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STRUCTURE AND ANTI-COMPLEMENTARY ACTIVITY OF PECTIC POLYSACCHARIDES ISOLATED FROM THE ROOT OF *Angelica acutiloba* Kitagawa*

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ABSTRACT

Four pectic polysaccharides (AR-2IIa–IId) with anti-complementary activity have been isolated from a hot-water extract of the root of *Angelica acutiloba* Kitagawa. Each of these polysaccharides contained a large proportion of GalA together with neutral sugars consisting mainly of Rha, Ara, and Gal. Digestion with endo-α-(1→4)-polygalacturonase indicated that AR-2IIa–IIc each contained a large proportion of enzyme-sensitive polygalacturonan regions, and that AR-2IId contained a large proportion of enzyme-resistant regions. When AR-2IId was de-esterified, it became sensitive to the enzyme. These polysaccharides also contained small proportions of enzyme-resistant regions (PG-1) which were rich in neutral sugars. Methylation analysis and base-catalysed β-elimination studies suggested that each PG-1 contained a rhamnogalacturonan moiety in which 2,4-disubstituted Rha was attached to 4-substituted GalA through position 2 of Rha.

Carboxyl-reduction and methyl- and de-esterification of these polysaccharides modulated their anti-complementary activities. Digestion with endo-α-(1→4)-polygalacturonase decreased the activities of AR-2IIa and -2IIb, but not those of AR-2IIc and -2IId. Although PG-1 fractions from AR-2IIa–IIc were more active than the original polysaccharides, oligogalacturonide fragments obtained by enzymic digestion had weak or negligible activity. AR-2IIa–IIc expressed their anti-complementary activities mainly *via* the classical pathway, but AR-2IId and each PG-1 expressed their activities *via* both the classical and alternative pathways.

INTRODUCTION

The root of *Angelica acutiloba* Kitagawa is a well known medicinal herb used in the treatment of gynecological diseases and arthritis. We have purified and characterised two pectic arabinogalactans (AGIIa[2] and AGIIb-1[3]) present in the root

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and which have potent anti-complementary activity. Each of these polysaccharides[2,4] is composed mainly of an arabino-3,6-galactan together with small amounts of uronic acids, and the (1→3,6)-β-galactan moiety is essential for anti-complementary activity. *A. acutiloba* root also contains another pectic polysaccharide fraction (AR-2)[5] which has moderate anti-complementary activity and contains a large proportion of GalA together with small amounts of neutral sugars[5]. Tomoda *et al.*[6] purified and characterised a major acidic polysaccharide (Angelica-pectin A) from the same fraction as AR-2, and showed it to be characteristic[7] of the crude drug. However, the detailed structure of Angelica-pectin A has not been reported and its biological activity is not known.

We have studied the structure and anti-complementary activity of the pectic polysaccharides isolated from AR-2, and have attempted to clarify the structure–activity relationships.

EXPERIMENTAL

Materials. — The root of *A. acutiloba* Kitagawa was purchased from Tochimoto Tenkaidoh Co. Ltd. (Japan). DEAE-Sephadex A-50, Sephadex G-50 and G-100, and Sepharose CL-6B were obtained from Pharmacia, and Bio-gel P-2 (200–400 mesh) and P-4 (-400 mesh) from Bio-Rad. Pectinase from *Aspergillus niger* was purchased from Sigma and endo-α-(1→4)-polygalacturonase was purified by using the procedure of Thibault and Mercier[8].

General. — Total carbohydrate, uronic acid, and protein were assayed by the phenol–sulfuric acid[9], *m*-hydroxybiphenyl[10], and Lowry methods[11], respectively, using Gal, GalA, and bovine serum albumin as the respective standards. Methyl and acetyl ester groups were assayed by the methods of Wood *et al.*[12] and McComb *et al.*[13], respectively, using methanol and D-glucose penta-acetate as the respective standards. Optical rotations were determined at 22° with a JASCO DIP-Digital polarimeter. Molecular weights of the polysaccharides were estimated from the calibration curve of the elution volumes of standard dextrans (T-2000, 500, 70, 40, and 10) from Sepharose CL-6B in 50mM acetate buffer (pH 5.6). Electrophoresis was performed (cellulose acetate membrane, Fuji Film Co. Ltd.) in acid (0.08M pyridine–0.04M acetic acid buffer, pH 5.4) or alkali (0.026M borate buffer, pH 9.2) at 70 V for 30 min with detection[7] using Toluidine Blue. Polysaccharides were hydrolysed with 2M trifluoroacetic acid at 121° for 1.5 h, and the hydrolysates were analysed by t.l.c. on cellulose (Merck) with ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Reducing sugars were detected with alkaline silver nitrate[14] and uronic acids with *p*-anisidine hydrochloride[15]. Sugars were converted conventionally 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. x 200 cm) packed with 1% of 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.

Purification of the acidic polysaccharides, AR-2IIa–IId. — The crude AR-2 fraction[5], prepared from the root of *A. acutiloba* Kitagawa by hot-water extraction, and ethanol and Cetavlon (cetyltrimethylammonium bromide) precipitation, was further fractionated on a column (5.4 x 37 cm) of DEAE-Sephadex A-50 (HCO_3^- form). Stepwise elution with 0.2, 0.3, 0.4, and 0.5M ammonium hydrogencarbonate, respectively, gave AR-2IIa–IId.

Digestion with endo-α-(1→4)-polygalacturonase. — Each polysaccharide (50–200 mg) was digested with endo-α-(1→4)-polygalacturonase[8] (0.1 U) in 50mM acetate buffer (pH 4.2, 6–20 mL) for 4 days at 30°. After neutralisation, each mixture was heated to 100° for 10 min to inactivate the enzyme, and then lyophilised. For de-esterification, a solution of AR-2IId (20 mg) in 0.5M sodium hydroxide (2 mL) was kept for 2 h at room temperature and then neutralised with acetic acid, and the product was digested with endo-α-(1→4)-polygalacturonase as described above.

Methylation analysis. — Acidic polysaccharides were methylated once by the method[16] of Hakomori in order to prevent β-elimination[17]. Methylated polysaccharides were recovered using a Sep-pak C_{18} cartridge (Waters Associates) by the procedure of Waeghe and Albersheim[18] except that the samples were eluted with ethanol. Uronic acids of methylated acidic polysaccharides were reduced[18] with sodium borodeuteride in tetrahydrofuran–ethanol (7:3) for 18 h at room temperature followed by incubation for 1 h at 75°. The methylated acidic or carboxyl-reduced polysaccharides were hydrolysed, and the products were reduced with sodium borohydride followed by acetylation. The resulting alditol acetates were analysed by g.l.c. and g.l.c.–m.s. G.l.c. was performed on a Hewlett–Packard model 5840A gas chromatograph equipped with a DB-1 capillary column (0.25-μm film, 30 m x 0.25 mm i.d., J & W Scientific) with splitless injection. The carrier gas (helium) was at 0.9 mL/min and the temperature programme was 60° for 1 min, →150° at 18°/min, then →210° at 2°/min. G.l.c.–m.s. (70 eV) was performed on a JEOL DX-300 instrument equipped with an SPB-1 capillary column (0.25-μm film, 25 m x 0.25 mm i.d., SPELCO), and the temperature programme was 120 → 210° at 2°/min.

β-Elimination[17] of methylated PG-1 (derived from the enzymic digests of the acidic polysaccharides). — To a solution of each dry methylated PG-1 (1–2 mg) in methyl sulfoxide (1 mL) was added methylsulfinylmethanide, and the mixture was stirred for 24 h at room temperature. To 50% of each sample was added an excess of ethyl iodide, the mixture was kept overnight at room temperature, the ethyl iodide was evaporated, and the product (R_2) was recovered by using a Sep-pak C_{18} cartridge as described above. The remainder of the sample was treated 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 analysed as alditol acetates by g.l.c. and g.l.c.–m.s. as described above.

Preparation of the carboxyl-reduced, methyl-esterified, and de-esterified acidic polysaccharides. — The carboxyl groups of GalA in AR-2IIa–IId were reduced

by the modified procedure[19,20] of Taylor and Conrad. Each polysaccharide (5 mg) was reduced by using 1-cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulfonate (CMC, 50 mg) and sodium borohydride (800 mg), and the products were isolated by dialysis and lyophilisation (yield, 70–80%). Carboxyl-reduced AR-2IIa and AR-2IIb were soluble in water, but carboxyl-reduced AR-2IIc and AR-2IId were almost insoluble in water. Of the GalA in AR-2IIa and AR-2IIb, ~90% was reduced to Gal by this procedure.

Each acidic polysaccharide (5 mg) was suspended in methanol (1 mL) and diazomethane in ether (5 mL) was added. Each suspension was stirred for 1 h at 4°, the solvent was removed in a stream of air, and a solution of the residue in water was lyophilised to give the methyl-esterified polysaccharide.

A solution of each acidic polysaccharide in 0.2M sodium hydroxide was stored for 2 h at room temperature, then neutralised with acetic acid, and dialysed. The de-esterfied polysaccharides were recovered by lyophilisation of the non-diffusible fractions.

Anti-complementary activity. — Various dilutions of the sample in water (50 μL) were mixed with 50 μL each of normal human serum (NHS) and Gelatin–veronal-buffered saline (pH 7.4) containing 500μM magnesium chloride and 150μM calcium chloride (GVB^{2+}). The mixtures were pre-incubated for 30 min at 37° and the residual hemolytic complement (TCH_{50}) was determined by a method using IgM-hemolysin-sensitised sheep erythrocytes (EA) at 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} (%) given by

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

Determination of the complement hemolysis through the alternative pathway (ACH_{50}). — ACH_{50} was determined[21] in 10mM EGTA containing 2mM magnesium chloride in Gelatin–veronal-buffered saline (Mg^{2+}-EGTA-GVB^{2-}). A sample was pre-incubated with Mg^{2+}-EGTA-GVB^{2-} and NHS at 37° for 30 min, and the residual complement of the mixtures was measured by the hemolysis of rabbit erythrocytes (5×10^7 cells/mL) incubated with Mg^{2+}-EGTA-GVB^{2-}.

Determination of C4. — Titration of C4 was performed[22] using intermediate cells $EAC1^{gp}$ for C4. $EAC1^{gp}$ cells were prepared from EA (10^8 cells/mL) incubated with C1 solution (10^{12} SFU/mL) in the ratio of 28:1 for 1 h at 4°.

RESULTS

Isolation and properties of the acidic polysaccharides. — The crude anti-complementary polysaccharide fraction (AR-2) was fractionated on DEAE-Sephadex A-50 (Fig. 1), to give four acidic polysaccharide fractions (AR-2IIa–IId), by eluting with 0.2, 0.3, 0.4, and 0.5M ammonium hydrogencarbonate, respectively. Each frac-

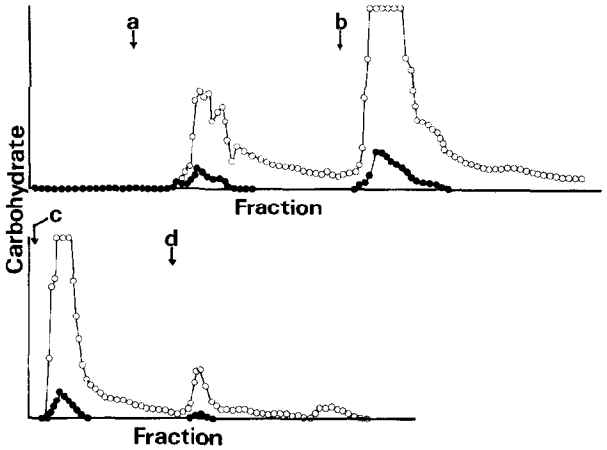


Fig. 1. Anion-exchange chromatography of AR-2 on DEAE-Sephadex. Acidic polysaccharides (AR-2IIa–IId) were eluted with 0.2 (a), 0.3 (b), 0.4 (c), and 0.5M (d) ammonium hydrogencarbonate, respectively: -●-, carbohydrate (490 nm); -○-, uronic acid (520 nm).

tion gave a single spot in electrophoresis on cellulose acetate in either acid or alkali, and was eluted as a single peak from Sepharose CL-6B. Thus, each fraction was a homogeneous polysaccharide.

The physicochemical properties of AR-2IIa–IId are summarised in Table I. AR-2IIa and -2IIb showed different molecular weights, whereas AR-2IIc and -2IId showed similar molecular weights. Each polysaccharide was markedly dextrorotato-

TABLE I

PHYSICOCHEMICAL PROPERTIES OF THE ACIDIC POLYSACCHARIDES

	AR-2IIa	*AR-2IIb*	*AR-2IIc*	*AR-2IId*
Molecular weight[a]	4.2×10^4	9.5×10^4	24.5×10^4	21.6×10^4
$[\alpha]_D$ (*c* 1, water)(°)	+170.45	+211.25	+197.51	+142.05
Total carbohydrate (%)	45.2	34.2	39.3	40.1
Uronic acid (%)	91.7	98.6	97.2	97.0
Protein (%)	2.1	1.4	1.8	6.0
Methyl ester[b] (%)	55.0	47.6	33.3	33.3
Acetyl ester[c] (%)	5.3	3.6	9.8	8.9
Neutral sugar (mol%)				
Rha	23.1	30.9	15.6	15.4
Ara	23.1	35.7	39.3	35.4
Xyl	7.6	4.8	7.8	15.4
Man	4.0		15.6	15.4
Gal	38.4	23.8	15.6	15.4
Glc	4.0	4.8	6.1	3.0

[a]Calculated by gel filtration on Sepharose CL-6B in 50mM acetate buffer (pH 5.6). [b]Expressed as percentages of methyl-esterified GalA in total GalA. [c]Calculated from weights of acetyl ester and polysaccharides.

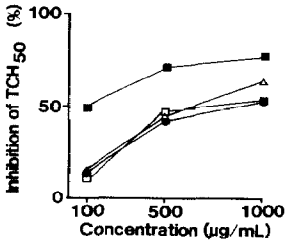


Fig. 2. Anti-complementary activity of AR-2IIa (•), AR-2IIb (□), AR-2IIc (Δ), and AR-2IId (■).

ry, contained a large proportion of GalA, small amounts of neutral sugars (mainly Rha, Ara, and Gal) and protein, and methyl and acetyl ester groups. Of the GalA, ~50% was methyl-esterified in AR-2IIa and -2IIb, and ~33% in AR-2IIc and -2IId. Methylation analysis showed that each acidic polysaccharide contained a large proportion of 4-linked GalA (data not shown) and was a pectin-like polysaccharide.

Anti-complementary activity of the acidic polysaccharides. — The anti-complementary activities of AR-2IIa–IId were tested (Fig. 2). AR-2IId had the highest activity, whereas AR-2IIa–IIc had similar moderate activities.

Enzymic digestion of the acidic polysaccharides. — AR-2IIa–IId were digested with endo-α-(1→4)-polygalacturonase[8] from *Aspergillus niger*, and each digest was fractionated on Sephadex G-50. AR-2IIa–IIc (Fig. 3A–C) each gave a small fraction (PG-1) eluted in the void volume, an intermediate fraction (PG-2), and a large

TABLE II

COMPONENT SUGARS AND MOLECULAR WEIGHTS OF ENZYMIC DIGESTION PRODUCTS (PG-1–3) FROM THE ACIDIC POLYSACCHARIDES

Polysaccharide		*Molar ratio*							*Mol.wt.*[b]
		Rha	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*GalA*[a]	
AR-2IIa	PG-1	0.4	0.5	0.06		1.0		+	3.2×10^4
	PG-2							+ + +	
	PG-3							+ + +	
AR-2IIb	PG-1	0.9	0.9	0.09	trace	1.0	trace	+	2.3×10^4
	PG-2							+ + +	
	PG-3							+ + +	
AR-2IIc	PG-1	1.6	1.5	0.1		1.0		+	1.1×10^4
	PG-2							+ + +	
	PG-3							+ + +	
AR-2IId	PG-1	0.7	1.6	0.09	trace	1.0	0.3	+	1.0×10^4
	PG-2							+ + +	
	PG-3							+ + +	

[a]Key: +, small amount; + + +, large amount.
[b]Calculated by gel filtration on Sepharose CL-6B in 50mM acetate buffer (pH 5.6).

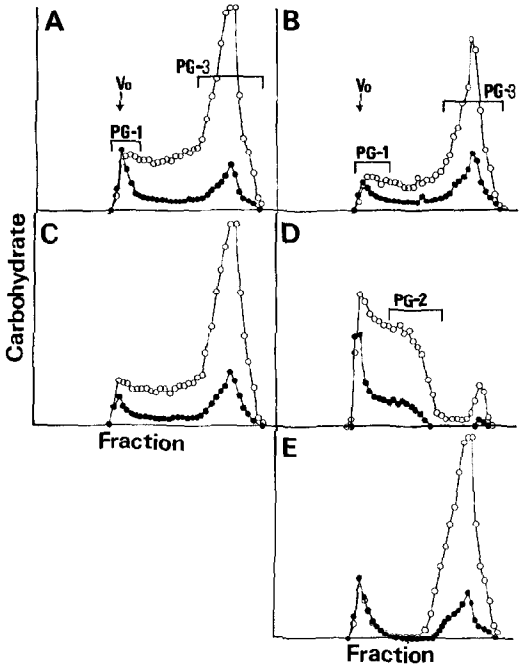


Fig. 3. Gel-filtration patterns on Sephadex G-50 of the acidic polysaccharides after digestion with endo-α-(1→4)-polygalacturonase: A, AR-2IIa; B, AR-2IIb; C, AR-2IIc; D, AR-2IId; and E, de-esterified AR-2IId. Symbols as in Fig. 1; V_o, void volume.

amount of lowest molecular weight fraction (PG-3), whereas AR-2IId (Fig. 3D) gave large amounts of PG-1 and PG-2, and a small amount of PG-3. However, when AR-2IId was de-esterified with 0.5M sodium hydroxide prior to digestion with the enzyme, it gave (Fig. 2E) a large amount of PG-3, but not of PG-2. Each PG-1 (Table II) consisted mainly of Rha, Ara, Gal, and GalA, and the ratio of Ara and Gal increased from AR-2IIa to AR-2IId. PG-2 and PG-3 were mostly composed of GalA. Each PG-3 was eluted from Bio-gel P-4 in the regions for mono- to octa-galacturonides (data not shown). The PG-1 products (Table II) derived from AR-2IIa and -2IIb had different molecular weights, but those from AR-2IIc and -2IId had similar molecular weights.

Methylation analysis (Table III, before elimination) showed that each PG-1 contained terminal, 4- or 5- and 3-linked, and 3,4- or 3,5-disubstituted Ara; terminal, 2-linked, and 2,4-disubstituted Rha; terminal, 3-, 4- and 6-linked, 4,6- and 3,6-disubstituted, and 3,4,6-trisubstituted Gal. Each PG-1, except that from AR-2IId, contained, in addition, terminal Glc, 2,4-disubstituted Gal, and 3,4-disubstituted Xyl. Methylation analysis showed that each PG-3 was composed of terminal and 4-linked GalA (data not shown). This result indicated that PG-2 and PG-3 were derived from the (1→4)-poly-α-D-galacturonan region of the acidic polysaccharides by the enzymic digestion. These results suggested that each acidic polysaccharide

was composed of two carbohydrate blocks, namely, a (1→4)-poly-α-D-galacturonan termed[23] a "smooth" (polygalacturonan) region, and a neutral-sugar-enriched block (PG-1) termed a "hairy" region.

Identification of the rhamnogalacturonan core in the "hairy" regions from the acidic polysaccharides by base-catalysed β-elimination[17]. — In order to confirm the presence of the rhamnogalacturonan core in each "hairy" region, base-catalysed β-elimination[17] of the methylated PG-1 was carried out and the exposed hydroxyl groups in the product (R_1) were ethylated to give R_2.

Methylation analysis (Table III, R_1) showed a loss of 2,4-disubstituted Rha (~54.8% in AR-2IIa, ~66.7% in IIb, ~59.0% in IIc, and ~76.5% in IId) from each PG-1. 1,4,5-Tri-*O*-acetyl-2-*O*-ethyl-3-*O*-methylrhamnitol and 1,5-di-*O*-acetyl-6-*O*-ethyl-2,3,4-tri-*O*-methylgalactitol were formed in the methylation analysis of R_2 (Table III). These results indicated that a part of 2,4-disubstituted Rha was attached to C-4 of GalA in each PG-1 (**1**), and that 4-substituted GalA was linked to position 2 of the 2,4-disubstituted Rha (**2**). Therefore, each PG-1 possessed the partial structure **3**. In addition, 4-substituted GalA was linked to position 6 of the terminal Gal (**4**). Other glycosyl residues, such as 3-linked Ara*p*, 2-linked Rha, and terminal Gal, were decreased by base-catalysed β-elimination (Table III, R_1), and the partial structures **5**–**7** were present in each PG-1.

TABLE III

LINKAGE COMPOSITION OF ENZYMIC DIGESTION PRODUCTS (PG-1) FROM THE ACIDIC POLYSACCHARIDES BOTH BEFORE AND AFTER BASE-CATALYSED β-ELIMINATION OF URONIC ACID RESIDUES.

Methylated sugar	*Before elimination*			
	AR-2IIa (mol %)	*AR-2IIb*	*AR-2IIc*	*AR-2IId*
2,3,5-Me_3-Ara	4.0	6.3	8.5	6.5
2,3,4-Me_3-Ara	1.7	2.4	2.7	1.4
2,3,4-Me_3-Rha	1.8	4.5	4.4	2.3
2,3-Me_2-Ara	3.4	9.9	12.4	14.2
2,4-Me_2-Ara	3.2	9.4	13.4	11.3
3,4-Me_2-Rha	1.7	4.1	3.4	5.2
2,3,4,6-Me_4-Glc	0.3	1.0	1.2	
2,3,4,6-Me_4-Gal	4.4	8.8	10.6	10.9
2-Me-Ara	1.0	3.3	6.1	13.3
2-Me-Xyl	1.8	3.0	2.0	
3-Me-Rha	4.2	6.6	7.8	9.8
2,3,6-Me_3-Gal	3.2	3.9	4.8	5.4
2,4,6-Me_3-Gal	3.4	3.9	3.4	5.3
2,3,4-Me_3-Gal	10.7	6.3	3.5	6.2
3,6-Me_2-Gal	6.0	10.7	7.3	
2,3-Me_2-Gal	6.5	4.3	3.2	1.3
2,4-Me_2-Gal	21.3	8.9	4.3	5.4
2-Me-Gal	20.8	2.8	1.2	1.4

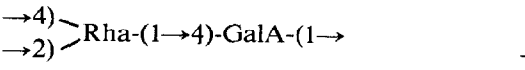
TABLE III (continue)

Methylated sugar	*After elimination*			
	R_1 *AR-2IIa (mol %)*	*AR-2IIb*	*AR-2IIc*	*AR-2IId*
2,3,5-Me_3-Ara	5.2	4.8	7.1	3.1
2,3,4-Me_3-Ara	1.1	1.3	3.0	0.8
2,3,4-Me_3-Rha	2.5	6.1	5.5	0.3
2,4-Me_2-Ara	3.0	8.4	14.1	18.5
2,4-Me_2-Ara	1.7	4.4	4.6	2.5
3,4-Me_2-Rha	1.1	3.2	1.7	2.4
2,3,4,6-Me_4-Glc				
2,3,4,6-Me_4-Gal	2.8	5.1	5.4	3.5
2-Me-Ara	2.0	6.7	11.4	22.4
2-Me-Xyl	2.7	6.2	5.3	
3-Me-Rha	1.9	2.2	3.2	2.3
2,3,6-Me_3-Gal	2.7	2.4	2.6	6.1
2,4,6-Me_3-Gal	4.5	6.3	3.6	4.8
2,3,4-Me_3-Gal	15.1	12.6	7.9	16.6
3,6-Me_2-Gal	5.6	15.9	14.2	
2,3-Me_2-Gal	4.4			
2,4-Me_2-Gal	25.1	12.6	8.4	12.2
2-Me-Gal	18.7	1.8	2.0	3.1
	R_2[a]			
2-Et-3-Me-Rha	+	+	+	+
6-Et-2,3,4-Me_3-Gal	+	+	+	+

[a]Partially ethylated and methylated alditol acetates were detected by g.l.c.–m.s.: + indicates the detectable residues, but their molar ratios could not be calculated because of trace amounts.

Effects of carboxyl-reduction and methyl- and de-esterification on the anti-complementary activity of the acidic polysaccharides. — When the carboxyl groups of GalA in the acidic polysaccharides were reduced[19,20], 90% of the GalA in AR-2IIa and -2IIb was converted into Gal. Because AR-2IIc and -2IId were difficult to solubilise in water after reduction, the anti-complementary activity was measured only for reduced AR-2IIa and -2IIb (Table IV). Carboxyl-reduction of AR-2IIa and -2IIb remarkably decreased their anti-complementary activities.

The GalA in the acidic polysaccharides was methyl-esterified by diazomethane. Treatment of the acidic polysaccharides with 0.2M sodium hydroxide decreased the methyl-ester contents to 8.8% in AR-2IIa, 3.7% in AR-2IIb, 2.7% in AR-2IIc, and 3.5% in AR-2IId. Methyl-esterification did not affect the anti-complementary activities (Table IV) of AR-2IIa and -2IIb, increased that of AR-2IIc, and reduced that of AR-2IId by ~10%. Methyl-esterified AR-2IIc and -2IId had similar activities. De-esterification of methyl-ester reduced the anti-complementary activities of AR-2IIa, -2IIc, and -2IId by 19–5, 17–13, and ~34%, respectively, but did not affect that of AR-2IIb. De-esterified AR-2IIb and -2IId had similar activi-



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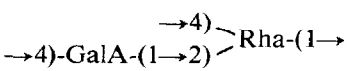

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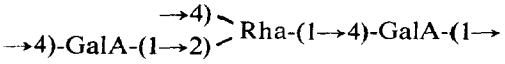

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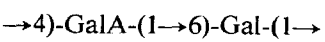

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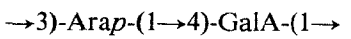

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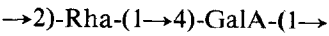

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ties.

These results suggested that carboxyl and methyl ester groups of GalA in the acidic polysaccharides affected their anti-complementary activities.

Effect of enzymic treatment on the anti-complementary activity of the acidic polysaccharides. — When the acidic polysaccharides (AR-2IIa–IId) were digested with endo-α-(1→4)-polygalacturonase, the anti-complementary activities (Table IV) of AR-2IIa and -2IIb were reduced by ~30% and ~20%, respectively, but those of AR-2IIc and -2IId were not affected.

When each digest was fractionated on Sephadex G-50, three carbohydrate fractions (PG-1–3) were obtained (Fig. 2). Each PG-1, except that from AR-2IId (Table V), had a higher anti-complementary activity than the original polysaccharide, and the magnitude of the activity was similar to that of AR-2IId. Oligogalacturonides (PG-2 and PG-3) from these polysaccharides had weak or negligible activities. Poly-galacturonic acid from apple also showed a weak activity as well as PG-2 (data not shown). These results suggested that the combination of PG-1 and polygalacturonan are involved in the expression of anti-complementary activity in AR-2IIa and -2IIb.

Mode of action of the acidic polysaccharides and their "hairy" regions. — AR-2IIa–IId were incubated with NHS in GVB^{2+}, and the residual activity of C4, involving the classical pathway of the complement system, was measured by C4 titration (Table VI). When 1000 μg/mL of these polysaccharides were used for the titration, 85–95% of the hemolytic titre of C4 was consumed. These polysaccharides were incubated with NHS in Mg^{2+}–EGTA–GVB^{2-}, and the residual complement components, involving the alternative pathway of the complement system, were assayed by ACH_{50} (Table VII). AR-2IId showed the highest anti-complementary

TABLE IV

EFFECTS OF CARBOXYL-REDUCTION, METHYL-ESTERIFICATION, DE-ESTERIFICATION, AND ENZYMIC DIGESTION ON ANTI-COMPLEMENTARY ACTIVITY OF THE ACIDIC POLYSACCHARIDES.

Treatment	*Concentration (μg/mL)* 1000	500	100
	Inhibition of TCH_{50} (%)		
1 AR-2IIa			
No treatment	76.5	66.7	23.5
Carboxyl-reduced	61.6	35.2	7.0
AR-2IIb			
No treatment	76.0	72.8	17.5
Carboxyl-reduced	52.5	24.0	6.0
2 AR-2IIa			
No treatment	43.0	30.5	9.2
Methyl-esterified	39.0	27.0	11.5
De-esterified	24.0	12.6	4.8
AR-2IIb			
No treatment	44.0	35.0	7.5
Methyl-esterified	43.8	35.0	7.2
De-esterified	41.2	34.0	10.5
AR-2IIc			
No treatment	36.6	26.2	n.d.[b]
Methyl-esterified	46.8	37.8	14.2
De-esterified	24.0	11.8	2.6
AR-2IId			
No treatment	76.2	74.0	44.2
Methyl-esterified	56.2	50.0	35.2
De-esterified	42.6	36.5	10.5
3 AR-2IIa			
No treatment	59.5	30.8	0.6
Enzymic digest[a]	29.0	11.8	1.0
AR-2IIb			
No treatment	62.6	31.8	2.0
Enzymic digest[a]	42.8	27.8	1.8
AR-2IIc			
No treatment	54.3	29.2	3.0
Enzymic digest[a]	60.0	34.0	1.2
AR-2IId			
No treatment	84.5	85.0	52.5
Enzymic digest[a]	83.2	79.8	18.0

[a]Digested with endo-α-(1→4)-polygalacturonase. [b]Not determined.

TABLE V

ANTI-COMPLEMENTARY ACTIVITY OF ENZYMIC DIGESTION PRODUCTS (PG-1–3) FROM THE ACIDIC POLYSACCHARIDES.

Product	*Concentration (μg/mL)* *1000*	*500*	*100*
	Inhibition of TCH_{50} (%)		
AR-2IIa			
Original	59.5	30.8	0.6
PG-1	82.5	74.5	25.0
PG-2	38.0	23.8	2.0
PG-3	19.2		
AR-2IIb			
Original	62.6	31.8	2.0
PG-1	82.2	74.5	25.5
PG-2	22.0	2.0	
PG-3	4.8	1.5	
AR-2IIc			
Orginal	54.3	29.2	3.0
PG-1	84.5	82.8	41.8
PG-2	28.5		
PG-3	1.8		
AR-2IId			
Original	84.5	85.0	52.5
PG-1	48.8	81.8	40.0
PG-2	33.0	10.0	
PG-3	n.d.[a]	n.d.	n.d.

[a]Not determined.

activity on ACH_{50} (ACP activity), but the activities of AR-2IIa–IIc were weak or negligible (Table VII, Expts. 1 and 2). These results indicated that AR-2IId expressed the anti-complementary activity mainly *via* both the classical and alternative pathways of the complement system, whereas AR-2IIa–IIc expressed the activity mainly *via* the classical pathway.

When 1000 μg/mL of PG-1 from AR-2IIa–IId were used for C4 titration, 90–100% of the hemolytic titre of the C4 was consumed (Table VI). These PG-1

TABLE VI

ANTI-COMPLEMENTARY ACTIVITY OF THE ACIDIC POLYSACCHARIDES AND THE ENZYMIC DIGESTION PRODUCTS (PG-1) THROUGH THE CLASSICAL PATHWAY

Polysaccharide		*Concentration (μg/mL)*	
		1000	*500*
		Comsumption of C4 (%)	
AR-2IIa	Original	86.5	78.2
	PG-1	90.0	86.0
AR-2IIb	Original	87.0	74.6
	PG-1	100.0	91.2
AR-2IIc	Original	90.0	76.2
	PG-1	100.0	98.8
AR-2IId	Original	95.0	94.8
	PG-1	100.0	97.0

fractions also had remarkably higher ACP activities than the original polysaccharides, except PG-1 from AR-2IId (Table VII, Expt. 2). These results indicated that "hairy" regions of these polysaccharides expressed the anti-complementary activity *via* the classical and alternative pathways of the complement system.

DISCUSSION

The present results showed that the four acidic polysaccharides, AR-2IIa–IId, isolated from a hot-water extract of the root of *A. acutiloba* were structurally related pectin-like polysaccharides. The general structure of pectin has been proposed[23] to consist both of "hairy" and "smooth" (polygalacturonan) regions on the basis of the result of digestion with endo-α-(1→4)-polygalacturonase. The results of the enzymic digestion (Table VIII) showed AR-2IIa–IIc and de-esterified AR-2IId to contain small proportions of "hairy" regions and large proportions of polygalacturonan regions in similar ratios. It was assumed that AR-2IIa–IId consisted similary of approximately three "hairy" regions and twenty polygalacturonan regions. Thibault *et al.* reported[24] that endo-α-(1→4)-polygalacturonase from *A. niger* slowly hydrolysed highly methyl-esterified polygalacturonans. Therefore, the enzyme resistance of the polygalacturonan region of AR-2IId could be due to methyl-ester-

TABLE VII

ANTI-COMPLEMENTARY ACTIVITY OF THE ACIDIC POLYSACCHARIDES AND THE ENZYMIC DIGESTION PRODUCTS (PG-1) THROUGH THE ALTERNATIVE PATHWAY

Polysaccharide		*Concentration (μg/mL)* 1000	500	100
		Inhibition of ACH_{50} (%)		
Experiment 1				
AR-2IIa		4.5	4.0	4.8
AR-2IIb		5.0	5.0	2.4
AR-2IIc		3.5	2.5	3.5
AR-2IId		39.5	28.0	9.5
Experiment 2				
AR-2IIa	Original	11.0	9.0	9.5
	PG-1	25.0	22.0	14.5
AR-2IIb	Original	9.5	10.2	5.0
	PG-1	36.0	35.0	10.5
AR-2IIc	Original	17.5	11.5	10.5
	PG-1	44.0	37.2	26.0
AR-2IId	Original	41.5	38.2	37.0
	PG-1	40.0	39.0	26.0

ification of the carboxyl groups of GalA. Fractions PG-3, produced from AR-2IIa–IIc by digestion with the endo-polygalacturonase, were composed of mono- to octa-galacturonides, and the preponderance of oligogalacturonides over tetragalacturonide in each PG-3 was ascribed to a high level of methyl-esterification because this enzyme hydroyses[24] polygalacturonic acid into mono- to tri-galacturonides. It has been proposed[25,26] that some polygalacturonan regions are highly methyl-esterified and others are relatively free from methyl ester groups. Thus, it is suggested that AR-2IIa–IIc contained shorter polygalacturonan regions (frequently methyl-esterified) than AR-2IId. Since the sizes of "hairy" and polygalacturonan regions in AR-211c and -2IId were similar, it is assumed that AR-2IIc and -2IId differ in the patterns of distribution of the methyl ester groups in the polygalacturonan regions (Fig. 4).

AR-2IId had the highest anti-complementary activity. AR-2IId expressed the

TABLE VIII

RATIOS OF "HAIRY" AND POLYGALACTURONAN REGIONS OF THE POLYSACCHARIDES

Polysaccharide	*Ratio of yield*			*Ratio of "hairy" and polygalacturonan regions*[a]
	PG-1	*PG-2*	*PG-3*	
AR-2IIa	0.2	0.4	1.0	0.14:1.0 (3:20)
AR-2IIb	0.2	0.3	1.0	0.15:1.0
AR-2IIc	0.2	0.3	1.0	0.15:1.0
AR-2IId	3.1	6.0	1.0	0.40:1.0
De-esterified AR-2IId	0.14	trace	1.0	0.14:1.0

[a] "Hairy" region expressed as the yield of PG-1, and polygalacturonan region expressed as the combined yields of PG-2 and PG-3.

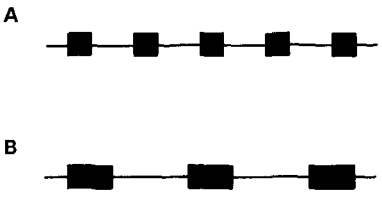

Fig. 4. Distribution of methyl ester groups in the polygalacturonan region of AR-2IIc and -2IId. The average chain-length of the frequently methyl-esterified polygalacturonan portion was <8 in AR-2IIc (A), and >8 in AR-2IId (B): ——, portion relatively free from methyl-esters; ■, portion frequently methyl-esterified.

activity *via* both the classical and alternative pathways, and the other polysaccharides mainly *via* the classical pathway. However, the "hairy" regions isolated from these polysaccharides had similar activities *via* both the classical and alternative pathways. These facts suggested that polygalacturonan regions of AR-2IIa–IIc, but not of AR-2IId, suppressed the expression of the anti-complementary activity of their "hairy" regions *via* the alternative pathway and that the different anti-complementary activities reflect different distributions of methyl ester groups.

Pectic polysaccharides[26] can form three-dimensional networks by interactions of the "hairy" regions and/or by chelation between carboxyl groups of GalA and

Ca^{2+} ion ("egg-box" structure). Methyl-esterification of GalA inhibits the formation of the "egg-box" structure. The different distributions of methyl ester groups in AR-2IIa–IId could result in the formation of different three-dimensional networks and thereby modulate the anti-complementary activities.

The "hairy" regions in AR-2II–IId consist of a rhamnogalacturonan core with some arabinosyl and galactosyl side-chains with the proportion of the former increasing from AR-2IIa to -2IId. It has been suggested that, for some pectic polysaccharides[2,4,27-30] from medicinal plants, the neutral side-chains attached to the rhamnogalacturonan core might be important for the expression of anti-complementary activity. However, the stucture and the role of the neutral side-chains in the "hairy" regions of the present polysaccharides in anti-complementary activity must await further study.

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REFERENCES

1 H. KIYOHARA, H. YAMADA, AND Y. OTSUKA, *Carbohydr. Res.*, 167 (1987) 221–237.
2 H. YAMADA, H. KIYOHARA, J-C. CYONG, AND Y. OTSUKA, *Mol. Immunol.*, 22 (1985) 295–304.
3 H. KIYOHARA, H. YAMADA, J-C. CYONG, AND Y. OTSUKA, *J. Pharmacobio-Dyn.*, 9 (1986) 339–346.
4 H. YAMADA, H. KIYOHARA, J-C. CYONG, AND Y. OTSUKA, *Carbohydr. Res.*, 159 (1987) 275–291.
5 H. YAMADA, H. KIYOHARA, J-C. CYONG, Y. KOJIMA, Y. KUMAZAWA, AND Y. OTSUKA, *Planta Med.*, (1984) 163–167.
6 M. TOMODA, M. ICHIKAWA, AND N. SHIMIZU, *Chem. Pharm. Bull.*, 34 (1986) 4992–4996.
7 M. TOMODA AND K. KATOH, *Shoyakugaku Zasshi*, 36 (1982) 319–324.
8 J. F. THIBAULT AND C. MERCIER, *J. Solid-Phase Biochem.*, 2 (1977) 295–304.
9 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
10 N. BLUMENKRANTZ AND G. ASBOE-HANSEN, *Anal. Biochem.*, 54 (1973) 484–489.
11 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.
12 P. J. WOOD AND I. R. SIDDIQUI, *Anal. Biochem.*, 39 (1971) 418–428.
13 E. A. MCCOMB AND R. M. MCCREADY, *Anal. Chem.*, 29 (1957) 819–821.
14 W. E. TREVELYAN, D. P. PROCTER, AND J. S. HARRISON, *Nature (London)*, 166 (1950) 444–445.
15 L. HOUGH, J. K. N. JONES, AND W. H. WADMAN, *J. Chem. Soc.*, (1950) 1702–1706.
16 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
17 M. MCNEIL, A. G. DARVILL, AND P. ALBERSHEIM, *Plant Physiol.*, 66 (1980) 1128–1134.
18 T. J. WAEGHE, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 123 (1983) 281–304.
19 R. E. TAYLOR AND H. E. CONRAD, *Biochemistry*, 11 (1972) 1383–1388.
20 W. S. YORK, A. G. DARVILL, M. MCNEIL, T. T. STEVENSON, AND P. ALBERSHEIM, *Methods Enzymol.*, 118 (1986) 3–41.
21 M. POLLY AND H. MÜLLER-EBERHARD, *J. Exp. Med.*, 126 (1967) 1013–1025.

22 M. WILSON AND D. C. MORRISON, *Eur. J. Biochem.*, 128 (1982) 137–141.
23 J. A. DE VRIES, F. M. ROMBOUTS, A. G. J. VORAGEN, AND W. PILNIK, *Carbohydr. Polym.*, 2 (1982) 25–33.
24 J. F. THIBAULT AND C. MERCIER, *J. Food Biochem.*, 2 (1978) 379–393.
25 A. DARVILL, M. MCNEIL, P. ALBERSHEIM, AND D. P. DELMER, IN N. E. TOLBERT (Eds.), *The Biochemistry of Plants*, Academic Press, New York, 1980, pp. 91–162.
26 P. M. DEY AND K. BRINSON, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 265–382.
27 H. YAMADA, K. OHTANI, H. KIYOHARA, J-C. CYONG, Y. OTSUKA, Y. UENO, AND S. ŌMURA, *Planta Med.* (1985) 121–125.
28 H. YAMADA, H. KIYOHARA, AND Y. OTSUKA, *Carbohydr. Res.*, 170 (1987) 181–191.
29 N. SHIMIZU AND M. TOMODA, *Chem. Pharm. Bull.*, 31 (1983) 499–506.
30 H. YAMADA, T. NAGAI, J-C. CYONG, Y. OTSUKA, M. TOMODA, N. SHIMIZU, AND R. GONDA, *Carbohydr. Res.*, 156 (1986) 137–145.