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STRUCTURAL CHARACTERISATION OF AN ANTI-COMPLEMENTARY PECTIC POLYSACCHARIDE FROM THE ROOTS OF *Bupleurum falcatum* L.

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ABSTRACT

An anti-complementary pectic polysaccharide (BR-2-IIb), isolated from the roots of *Bupleurum falcatum* L., has an average molecular weight of 36,000 (gel filtration), and was subjected to methylation analysis before and after carboxyl-reduction, digestion with endo-polygalacturonase, base-catalysed β-elimination, and partial acid hydrolysis. BR-2-IIb consisted mainly of galacturonic acid, arabinose, rhamnose, and galactose in the molar ratios 13.0:2.1:1.4:1.0 and contained a large enzyme-sensitive polygalacturonan region. The enzyme-resistant region (PG-1) was rich in neutral sugars and contained a backbone of 4-linked GalA and 2-linked Rha to which a highly branched arabinogalactan was attached to position 4 of some 2-linked Rha units. Partial acid hydrolysis of BR-2-IIb gave Ara-(1→3)-Ara, Ara-(1→4)-Ara*p*, Ara-(1→5)-Ara*f*, Ara-(1→6)-Gal, Gal-(1→4)-Gal, GalA-(1→2)-Rha, GalA-(1→4)-Rha, GalA→Rha→Rha, Gal→Rha→Rha, and GalA-(1→6)-Gal in addition to (1→4)linked oligogalacturonides. The anti-complementary activity of BR-2-IIb was enhanced by de-esterification, but carboxyl-reduction decreased the activity.

INTRODUCTION

The root of *Bupleurum falcatum* L. (Japanese name, Saiko) has been used in Chinese and Japanese herbal medicine for the treatment of chronic hepatitis, nephrosis syndrome, and autoimmune diseases. The hot-water extract from the root of *B. falcatum* L. also has been reported to enhance antibody response and to inhibit mitogen-induced lymphocyte transformation[1].

We have reported[2] that the major neutral anti-complementary polysaccharides in the hot-water extract of the root of *B. falcatum* were a 3,5-α-arabinan, an amylose-type α-glucan, and an α-arabinoglucan. We have now isolated a pectic polysaccharide from the same extract and report on its structure and anti-complementary activity.

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EXPERIMENTAL

Materials. — The roots of *B. falcatum* L. were purchased from Uchida Wakanyaku Co. Ltd. Sepharose CL-6B, and Sephadex G-100, G-50, and G-10 were obtained from Pharmacia, and Bio-gel P-2 (200–400 mesh) and P-4 (−400 mesh) from Bio-Rad. Sep-Pak C_{18} cartridges were purchased from Waters Associates Inc. Pectinase from *Aspergillus niger* was purchased from Sigma and endo-(1→4)-α-polygalacturonase was purified using the procedure of Thibault and Mercier[3].

General. — Total carbohydrate, uronic acid, and protein contents were assayed by the phenol–sulfuric acid[4], *m*-hydroxybiphenyl[5], and Lowry[6] methods, respectively, using arabinose, galacturonic acid, and bovine serum albumin as the respective standards. Methyl ester groups were assayed by the method[7] of Wood and Siddiqui, using methanol as the standard. Optical rotations were determined at 23° with a JASCO DIP digital polarimeter. Acidic polysaccharides were hydrolysed with 2M trifluoroacetic acid for 1.5 h at 121°, and the resulting neutral sugars and uronic acids were converted[8] into the corresponding alditol acetates and analysed by g.l.c. T.l.c. was performed on cellulose (Merck) with ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Reducing sugars were detected with alkaline silver nitrate[9], and uronic acids with *p*-anisidine hydrochloride[10]. 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% OV-225 on Uniport HP. The molar ratios of neutral sugars were calculated from the peak areas and molecular weights of the corresponding alditol acetates. The molar ratios of uronic acid and neutral sugars were calculated from the contents of uronic acid. H.p.l.c. was performed[11] on a Waters Model ALC/GPC 244 instrument equipped with columns (0.76 × 50 cm) of Asahipak GS 510 + GS 320 (Asahi Chemical Industry Co. Ltd.) and developed with 0.2M sodium chloride. Electrophoresis (cellulose acetate membrane, Fuji Film Co, Ltd.) was performed in 0.08M pyridine–0.04M acetate buffer (pH 5.4) at 70 V for 30 min with detection using Toluidine Blue.

Fractionation and purification of BR-2-IIb. — The crude polysaccharide fraction (BR-1), prepared[2] by hot-water extraction and precipitation with ethanol from *B. falcatum* L., was fractionated[12] by treatment with cetyltrimethylammonium bromide (Cetavlon), and the acidic polysaccharide fraction (BR-2) was obtained as the precipitate.

BR-2 (500 mg) was further fractionated on a column (3.8 × 40 cm) of DEAE-Sepharose CL-6B (HCO_3^- form), which was equilibrated with water, by stepwise elution with water, 0.1, 0.3, 0.5, 1, and 2M ammonium hydrogencarbonate.

The major polysaccharide fraction, eluted with 0.3M ammonium hydrogencarbonate, was dialysed and lyophilised, to give BR-2-IIb (33.1% from BR-2).

Preparation of de-esterified[13] *and carboxyl-reduced BR-2-IIb.* — A solution of BR-2-IIb (50 mg) at pH 12 was stored for 2 h at 4°, then the de-esterified product

(45 mg) was recovered after neutralisation and dialysis. The carboxyl groups of GalA in de-esterified BR-2-IIb (10 mg/4 mL of D_2O) were reduced by the modified procedure[14] of Taylor and Conrad[15], using 1-cyclohexyl-3-(2-morpholinoethyl)-carbodi-imide metho-*p*-toluenesulfonate (CMC, 100 mg) and sodium borodeuteride (1.6 g/10 mL of D_2O), and the product was isolated by dialysis and lyophilisation (yield, 86%). The remaining trace of boric acid was removed by evaporating 10% acetic acid in methanol (4 times) and methanol (2 times) from the product.

Digestion with endo-(1→4)-α-polygalacturonase. — BR-2-IIb (70 mg) was digested with endo-(1→4)-α-polygalacturonase[3] (1750 nkat) for 2 days at 30° in 20mM acetate buffer (pH 4.3, 10 mL) in the presence of toluene. After neutralisation, the mixture was heated for 5 min at 100° and then lyophilised, and the residue was eluted from a column of Sephadex G-50 to give three fractions (PG-1–PG-3). Each fraction was collected and desalted with AG50W-X8 (H^+) resin.

Partial acid hydrolysis. — BR-2-IIb (100 mg) was hydrolysed with 0.1M trifluoroacetic acid for 1 h at 100°, the hydrolysate was lyophilised, and the residue was applied to a column (3 × 35 cm) of DEAE-Sephadex A-25 ($HCOO^-$ form). The neutral fraction was obtained by elution with water, and the acidic fractions with 5M formic acid and M sodium chloride. The neutral fraction was eluted from a column (1.1 × 46.5 cm) of Bio-gel P-2 with water to give a fraction (PN-1) in the void volume and four oligosaccharide fractions (PN-2–PN-5). The first acidic fraction was neutralised with M sodium hydroxide, then centrifuged, and the supernatant solution was desalted on a column (5.3 × 20 cm) of Sephadex G-10 and lyophilised to give the carbohydrate fraction (PA-1, 30 mg). The second acidic fraction was also desalted and lyophilised to give PA-2 (10 mg). PA-1 was hydrolysed with aqueous 70% formic acid for 1 h at 70°, the hydrolysate was lyophilised, and the residue was eluted from a column (1.1 × 46.5 cm) of Bio-gel P-2 with 0.2M acetate buffer (pH 5.6) to give a fraction (PA-1-V) in the void volume and three oligosaccharide fractions (PA-1-II–PA-1-IV). The fraction eluted in the void volume was further hydrolysed with 0.1M trifluoroacetic acid for 1.5 h at 121°, and the hydrolysate was fractionated on a column (1.1 × 47 cm) of Bio-gel P-4 with 0.2M acetate buffer (pH 5.6) to give four oligosaccharide fractions (PA-1-V-2–PA-1-V-5).

Methylation analysis. — (*a*) BR-2-IIb, de-esterified BR-2-IIb, carboxyl-reduced BR-2-IIb, PG-1, and PG-3 were methylated once (Hakomori[16]) in order to prevent β-elimination, but methylsulphinylcarbanion was added two or three times until the conversion of the polysaccharide into a polyalkoxide was complete as checked by using triphenylmethane[17]. The methylated polysaccharides were purified[18] on a Sep-Pak C_{18} cartridge by elution with acetonitrile followed by ethanol.

Each methylated polysaccharide was hydrolysed with 2M trifluoroacetic acid for 1 h at 121°, and the products were reduced with sodium borohydride followed by acetylation. The resulting methylated alditol acetates were analysed by g.l.c.

and g.l.c.–m.s. with an SPB-1 capillary column (0.25-μm film thickness, 25 m × 0.25 mm i.d., SUPELCO).

Uronic acids of methylated PG-1 and PG-3 were reduced[18] with sodium borodeuteride in tetrahydrofuran–ethanol (7:3) for 18 h at room temperature followed by incubation for 1 h at 80°. The excess of sodium borodeuteride was decomposed with acetic acid, the mixture was concentrated, the borate was removed by repeated evaporation of 10% acetic acid in methanol and then methanol from the residue, and sodium acetate was removed on AG50W-X8 (H^+) resin. The products were analysed as methylated alditol acetates.

(*b*) Each acidic oligosaccharide was reduced with sodium borodeuteride, methylated, carboxyl-reduced[18] with sodium borodeuteride, and remethylated.

(*c*) The methylated oligosaccharide-alditols from PN-2 and -3 were each fractionated on a Sep-pak C_{18} cartridge. Elution with acetonitrile–water (3:17) gave PN-2a and -3a, and with acetonitrile–water (1:4) gave PN-2b and -3b.

Mass spectrometry. — A JEOL DX-300 mass spectrometer was used: e.i. 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.[19] and e.i. fragment ions [A, J, and alditol (ald)][20] were used to determine the structure of the methylated oligosaccharide-alditols.

β-Elimination[21,22] of methylated PG-1. — To a solution of dried methylated PG-1 (2 mg) in methyl sulfoxide (1 mL) was added methylsulfinylmethanide, and the mixture was stirred for 24 h at room temperature. Part (70%) of the sample was stirred with an excess of ethyl iodide for 24 h at room temperature, the ethyl iodide was evaporated, and the product was recovered using a Sep-Pak C_{18} and fractionated[22] on a column (1.0 × 25 cm) of Sephadex LH-20 to give a material (R_2) of high molecular weight. The remainder of the sample was neutralised with aqueous 50% acetic acid and the product (R_1) was obtained as described above. R_1 and R_2 were each hydrolysed and the products were converted into the methylated alditol acetates. The linkage composition of the sample was then determined by g.l.c. and g.l.c.–m.s.

N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra were recorded at 80° for solutions in D_2O on a Varian EM-390 spectrometer (90 MHz). Chemical shifts were expressed in p.p.m. from the signal of sodium 3-(trimethylsilyl)propanesulfonate (TSP).

Anti-complementary activity. — This was measured as described[23].

RESULTS

Isolation and properties of BR-2-IIb. — The crude polysaccharide fraction (BR-1) was fractionated into BR-2–BR-5, using Cetavlon. BR-2, which had the highest anti-complementary activity (data not shown), was fractionated further on DEAE-Sepharose CL-6B to give BR-2-IIa–IIe, which were eluted with 0.1, 0.3, 0.5, 1, and 2M ammonium hydrogencarbonate, respectively. BR-2-IId had the highest anti-complementary activity, but was obtained in the lowest yield. BR-2-

IIb, obtained in the highest yield and the second highest activity, gave a single spot in cellulose acetate membrane electrophoresis (staining with Toluidine Blue), was eluted from Sepharose CL-6B as a single peak (which was co-eluted with uronic acid), and gave a single peak in h.p.l.c. using Asahi-pak GS-510 + GS-320. BR-2-IIb had $[\alpha]_D$ +120° (*c* 1, water), contained 75.5% of uronic acid (45.7% was methyl-esterified), 23.4% of hexose, 1.1% of protein, and 1.6% of N but no S, had a molecular weight of 36,000 (gel filtration on Sepharose CL-6B), and contained GalA, Ara, Rha, and Gal in the molar ratios 13.0:2.1:1.4:1.0 together with traces of Xyl and Glc.

The ^{1}H-n.m.r. spectrum of BR-2-IIb contained signals at 1.4 (Rha, Me), 2.1 (Ac), and 3.8 p.p.m. (COOMe).

Methylation analysis of BR-2-IIb and its carboxyl-reduced products. — De-esterified BR-2-IIb was methylated (Hakomori[16]), the product was hydrolysed, and the resulting partially methylated alditol acetates were analysed by g.l.c. and g.l.c.–m.s. (Table I). De-esterified BR-2-IIb contained 4- or 5-linked and 3,4- or 3,5-di-substituted Ara, 2-linked and 2,4-disubstituted Rha, terminal, 4- and 6-linked, 2,4- and 3,6-disubstituted, and 3,4,6-trisubstituted Gal, and 3,4-disubstituted Xyl. Carboxyl-reduction of BR-2-IIb with sodium borodeuteride, after de-esterification, gave a product that contained Ara, Rha, and Gal in the molar ratios 1.0:1.6:18.2 together with a trace of Xyl. The presence of a large amount of 4-linked GalA was suggested by the increase of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol-6,6-d_2 in

TABLE I

METHYLATION ANALYSIS OF DE-ESTERIFIED AND CARBOXYL-REDUCED BR-2-IIb

Residue	*Position of OMe groups*	*Linkages*	*Composition (mol %)*	
			De-esterified BR-2-IIb	*Carboxyl-reduced BR-2-IIb*
Ara	2,3	4 or 5	3.2	1.1
	2	3,4 or 3,5	4.6	7.6
Rha	3,4	2	1.3	1.5
	3	2,4	3.1	1.7
Xyl	2	3,4	1.6	n.d.[a]
Glc	2,3,4,6	terminal	0.1	0.2
Gal	2,3,4,6	terminal	1.6	2.7[b]
	2,3,6	4	3.4	66.1[c]
	2,3,4	6	0.8	1.5
	3,6	2,4	1.9	9.1
	2,4	3,6	0.7	0.5
	2	3,4,6	1.0	8.0
Total neutral sugar (%)			23.4[d]	100.0

[a]Not detected. [b]Detected as a mixture of galactitol-6,6-d_2 (*m/z* 47 and 207), and galactitol (*m/z* 46 and 205). [c]Mostly detected as galactitol-6,6-d_2 (*m/z* 47 and 235). [d]Percentage of the neutral sugars.

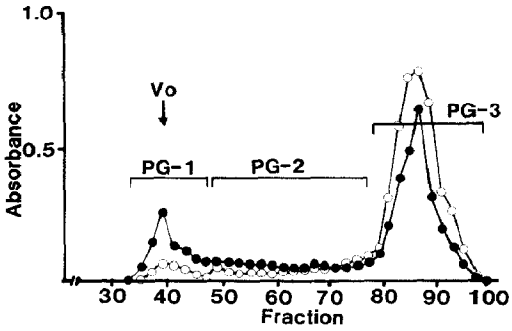


Fig. 1. Gel filtration on Sephadex G-50 of the products of digestion of BR-2-IIb with endo-(1→4)-α-polygalacturonase: ●, carbohydrate; ○, uronic acid; V_0, void volume.

carboxyl-reduced BR-2-IIb (Table I). The presence of terminal GalA was also suggested by the detection of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol-*6,6-d*$_2$.

Enzymic digestion of BR-2-IIb. — BR-2-IIb was digested with endo-(1→4)-α-polygalacturonase. Elution of the products from Sephadex G-50 (Fig. 1) gave a

TABLE II

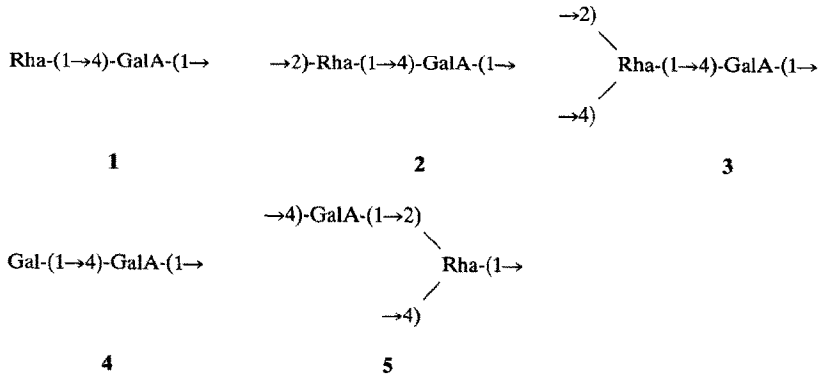
METHYLATION ANALYSIS OF PG-1 BEFORE AND AFTER BASE-CATALYSED β-ELIMINATION

Residue	*Position of OMe groups*	*Position of OEt groups*	*Linkages*	*Composition (mol %)*		
				Before elimination	*After elimination*	
				PG-1	*R_1-HexA*	*HexA-R_2*
Ara	2,3,5		terminal (furanosyl)	0.2	1.8	
	2,3,4		terminal (pyranosyl)	0.4	trace	
	2,3		4 or 5	3.1	6.8	
	2		3,4 or 3,5	9.2	21.0	
Rha	2,3,4		terminal	5.5	trace	
	3,4		2	4.5	trace	
	3		2,4	5.6	2.4	
	3	2	2,4	n.d.[a]	n.d.	+[b]
Xyl	2		3,4	trace	trace	
Glc	2,3,4,6		terminal	2.7	3.2	
Gal	2,3,4,6		terminal	4.7	2.6	
	2,3,6		4	4.1	9.1	
	2,4,6		3	4.7	7.7	
	2,3,4		6	8.3	10.3	
	3,6		2,4	6.0	3.1	
	2,3		4,6	4.4	4.4	
	2,4		3,6	5.7	15.0	
	2		3,4,6	7.3	12.5	
GalA[c]	2,3		4	16.4		

[a]Not detected. [b]Detected. [c]Identified as 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylgalactitol-*6,6-d*$_2$.

small fraction (PG-1) in the void volume, an intermediate fraction (PG-2), and a major fraction (PG-3) having the lowest molecular weight. PG-1 contained Ara, Gal, Rha, and Xyl in the molar ratios 1.0:0.8:0.6:0.1, and contained 33.7% of GalA. PG-2 contained mainly GalA with traces of Rha, Ara, and Gal. PG-3 contained mainly GalA (91.5% after de-esterification). PG-1 and PG-3 were each methylated, carboxyl-reduced with sodium borodeuteride, and converted into methylated alditol acetates. Methylation analysis (Table II, before β-elimination) showed that PG-1 contained glycosidic linkages similar to those in BR-2-IIb except for terminal Ara*f*, Ara*p*, and Rha*p* derivatives. PG-3 contained terminal GalA and 4-linked GalA since 2,3,4-tri-*O*-methylgalactitol-*6,6-d*$_2$ and 2,3-di-*O*-methylgalactitol-*6,6-d*$_2$ were detected by g.l.c.–m.s. PG-3 was resolved into seven oligogalacturonides by gel filtration on Bio-gel P-4. These results suggested that BR-2-IIb was a pectic polysaccharide containing a (1→4)-α-D-polygalacturonan and a "ramified" region (PG-1) enriched in neutral sugars.

Identification of the rhamnogalacturonan core in the "ramified" region by base-catalysed β-elimination. — Base-catalysed β-elimination of methylated PG-1 exposed hydroxyl groups, and the product (R_1) was ethylated to give R_2. Methylation analysis (Table II) showed losses of terminal Rha (~100%), 2-linked (~100%) and 2,4-disubstituted Rha (~57%), and terminal Gal (~45%) from PG-1. 1,4,5-Tri-*O*-acetyl-2-*O*-ethyl-3-*O*-methylrhamnitol was formed in the methylation analysis of R_2. These results indicated that terminal Rha (**1**), 2-linked Rha (**2**), a part of 2,4-disubstituted Rha (**3**), and terminal Gal (**4**) were attached to position 4 of GalA in PG-1, and that 4-substituted GalA was linked to position 2 of 2,4-disubstituted Rha (**5**).



Partial acid hydrolysis of BR-2-IIb. — Hydrolysis of BR-2-IIb with 0.1M trifluoroacetic acid for 1 h at 100°, followed by chromatography on DEAE-Sephadex, gave one neutral and two acidic carbohydrate fractions (PA-1 and -2). The neutral fraction gave material (PN-5) eluted near the void volume, three oligosaccharide fractions (PN-2–PN-4), and a monosaccharide fraction (PN-1) on Bio-gel P-2 (data not shown). PN-1 contained mainly Ara.

Analysis of neutral oligosaccharides from BR-2-IIb. — PN-2–PN-5 contained mainly Ara with traces of Rha and Gal. Methylation analysis (Table III) showed that PN-4 mainly contained terminal Ara*p*, 4- or 5-linked Ara, 3-linked Ara*p*, 3,4- or 3,5-disubstituted Ara, and 6-linked Gal, whereas PN-5 mainly contained 3-linked Ara*p*, 4- or 5-linked Ara, 3,4- or 3,5-disubstituted Ara, and terminal Glc, and 3- and 6-linked, 2,4- and 4,6-disubstituted, and 3,4,6-trisubstituted Gal. PN-2 and -3 were each reduced with sodium borodeuteride, methylated, and then fractionated on Sep-Pak C_{18} cartridges. Elution[18] with acetonitrile–water (3:17) gave PN-2a and -3a-, and with acetonitrile–water (1:4) gave PN-2b and -3b. Methylation analysis (Table IV) showed that PN-2a, -2b, -3a, and -3b mainly contained terminal Ara*f* and Ara*p*, 4- or 5-linked Ara, and 3-linked Ara*p*. 3-*O*-Acetyl-1,2,4,5-tetra-*O*-methylarabinitol-*1-d* was also formed from PN-2a, -2b, and -3a, 2-*O*-acetyl-1,3,4,5-tetra-*O*-methylarabinitol-*1-d* from PN-3b, and 2,4-di-*O*-acetyl-1,3,5-tri-*O*-methylarabinitol-*1-d* from PN-2a and -2b. G.l.c.–c.i.-m.s. (Table V) showed that PN-2a contained seven pentosyl→pentitols, three pentosyl→6-deoxyhexitols, and two pentosyl→hexitols, as did PN-2b and -3b (data not shown). E.i.-m.s. (Table VI) showed that PN-2a contained pentosyl-(1→3)-pentitol-*1-d* and pentosyl-(1→5)-pentitol-*1-d*, and PN-2b contained pentosyl-(1→4)-pentitol-*1-d*. Methylation and sugar analysis suggested that pentosyl, 6-deoxyhexosyl, and hexosyl residues in

TABLE III

METHYLATION ANALYSIS DATA FOR THE PRODUCTS OF PARTIAL ACID HYDROLYSIS OF BR-2-IIb

Residue	*Position of OMe groups*	*Linkages*	*Composition (mol %)*		
			PN-4	*PN-5*	*PA-1*
Ara	2,3,5	terminal (furanosyl)	4.0	1.4	0.2
	2,3,4	terminal (pyranosyl)	23.5	4.3	n.d.
	2,3	4 or 5	15.2	5.5	0.4
	2,4	3 (pyranosyl)	18.1	10.6	n.d.
	2	3,4 or 3,5	5.1	7.2	1.2
Rha	2,3,4	terminal	4.2	0.5	0.8
	3,4	2	2.8	4.6	0.4
	3	2,4	2.8	5.2	1.6
Xyl	2	3,4	0.6	2.7	n.d.
Glc	2,3,4,6	terminal	1.5	6.1	2.2
Gal	2,3,4,6	terminal	4.4	3.3	2.5
	2,3,6	4	3.0	5.0	3.3
	2,4,6	3	1.5	6.6	2.1
	2,3,4	6	5.1	6.1	2.1
	3,6	2,4	1.6	10.2	4.3
	2,3	4,6	2.7	8.0	n.d.
	2,4	3,6	2.6	n.d.	6.7
	2	3,4,6	1.4	12.7	17.8
GalA	2,3,4	terminal	n.d.[a]	n.d.	6.3
	2,3	4	n.d.	n.d.	48.9

[a]Not detected.

TABLE IV

METHYLATION ANALYSIS OF NEUTRAL OLIGOSACCHARIDE FRACTIONS (PN-2a, -2b, -3a, AND -3b) OBTAINED FROM BR-2IIb BY PARTIAL ACID HYDROLYSIS

Residue	*Positon of OMe groups*	*Linkages*	*Composition (mol %)*			
			PN-2a	*PN-2b*	*PN-3a*	*PN-3b*
Ara	1,3,4,5	2 (reducing terminal)	n.d.[a]	n.d.	n.d.	trace
	1,2,4,5	3 (reducing terminal)	trace	n.d.	trace	trace
	1,3,5	2,4 (reducing terminal)	trace	trace	n.d.	n.d.
	2,3,5	terminal (furanosyl)	11.5	25.8	13.1	21.7
	2,3,4	terminal (pyranosyl)	63.7	50.0	50.5	35.0
	2,3	4 or 5	8.9	12.1	15.2	21.7
	2,4	3 (pyranosyl)	15.9	12.1	21.2	21.7

[a]Not detected.

TABLE V

DIAGNOSTIC C.I.-M.S. FRAGMENT IONS OF DISACCHARIDE-ALDITOLS FROM NEUTRAL OLIGOSACCHARIDE FRACTION PN-2a

Peak	*Fragment ions* [m/z *(relative abundance)*]						*Oligosaccharide-alditol*
	$(M + H)^+$	$(M + H)^+ - MeOH$	bA_1	bA_2	aJ_2	aJ_2OH_2	
a	384 (31.8)		175 (75.2)	143 (17.5)		210 (100)	pentosyl→pentitol-*1-d*
b	384 (19.6)	352 (5.6)	175 (29.7)			210 (63.6)	pentosyl→pentitol-*1-d*
c	384 (19.0)	352 (19.0)	175 (36.7)	143 (21.2)	192 (49.8)	210 (100)	pentosyl→pentitol-*1-d*
d-1	384 (3.6)	352 (3.6)	175 (67.5)		192 (59.8)	210 (100)	pentosyl→pentitol-*1-d*
d-2	398 (3.6)	366 (3.6)	175 (67.5)			224 (34.6)	pentosyl→6-deoxyhexitol-*1-d*
e	398 (24.8)	366 (31.8)	175 (75.2)			224 (100)	pentosyl→6-deoxyhexitol-*1-d*
f-1	384 (31.5)		175 (99.3)		192 (100)	210 (33.6)	pentosyl→pentitol-*1-d*
f-2	398 (10.5)		175 (99.3)		206 (80.1)	224 (42.1)	pentosyl→6-deoxyhexitol-*1-d*
g	384 (76.6)	352 (19.9)	175 (90.9)		192 (100)	210 (75.5)	pentosyl→pentitol-*1-d*
h	384 (9.5)		175 (100)		192 (59.9)	210 (20.4)	pentosyl→pentitol-*1-d*
i	428 (37.8)		175 (29.4)		236 (100)	254 (21.0)	pentosyl→hexitol-*1-d*
j	428 (40.9)	396 (4.5)	175 (32.9)		236 (100)	254 (28.3)	pentosyl→hexitol-*1-d*

TABLE VI

DIAGNOSTIC E.I.-M.S. FRAGMENT IONS OF METHYLATED DISACCHARIDE-ALDITOLS FROM NEUTRAL OLIGOSACCHARIDE FRACTIONS PN-2a AND PN-2b

Peak	*Fragment ions* [m/z *(relative abundance)*]									*Oligosaccharide-alditols*
	bA_1	bA_2	aJ_1	aJ_2	*ald*					
PN-2a-1	175	143	252	192	338	306	294			Ara-(1→3)-Ara-ol-*1-d*
	(80.1)	(100)	(57.8)	(27.5)	(0.9)	(0.7)	(0.8)			
-2	175	143	252	192	305	293	178	146	134	Ara-(1→5)-Ara-ol-*1-d*
	(47.0)	(75.3)	(2.8)	(100)	(1.0)	(0.7)	(2.1)	(23.5)	(3.7)	
-3	175	143		236	293	178	146	134		Ara-(1→6)-Gal-ol-*1-d*
	(72.8)	(100)		(57.1)	(1.4)	(15.3)	(59.4)	(26.5)		
PN-2b-1	175	143		192	306	305	294			Ara-(1→4)-Ara-ol-*1-d*
	(74.9)	(100)		(23.0)	(1.3)	(0.7)	(1.4)			
-2	219	187	296	236	349	134				Gal-(1→4)-Gal-ol-*1-d*
	(10.8)	(69.7)	(1.9)	(31.7)	(2.4)	(17.1)				

these disaccharide-alditols were Ara, Rha, and Gal, respectively, and that the arabinodisaccharide-alditols might consist of Ara*f* and Ara*p* in various combinations such as Ara*f*(*p*)→2Ara-ol-*1-d*, Ara*f*(*p*)→3Ara-ol-*1-d*, Ara*f*(*p*)→4Ara-ol-*1-d*, and Ara*f*(*p*)→5Ara-ol-*1-d*. The low recoveries of monosubstituted arabinitol derivatives might have been a consequence of their volatility. The result of e.i.-m.s. also indicated that PN-2a contained Ara-(1→6)-Gal and PN-2b contained Gal-(1→4)-Gal.

Analysis of the acidic fragments from BR-2-IIb. — The acidic fragments PA-1 and PA-2 each gave a single peak in gel filtration on Sephadex G-100, and their molecular weights were estimated to be 5,300 and 13,500, respectively. The major acidic fragment PA-1 contained 56.3% of GalA and 23.4% of neutral sugars comprising Gal, Rha, and Ara in the molar ratios 16.4:1.6:1.0 together with traces of Glc and Xyl. Methylation analysis (Table III) showed that PA-1 mainly contained 4-linked GalA and terminal GalA, and 3,6-disubstituted Gal and 3,4,6-trisubstituted Gal.

PA-1 was hydrolysed with aqueous 70% HCOOH for 1 h at 70°. Elution of the products from Bio-gel P-2 (Fig. 2) gave fraction PA-1-V in the void volume and oligosaccharide fractions PA-1-II–IV. PA-1-V was hydrolysed with 0.1M trifluoroacetic acid for 1.5 h at 121°, and the products were eluted from Bio-gel P-4 (Fig. 3) to give four oligosaccharide fractions PA-1-V-2–5. Methylation analysis (Table VII) showed that PA-1-IV mainly contained terminal and 4-linked GalA, and that PA-1-V-4 and -V-5 mainly contained terminal Ara*f*, 3,6-disubstituted Gal, and terminal GalA. PA-1-V-4 also contained a large amount of 4-linked GalA. Small amounts of 3,4-disubstituted and 2,4-disubstituted GalA were detected in PA-1-IV and PA-1-V-5, respectively. The acidic fractions (PA-1-II and -III, and PA-1-V-2 and -V-3) were reduced with sodium borodeuteride, methylated, carboxyl-reduced with sodium borodeuteride, and methylated. G.l.c.–m.s. (Table VIII) of the resulting methylated oligosaccharide-alditols indicated that PA-1-II, -III, and -V-3 gave the same major peak, which showed the fragment ions due to bA_1 (*m/z* 221), aJ_2 (*m/z* 206), and the ald series either of the 2-linked rhamnitol

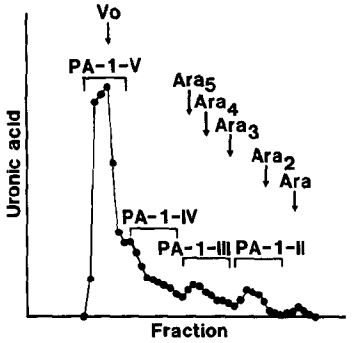


Fig. 2. Gel filtration on Bio-gel P-2 of the products of partial acid hydrolysis of PA-1: ●, uronic acid; Ara, arabinose; Ara_2–Ara_5, di- to penta-arabinosaccharides.

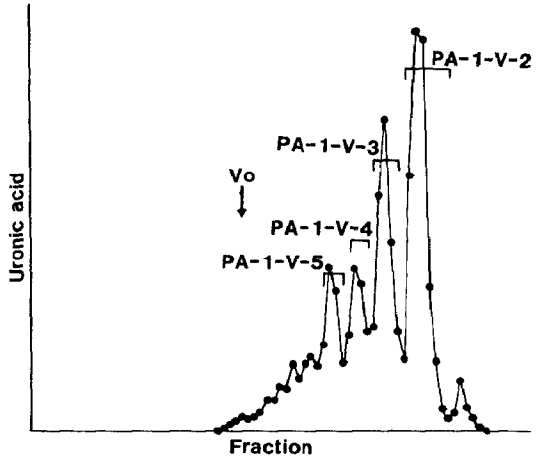


Fig. 3. Gel filtration on Bio-gel P-4 of products of partial acid hydrolysis of PA-1-V (Fig. 2). The symbols are the same as those in Fig. 2.

TABLE VII

METHYLATION ANALYSIS OF ACIDIC OLIGOSACCHARIDE FRACTIONS PA-1-IV, PA-1-V-4, AND PA-1-V-5 OBTAINED BY PARTIAL ACID HYDROLYSIS OF BR-2-IIb

Residue	*Position of OMe groups*	*Linkages*	*Composition (mol %)*		
			PA-1-IV	*PA-1-V-5*	*PA-1-V-4*
GalA	2,3,4	terminal	13.1	28.4	25.8
	2,3	4	31.9	4.0	41.6
	2	3,4	4.4	n.d.	n.d.
	3	2,4	n.d.[a]	3.0	n.d.
Ara	2,3,5	terminal (furanosyl)	3.7	17.6	11.3
	2,3,4	terminal (pyranosyl)	0.5	1.1	0.3
	2,3	4 or 5	1.1	3.1	1.3
	2,4	3 (pyranosyl)	n.d.	1.4	n.d.
	2	3,4 or 3,5	0.5	1.4	n.d.
Rha	2,3,4	terminal	0.3	0.6	0.2
	3	2,4	0.8	2.0	0.2
Gal	2,3,4,6	terminal	5.1	1.1	0.1
	2,3,6	4	8.2	6.5	0.1
	2,4,6	3	2.9	2.0	0.1
	2,3,4	6	2.1	n.d.	n.d.
	2,6	3,4	2.8	3.1	1.5
	2,3	4,6	3.6	n.d.	n.d.
	2,4	3,6	4.5	12.5	10.5
	2	3,4,6	4.4	7.7	6.8
	3	2,4,6	n.d.	1.5	n.d.
Glc	2,3,4,6	terminal	2.2	2.0	0.4
Xyl	2	3,4	0.3	0.9	0.1

[a]Not detected.

TABLE VIII

DIAGNOSTIC E.I.-M.S. FRAGMENT IONS OF METHYLATED OLIGOSACCHARIDE-ALDITOLS FROM PRODUCTS PA-1-II, PA-1-III, PA-V-2, AND PA-1-V-3 OBTAINED BY PARTIAL ACID HYDROLYSIS OF BR-2-IIb

Product	*Fragment ions* [m/z *(relative abundance)*]									*Oligosaccharide-alditol*
	bA_1	bA_2	aJ_1	aJ_2	*ald*					
PA-1-II	221	189	266	206	352	340	309	308		GalA-(1→2 and 4)-Rha-ol-*1-d*
PA-1-III	(18.5)	(100)	(18.8)	(38.3)	(0.4)	(0.1)	(0.7)	(0.4)		
PA-1-V-3					353	321	277	134		
					(0.3)	(0.9)	(1.0)	(15.0)		
PA-1-V-2	221	189	266	206	353	321	309	277	134	GalA-(1→4)-Rha-ol-*1-d*
	(7.0)	(100)	(0.1)	(18.4)	(1.5)	(0.8)	(0.7)	(1.9)	(16.4)	
PA-1-V-3	221	189		236	339	178	146	134		GalA-(1→6)-Gal-ol-*1-d*
	(12.8)	(100)		(79.3)	(36.1)	(19.9)	(76.7)	(28.6)		
	bA_1	bA_2	cbA_1	cbA_2	aJ_1	aJ_2	abJ_1	abJ_2		
PA-1-II	219	187	393	361	266	206		380		Gal→Rha→Rha-ol-*1-d*
	(80.1)	(100)	(87.2)	(1.7)	(13.6)	(100)		(10.1)		
PA-1-V-3	221	189	395	363		206		380		GalA→Rha→Rha-ol-*1-d*
	(30.5)	(49.2)	(6.0)	(1.1)		(100)		(0.2)		
	221	189			298	238		444		GalA→GalA→GalA-ol-*1-d*
	(31.2)	(100)			(5.3)	(47.4)		(24.1)		

TABLE IX

EFFECTS OF CHEMICAL AND ENZYMIC TREATMENTS ON THE ANTI-COMPLEMENTARY ACTIVITY OF BR-2-IIb

Treatment		*Concentration (μg/mL)*		
		1000	*500*	*100*
		Anti-complementary activity (%)		
1[a] No treatment		73	37	19
De-esterified		85	84	29
Carboxyl-reduced		66	50	22
2[a] No treatment		73	63	29
Endo-polygalacturonase treated	digest	84	82	62
	PG-1	90	89	84
	PG-2	63	43	23
	PG-3	17	8	7
Partially acid-hydrolysed[b]	PA-1	39	32	18
	PA-2	21	10	5

[a]Different sera were used. [b]100mM Trifluoroacetic acid, 100°, 1 h.

portion (*m/z* 352, 340, and 308) or the 4-linked rhamnitol portion (*m/z* 353, 321, 309, 277, and 134). These data suggested that this peak contained GalA-(1→2)-Rha-ol-*1-d* and GalA-(1→4)-Rha-ol-*1-d*. PA-1-V-2 gave the peak containing only GalA-(1→4)-Rha-ol-*1-d*, and PA-1-V-3 also gave the peak containing GalA-(1→6)-Gal-ol-*1-d*. G.l.c.–m.s. also suggested that PA-1-II contained Gal-→Rha→Rha and that PA-1-V-3 contained GalA→Rha→Rha and GalA→GalA→GalA.

Effects of chemical and enzymic treatments on the anti-complementary activity of BR-2-IIb. — The results of tests for anti-complementary activity are shown in Table IX. De-esterification of BR-2-IIb enhanced the anti-complementary activity, but subsequent carboxyl-reduction decreased the activity to the original level. These results suggested that carboxyl groups in GalA of BR-2-IIb contributed to its anti-complementary activity. Of the products of digestion with endo-(1→4)-α-polygalacturonase, PG-1 and PG-2 showed enhanced and decreased activities, respectively. The activity of PG-3 was negligible. The acidic fragments, PA-1 and PA-2, obtained by partial hydrolysis of BR-2-IIb, had activities weaker than that of PG-2, and PA-2 showed negligible activity. These results also suggested that the "ramified" region is essential for the anti-complementary activity of BR-2-IIb.

DISCUSSION

The general structure of pectin has been proposed[24,25] to consist both of "ramified" (rhamnogalacturonan with neutral carbohydrate chains) and poly-

galacturonan regions on the basis of the result of digestion with endo-(1→4)-α-polygalacturonase. Enzymic digestion (Fig. 1) showed BR-2-IIb to contain a small proportion of enzyme-resistant "ramified" region (PG-1) and a large proportion of polygalacturonan region. It has been proposed[24,25] that some polygalacturonan regions are highly methyl-esterified and others are relatively free from methyl-ester groups. The methyl-ester groups in BR-2-IIb were distributed widely in the polygalacturonan region, because the endo-(1→4)-α-polygalacturonase-sensitive region (PG-3) contained seven (1→4)-linked oligogalacturonides of different sizes in spite of the fact that this enzyme can hydrolyse[26] polygalacturonic acid into mono- to tri-galacturonides.

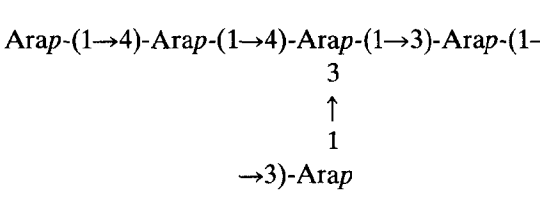
Oligosaccharide analysis of BR-2-IIb and base-catalysed β-elimination studies of PG-1 suggesed that BR-2-IIb contained a rhamnogalacturonan core. BR-2-IIb might contain a dirhamnosyl unit, because GalA→Rha→Rha and Gal→Rha→Rha were identified in PA-1-II and PA-1-V-3 which were obtained by partial hydrolysis of BR-2-IIb. A dirhamnosyl unit was present in some pectic polysaccharides that were obtained from the roots of *Angelica acutiloba* (AGIIb-1[22]) and the leaves of *Artemisia princeps* (AAFIIb-3[27]). Partial acid hydrolysis showed the presence of the glycosidic sequence →4)-GalA-(1→4)-Rha-(1→ in several oligosaccharide fractions, strongly suggesting that position 4 of 2,4-disubstituted Rha in the rhamnogalacturonan core of BR-2-IIb was substituted partially with 4-linked GalA. Some pectic polysaccharides from *A. acutiloba* (AGIIb-1[22]) and *A. princeps* (AAFIIb-3[27]) contain this sequence. It is suggested that the neutral carbohydrate side-chains might be linked either to position 4 of GalA which substitutes the rhamnogalacturonan core directly through position 4 of 2,4-disubstituted Rha.

Recently, the elimination of the neutral carbohydrate side-chains from position 4 of GalA in the acidic core of a pectic polysaccharide by a base-catalysed β-elimination reaction in the presence of sodium borodeuteride without prior methyl-etherification was reported[28,29,30] and indicated[28,30] that various sizes of galactosyl and arabinosyl side-chains substituted their rhamnogalacturonan core through 4-linked GalA in the pectic polysaccharides from *A. acutiloba*. Lau *et al.* also concluded[31] that neutral oligosaccharide side-chains in Rhamnogalacturonan I substituted the rhamnogalacturonan core in a similar manner. The sequences Gal→Rha→Rha and Ara→Rha were detected in the products of partial acid hydrolysis of BR-2-IIb. Some galactosyl and arabinosyl chains may be attached directly to position 4 of 2,4-disubstituted Rha in the rhamnogalacturonan core of BR-2-IIb. Thus, BR-2-IIb contained the possible partial structure **6**.

Methylation analysis indicated that the galactan moiety contained various linkages and could be present as an arabinogalactan, because Ara-(1→6)-Gal was present in a partial hydrolysate of BR-2-IIb. Most of the arabinan moiety was assumed to consist of Ara*p* as shown in **7**, because large amounts of terminal Ara*p* and 3-linked Ara*p* were detected in the neutral fragments obtained from BR-2-IIb by partial hydrolysis (Tables III and IV).

→4)-GalA-(1→2)-Rha-(1→2)-Rha-(1→4)-GalA-(1→2)-Rha-(1→4)-GalA-(1→2)-Rha-(1→4)-MeGalA-(1→2)-Rha-(1→
4
↑
Gal
↑
(Gal)n
↑
galactan or
arabinogalactan
4
↑
Gal
4
↑
GalA
4
↑
Gal
↑
galactan or
arabinogalactan
4
↑
(Ara)n
arabinan

6



7

It has been suggested that, for pectic polysaccharides from medicinal plants such as the roots of *A. acutiloba* (AGIIb-1[32]), the seed of *Coix lacryma-jobi* (CA-1 and -2[33]), the leaves of *A. princeps* (AAFIIb-2 and -3[27]), and the leaves of Panax ginseng (GL-PI, -PII, -PIII, and -PIV[34]), the neutral carbohydrate side-chains attached to the rhamnogalacturonan core might be important for the expression of their anti-complementary activities. The anti-complementary activity of PG-1 was greater than that of BR-2-IIb, but the small galacturonan (PA-2), obtained from partial acid hydrolysates, showed negligible activity. These observations suggested that the anti-complementary activity of BR-2-IIb also depends on the neutral carbohydrate side-chains and rhamnogalacturonan backbone. It has been postulated[25] that methyl-esterification of GalA in the polygalacturonan region inhibits the formation of an "egg-box" structure that forms three-dimensional networks by chelation between carboxyl groups of GalA and Ca^{2+} ion, and that de-esterification or carboxyl-reduction might result in the formation of different three-dimensional networks and thereby modulate the activities.

The detailed structure and the role of the neutral carbohydrate side-chains in the "ramified" region of BR-2-IIb for the expression of anti-complementary activity remain to be determined.

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