5.17/4.30 ppm.

The ratios of H1 signal intensities of Ara residues based on the ^{1}H NMR spectrum of AS-A-S were used to determine the ratios of monomer units. The data suggest that almost 43% of the Ara residues in AS-A-S are present as 1,5-linked Ara residues (H1, δ = 5.08 ppm). The percentage of terminal Ara residues (H1, δ = 5.14, 5.17, 5.21 ppm) is 33%. The amount of branching points represented by 2,5-di-*O*- and 2,3,5-tri-*O*-substituted Ara residues (H1, δ = 5.23 ppm) is 14% of the total Ara amount. Additionally, 3-*O*- and 3,5-di-*O*-substituted Ara residues (H1, δ = 5.10, 5.11 ppm) account for 8.9% of the total Ara content. Together, these NMR data showed that AS-A-S contains side chains made of mainly 1,5-linked Ara residues, which are 3-*O*- or 2,3-di-*O*-substituted with terminal Ara residues.

Thus, NMR spectral data confirmed that AS-A-S appeared to be a branched pectic polysaccharide. The backbone of the polysaccharide contains short lengths of α-1,4-D-galacturonan with some existing as methyl esters. The side chains of AS-A-S consisted of terminal, 1,5-, and 1,3-linked α-L-arabinofuranose residues. The branching points of the side chains appeared to be 2,5-, 3,5-di-*O*-substituted and 2,3,5-tri-*O*-substituted α-L-arabinofuranosyl residues.

3.7. NMR spectroscopy of fragment AS-A-H-1

The data of the fragment AS-A-H-1 (Table 3) revealed that 1,4-α-D-galacturonan is present in its sugar chains. The chemical shifts of carbon atoms of GalA residues coincide with those in the ^{13}C NMR spectrum of authentic 1,4-α-D-galactopyranosyluronan (Caffall & Mohnen, 2009; Ovodova, Bushneva, Shashkov, Chizhov, & Ovodov, 2005; Schols, Posthumus, & Voragen, 1990; Taboada et al., 2010). The ^{13}C- and ^{1}H NMR spectra of AS-A-H-1 were interpreted using two-dimensional spectroscopic techniques such as TOCSY, COSY, HSQC, and ROESY.

The presence of methyl esters and *O*-acetyl groups in AS-A-H-1 was confirmed by the correlation peaks in the $^{1}H/^{13}C$ HSQC spectrum (Fig. 5 and Table 3). The $^{1}H/^{13}C$ HSQC signal of C3/H3 of GalA residues at 72.0/5.11 ppm demonstrated that the GalA residues in AS-A-H-1 contained 3-*O*-acetyl groups (Caffall & Mohnen, 2009; Ridley et al., 2001). The correlation peak of H1/H4 of 4-*O*-substituted GalA residues at 5.09/4.45 ppm and the correlation peak of H1/H4 of 4-*O*-substituted GalA methyl ester residues at 4.96/4.45 ppm were also detected in the ROESY spectrum. These correlation peaks indicated the occurrence of these fragments: …→4)-α-D-Gal*p*A-(1→… and …→4)-α-D-Gal*p*A(CO_2Me)-(1→…. The correlation peaks of H1 of 4-*O*-substituted GalA residues with H1 of 4-*O*-substituted GalA methyl ester residues at 5.09/4.96 ppm were observed in the ROESY spectrum and indicated the presence of these fragments: …→4)-α-D-Gal*p*A-(1→4)-D-Gal*p*A(CO_2Me)-(1→…. These data suggested the presence of regions of 1,4-α-D-galacturonan substituted partially with methyl esters and *O*-acetyl groups in the abienan macromolecule.

Intense resonances from C1/H1 of Rha residues at 100.3/5.21 and 100.3/5.20 ppm were observed in the $^{1}H/^{13}C$ HSQC spectrum of AS-A-H-1. The high-field resonance of $^{1}H/^{13}C$ HSQC showed the correlation peaks of C6/H6 of the α-rhamnopyranose methyl group at 18.0/1.25 and 18.0/1.32 ppm. The HSQC spectrum also showed the α-configuration for the Rha residues (C5/H5 correlation peaks at 70.7/3.64 and 69.1/3.73 ppm) (Fig. 5).

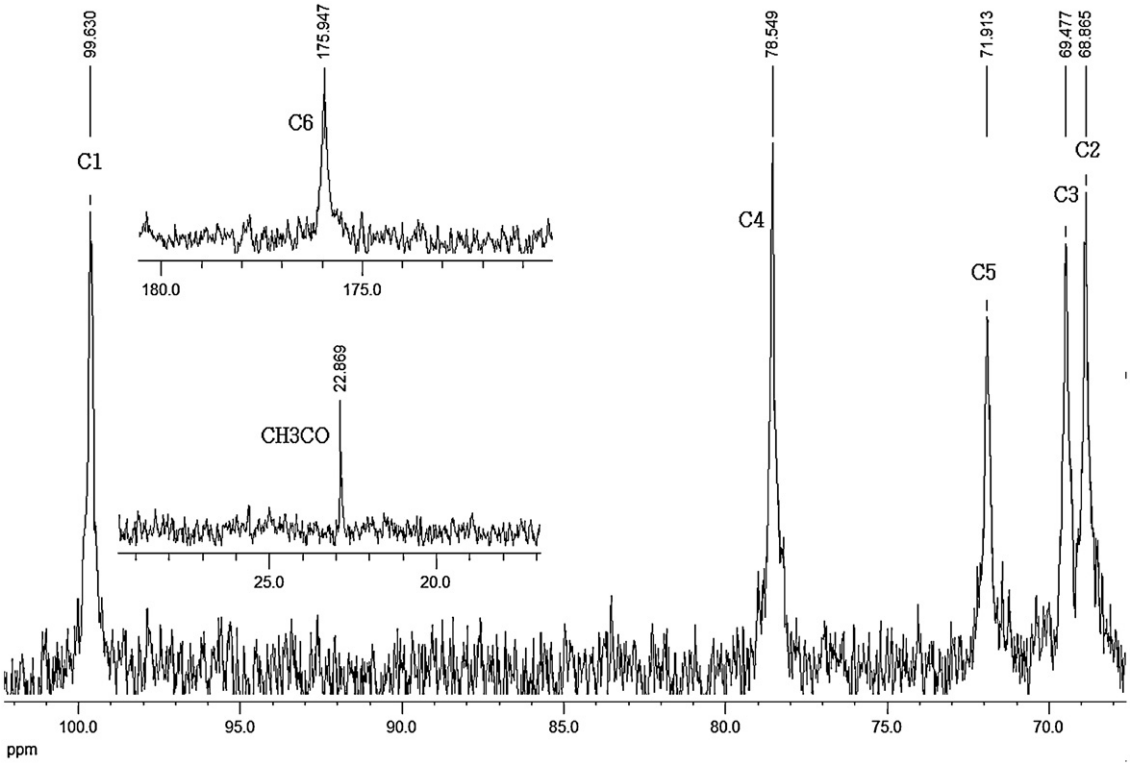

Fig. 2. ^{13}C NMR spectrum of galacturonan AS-A-H-3.

The ^{1}H/^{13}C HSQC spectrum showed the signals of C2/H2 of 2-*O*-glycosylated Rha residues at 77.8/4.08 ppm. Furthermore, the correlation peaks of C2/H2 at 77.8/4.12 ppm and C4/H4 at 80.2/3.68 ppm confirmed the substitution of the 2-*O*-glycosylated Rha residues at the fourth position (Fig. 5). Here, 2,4-di-*O*-substituted Rha residues appeared to be the branching points of the abienan AS_2 backbone. The data from two-dimensional spectroscopic experiments (TOCSY, COSY, HSQC, and ROESY) confirmed the occurrence of Rha residues (Table 3).

The ratio of H-6 signal intensities of Rha residues in the ^{1}H NMR spectrum of AS-A-H-1 demonstrated that the proportion of branched Rha residues and linear Rha residues were 27% and 73%,

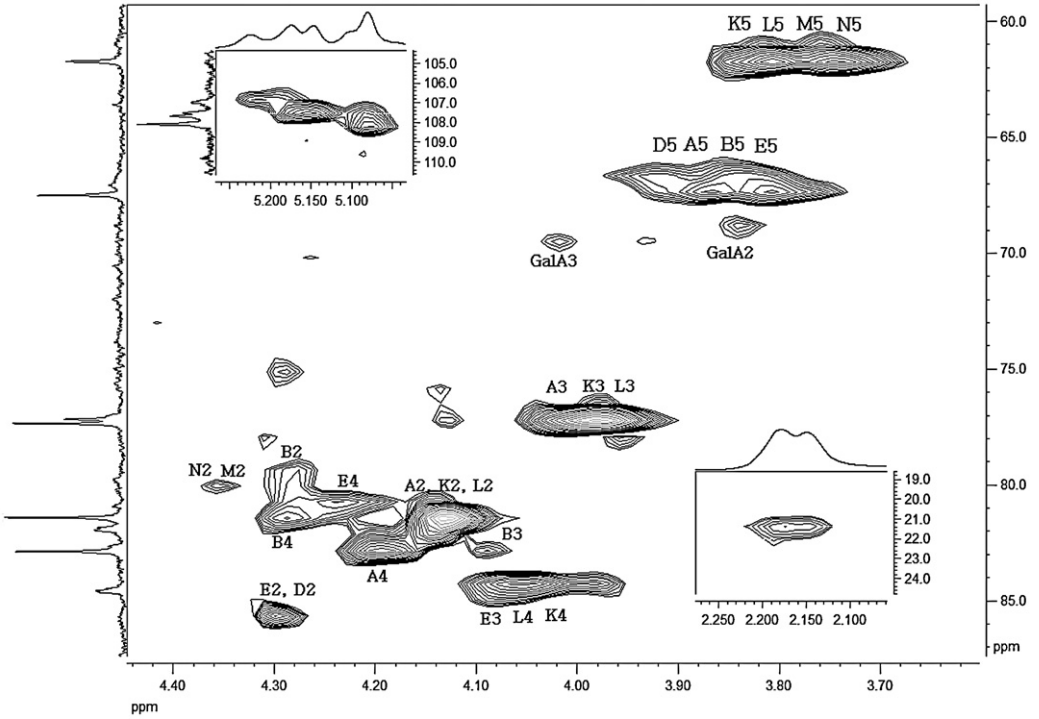

Fig. 3. ^{1}H/^{13}C HSQC spectrum of polysaccharide AS-A-S. Correlation peaks are marked on the spectrum (abbreviations as in Table 2).

Table 2
Chemical shifts of the ^{13}C and ^{1}H NMR spectrum of the polysaccharide fragment AS-A-S.

Residue		C-1 *H-1*	C-2 *H-2*	C-3 *H-3*	C-4 *H-4*	C-5 *H-5,5′*	C-6 *H-6,6′*
→4)-α-D-GalA*p*-(1→		100.0 *5.04*	68.8 *3.84*	69.6 *4.00*	79.5 *4.46*	n.d.	n.d.
→4)-α-D-GalA*p*(OMe)-(1→		100.0 *4.96*	68.3 *3.75*	69.6 *4.01*	80.1 *4.46*	n.d.	n.d.
→2)-α-Rha*p*-(1→		n.d.	77.3 *4.13*	n.d.	n.d.	n.d.	17.5 *1.25*
→5)-α-L-Ara*f*-(1→	A	108.1 *5.08*	81.8 *4.13*	77.3 *4.01*	83.0 *4.19*	67.5 *3.87;3.81*	
→3,5)-α-L-Ara*f*-(1→	B	107.7 *5.11*	80.0 *4.28*	82.9 *4.07*	81.4 *4.29*	67.0 *3.93;3.83*	
→3)-α-L-Ara*f*-(1→	C	107.7 *5.10*	80.0 *4.28*	n.d. *4.03*	82.9 *4.21*	61.7 *3.89;3.78*	
→2,5)-α-L-Ara*f*-(1→	D	106.9 *5.24*	85.8 *4.30*	n.d.	83.0 *4.20*	67.0 *3.94;3.85*	
→2,3,5)-α-L-Ara*f*-(1→	E	106.9 *5.23*	85.8 *4.30*	84.5 *4.08*	80.8 *4.24*	67.0 *3.93;3.83*	
α-L-Ara*f*-(1→3	K	107.5 *5.14*	81.9 *4.14*	77.1 *3.98*	84.5 *4.04*	61.7 *3.84;3.73*	
α-L-Ara*f*-(1→2	L	107.5 *5.17*	81.9 *4.14*	77.1 *3.99*	84.5 *4.07*	61.7 *3.84;3.73*	
α-L-Ara*f*-(1→5	M	107.0 *5.17*	80.0 *4.34*	77.1 *3.93*	83.0 *4.21*	61.7 *3.87;3.78*	
α-L-Ara*f*-(1→5	N	107.0 *5.21*	80.0 *4.35*	77.1 *3.96*	83.0 *4.17*	61.7 *3.87;3.78*	

respectively. These data confirmed that the fragment contains parts of linear and branched rhamnogalacturonan of abienan AS-A.

The correlation peaks of H1 of 1,4-linked GalA methyl ester residues and terminal GalA residues with H2 of Rha residues at 4.96/4.12 ppm and at 5.03/4.12 ppm, respectively, were observed in the ROESY spectrum of AS-A-H-1. These data indicated the presence of the GalA residues linked to the 2-position of Rha residues. The correlation peak of H1/H4 of Rha residues and 4-*O*-substituted GalA residues at 5.21/4.45 ppm and the correlation peak of H1/H1 of Rha and 1,4-linked GalA methyl ester residues at 5.21/4.96 ppm were also detected in the spectrum. These data indicated the presence of the Rha residues linked to the 4-position of GalA residues. Thus, the NMR data for AS-A-H-1 demonstrated the presence of the linear rhamnogalacturonan in abienan AS-A.

The nearby resonances from the C1-atoms of terminal (C1 104.9, 105.2 ppm; C4 69.8 ppm), 3-*O*-substituted (C1 105.0 ppm) and 4-*O*-substituted (C1 105.2, 105.5 ppm; C4 78.8 ppm) Gal residues were observed in the $^{1}H/^{13}C$ HSQC spectrum (Fig. 5).

Furthermore, the ROESY spectrum showed the correlation peaks of H1/H2, H1/H3, H1/H4, H1/H5, and H1/H6, H6′ of 4-*O*-substituted

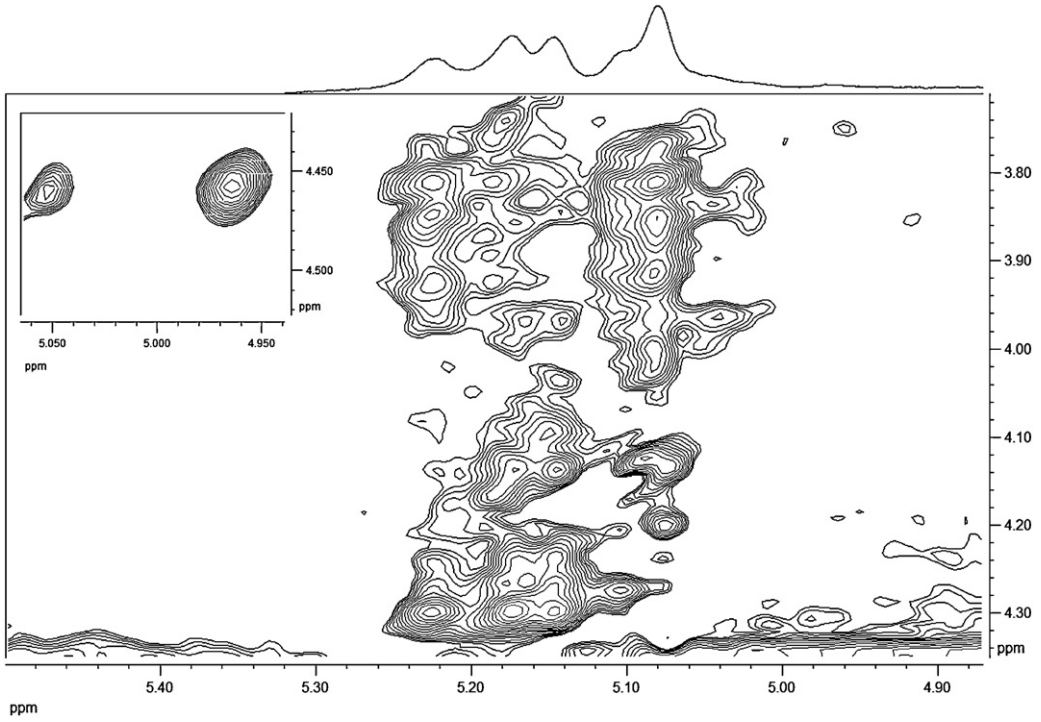

Fig. 4. ROESY spectrum of polysaccharide AS-A-S.

Table 3
Chemical shifts of the ^{13}C and ^{1}H NMR spectrum of the polysaccharide fragment AS-A-H-1.

Residue	C-1 *H-1*	C-2 *H-2*	C-3 *H-3*	C-4 *H-4*	C-5 *H-5,5′*	C-6 *H-6,6′*	CH_3O	CH_3CO
α-D-GalA*p*-(1→	100.8	70.0	69.9	71.5	71.5	174.0		
	5.03	*3.83*	*3.96*	*4.34*	*4.97*			
→4)-α-D-GalA*p*-3-OAc-(1→	99.3	69.5	72.0	78.9	n.d.	174.0		21.8
	5.05	*3.83*	*5.11*	*4.60*				*2.18*
→4)-α-GalA*p*-6-OMe-(1→	101.3	69.4	69.5	79.7	72.0	171.7	54.0	
	4.96	*3.72*	*4.00*	*4.45*	*5.05*		*3.80*	
→4)-α-D-GalA*p*-(1→	101.3	69.4	69.4	79.9	71.5	174.0		
	5.09	*3.73*	*4.00*	*4.45*	*4.97*			
→2)-α-Rha*p*-(1→	100.3	77.8	72.6	73.0	70.7	18.0		
	5.21	*4.12*	*4.02*	*3.44*	*3.64*	*1.25*		
→2,4)-α-Rha*p*-(1→	100.3	77.8	72.6	80.2	69.1	18.0		
	5.20	*4.08*	*4.02*	*3.68*	*3.73*	*1.32*		
β-Gal*p*-(1→	104.9	72.6	73.4	69.8	76.2	62.1		
	4.42	*3.56*	*3.64*	*3.92*	*3.68*	*3.78;3.72*		
→4)-β-Gal*p*-(1→4	105.2	72.6	74.5	78.8	76.0	62.1		
	4.63	*3.68*	*3.80*	*4.15*	*3.70*	*3.81;3.78*		
→4)-β-Gal*p*-(1→	105.5	72.6	74.5	78.8	76.0	62.1		
	4.61	*3.52*	*3.79*	*4.15*	*3.70*	*3.81;3.78*		
β-Gal*p*-(1→	105.2	72.6	73.4	69.8	76.0	62.1		
	4.59	*3.63*	*3.67*	*3.91*	*3.69*	*3.78;3.72*		
→3)-β-Gal*p*-(1→	105.0	72.6	83.0	n.d.	76.0	61.9		
	4.68	*3.82*	*3.87*	*4.20*	*3.70*	*3.78*		
→4)-β-Xyl*p*-(1→	101.5	74.2	74.8	77.8	n.d.			
	4.48	*3.30*	*3.58*	*3.81*	*4,18;3.44*			
β-Xyl*p*-(1→	101.5	74.2	n.d.	69.3	n.d.			
	4.54	*3.38*	*3.65*	*3.73*	*4.12;3.38*			
→2,3,5)-α-L-Ara*f*-(1→	108.0	86.4	85.2	81.5	67.9			
	5.23	*4.29*	*4.08*	*4.24*	*3.94;3.84*			
→5)-α-L-Ara*f*-(1→	108.9	82.5	77.8	83.6	67.9			
	5.08	*4.12*	*4.01*	*4.19*	*3.86;3.81*			
→3,5)-α-L-Ara*f*-(1→	108.6	80.5	82.7	82.2	67.7			
	5.10	*4.27*	*4.09*	*4.28*	*3.93;3.83*			
→3)-α-L-Ara*f*-(1→	108.6	80.5	82.7	83.6	61.7			
	5.10	*4.27*	*4.03*	*4.21*	*3.87;3.78*			
→2,5)-α-L-Ara*f*-(1→	108.0	86.4	77.8	83.5	67.9			
	5.22	*4.29*	*4.07*	*4.20*	*3.94;3.84*			
α-L-Ara*f*-(1→3	108.2	82.4	77.8	85.2	62.4			
	5.14	*4.13*	*3.96*	*4.03*	*3.85;3.75*			
α-L-Ara*f*-(1→2	108.2	82.4	77.8	85.2	62.4			
	5.17	*4.13*	*3.97*	*4.07*	*3.85;3.75*			
α-L-Ara*f*-(1→5	108.2	81.0	77.8	83.0	62.4			
	5.17	*4.34*	*3.95*	*4.21*	*3.88;3.78*			
α-L-Ara*f*-(1→5	108.2	81.0	77.8	83.0	62.4			
	5.21	*4.35*	*3.97*	*4.17*	*3.88;3.78*			
→4)-β-Man*p*-(1→	101.5	71.0	72.6	77.8	76.4	62.2		
	4.73	*4.12*	*3.81*	*3.81*	*3.55*	*3.91;3.73*		

Gal residues at 4.63/3.68, 4.63/3.80, 4.63/4.15, 4.63/3.70, and 4.63/3.81, 3.78 ppm, which corresponded to the fragment: . . .→4)-β-Gal*p*-(1→4)-β-Gal*p*-(1→. . .. The correlation peak of H1/H4 of 4-*O*-substituted Gal residues and 2-*O*-substituted Rha residues at 4.61/3.68 ppm in the ROESY spectrum of AS-A-H-1 showed the connection of some of the 1,4-linked Gal residues to the 4-*O*-position of the 2-*O*-substituted Rha residues (Mikshina et al., 2012). Moreover, the ROESY spectrum showed the correlation peaks of H1/H2, H1/H3, and H1/H5 of 3-*O*-substituted Gal residues at 4.68/3.82, 4.68/3.87, and 4.68/3.70 ppm, which indicated the presence of the fragment: . . .→3)-β-Gal*p*-(1→. . . (Capek et al., 2010).

The ratio of H1 signal intensities of Gal residues based on the ^{1}H NMR spectrum of the fragment AS-A-H-1 were used to determine the ratios of monomer units. The data suggest that 76% the Gal residues in AS-A-H-1 are present as 1,4-linked Gal residues (H1, δ = 4.61, 4.63 ppm). The percentage of terminal (H1, δ = 4.42, 4.59 ppm) and 1,3-linked Gal residues (H1, δ = 4.68 ppm) is 24%. Thus, the RG-I of abienan AS-A was found to contain side chains of galactan consisting mainly of 1,4-linked β-galactopyranosyl residues.

The two-dimensional NMR spectra of AS-A-H-1 showed the presence of terminal, 1,4-linked β-Xyl*p* and 1,4-linked β-Man*p* residues (Table 3).

Analysis of the ^{13}C- and ^{1}H NMR spectra revealed residues of terminal, 1,3-, and 1,5-linked Ara residues as well as 2,5-di-*O*-, 3,5-di-*O*-, and 2,3,5-tri-*O*-substituted Ara residues in the side chains of AS-A-H-1 (Table 4) (Capek et al., 2010; Cardoso et al., 2002; Dourado et al., 2006; Khramova et al., 2011; Nunes et al., 2008; Westphal et al., 2010). The chemical shifts of Ara residue signals in the NMR spectra of AS-A-H-1 were compared with the resonances in the NMR spectra of the pectic polysaccharide AS-A-S (Tables 2 and 3). A significant overlap of chemical shifts was observed in the NMR spectra of these polymers. In particular, the ROESY spectra of these pectic polysaccharides contained very similar correlation peaks of H atoms of the Ara residues. Thus, the RG-I of abienan AS-A was found to contain arabinose side chains composed of branched arabinan. In addition, structures analogous to the arabinan structure contained in AS-A-H-1 and AS-A-S were observed. These data may confirm that the polysaccharide AS-A-S represents a component of abienan AS-A.

3.8. NMR spectroscopy of fraction AS-A-D-0

The data of the ^{13}C- and ^{1}H NMR spectra confirmed that the fraction AS-A-D-0 contained residues of α-1,4-linked GalA, 2-*O*-substituted Rha, and terminal, 1,5-linked, 3,5-di-*O*-, and

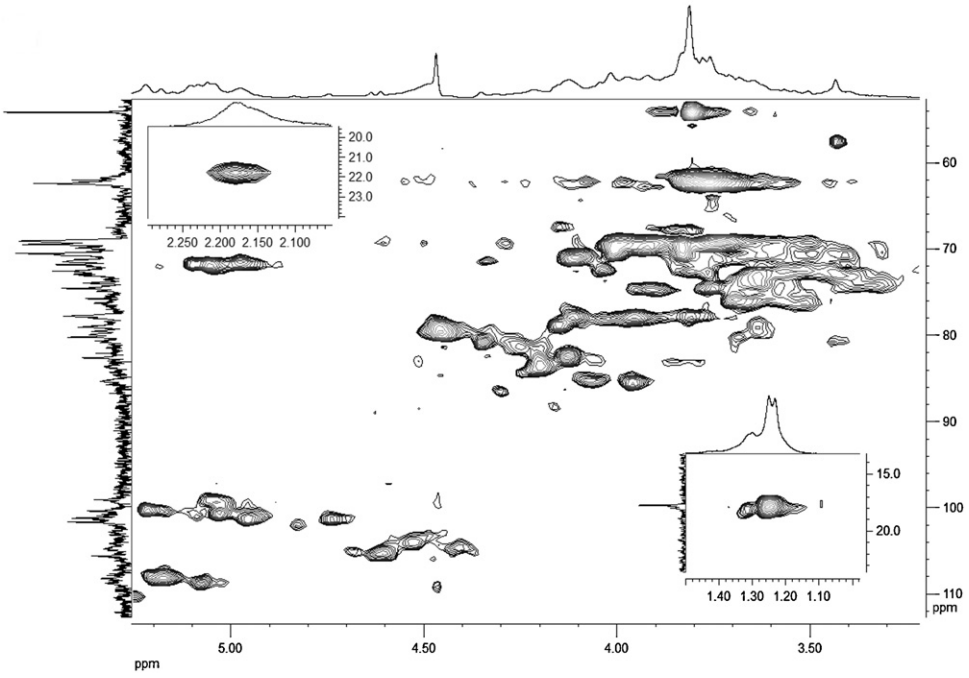

Fig. 5. $^{1}H/^{13}C$ HSQC spectrum of polysaccharide fragment AS-A-H-1.

Table 4
Chemical shifts of the ^{13}C and ^{1}H NMR spectrum of the polysaccharide fraction AS-A-D-0.

Residue	C-1 *H-1*	C-2 *H-2*	C-3 *H-3*	C-4 *H-4*	C-5 *H-5,5′*	C-6 *H-6,6′*
→4)-α-D-GalA*p*-(1→	102.5 *5.03*	71.0 *3.83*	72.0 *4.01*	81.9 *4.47*	n.d.	n.d.
→2)-α-Rha*p*-(1→	n.d.	79.3 *4.11*	n.d. *3.87*	75.6 *3.43*	71.8 *3.68*	n.d. *1.30*
→3,5)-α-L-Ara*f*-(1→	110.3 *5.10*	82.3 *4.28*	85.0 *4.09*	83.6 *4.28*	69.7 *3.93;3.84*	
→5)-α-L-Ara*f*-(1→	110.3 *5.07*	83.6 *4.12*	79.5 *4.00*	85.1 *4.20*	69.7 *3.86;3.81*	
→2,3,5)-α-L-Ara*f*-(1→	109.4 *5.22*	88.0 *4.30*	86.7 *4.09*	83.0 *4.23*	69.7 *3.93;3.85*	
α-L-Ara*f*-(1→3	109.9 *5.14*	83.6 *4.13*	79.5 *3.97*	86.8 *4.05*	63.9 *3.85;3.75*	
α-L-Ara*f*-(1→2	109.9 *5.17*	83.6 *4.13*	79.5 *3.98*	86.8 *4.07*	63.9 *3.85;3.75*	
α-L-Ara*f*-(1→5	109.9 *5.17*	82.5 *4.34*	79.5 *3.95*	85.1 *4.21*	63.9 *3.87;3.78*	
α-L-Ara*f*-(1→5	109.9 *5.20*	82.5 *4.35*	79.5 *3.97*	85.1 *4.17*	63.9 *3.87;3.78*	
→4)-β-Man*p*-(1→	103.0 *4.73*	73.0 *4.11*	73.8 *3.81*	79.2 *3.81*	n.d. *3.54*	63.8 *3.94;3.76*

2,3,5-three-*O*-substituted Ara (Table 4). The presence of these residues was determined by the comparative analysis of experimental chemical shifts in the NMR spectra of AS-A-D-0, AS-A-H-1 and AS-A-S (Tables 2–4).

A small amount of 1,4-linked mannose residues indicated by NMR data from AS-A-D-0 was due to the water-soluble component of mannan hemicellulose found with abienan in Siberian fir wood greenery.

4. Conclusions

From this study, the macromolecule abienan AS-A, a pectic polysaccharide from Siberian fir wood greenery, was shown to be composed of blocks of linear and branched regions. The linear regions were found to consist of 1,4-α-D-galactopyranosyluronan and rhamnogalacturonan. The 1,4-α-D-galacturonan residues were partially substituted with methyl esters or 3-*O*-acetyl groups. Rhamnogalacturonan consists of separate parts of 1,4-α-D-galacturonan methyl ester residues and rhamnopyranose residues containing 1,2-linkages. Considerable blocks of the branched region of abienan AS-A are present in RG-I. The side chains of RG-I were found to be made of blocks of 1,4-β-galactan and 1,5-α-arabinan that is 3,5-di-*O*- or 2,3,5-tri-*O*-substituted by terminal α-L-arabinofuranose residues. The backbone and the side chains of the hairy region of abienan AS-A were shown to be covalently linked. Some of the 2-*O*-substituted α-rhamnopyranose residues of the hairy region backbone were shown to be branching points bearing 4-*O*-substituted β-galactopyranose residues on the 4-position.

Acknowledgements

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