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Heterogeneity and characterisation of mitogenic and anti-complementary pectic polysaccharides from the roots of *Glycyrrhiza uralensis* Fisch *et* D.C.

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ABSTRACT

Two anti-complementary polysaccharide fractions (GR-2IIa and GR-2IIb), isolated from the roots of *Glycyrrhiza uralensis* Fisch *et* D.C., each gave five anti-complementary polysaccharides (GR-2IIa-1–5 and GR-2IIb-1–5) on h.p.l.c.; likewise, GR-2IIc gave two anti-complementary and mitogenic polysaccharides (GR-2IIc-1-2A and -2IIc-2) by gel filtration and h.p.l.c. GR-2IIc-1-2A showed the most potent anti-complementary activity. GR-2IIa-1–5 and GR-2IIb-1–5 contained 40–85% and 50–90% of GalA, respectively, in addition to Rha, Ara, and Gal. GR-2IIc-1-2A and -2IIc-2 mainly comprised Glc, Gal, GalA, and GlcA in addition to Rha, Fuc, Xyl, Ara, and Man. Methylation analysis and digestion with endo-α-(1→4)-polygalacturonase indicated that all of the polysaccharides contained polygalacturonan regions which were frequently methyl-esterified. GR-2II-a, -2IIb, and -2IIc gave enzyme-resistant fractions of large and intermediate sizes, in addition to oligogalacturonides. Each large fraction from GR-2IIa and -2IIb consisted mainly of Ara, Gal, and GalA, whereas the intermediate fractions were composed of small proportions of 2-Me-Fuc, 2-Me-Xyl, and apiose (Api), in addition to Rha, Ara, Gal, and GalA. The large fraction from GR-2IIc mainly contained Rha, Man, Gal, and GalA in addition to Fuc, Ara, Xyl, and Glc, whereas the intermediate fraction consisted of 2-Me-Fuc, 2-Me-Xyl, and Api, in addition to Rha, Ara, GalA, Fuc, Xyl, Man, Gal, and Glc. Base-catalysed β-elimination followed by ethylation indicated that all the polysaccharides except GR-2IIc-2 contained a 4-linked uronic acid attached to position 2 of 2,4-linked Rha. Single radial gel diffusion, using the β-D-glucosyl-Yariv antigen, indicated that GR-2IIa-1 and GR-2IIc-2 contained relatively large proportions of (1→3,6)-β-D-galactan moieties. The anti-complementary activities of GR-2IIa-3, GR-2IIa-4, and GR-2IIb-4 decreased after de-esterification followed by digestion with endo-α-(1→4)-polygalacturonase. The large fractions from GR-2IIa–2IIc showed more potent anti-complementary activities than the original polysaccharide fractions, whereas the intermediate fractions and oligogalacturonides were inactive. The large fraction from GR-2IIc had more potent mitogenic acitivity than GR-2IIc, whereas the intermediate fraction and oligogalacturonides from GR-2IIc were inactive.

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INTRODUCTION

The roots of *Glycyrrhiza uralensis* Fisch. *et* D.C. (Japanese name Kanzo) have been a component herb in many kinds of Japanese herbal (kampo) medicines clinically used for the treatment of inflammation, allergy, gastric ulcer, *etc.* The polysaccharide fraction from the roots of *G. uralensis* plays[1] an important role in the expression of immunomodulating activity of one kampo medicine, Juzen-Taiho-To[2-5]. Three immunomodulating polysaccharides (GR-2IIa, -2IIb, and -2IIc) have been isolated[6] from the hot-water extract of *G. uralensis*. GR-2IIa and GR-2IIb had moderate anti-complementary activities, whereas GR-2IIc had extremely potent mitogenic and anti-complementary activities[6]. These activities decreased[6] on digestion with endo-α-(1→4)-polygalacturonase, which suggests that pectic polysaccharide moieties were involved. These polysaccharides were highly heterogeneous and have yielded 12 kinds of active polysaccharides which are now reported.

EXPERIMENTAL

Materials. — The roots of *G. uralensis* were obtained from Tsumura & Co. Endo-α-(1→4)-polygalacturonase [poly(1,4-α-D-galacturonide) glycanohydrolase; EC 3.2.1.15] from *Aspergillus niger* was purified as described[7]. The α- and β-D-glucosyl Yariv antigens were gifts from Dr. A. E. Clarke (Plant Cell Biology Research Center, School of Botany, University of Melbourne).

General. — Total carbohydrate, uronic acid, and protein contents were determined by the phenol–sulfuric acid[8], *m*-hydroxybiphenyl[9], and Lowry methods[10], respectively, using Gal, GalA, and bovine serum albumin as the respective standards. Methyl ester groups were assayed by the method of Wood and Siddiqui[11], using methanol as the standard. Polysaccharides were hydrolysed with 2M trifluoroacetic acid for 1.5 h at 121°, and the hydrolysates were analysed by t.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[12] and uronic acids with *p*-anisidine hydrochloride[13]. The sugars in hydrolysates were converted[14] into the corresponding alditol acetates, and analysed by g.l.c. G.l.c. was carried out at 120→195° (3°/min) using a Shimadzu GC-6A gas chromatograph equipped with an SPB-5 capillary column (0.25-μm film, 30 m × 0.25 mm i.d., Supelco). The molar ratios of neutral sugars were calculated from the peak areas and molecular factors on f.i.d., estimated from molecular weights of the corresponding alditol acetates. The molar ratios of uronic acids and neutral sugars were calculated from the contents of uronic acids. 2-Me-Fuc, 2-Me-Xyl, and apiose (Api) in hydrolysates were detected as their alditol acetates by g.l.c. and g.l.c.–m.s. on an SP-2380 capillary column (0.2-μm film, 30 m × 0.25 mm i.d., Supelco). G.l.c. was carried out using a Hewlett–Packard model 5890A gas chromatograph and temperature program: 60° (1 min), 60°→210° (18°/min), 210° (9.67 min), 210°→250° (8°/min), 250° (12 min). E.i.-m.s. was performed with a Hewlett–Packard 5970B mass spectrometer, and c.i. (isobutane)-m.s. with a JEOL DX-300 mass spectrometer. Electrophoresis on

glass-fiber paper (Toyo-Roshi GA-100) was performed in 26mM borate buffer (pH 9.6) at 5 mA/cm for 30 min with detection[15] using 1-naphthol–sulfuric acid or on a cellulose acetate membrane in 0.08M pyridine–0.04M acetate buffer (pH 5.4) at 200 mA/cm for 30 min with detection[16] using Toluidine Blue. H.p.l.c. was performed on a Waters Model ALC/GPC 244 equipped with columns (0.76 × 50 cm) of Asahi-pak GS-510 + GS-320 (Asahi Chemical Industry Co. Ltd.) and developed with 0.2M sodium chloride. Molecular weights of polysaccharides were estimated from the calibration curve of the elution volume of standard pullulans (P-400, 200, 100, 50, 20, and 5; Showa Denko Co. Ltd.) from Asahi-pak GS-510 + GS-320 in 0.2M sodium chloride.

Preparation of immunomodulating polysaccharide fractions[6]. — The crude fraction GR-1, obtained[1] by hot-water extraction followed by methanol extraction and ethanol precipitation from the roots of *G. uralensis*, was fractionated further[6] into the subfractions GR-2–4, GR-5a, and GR-5b by the precipitation method using cetyltrimethylammonium bromide (Cetavlon) and boric acid. The most acidic fraction (GR-2), which had anti-complementary and mitogenic activities, was applied to a column of DEAE-Sepharose CL-6B (Cl^- form), and anti-complementary fractions (GR-2IIa and GR-2IIb) and an anti-complementary and mitogenic fraction (GR-2IIc) were obtained[6] as absorbed fractions.

Purification of the anti-complementary polysaccharides from GR-2IIa and GR-2IIb. — GR-2IIa and GR-2IIb (100 mg) were each fractionated on a column (2.6 × 95 cm) of Sepharose CL-6B equilibrated with 0.2M sodium chloride. Each fraction was analysed by h.p.l.c. on Asahi-pak GS-510 + GS-320 in 0.2M sodium chloride. Fractions which showed a single peak and the same retention time were combined, and dialysed to obtain the purified polysaccharides GR-2IIa-1–5 from GR-2IIa and GR-2IIb-1–5 from GR-2IIb.

Purification of the anti-complementary and mitogenic polysaccharides from GR-2IIc. — (*a*) *H.p.l.c.* GR-2IIc (55 mg) was fractionated by h.p.l.c. on Asahi-pak GS-510 + GS-320 in 0.2M sodium chloride. A fraction (GR-2IIc-1, 10 mg) eluted in the void volume and a lower-molecular-weight fraction (GR-2IIc-2, 13.5 mg) were obtained.

(*b*) *Gel filtration on Sepharose CL-2B.* GR-2IIc-1 from (*a*) was fractionated on a column (2.6 × 90 cm) of Sepharose CL-2B in 0.2M sodium chloride. A fraction eluted in the void volume (GR-2IIc-1-1, trace) and a relatively lower-molecular-weight fraction (GR-2IIc-1-2, 8.3 mg) were obtained.

(*c*) *Fractionation on a Sep-pak C_{18} cartridge.* A solution of GR-2IIc-1-2 (8.3 mg) from (*b*) in water was applied to a Sep-pak C_{18} cartridge (Waters Assoc.) and washed with water to give an unabsorbed fraction (GR-2IIc-1-2A, 8 mg) and with ethanol to give an absorbed fraction (GR-2IIc-1-2B, 0.2 mg).

Methylation analysis. — Each polysaccharide was de-esterified with 0.2M sodium hydroxide for 2 h at room temperature, then methylated (Hakomori[17]) once in order to prevent[18] β-elimination, but methylsulphinylmethanide was added two or three times until the conversion of the polysaccharides into polyalkoxides was complete, as checked[19] by using triphenylmethane. Each methylated polysaccharide was recovered[20]

using a Sep-pak C_{18} cartridge and eluted with ethanol, and the uronic acids were reduced[20] with sodium borodeuteride in tetrahydrofuran–ethanol (7:3). Methylated polysaccharides were hydrolysed with 2M trifluoroacetic acid for 1.5 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. G.l.c. was performed with a Hewlett–Packard model 5840A gas chromatograph equipped with an SP-2380 capillary column (30 m × 0.25 mm i.d., 0.2-μm film, Supelco) with splitless injection. G.l.c.–m.s. was performed on a Hewlett–Packard 5890A gas chromatograph and 5970B mass spectrometer equipped with an SP-2380 capillary column. The carrier gas was He (0.9 mL/min in g.l.c., 0.5 mL/min in g.l.c.–m.s.) and the temperature programs were as follows: 60° for 1 min, 60→180° at 30°/min, 180→250° at 1.5°/min, and 250° for 5 min (in g.l.c.); 60° for 1 min, 60→150° at 30°/min, and 150→250° at 1.5°/min (in g.l.c.–m.s.). Methylated alditol acetates were identified by their fragment ions in m.s. and relative retention times in g.l.c., and their molar ratios were estimated from the peak areas and response factors[21].

β-Elimination[18] of methylated polysaccharides. — To a solution of each dry methylated polysaccharide (0.5 mg) in methyl sulfoxide (500 μL) was added 2M methylsulfinylmethanide, and the mixture was stirred for 24 h at room temperature. Excess of ethyl iodide was added, the mixture was kept overnight at room temperature, and the products were recovered using Sep-pak C_{18} cartridges as described above, and fractionated[22] on a column (1 × 30 cm) of Sephadex LH-20 (chloroform–methanol, 1:1) to give a Molisch reagent[15]-positive material in the void volume. The material was hydrolysed and the products were analysed by g.l.c. and g.l.c.–m.s. on an SP-2380 capillary column as alditol acetates.

Single radial gel-diffusion[23] of polysaccharides using the Yariv antigen. — β-D-Glucosyl-Yariv antigen [1,3,5-tri-(4-β-D-glucopyranosyloxyphenylazo)-2,4,6-trihydroxybenzene] was used as a positive reagent and the α-D-glucosyl Yariv antigen was a negative reagent. Each sample (10 μg) was applied to an agarose plate containing the Yariv antigen (10 μg/mL), and incubated overnight at room temperature.

Digestion of polysaccharides with endo-α-(1→4)-polygalacturonase. — GR-2IIa-2, GR-2IIa-4, and GR-2IIb-4 (1–3 mg each) were each digested with endo-α-(1→4)-polygalacturonase (0.1 U) in 50mM acetate buffer (pH 4.2, 1–3 mL) for 4 days at 37°. After neutralisation, the solution was lyophilised, then fractionated on Sephadex G-50 in water.

GR-2IIa (100 mg), GR-2IIb (100 mg), and GR-2IIc (12 mg) were each de-esterified in 0.5M sodium hydroxide (20, 20, and 5 mL, respectively) for 2 h at room temperature, and neutralised with acetic acid. The products were digested with endo-α-(1→4)-polygalacturonase by the above procedure, and fractionated on Sephadex G-50.

Immunomodulating activities. — (*a*) *Anti-complementary activity.* This activity was measured as described[24].

(*b*) *Mitogenic activity.* The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used as described[1].

RESULTS

Purification and properties of the anti-complementary polysaccharides isolated from GR-2IIa and GR-2IIb. — GR-2IIa and GR-2IIb each gave a single spot on electrophoresis on glass-fiber paper and cellulose acetate membrane (data not shown). They were each eluted as a single peak from Sepharose CL-6B and the uronic acid was also co-eluted with the hexose peak (Fig. 1A and B). H.p.l.c. of each eluate on Asahi-pak GS-510 + GS-320 in 0.2M sodium chloride revealed five polysaccharides (GR-2IIa-1–5 and GR-2IIb-1–5) with different molecular weights (Fig. 1C and D). The order of the anti-complementary activities was GR-2IIa-1 and GR-2IIb-1 > GR-2IIa-2 and GR-2IIb-2 > GR-2IIa-3 and GR-2IIb-3 > GR-2IIa-4 and GR-2IIb-4 > GR-2IIa-5 and GR-2IIb-5 (Table I). The molecular weights of GR-2IIa-1–5 and GR-2IIb-1–5 were 1.9 $\times$ 10^5–8.0 $\times$ 10^3 and 1.9 $\times$ 10^5–2.7 $\times$ 10^4, respectively (Tables II and III). The potent anti-complementary polysaccharides GR-2IIa-1, 2IIa-2, 2IIb-1, and 2IIb-2 contained 42–67% of GalA and 25–34% of neutral sugars, whereas the less active polysaccharides GR-2IIa-3–5 and GR-2IIb-3–5 comprised 76–90% of GalA and 18–13% of neutral sugars. Much (43–97%) of the GalA in these polysaccharides was methyl-esterified. The main neutral sugars were Ara and Gal in GR-2IIa-1, Rha, Ara, Man, and Gal in GR-2IIa-2–4; Rha, Ara, Man, Glc, and Gal in GR-2IIa-5; Rha, Ara, Man, and Gal in GR-2IIb-1–3; and Rha, Ara, and Gal in GR-2IIb-4 and -5.

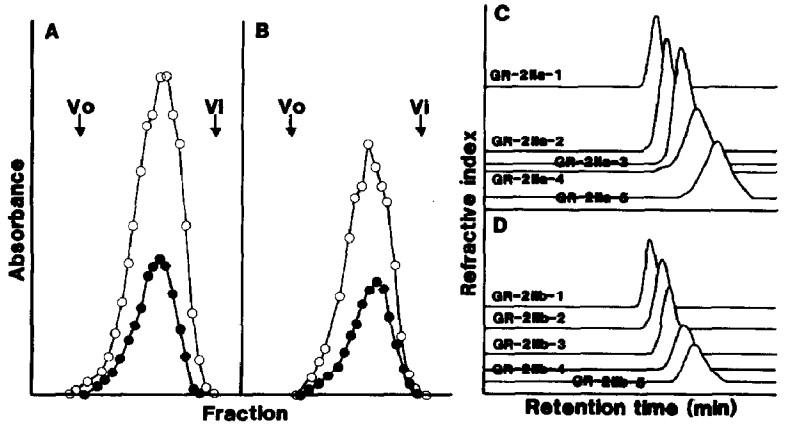

Fig. 1. Elution (0.2M sodium chloride) patterns on Sepharose CL-6B of GR-2IIa (*A*) and GR-2IIb (*B*), and on h.p.l.c. on Asahi-pak GS-510 + GS-320 of GR-2IIa (*C*) and GR-2IIb (*D*): carbohydrate (490 nm), ●; uronic acid (520 nm), ○; Vo, void volume; Vi, inner volume.

TABLE I

Anti-complementary activity (%) of polysaccharides from GR-2IIa and GR-2IIb

Polysaccharides	*Concentration (μg/mL)*		
	333	*167*	*33*
GR-2IIa	93.0	91.2	55.4
GR-2IIa-1	100.0	97.0	78.5
GR-2IIa-2	98.3	91.0	71.5
GR-2IIa-3	79.6	77.5	48.0
GR-2IIa-4	51.5	34.5	2.0
GR-2IIa-5	30.0	22.5	n.d.[a]
GR-2IIb	94.0	91.5	56.0
GR-2IIb-1	100.0	99.0	68.5
GR-2IIb-2	96.0	88.5	59.5
GR-2IIb-3	78.0	76.0	42.8
GR-2IIb-4	54.5	37.4	3.5
GR-2IIb-5	36.5	24.0	n.d.

[a] Not determined.

TABLE II

Physicochemical properties of polysaccharides from GR-2IIa

	GR-2IIa-1	*GR-2IIa-2*	*GR-2IIa-3*	*GR-2IIa-4*	*GR-2IIa-5*
Yield (%)[a]	0.3	1.5	1.5	1.4	0.06
Molecular weight	1.9×10^5	9.5×10^4	4.4×10^4	2.0×10^4	8.0×10^4
Neutral sugar (%)	34.2	33.1	3.4	9.8	n.d.[b]
GalA (%)	41.8	50.7	62.2	85.2	n.d.
Protein (%)	2.5	0.05	0.02	0.05	n.d.
Methyl ester (%)	7.7	9.4	9.6	8.6	n.d.
Reactivity with β-D-glucosyl-Yariv antigen	++	+	±	±	±
Neutral sugar composition					
Rha	7.2	12.4	28.7	34.9	39.2
Fuc	tr[c]	tr	tr	tr	tr
Xyl	tr	tr	tr	tr	tr
Ara	47.0	46.7	36.4	26.0	27.5
Man	8.9	10.7	15.4	18.2	9.8
Glc	6.6	3.5	tr	2.6	11.0
Gal	30.2	26.7	19.6	18.2	12.5

[a] Calculated from GR-1 (crude polysaccharide fraction). [b] Not determined. [c] Trace.

TABLE III

Physicochemical properties of polysaccharides from GR-2IIb

	GR-2IIb-1	*GR-2IIb-2*	*GR-2IIb-3*	*GR-2IIb-4*	*GR-2IIb-5*
Yield (%)[a]	0.2	0.3	1.8	1.8	0.4
Molecular weight	1.9×10^5	1.6×10^5	1.1×10^5	4.2×10^4	2.7×10^4
Neutral sugar (%)	32.0	24.6	17.7	14.5	2.9
GalA (%)	54.5	66.6	76.2	79.5	90.4
Protein (%)	4.3	1.3	0.3	0.1	0.8
Methyl ester (%)	9.2	7.5	5.8	5.9	5.9
Reactivity with β-D-glucosyl-Yariv antigen	+	+	+	±	±
Neutral sugar composition					
Rha	15.5	17.7	39.7	43.4	49.1
Fuc	tr[b]	tr	tr	tr	tr
Xyl	tr	tr	tr	tr	tr
Ara	49.6	47.2	29.8	35.5	34.9
Man	10.3	9.4	8.8	4.1	tr
Glc	4.5	1.3	tr	4.5	tr
Gal	20.0	24.4	21.7	12.4	16.0

[a] Calculated from GR-1 (crude polysaccharide fraction). [b] Trace.

Purification and properties of the anti-complementary and mitogenic polysaccharides isolated from GR-2IIc. — GR-2IIc, which had the highest anti-complementary and mitogenic activities, gave a long spot in electrophoresis on a cellulose acetate membrane. H.p.l.c. on Asahi-pak GS-510 + GS-320 in 0.2M sodium chloride gave two fractions, GR-2IIc-1 and -2 (Fig. 2A), which showed anti-complementary and mitogenic activities (GR-2IIc-1 > GR-2IIc-2) (Tables IV and V). GR-2IIc-1 gave a trace of a fraction (GR-2IIc-1-1) that was eluted in the void volume, and a large proportion of a lower-molecular-weight fraction (GR-2IIc-1-2), by gel filtration on Sepharose CL-2B (Fig. 2B), which had similar mitogenic activities (data not shown). GR-2IIc-1-2 had a pale-brown colour, and, when passed through a Sep-pak C_{18} cartridge, gave unbound (GR-2IIc-1-2A) and bound (GR-2IIc-1-2B) fractions in the ratio 40:1 by elution with water then ethanol. GR-2IIc-1-2A and -2B had similar mitogenic activities and were more potent than GR-2IIc-1 (Table V). GR-2IIc-1-2A also showed extremely potent anti-complementary activity (Table IV), which was higher than those of GR-2IIa-1–5 and GR-2IIb-1–5 (data not shown). GR-2IIc-1-2A and 2IIc-2 each gave a single spot in electrophoresis, was eluted as a single peak on gel filtration, and had a large molecular weight ($\geqslant 3.38 \times 10^5$ and 1.6×10^5, respectively) (Table VI). They each contained ~10% of GlcA and GalA, and larger proportions of neutral sugars than GR-2IIa-1–5 and GR-2IIb-1–5, which were composed mainly of Glc and Gal in addition to Rha, Fuc, Ara, and Man. However, GR-2IIc-1-2B could not be analysed further because of the small amount available.

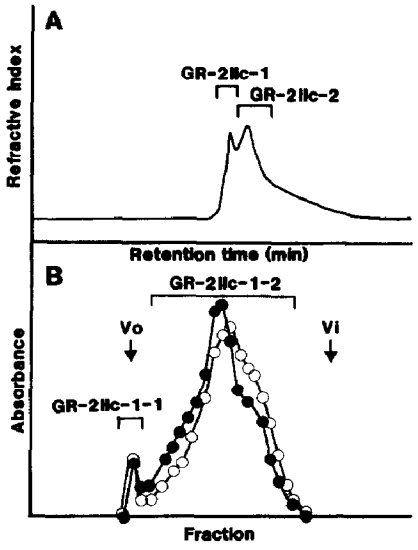

Fig. 2. Elution (0.2M sodium chloride) patterns *A*, on h.p.l.c. on Asahi-pak GS-510 + GS-320 of GR-2IIc; *B*, on Sepharose CL-2B of GR-2IIc-1 from *A*: carbohydrate (490 nm), ●; uronic acid (520 nm), ○; Vo, void volume; Vi, inner volume.

TABLE IV

Anti-complementary activity (%) of polysaccharides from GR-2IIc

Polysaccharides	*Concentration (μg/mL)*		
	33	*17*	*3.3*
Experiment 1			
GR-2IIc	97.0	96.0	57.6
GR-2IIc-1	100.0	99.1	88.3
GR-2IIc-2	97.9	97.1	81.3
	Concentration (μg/mL)		
	3.3	*1.7*	*0.3*
Experiment 2			
GR-2IIc-1	87.0	42.1	20.5
GR-2IIc-1-2A	90.4	49.0	26.6
GR-2IIc-1-2B	n.d.[a]	n.d.	n.d.

[a] Not determined because of the small amount of sample available.

TABLE V

Mitogenic activity of polysaccharides from GR-2IIc

Polysaccharide	*Dose (μg/mL)*	*Proliferation*[a]
Control		0.189 ±0.006
GR-2IIc	30	0.836 ±0.029
	100	1.073 ±0.036
GR-2IIc-1	30	1.011 ±0.010
	100	1.318 ±0.011
GR-2IIc-2	30	0.467 ±0.000
	100	0.580 ±0.006
GR-2IIc-1-2A	30	1.316 ±0.006
	100	1.619 ±0.008
GR-2IIc-1-2B	30	1.312 ±0.002
	100	1.588 ±0.024

[a] Proliferation of spleen cells were measured by the MTT assay.

TABLE VI

Physicochemical properties of polysaccharides from GR-2IIc

	GR-2IIc-1-2A	*GR-2IIc-2*
Yield (%)[a]	0.08	0.13
Molecular weight	$\geqslant 3.38 \times 10^5$	1.6×10^5
Neutral sugar (%)	70.4	68.0
GalA (%)	9.1	11.0
GlcA (%)	13.5	11.5
Protein (%)	4.2	4.6
Methyl ester (%)	2.8	n.d.[b]
Reactivity with β-D-glucosyl-Yariv antigen	+	+++
Neutral sugar composition		
Rha	7.8	11.6
Fuc	7.6	6.8
Xyl	tr[c]	tr
Ara	9.5	9.0
Man	9.8	9.6
Glc	21.1	14.1
Gal	44.2	48.8

[a] Calculated from GR-1 (crude polysaccharide fraction). [b] Not determined. [c] Trace.

Glycosyl linkages of the immunomodulating polysaccharides. — GR-2IIa-1–5 and GR-2IIb-1–5 were subjected to methylation analysis, and the results are summarised in Tables VII and VIII. Each polysaccharide consisted mainly of 4- and 2,4-linked GalA. GR-2IIa-1 and -2 each contained relatively large proportions of terminal Ara*f* and Ara*p* and 4- or 5-linked Ara, and, in addition, GR-2IIa-2 contained terminal Rha. GR-2IIa-3 and -4 each contained mainly terminal Ara*p*, 4- or 5-linked Ara, and terminal Rha, and GR-2IIa-5 contained terminal Ara*f*, 4- or 5-linked Ara, and terminal Rha. GR-2IIb-1–5 contained relatively large proportions of terminal Ara*f* and 4- or 5-linked Ara. In addition, GR-2IIb-2 contained 2-linked Rha, and GR-2IIb-3 contained terminal Ara*p*, whereas GR-2IIb-4 and -5 each contained terminal and 2-linked Rha.

GR-2IIc-2 contained 4- and 2,4-linked GalA and 4-linked GlcA, whereas GR-2IIc-1-2A contained terminal, 3-, 4-, and 2,4-linked GlcA, and 4-linked GalA (Table IX). GR-2IIc-1-2A also contained $\geqslant 7\%$ of 3- and 3,4-linked Gal, and 3- and 4-linked Glc, whereas GR-2IIc-2 contained 3,6-linked Gal, and 3- and 4-linked Glc (Table IX).

The methylated polysaccharides were also subjected to base-catalysed β-elimination using methanesulfinylmethanide. 2-*O*-Ethyl-3-*O*-methylrhamnitol triacetate was produced from every polysaccharide except GR-2IIc-2 after base-catalysed β-elimination followed by ethylation (data not shown), which suggested that they each contained the sequence →4)-HexA-(1→2)-[→4)-Rha-(1→]. Base-catalysed β-elimination also decreased by $\geqslant 80\%$ the proportions of the following constituents (Tables VII–IX): 2-linked Rha (in GR-2IIa-1, 2IIa-2, 2IIb-1, and 2IIc-2), 3-linked Rha (in GR-2IIa-1–5), 2,3-linked Rha (in GR-2IIc-1-2A), 2,4-linked Rha (in GR-2IIa-1–5 and GR-2IIb-5), 2-linked Gal (in GR-2IIb-4), terminal Glc (in GR-2IIb-2), 3-linked Glc (in GR-2IIb-1), 4-linked Glc (in GR-2IIa-4 and -5), 4-linked Man (in GR-2IIa-5), 3,4-linked Xyl (in GR-2IIa-2), and 4,6-linked Gal (in GR-2IIa-1). These results suggested that the above glycosyl residues might be attached to position 4 of uronic acid.

Reactivity of the immunomodulating polysaccharides with the β-D-glucosyl-Yariv antigen. — Since the β-D-glucosyl-Yariv antigen reacts[25,26] with (1→3,6)-β-D-galactan to form a red dye, the reactivities of the immunomodulating polysaccharides were tested by single radial gel diffusion[23]. GR-2IIa-1 and GR-2IIc-2 reacted relatively strongly with the antigen; GR-2IIa-2, GR-2IIb-1–3, and GR-2IIc-1-2A reacted slightly; whereas GR-2IIa-3–5 and GR-2IIb-4 and -5 reacted weakly or negligibly (Tables II, III, and VI). These polysaccharides did not react with the α-D-glucosyl-Yariv antigen (negative control, data not shown).

Digestion of the immunomodulating polysaccharides with endo-α-(1→4)-polygalacturonase. — After digestion of GR-2IIa-2–4 and GR-2IIb-4 (the relatively major polysaccharides) with endo-α-(1→4)-polygalacturonase, as shown in Fig. 3A–D, large proportions of GalA were still present in the fractions of the void volume obtained on gel filtration on Sephadex G-50. However, when the polysaccharides were digested with the enzyme after de-esterification with 0.2M sodium hydroxide, only small proportions of fractions (PG-1) eluted in the void volume and intermediate fractions (PG-2) were obtained, in addition to large proportions of low-molecular-weight fractions (PG-3)

(Fig. 3E–H). PG-1 from GR-2IIa-2–4 and GR-2IIb-4 mainly contained Rha, Ara, and Gal, in addition to small and intermediate proportions of GalA, respectively, whereas PG-3 mainly contained GalA. These results suggested that GR-2IIa-2–4 and GR-2IIb-4 mainly contained polygalacturonan; the other polysaccharides could not be analysed because of the small amounts available. Since these results suggested that GR-2IIa-1–5 and GR-2IIb-1–5 contained polygalacturonan moieties, GR-2IIa and GR-2IIb were each de-esterified and then digested with endo-α-(1→4)-polygalacturonase. Elution of the products from Sephadex G-50 gave small proportions of fractions (PG-1a and -1b) eluted in the void volume and intermediate fractions (PG-2a and -2b), and large proportions of lower-molecular-weight fractions (PG-3a and -3b) (Fig. 4A and B). PG-2a and -2b were each de-esterified, then treated with the enzyme again, and the products were fractionated on Bio-Gel P-10 to give PG-2a-1 and PG-2b-1 (eluted in the void volume) and PG-2a-2 and PG-2b-2 (lower-molecular-weight fraction) without production of oligogalacturonides (Fig. 4D and E). PG-1a and -1b were each composed mainly of Ara, Gal, and GalA, in addition to small proportions of Rha, Fuc, Xyl, Man, and Glc (Table X), whereas PG-3a and -3b each contained mainly GalA. PG-2a-1 and -2 each contained mainly Rha, Ara, and Gal, and PG-2b-1 mainly contained Rha, whereas PG-2b-2 mainly contained Ara (Table X). PG-2a-2 and PG-2b-2 gave three unusual

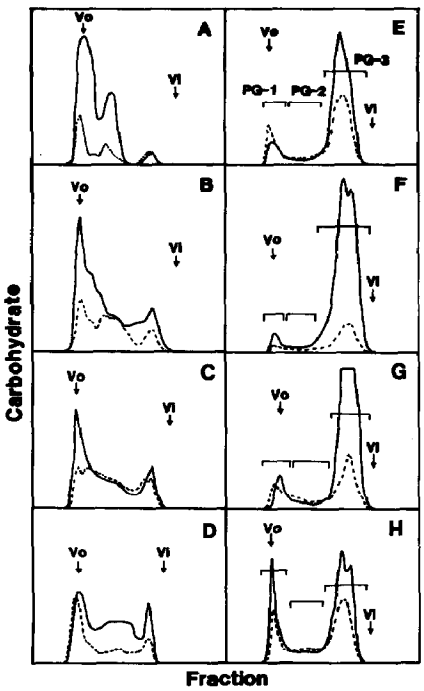

Fig. 3. Elution patterns on Sephadex G-50 of the products obtained from polysaccharides by digestion with endo-α-(1→4)-polygalacturonase before (*A–D*) and after de-esterification (*E–H*); *A* and *E*, GR-2IIa-2; *B* and *F*, GR-2IIa-3; *C* and *G*, GR-2IIa-4; *D* and *H*, GR-2IIb-4: carbohydrate (490 nm), ----; uronic acid (520 nm), —; Vo, void volume; Vi, inner volume.

TABLE VII

Methylation analysis (mol. %) before and after base-catalysed β-elimination of polysaccharides from GR-2IIa

Glycosyl residue[a]	*Linkage*	*Before elimination*					*After elimination*				
		GR-2IIa-1	*GR-2IIa-2*	*GR-2IIa-3*	*GR-2IIa-4*	*GR-2IIa-5*	*GR-2IIa-1*	*GR-2IIa-2*	*GR-2IIa-3*	*GR-2IIa-4*	*GR-2IIa-5*
Ara	Terminal (furanosyl)	13.5	15.1	8.2	5.8	14.5	11.0	23.3	5.1	30.8	7.0
	Terminal (pyranosyl)	18.0	13.3	18.3	17.6	8.6	18.0	11.7	7.1	5.4	9.1
	4 or 5	33.5	22.4	23.6	27.1	12.8	28.0	21.0	41.1	14.4	18.7
Rha	Terminal	2.8	10.5	15.5	11.8	12.8	2.5	9.2	9.5	14.5	25.5
	2	1.5	3.3	4.5	5.0	7.2	tr[b]	tr	4.1	2.7	6.3
	3	0.5	1.6	1.4	2.6	3.6	tr	tr	tr	tr	tr
	2,4	1.9	2.7	4.5	5.8	9.3	tr	tr	tr	tr	tr
Gal	Terminal	3.8	6.5	8.2	7.7	7.6	9.6	5.4	4.2	5.4	6.0
	3	4.0	4.5				3.3	7.5			
	2	4.2	3.9				6.6	4.3			
	4,6	1.7	3.3			2.1	tr	2.1	4.1		4.8
	3,6	4.2	5.5	2.6	3.8	0.7	3.5	4.6	6.3	5.9	1.5
Glc	Terminal	1.7				1.1	3.3				2.9
	4				0.9	5.9					tr
	6	3.4	1.4	0.9			5.8	1.8	0.8	1.6	
Man	4	1.1	1.5	2.9	5.0	1.2	1.2	2.1	3.1	5.8	tr
	2,4	2.2	3.3	4.5	3.8	4.1	1.8	3.3	1.6	3.1	3.7

Xyl	4					6.2					4.8
	3,4	1.3	1.6	1.8		1.1	2.6	tr	3.0		1.5
Fuc	3,4	1.2		3.6	3.1	1.3	2.7		5.2	4.7	1.5
		Molar ratio									
GalA	4	4.1	4.8	6.1	6.9	6.1					
	2,4	1.2	0.7	2.5	1.2	1.2					

[a] Alditol acetates derived from neutral sugars and GalA were separately analysed because many alditol acetates derived from neutral sugars could not be detected in analysis of alditol acetates from GalA. [b] Trace.

TABLE VIII

Methylation analysis (mol. %) before and after base-catalysed β-elimination of polysaccharides from GR-2IIb

Glycosyl residue[a]	*Linkage*	*Before elimination*					*After elimination*				
		GR-2IIb-1	*GR-2IIb-2*	*GR-2IIb-3*	*GR-2IIb-4*	*GR-2IIb-5*	*GR-2IIb-1*	*GR-2IIb-2*	*GR-2IIb-3*	*GR-2IIb-4*	*GR-2IIb-5*
Ara	Terminal (furanosyl)	12.4	12.7	19.0	23.1	15.0	16.4	9.5	17.7	6.5	15.2
	Terminal (pyranosyl)	2.4	7.1	17.2	5.3	4.1	0.9	3.2	4.3	8.9	10.4
	4 or 5	31.7	13.0	19.0	13.6	11.7	30.1	25.8	26.4	22.0	9.0
Rha	Terminal	1.2	8.7	6.1	18.3	12.3	2.0	5.4	3.4	7.9	18.9
	2	7.5	10.2	4.8	10.5	11.6	tr[b]	2.6	2.9	6.1	8.6
	3	0.8	2.7	1.3	2.6	5.2	1.0	0.6	1.4	1.5	2.8
	2,4	4.9	2.7	4.8	4.2	2.1	1.7	1.7	1.4	1.9	tr
Gal	Terminal	3.8	6.7	3.0	5.6	6.9	3.5	7.0	2.9	5.2	5.4
	3	2.4	7.5	1.3	0.9		2.4	2.7	2.0	1.5	
	6	2.2	1.3				2.0	3.1		6.1	
	2	2.4	2.5	1.3	2.0		2.2	2.5	3.8		
	4,6	0.8	2.6	1.8	2.8	2.1	0.4	1.0	0.9	1.8	2.5
	3,4	2.4	1.3	3.9	3.5		3.5	3.1	7.7	1.8	
	3,6	3.5	4.5	0.5	1.4	1.1	3.5	2.7	0.5	0.9	1.9
	2,4	0.8	4.5	3.9	5.4	6.4	1.2	5.9	2.9	7.1	5.3
	2,6	1.1	2.5	0.8	3.1		0.6	0.7	2.4	1.8	
Glc	Terminal	0.8					2.6				
	4	3.1	2.7	3.1	2.0		3.7	0.6	3.4	2.8	
	6	2.8	2.9		0.9	2.1	4.7	5.3		2.6	4.4
	3	3.8	2.1	1.8	2.0		0.6	0.7	2.4	1.8	

Man	4	4.7	2.9	1.8	0.9	4.1	7.5	3.0	3.8	1.8	1.9
	3	1.4	0.8	0.8		2.7	1.8	0.6	0.8		1.9
Xyl	4	2.0	2.1	1.8		5.1	3.1	6.7	2.8		4.4
Fuc	3,4	0.8	1.9	1.8	3.1	6.4	1.4	2.5	3.0	6.5	4.2
		Molar ratio									
GalA	4	4.2	4.4	5.5	5.7	7.1					
	2,4	1.0	1.8	1.8	2.0	0.6					

[a] Alditol acetates derived from neutral sugars and GalA were separately analysed because many alditol acetates derived from neutral sugars could not be detected in analysis of alditol acetates from GalA. [b] Trace.

TABLE IX

Methylation analysis (mol. %) before and after base-catalysed β-elimination of polysaccharides from GR-2IIc

Glycosyl residue	*Linkage*	*Carboxyl-reduced*		*Before elimination*		*After elimination*	
		GR-2IIc-1-2A	*GR-2IIc-2*	*GR-2IIc-1-2A*	*GR-2IIc-2*	*GR-2IIc-1-2A*	*GR-2IIc-2*
Ara	Terminal (furanosyl)	3.2	2.2	2.3	3.0	2.5	2.2
	Terminal (pyranosyl)	1.0		tr[a]		tr	
	4 or 5	1.2	4.2	1.5	5.6	2.0	7.7
	3,4 or 3,5		tr		0.3		0.5
Rha	Terminal	1.1		2.6		1.2	
	2	2.4	3.7	2.6	4.4	1.4	0.8
	3	0.7		1.3		2.4	
	4	3.2		2.0		1.4	
	2,3	0.9		0.5		tr	
	2,4	tr	2.3	tr	3.8	tr	2.4
Gal	Terminal	2.4	0.6	3.4	1.9	6.1	1.6
	2		7.5		4.7		3.8
	3	7.0	3.3	9.3	4.9	5.6	3.0
	4	2.8	4.1	5.8	5.5	3.5	4.9
	3,4	4.0		7.1		5.1	
	4,6	8.0	2.4	5.3	5.2	4.8	2.0
	3,6	4.6	7.5	2.4	13.3	7.5	5.5
	2,4,6		1.0		2.0		0.6
	3,4,6	2.6	0.9	1.2	1.9	5.6	0.4
Glc	Terminal	3.2		3.7		1.3	
	3	4.7	8.1	7.2	10.8	4.8	12.0
	2	1.9		2.2		1.6	
	6	5.8		5.8		6.2	
	4	9.7	13.6	7.0	20.3	8.3	28.6
	3,6	0.8		1.8		2.2	

	4,6	1.7	1.7	2.2	2.5	5.1	3.1
	3,4,6	1.9	2.7	1.5	1.9	2.2	0.4
	2,4,6	0.9		1.9		1.7	
Man	Terminal		2.9		2.7		5.8
	4	2.8		3.1		4.0	
	3,6	3.0		5.8		4.5	
	4,6	2.1	6.1	3.0	1.5	3.5	1.6
	3,4,6	1.8	0.7	2.4	0.8	4.0	0.2
Xyl	3		1.9		1.6		2.6
Fuc	4	3.2		3.2		4.1	
GalA	4	1.6	15.9				
	2,4		1.3				
GlcA	Terminal	2.5					
	3	0.8					
	4	5.3	4.4				
	2,4	0.9					

[a] Trace.

TABLE X

Component sugars (mol. %) of products from polysaccharide fractions (GR-2IIa, GR-2IIb, and GR-2IIc) by digestion with endo-α-(1→4)-polygalacturonase

Products	*Rha*	*Fuc*	*Ara*	*Xyl*	*Man*	*Gal*	*Glc*	*2-Me-Fuc*	*2-Me-Xyl*	*Api*	*GalA*	*GlcA*
GR-2IIa												
PG-1a	4.5	tr[b]	18.3	tr	6.2	28.1	5.1				37.9	
PG-2a-1	19.5	1.2	15.1	tr	2.9	16.3	2.3				39.0	
PG-2a-2	16.1	2.9	19.3	tr	1.8	10.3	4.8	3.1	3.3	3.5	55.0	
PG-3a	3.0		3.7		tr	7.4	3.0				82.9	
GR-2IIb												
PG-1b	6.8	tr	15.3		1.2	31.3	7.6				37.8	
PG-2b-1	33.5	2.1	8.6	tr	1.9	9.7	1.2	n.d.[a]	n.d.	n.d.	38.7	
PG-2b-2	9.9	6.2	13.3	tr	1.9	7.1	1.1	7.4	2.8	5.1	44.3	
PG-3b	3.8		5.1		2.2	3.2	1.7				83.4	
GR-2IIc												
PG-1c	16.3	1.4	5.9	4.3	10.2	27.0	9.7				17.9	7.3
PG-2c	17.4	3.2	11.6	tr	2.7	9.6	4.1	2.7	1.2	3.9	42.7	
PG-3c	0.6	tr	2.3		3.1	5.2	4.2				84.6	

[a] Not determined. [b] Trace.

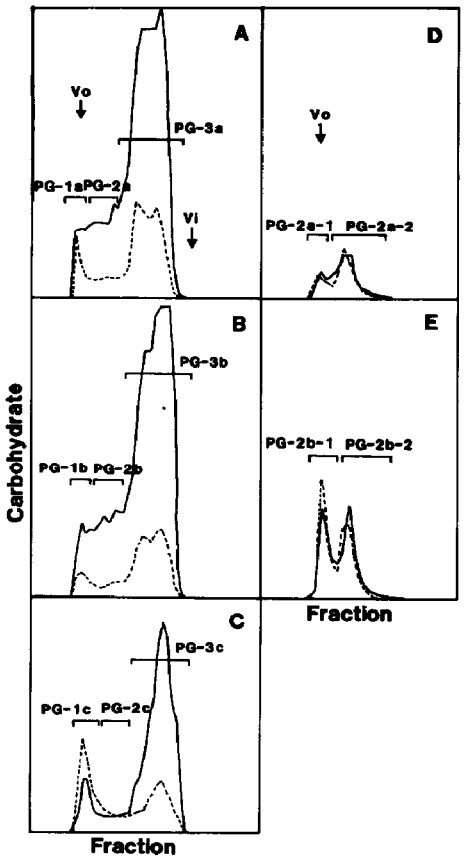

Fig. 4. Elution patterns on Sephadex G-50 of the products obtained from GR-2IIa (*A*), GR-2IIb (*B*), and GR-2IIc (*C*) by digestion with endo-α-(1→4)-polygalacturonase after de-esterification, and on Bio-gel P-10 of PG-2a (*D*) and PG-2b (*E*) from *A* and *B*, respectively: carbohydrate (490 nm), ----; uronic acid (520 nm), —; Vo, void volume; Vi, inner volume.

alditol acetates that were identified by g.l.c.–c.i.- and e.i.-m.s. as derivatives of 2-Me-Fuc, 2-Me-Xyl, and apiose (Api) (data not shown). PG-2a-2 and PG-2b-2 contained small proportions of 2-Me-Fuc, 2-Me-Xyl, and Api, whereas PG-2a-1 did not contain these residues (Table X).

Methylation analysis suggested that GR-2IIc-1-2A and 2IIc-2 also contained galacturonan groups, but they could not be analysed further because of the small amounts available. Therefore, GR-2IIc, which mainly contained GR-2IIc-1-2A and IIc-2, was de-esterified and then digested with endo-α-(1→4)-polygalacturonase. Gel filtration on Sephadex G-50 of the products gave a fraction (PG-1c) eluted in the void volume, an intermediate fraction (PG-2c), and a low-molecular-weight fraction (PG-3c) (Fig. 4C). PG-1c mainly contained Rha, Man, Gal, and GalA, in addition to Glc and GlcA, whereas PG-3c mainly contained GalA (Table X). PG-2c mainly contained Rha, Ara, and GalA, together with small proportions of 2-Me-Fuc, 2-Me-Xyl, and Api.

TABLE XI

Effect of digestion with endo-α-(1→4)-polygalacturonase on anti-complementary activity (%) of polysaccharides

Polysaccharides	*Treatment*	*Concentration (μg/mL)*		
		333	*167*	*33*
GR-2IIa-2	No treatment	88.0	65.0	24.0
	Digested[a]	100.0	75.0	34.0
GR-2IIa-3	No treatment	75.0	40.0	12.0
	Digested	33.0	25.0	18.0
GR-2IIa-4	No treatment	40.0	12.0	n.d.[b]
	Digested	25.0	18.0	n.d.
GR-2IIb-4	No treatment	42.0	16.0	n.d.
	Digested	27.0	5.0	n.d.

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

Effect of enzymic treatment on the anti-complementary and mitogenic activities. — The anti-complementary activity was increased slightly after digestion of GR-2IIa-2 with endo-α-(1→4)-polygalacturonase, whereas ~50% of the activities of GR-2IIa-3, 2IIa-4, and 2IIb-4 was lost by the digestion (Table XI). Because the amounts of the products (PG-1–3) of enzymic digestion of these purified polysaccharides were too small for measurement of anti-complementary activity, the corresponding fragments from GR-2IIa–IIc were assayed. PG-1a and -1b each showed remarkably potent anti-complementary activity, which was higher than those of GR-2IIa and GR-2IIb, whereas PG-3a and -3b were inactive (Table XII). Although PG-2a and -2b each had moderate anti-complementary activity, only the subfractions, PG-2a-1 and PG-2b-1, were active; PG-2a-2 and PG-2b-2 were inactive (Table XII). PG-1c showed more potent anti-complementary activity than GR-2IIc, PG-1a and -1b showed weak activity, and PG-2c and -3c were inactive (Table XII).

Of the polysaccharide fractions from *G. uralensis*, only GR-2IIc showed mitogenic activity. Of the fragments (PG-1c–3c) from GR-2IIc, PG-1c showed more potent mitogenic activity than GR-2IIc, whereas PG-2c and PG-3c were inactive (Table XIII).

DISCUSSION

Three immunomodulating polysaccharide fractions, GR-2IIa–IIc, have been obtained[6] from the roots of *G. uralensis* Fisch *et* D.C., of which GR-2IIa and 2IIb each had moderate anti-complementary activity, whereas GR-2IIc had potent mitogenic and anti-complementary activities. Although GR-2IIa and 2IIb each appear homogeneous on gel filtration and electrophoresis, five polysaccharides (GR-2IIa-1–5 and GR-2IIb-1–5) were obtained by h.p.l.c.

TABLE XII

Anti-complementary activity (%) of products from GR-2IIa, GR-2IIb, and GR-2IIc by digestion with endo-α-(1→4)-polygalacturonase

Polysaccharide	*Product*	*Concentration (μg/mL)*		
		333	*167*	*33*
Experiment 1				
GR-2IIa	Original	100.0	93.0	52.0
	PG-1a	100.0	100.0	99.8
	PG-2a	74.0	32.0	4.6
	PG-3a	1.0	0.5	0.0
GR-2IIb	Original	100.0	93.0	54.0
	PG-1b	100.0	100.0	99.5
	PG-2b	80.5	43.0	5.0
	PG-3b	4.0	1.0	0.0
Experiment 2				
GR-2IIa	PG-2a	100.0	56.0	32.0
	PG-2a-1	100.0	73.5	44.0
	PG-2a-2	1.0	1.0	0.0
GR-2IIb	PG-2b	100.0	59.0	38.5
	PG-2b-1	100.0	80.0	59.5
	PG-2b-2	3.0	2.0	0.0
		Concentration (μg/mL)		
		3.3	*1.67*	*0.33*
Experiment 3				
GR-2IIc	Original	32.5	21.5	9.4
	PG-1c	80.3	64.5	33.4
	PG-2c	0.0	0.0	0.0
	PG-3c	0.0	0.0	0.0
GR-2IIa	PG-1a	11.5	0.0	0.0
GR-2IIb	PG-1b	9.8	0.0	0.0

The chemical properties suggest that the anti-complementary polysaccharides are pectic polysaccharides. Pectic polysaccharides consist[27,28] of a "ramified" moiety (rhamnogalacturonan possessing neutral side chains) and a (1→4)-α-galacturonan. The present study strongly suggests that GR-2IIa-1–5 and GR-2IIb-1–5 each consisted mainly of (1→4)-α-galacturonan with many methyl-esterified carboxyl groups, and with two types of enzyme-resistant region. Base-catalysed β-elimination indicated that some of the enzyme-resistant regions contained sequences such as →4)-GalA-(1→2)-[→4)-Rha-(1→], present in the rhamnogalacturonan moieties. The enzyme-resistant regions also contained chains of neutral carbohydrates consisting mainly of Rha, Ara, and Gal, in addition to various proportions of (1→3,6)-β-D-galactan moieties, and

TABLE XIII

Mitogenic activity of products from GR-2IIc by digestion with endo-α-(1→4)-polygalacturonase

Product		*Concentration (μg/mL)*	*Mitogenic activity*[a]
Control			0.106 ±0.005
GR-2IIc		10	0.179 ±0.009
		30	0.217 ±0.010
		100	0.288 ±0.002
GR-2IIc	PG-1c	10	0.224 ±0.007
		30	0.294 ±0.006
		100	0.408 ±0.002
	PG-2c	10	0.127 ±0.004
		30	0.140 ±0.004
		100	0.157 ±0.004
	PG-3c	10	0.133 ±0.002
		30	0.151 ±0.003
		100	0.170 ±0.002

[a] Mitogenic activity was assayed by the MTT method.

which might be the "ramified" regions. However, one kind of resistant region (obtained as relatively lower-molecular-weight fractions by gel filtration on Bio-gel P-10) (Fig. 4D and E) also contained 2-Me-Fuc, 2-Me-Xyl, and apiose (Api). These unusual sugars have been detected[29] in rhamnogalacturonan II (RGII) of plant cell-wall polysaccharides together with unusual acidic sugars such as aceric acid (AceA), 3-deoxy-D-*lyxo*-2-heptulosaric acid (Dha), and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo). Although AceA, Dha, and Kdo were not analysed in the present study, the resistant regions of lower molecular weight appeared to contain RGII-like moieties. Yamada *et al.*[30] have reported the presence of an RGII-like region in an anti-ulcer pectic polysaccharide isolated from the roots of *Bupleurum falcatum*, and suggested that it is combined with a "ramified" region through a (1→4)-α-galacturonan. The present results also indicate that GR-2IIa-1–5 and GR-2IIb-1–5 consisted of (1→4)-α-galacturonans, "ramified" regions, and RGII-like regions.

Purification of GR-2IIc by gel filtration and h.p.l.c. gave two active polysaccharides (GR-2IIc-1-2A and 2IIc-2). The present results suggest that each was grouped into pectic polysaccharides containing RGII-like regions. Base-catalysed β-elimination did not confirm the presence of the rhamnogalacturonan moieties, but GR-2IIc-1-2A and 2IIc-2 were concluded to contain sequences such as →4)-HexA-(1→2)-[→4)-Rha-(1→] and →2)-Rha-(1→4)-HexA-(1→, respectively, and it was inferred that the polysaccharides might contain rhamnogalacturonan moieties. Single radial gel diffusion indicated that GR-2IIc-2 contained a larger proportion of (1→3,6)-β-D-galactan regions than GR-2IIc-1-2A.

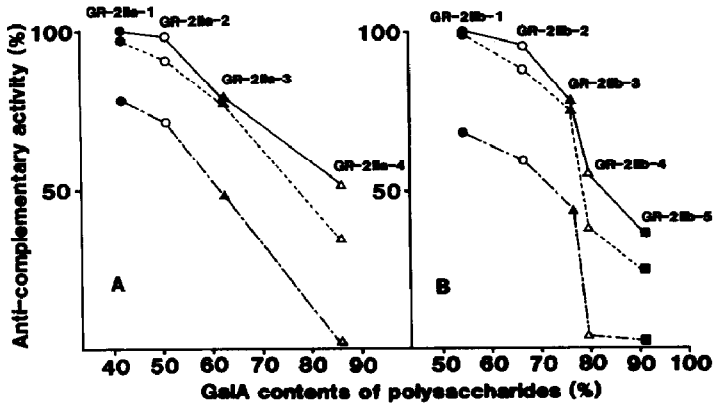

Fig. 5. Correlation between contents of polygalacturonans and anti-complementary activity of polysaccharides from GR-2IIa (*A*) and GR-2IIb (*B*); GR-2IIa-1 and GR-2IIb-1, ●; GR-2IIa-2 and GR-2IIb-2, ○; GR-2IIa-3 and GR-2IIb-3, ▲; GR-2IIa-4 and GR-2IIb-4, △; GR-2IIb-5, ■; at 333 (—), 167 (----), and 33 μg/mL (—·—).

The present study strongly suggests that the enzyme-resistant regions ("ramified" regions) of the polysaccharides from GR-2IIa and 2IIb were each involved in the expression of anti-complementary activity, but that the RGII-like moiety was not involved. Figure 5 shows that the anti-complementary activities decreased as the contents of polygalacturonans increased. The enzyme-resistant regions ("ramified" regions) of GR-2IIc-1-2A and IIc-2 may also be involved in the expression of mitogenic activity.

Kiyohara *et al.*[31] reported that the fractions of neutral carbohydrate side chains, derived from a "ramified" region of anti-complementary pectic polysaccharide of *A. acutiloba*, and which contained (1→3,6)-β-D-galactan or some (1→6)-linked galactooligosaccharides, have anti-complementary activities. It was proposed that the combination of these galactosyl chains and the rhamnogalacturonan core was essential for the expression of the potent anti-complementary activity. Because the present results indicated that "ramified" regions of GR-2IIa-1–5 and GR-2IIb-1–5 were composed of some galactosyl chains together with large proportions of arabinosyl chains, it is assumed that some galactosyl chains might be involved in the expression of the anti-complementary activity. Although the "ramified" region of GR-2IIc-1-2A contains a proportion of (1→3,6)-β-D-galactan units lower than that of GR-2IIc-2, it showed more potent anti-complementary and mitogenic activities than GR-2IIc-2. Since GR-2IIa-1–5 and GR-2IIb-1–5 had no mitogenic activity, it is assumed that neutral chains other than (1→3,6)-β-D-galactans in GR-2IIc-1-2A might be involved in the expression not only of mitogenic activity but also of anti-complementary activity.

Further structural analysis of "ramified" regions in these immunomodulating pectic polysaccharides is in progress.

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REFERENCES

1 H. Yamada, H. Kiyohara, N. Takemoto, J.-F. Zhao, H. Kawamura, Y. Komatsu, J.-C. Cyong, M. Aburada, and E. Hosoya, *Planta Med.*, in press.
2 Y. Komatsu, N. Takemoto, H. Maruyama, H. Tsuchiya, M. Aburada, E. Hosoya, S. Shinohara, and H. Hamada, *Jpn. J. Inflammation*, 6 (1986) 405–408.
3 H. Maruyama, H. Kawamura, N. Takemoto, Y. Komatsu, M. Aburada, and E. Hosoya, *Jpn. J. Inflammation*, 8 (1988) 65–66.
4 N. Takemoto, H. Maruyama, H. Kawamura, Y. Komatsu, M. Aburada, and E. Hosoya, *Jpn. J. Inflammation*, 9 (1989) 137–140.
5 N. Takemoto, H. Kawamura, H. Maruyama, Y. Komatsu, M. Aburada, and E. Hosoya, *Jpn. J. Inflammation*, 9 (1989) 49–52.
6 J.-F. Zhao, H. Kiyohara, X.-B. Sun, T. Matsumoto, J.-C. Cyong, H. Yamada, N. Takemoto, and H. Kawamura, *Phytotherapy Res.*, in press.
7 H. Kiyohara, J.-C. Cyong, and H. Yamada, *Carbohydr. Res.*, 182 (1988) 259–275.
8 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
9 N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.*, 54 (1973) 484–489.
10 O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265–275.
11 P. J. Wood and I. R. Siddiqui, *Anal. Biochem.*, 39 (1971) 418–428.
12 W. E. Trevelyan, D. P. Procter, and J. S. Harrison, *Nature (London)*, 166 (1950) 444–445.
13 L. Hough, J. K. N. Jones, and W. H. Wadman, *J. Chem. Soc.*, (1950) 1702–1706.
14 T. M. Jones and P. Albersheim, *Plant Physiol.*, 49 (1972) 926–936.
15 Z. Dische, *Methods Carbohydr. Chem.*, 1 (1962) 478–481.
16 M. Tomoda and K. Katoh, *Shoyakugaku Zasshi*, 36 (1982) 319–324.
17 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
18 M. McNeil, A. G. Darvill, and P. Albersheim, *Plant Physiol.*, 66 (1980) 1128–1134.
19 H. Rauvala, *Carbohydr. Res.*, 72 (1979) 257–260.
20 T. J. Waeghe, A. G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.
21 D. P. Sweet, R. H. Shapiro, and P. Albersheim, *Carbohydr. Res.*, 40 (1975) 217–225.
22 H. Kiyohara, H. Yamada, and Y. Otsuka, *Carbohydr. Res.*, 167 (1987) 221–237.
23 G.-J. Holst and A. E. Clarke, *Anal. Biochem.*, 148 (1985) 446–450.
24 H. Yamada, H. Kiyohara, J.-C. Cyong, and Y. Otsuka, *Carbohydr. Res.*, 159 (1987) 275–291.
25 M. A. Jermyn and Y. M. Yeow, *Aust. J. Plant Physiol.*, 2 (1975) 501–531.
26 H. Kiyohara and H. Yamada, *Carbohydr. Res.*, 187 (1989) 255–265.
27 A. Darvill, M. McNeil, P. Albersheim, and D. P. Delmer, in N. E. Tolbert (Ed.), *The Biochemistry of Plants*, Academic Press, New York, 1980, pp. 91–162.
28 P. M. Dey and K. Brinson, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 265–382.
29 J. R. Thomas, A. G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 185 (1989) 261–277.
30 H. Yamada, M. Hirano, and H. Kiyohara, *Carbohydr. Res.*, 219 (1991) 173–192.
31 H. Kiyohara, J.-C. Cyong, and H. Yamada, *Carbohydr. Res.*, 193 (1989) 201–214.