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Rhamnogalacturonan II from the leaves of *Panax ginseng* C.A. Meyer as a macrophage Fc receptor expression-enhancing polysaccharide

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Abstract

A complex pectic polysaccharide (GL-4IIb2) has been isolated from the leaves of *Panax ginseng* C.A. Meyer, and shown to be a macrophage Fc receptor expression-enhancing polysaccharide. The primary structure of GL-4IIb2 was elucidated by composition, ^{1}H NMR, methylation, and oligosaccharide analyses. GL-4IIb2 consisted of 15 different monosaccharides which included rarely observed sugars, such as 2-*O*-methylfucose, 2-*O*-methylxylose, apiose, 3-*C*-carboxy-5-deoxy-L-xylose (aceric acid, AceA), 3-deoxy-D-*manno*-2-octulosonic acid (Kdo), and 3-deoxy-D-*lyxo*-2-heptulosaric acid (Dha). Methylation analysis indicated that GL-4IIb2 comprised 34 different glycosyl linkages, such as 3,4-linked Fuc, 3- and 2,3,4-linked Rha, and 2-linked GlcA, which are characteristic of rhamnogalacturonan II (RG-II). Sequential degradation using partial acid hydrolysis indicated that GL-4IIb2 contained α-Rha*p*-(1 → 5)-Kdo and Ara*f*-(1 → 5)-Dha structural elements, an AceA-containing oligosaccharide, and uronic acid-rich oligosaccharide chains in addition to an α-(1 → 4)-galacturono-oligosaccharide chain. FABMS and methylation analyses suggested that the AceA-containing oligosaccharide was a nonasaccharide in which terminal Rha was additionally attached to position 3 of 2-linked Ara*p* of the octasaccharide chain observed in sycamore RG-II. Component sugar and methylation analyses assumed that the uronic acid-rich oligosaccharides possessed a similar structural feature as those in sycamore RG-II. GL-4IIb2 had a larger molecular mass (11,000) than sycamore RG-II (~ 5000). © 1997 Elsevier Science Ltd.

Keywords: *Panax ginseng*; Araliaceae; Rhamnogalacturonan II; Macrophage Fc receptor expression-enhancing activity

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1. Introduction

The roots of *Panax ginseng* C.A. Meyer are a well-known Chinese crude drug widely used clinically for the treatment of gastrointestinal disorders as well as an erythropoietic and a tonic. The roots of *P. ginseng* are valuable because it takes 4–6 years for its harvest, whereas the leaves of *P. ginseng* can be harvested every year. Therefore, if the leaves have a similar pharmacological activity as the roots, they will be available for clinical uses as well as the roots. Several pharmacologically active polysaccharides [1–3] have been reported in the roots of *P. ginseng*. In earlier studies we have described the isolation of polysaccharides from the leaves having potent anti-complementary [4] and anti-ulcer activity [5], and suggested the clinical value of the leaves. Recently, we have also purified another unique polysaccharide (GL-4IIb2) from the leaves, and observed a potent immune complex clearance-enhancing activity which is due to the increment of the de novo synthesis of a macrophage Fc receptor [6]. GL-4IIb2 contained several unusual monosaccharide units, such as 2-*O*-methylfucose (2-MeFuc), 2-*O*-methylxylose (2-MeXyl), apiose (Api), 3-*C*-carboxy-5-deoxy-L-xylose (aceric acid, AceA), 3-deoxy-D-*lyxo*-2-heptulosaric acid (Dha), and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo). Since these unusual sugars are characteristics of rhamnogalacturonan II (RG-II) of plant cell wall polysaccharides [7], GL-4IIb2 has been assumed to contain RG-II [6].

In the present paper, we describe the structural characterization of GL-4IIb2 with macrophage Fc receptor expression-enhancing activity from the leaves of *P. ginseng* C.A. Meyer.

2. Experimental

Materials.—The leaves of *P. ginseng* C.A. Meyer were cultured and collected (September, 1987) in Chan Bai Mountain, Jilin, China. Pectinase from *Aspergillus niger* was purchased from Sigma (St. Louis, USA) and endo-α-(1 → 4)-polygalacturonase [poly (1,4-α-D-galacturonide) glycohydrolase; EC 3.2.1.15] was purified from pectinase by the procedure of Thibault and Mercier [8]. The β-D-glucosyl-Yariv antigen was obtained from Biosupplies (Australia). DEAE-Sepharose CL-6B, Q-Sepharose, and QAE-Sephadex A-25 were purchased from Pharmacia (Sweden), AG-50WX8 and Bio-Gel P-30, P-10, and P-6 from Bio-Rad (USA), and Sep-pak C_{18} cartridges from Waters Associates (USA).

General.—Total carbohydrate, uronic acid, and protein contents were assayed by the phenol–H_2SO_4 [9], *m*-hydroxybiphenyl [10], and Lowry [11] methods, respectively, using Gal, GalA, and bovine serum albumin as the respective standards. The contents of Kdo and Dha were determined colourimetrically by a modified thiobarbituric acid (TBA) assay [12]. Poly- and oligo-saccharides released by partial acid hydrolysis of GL-4IIb2 were analyzed by GLC for their monosaccharide constituents, making use of alditol acetates or Me_3Si methyl ethers. Alditol acetates were prepared after hydrolysis with 2 M CF_3CO_2H for 1.5 h at 121 °C [13], and analyzed by GLC as described in ref. [14]; Me_3Si methyl ethers were prepared and analyzed by GLC according to the method of York et al. [15]. The contents of Kdo and Dha were determined by GLC by using alditol acetates according to modified methods of York et al. [12] and Stevenson et al. [16]. Briefly, for Kdo the sample was hydrolyzed partially under mild acid conditions (aq 1% HOAc, 100 °C, 2 h), and the resulting hydrolysates were reduced with $NaBD_4$. For Dha, the sample was hydrolyzed with 0.1 M CF_3CO_2H for 1 h at 100 °C, followed by reduction with $NaBD_4$. The samples were then treated with 2 M CF_3CO_2H for 1 h at 121 °C, and the resulting lactones were reduced with $NaBD_4$ under neutral conditions. The products were further treated with 2 M CF_3CO_2H and with $NaBD_4$ to reduce Dha completely. After acetylation, the resulting carboxyl-reduced alditol acetates were analyzed by GLC–MS using an SP-2380 capillary column. The molar ratios were calculated from the peak areas and response factors using a flame-ionization detector (FID). Polysaccharide material was analyzed by HPLC on a Shimadzu LC-6A instrument equipped with columns (0.76 × 50 cm each) of Asahi-pak GS-510 + GS-320 or GS-320 + GS-220 (Asahi Chemical Industry Co. Ltd., Japan) in 0.2 M NaCl, and a molecular mass calibration curve was made from the elution volumes of standard pullulans (P-400, 200, 100, 50, 20, and 5; Showa Denko Co. Ltd.). Single radial gel diffusion using the β-D-glucosyl-Yariv antigen was performed according to the method of Holst and Clarke [17].

Purification of GL-4IIb2.—A crude polysaccharide fraction (GL-2) was prepared from the leaves of *P. ginseng* by hot water extraction, EtOH precipitation, and dialysis [4]. GL-2 was fractionated by Cetavlon (cetyltrimethylammonium bromide) precipitation, and a weakly acidic polysaccharide fraction (GL-4) was obtained. The Fc receptor expression-enhancing polysaccharide (GL-4IIb2) was purified from

GL-4 by anion-exchange chromatography on DEAE-Sepharose CL-6B as described previously [6]. In order to remove the coloured materials in the polysaccharide, GL-4IIb2 was further purified on a Q-Sepharose column (Cl^- form). The column was washed with water and eluted sequentially with 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 M NaCl. The major fraction, which was eluted with 0.3 M NaCl, was further fractionated by gel filtration on a Bio-Gel P-30 column to obtain the purified GL-4IIb2 (yield: 5.8 mg/kg dry leaves).

Sequential chemical and enzymic degradation of GL-4IIb2.—(a) Procedure 1: GL-4IIb2 was hydrolyzed with 0.1 M CF_3CO_2H for 30 min at 60 °C. The hydrolysate was applied to a column (2.5 × 65 cm) of Bio-Gel P-10, and fractions PA-1 and PA-2 were obtained by eluting with 50 mM NaOAc buffer (pH 5.2). Fraction PA-2 was desalted with AG-50WX8 (H^+ form) resin and then loaded onto a column (10 mL) of QAE-Sephadex A-25 ($HCOO^-$ form). After the column had been washed with H_2O, bound materials were eluted with a linear concn gradient of 0.0 → 1.0 M $HCOONH_4$ (pH 7.0). The major carbohydrate fraction was desalted by passing through AG-50WX8 (H^+ form) resin, and the eluates were lyophilized to give PA-2′.

(b) Procedure 2: Fraction PA-1, which was obtained by Procedure 1, was treated with 0.1 M CF_3CO_2H for 24 h at 40 °C. Partially hydrolyzed PA-1 was fractionated on a column (1.7 × 92 cm) of Bio-Gel P-6 equilibrated with 50 mM NaOAc buffer (pH 5.2), and three major peaks, PA-1-I, PA-1-II, and PA-1-III, were obtained. Each fraction was desalted by using an electrophoretic dialyzer (Microacylizer, Asahi Chemical Industry Co. Ltd.) and lyophilized.

(c) Procedure 3: Fraction PA-1-I, obtained as described above, was further treated with 0.1 M CF_3CO_2H for 84 h at 40 °C. The hydrolysates were fractionated on a Bio-Gel P-6 column as described above, and four subfractions, PA-1-Ia, PA-1-Ib, PA-1-Ic,and PA-1-Id, were obtained.

(d) Procedure 4: Fraction PA-1-Ia was digested with endo-α-(1 → 4)-polygalacturonase (0.1 U) for 4 days at 37 °C in 50 mM NaOAc buffer (pH 4.2). After neutralization, the products were fractionated on a Bio-Gel P-6 column in 50 mM NaOAc buffer (pH 5.2) to give PG-1, PG-2, and PG-3.

(e) Procedure 5: Fraction PG-1 was rehydrolyzed with 0.1 M CF_3CO_2H for 48 h at 50 °C, and the hydrolysates were fractionated on Bio-Gel P-6 in 50 mM NaOAc buffer (pH 5.2) to give three fractions, PG-1A, PG-1B, and PG-1C.

Methylation analysis.—Samples (200–500 μg) were methylated according to Hakomori [18] only once in order to prevent β-elimination [19], but methylsulfinyl carbanion was added two or three times until conversion of the saccharides into alkoxides was complete, as checked by using triphenylmethane [20]. For the methylation of samples which contained 2-MeFuc and/or 2-MeXyl, CD_3I was used instead of CH_3I [21], and oligosaccharides were pre-reduced with $NaBD_4$ prior to methylation [12]. The methylated samples were recovered using a Sep-pak C_{18} cartridge by the procedure of Waeghe et al. [22], except that samples were eluted with EtOH. Carboxylmethyl groups in methylated products were reduced with lithium triethylborodeuteride in THF (Super-Deuteride, Aldrich) at room temperature for 1 h [15], and the reduced products were recovered by a Sep-pak C_{18} cartridge. The products were hydrolyzed with 1 M CF_3CO_2H for 2 h at 121 °C, and reduced with $NaBH_4$ or $NaBD_4$, followed by acetylation. The glycosyl linkage of AceA was determined by the modified procedure of Spellman et al. [23]. Briefly, per-*O*-methylated polysaccharide containing AceA was hydrolyzed and the products were reduced with $NaBD_4$. Then the reaction mixture was desalted by passing through a column (2 mL) of AG-50WX8 (H^+ form) resin after decomposition of the excess $NaBD_4$. This procedure yielded a mono-*O*-methyl lactone derivative of AceA. The lactone was carboxyl-reduced and per-*O*-acetylated. The glycosidic linkages of Kdo in oligosaccharides were determined by the method of York et al. [12]. After reduction of the Kdo residue in the oligosaccharide with $NaBD_4$ in 1 M NH_4OH (room temperature, 1 h), the resulting product was permethylated. The carboxylmethyl group was reduced with $NaBD_4$ in EtOH, and the product was hydrolyzed with 1 M CF_3CO_2H for 30 min at 100 °C, then converted into partially methylated alditol acetates, which were analyzed by GLC and GLC–EIMS. GLC and EIMS were performed on a Hewlett–Packard model 5890A gas chromatograph equipped with a 5970B mass selective detector. The carrier gas was He (0.9 mL/min in GLC and 0.5 mL/min in GLC–EIMS); the temperature program was: 60 °C for 1 min, 60 → 150 °C (30 °C/min), 150 → 250 °C (1.5 °C/min), and 250 °C for 5 min. Methylated alditol acetates were identified by their fragment ions and relative retention times on GLC, and their molar ratios were estimated from the peak areas and response factors in FID [24].

GLC–EIMS of methylated oligosaccharide-alditols.—Solutions of methylated oligosaccharide-alditols in

acetone were analyzed on an SP-2380 capillary column (0.2 μm film thickness, 0.25 mm i.d. × 15 m, Supelco) making use of an on-column injector and a temperature program as follows: 100 °C for 1 min, 100 → 150 °C (30 °C/min) and 150 → 270 °C (2 °C/min). EIMS was carried out on a Hewlett–Packard model 5970B mass selective detector. EIMS fragment ions [A, J, and alditol (ald)] [25] were used to determine the structures of the methylated oligosaccharide-alditols.

Fast - atom - bombardment mass spectrometry (FABMS).—A JEOL JMS-AX505 HA mass spectrometer interfaced with an OA-5000 computer was used. Xenon was used as the bombarding gas, and an atom gun was operated at 3 kV, 10 mA. The instrument was scanned at m/z 0–1500 with a scan rate of 20 s/decade. The accelerating voltage was 3 kV. A mixture of 1:1 glycerol–monothioglycerol was used as a matrix. One μL of a soln (1–20 mg/mL aq 5% HOAc) of oligosaccharide was placed on the target of the probe and was mixed with 1 μL matrix. B/E (daughter ions) linked scans were performed by using a linked scan unit at a scan rate of 120 s/decade using He as a collisional gas; the registration was carried out with an UV oscillograph.

NMR spectrometry.—The ^{1}H NMR (300 MHz) spectrum was recorded for a soln in D_2O with a Varian XL-300 spectrometer at 50 °C.

Assay procedure of macrophage Fc receptor expression-enhancing activity.—The determination of macrophage Fc receptor expression-enhancing activity was performed as described previously [6,26]. Briefly, macrophages were cultured in the absence or presence of samples for 15 h at 37 °C. Thereafter, the macrophage monolayer was washed with phosphate buffered-saline containing 1% BSA (PBS–1% BSA). Glucose oxidase–anti-glucose oxidase complexes (GAG, Lot No. 0012) were obtained from ICN ImmunoBiologicals (Costa Mesa, CA 92626, USA). In contrast to the earlier used GAG [6,26], which consisted of polyclonal antibodies of anti-glucose oxidase, the GAG used in the present study comprised a monoclonal antibody of the IgG2a subclass. One hundred μL of this GAG soln was added to each well and incubated for 240 min at 4 °C. The incubation was terminated by aspirating the GAG soln. After the macrophages were washed with PBS–1% BSA, they were solubilized by the addition of 50 μL 1% Nonidet P-40. The reacting fluid [1 U/mL of horseradish peroxidase, 50 mg/mL Glc, 1 mg/mL 2,2′-azinobis(3-ethylbenzthizoline sulfonic acid) (ABTS)] was added to each well (150 μL) and incubated at room temperature. After 30 min, the extinction due to glucose oxidase activity based on GAG bound to the cells was measured at 405 nm.

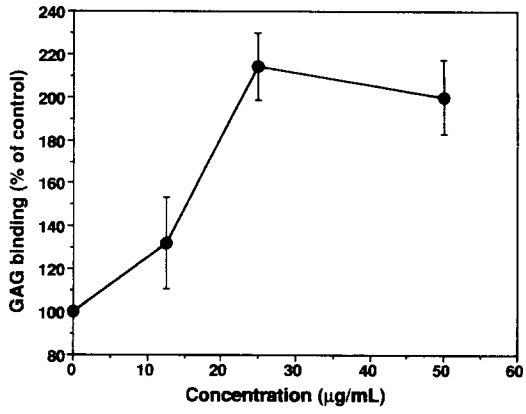


Fig. 1. Macrophage Fc receptor expression-enhancing activity of purified GL-4IIb2.

3. Results

Property of GL - 4IIb2.—GL-4IIb2 was purified from a hot water extract without any digestion with enzymes such as endo-polygalacturonase. A further purification was carried out by anion-exchange chromatography and gel filtration to remove coloured materials. The purified GL-4IIb2 was tested for macrophage Fc receptor expression-enhancing activity by using GAG, composed of monoclonal anti-glu-

Table 1
Monosaccharide constituents of GL-4IIb2 and derived fragments obtained by partial acid hydrolysis

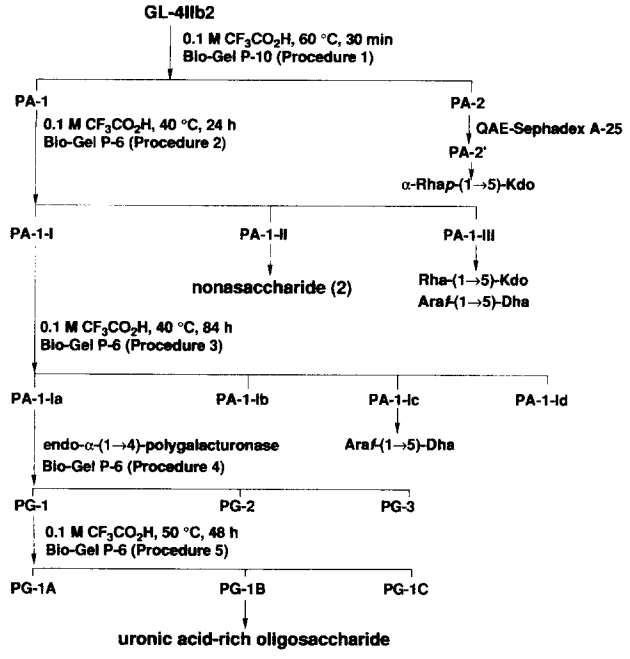
Monosaccharides	Mol % [a]				
	GL-4IIb2	PA-2′	PA-1-II	PA-1-Ia	PG-1B
2-MeFuc	5.2	–	19.7	–	–
Rha	16.5	46.0	35.1	12.1	14.4
Fuc	3.9	–	2.5	7.8	11.5
2-MeXyl	3.8	–	1.8	6.5	9.6
Ara	8.8	3.2	13.6	10.1	1.5
Xyl	0.5	–	–	–	–
Api	4.3	–	7.6	3.2	5.1
Man	1.4	–	0.3	0.8	0.4
AceA	1.6	–	4.1	–	–
Gal	10.2	–	14.7	6.7	8.9
Glc	1.6	–	0.7	2.6	7.5
Dha	4.1	–	–	1.2	–
Kdo	5.4	38.2	–	–	–
GalA	25.1	5.7	–	31.2	26.4
GlcA	7.8	7.0	–	17.7	14.6

[a] Monosaccharides were analyzed using alditol acetates. Mol % was calculated from the total carbohydrate detected.

cose oxidase. The purified GL-4IIb2 showed Fc receptor expression-enhancing activity at concentrations of 12.5–50 μg/mL (Fig. 1). GL-4IIb2 eluted as a single peak on Bio-Gel P-10. It also gave a single HPLC peak on Asahi-pak GS-510 + GS-320 and GS-320 + GS-220 columns, and its molecular mass was estimated to be 11,000 (HPLC). GL-4IIb2 contained ~65% total carbohydrate, 33.7% uronic

Table 2
Methylation analysis (mol %) of GL-4IIb2 and derived fragments obtained by partial acid hydrolysis

Glycosyl residue	Deduced linkage	Mol %				
		GL-4IIb2	PA-2′	PA-1-II	PA-1-Ia	PG-1B
Ara	Terminal (*f*)	9.1		2.5	4.5	
	2 (*p*)	1.4		7.6		
	2,3 (*p*)	1.7		5.8		
Xyl	Terminal	2.9				
	2	1.0			4.6	
2-MeXyl	Terminal	3.0	4.2	2.4	4.7	6.1
Fuc	3,4	4.3		2.9	7.6	13.4
2-MeFuc	Terminal	4.5		18.7		
Rha	Terminal	6.9	43.2	11.3		
	2	1.6	0.8	2.1	2.4	
	3	4.5	5.1	15.2		
	2,3	1.1		trace		
	2,4	0.5				
	2,3,4	5.2			12.7	16.5
Gal	Terminal	4.7	3.3	3.2	8.4	15.9
	2	1.3		1.0	0.4	
	3	0.5				
	6	1.4				
	2,4	3.5	2.6	19.4	0.4	
	2,6	0.5				
	4,6	0.6				
	2,4,6	1.3				
Glc	4	0.4			2.0	
	3,4,6	0.6				
Man	3	0.5				
	3,4	0.3				
Api	3′	4.2		1.0	8.3	13.5
	2,3,3′	3.4				
GlcA	2	4.5			7.3	10.7
GalA	Terminal	11.5			20.3	23.8
	2	1.1			10.0	
	4	2.8			0.4	
	2,4	4.8			1.3	
	3,4	4.6			4.8	
AceA	2			6.8		
Kdo	5		40.8			



Scheme 1. Procedure of sequential degradation of GL-4IIb2.

acid, whereas the acetyl-group content was 0.82%; in addition a small proportion of protein was present (2.2%). Monosaccharide analysis indicated that GL-4IIb2 consisted of 6 kinds of unusual sugars, namely, 2-MeFuc, 2-MeXyl, Api, AceA, Kdo, and Dha, which are characteristic monosaccharide constituents of RG-II [7] of plant cell-wall polysaccharides, in addition to Fuc, Ara, Xyl, Rha, Man, Gal, Glc, GlcA, and GalA (Table 1). GL-4IIb2 did not react with the β-D-glucosyl-Yariv antigen on single radial gel diffusion (data not shown). GL-4IIb2 did not release any oligogalacturonide by digestion with endo-α-(1 → 4)-polygalacturonase, and its molecular mass did not change after the digestion (data not shown). Methylation analysis indicated that GL-4IIb2 consisted of 32 kinds of glycosyl linkages such as 3,4-linked Fuc, 3- and 2,3,4-linked Rha, and 2-linked GlcA, which are characteristic of RG-II (Table 2). Very recently, Pellerin et al. [27] and O'Neil et al. [28] have found that RG-II from red wine, sycamore, and pea contained 2,3,3′-linked Api which contributed to the dimerization of RG-II. Methylation analysis also indicated that GL-4IIb2 contained 2,3,3′-linked Api (Table 2).

Sequential degradation of GL-4IIb2.—It has been reported that partial acid hydrolysis of RG-II liberates characteristic oligosaccharides [7], and therefore GL-4IIb2 was degraded sequentially using acid hydrolysis in order to elucidate whether GL-4IIb2 comprised the RG-II structure (Scheme 1). First, partial acid hydrolysis of GL-4IIb2 with 0.1 M trifluoroacetic acid (30 min, 60 °C) gave the products PA-1 and PA-2, and about 35% of the TBA-positive material in GL-4IIb2 was found back in PA-2 (Fig. 2A). Fraction PA-2 was further purified on QAE-Sephadex A-25, yielding a single peak PA-2′ containing TBA-positive material (Fig. 2B). Monosaccharide analysis indicated that PA-2′ consisted of almost equal amounts of Rha and Kdo in addition to small proportions of Ara, GalA, and GlcA (Table 1). When permethylated oligosaccharide-alditols derived from PA-2′ were analyzed by GLC–EIMS, a single derivative with a retention time of 25.02 min was detected (Fig. 3A). EIMS of the GLC peak indicated fragment ions at *m/z* 189 (bA_1, 6-deoxyhexose) and at *m/z* 308 (aJ_2) and 368 (aJ_1) (alditol from Kdo) (Fig. 3B). The GLC peak also gave the characteristic ion at *m/z* 162 due to the fragmentation of the alditol portion, but it did not give the fragment ion at *m/z* 177, suggesting that Kdo is substituted at C-5 but not at C-4. The mass spectrum was indistinguishable from that of α-L-Rhap-(1 → 5)-D-Kdo which has been reported by Schmidt and Jann [29]. Methylation analysis indicated that PA-2′ was mainly composed of equal amounts of terminal Rhap and 5-substituted Kdo (Table 2). ^1H NMR analysis of the reduced product from PA-2′ gave signals at 5.09 ppm (H-1 of α-Rha), 1.34 ppm (CH_3 of Rha), and 1.96 and 2.28 ppm

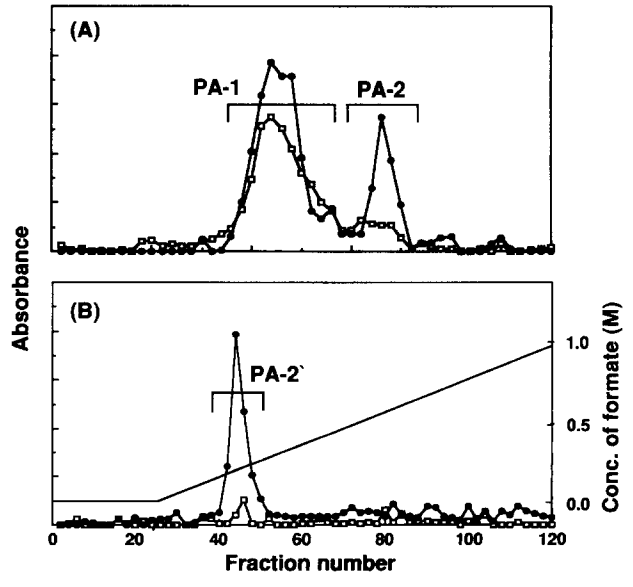


Fig. 2. (A) Elution pattern on Bio-Gel P-10 of products derived from GL-4IIb2 by partial acid hydrolysis (0.1 M CF_3CO_2H, 60 °C, 30 min). (B) Elution pattern on QAE-Sephadex of PA-2 from (A). □, Total carbohydrate (490 nm); ●, TBA-positive material (548 nm).

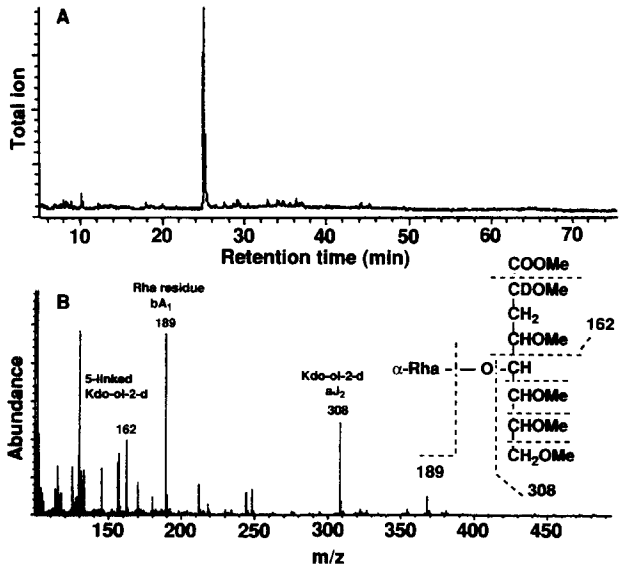


Fig. 3. (A) Total-ion chromatography on GLC–MS of permethylated oligosaccharide-alditols derived from PA-2′. (B) Fragment ions of a permethylated disaccharide-alditol derived from PA-2′ on EIMS.

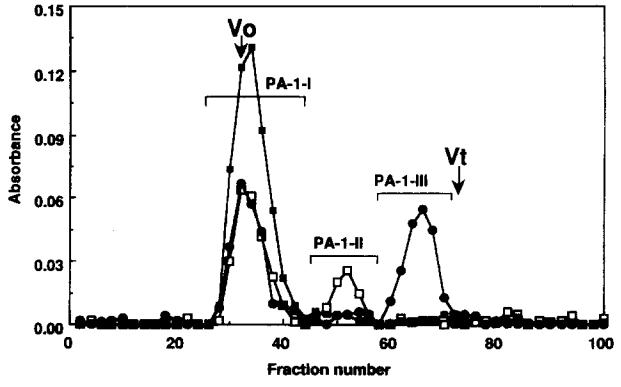


Fig. 4. Elution pattern on Bio-Gel P-6 of products derived from PA-1 by partial acid hydrolysis (0.1 M CF_3CO_2H, 40 °C, 24 h). □, Total carbohydrate (490 nm); ■, uronic acid (520 nm); ●, TBA-positive material (548 nm).

(H-3ax en H-3eq of Kdo). These results indicated that PA-2′ mainly contained α-Rha*p*-(1 → 5)-Kdo.

Fraction PA-1 was further treated with 0.1 M trifluoroacetic acid (24 h, 40 °C), and the products were fractionated on Bio-Gel P-6 (Scheme 1). Three carbohydrate peaks (PA-1-I–PA-1-III) were obtained as shown in Fig. 4. Fraction PA-1-II was composed mainly of neutral sugar, but PA-1-III consisted mainly of TBA-positive material. About 50% of the TBA-positive material was released from PA-1 by the acid hydrolysis. Fraction PA-1-III mainly comprised Rha and Kdo, and small proportions of Ara and Dha. GLC–MS analysis of permethylated oligosaccharide-alditols, prepared from PA-1-III, suggested that PA-1-III contained Rha-(1 → 5)-Kdo (peak 1P) and Ara*f*-(1 → 5)-Dha (peaks 2P and 3P) (Table 3).

The intermediate-size fraction, PA-1-II consisted mainly of 2-MeFuc, Rha, Ara, Api, and Gal, and minor amounts of Fuc, 2-MeXyl, Man, AceA, and Glc (Table 1). Methylation analysis indicated that PA-1-II contained 15 different glycosidic linkages such as terminal 2-MeFuc and Rha, 3-linked Rha, 2,4-linked Gal, and 2-linked AceA (Table 2). The monosaccharide and linkage analyses of PA-1-II were similar to those of the AceA-containing oligosaccharide in sycamore RG-II [30]. The presence of an AceA-containing oligosaccharide in PA-1-II was confirmed by FABMS (Fig. 5). Negative-ion FABMS of PA-1-II gave pseudo-molecular ions ($[M-H]^-$) at *m/z* 1055, 1187 and 1201, which are suggested to be due to non-acetylated hepta- and octa-saccharides, respectively (Fig. 5). PA-1-II also gave $[M-H]^-$ ions of monoacetylated hepta- (*m/z* 1097) and octa-

Table 3
Diagnostic fragment ions on EIMS of permethylated disaccharide-alditols derived from PA-1-III

Peak	Retention time (min)	Fragment ions [*m/z* (relative abundance)]									Structure inferred
		aJ_1	aJ_0	aJ_2	bA_1	bA_2	bA_3	ald			
1P	25.07	368 (4.6)	354 (1.3)	308 (23.8)	189 (48.7)	157 (15.7)	125 (12.2)	162 (19.2)			Rha-(1 → 5)-Kdo-ol-2-*d*
2P	30.74	338 (2.3)		278 (2.5)	175 (10.6)	143 (16.2)	111 (7.2)	162 (7.8)	308 (0.4)	366 (0.5)	Ara-(1 → 5)-Dha-ol-2-*d*
3P	32.15	338 (1.9)		278 (2.7)	175 (11.7)	143 (15.8)	111 (9.1)	162 (7.2)	308 (1.3)	366 (0.5)	Ara-(1 → 5)-Dha-ol-2-*d*

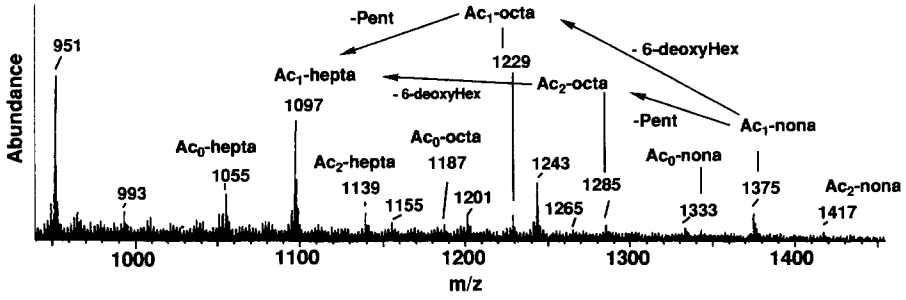


Fig. 5. Negative-ion FAB mass spectrum of PA-1-II. Series of ions at *m/z* 1187 and 1229 were due to octasaccharide which was derived from nonasaccharide by elimination of one 6-deoxyhexosyl unit. Series of ions at *m/z* 1201, 1243, and 1285 were due to octasaccharide, which was derived from nonasaccharide by elimination of one pentosyl unit.

saccharides (*m/z* 1229 and 1243), and diacetylated hepta- (*m/z* 1139) and octa-saccharides (*m/z* 1285). The positive-ion spectrum also showed pseudo-molecular ions ($[M + Na]^+$) due to non-, mono-, and di-acetylated heptasaccharide [*m/z* 1079, 1121, and 1163 (Ac_0-, Ac_1-, and Ac_2-heptasaccharide, respectively)] and octasaccharide [*m/z* 1225, 1267, and 1309 (Ac_0-, Ac_1-, and Ac_2-octasaccharide, respectively)] (data not shown). The major pseudo-molecular ion of the heptasaccharide (*m/z* 1097) was analyzed by a CAD spectrum using B/E linked scans. The CAD spectrum gave fragment ions at *m/z* 965, 819, 659, 337, and 205 due to successive eliminations of pentosyl (minus 132 mu), 6-deoxyhexosyl (minus 146 mu), hexosyl (minus 160 mu), hexosyl and monomethyl-6-deoxyhexosyl (minus 322 mu), and pentosyl (minus 132 mu) units from the heptasaccharide (data not shown). These results suggested that the monoacetylated heptasaccharide element in GL-4IIb2 possessed the same glycosyl sequence as in other RG-IIs [30].

R-(1→3)
Ara-(1→2)-Rha-(1→2)-Ara-(1→4)-Gal-(1→2)-AceA-(1→3)-Rha-(1→3`)-Api
2-MeFuc-(1→2)

1: R = OH
2: R = Rha

Recently, Whitcombe et al. [31] have reported that the AceA-containing oligosaccharide in sycamore RG-II exists as an octasaccharide as shown in **1**. However, PA-1-II derived from GL-4IIb2 also gave pseudo-molecular ions at *m/z* 1333, 1375, and 1417 due to non-, mono-, and di-acetylated nonasaccharides (Fig. 5). PA-1-II further showed peaks due to two octasaccharides (*m/z* 1187 and 1201) which are suggested to be derived from non-acetylated nonasaccharide by elimination either of terminal 6-deoxyhexosyl or pentosyl residues, respectively (Fig. 5). Methylation analysis indicated that PA-1-II contained 2,3-linked Ara and terminal Rha groups as well as terminal Ara*f* and 2-linked Rha residues (Table 2). From these results, it is proposed that GL-4IIb2 comprised AceA-containing oligosaccharide chains possessing a nonasaccharide unit (**2**), in which terminal Rha was additionally attached to position 3 of 2-linked Ara*p* of an octasaccharide chain observed in sycamore RG-II.

PA-1-I was further treated with 0.1 M trifluoroacetic acid (84 h, 40 °C), affording four subfractions PA-1-Ia, PA-1-Ib, PA-1-Ic, and PA-1-Id on Bio-Gel P-6 (Fig. 6A). About 50% of the TBA-positive material in PA-1-I was found back in the fractions PA-1-Ic and PA-1-Id, and PA-1-Ic was identified as Rha-(1 → 5)-Kdo as described above (data not shown). Response factors for Kdo and Dha using the TBA method were determined to be 1.0 and 0.39, and the Kdo/Dha ratio in GL-4IIb2 was calculated to be 1.8:1.0 from the amount of TBA-positive material in the fractions obtained by the present sequential degradation. Since the response factors for Kdo and Dha using the alditol acetate method were calculated to be 1.0 and 0.26, the Kdo/Dha ratio in GL-4IIb2 was determined to be 1.3:1.0 by this method. Kdo/Dha ratios of RG-II from sycamore and sugar beet pulp were almost 1:1 [32,33]. From these, GL-4IIb2 was assumed to comprise a slightly higher proportion of α-Rha*p*-(1 → 5)-Kdo side-chains than Ara*f*-(1 → 5)-Dha, compared with other RG-IIs. The high-molecular-mass fraction, PA-1-Ia, consisted mainly of Rha, Ara, GalA, and GlcA with smaller amounts of Fuc, 2-MeXyl, Api, Man, Gal, Glc, and Dha (Table 1). Methylation analysis indicated that PA-1-Ia contained characteristic glycosidic linkages such as terminal Ara*f*, 3,4-linked Fuc, 2,3,4-linked Rha, terminal Gal, 3′-linked Api, 2-linked GlcA, and

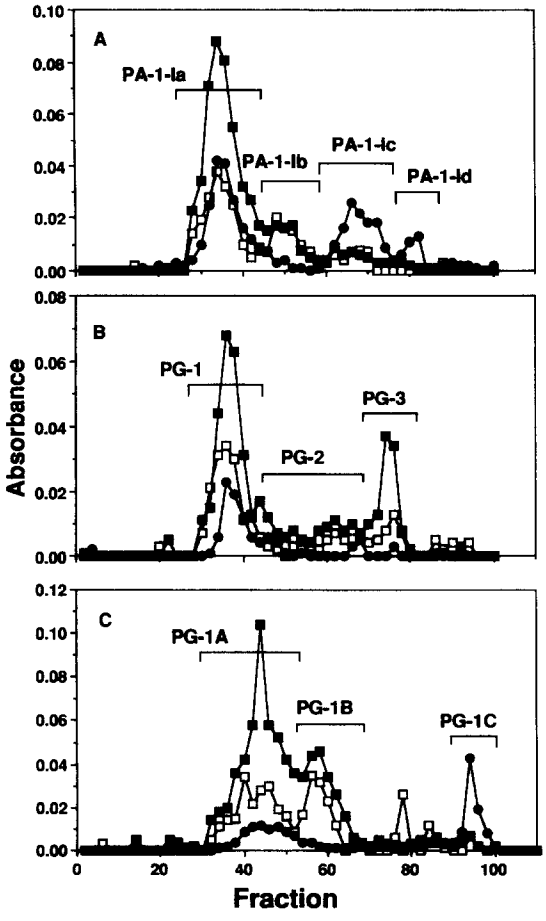


Fig. 6. (A) Elution pattern on Bio-Gel P-6 of products derived from PA-1-I by partial acid hydrolysis (0.1 M CF_3CO_2H, 40 °C, 84 h). (B) Elution pattern on Bio-Gel P-6 of products from PA-1-Ia by endo-α-(1 → 4)-polygalacturonase digestion. (C) Elution pattern on Bio-Gel P-6 of products from PG-1 by partial acid hydrolysis (0.1 M CF_3CO_2H, 50 °C, 48 h). □, Total carbohydrate (490 nm); ■, uronic acid (520 nm); ●, TBA-positive material (548 nm).

terminal GalA (Table 2). From these results, it was assumed that PA-1-Ia contained an uronic acid-rich octasaccharide [32,34], which also has been found in RG-II in addition to Ara*f*-(1 → 5)-Dha.

Digestion with endo-polygalacturonase of PA-1-Ia gave a small oligosaccharide fraction (PG-3) in addition to the resistant (PG-1) and the intermediate-size fraction (PG-2), although GL-4IIb2 was not digested with the enzyme (Fig. 6B). Fraction PG-2 contained a large amount of GalA and a small proportion of Rha, Fuc, 2-MeXyl, Ara, Gal, GalA, and GlcA, whereas PG-3 consisted only of GalA. High-performance anion-exchange chromatography equipped with pulsed electrochemical analysis indicated that PG-3 contained GalA and a galacturonodisaccharide (data not shown).

Fraction PG-1 was further treated with 0.1 M trifluoroacetic acid (48 h, 50 °C) in order to generate an uronic acid-rich oligosaccharide from PG-1, and the products were fractionated on Bio-Gel P-6. The products gave three fragments PG-1A, PG-1B, and PG-1C (Fig. 6C). Among the fractions, PG-1B was found to comprise Api, Rha, Fuc, 2-MeXyl, Gal, GalA, and GlcA (Table 1). Methylation analysis indicated that PG-1B consisted of terminal 2-MeXyl, Gal and GalA, 3′-linked Api, 2-linked GlcA, 3,4-linked Fuc, and 2,3,4-linked Rha (Table 2). The monosaccharide and linkage analyses were almost identical with those of the uronic acid-rich octasaccharide in RG-II [32,34]. Therefore, it is assumed that GL-4-IIb2 comprises similar structural units of uronic acid-rich octasaccharide side-chains as RG-II.

4. Discussion

Rhamnogalacturonan II (RG-II) is widely distributed in plant cell walls so far examined in sycamore (*Acer pseudoplatanus*) [35], Douglas fir (*Pseudotsuga menziensii*) [36], onion (*Allium cepa*) [37], rice (*Oryza sativa*) [38], kiwi fruits (*Actinidia deliciosa*) [39], medicinal plants such as *Bupleurum falcatum*, *Angelica acutiloba*, and *Glycyrrhiza uralensis* [40,41], and sugar beet [33], and have also been found in the medium of cultured sycamore cells [21], a commercial enzyme preparation (Pectinol AC) [23], and a red wine [27,42]. It has generally been expected that RG-II conjugates with homogalacturonan and rhamnogalacturonan I (same as hairy region or 'ramified' region) since RG-IIs have been released from cell walls by endo-polygalacturonase digestion [7]. Hirano et al. also have found that some pectins comprised the RG-II structure but pectic arabinogalactan does not [41]. It has been reported that RG-IIs have a highly conserved structure, even though they are highly branched and extremely complex polysaccharides [7]. Recently, RG-II has been found to inhibit the incorporation of leucine into cells by using a tomato cell culture [43]; however, the functions of RG-II are known neither in plants nor in humans [7]. The present study indicates that GL-4IIb2 belongs to the RG-II group, and is a first finding for pharmacological activity [6] in RG-II-type polysaccharides. GL-4IIb2 was obtained from the leaves of *P. ginseng* without endo-polygalacturonase digestion, and therefore it is suggested that GL-4IIb2 exists as the free form of RG-II-type polysaccharide in the leaves but not as the conjugated form with

rhamnogalacturonan I and homogalacturonan. It is also possible that the leaves contain potent endo-polygalacturonase activity, and therefore GL-4IIb2 may be generated as a free form by the endogenous enzyme. Because other RG-II-like structural units, which have been isolated from the pectin of *B. falcatum*, did not show Fc receptor expression-enhancing activity [26], there might be a possibility that GL-4IIb2 comprises a certain characteristic structure for expression of the activity.

The present study strongly suggests that GL-4IIb2 comprises an α-(1 → 4)-linked oligogalacturonide backbone and mainly four different side-chains such as Ara*f*-(1 → 5)-Dha, α-Rha*p*-(1 → 5)-Kdo, AceA-containing oligosaccharides, and an uronic acid-rich octasaccharide. These structural units have also been found in RG-II from sycamore [7]. In the present study a detailed structure of the uronic acid-rich oligosaccharide was not established, but monosaccharide and methylation analyses suggest that the structure of the oligosaccharide chain is similar to that from sycamore RG-II.

The present results indicate that GL-4IIb2 contains a branched nonasaccharide chain (**2**), of which the structure is suggested to correlate with the octasaccharide chain detected in sycamore RG-II [31] as an AceA-containing oligosaccharide side-chain. Whitcombe et al. have reported that sycamore RG-II released a nonasaccharide chain in addition to structurally related hexa- to octa-saccharide chains in the form of AceA-containing side-chains, and that this nonasaccharide chain fraction contains 2,3-disubstituted Ara but not terminal Rha [31]. Therefore, it has been proposed that the structure of the nonasaccharide chain did not correlate to those of the hexa- to octa-saccharide chains in sycamore RG-II [31]. From these observations, sycamore RG-II is assumed not to contain the nonasaccharide chain corresponding to **2**. It was also indicated that the molecular mass of GL-4IIb2 (MW 11,000) was larger than that of sycamore RG-II (MW ~ 5000) [32]. During the preparation of the manuscript, Kobayashi et al., Ishii and Matsunaga, and O'Neill et al. have reported that RG-II from radish [44], sugar beet pulp [33], and sycamore and pea stem [28] dimerize by forming a complex with boron, and the molecular mass (MW 10,500) of RG-II from sugar beet was similar to that of GL-4IIb2 [33]. We also have found that the molecular mass of GL-4IIb2 is reduced to about 7000 on HPLC after acidic treatment (> pH 2.0) [45]. In the present study methylation analysis indicated that GL-4IIb2 consisted of 2,3,3′-linked Api, which is known to contribute to the dimerization of RG-IIs. It was therefore assumed that GL-4IIb2 also formed a complex. The research on structure–activity relationships of GL-4IIb2 must await further study.

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