Carbohydrate Research, 167 (1987) 221–237
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

UNIT STRUCTURE OF THE ANTI-COMPLEMENTARY ARABINOGALACTAN FROM *Angelica acutiloba* Kitagawa*,†

HIROAKI KIYOHARA, HARUKI YAMADA**, AND YASUO OTSUKA
Oriental Medicine Research Center of the Kitasato Institute, Minato-ku, Tokyo 108 (Japan)
(Received April 29th, 1986; accepted for publication, November 20th, 1986)

ABSTRACT

Mild acid hydrolysis of an anti-complementary arabinogalactan (AGIIb-1), isolated from the roots of *Angelica acutiloba* Kitagawa, gave one neutral (N-I) and two acidic arabinogalactans (A-I and A-II) and one neutral arabinan (N-II). Likewise, the product (AF-AGIIb-1) of digestion with exo-α-L-arabinofuranosidase gave four carbohydrate units. Methylation analysis showed that N-I was a (1→6)-linked galactan with unbranched short side-chains of Ara*f* attached at position 3 and that A-I and A-II contained, in addition, 4-linked Gal*p*. Methylation analysis and oligosaccharide analysis showed that A-I and A-II also contained highly branched Ara chains possessing Ara*f* side-chains attached at positions 3 of some 4- or 5-linked Ara and that a small proportion of Ara*p* was present in each acidic unit. Base-catalysed β-elimination and oligosaccharide analysis indicated that A-I and A-II also contained a rhamnogalacturonan moiety in which 2,4-disubstituted Rha residues were attached to 4-substituted Gal*p*A through position 2 of Rha. Methylation analysis, ^{1}H- and ^{13}C-n.m.r. studies, and enzymic hydrolysis showed N-II to be a highly branched arabinan containing a backbone of (1→5)-linked α-L-Ara*f* with α-L-Ara*f* side-chains attached to positions 3.

INTRODUCTION

The anti-complementary arabinogalactan (AGIIb-1), isolated from the roots of *Angelica acutiloba* Kitagawa (Japanese name, Yamato-Tohki)[1,2], is a complex pectic arabinogalactan containing 14–20% of 2,4-disubstituted Rha*p* and (1→4)-linked Gal*p*A in addition to the major constituent, namely, a (1→6)-linked galactan, to which are attached highly branched Ara side-chains at positions 3. Mild acid hydrolysis[1] of AGIIb-1 decreased its anti-complementary activity, and more rigorous treatment yielded one neutral and two acidic arabinogalactans

*Studies on Polysaccharides from *Angelica acutiloba*, Part VII. For Part VI, see ref. 1.
†Presented at the XIIIth International Carbohydrate Symposium, Ithaca, August 10–15, 1986.
**To whom correspondence should be addressed.

0008-6215/87/$ 03.50

together with highly branched arabino-oligosaccharides. When AGIIb-1 was digested with exo-α-L-arabinofuranosidase, the branched Ara side-chains were mostly converted into 4- or 5-linked Ara chains, and the anti-complementary activity was markedly enhanced[1]. AGIIb-1 may consist of different arabinogalactan units joined through acid-labile linkage(s), and the structure of Ara*f* chains and acid-labile linkages may be responsible for the expression of the anti-complementary activity. The above arabino-oligosaccharides have been characterised[1] and we now report on the structure of the four carbohydrate units isolated from AGIIb-1 by a mild acid hydrolysis more appropriate than that of the previous study[1].

EXPERIMENTAL

Materials and methods. — The roots of *A. acutiloba* Kitagawa were purchased from Uchida Wakanyaku Co. Ltd. (Japan). DEAE-Sephadex and Sephadex G-10 and G-100 were obtained from Pharmacia, and Bio-gel P-2 (200–400 mesh) from Bio-Rad. SPECTRA/PORE 6 cellulose dialysis-tubing was purchased from Spectrum Medical Industries Inc. (U.S.A.).

Uronic acid content was assayed by the *m*-hydroxybiphenyl method[3], using D-galacturonic acid as the standard. The carbohydrate and pentose in the column eluates were monitored by the phenol–sulfuric acid[4] and phloroglucinol[5] methods. Neutral sugars of polysaccharides were analysed[1] as the corresponding alditol acetates by g.l.c. Uronic acids were converted into the corresponding reduced products and analysed[6] by g.l.c. The anti-complementary arabinogalactan, AGIIb-1, was isolated from *A. acutiloba* as described previously[1,2]

Purification of the polysaccharide units from AGIIb-1. — A solution of AGIIb-1 (30.2 mg) in 10mM hydrochloric acid (6 mL) was heated for 10 min at 100°, then cooled, neutralised with 100mM sodium hydroxide, and applied to a column (1.9 × 6.5 cm) of DEAE-Sephadex A-25 ($HCOO^-$ form). The neutral and two acidic carbohydrate fractions were obtained by eluting with water, 2M formic acid, and 2M sodium chloride, respectively. The first acidic fraction was neutralised with M sodium hydroxide, and eluted from Sephadex G-10 with water to give A-I (4.5 mg). Likewise, A-II (2.8 mg) was obtained from the second acidic fraction. The neutral fraction was eluted from a column (2.6 × 90 cm) of Sephadex G-100 with 0.2M sodium chloride, to give a hexose-rich fraction in the void volume and a pentose-rich fraction as a broad peak. The fractions were dialysed against water using cellulose tubing (Visking Company) and SPECTRA/PORE 6 (mol. wt. cut-off, 1000), respectively, to give, as the non-dialysable portions, N-I (5.5 mg) and N-II (5.4 mg); weight ratio N-I:N-II:A-I:A-II = 4:4:3:2.

In addition, AGIIb-1 (91.2 mg) was dissolved in 0.1M acetate buffer (75 mL, pH 4.0) and *Rhodotolura flava* exo-α-L-arabinofuranosidase (0.7 unit) was added. The mixture was incubated at 50° for 48 h in the presence of one drop of toluene, then neutralised to inactivate the enzyme, and lyophilised. Elution of the residue from a column (2.6 × 95 cm) of Sephadex G-10 with water gave AF-AGIIb-1 (55.6

mg) in the void volume. Treatment of AF-AGIIb-1 with 10mM hydrochloric acid, as for AGIIb-1, gave two neutral (AF-N-I and AF-N-II, 4.0 and 9.6 mg) and two acidic (AF-A-I and AF-A-II, 14.2 and 5.8 mg) carbohydrate fractions by chromatography of the hydrolysate on DEAE-Sephadex and Sephadex G-100; weight ratio AF-N-I:AF-N-II:AF-A-I:AF-A-II = 2:5:7:3.

Partial acid hydrolysis. — A solution of AF-A-I (14 mg) in 10mM hydrochloric acid (4 mL) was kept at 100° for 1 h, then neutralised, and applied to a column (1.5 × 3.2 cm) of DEAE-Sephadex A-25 ($HCOO^-$ form). The neutral fraction was obtained by elution with water and the acidic fraction with 2M formic acid. The neutral fraction was further fractionated by elution from a column (2.5 × 50 cm) of Bio-gel P-2 at 55° with water, to give fraction (PN-1) in the void volume and three oligosaccharide fractions (PN-2, PN-3, and PN-4) eluted in the regions for polymers, trisaccharides, and disaccharides, respectively. The acidic fraction was hydrolysed with 0.1M trifluoroacetic acid at 121° for 1 h to give a neutral and an acidic fraction as described above. This neutral fraction was eluted with water from a column (2.5 × 50 cm) of Bio-gel P-2 to give two fractions, namely, in the disaccharide region (PN-5) and the included volume. The final acidic fraction was eluted from a column (1.3 × 43 cm) of Sephadex G-25 with 50mM acetate buffer (pH 5.2) to give four acidic oligosaccharide fractions (PA-1, PA-2, PA-3, and PA-4). P.c. (ethyl acetate–formic acid–acetic acid–water, 18:1:3:4) of PA-4 gave one major oligosaccharide (PA-4a).

Methylation analysis. — Each carbohydrate unit and PN-1 and PN-2 were methylated once (Hakomori[7]) in order to prevent β-elimination[10], and the partially methylated alditol acetates were analysed as described previously[1]. PN-3, PN-4, PN-5, PA-3, and PA-4a were reduced with sodium borodeuteride and then methylated. The methylated acidic oligosaccharide-alditols were reduced with sodium borodeuteride in tetrahydrofuran–ethanol (7:3) at room temperature for 18 h followed[8] by incubation at 75° for 1 h in order to effect carboxyl-reduction. Each mixture was acidified with acetic acid and desalted with AG50W-X8 (H^+) resin equilibrated with 95% ethanol. The products from PA-4a were further methylated. PA-1 and PA-2 were methylated, carboxyl-reduced, and hydrolysed, and the products were converted into the corresponding alditol acetates.

β-Elimination of the methylated acidic carbohydrate units[9,10]. — To solutions of dry methylated AF-A-I and AF-A-II (2 mg) in methyl sulfoxide (1 mL) was added methylsulfinylcarbanion, and each mixture was stirred for 24 h at room temperature. To 70% of each sample was added excess of ethyl iodide, and the mixture was kept overnight at room temperature. The ethyl iodide was then evaporated, and the product was recovered using a Sep-pak C_{18} cartridge (Waters Assoc.) by the procedure of Waeghe and Albersheim[8] except that the samples were eluted with ethanol. Each product was fractionated on a column (1.0 × 25 cm) of Sephadex LH-20 equilibrated with chloroform–methanol (1:1), and fractions of high (R_2-a) and low (R_2-b) molecular weight were obtained (detection with the 1-naphthol–sulfuric acid reagent[11]). The remainder of the sample was neutralised

with aqueous 50% acetic acid and the product (R_1) was obtained as described above. R_1, R_2-a, and R_2-b were each hydrolysed with 2M trifluoroacetic acid at 121° for 1.5 h, and the products were reduced with sodium borohydride and then acetylated[1]. Linkage analysis of each sample was then effected by g.l.c. and g.l.c.–m.s., using a JEOL DX-300 instrument equipped with a SPB-5 capillary column (0.25-μm film thickness, 25 m × 0.25 mm i.d., SPELCO), an ionisation voltage of 70 eV, helium as the carrier gas at 0.9 mL/min, and the temperature programme 120→210° at 2°/min.

G.l.c.–m.s. of the methylated oligosaccharide-alditols. — Samples were dissolved in acetone and injected into a SPB-5 capillary column with splitless injection. The gas chromatograph was programmed at 120° for 3 min, →190° at 30°/min, and →310° at 4°/min. M.s. was performed with a JEOL DX-300 mass spectrometer, e.i.-m.s. at 70 eV with an ionisation current of 300 μA, and c.i. (isobutane) at 250 eV and an accelerating voltage of 3 kV. C.i.[12] and e.i. fragment ions [A, J and alditol *(ald)*][13] were used to determine the structure of the methylated oligosaccharide-alditols.

N.m.r. spectroscopy. — ^{1}H- (400 MHz) and ^{13}C-n.m.r. (100 MHz) spectra of N-II and AF-N-II were obtained for 0.5% solution in D_2O at 80°, using a Varian XL-400 F.t. spectrometer. Chemical shifts were expressed relative to that of sodium 3-(trimethylsilyl)propane-1-sulphonate-d_4 (TSP).

RESULTS

Isolation and properties of the carbohydrate units. — AGIIb-1 and the product (AF-AGIIb-1) of digestion with exo-α-L-arabinofuranosidase were treated with 10mM hydrochloric acid at 100° for 10 min, and each hydrolysate was fractionated on DEAE-Sephadex to give (Figs. 1A and 2A) one neutral and two acidic (A-I and A-II from AGIIb-1, and AF-A-I and AF-A-II from AF-AGIIb-1) carbohydrate fractions. When each neutral fraction was purified on Sephadex G-100 (0.2M NaCl), a hexose-rich fraction (N-I from AGIIb-1, AF-N-I from AF-AGIIb-1) was obtained in the void volume, and a pentose-rich fraction (N-II from AGIIb-1 and AF-N-II from AF-AGIIb-1, Figs. 1B and 2B) was obtained as a broad peak. The fractions (N-III and AF-N-III) eluted in the region of lowest molecular weight contained mainly Ara.

N-I, N-II, A-I, and A-II were eluted as almost single peaks from Sepharose CL-6B by 0.2M NaCl, but the positions of elution of these fractions from AGIIb-1 (except N-II) were almost the same as that of AGIIb-1 (data not shown). N-I contained mainly Ara and Gal, and N-II mainly Ara (Table I). A-I and A-II contained Ara, Gal, Rha, and GalA, and a trace of GlcA. Although the uronic acid content of A-I was slightly higher than that of A-II, the ratio of GlcA to GalA in A-II was higher than that in A-I. The Ara content (60–80%) in the carbohydrate fraction from AGIIb-1 was less in those from AF-AGIIb-1 (Table I). AF-N-II contained mainly Ara, and AF-A-I and AF-A-II contained more Ara than AF-N-I.

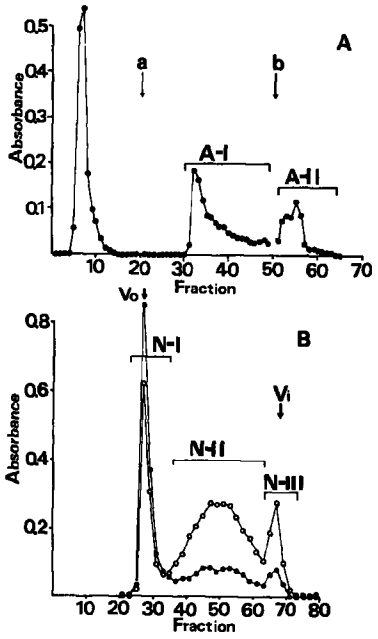


Fig. 1. A, Chromatography on DEAE-Sephadex A-25 of the products obtained on treatment of AGIIb-1 with 10mM hydrochloric acid at 100° for 10 min [stepwise elution with water and then (a) 2M formic acid, (b) 2M sodium chloride]; B, elution of the neutral fraction in A from Sephadex G-100 with 0.2M sodium chloride: carbohydrate, 490 nm (●); and pentose, 552–510 nm (○).

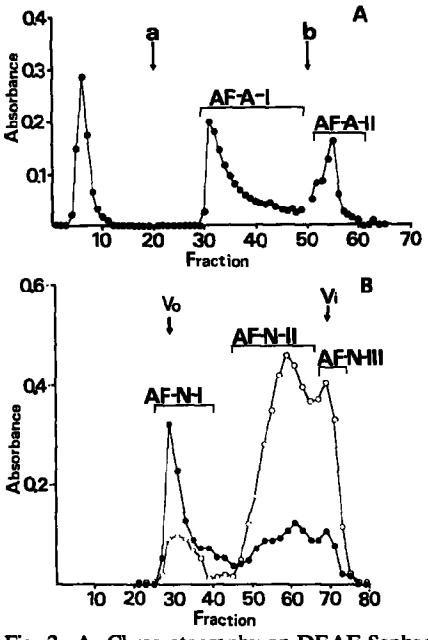


Fig. 2. A, Chromatography on DEAE-Sephadex A-25 of the products obtained on treatment of AF-AGIIb-1 with 10mM hydrochloric acid at 100° for 10 min; B, elution of the neutral fraction in A from Sephadex G-100. Symbols same as in Fig. 1.

TABLE I

PROPERTIES OF THE CARBOHYDRATE UNITS

	N-I	*N-II*	*A-I*	*A-II*	*AF-N-I*	*AF-N-II*	*AF-A-I*	*AF-A-II*
Yield (%)	18.1[a]	17.9[a]	14.9[a]	9.3[a]	7.2[b]	17.3[b]	25.5[b]	10.5[b]
Uronic acid (%)	—	—	15.1	12.1	—	—	18.2	14.2
Component sugar (molar ratio)								
Rha	0.1	—	0.3	0.4	0.2	—	0.3	0.2
Ara	1.2	25.7	1.7	1.5	0.2	10.1	0.7	0.5
Gal	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
GalA	—	—	0.4	0.2	—	—	0.3	0.2
GlcA	—	—	0.1	0.1	—	—	0.1	0.1

[a]Calculated from AGIIb-1. [b]Calculated from AF-AGIIb-1.

These results suggested that AGIIb-1 consisted of one neutral and two different acidic arabinogalactan units and one neutral arabinan unit linked through acid-labile linkage(s). Digestion of AGIIb-1 with exo-α-L-arabinofuranosidase did not hydrolyse all of the arabinan unit.

Methylation analysis of the carbohydrate units. — The data for the neutral sugar moieties from AGIIb-1 and AF-AGIIb-1 (N-I and N-II, respectively) are given in Table II. Thus, N-I is shown to be a typical arabino-3,6-galactan with a main chain of (1→6)-linked Gal units and unbranched short chains of Ara units attached to positions 3, and N-II to be a (1→4)- or (1→5)-linked arabinan possessing Ara*f* side-chains attached to positions 3. This highly branched arabinan unit was converted into a linear arabinan unit on digestion with exo-α-L-arabinofuranosidase.

A-I and A-II each contained 3,4- or 3,5-disubstituted Ara, 2,4-disubstituted Rha*p*, and 4-linked Gal*p* in addition to the same glycosidic linkages as N-I. The Ara*f* side-chains of A-I and A-II were attached mainly to positions 3 of 6-linked Gal*p* as in N-I. These results suggested that the three arabinogalactan units in AGIIb-1 were arabino-3,6-galactans with Ara*f* side-chains attached mainly at positions 3 of some 6-linked Gal*p*.

In AF-A-I and AF-A-II, the contents of terminal Ara*f* and branched Ara units had been reduced to negligible levels, but the contents of 4- or 5-linked Ara had increased significantly in comparison with those of A-I and A-II.

Thus, A-I and A-II contained highly branched Ara chains of which Ara*f* side-chains were attached to positions 3 of 4- or 5-linked Ara.

N.m.r. spectroscopy of N-II and AF-N-II. — The ^{1}H-n.m.r. spectrum of AF-N-II contained a signal for anomeric protons at 5.10 p.p.m. [→5)-α-L-Ara*f*-(1→] (Fig. 3A), and that of N-II contained three signals for such protons at 5.10 [→5)-α-L-Ara*f*-(1→], 5.12 and 5.17 p.p.m. $[\rightarrow^{5}_{3})$-α-L-Ara*f*-(1→] or [α-L-Ara*f*-(1→] (Fig.

TABLE II

METHYLATION ANALYSIS OF THE CARBOHYDRATE UNITS

Sugar linkage[a]	*N-I*	*AF-N-I*	*N-II*	*AF-N-II*	*Mol. %*							
					A-I[b]		*AF-A-I*[b]		*A-II*[b]		*AF-A-II*[b]	
					Total	*Galactan*	*Total*	*Galactan*	*Total*	*Galactan*	*Total*	*Galactan*
Terminal Ara*f*	33.9	9.7	35.7	5.6	23.2		6.1		23.3		7.5	
4- or 5-Ara	11.6	2.4	25.8	84.6	12.0		23.8		15.8		23.0	
Terminal Gal*p*	2.6	11.0	—	—	12.7	32.7	16.1	29.0	9.5	24.8	15.7	26.9
3,4- or 3,5-Ara	trace	—	38.5	2.5	14.2		0.8		14.4		—	
2,4-Rha	—	—	—	—	11.9		14.0		8.1		11.0	
4-Gal*p*	—	—	—	—	8.7	22.4	10.0	18.0	8.5	22.2	9.9	17.0
3-Gal*p*	5.5	6.2	—	—	3.4	8.8	3.7	6.7	3.8	9.9	3.1	5.3
6-Gal*p*	10.7	54.1	—	—	3.7	9.5	17.2	31.0	4.0	10.4	21.4	36.6
3,6-Gal*p*	27.9	13.9	—	—	8.1	20.9	7.1	12.8	9.4	24.5	6.9	11.8
3,4,6-Gal*p*	4.9	2.6	—	—	2.2	5.7	1.4	2.5	3.1	8.1	1.4	2.4

[a]4- or 5-Ara = 4- or 5-linked arabinosyl residue. [b]Calculated from all glycosyl residues (total) or from galactosyl residues only (galactan).

TABLE III

^{13}C-N.M.R. DATA

Sugar	*Chemical shift (p.p.m.)*				
	C-1	*C-2*	*C-3*	*C-4*	*C-5*
Methyl α-L-arabinopyranoside[a]	106.51	73.24	74.91	70.60	68.36
Methyl α-L-arabinofuranoside[a]	110.96	83.43	79.16	86.32	63.86
Methyl β-L-arabinofuranoside[a]	104.77	79.15	77.43	84.59	65.76
Arabinan I[a]	109.90				69.45
	109.21				69.15
AF-N-II	110.45 (A)	83.81 (C)	79.81 (D)	85.17 (B)	69.95 (E)
N-II	110.44 (A′)				69.40 (D′)
	110.33 (B′)				69.07 (E′)
	109.96 (C′)				63.98 (F′)

[a]Values have been assigned by Joseleau *et al.*[14].

3B)[14] with integrated intensities in the ratios 2:3:3 which accorded with the result of methylation analysis. The ^{13}C-n.m.r. spectrum of AF-N-II contained a signal (A) for Ara*f* in →5)-α-L-Ara*f*-(1→ and that for N-II contained signals (A′, B′, C′) for Ara*f* in →5)-α-L-Ara*f* (1→, $\rightarrow^{5}_{3}$)α-L-Ara*f*-(1→, or α-L-Ara*f*-(1→ (Table III). No signals for Ara*p* were observed in the ^{1}H- and ^{13}C-n.m.r. spectra. The assignments in the ^{13}C-n.m.r. spectra (Table III) accorded with the results of methylation analysis and the ^{1}H-n.m.r. data, and indicated that N-II and AF-N-II were virtually composed of α-L-Ara*f*.

Partial acid hydrolysis of AF-A-I. — Hydrolysis of AF-A-I (which was obtained in the highest yield from AF-AGIIb-1) with 10mM hydrochloric acid at 100°

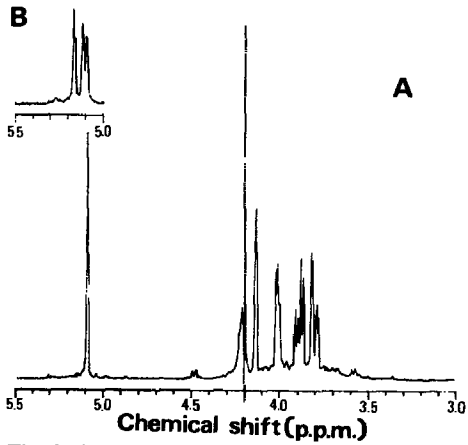


Fig. 3. ^{1}H-N.m.r. spectra of AF-N-II (A) and N-II (B).

TABLE IV

METHYLATION ANALYSIS OF NEUTRAL AND ACIDIC OLIGOSACCHARIDES FROM ACIDIC ARABINOGALACTAN UNIT (AF-A-I) OF ARABINOFURANOSIDASE-DIGESTED AGIIb-1

Glycosyl residue	*Position of* O-*methyl groups*	*Deduced glycosidic linkages*	*Mol.%*				
			PN-1	*PN-2*	*PA-1*	*PA-2*	*PA-3*[a]
Rhamnosyl	1,3,4,5[b]	Reducing terminal	—	—	—	—	4.0
	3,4	2	—	—	53.5	51.6	22.6
	2,3	4	—	—	1.5	—	—
	3	2,4	—	—	2.4	1.6	—
Arabinosyl	2,3,5	Terminal	6.7	17.5	—	—	—
	2,3	4 or 5	8.9	49.9	—	—	—
Galactosyl	2,3,4,6	Terminal	4.5	4.2	—	1.0	—
	2,3,6	4	6.7	9.8	—	—	—
	2,3,4	6	58.5	10.9	—	3.8[d]	17.6[d]
	2,4	3,6	14.7	2.1	—	0.6	—
Galacturonosyl[c]	2,3,4	Terminal	—	—	9.1	11.0[d]	19.8[d]
	2,3	4	—	—	33.5	30.0	29.3
	2,4	3	—	—	—	0.4	—
Glucuronosyl[c]	2,3,4	Terminal	—	—	—	—	6.7

[a]Pre-reduced with sodium borodeuteride before methylation to convert into oligosaccharide-alditols. [b]Detected as 2-*O*-acetyl-1,3,4,5-tetra-*O*-methyl-rhamnitol-*1-d*. [c]Detected as partially methylated galactitol-*6,6-d*$_2$ and glucitol-*6,6-d*$_2$ acetates. [d]Calculated from the ratio of the intensities of the fragment ions at *m/z* 233 and 235.

TABLE V

DIAGNOSTIC IONS OF C.I.-M.S. OF METHYLATED OLIGOSACCHARIDE-ALDITOLS

Oligosaccharide fraction	*Fragment*	*Oligosaccharide*	*Chemical-ionisation mass-spectral fragment ions* [m/z *(relative abundance)*]			
			$(M + H)^+$	AOH_3^+	A^+	G^+
PN-4	a	Pentosyl-pentitol	384 (100)	210 (43.4)	192 (15.7)	175 (74.8)
	b	Hexosyl-methylpentitol	442 (24.5)	224 (11.5)	206 (35.0)	219 (97.6)
	c	Hexosyl-hexitol	472 (63.1)	254 (12.6)	236 (100)	219 (48.6)
PA-4a	d	Hexuronosyl-hexitol	474 (2.8)	254 (48.1)	236 (64.7)	221 (10.7)
PN-5	g	Hexosyl-hexitol	472 (8.4)	254 (41.8)	236 (28.3)	219 (53.3)
	h	Hexosyl-hexitol	472 (7.0)	254 (42.0)	236 (33.9)	219 (59.1)
	i	Hexosyl-hexitol	472 (12.9)	254 (16.1)	236 (94.4)	219 (45.6)

TABLE VI

DIAGNOSTIC IONS OF E.I.-M.S. OF METHYLATED OLIGOSACCHARIDE-ALDITOLS

Oligosaccharide fraction	*Fragment*	*Oligosaccharide*	*E.i. mass-spectral fragment ions* [m/z *(relative abundance)*]							
			aJ_1	aJ_2	bA_1	bA_2	*ald*			
PN-4	a	Ara-(1→4)-Ara-(1→	252 (22.7)	192 (45.8)	175 (100)	143 (94.8)	338 (1.0)	293 (14.1)	249 (15.6)	
	b	Gal-(1→4)-Rha-(1→	266 (52.1)	206 (100)	219 (27.8)	187 (76.0)	319 (5.4)	275 (1.7)	134 (26.0)	
	c	Gal-(1→6)-Gal-(1→	296 (1.0)	236 (100)	219 (15.6)	187 (40.3)	337 (3.8)	305 (0.7)	178 (8.3)	146 (25.7)
PA-4a	d	HexA-(1→6)-Gal-(1→	296 (10.5)	236 (43.7)	221 (3.8)	189 (42.0)	339 (1.9)	307 (0.4)	178 (8.7)	146 (28.7)
	e	HexA(1→4)-Rha-(1→	—	206 (89.5)	221 (12.1)	189 (42.0)	309 (26.2)	277 (7.3)	134 (35.0)	
	f	HexA-(1→2)-Rha-(1→	266 (6.5)	206 (28.0)	221 (58.9)	—	147 (35.9)			
PN-5	g	Gal-(1→3)-Gal-(1→	296 (56.7)	236 (85.8)	219 (44.7)	187 (100)	426 (0.5)	381 (10.9)	338 (1.0)	133 (23.3)
	h	Gal-(1→4)-Gal(1→	296 (66.1)	236 (100)	219 (41.0)	187 (87.3)	305 (2.7)	134 (25.0)		
	i	Gal-(1→6)-Gal-(1→	296 (1.7)	236 (100)	219 (14.0)	187 (34.6)	337 (3.3)	178 (7.3)	146 (24.1)	

for 1 h, and fractionation of the neutral products on Bio-gel P-2 gave PN-1 (Gal, Ara, and Rha in the molar ratios of 1.0:0.2:0.1) eluted in the void volume, a high-molecular-weight fraction PN-2 (Ara:Gal 4.1:1.,0), and PN-3 (Rha:Ara:Gal 0.4:12.1:1.0) and PN-4 (Rha:Ara:Gal 0.5:13.1:1.0) eluted in regions for tri- and di-saccharides, respectively.

Methylation analysis (Table IV) indicated PN-1 to consist mainly of 6-linked and 3,6-di-substituted Gal*p* together with small proportions of terminal Ara*f* and 4- or 5-linked Ara, and terminal, 4-linked Gal*p*. The partially methylated Rha derivatives were lost, probably because of their volatility. PN-2 contained a large proportion of 4- or 5-linked Ara. PN-3 and PN-4 were each reduced with sodium borodeuteride, and the resulting oligosaccharide-alditols were methylated and then analysed by g.l.c.–c.i.- and e.i.-m.s. C.i.-m.s. of the product from PN-4 indicated the presence of pentosyl-pentitol (fragment [a]), hexosylmethyl-pentitol (fragment [b]), and hexosyl-hexitol (fragment [c]) (Table V). E.i.-m.s. of the fragments from reduced PN-4 showed fragment ions of the *ald* series at *m/z* 338, 293, and 249 in [a], suggesting it to be Ara-(1→4)-Ara-ol (Table VI). Similarly [b] was identified as Gal-(1→4)-Rha-ol and [c] as Gal-(1→6)-Gal-ol. C.i.-m.s. of methylated oligosaccharide-alditol fractions from PN-3 indicated an arabinotrisaccharide-alditol, but this could not be characterised because the specific fragment ions of the *ald* series required to characterise the glycosidic linkage of pentitol were not observed (data not shown).

The acidic fraction obtained by the first partial hydrolysis of AF-A-1, when hydrolysed further with 0.1M trifluoroacetic acid, gave neutral and acidic products. Fractionation of the neutral products on Bio-gel P-2 gave mainly monosaccharide and a small proportion of disaccharide (PN-5). Both mono- and di-saccharide fractions were composed mainly of galactose. C.i.- and e.i.-m.s. (Tables V and VI) of the methylated oligosaccharide-alditols derived from PN-5 indicated the presence originally of Gal-(1→4)-Gal, Gal-(1→3)-Gal, and Gal-(1→6)-Gal. Fractionation (Sephadex G-25) of the acidic product gave four oligosaccharide fractions (PA-1,2,3,4), and p.c. of PA-4 gave one major oligosaccharide (PA-4a). The higher acidic oligosaccharide fractions, PA-1 and PA-2, were methylated, then carboxyl-reduced with sodium borodeuteride, and converted into the partially methylated alditol acetates. Methylation analysis (Table IV) showed that PA-1 and PA-2 consisted mainly of 2-linked Rha, and terminal and 4-linked Gal*p*A. Likewise, the intermediate acidic oligosaccharide fraction PA-3 was shown to be composed mainly of 2-linked Rha as the reducing end-group, and 6-linked Gal*p* and terminal Glc*p*A in addition to the glycosidic linkages observed in PA-1 and PA-2. The carboxyl-reduced methylated oligosaccharide-alditols derived from the lower acidic oligosaccharide fraction, PA-4a, were further methylated before analysis by g.l.c.–m.s. (Tables V and VI). C.i.- and e.i.-m.s. showed that PA-4a contained three oligosaccharide fragments, [d], [e], and [f], which were HexA-(1→6)-Gal-ol, HexA-(1→4)-Rha-ol, and HexA-(1→2)-Rha-ol, respectively, on the basis of specific fragment ions. However, the hexuronosyl residues could not be assigned as GlcA or GalA.

TABLE VII

LINKAGE COMPOSITION OF ACIDIC ARABINOGALACTAN UNITS OF ARABINOFURANOSIDASE-DIGESTED AGIIb-1 (AF-A-I AND AF-A-II) BOTH BEFORE AND AFTER BASE-CATALYSED β-ELIMINATION OF URONIC ACID RESIDUES

Glycosyl residues	*Position of O-methyl groups*	*Position of O-ethyl groups*	*Deduced glycosidic linkages*	*Before elimination*		*After elimination*					
				(mol. %)		*(mol. %)*				*(molar ratios)*	
						R_1-HexA		*HexA-R_2a*		*HexA-R_2b*	
				AF-A-I	*AF-A-II*	*AF-A-I*	*AF-A-II*	*AF-A-I*	*AF-A-II*	*AF-A-I*	*AF-A-II*
Arabinosyl	2,3,5	—	Terminal	5.8	6.1	4.7	7.6	6.4	7.2	—	—
	2,3	—	4 or 5	21.1	20.1	27.3	31.5	24.6	34.9	—	—
	2	—	3,4 or 3,5	0.5	trace	trace	trace	trace	trace	—	—
Rhamnosyl	3	—	2,4	13.4	11.7	5.3	3.4	3.3	0.6	—	—
	3	2	2,4	—	—	—	—	1.3	0.6	—	0.5
Galactosyl	2,3,4,6	—	Terminal	17.2	16.0	11.8	9.3	10.1	7.6	detected[a]	1.0
	2,3,6	—	4	10.3	9.7	11.5	8.2	12.0	8.5	—	—
	2,4,6	—	3	3.9	3.2	3.3	3.3	7.9	3.0	—	—
	2,3,4	—	6	18.9	23.6	25.0	26.1	22.5	25.2	—	—
	2,3,4	6	6	—	—	—	—	2.8	3.0	—	—
	2,4	—	3,6	7.2	8.0	9.5	8.6	8.2	8.8	—	—
	2	—	3,4,6	1.7	1.6	1.6	1.9	0.8	0.3	—	—

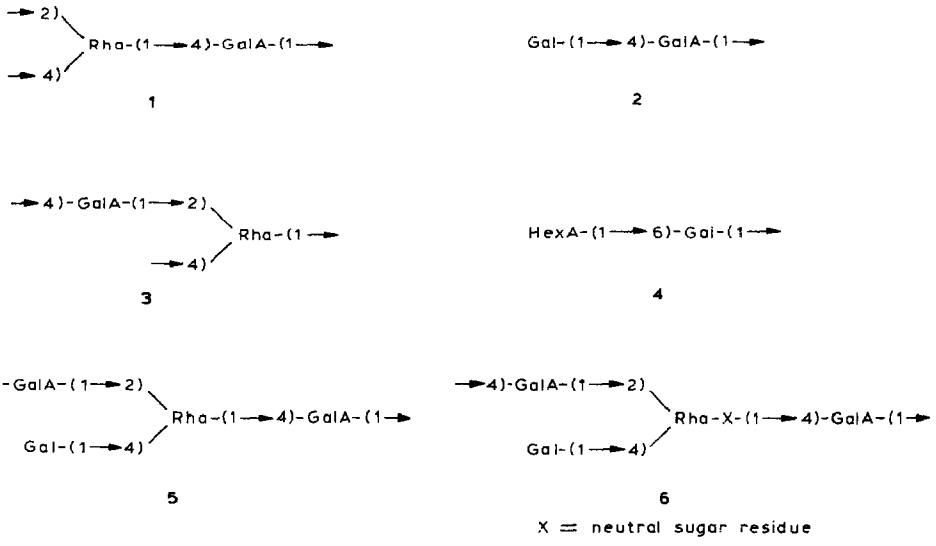
[a]Only 2,3,4,6-tetra-*O*-methylgalactitol diacetate was detected.

These results suggested that the major acidic arabinogalactan unit, AF-A-I, contained 4-linked Ara*p* chains, a backbone repeating-unit →4)-GalA-(1→2)-Rha-(1→, and GlcA-(1→6)-Gal-(1→ as a partial structural moiety.

Identification of rhamnogalacturonan core in acidic units by base-catalysed β-elimination. — The base-catalysed β-elimination of methylated AF-A-I and AF-A-II was performed by the modified procedure of McNeil *et al.*[10], and the exposed hydroxyl groups were ethylated. Two partially methylated and ethylated β-elimination products (R_2-a and R_2-b) of high and low molecular weight were obtained by gel filtration on Sephadex LH-20.

Methylation analysis (Table VII) showed losses of ~60% of 2,4-disubstituted Rha*p* and ~31% of terminal Gal*p* from AF-A-I. The corresponding losses from AF-A-II were ~70% and ~42%. These results indicated that 60% or more of the 2,4-disubstituted Rha was attached to C-4 of Gal*p*A in A-I and 70% or more in A-II (**1**), and also suggested some terminal Gal*p* was attached to C-4 of Gal*p*A in both A-I and A-II (**2**). 1,4,5-Tri-*O*-acetyl-2-*O*-ethyl-3-*O*-methylrhamnitol was formed in the methylation analysis of R_2-a from both AF-A-I (10% of Rha) and AF-A-II (5% of Rha), as was 1,5-di-*O*-acetyl-6-*O*-ethyl-2,3,4-tri-*O*-methylgalactitol. 2,4-Disubstituted Rha was also detected (25% from AF-A-I, 5% from AF-A-II) in R_2-a. R_2-b from AF-A-I contained only terminal Gal*p*, whereas R_2-b from AF-A-II gave 1,4,5-tri-*O*-acetyl-2-*O*-ethyl-3-*O*-methylrhamnitol and terminal Gal*p* in the molar ratio 0.5:1.0.

These results suggested that 4-substituted Gal*p*A was linked to position 2 of the 2,4-disubstituted Rha (**3**) in A-I (10%) and A-II (5%), respectively, and that uronic acid residues were also attached to C-6 of Gal*p* both in A-I and A-II (**4**). The analysis of R_2-b suggested that A-I and A-II possessed the partial structure shown in **5** and **6**, respectively.



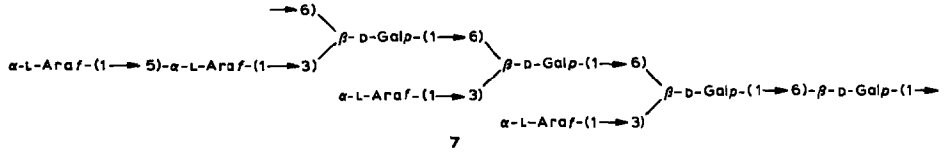
DISCUSSION

The present results suggested that AGIIb-1 consisted of one neutral and two acidic arabinogalactans and one neutral arabinan, which were linked to each other by acid-labile linkages that were broken by the mild acid treatment which decreased the anti-complementary activity[1] of AGIIb-1.

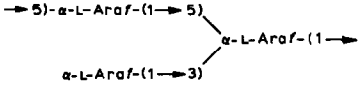
Some plant and microbial polysaccharides contain such acid-labile linkages as phenolic acid esters[15] and phosphodiesters[16]. Recently, York *et al.*[17] reported that 3-deoxy-D-*manno*-2-octulosonic acid (KDO) was present in purified cell walls of several plants and is released by mild acid hydrolysis[17]. However, under mildly alkaline[18] or weakly acid conditions[17] (M acetic acid, 40°, 6 h), no carbohydrate units were liberated from AGIIb-1. AGIIb-1 contained a large proportion of acid-labile Ara*f* linkages, and treatment with 10mM hydrochloric acid at 100° for 10 min released a significant amount of Ara in addition to the major four units (Figs. 1 and 2), suggesting that they might be connected by Ara*f* linkages.

The neutral arabinogalactan unit (N-I) appeared to be a typical arabino-3,6-galactan with short unbranched Ara*f* side-chains mainly attached at positions 3 of the (1→6)-linked galactan chain as shown in **7**, whereas the neutral arabinan unit (N-II) appeared to be a (1→5)-α-L-arabinan possessing numerous Ara*f* side-chains at positions 3 as shown in **8**.

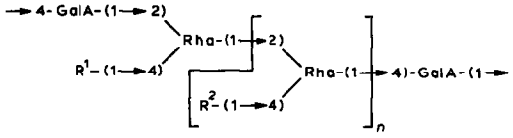
The previous study[1] suggested AGIIb-1 to be a pectic arabinogalactan and AGIIb-1 to have a rhamnogalacturonan core with an arabinogalactan moiety attached at position 4 of Rha as found[10] in rhamnogalacturonan I. Oligosaccharide analysis and base-catalysed β-elimination studies suggested that two acidic arabinogalactan units (A-I and A-II) contained a rhamnogalacturonan core as



7



8



9 R^1, R^2 = Galp-(1→4)-GalA-(1→ or Galp-(1→ or arabino-3,6-galactan

A-I, R^2 = Galp; A-II, R^1 or R^2 = Galp

shown in **9**. A-I and A-II units might contain dirhamnosyl units because PA-1 and PA-2, which were obtained by partial acid hydrolysis from AF-A-I, consisted mainly of 2-substituted Rha and 4-substituted Gal*p*A in the molar ratio ~2:1. This result accords with those of base-catalysed β-elimination of methylated AF-A-I and AF-A-II.

Base-catalysed β-elimination studies and partial acid hydrolysis suggested that the arabinogalactan moiety might be linked to position 4 of 2,4-disubstituted Rha and that 6-substituted Gal*p* might be attached to the rhamnogalacturonan inner core. Thus, the arabino-3,6-galactan moiety could be attached to the core through position 4 of 2,4-disubstituted Rha*p* in A-I and A-II. Some 6-substituted Gal*p* of the arabinogalactan chain was terminated by Glc*p*A, and some 4-substituted Gal*p* might also be associated with Ara chains which contained a small proportion of Ara*p* near the reducing terminals. Because 4-substituted Gal*p* was detected in the neutral oligosaccharide fractions (PN-1, PN-2, and PN-5) derived from A-I by mild acid hydrolysis, 4-substituted Gal*p* chains might be linked to both with 6-substituted Gal*p* chains and the rhamnogalacturonan core. Methylation analysis could not elucidate the difference between the structures of A-I and A-II, but the proportion of terminal Glc*p*A to 4-substituted Gal*p*A was higher in A-II than in A-I, suggesting heterogeneity in the content of terminal Glc*p*A.

When AGIIb-1 was digested with exo-α-L-arabinofuranosidase, a significant amount of linear (1→5)-linked-α-L-Ara remained intact, although Uesaka *et al.*[19] reported that this enzyme was able to attack linear (1→5)-α-L-arabinans. These results suggested that some other sugar residues may be attached to the non-reducing terminal of the linear (1→5)-α-L-arabinan. Similar resistance to exo-α-L-arabinofuranosidase has been observed in the pectic polysaccharides of rice endosperm cell walls[20] and the leaves of *Artemisia princeps* PAMP[21]. Therefore, the neutral arabinogalactan unit may be attached to the arabinan unit. Talmadge *et al.*[22] proposed that the 2,4-disubstituted Rha in rhamnogalacturonan was linked with other neutral sugar moieties (*e.g.*, arabinogalactan). The neutral arabinogalactan and the arabinan units in AGIIb-1 may be directly or indirectly linked to the rhamnogalacturonan core of acidic arabinogalactan units (Fig. 4).

The details of the total structure of AGIIb-1 must await further studies.

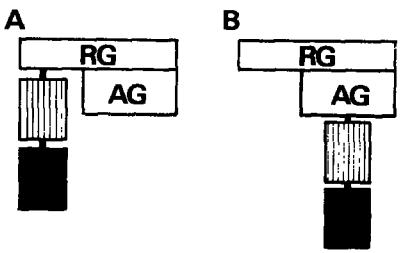


Fig. 4. Possible structures of AGIIb-1: ▬, neutral arabinogalactan unit (N-I); ■, neutral arabinan unit (N-II); ▭, acidic arabinogalactan units (A-I and A-II); ——, acid-labile linkage; RG, rhamnogalacturonan moiety; AG, acidic arabinogalactan moiety.

ACKNOWLEDGMENTS

The authors thank Dr. N. Shibuya (National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Japan) for a gift of exo-α-L-arabinofuranosidase from *R. flava*, Ms. A. Nakagawa and Ms. C. Sakabe for assistance with g.l.c.–m.s., and Dr. J.-C. Cyong for his encouragement. A part of this work was supported by the fund of Tsumura-Juntendo Co. Ltd., Tokyo.

REFERENCES

1 H. YAMADA, H. KIYOHARA, J.-C. CYONG, AND Y. OTSUKA, *Carbohydr. Res.*, 159 (1987) 275–291.
2 H. KIYOHARA, H. YAMADA, J.-C. CYONG, AND Y. OTSUKA, *J. Pharmacobio-Dynamics*, 9 (1986) 339–346.
3 N. BLUMENKRANTZ AND G. ASBOE-HANSEN, *Anal. Biochem.*, 54 (1973) 484–489.
4 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
5 Z. DISCHE AND E. BORENFREUND, *Biochim. Biophys. Acta*, 23 (1957) 639–642.
6 T. M. JONES AND P. ALBERSHEIM, *Plant Physiol.*, 49 (1972) 926–936.
7 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
8 T. Z. WAEGHE, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 123 (1983) 281–304.
9 B. LINDBERG, J. LÖNNGREN, AND J. L. THOMPSON, *Carbohydr. Res.*, 28 (1973) 351–357.
10 M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Plant Physiol.*, 66 (1980) 1128–1134.
11 Z. DISCHE, *Methods Carbohydr. Chem.*, 1 (1962) 478–481.
12 O. S. CHIZHOV, V. I. KADENTSEV, A. A. SOLOVYOV, P. F. LEVONOWICH, AND R. C. DOUGHERTY, *J. Org. Chem.*, 41 (1976) 3425–3428.
13 N. K. KOCHETKOV AND O. S. CHIZHOV, *Adv. Carbohydr. Chem.*, 21 (1966) 39–93.
14 J.-P. JOSELEAU, G. CHAMBAT, M. VIGNON, AND F. BARNOUD, *Carbohydr. Res.*, 58 (1977) 165–175.
15 P. J. HARRIS AND R. D. HARTLEY, *Biochem. Syst. Ecol.*, 8 (1981) 189–203.
16 T. R. THIEME AND C. E. BALLOU, *Biochemistry*, 10 (1971) 4121–4129.
17 W. S. YORK, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 138 (1985) 109–126.
18 S. C. FRY, *Biochem. J.*, 203 (1982) 493–504.
19 E. UESAKA, M. SATO, M. RAIJU, AND A. KAJI, *J. Bacteriol.*, 133 (1978) 1073–1077.
20 N. SHIBUYA AND R. NAKANE, *Phytochemistry*, 23 (1984) 1425–1429.
21 H. YAMADA, Y. OTSUKA, AND S. ŌMURA, *Planta Med.*, (1986) 311–314.
22 K. W. TALMADGE, K. KEEGSTRA, W. D. BAUER, AND P. ALBERSHEIM, *Plant Physiol.*, 51 (1973) 158–173.