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Rhamnogalacturonan II dimers cross-linked by borate diesters from the leaves of *Panax ginseng* C.A. Meyer are responsible for expression of their IL-6 production enhancing activities

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Abstract

Leaves of *Panax ginseng* C.A. Meyer contain larger amounts of thiobarbituric acid (TBA)-positive material, suggested to be rhamnogalacturonan II (RG-II), than the roots. Starting from leaves, three different polysaccharides, GL-RI, GL-RII and GL-RIII, were isolated from polysaccharide subfraction GL-5 by subsequent anion-exchange and size-exclusion chromatography. Sugar and methylation analyses indicated that the three polysaccharides consisted of the same substitution patterns of 2-*O*-methyl-fucose, 2-*O*-methyl-xylose, 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), being characteristic monosaccharides of RG-II, but no other pectic components. Another RG-II (GL-4IIb2) has also been isolated without endo-polygalacturonase digestion from leaves of *P. ginseng*, and turned out to be a macrophage Fc receptor expression enhancing polysaccharide [K.-S. Shin, H. Kiyohara, T. Matsumoto, and H. Yamada, *Carbohydr. Res.*, 300 (1997) 239–249]. GL-4IIb2 and GL-RIII had relatively potent interleukin-6 (IL-6) production enhancing activity of macrophages, however, GL-RI and GL-RII had negligible and weak enhancing activities, respectively. Partial acid hydrolysis (0.1 M trifluoroacetic acid, 40°C, 24 h) of GL-RI, GL-RII and GL-RIII gave in each case a large-size fraction (AH-1), an intermediate-size fraction (AH-2) and a short oligosaccharide fraction (AH-3). EIMS and FABMS analyses indicated that AH-3 consisted of Rha-(1→5)-Kdo and Ara-(1→5)-Dha, and that AH-2 comprised non-, mono- and di-acetylated AceA-containing hexa- to nona-saccharides. Also for AH-1 no significant structural differences were observed among the polysaccharides, but the presence of microheterogeneity is suggested. The AH-1 fractions did not show any IL-6 production enhancing activity. Size-exclusion HPLC indicated that GL-RIII mainly comprised RG-II of higher molecular mass (12,000), and that GL-RI and GL-RII mainly contained RG-II of lower molecular mass (7000). Boron content and ^{11}B NMR analyses indicated that the higher molecular weight polysaccharide in GL-RIII was mainly present as a RG-II dimer cross-linked by borate diesters.

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Dissociation of the RG-II dimer containing borate diester in GL-RIII to the monomer by mild acid treatment significantly decreased its IL-6 production enhancing activity whereas re-dimerization of dissociated GL-RIII recovered the enhancing activity. Artificial re-formation of the RG-II dimer containing borate diester in GL-RI also potentiated the IL-6 production enhancing activity. The present observation is a first report of biological activity related to the RG-II dimer.

Keywords: *Panax ginseng*; Araliaceae; Rhamnogalacturonan II; RG-II dimer containing borate diester; Interleukin-6 (IL-6) production enhancing activity

1. Introduction

The roots of *Panax ginseng* C.A. Meyer are a well-known component herb of Japanese herbal (Kampo) medicines widely used clinically for the treatment of gastrointestinal disorders and as an erythropoietic and a tonic. The roots of *P. ginseng* are valuable because it takes 4–6 years for their harvest, whereas the leaves of *P. ginseng* can be harvested every year. In earlier studies we have reported that polysaccharides from the leaves have more potent anti-complementary and anti-ulcer activities than those from the roots [1,2]. Another polysaccharide (GL-4IIb2), purified from the leaves, has been shown to be a Fc receptor expression enhancing polysaccharide [3]. Very recently, GL-4IIb2 has been found to be a rhamnogalacturonan-II (RG-II) having unique characteristics [4]: (1) GL-4IIb2 exists as a free RG-II in the leaves although it has generally been believed that other RG-IIs in plant cell walls conjugate covalently with other pectic components such as rhamnogalacturonan I and galacturonan since RG-IIs have been released from cell walls by endo-polygalacturonase digestion [5]; (2) GL-4IIb2 contains aceric acid (AceA)-containing nonasaccharide side chains with non-, mono- and di-acetyl groups, which have not been found in other RG-IIs [6].

In the present paper, we describe the isolation of three other RG-IIs (GL-RI, GL-RII and GL-RIII) from the leaves of *P. ginseng* having a different degree of interleukin-6 (IL-6) production enhancing activity for macrophages. Furthermore, a correlation between the structures and the activities of these RG-IIs is presented.

2. Experimental

Materials.—The leaves of *P. ginseng* C.A. Meyer were cultured and collected (September 1987) in Chan Bai Mountain, Jilin, China. A crude polysaccharide fraction (GL-2) was prepared from the leaves by hot water extraction, EtOH precipitation and dialysis [1], and GL-2 was further fractionated by cetyltrimethylammonium bromide (cetavlon) according to the method of Gao et al. [1] to obtain strongly acidic (GL-3), weakly acidic (GL-4), and the remaining (GL-5) polysaccharide fractions. GL-4IIb2 was purified from GL-4 as described in [3] and [4]. 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 [7]. DEAE-Sepharose FF was obtained from Pharmacia (Sweden), AG50-WX8 and Bio-Gel P-6 and P-30 from Bio-Rad (USA), and Sep-Pak C_{18} cartridges from Waters Associates (USA). Thioglycollate medium was purchased from Eiken (Tokyo, Japan).

General.—Total carbohydrate, uronic acid, and protein contents were assayed by the phenol-H_2SO_4 [8], *m*-hydroxybiphenyl [9], and Lowry [10] methods, respectively, and contents of Kdo and Dha were determined colorimetrically by the modified thiobarbituric acid (TBA) assay [11]. Monosaccharide constituents of poly- and oligosaccharides were analyzed by GC of their alditol acetates as described in [12] and [13]. The contents of Kdo and Dha were also determined by GC according to a modified method of York et al. [11] and Stevenson et al. [14].

Purification of GL-RI, GL-RII and GL-RIII.—GL-5 (4 g) was fractionated on a column (5.6×35 cm) of DEAE-Sepharose FF (Cl^- form). The column was washed with water to obtain GL-5I (442 mg), and the absorbed polysaccharide fractions were eluted sequentially with 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1.0 and 2.0 M NaCl to give fractions GL-5IIa (371 mg), IIb (278 mg), IIc, IId, IIe (392 mg), IIf (322 mg), IIg (398 mg), and IIh

(77 mg), respectively. GL-5IIc and IId each contained a retarded fraction in addition to a faster-eluted fraction. TBA-positive material was found in both the faster-eluted (GL-5IIc1, 357 mg) and the retarded fraction (GL-5IIc2, 493 mg) in GL-5IIc, and in the faster-eluted fraction (GL-5IId1, 224 mg) in GL-5IId. The subfractions GL-5IIc1, IIc2 and IId1 each were applied to a column (2.5×90 cm) of Bio-Gel P-30 equilibrated with 50 mM acetate buffer (pH 5.2), and were eluted with the same buffer. All TBA-positive material in GL-5IIc1, IIc2 and IId1 was eluted only in the respective intermediate fraction, and three major purified polysaccharides (GL-RI, RII and RIII) were obtained, and lyophilized after desalting by dialysis using Spectrapor MWCO 1000 (molecular mass cut off; 1000) (yields: GL-RI, 3.4% from GL-5; GL-RII, 7.3% from GL-5; GL-RIII, 2.0% from GL-5).

Mild acid treatment of GL-RI, RII and RIII.—GL-RI (40 mg), RII (40 mg) and RIII (30 mg) were each treated with 0.1 M CF_3CO_2H (30–40 mL) for 24 h at 40°C as described in [4]. The hydrolysates were fractionated by Bio-Gel P-6 with 50 mM acetate buffer (pH 5.2).

Methylation analysis.—Glycosidic linkages were analyzed as described previously [4] by using the method of Hakomori [15].

GC-EIMS of methylated oligosaccharide-alditols.—Structural features of methylated oligosaccharide-alditols were analyzed by GC-EIMS on an SP-2380 capillary column as described previously [4].

Negative-ion fast-atom bombardment mass spectrometry (negative FABMS).—FABMS spectra were obtained on a JEOL JMS-AX505 HA mass spectrometer using as a matrix 1:1 glycerol-monothioglycerol as described previously [4].

Boron determination.—The B concentration in each sample was determined with an inductive coupled plasma-emission spectrometer (ICP, Leeman Labs PS 3000 μV) after ashing at 500°C. The ashed samples were dissolved in 6 M HNO_3 and then placed on a hot plate to dry. The dried ashes were dissolved in 1 M HNO_3.

^{11}B NMR spectroscopy.—The ^{11}B NMR spectrum of each sample was recorded at 25°C on a JEOL JNM-A600 spectrometer operated at 192.6 MHz without field lock using 5-mm diameter quartz-glass tubes. Chemical shifts (δ) are reported relative to external H_3BO_3 (0.1 M) at 0.0 ppm. NMR conditions were as follows: number of data points, 2048; observation frequency range, 28,900 Hz; pulse width, 15 μsec (90°); pulse repetition time, 0.11 s; exponential broadening factor, 15 Hz.

Hydrolysis and artificial formation of borate ester.—Hydrolysis and re-formation of an RG-II dimer containing borate diester was performed according to the modified method of O'Neill et al. [16]. GL-RIII (3.5 mg) was treated with 0.1 M HCl (1.2 mL) at 20°C for 40 min. After neutralization of the solution with 0.5% NaOH, the reaction product was desalted by Microacylizer, and lyophilized to obtain partially monomerized GL-RIII (mGL-RIII).

mGL-RIII (3.5 mg) and GL-RI (1.7 mg) were each dissolved in 1 mL 50 mM acetate buffer (pH 3.3) containing 1.2 mM H_3BO_3 and 0.5 mM $Pb(NO_3)_2$. Reaction mixtures were incubated at room temperature for 24 h, desalted by Microacylizer, and lyophilized to obtain dimerized polysaccharides (dGL-RIII and dGL-RI).

Size-exclusion HPLC of the samples was performed on a Shimadzu LC 6A instrument equipped with combined columns (0.76×50 cm each) of Asahi-pak GS-320 + GS-220 or GS-510 + GS-320 + GS-220 (Asahi Chemical Industry Co. Ltd., Japan) and eluted with 0.2 M NaCl. Apparent molecular mass calibration curves were made with standard pullulans (P-400, 200, 100, 50, 20, and 5; Showa Denko Co. Ltd., Japan).

Measurement of IL-6.-(a) Animals.—Male C3H/HeJ mice, weighing 30–35 g, were purchased from SLC (Shizuoka, Japan), and were housed and maintained at 25°C constant humidity (55%). They had free access to food (CE-2 from CLEA, Japan) and water from the beginning of the experiment. (b) *Preparation of macrophage monolayer.*—The thioglycollate elicited peritoneal macrophages were obtained from the mice, and the macrophage monolayer was prepared as described previously [17]. More than 95% of the adherent cells showed typical macrophage morphology with characteristic staining. (c) *Measurement of IL-6 production of macrophages.*—Macrophages were cultured in the absence or presence of samples for 24 h at 37°C, and IL-6 contents in the culture supernatant were measured by a bioassay using IL-6 dependent murine hybridoma (MH60. BSF2) [18]. Briefly, the MH60. BSF2 cells were washed three times with RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (RPMI-1640-FCS), and then seeded in microplates at a cell density of 5×10^3

cells/well in 100 μL of RPMI-1640-FCS. MH 60. BSF2 cells were added to the culture supernatants of macrophages obtained as above, and then cultured for 72 h at 37°C. Cell proliferation of MH 60. BSF2 cells was measured using the Alamar Blue reduction assay [19]. Five hours prior to culture termination of MH 60. BSF2 cells, 20 μL of Alamar Blue™ solution was added to each well of the culture plate, and then the cells were continuously cultured at 37°C. The fluorescence intensity of the solution in each well was measured by a Fluoroskan II (Labosystems, Japan) at an excitation wavelength of 544 nm and emission wavelength of 590 nm.

3. Results

Analysis of RG-IIs in roots and leaves of P. ginseng.—In order to compare the contents of RG-IIs in roots and leaves of *P. ginseng*, the TBA reaction was performed on crude polysaccharide fractions from roots (GR-2) and leaves (GL-2). As shown in Fig. 1, GL-2 strongly reacted with TBA whereas GR-2 did not contain significant amounts of TBA-positive material such as Kdo and Dha which are characteristic monosaccharide constituents of RG-II [5]. In earlier studies, GL-2 was fractionated to give three polysaccharide fractions, GL-3, GL-4 and GL-5, by cetavlon fractionation [1]. All these subfractions showed a similar strong reactivity with TBA as GL-2 (Fig. 1). GL-3, GL-4 and GL-5 were each subfractionated by size-exclusion HPLC using combined columns of Asahi-pak GS-510 + GS-320 + GS-220 in 0.2 M NaCl, and the eluted fractions were analyzed for carbohydrate, uronic acids and TBA-positive material. All subfractions gave peaks with an apparent molecular mass around 10,000 that reacted positively with TBA (Fig. 2). All carbohydrate and uronic acid in GL-5 seemed to co-elute with the TBA-positive peak (Fig. 2C) whereas GL-3 and GL-4 also contained carbohydrate peaks which were free from TBA-positive material (Fig. 2A and B).

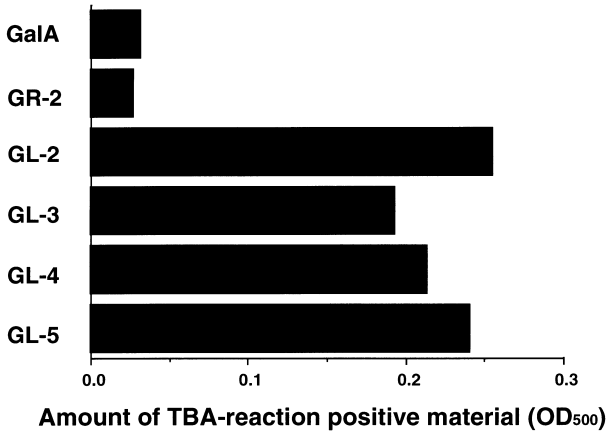

Fig. 1. Comparison of the content of TBA-positive material in polysaccharide fractions obtained from roots and leaves of *P. ginseng* C.A. Meyer.

Isolation and characterization of RG-IIs from GL-5.—Fraction GL-5 was subfractionated by anion-exchange chromatography on DEAE-Sepharose FF (Cl^- form) and the major three TBA-positive fractions, GL-5IIc1, IIc2 and IId1, were obtained as the fractions eluted by 0.2 and 0.3 M NaCl (data not shown). When these fractions were further fractionated by Bio-Gel P-30, the respective intermediate-size fractions GL-RI, GL-RII and GL-RIII containing TBA-positive material were obtained (data not shown).

GL-RI, RII and RIII mainly contained neutral sugar and uronic acid in addition to small amounts of protein (Table 1). Digestion with endo-α-(1→4)-polygalacturonase from *A. niger* had no apparent effect on these polysaccharides (data not shown).

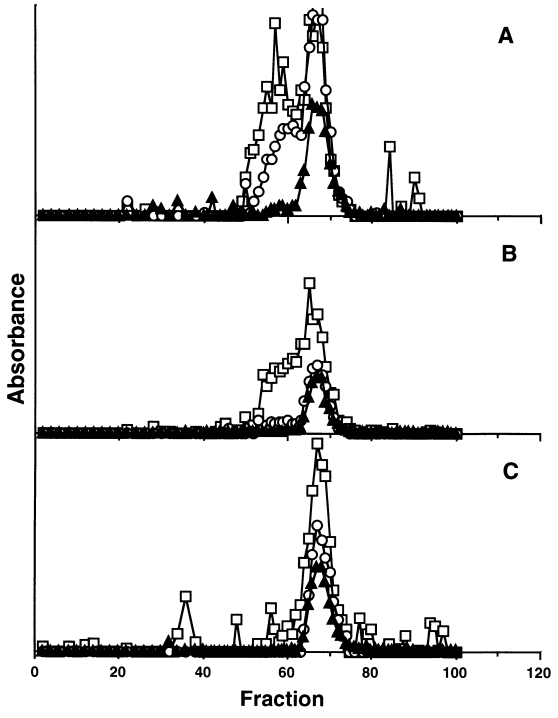

Fig. 2. Elution patterns of polysaccharide fractions [GL-3 (A), GL-4 (B) and GL-5 (C)] from leaves of *P. ginseng* on size-exclusion HPLC using Asahi-pak GS-510 + GS-320 + GS-220. □, carbohydrate (490 nm); ○, uronic acid (520 nm); ▲, TBA-positive material (500 nm).

Table 1
Chemical properties of GL-RI, RII and RIII

	GL-RI	GL-RII	GL-RIII
Total carbohydrate (%)[a]	37.5	37.1	26.0
Uronic acid (%)[b]	22.1	21.1	14.1
Protein (%)[c]	0.5	0.4	0.4
Boron (%)	0.08	0.02	0.08
Monosaccharide (mol %)			
2-Me-Fuc	4.7	4.8	4.3
Rha	18.6	18.7	24.0
Fuc	4.5	4.1	3.9
2-Me-Xyl	4.6	4.0	3.6
Ara	12.3	10.0	10.0
Api	6.5	5.3	5.0
Man	1.0	0.5	0.8
AceA	1.4	1.4	1.2
Gal	9.4	7.8	10.2
Glc	3.0	1.0	1.5
Dha	6.7	10.2	5.3
Kdo	6.3	8.0	5.1
GalA	17.6	19.4	22.0
GlcA	3.4	4.8	3.1

[a]Calibrated as Gal.
[b]Calibrated as GalA.
[c]Calibrated as bovine serum albumin.

Sugar analysis indicated that these polysaccharides consisted of at least 14 kinds of monosaccharides such as 2-*O*-methyl-fucose (2-Me-Fuc), 2-*O*-methyl-xylose (2-Me-Xyl), 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) in addition to Fuc, Ara, Rha, Man, Gal, Glc, GlcA and GalA (Table 1). Methylation analysis of GL-RI, RII and RIII showed, among others, the occurrence of terminal 2-Me-Xyl, terminal 2-Me-Fuc, 2,3,4-linked Rha, 3′-linked Api, 2,3,3′-linked Api, being characteristic glycosidic linkages in RG-II; no significant difference was observed in the composition of the glycosidic linkages among the three polysaccharides (data not shown). These results suggest that GL-RI, RII and RIII mainly comprise RG-II structures, and that these polysaccharides also exist without other pectic components in the leaves of *P. ginseng* similar as GL-4IIb2 [4].

GL-4IIb2, GL-RI, GL-RII and GL-RIII were tested for their effects on IL-6 production of macrophages. Although GL-4IIb2 had the most potent enhancing activity of IL-6 production, GL-RIII showed the second most potent enhancing activity, and GL-RII expressed weaker enhancing activity than GL-RIII, however GL-RI seemed to have no effect on IL-6 production (Fig. 3). Since the assay of IL-6 production was tested using IL-6 dependent murine hybridoma (MH 60. BSF2 cells), the results arised a possibility that RG-IIs from *P. ginseng* directly stimulate a proliferation of MH 60. BSF2 cells. However, when MH 60. BSF2 cells were incubated with 0.1–10000 ng/mL of GL-RIII for 72 h, no stimulating effect was observed on a proliferation of MH 60. BSF2 cells (data not shown). These results indicate that some RG-IIs from *P. ginseng* have IL-6 production enhancing activity and it is expected that these RG-IIs may have certain different primary structures corresponding to the degree of IL-6 production enhancing activity.

When GL-RI, RII and RIII were treated with 0.1 M trifluoroacetic acid at 40°C for 24 h, three major carbohydrate peaks (AH-1, AH-2 and AH-3) were obtained by gel filtration on Bio-Gel P-6 from the respective hydrolysates. Although these polysaccharides gave similar elution patterns, the proportions of total carbohydrate, uronic acid and TBA-positive material in AH-1 were different from each other (Fig. 4). Sugar, methylation and GC-MS analyses of (methylated) oligosaccharide-alditols derived from AH-3 indicated that AH-3 from GL-RI, RII and RIII contained Rha-(1→5)-Kdo and Ara-(1→5)-Dha, and no significant difference was observed among AH-3 from these RG-IIs (data not shown). Negative FABMS analysis of AH-2 from the three RG-IIs gave characteristic ions at m/z 1333, 1375 and 1417, which might be assigned to non-, mono-, and di-acetylated non-asaccharides as reported previously [4] (data not shown). The sequences derived from these ions were determined by collisional activated dissociation (CAD) mass spectrometry using B/E-linked scans in order to confirm the structures. The CAD

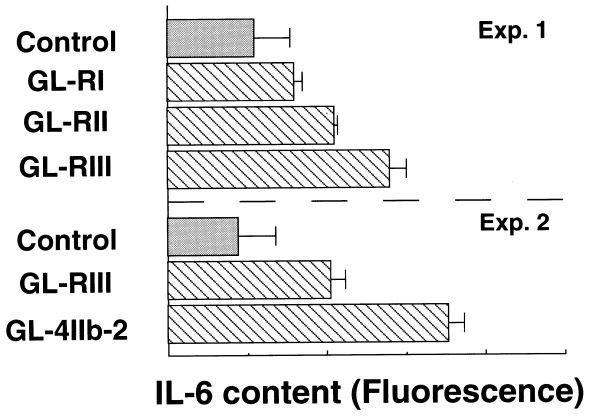

Fig. 3. Effect of RG-IIs from leaves of *P. ginseng* on IL-6 production of macrophages. Macrophages were treated with 10 μg/mL of polysaccharides.

spectrum of the monoacetylated nonasaccharide (m/z 1375) gave daughter fragment ions at m/z 1243, 1097, 951, 819, 617 and 455, which arose by the successive eliminations of glycosyl residues from the non-reducing terminus of the monoacetylated nonasaccharide (Fig. 5), suggesting that

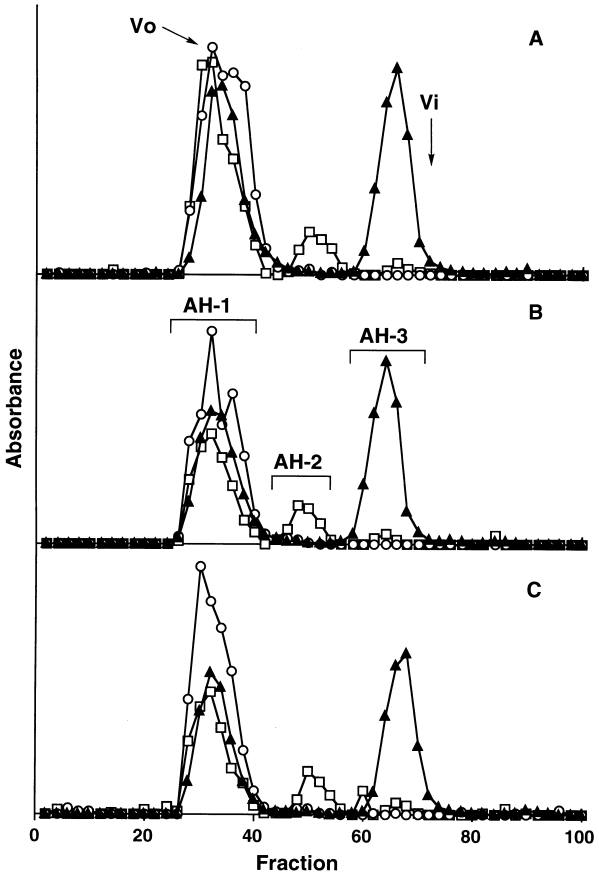

Fig. 4. Elution patterns of partially hydrolyzed GL-RI (A), RII (B) and RIII (C) on Bio-Gel P-6. Symbols are the same as in Fig. 2.

the pseudomolecular ion at m/z 1375 possessed the nonasaccharide sequence as shown in Fig. 5, and reported previously [4]. Comparison of the negative FABMS spectra also indicated that AH-2 from the three RG-IIs comprised the same AceA-containing oligosaccharide chains (data not shown) [4].

When AH-1 from GL-RI, RII and RIII were further partially hydrolyzed with 0.1 M trifluoroacetic acid (50°C, 48 h) in order to generate uronic acid-rich oligosaccharides, and the products were fractionated on Bio-Gel P-6, quite different elution patterns were obtained, assuming that AH-1 of these polysaccharides might possess different carbohydrate structures (data not shown). However, the three AH-1 fractions did not show any IL-6 production enhancing activity (data not shown).

Contribution of P. ginseng *RG-II dimers containing borate ester to the IL-6 production enhancing activity*.—When GL-RI, RII and RIII were analyzed by size-exclusion HPLC, GL-RII gave two peaks having an apparent molecular mass of 12,000 and 7000 (Fig. 6B). These fractions contained the same monosaccharide constituents, and included characteristic monosaccharides of RG-II such as 2-Me-Fuc, 2-Me-Xyl, Api, AceA, Kdo and Dha (data not shown). In each case GL-RI and RIII was eluted as peaks having shoulders on sides of high molecular mass and low molecular mass, respectively (Fig. 6A and C). During the present study, Kobayashi et al., Ishii and Matsunaga, O'Neill et al., and Kaneko et al. have reported that RG-II from radish [20], sugar beet pulp [21], sycamore and pea stem [16], and bamboo shoot [22] dimerize by forming a complex with borate, and that the 2,3,3′-branched Api in RG-II is involved in the formation of the RG-II dimer containing borate diester [16]. Matoh et al. also have reported the wide distribution of the borate-RG-II complex in higher plants [23]. After GL-RI, RII and RIII had been treated with diluted HCl for 30 min, the products were analyzed on HPLC. The peaks of high molecular mass (12,000) in all polysaccharides disappeared, and single peaks having an apparent molecular mass of 7000 were obtained (Fig. 6D–F). Methylation analysis indicated that GL-RI, RII and RIII also contained 2,3,3′-branched Api, which has been suggested to form a complex with borate in RG-II [16] (data not shown). Therefore it was assumed that these polysaccharides from *P. ginseng* also might contain borate-RG-II complexes as RG-IIs obtained from other sources. The

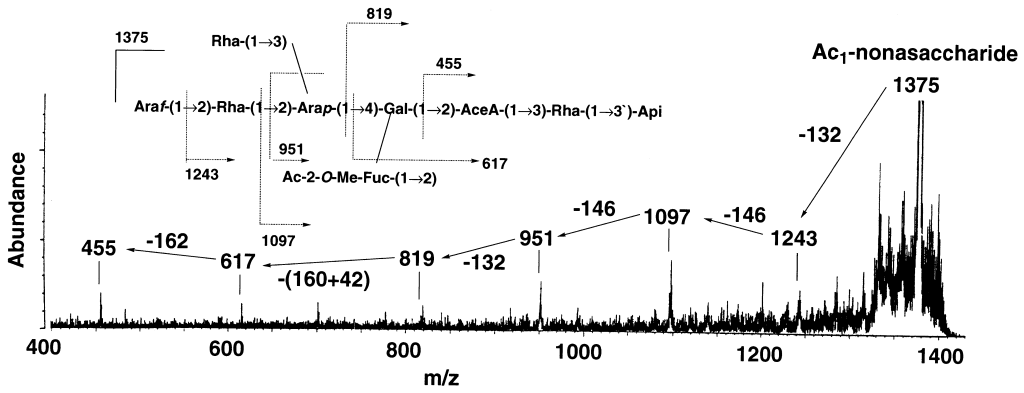

Fig. 5. Collisional-activated dissociation mass spectrum of the pseudomolecular ion m/z 1375 of the monoacetylated nonasaccharide in GL-RI.

boron contents of GL-RI, RII and RIII were estimated by ICP, affording 0.08, 0.02 and 0.08% (w/w) of boron, respectively (Table 1). It has been reported that ^{11}B NMR spectroscopy is a useful technique in characterizing esters formed by interaction of boric acid and diols [21,24]. GL-RI and RIII were analyzed by ^{11}B NMR, and both gave signals at δ −9.60 and 0.00, which were assigned to belong to tetrahedral borate diol diester and free boric acid, respectively [21] (Fig. 7). The results suggest that GL-RI contained a lower amount of borate diester than GL-RIII. Figure 6 also indicated that GL-RIII contained a higher amount of dimerized polysaccharide than GL-RI, and the dimer content in the polysaccharides seemed to correlate well with the degree of IL-6 production enhancing activity of the polysaccharides.

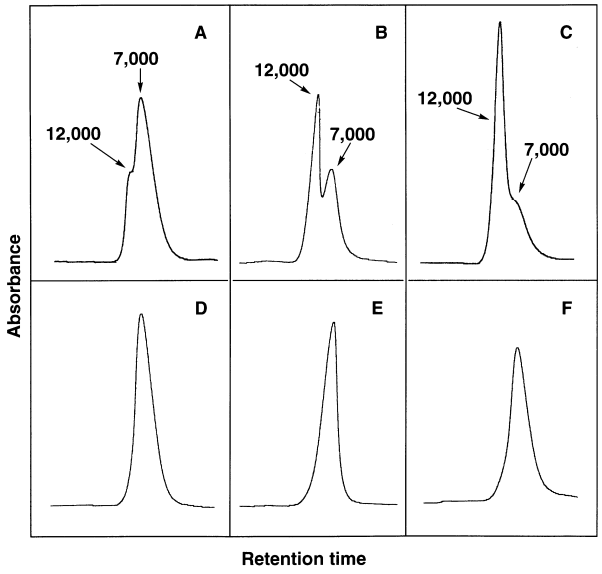

Fig. 6. Elution patterns on size-exclusion HPLC using Asahi-pak GS-320+GS-220 of GL-RI, RII and RIII before and after treatment with mild acid. (A-C) before treatment; (D-E) after treatment. (A, D) GL-RI; (B, E) GL-RII; (C, F) GL-RIII.

Recently, O'Neill et al. have found that monomerized RG-II from sycamore can be re-dimerized under acidic conditions (pH 3.0–3.4) in the presence of boric acid and divalent cations such as Sr^{2+}, Pb^{2+} or Ba^{2+} [16]. When GL-RIII, which showed potent IL-6 production enhancing activity (Fig. 3), was treated with 0.1 M HCl (20°C, 40 min), on HPLC the peak of higher molecular mass (12,000) was partially dissociated to the peak of lower molecular mass (7000) (Fig. 8A and B). The IL-6 production enhancing activity of the acid-treated GL-RIII was significantly reduced in comparison with the untreated GL-RIII (Fig. 9A). However, when the acid-treated GL-RIII (mGL-RIII) was incubated at pH 3.3 with BO_3^{3-} and Pb^{2+} ions (room temperature, 24 h), the peak of lower molecular mass was re-dimerized to form the peak of high molecular mass (Fig. 8C), and the activity of mGL-RIII was significantly increased by re-dimerization of the polysaccharide having the lower molecular mass (Fig. 9A). In order to confirm whether dimerization of GL-RI, which showed negligible activity, also increased IL-6 production enhancing activity, GL-RI was treated at pH 3.3 with BO_3^{3-} and Pb^{2+} ions as above. As shown in Fig. 9B, the activity of GL-RI significantly enhanced by the dimerization, however dimerized GL-RI did not have the similar activity as GL-RIII.

4. Discussion

RG-II is widely distributed in plant cell walls such as sycamore (*Acer pseudoplatanus*) [25], Douglas fir

(*Pseudotsuga menziensii*) [26], onion (*Allium cepa*) [27], rice (*Oryza sativa*) [28], kiwi fruits (*Actinidia deliciosa*) [29], medicinal plants such as *Bupleurum falcatum* [30], *Angelica acutiloba* [31] and *Glycyrrhiza uralensis* [13,31], and sugar beet [21]; and has also been found in the medium of cultured sycamore cells [32], in a commercial enzyme preparation (Pectinol AC) [33] and in red wines [34,35]. Recently, we have isolated RG-II (GL-4IIb2) as murine macrophage Fc receptor expression enhancing polysaccharide from leaves of *P. ginseng* [3,4]. The present study suggests that leaves of *P. ginseng* contain more RG-IIs than the roots, and we succeeded in isolating three other RG-II preparations, GL-RI, RII and RIII. RG-II has been found to inhibit the incorporation of leucine into the cells by using tomato cell cultures [36]; however, the functions of RG-II are not known in plants nor in humans [5]. Because GL-4IIb2 was suggested to modulate the function of macrophages, resulting in the enhancement of the de novo synthesis of Fc receptor [3], we attempted to compare the effect of RG-IIs from the leaves on the production of IL-6 as one of the macrophage functions. The present results indicate that the RG-IIs from *P. ginseng* had different degrees of IL-6 production enhancing activity against macrophages. RG-IIs have been reported to have a highly conserved structure, even though they are highly branched and extremely complex polysaccharides [5]. GL-4IIb2 has been found to possess the unique AceA-containing nonasaccharide side chain, assuming the possibility that there was a difference in a specific carbohydrate structure among the RG-IIs from *P. ginseng*. However, the present results denied this possibility because structures of the side chains which were liberated by partial acid hydrolysis were almost the same among the RG-IIs, and the large size oligosaccharide fraction (AH-1) had no activity.

Recently, several research groups have reported the presence of a borate-RG-II dimer in plant cell walls [16,20–23]. The present results strongly suggest that GL-RI, RII and RIII also contain RG-II dimers cross-linked by borate diesters to a differing

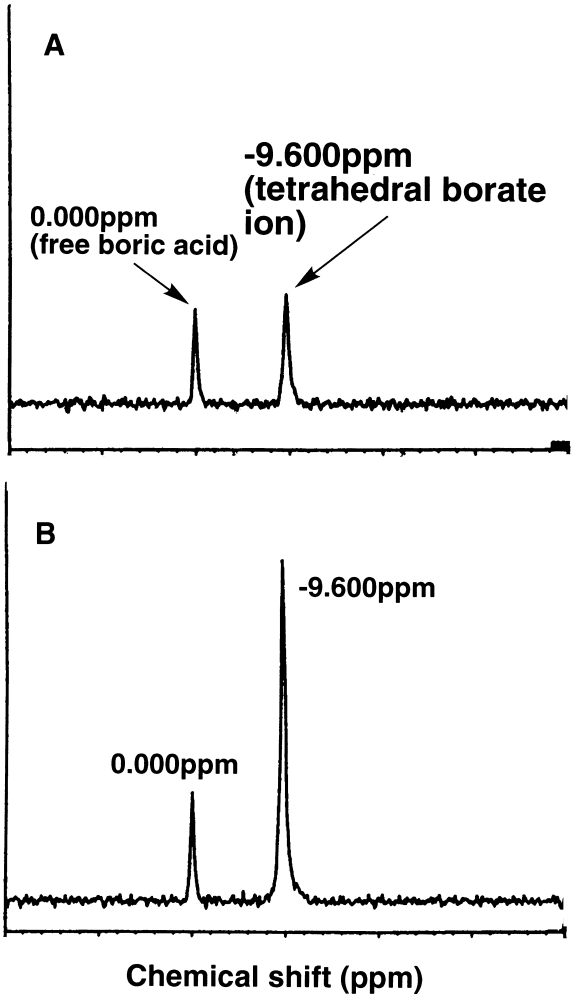

Fig. 7. ^{11}B NMR spectra of GL-RI (A) and GL-RIII (B).

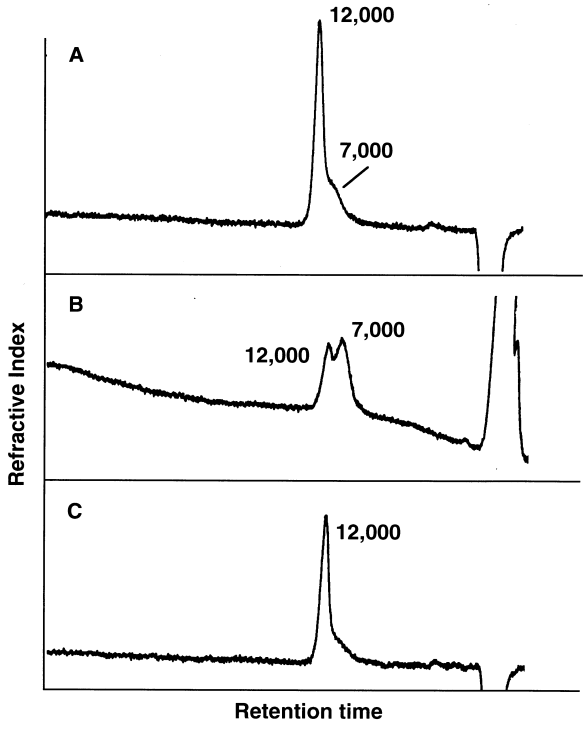

Fig. 8. Changes of elution patterns of GL-RIII on size-exclusion HPLC using Asahi-pak GS-320 + GS-220 after acidic treatment and artificial dimerization. (A) intact GL-RIII; (B) GL-RIII treated with 0.1 M HCl at 20°C for 40 min.; (C) GL-RIII from (B) treated in 50 mM acetate buffer (pH 3.3) with boric acid and Pb^{2+} ions at room temperature for 24 h.

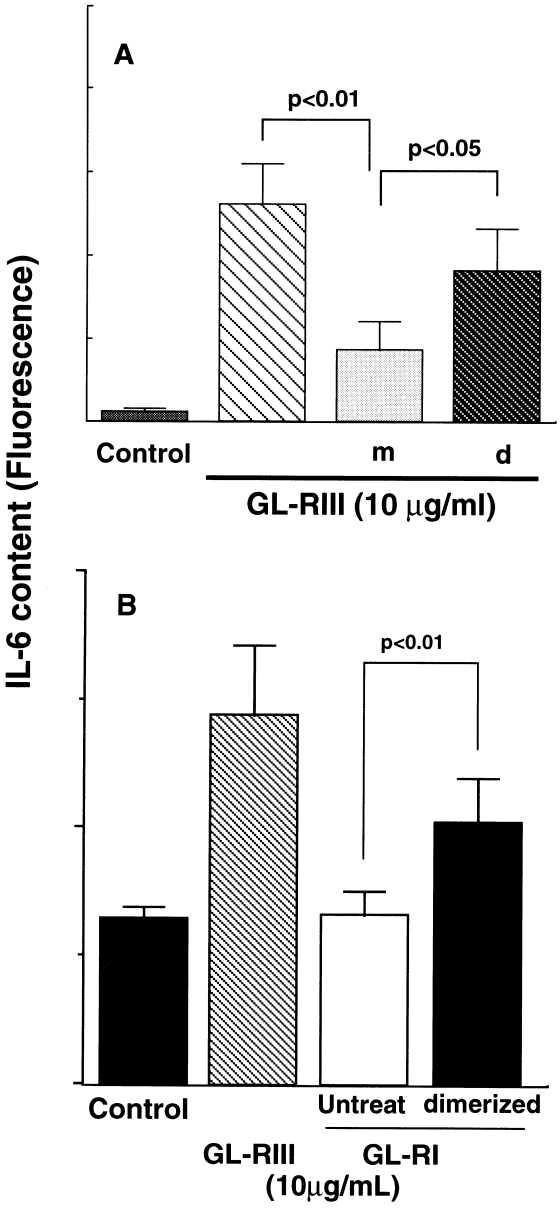

Fig. 9. (A) Effect of monomerization and artificial re-dimerization of GL-RIII on IL-6 production enhancing activity. m, partially monomerized GL-RIII from Fig. 8B; d, dimerized GL-RIII from Fig. 8C. (B) Effect of artificial dimerization of GL-RI on IL-6 production enhancing activity.

extent, and GL-RIII, which had a relatively potent IL-6 production enhancing activity, contained the highest content of the dimer among GL-RI, RII and RIII. GL-4IIb2 also contained 0.09% of boron. GL-4IIb2 was eluted as an almost single peak having an apparent molecular mass of 11,000 on HPLC and the peak was completely down shifted to a molecular mass of 7000 when GL-4IIb2 was treated with diluted HCl (data not shown), indicating that GL-4IIb2 having the most potent activity might contain the highest amount of RG-II dimer containing borate diester among the RG-IIs from *P. ginseng*.

Examination of the monomerization and re-dimerization of GL-RIII, which had potent IL-6 production enhancing activity, clearly indicated that the formation of a RG-II dimer containing borate diester strongly contribute to the expression of the activity. The same phenomenon was also observed in GL-RI. Although redimerized RG-II is indistinguishable from naturally occurring cross-linked RG-II by size-exclusion HPLC, re-dimerized GL-RI and RIII showed slightly weaker activity than intact GL-RIII in the present study. O'Neill et al. have proposed that the borate complex is formed through Api in RG-II [16], but it is not known whether the artificial dimerization also represents the same borate complex as the intact RG-II because Api is present both in reducing terminal sides of AceA-containing oligosaccharide and uronic acid-containing oligosaccharide side chains [5]. Therefore the present observations are not able to deny a possibility that re-dimerized RG-II had different conformations from the intact ones, resulting in the slightly weaker activity.

Hirano et al. have found that anti-ulcer pectins (bupleuran 2IIb and 2IIc) from roots of *B. falcatum* contain RG-II units which are liberated by endo-polygalacturonase digestion [30,31], and size-exclusion HPLC has suggested that these RG-II units mainly contain the dimerized form [31]. However, the RG-IIs from *B. falcatum* had no IL-6 production enhancing activity (Sakurai et al., personal communication). The present results assumed that there might be a microheterogeneity in the structures of the large size fractions (AH-1) obtained from GL-RI, RII and RIII. Edashige and Ishii also have reported the possibility that side chain structures of RG-II from poplar cell walls might contain more complicated structures than those proposed until now [37]. It is not known now whether microheterogeneity of RG-IIs affects the formation of dimer containing borate diesters and expression of the activity. The clarification of these possibilities must await further study.

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