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STRUCTURAL CHARACTERISATION OF AN ANTI-COMPLEMENTARY ARABINOGALACTAN FROM THE ROOTS OF *Angelica acutiloba* KITAGAWA*

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ABSTRACT

An anti-complementary arabinogalactan (AGIIb-1), isolated from the roots of *Angelica acutiloba* Kitagawa, has been subjected to methylation analysis, digestion with α-L-arabinofuranosidase, controlled Smith-degradation, and partial acid hydrolysis. AGIIb-1 consisted of arabinose, galactose, rhamnose, galacturonic acid, and glucuronic acid in the molar ratios 1.8~2.2:1.0:0.2~0.3:0.2~0.4:0.1. AGIIb-1 contained mainly an arabino-3,6-galactan moiety, and most of the Ara was present as α-L-arabinofuranosyl residues in the non-reducing terminals and the highly polymerised and branched side-chains which were attached mainly to positions 3 and 6 of (1→6)- and (1→3)-linked Gal, respectively. Some Ara-containing chains were also attached to (1→4)-linked Gal residues. The ^{13}C-n.m.r. data for AGIIb-1 showed that the Gal*p* was β. Mild acid hydrolysis of AGIIb-1 yielded several linear and highly branched arabino-oligosaccharides, a neutral arabinogalactan, and two acidic arabinogalactans. Some arabino-oligosaccharides contained a (1→4)-linked Ara*p* at the reducing terminal. The neutral arabinogalactan contained (1→3)-, (1→4)-, and (1→6)-linked and 3,6-di-*O*-substituted Gal, whereas the acidic arabinogalactans contained, in addition, non-reducing terminal GlcA, (1→4)-linked GalA, and 2,4-di-*O*-substituted Rha. The anti-complementary activity was decreased when AGIIb-1 was partially hydrolysed with mild acid (10mM HCl, 100°, 10 min), but treatment with exo-α-L-arabinofuranosidase markedly enhanced the activity.

INTRODUCTION

The complement system plays an important role in host defence, inflammation, and allergic reactions, and the anti-complementary substance is able to prevent hemolytic activity by the activation or inhibition of the complement system. Anti-complementary polysaccharides, for example, lipopolysaccharide[2–4],

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(1→3)-β-D-glucan[5,6], 6-branched (1→3)-β-D-glucan[5,6], inulin[7], arabinan[8], and highly branched *O*-acetylated acidic polysaccharides[8], have been isolated from bacteria, fungi, and plants. We have found potent anti-complementary activity in the polysaccharide fraction from a Chinese herb, the root of *Angelica acutiloba* Kitagawa[9] (Japanese name = Yamato-Tohki), which is a well known crude drug used in the treatment of gynaecological diseases and arthritis in Sino-Japanese herbal medicine. Recently, two kinds of anti-complementary arabinogalactans (AGIIa[10] and AGIIb-1[1]) were purified from the hot-water extract of *A. acutiloba*. AGIIa was suggested[10] to contain a backbone of (1→6)-linked Gal with (1→5)-linked α-L-Ara*f* and (1→3)-linked Gal*p* attached to positions 3 of Gal*p* of the backbone. AGIIb-1 tended to self-aggregate[1] and this property was suggested to be important in the expression of anti-complementary activity[1].

We now report further on the structure of AGIIb-1 and its anti-complementary activity.

EXPERIMENTAL

Materials. — The roots of *A. acutiloba* Kitagawa were purchased from Uchida Wakanyaku Co. Ltd., Japan. Sepharose CL-2B and CL-6B, and Sephadex G-100 and G-10 were obtained from Pharmacia, and Bio-gels P-2 (200–400 mesh) and P-4 (−400 mesh) from Bio-Rad. *Rhodotolura flava* exo-α-L-arabinofuranosidase[11] was a gift from Dr. Naoto Shibuya (National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Japan).

General. — Total carbohydrate, uronic acid, and protein contents were assayed by the phenol–sulfuric acid[12], *m*-hydroxybiphenyl[13], and Lowry[14] methods, respectively, using a 1.8:1.0 mol mixture of arabinose and galactose, galacturonic acid, and bovine serum albumin as the respective standards. Reducing power was measured by the procedure of Park and Johnson[15], using arabinose as the standard. Optical rotations were determined at 22° with a JASCO DIP-Digital polarimeter. Neutral polysaccharides were hydrolysed with 2M trifluoroacetic acid at 121° for 1 h (1.5 h for acidic polysaccharides). Sugars were converted conventionally[16] into the alditol acetates. G.l.c. was carried out at 190° using a Shimadzu GC-6A gas chromatograph equipped with a flame-ionisation detector and a glass column (3 mm i.d. × 200 cm) packed with 1% of OV-225 on Uniport HP. The molar ratios of neutral sugars were calculated from the peak areas and mol. wts. of the corresponding alditol acetates. The molar ratios of uronic acid and neutral sugars were calculated from the content of uronic acid. T.l.c. of acid hydrolysates was performed on cellulose-coated plastic sheets (Merck), using *A*, ethyl acetate–pyridine–acetic acid–water (5:5:1:3). P.c. was performed on No. 51 paper (Toyo-Roshi Co. Ltd., Japan), using *A* or *B*, 1-butanol–acetic acid–water (4:1:5, upper layer). Reducing sugars were detected with alkaline silver nitrate[17], and uronic acid with *p*-anisidine hydrochloride[18].

Anti-complementary arabinogalactan, AGIIb-1. — (a) Purification. The crude

polysaccharide fraction (AR-1), prepared[9] by hot-water extraction and ethanol precipitation from *A. acutiloba* Kitagawa, was further fractionated by treatment with cetyltrimethylammonium bromide, and the active fraction, AR-4, was purified[9] on DEAE-Sephadex A-50. Fraction AR-4IIb from the absorbed fraction was further purified[1] by gel filtration on Sephadex G-100 and by affinity chromatography on *Ricinus communis* agglutinin (RCA)-conjugated Sepharose; AGIIb-1 was obtained as the RCA-unbound fraction.

(b) Digestion with exo-α-L-arabinofuranosidase. AGIIb-1 (6.4 mg) was digested with exo-α-L-arabinofuranosidase[11] (0.7 U) in 0.1M citrate phosphate buffer (pH 3.0, 5.0 mL) at 50° for 20 h. The mixture was neutralised with M sodium hydroxide and lyophilised, and the residue was eluted from a column (2.5 × 50 cm) of Bio-gel P-2 at 55° with water. The carbohydrate fractions eluted in the void volume (AF-AGIIb-1) and the included volume were collected.

(c) Periodate oxidation and Smith degradation. AGIIb-1 (18.9 mg) was oxidised with 20mM sodium metaperiodate (15 mL) at 4° in the dark. Periodate consumption was determined by the method of Avigad[19]. After 118 h, ethylene glycol was added to reduce the excess of periodate, the oxidised AGIIb-1 was reduced with sodium borohydride for 4 h at room temperature, the mixture was dialysed against water for 2 days, and AGIIb-1 polyalcohol was isolated by freeze-drying. A portion of AGIIb-1 polyalcohol was hydrolysed with acid (see above), and the products were converted into the alditol acetates and analysed by g.l.c. (130°→210° at 10°/min).

(d) Controlled Smith-degradation. A solution of AGIIb-1 polyalcohol in 0.1M sulfuric acid was stirred for 24 h at room temperature, then neutralised ($BaCO_3$), and desalted with AG50W-X8 (H^+) resin. Elution of the product from a column of DEAE-Sephadex A-25 ($HCOO^-$ form) with water gave the neutral fraction (SD-N), and elution with 2M formic acid gave the acidic fraction (SD-A).

(e) Partial acid hydrolysis. A solution of AGIIb-1 (87.2 mg) in 10mM hydrochloric acid (10 mL) was heated at 100° for 1 h, then neutralised with 0.1M sodium hydroxide, and lyophilised. Elution of the residue from a column of DEAE-Sephadex A-25 ($HCOO^-$ form) with water gave the neutral fraction, and elution with 2M formic acid and lyophilisation gave A-1 (26.6%). Elution with 2M sodium chloride and desalting on Sephadex G-10 gave A-2 (10.2%). Elution of the neutral fraction from a column (2.0 × 180 cm) of Bio-gel P-2 with water at 55° gave oligosaccharide fractions I–VI, and the carbohydrate fraction N in the void volume. Fraction I was further purified by p.c. (solvent *A*), as were fractions II–VI (solvent *B*). Fractionation of N on a column (2.0 × 100 cm) of Bio-gel P-4 at 55° gave N-1 (6.4%, eluted in the void volume) and N-2 (16.4%, eluted as a broad peak).

Carboxyl-reduction of uronic acid. — The method of Taylor and Conrad[20] was used. The reaction mixture of the carboxyl-reduced A-1 was desalted by elution from a column (2.6 × 95 cm) of Sephadex G-10 with water, and the fraction (RA-1-1) eluted in the void volume and that eluted in the included volume were obtained in the weight ratio 0.4:1.0 (Fig. 1A). When the latter fraction was eluted

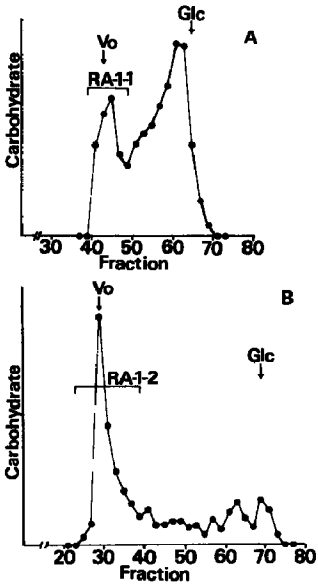


Fig. 1. Gel filtration of *A*, the carboxyl-reduced products from A-1 on Sephadex G-10; and *B*, on Bio-gel P-2, the fraction eluted in the included volume from *A*; V_0, void volume.

from a column of Bio-gel P-2, the major carbohydrate fraction (RA-1-2) appeared in the void volume (Fig. 1B). The major component sugars of RA-1-1 and RA-1-2 were Rha, Ara, and Gal in the molar ratios 0.3:0.4:1.0 and 0.3:0.2:1.0, respectively; a trace of glucose was also present. Carboxyl-reduced A-2 was fractionated into RA-2-1 (void volume) and RA-2-2 (included fraction) in the weight ratio 1.4:1.0 (data not shown). RA-2-1 and RA-2-2 were composed of Rha, Ara, and Gal in the molar ratios 0.2:0.3:1.0, together with a trace of glucose.

Methylation analysis. — Each poly- and oligo-saccharide was methylated once by the Hakomori method[21] in order to prevent β-elimination, and the completeness of the methylation was checked by using triphenylmethane[22]. The purified arabino-oligosaccharides were reduced conventionally with sodium borodeuteride in water (4 h, room temp.), and the resulting alditols were methylated and then purified using[23] a Sep-pak C_{18} cartridge (Waters Assoc.). The methylated polysaccharides and oligosaccharide-alditols were hydrolysed with acid (see above); the products were reduced with sodium borohydride or borodeuteride and then acetylated. The resulting alditol acetates were analysed by g.l.c. and g.l.c.–m.s.[24]. G.l.c. was performed on a Hewlett-Packard model 5840A gas chromatograph equipped with dual flame-ionisation detectors. Solutions of partially methylated alditol acetates in acetone were injected into a DB-1 capillary column (0.25-μm film thickness, 30 m × 0.25 mm i.d., J and W Scientific Inc.) with split (100:1) or unsplit injection. The carrier gas was helium at 0.9 mL/min and the temperature programme was 150° for 1 min and then 2°/min→210°. G.l.c.–m.s. (70 eV) was performed on a JEOL DX-300 instrument equipped with a glass column packed

with 1% of OV-225 on Uniport HP and operated at 170°. Peaks were identified on the basis of relative retention times and fragmentation patterns. The calibration of molar ratios for each sugar was performed from the peak areas and response factors[25] of f.i.d. in g.l.c. on DB-1.

Mass spectrometry. — F.a.b.-m.s. of oligosaccharide-alditols was performed with a Hitachi M-80 mass spectrometer interfaced with an M-003 computer. A solution of each oligosaccharide-alditol in aqueous 50% triethanolamine was loaded on to a silver plate, and excess of solvent was removed with air. The accelerating voltage was 3 kV and 5keV Xe^+ was used as the primary ion with an ion current of 5×10^{-8} A.

E.i. (70 eV; accelerating voltage, 3 kV) and c.i. (isobutane, 250 eV; accelerating voltage, 3 kV; sample evaporation, 50°→180°) mass spectra were recorded with a JEOL DX-300 mass spectrometer with direct insertion of each methylated oligosaccharide-alditol.

N.m.r. spectrometry. — ^{1}H- (90 MHz) and ^{13}C-n.m.r. (22.5 MHz) spectra of AGIIb-1 and reference substances were obtained for solutions in D_2O at 80°, using a JEOL JNM-FX 90Q spectrometer operated in the Fourier-transform mode. Chemical shifts were expressed in p.p.m. from the signal of sodium 3-(trimethylsilyl)propane-1-sulfonate-d_4 (TSP).

Mild acid treatment of AGIIb-1. — Solutions of AGIIb-1 (400 μg) in 10mM hydrochloric acid (1 mL) were stored at room temperature for 10 min, and at 100° for 1, 10, 30, 60, and 90 min. Each mixture was then concentrated to dryness at ~40° and the residue was stored over sodium hydroxide in *vacuo* to remove the residual acid. Anti-complementary activities were measured on aqueous solutions of the products.

Anti-complementary activity. — The procedure described previously[1] was used. Gelatin-veronal-buffered saline (pH 7.4) containing 500μM magnesium chloride and 150μM calcium chloride (GVB^{2+}) was prepared by the method of Kabat *et al.*[26], and normal human serum (NHS) was obtained from a healthy adult. Various dilutions of the sample in water (50 μL) were mixed with 50 μL of NHS and GVB^{2+}. The mixtures were pre-incubated at 37° for 30 min and 350 μL of GVB^{2+} was added. The residual hemolytic complement (TCH_{50}) was determined by a method using IgM-hemolysin-sensitised sheep erythrocytes (EA) at 1×10^8 cells/mL. NHS was incubated with water to provide a control. The anti-complementary activity of the sample was expressed as inhibition of TCH_{50} (%) =

$$\frac{TCH_{50}\text{ of control} - TCH_{50}\text{ treated with polysaccharide}}{TCH_{50}\text{ of control}} \times 100$$

RESULTS

Properties of AGIIb-1. — AGIIb-1 had $[\alpha]_D$ −57° (*c* 1, water), was soluble in water, contained 92.7% of neutral sugar, 8.7–12.2% of uronic acid, and 2.3% of

protein, and was composed of Ara, Gal, Rha, GalA, and GlcA in the molar ratios 1.8~2.2:1.0:0.2~0.3:0.2~0.4:0.1 in the different preparations. The ^{1}H-n.m.r. spectrum of AGIIb-1 contained signals at 1.33 (*J* 7.4 Hz, CMe of Rha), 2.11 and 2.16 (OAc), 4.5 and 4.65 (H-1β), and 5.10, 5.15, 5.25, and 5.40 p.p.m. (H-1α). The ratio of signal intensities for anomeric and acetyl protons was 15:0.37, indicating the *O*-acetyl content of AGIIb-1 to be ~2.4%. The ^{13}C-n.m.r. spectrum of AGIIb-1 contained signals due to anomeric carbons at 112.09, 110.29, 109.95, 107.17, and 106.24 p.p.m. The first three signals were assigned[27] to α-L-Ara*f* and the last two to β-D-Gal*p* (*cf.* 106.58 p.p.m. for C-1 of methyl β-D-galactopyranoside). Signals due to C-1 of other glycose residues were not observed because of their low abundance. Methylation analysis showed (Table I) that AGIIb-1 contained mainly Ara*f* non-reducing end-groups, 4- or 5-*O*-linked Ara, 3,4- or 3,5-di-*O*-substituted Ara, and 3,6-di-*O*-substituted Gal*p*. The presence of 3,4- or 3,5-di-*O*-substituted Ara was indicated by the identification of acetylated 2-*O*-methylarabinitol-*1-d*, which has[24] major primary fragments at *m/z* 118 and 261. AGIIb-1 also contained non-reducing Gal*p* end-groups, 4-, 3-, and 6-*O*-linked Gal*p*, and 2,4-di-*O*-substituted Rha*p*.

Enzymic digestion of AGIIb-1. — On digestion with *R. flava* exo-α-L-arabinofuranosidase, 37.2% of AGIIb-1 (calculated on the basis of reducing power) was hydrolysed and the only monosaccharide released (t.l.c., g.l.c.) was Ara. The α-L-arabinofuranosidase-resistant fraction (AF-AGIIb-1) was composed of Ara, Gal, and Rha in the molar ratios 0.8:1.0:0.2, and the decreased content of Ara corresponded to 56% of the Ara in AGIIb-1. Methylation analysis (Table I) of AF-AGIIb-1 showed that (*a*) the non-reducing terminal and branched Ara residues were decreased remarkably, whereas 4- or 5-*O*-linked Ara was increased significantly and a large proportion of 4- or 5-*O*-linked Ara still remained after the enzymic digestion; (*b*) 3,6-di-*O*-substituted Gal*p* decreased, whereas 6- and 3-*O*-linked Gal*p* increased significantly in comparison with AGIIb-1, when the molar ratios (%) were calculated from the linkages due to only Gal*p*. These results suggested that (*a*) the Ara*f* side-chains attached to position 3 of 5- or 4-*O*-linked Ara in AGIIb-1 were removed by the action of exo-α-L-arabinofuranosidase and that the remaining Ara was resistant, and (*b*) some Ara*f* was attached to either position 3 of 6-*O*-linked Gal*p* or position 6 of 3-*O*-linked Gal*p*. Although the decrease of 4-*O*-linked Gal*p* was observed, the molar percent of the non-reducing terminal Gal*p* did not increase significantly.

Smith and controlled Smith degradation of AGIIb-1. — AGIIb-1 consumed 0.82 mol of periodate per "anhydrohexose" unit, and the Smith degradation yielded glycerol, threitol, Ara, Gal, Rha, and uronic acid in the molar ratios 6.6:0.4:1.4:1.0:0.4:trace. When the oxidised AGIIb-1 was reduced with borohydride and then treated with a weak acid, fractionation of the product by anion-exchange chromatography gave unabsorbed (SD-N) and absorbed (SD-A) fractions in the ratio of 10:1. SD-N was further fractionated on Bio-gel P-2, to give a fraction (SD-N-1) eluted in the void volume and a fraction (SD-N-2) containing several oligosaccharide-alcohols (Fig. 2A). SD-N-2 yielded Ara and small

TABLE I

METHYLATION ANALYSIS OF AGIIb-1, THE α-L-ARABINOFURANOSIDASE DIGEST (AF-AGIIb-1), AND SMITH-DEGRADATION PRODUCTS (SD-N-1-1 AND SD-A)

Glycose	*Position of O-methyl groups*	*Deduced glycosidic linkages*	*Mol. %*					
			AGIIb-1[a]		*AF-AGIIb-1*[a]		*SD-N-1-1*	*SD-A*
			Total	*Galactan*	*Total*	*Galactan*		
Ara	2,3,5	terminal (furanose)	39.9		3.3		0.6	—
	2,3,4	terminal (pyranose)	—		—		—	3.8
	2,3	4 or 5	12.8		22.0		0.5	—
	2	3,4 or 3,5	17.5		trace		—	—
Rha	2,3,4	terminal	—		—		—	10.6
	3,4	2	—		—		—	49.0
	3	2,4	2.2		4.7		—	3.4
Gal	2,3,4,6	terminal	4.9	17.2	12.7	18.2	10.9	3.3
	2,3,6	4	3.6	12.8	4.7	6.7	—	—
	2,4,6	3	2.9	10.2	15.4	22.0	12.1	18.4
	2,3,4	6	3.7	12.9	28.8	41.0	52.9	28.0
	2,4	3,6	11.1	38.8	8.4	12.0	22.9	26.6
	2	3,4,6	2.3	8.1	trace	trace	trace	17.0

[a]Calculated from all glycose (total) and from galactose residues only (galactan).

proportions of glycerol and threitol on hydrolysis. When SD-N-2 was digested with exo-α-L-arabinofuranosidase, most of the product was eluted from Bio-gel P-2 in the included volume (Fig. 2B) and was mainly Ara. SD-N-1 was further fractionated on Bio-gel P-4, and a fraction (SD-N-1-1) eluted in the void volume and a fraction (SD-N-1-2) containing long-chain oligosaccharide-alcohols were obtained (data not shown). SD-N-1-1 was composed of Ara and Gal in the molar ratio 0.2:1.0, and SD-N-1-2 consisted of Ara and traces of glycerol and threitol. SD-N-1-2 might comprise highly branched, long chains of Ara, but further analysis was not possible because of the small quantity available. These results suggested that AGIIb-1 contained highly branched Ara*f* chains, and that some of these chains were attached to position 4 of Gal*p* at the reducing terminal, because threitol was detected in SD-N-2 and SD-N-1-2. SD-A was composed of glycerol, Rha, Ara, and Gal in the molar ratios 0.2:2.5:0.4:1.0. SD-A might contain the acidic components derived from uronic acid, but further analysis could not be carried out. The results of methylation analysis (Table I) showed that SD-N-1-1 mainly contained 6-*O*-linked Gal*p* in addition to non-reducing terminal, 3-*O*-linked, and 3,6-di-*O*-substituted Gal*p*.

These results indicated that AGIIb-1 contained a highly branched (1→6)-linked galactan with Gal and Ara*f* side-chains attached at position 3 and a highly branched (1→5)-linked Ara*f* polymer with Ara side-chains attached at position 3. SD-A contained a large proportion of 2-*O*-linked Rha and significant amounts of 3- and 6-*O*-linked and 3,6-di-*O*-substituted Gal*p* in addition of 2,4-di-*O*-substituted

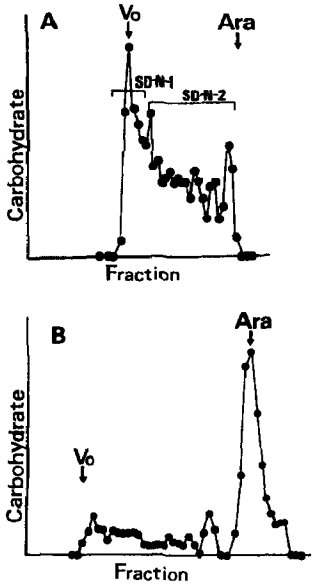


Fig. 2. Fractionation on Bio-gel P-2: *A*, the neutral fraction (SD-N) of the controlled Smith-degradation products from AGIIb-1; and *B*, after treatment of SD-N-2 in *A* with *R. flava* exo-α-L-arabinofuranosidase. The column (2.0 × 50 cm) was equilibrated and eluted with water at 55°.

Rha as the neutral sugar moieties. These results suggested that AGIIb-1 might contain highly branched (1→2)-linked Rha attached to the (1→3)- or (1→6)-linked galactan chain at positions 4. SD-A also contained a significant amount of 3,4,6-tri-*O*-substituted Gal and a small proportion of Ara*p* non-reducing end-groups.

Partial acid hydrolysis of AGIIb-1. — Hydrolysis with 10mM hydrochloric acid at 100° for 1 h, followed by anion-exchange chromatography, gave one neutral and two acidic carbohydrate fractions (A-1 and A-2) (Fig. 3A). The neutral fraction gave material (N) in the void volume and six oligosaccharide fractions (I–VI) by gel filtration on Bio-gel P-2 (Fig. 3B). Each oligosaccharide fraction was further purified by p.c., and seven oligosaccharides (I-A, I-B, II–VI) were obtained as major arabino-oligosaccharides. M.s. and methylation analysis data for the oligosaccharide-alditols are given in Table II. F.a.b.-m.s. indicated that oligosaccharides II–VI were tri- to hepta-saccharide, respectively. The oligosaccharides I-A, I-B, and II were reduced with borodeuteride and then methylated, and the products were analysed by c.i.(isobutane)-m.s. The results indicated that I-A and I-B were disaccharides and that II was a trisaccharide. I-A-alditol gave acetylated 1,2,3,5-tetra-*O*-methylarabinitol-*1-d* and 2,3,5-tri-*O*-methylarabinitol, and I-B-alditol gave acetylated 1,2,4,5-tetra-*O*-methylarabinitol-*1-d* and 2,3,5-tri-*O*-methylarabinitol. Thus, I-A was Ara*f*-(1→4)-Ara*p* and I-B was Ara*f*-(1→3)-Ara. The methylation analysis data also suggested that II–V contained 4-*O*-linked Ara*p* as reducing terminals because the fragment peaks at *m/z* 46, 87, and 101 were observed in the

TABLE II

ANALYTICAL DATA OF ARABINO-OLIGOSACCHARIDES FROM AGIIb-1

	Oligosaccharide						
	I-A	*I-B*	*II*	*III*	*IV*	*V*	*VI*
F.a.b.-m.s.[a]							
$(M + Na)^+$, *m/z*	—	—	440	572	704	836	968
C.i.-m.s.[b]							
$(M + H)^+$, *m/z*	384	384	544	—	—	—	—
D.p.[c]	2	2	3	4	5	6	7
Methylated sugars[d] *(molar ratio)*							
1,2,3,5-Me_4-Ara-*1-d*	0.6	—	0.4	0.5	0.5	0.3	—
1,2,4,5-Me_4-Ara-*1-d*	—	1.3	—	—	—	—	—
2,3,5-Me_3-Ara	1.0	1.0	1.0	1.0	1.0	1.0	—
2,5-Me_2-Ara	—	—	0.6	0.3	0.3	0.3	—
2,3-Me_2-Ara	—	—	0.3	0.6	0.6	0.8	—
2-Me-Ara	—	—	—	0.4	0.4	0.5	—

[a]Oligosaccharide-alditols obtained by reduction with sodium borodeuteride. [b]Methylated oligosaccharide-alditols obtained by reduction with sodium borodeuteride were measured. [c]The d.p. of each oligosaccharide was determined from the results of f.a.b.- and c.i.-m.s. [d]Methylated oligosaccharide-alditols obtained by reduction with sodium borodeuteride and then acetylation.

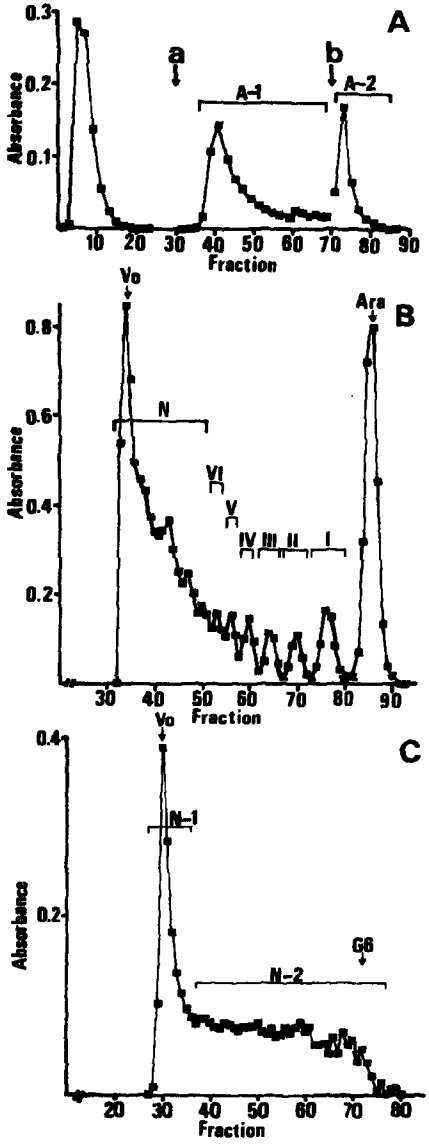


Fig. 3. *A*, Fractionation of the products of partial acid hydrolysis of AGIIb-1 on DEAE-Sephadex A-25 (formate form) [elution with water, 2M formic acid (a), and 2M sodium chloride (b)]; *B*, gel filtration on Bio-gel P-2 of the neutral fraction from *A*; *C*, gel filtration of fraction N from *B* on Bio-gel P-4; G_6, position of elution of glucohexaose.

corresponding g.l.c. peak. However, the d.p. of each oligosaccharide calculated from the results of methylation analysis did not accord with that calculated on the basis of the f.a.b.- and c.i.-m.s. data shown in Table II, which may be due to the low stability of Ara towards acid hydrolysis. E.i.-m.s. of methylated II-alditol gave fragment peaks[28,29] at *m/z* 192 (aJ_2), 335 (cbA_1), 352 (abJ_2), and 175 (cA_1), and methylation analysis showed II-alditol to contain 4- or 5-*O*-linked Ara, 3-*O*-linked Ara*f*, and non-reducing terminal Ara*f* in addition to the reducing terminal Ara-ol (Table II). These results suggested II to be a mixture of the two linear arabinotrisaccharides Ara*f*-(1→4 or 5)-Ara-(1→4)-Ara*p* and Ara*f*-(1→3)-Ara*f*-(1→4)-Ara*p*. The oligosaccharides larger than tetrasaccharides appeared to have a branch point at position 3 of 4- or 5-*O*-linked Ara.

Fractionation of N on Bio-gel P-4 gave N-1 (eluted in the void volume) and a highly polymerised oligosaccharide fraction (N-2) (Fig. 3C). N-1 was composed mainly of Ara and Gal in the molar ratio 2.7:1.0 together with a trace of Rha, whereas N-2 mainly consisted of Ara. Hydrolysis of AGIIb-1 with 10mM hydrochloric acid (100°, 1.5 h) gave an N-1-like fraction, which was purified as for N-1 and was composed of Rha, Ara, and Gal in the molar ratios 0.1:0.2:1.0. Methylation analysis suggested that N-2 was a mixture of linear polymers containing (1→4)- or (1→5)-linked Ara or highly branched oligosaccharides possessing Ara*f* side-chains at position 3, and that N-1 was a mixture of higher arabino-oligosaccharides and fragments containing mainly 4-, 3-, and 6-*O*-linked and 3,6-di-*O*-substituted Gal*p* (Table III).

A-1 and A-2 consisted mainly of Rha, Ara, Gal, and uronic acid in the molar ratios 0.3:0.4:1.0:0.4 and 0.1:0.4:1.0:0.3, respectively. Elution of A-1 or A-2 from Sephadex G-100 with 0.2M sodium chloride gave only material in the void volume (data not shown). Methylation analysis of A-1 and A-2 indicated signifiant amounts of terminal, 4- and 6-*O*-linked, and 3,6-di-*O*-substituted Gal*p* and 2,4-di-*O*-substituted Rha, and small amounts of terminal Ara*f*, 4- or 5-*O*-linked Ara, and 3,4- or 3,5-di-*O*-substituted Ara (Table III).

Carboxyl-reduction[20] of A-1 and A-2 caused partial degradation to reduced products with high (RA-1-1 and RA-2-1) and low molecular weights (RA-1-2 and RA-2-2). Methylation analysis (Table III) of the major products, RA-1-1, RA-1-2, and RA-2-1 suggested that both A-1 and A-2 contained terminal GlcA and 4-*O*-linked GalA since acetylated 2,3,4,6-tetra-*O*-methylglucitol-*6,6-d*$_2$ and 2,3,6-tri-*O*-methylgalactitol-*6,6-d*$_2$ were detected by g.l.c.–m.s.

Effects of mild acid and enzymic treatments on the anti-complementary activity of AGIIb-1. — Treatment of AGIIb-1 with 10mM hydrochloric acid at room temperature for 10 min or at 100° for 1 min reduced the anti-complementary activity of AGIIb-1 by ~15% (Table IV), treatment at 100° for 10 min reduced the activity by 30%, and a further gradual decrease was observed when the heating was continued for 90 min.

AGIIb-1 contains an exo-α-L-arabinofuranosidase-sensitive region, and most of the Ara*f* in the non-reducing terminals and the side chains was removed by

TABLE III

METHYLATION ANALYSIS DATA FOR THE PRODUCTS OF PARTIAL ACID HYDROLYSIS AND CARBOXYL-REDUCTION OF AGIIb-1

Glycose	*Position of O-methyl group*	*Deduced glycosidic linkages*	*Mol. %*						
			N-1	*N-2*	*A-1*	*RA-1-1*	*RA-1-2*	*A-2*	*RA-2-1*
Ara	2,3,5	terminal (furanose)	27.6	39.0	3.3	10.0	1.7	6.2	3.6
	2,3,4	terminal (pyranose)	—	—	—	—	5.7	—	—
	2,3	4 or 5	21.2	32.1	2.6	3.4	0.7	2.2	1.3
	2	3,4 or 3,5	21.6	7.9	5.1	1.4	—	trace	—
Rha	3,4	2	—	—	—	—	1.8	—	—
	3	2,4	—	—	12.6	5.3	7.0	10.3	15.3
Gal	2,3,4,6	terminal	0.9	—	17.5	6.2	14.2	4.7	19.0
	2,3,6	4	5.2	—	11.4	7.2	23.3	18.1	15.6
	2,4,6	3	8.1	—	7.0	32.2	2.8	3.8	2.3
	2,3,4	6	8.0	—	20.7	7.7	15.8	26.5	11.8
	2,4	3,6	7.3	—	17.0	12.3	5.8	14.6	9.3
	2,3	4,6	—	—	—	—	6.0	—	—
	2	3,4,6	0.3	—	2.6	6.3	trace	—	2.5
GlcA[a]	2,3,4,6	terminal	—	—	—	1.9	1.1	—	1.1
GalA[a]	2,3,6	4	—	—	—	8.2	13.9	—	18.3

[a]Acidic fractions were carboxyl-reduced (see RESULTS) and then methylated. The peaks due to uronic acid residues were identified by fragment ions at *m/z* 47, 131, and 207 for terminal Glc*p*A and at 47, 131, and 235 for 4-*O*-linked Gal*p*A. The molar ratios of uronic acid residues were calculated from the proportion of the intensities of the ions at *m/z* 205 and 207 for terminal Glc*p*A and at *m/z* 233 and 235 for 4-*O*-linked Gal*p*A.

TABLE IV

EFFECT OF ACID AND EXO-α-L-ARABINOFURANOSIDASE ON THE ANTI-COMPLEMENTARY ACTIVITY OF AGIIb-1

Treatment	*Concentration (μg/mL)*		
	1000	*500*	*100*
	Anti-complementary activity (%)		
AGIIb-1			
1[a] No treatment	53.1	44.6	12.7
10mM Hydrochloric acid, room temp., 10 min	37.8	35.5	15.3
100°, 1 min	38.3	29.8	15.0
100°, 10 min	23.0	16.3	8.8
100°, 30 min	17.7	11.8	7.6
100°, 60 min	15.0	10.5	6.1
100°, 90 min	15.4	6.8	3.7
2[a] No treatment	70.9	63.4	43.3
10mM Hydrochloric acid, 100°, 10 min	53.1	30.8	17.9
AF-AGIIb-1			
No treatment	100.0	87.1	52.4
10mM Hydrochloric acid, 100°, 10 min	54.4	46.2	28.2

[a]Different preparations of AGIIb-1.

enzymic digestion. The anti-complementary activity of the product (AF-AGIIb-1) was increased 30% in comparison with that of AGIIb-1 (Table IV).

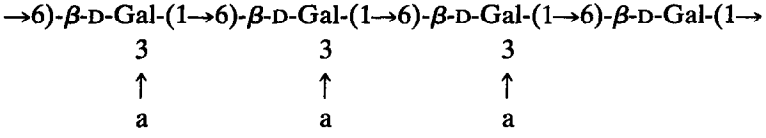
Treatment of AF-AGIIb-1 with 10mM hydrochloric acid at 100° for 10 min decreased the anti-complementary activity to the level of that obtained by similar treatment of AGIIb-1 with acid.

These results suggested that a certain acid-labile linkage(s) and the Ara*f* of AGIIb-1 contributed to its anti-complementary activity.

DISCUSSION

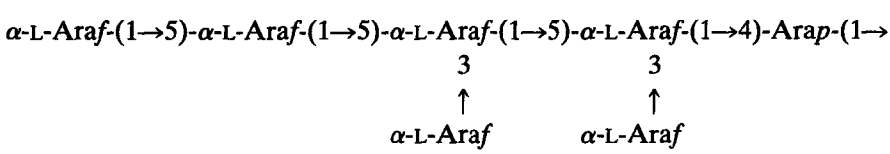
Previously, we have purified two kinds[1,10] of anti-complementary arabinogalactans (AGIIa and AGIIb-1) from the hot-water extract of the roots of *A. acutiloba*. AGIIa[10] is an arabino-3,6-galactan, but AGIIb-1 seems to be a pectic arabinogalactan because it contains[1] 14–20% of Rha and GalA in addition to the arabinogalactan moiety as the major constituent. Methylation analysis of AGIIb-1, before and after digestion with α-L-arabinofuranosidase, suggested that the arabinogalactan moiety **1** consisted mainly of a highly branched (1→6)-linked galactan with Ara*f* side-chains or (1→3)-linked Gal side-chains at position 3, most of which carried the terminal Ara*f*. N.m.r. studies indicated that most of the arabinosyl chains of AGIIb-1 contained α-L-furanosyl residues, but a trace of Ara*p*

was also present because small proportions of arabino-oligosaccharides (I–V) containing Ara*p* were obtained by the mild acid hydrolysis of AGIIb-1. The analytical data suggested that I–VI contain the sequence **2**. These arabinose chains consisted mainly of long chains of highly branched Ara possessing Ara*f* side-chains at position 3 of some (1→5)-linked Ara*f* with several different chain-lengths. Some long, highly branched Ara chains were attached tc (1→4)-linked Gal because the arabino-oligosaccharide alcohols, obtained by controlled Smith-degradation of AGIIb-1, contained threitol as the reducing terminal. From the results of this structural analysis, the partial structure **3** could be deduced.

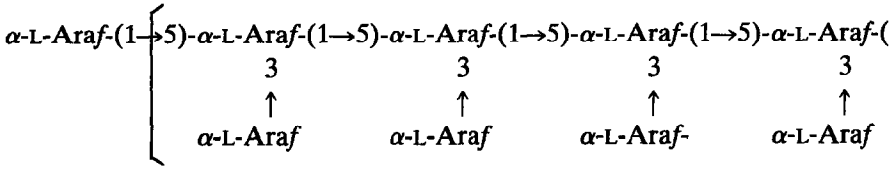


a = arabinosyl side-chains or α-L-Ara*f*-(1→ or β-D-Gal-(1→

1



2



b = →4)-Ara*p*-(1→4)-β-D-Gal*p*-(1→ and/or →4)-β-D-Gal*p*-(1→
and/or →5)-α-L-Ara*f*-(1→

3

Arabinogalactans can be grouped into three main structural types[30], namely, arabino-4-galactans (Type I by Aspinall[31]), arabino-3,6-galactans (Type II), and polysaccharides having arabinogalactan side-chains. AGIIb-1 is thought to belong to the last type[30] since it is a pectic arabinogalactan, but the arabinogalactan moiety of AGIIb-1 comprised arabino-3,6-galactan (major) and arabino-4-galactan (minor) constituents.

AGIIb-1 contained small proportions of 2,4-di-*O*-substituted Rha*p* and 4-*O*-linked GalA in approximately the same ratio. When AGIIb-1 was subjected to a

controlled Smith-degradation, a substantial amount of 2-*O*-linked Rha*p* was detected in the degraded acidic fraction. Therefore, AGIIb-1 may contain a rhamnogalacturonan core possessing major arabinogalactan chains attached to position 4 of Rha*p*. Similar rhamnogalacturonan chains have been observed in the pectic polysaccharides isolated from Cotyledon meal[32] and hulls of *Glycine max*[33], leaves and stems of *Medicago sativa*[34], suspension-cultured sycamore cell walls[35], the fruits of *Zizyphus jujuba* MILLAE var. inermis REHD[36], suspension-cultured cells and leaves of *Nicotiana tabacum*[37,38], and rice-endosperm cell walls[39]. However, these pectic polysaccharides contained a large proportion of GalA and significant amounts of 4-*O*-linked Gal. Only pectic arabinogalactans from *Cannabis sativa*[40] contained small proportions of Rha and GalA and significant amounts of (1→6)-, (1→3)-, and (1→4)-linked Gal*p*, but they did not contain branched Ara. AGIIb-1 contained a large proportion of branched Ara, but until now the presence of the branched Ara chain has been reported in the (1→4)-linkage-rich galactan. Thus, AGIIb-1 has a unique structure as an arabinogalactan-type polysaccharide. The structure of the branched arabinan moiety was also similar to that of anti-complementary acidic heteroglycans (AAFIIb-2 and IIb-3) from the leaves of *Artemisia princeps* PAMP[41], but both had a higher content of rhamnogalacturonan than AGIIb-1.

Typical arabino-3,6-galactans have been isolated from *Larix laricina*[42], suspension cultures of endosperm from *Lolium multiflorum*[43], gladiolus style mucilage[44], sugar cane[45], *Zea mays*[46] shoots, and the roots of *A. acutiloba*[10]. Their galactan backbones variously involved (1→3) and/or (1→6) linkages. AGIIb-1 contained a large proportion of (1→3,6)-linked Gal*p*. However, it is not yet known whether the backbone of the arabino-3,6-galactan moiety of AGIIb-1 is a (1→6)- and/or (1→3)-linked galactan.

The present results indicated that AGIIb-1 has acid-labile linkages which play an important role in the expression of the anti-complementary activity, and may consist of different arabinogalactans joined through acid-labile linkage(s), because mild acid hydrolysis liberated one neutral and two acidic arabinogalactans and higher arabino-oligosaccharides. When AGIIb-1 was digested with exo-α-L-arabinofuranosidase, the branching Ara side-chains were mostly converted into 4- or 5-*O*-linked Ara chains and the anti-complementary activity was enhanced. These results indicated that the structures of the Ara*f* chains are also related to the expression of the anti-complementary activity of AGIIb-1.

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REFERENCES

1 H. KIYOHARA, H. YAMADA, J.-C. CYONG, AND Y. OTSUKA, *J. Pharmacobio-Dynamics*, 9 (1986) 339–346.
2 L. PILLEMER, M. D. SCHOENBERG, L. BLUM, AND L. WURZ, *Science*, 122 (1955) 545–549.
3 R. L. MARCUS, H. S. SHIN, AND M. M. MAYER, *Proc. Natl. Acad. Sci. U.S.A.*, 68 (1971) 1351–1354.
4 H. GEWURZ, H. S. SHIN, AND S. E. MERGENHAGEN, *J. Exp. Med.*, 128 (1968) 1049–1057.
5 T. OKUDA, Y. YOSHIOKA, T. IKEKAWA, G. CHIHARA, AND K. NISHIOKA, *Nature (London), New Biol.*, 238 (1972) 59–60.
6 J. HAMURO, U. HADDING, AND D. BITTER-SUERMANN, *Immunology*, 34 (1978) 695–705.
7 O. GÖTZE AND H. J. MÜLLER-EBERHARD, *J. Exp. Med.*, 134 (1971) 90s–108s.
8 H. YAMADA, T. NAGAI, J.-C. CYONG, Y. OTSUKA, M. TOMODA, N. SHIMIZU, AND K. SHIMADA, *Carbohydr. Res.*, 144 (1985) 101–111.
9 H. YAMADA, H. KIYOHARA, J.-C. CYONG, Y. KOJIMA, Y. KUMAZAWA, AND Y. OTSUKA, *Planta Med.*, 50 (1984) 163–167.
10 H. YAMADA, H. KIYOHARA, J.-C. CYONG, AND Y. OTSUKA, *Mol. Immunol.*, 22 (1985) 295–304.
11 E. UESAKA, M. SATO, M. RAIJU, AND A. KAJI, *J. Bacteriol.*, 133 (1978) 1073–1077.
12 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
13 N. BLUMENKANTZ AND G. ASBOE-HANSEN, *Anal. Biochem.*, 54 (1973) 484–489.
14 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
15 J. T. PARK AND M. J. JOHNSON, *J. Biol. Chem.*, 181 (1949) 149–151.
16 P. ALBERSHEIM, D. J. NEVINS, P. D. ENGLISH, AND A. KARR, *Carbohydr. Res.*, 5 (1967) 340–345.
17 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444–445.
18 L. HOUGH, J. K. N. JONES, AND W. H. WADMAN, *J. Chem. Soc.*, (1950) 1702–1706.
19 G. AVIGAD, *Carbohydr. Res.*, 11 (1969) 119–123.
20 R. E. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
21 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
22 H. RAUVALA, *Carbohydr. Res.*, 72 (1979) 257–260.
23 T. J. WAEGHE, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 123 (1983) 281–304.
24 B. LINDBERG, *Methods Enzymol.*, 28 (1972) 178–195.
25 D. P. SWEET, R. H. SHAPIRO, AND P. ALBERSHEIM, *Carbohydr. Res.*, 40 (1975) 217–225.
26 E. A. KABAT AND M. M. MAYER (Eds.), *Experimental Immunochemistry*, Thomas, Illinois, 1964, pp. 133–240.
27 J.-P. JOSELEAU, G. CHAMBAT, M. VIGNON, AND F. BARNOUD, *Carbohydr. Res.*, 58 (1977) 165–175.
28 N. K. KOCHETKOV AND O. S. CHIZHOV, *Adv. Carbohydr. Chem.*, 21 (1966) 39–93.
29 V. KOVACIK, S. BAUER, J. ROSIK, AND P. KOVAC, *Carbohydr. Res.*, 8 (1968) 282–290.
30 A. E. CLARKE, R. L. ANDERSON, AND B. A. STONE, *Phytochemistry*, 18 (1979) 521–540.
31 G. O. ASPINALL, in F. A. LOEWUS (Eds.), *Biogenesis of Plant Cell Wall Polysaccharides*, Academic Press, New York, 1973, pp. 95–115.
32 G. O. ASPINALL, I. W. COTTRELL, S. V. EGAN, I. M. MORRISON, AND J. N. C. WHYTE, *J. Chem. Soc., C*, (1967) 1071–1080.
33 G. O. ASPINALL, K. HUNT, AND I. M. MORRISON, *J. Chem. Soc., C*, (1967) 1080–1086.
34 G. O. ASPINALL, B. GESTETNER, J. A. MOLLOY, AND M. UDDIN, *J. Chem. Soc., C*, (1968) 2554–2559.
35 M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Plant Physiol.*, 66 (1980) 1128–1134.
36 N. SHIMIZU AND M. TOMODA, *Chem. Pharm. Bull.*, 31 (1983) 499–506.
37 H. HORI, Y. TAKEUCHI, AND T. FUJII, *Phytochemistry*, 19 (1980) 2755–2756.
38 S. EDA, K. KATO, A. ISHIZU, AND J. NAKANO, *Agric. Biol. Chem.*, 46 (1982) 1783–1790.
39 N. SHIBUYA AND R. NAKANE, *Phytochemistry*, 23 (1984) 1425–1429.
40 A. HILLESTAD AND J. K. WOLD, *Phytochemistry*, 16 (1977) 1947–1951.

41 H. YAMADA, Y. OTSUKA, AND S. ŌMURA, *Planta Med.*, (1986) 311–314.
42 C. HAQ AND G. A. ADAMS, *Can. J. Chem.*, 39 (1961) 1563–1573.
43 R. L. ANDERSON, A. E. CLARKE, M. A. JERMYN, R. B. KNOX, AND B. A. STONE, *Aust. J. Plant Physiol.*, 4 (1977) 143–158.
44 P. A. GLEESON, AND A. E. CLARKE, *Biochem. J.*, 181 (1979) 607–621.
45 J. D. BLAKE, M. L. CLARKE, AND P. E. JANSSON, *Carbohydr. Res.*, 115 (1983) 265–272.
46 Y. KATO AND D. J. NEVINS, *Plant Physiol.*, 74 (1984) 562–568.