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Total fractionation and characterization of the water-soluble polysaccharides isolated from *Panax ginseng C. A. Meyer*

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ABSTRACT

Water-soluble polysaccharides isolated from the roots of *Panax ginseng C. A. Meyer* were completely fractionated into two neutral fractions (WGPN and WGPA-N) and six acidic fractions (WGPA-1-RG, WGPA-2-RG, WGPA-1-HG, WGPA-2-HG, WGPA-3-HG and WGPA-4-HG) by a combination of ethanol precipitation, ion-exchange and gel permeation chromatographies. The analytical results showed that WGPN was a starch-like glucan; WGPA-N was a mixture of starch-like glucan and arabinogalactan; WGPA-1-RG and WGPA-2-RG were composed of major neutral sugars and minor acidic sugars that belong to the type-I rhamnogalacturonan (RG-I)-rich pectins, while fractions WGPA-1-HG to WGPA-4-HG were mainly composed of galacturonic acid (GalA, 62.4–92.1%) and have been identified to be homogalacturonan (HG)-rich pectins with different degrees of methyl-esterification, ranging from 0% to 30%. High performance gel permeation chromatography (HPGPC) showed that the six acidic fractions were homogenous, with molecular weights approximately ranging from 3.5×10^3 to 1.1×10^5. Lymphocyte proliferation assays showed that both the neutral polysaccharides and acidic polysaccharides were potent B and T cell stimulators.

1. Introduction

Panax ginseng C. A. Meyer (P. ginseng) has been used in China as a traditional medicine for over 2000 years. It is believed to be a panacea and to promote longevity. P. ginseng contains many active components, including ginsenosides, ginseng peptides and ginseng polysaccharides. It has been reported that P. ginseng polysaccharides have immunomodulation, anti-tumor, anti-adhesive, anti-oxidant and hypoglycemic activities (Fu et al., 1994; Lee et al., 2006; Luo & Fang, 2008; Shin et al., 2004; Song et al., 2002; Suzuki & Hikino, 1989).

Since the first report from Ovodov and Solov'eva (1966), many studies have been conducted on P. ginseng polysaccharides regarding purification, structural analysis and bioactivities. It has been reported that P. ginseng polysaccharides contain starch-like polysaccharide and pectin (Ovodov & Solov'eva, 1966). The ginseng starch-like polysaccharide consists of 3-branched α-D-(1,6)-glucans (Luo & Fang, 2008; Oshima, Konno, & Hikino, 1985; Tomoda, Shimada, Konno, & Hikino, 1985; Tomoda, Shimada, Konno, Sugiyama, & Hikino, 1984) and 6-branched α-D-(1,4)-glucans (Fu et al., 1994). The ginseng pectin mainly consists of galactouronic acid (GalA), galactose (Gal) and arabinose (Ara). In addition, it also contains minor amounts of glucose (Glc), rhamnose (Rha), mannose (Man) and glucouronic acid (GlcA). Structural analyses have revealed that P. ginseng pectin contains α-(1,4)-linked galacturonopyranosyl residues, β-(1,3)-, β-(1,4)- and β-(1,6)-linked galactopyranosyl residues and α-(1,3)- and (1,5)-linked arabinofuranosyl residues (Solov'eva et al., 1969; Tomoda, Hirabayashi, Shimizu, Gonda, & Ohara, 1994; Tomoda et al., 1993a, 1993b).

The Changbai Mountain region of China is one of the main regions for commercial P. ginseng harvesting. The relatively large supply of ginseng makes it possible to produce P. ginseng on an industrial scale. During ginseng processing, the valuable water-soluble polysaccharides are usually discarded, despite the fact that they may be widely used in the food industry and medicine. Use of these polysaccharides is restricted for various reasons, where one of the main reasons is that studies on ginseng polysaccharides are not sufficient to support the applications. Although there have been quite a few studies on P. ginseng polysaccharides, there is a lack of understanding of all of the ginseng polysaccharides. Up until now, it has been unclear how many polysaccharide fractions are present in the water-soluble ginseng polysaccharides and what their structure-activity relationships are. Thus, there is a real need to develop a practical procedure based on which ginseng polysaccharides could be completely fractionated into well-defined polysaccharide fractions in sufficient amounts to obtain further insights into their biological activities.

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To exploit ginseng polysaccharides for food and medicinal applications, we recently designed a research program to completely fractionate P. ginseng polysaccharides and then systematically study the bioactivities of each fraction. We now report the first part of the results of the research program, the total fractionation of the water-soluble ginseng polysaccharides by a combination of anion-exchange and gel permeation chromatographies. Furthermore, we also show the macromolecular features and initial immunological activities of these fractions.

2. Experimental

2.1. Materials

The roots of P. ginseng were cultivated and collected from Changbai Mountain, Jilin, China. The DEAE-cellulose, Sepharose CL-6B, Sephadex G-75, α-amylase (E.C.3.2.1.1) from *Bacillus* sp., concanavalin A (ConA) and lipopolysaccharide (LPS) were purchased from Sigma. The pectinase (Pectinex® Ultra SP-L) was obtained from Novozyme Company. All other chemicals were of analytical grade.

2.2. General methods

Total carbohydrate content was determined by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The standards used in this assay were prepared from monosaccharides that constituted the polysaccharide to be tested, according to the sugar composition. Uronic acid contents were determined by the *m*-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973), using galacturonic acid as a standard. Gel permeation and anion-exchange chromatographies were monitored by assaying the total sugar and uronic acid contents. Protein content was determined by the Bradford assay (Sedmark & Grossberg, 1979), using bovine serum albumin as the standard.

2.3. Extraction

The roots of P. ginseng (500 g) were extracted with 7.0 L distilled water at 100 °C for 4 h and filtered through four sheets of gauze. The solid material was extracted twice again under the same conditions. The filtrates were combined, centrifuged to remove water-insoluble materials, concentrated to 600 mL and precipitated by the addition of 95% ethanol (4 volumes). After centrifugation, the precipitate was dried by solvent exchange, first using 95% ethanol, then absolute ethanol and finally ether. Three identical extractions were carried out and the crude polysaccharide was obtained (152.9 g). The crude polysaccharide (150 g) was re-dissolved in distilled water (1500 mL) and treated with Sevag reagent (1:4 *n*-butanol:chloroform, v/v, 375 mL) to remove proteins (Sevag, Lackman, & Smolens, 1938). After precipitation by ethanol and drying by solvent exchange, the deproteined polysaccharide fraction WGP (129 g) was obtained. The procedure for the extraction and fractionation of P. ginseng polysaccharides is shown in Fig. 1.

2.4. Chromatography on DEAE-Cellulose

2.4.1. Analytical chromatography on DEAE-Cellulose

WGP or WGPA (10 mg) was dissolved in distilled water (1 mL) and loaded on a DEAE-Cellulose (Cl^-) column (1.5 × 14 cm) pre-equilibrated with distilled water. The column was eluted first with 50 mL distilled water at 1.0 mL/min and then with a linear gradient from 0.0 to 1.0 M NaCl within 300 mL. The eluate was collected at 4 mL per tube and assayed for total sugar and uronic acid contents.

2.4.2. Semi-preparative chromatography on DEAE-Cellulose

WGP (25 g) was dissolved in distilled water (250 mL) and loaded on a DEAE-Cellulose column (8.0 × 20 cm, Cl^-) pre-equilibrated with distilled water. The column was eluted first with 2 L of distilled water at a flow rate of 25 mL/min to obtain the unbound fraction (WGPN) and then with 2 L of 0.5 M NaCl to obtain the bound fraction (WGPA). The eluate was collected at 50 mL per tube and assayed for the distribution of total sugars and uronic acids. The appropriate fractions were combined, concentrated, dialyzed against distilled water and lyophilized to give the neutral fraction WGPN (15.1 g) and the acidic fraction WGPA (4.3 g).

WGPA (15 g) was dissolved in distilled water (150 mL), loaded on a DEAE-Cellulose column (10 × 20 cm, Cl^-) and eluted by a stepwise gradient of NaCl aqueous solutions (0.0, 0.1, 0.2, 0.3 and 0.5 M) at a flow rate of 40 mL/min. The eluate was collected at 150 mL per tube and assayed as described above. The appropriate fractions were combined, dialyzed and lyophilized to get five frac-

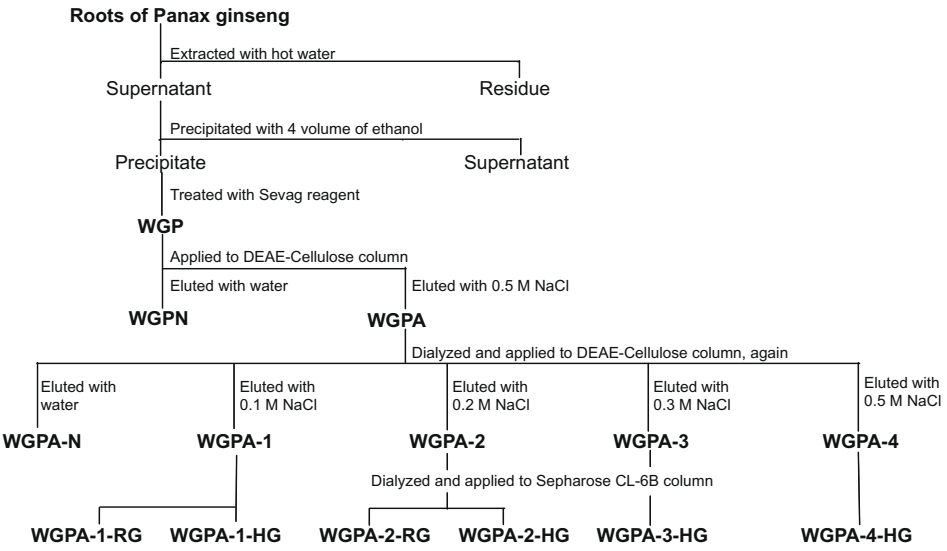

Fig. 1. Fractionation scheme for ginseng polysaccharides by anion-exchange and size-exclusion chromatographies.

tions, WGPA-N (3.8 g), WGPA-1 (1.4 g), WGPA-2 (4.0 g), WGPA-3 (3.0 g) and WGPA-4 (1.9 g).

2.5. Gel permeation chromatography on Sepharose CL-6B

2.5.1. Analytical chromatography on Sepharose CL-6B

Each sample (5–10 mg) was dissolved in 0.15 M NaCl (1 mL), loaded onto a Sepharose CL-6B column (1.5 × 90 cm) and eluted with 0.15 M NaCl at a flow rate of 0.15 mL/min. The eluate was collected at 3 mL per tube and assayed for total sugar and uronic acid contents.

2.5.2. Semi-preparative chromatography on Sepharose CL-6B

WGPA-1, WGPA-2, WGPA-3 and WGPA-4 each was applied to a preparative Sepharose CL-6B column (3.0 × 90 cm) and eluted with 0.15 M NaCl at 0.5 mL/min. The eluate (10 mL per tube) was collected and assayed for total sugar and uronic acid contents. The appropriate fractions were combined, concentrated, dialyzed against distilled water and lyophilized to give WGPA-1-RG, WGPA-1-HG, WGPA-2-RG, WGPA-2-HG, WGPA-3-HG and WGPA-4-HG.

2.6. High performance gel permeation chromatography

High performance gel permeation chromatography was carried out at 40 °C using a TSK-gel G-3000PW$_{XL}$ column (7.8 × 300 mm, TOSOH, Japan) connected to a Shimadzu HPLC system. The column was pre-calibrated with standard dextrans. Twenty microliters of sample (5 mg/mL) was injected, eluted with 0.2 M NaCl at a flow rate of 0.6 mL/min and monitored using a refractive index RID-10A detector (Shimadzu, Tokyo, Japan).

2.7. Sugar composition analysis

Polysaccharide samples (2 mg) were hydrolyzed first with anhydrous methanol containing 1 M HCl at 80 °C for 16 h and then with 2 M TFA at 120 °C for 1 h. The resulting hydrolysates were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) according to the method in the literature (Yang, Zhao, Wang, Wang, & Mei, 2005) and analyzed on a DIKMA Inertsil ODS-3 column (4.6 × 150 mm) connected to a Shimadzu HPLC system (LC-10ATvp pump and SPD-10AVD UV–VIS detector). The PMP derivative (20 μL) was injected, eluted with 82.0% PBS (0.1 M, pH 7.0) and 18.0% acetonitrile (v/v) at a flow rate of 1.0 mL/min and monitored by UV absorbance at 245 nm.

2.8. De-esterification

The de-esterification was performed according to the method of Thibault, Renard, Axelos, Roger, and Crepeau (1993). WGPA-3-HG (50 mg) was dissolved in 10 mL distilled water and cooled to 0 °C using an ice bath. The pH was adjusted to 12.0 by drop-wise addition of 0.1 M NaOH under agitation. The solution was stirred at constant pH and temperature for 24 h. Then 0.1 M HCl was added drop-wise to the mixture to lower the pH to 4.5. After dialysis against distilled water and lyophilization, the de-esterified WGPA-3-HG was obtained.

2.9. Enzymatic hydrolysis

The neutral fractions WGPN and WGPA-N were treated with α-amylase as described in the following procedure. WGPN or WGPA-N (50 mg) was dissolved in distilled water (1 mL) and incubated with α-amylase (25 U) at 37 °C for 16 h. Then the enzyme was inactivated by heating the reaction mixture at 100 °C for 10 min and removed by centrifugation.

The acidic fractions WGPA-1-RG, WGPA-2-RG, WGPA-1-HG, WGPA-2-HG, WGPA-3-HG and WGPA-4-HG were treated with pectinase as follows. Each sample (50 mg) was dissolved in distilled water (1 mL) and incubated with pectinase (10 μL, 260 U) at room temperature for 1 h. Subsequently, the enzyme was inactivated as above and removed by centrifugation.

The hydrolysates of both α-amylase and pectinase were analyzed on Sepharose CL-6B.

2.10. NMR spectra

The ^{13}C NMR spectra were obtained on a Bruker AV600 spectrometer at 150 MHz. The samples (20 mg) were dissolved in D_2O (1 mL, 99.8%) with overnight stirring at room temperature. The spectra were recorded at 25 °C after 57,000 scans.

2.11. In vivo lymphocyte proliferation assay

Male ICR mice (18–22 g) were purchased from the Pharmacology Experimental Center of Jilin University, China. The mice were housed with a light–dark cycle of 12–12 h and had access to food and water *ad lib*. The mice were classified into test and control groups (8 mice/group). The polysaccharide samples WGP, WGPN and WGPA were dissolved in physiological saline and administered intraperitoneally into mice at doses of 1, 5, 10 and 50 mg/kg for 14 consecutive days. Control mice were given physiological saline instead of the polysaccharide solution. The dose volume was 0.2 mL.

After the 14-day treatment, the mice were sacrificed by cervical dislocation. Spleens were taken from the mice of each group and milled in RPMI 1640 medium (Gibco), supplemented with 10% new-born calf serum (Gibco), 100 kU/L benzylpenicillin (Hyclone), 100 mg/L streptomycin (Hyclone) and 10 μM HEPES (Sigma), pH 7.2. Spleen cells (5×10^6/mL) were seeded in a 96-well plate in the presence of ConA (5.0 μg/mL) or LPS (10.0 μg/mL) and incubated at 37 °C for 44 h in a humidified 5% CO_2 incubator. MTT (10 μL, 5 mg/mL) was added to each well and the plate was incubated for another 4 h. After aspirating the supernatant from the wells, DMSO (100 μL) was added for dissolution of the formazan crystals. The absorbance at 570 nm was measured using a microplate reader (Han et al., 1998).

3. Results and discussion

3.1. Isolation of ginseng polysaccharides

The water-soluble polysaccharides were extracted from the roots of P. ginseng with hot water and precipitated by the addition of 4 volumes of 95% ethanol. After deproteination using the Sevag method, a crude polysaccharide fraction, referred to as WGP (for Water-soluble Ginseng Polysaccharides), was obtained with a yield of 10.7% (w/w). WGP contained 77.1% total sugar, 10.0% uronic acid and less than 1% protein. Sugar composition analysis by HPLC indicated that WGP consisted of Glc (77.9%), Gal (6.8%), Ara (4.6%), Man (1.0%), Rha (1.1%) and GalA (8.7%).

WGP showed a wide molecular weight distribution on Sepharose CL-6B. It contained both neutral and acidic polysaccharides. Therefore, a method was designed to fractionate WGP by a combination of anion-exchange and gel permeation chromatographies. The procedure for WGP fractionation is summarized in Fig. 1.

3.2. Fractionation by DEAE-Cellulose chromatography

3.2.1. Preliminary separation on DEAE-Cellulose column

Before fractionation on a semi-preparative scale, we analyzed WPG using an analytical DEAE-Cellulose column (Fig. 2a). The

profile showed an unbound portion eluted with water and a bound portion eluted with a linear gradient of NaCl. It failed to separate all fractions in a single run of DEAE-Cellulose chromatography with a 0.0–0.5 M NaCl linear gradient elution, because the abundant unbound neutral polysaccharides interfered the fine separation of the acidic polysaccharides. Thus, we separated WGP first into two fractions on a semi-preparative scale: an unbound fraction (WGPN) by water elution with a recovery of 60.5% and a bound fraction (WGPA) by 0.5 M NaCl elution with a recovery of 17.1% (Fig. 2b). As expected, the unbound portion WGPN did not contain uronic acid and was not able to be separated further by anion-exchange chromatography. The bound portion WGPA contained uronic acid and could be fractionated continually by a second step of DEAE-cellulose chromatography.

3.2.2. The second run of the separation on DEAE-Cellulose column

In the second run of DEAE-Cellulose chromatography, the bound portion (WGPA) was separated as follows. First, analytical DEAE-Cellulose chromatography of WGPA was carried out (Fig. 2c). The elution profile indicated that WGPA contained a small proportion of neutral polysaccharides and a large proportion of acidic polysaccharides. Compared to the elution profile of WGP (Fig. 2a), the elution profile of WGPA (Fig. 2c) was significantly simpler. However, elution with a linear gradient of NaCl was still unlikely to produce enough separation for a practical pooling strategy. We decided to separate WGPA on a preparative DEAE-Cellulose column by elution with a stepwise gradient of NaCl. With these elution steps, WGPA was separated into five fractions (Fig. 2d): WGPA-N (25.6%), WGPA-1 (9.5%), WGPA-2 (26.8%), WGPA-3 (20.2%) and WGPA-4 (12.7%) corresponding to 0.0, 0.1, 0.2, 0.3 and 0.5 M NaCl, respectively. Total sugar and uronic acid assays indicated that WGPA-N was a neutral polysaccharide fraction and WGPA-1, WGPA-2, WGPA-3 and WGPA-4 were acidic polysaccharide fractions.

3.3. Sugar composition of the fractions separated by DEAE-Cellulose chromatography

The monosaccharide compositions and yields of the collected fractions separated from WGP by DEAE-Cellulose chromatography are listed in Table 1. As shown, WGPN and WGPA-N were composed of neutral sugars, since they are neutral polysaccharides. WGPN contained Glc (95.3%) as the main component and Gal (3.3%) and Ara (1.3%) as minor components. WGPA-N contained Glc (66.3%), Gal (18.0%) and Ara (15.7%). GalA was not detected in WGPA-N, but it eluted together with acidic polysaccharides from the semi-preparative DEAE-Cellulose column. The reason might be that in the previous separation on the DEAE-Cellulose column (Fig. 2b), the water elution step was not long enough, which resulted in some neutral sugars still eluting when the 0.5 M NaCl step began. Alternatively, WGPA-N might contain such a minor amount of GalA that it could not be determined by the HPLC method used in this paper.

WGPA-1, WGPA-2, WGPA-3 and WGPA-4 contained 20.0%, 41.3%, 54.5% and 56.2% GalA, respectively. The elution sequence from the DEAE-Cellulose column was related to the amount of negatively charged sugars and also could be modulated by different levels of methylation, which could modify the overall net charge.

According to the recovery of each fraction in the two separations on DEAE-Cellulose (Table 1), the neutral polysaccharides were estimated to account for ~70% of WGP by weight and the acidic polysaccharides for ~15%.

3.4. Fractionation by Sepharose CL-6B chromatography

All collected fractions from the DEAE-Cellulose column were further separated on a Sepharose CL-6B column. As shown in Fig. 3a, WGP gave a wide distribution, almost from the void volume to the total volume. By the first separation on DEAE-cellulose, the degree of homogeneity of WGPN and WGPA was not significantly

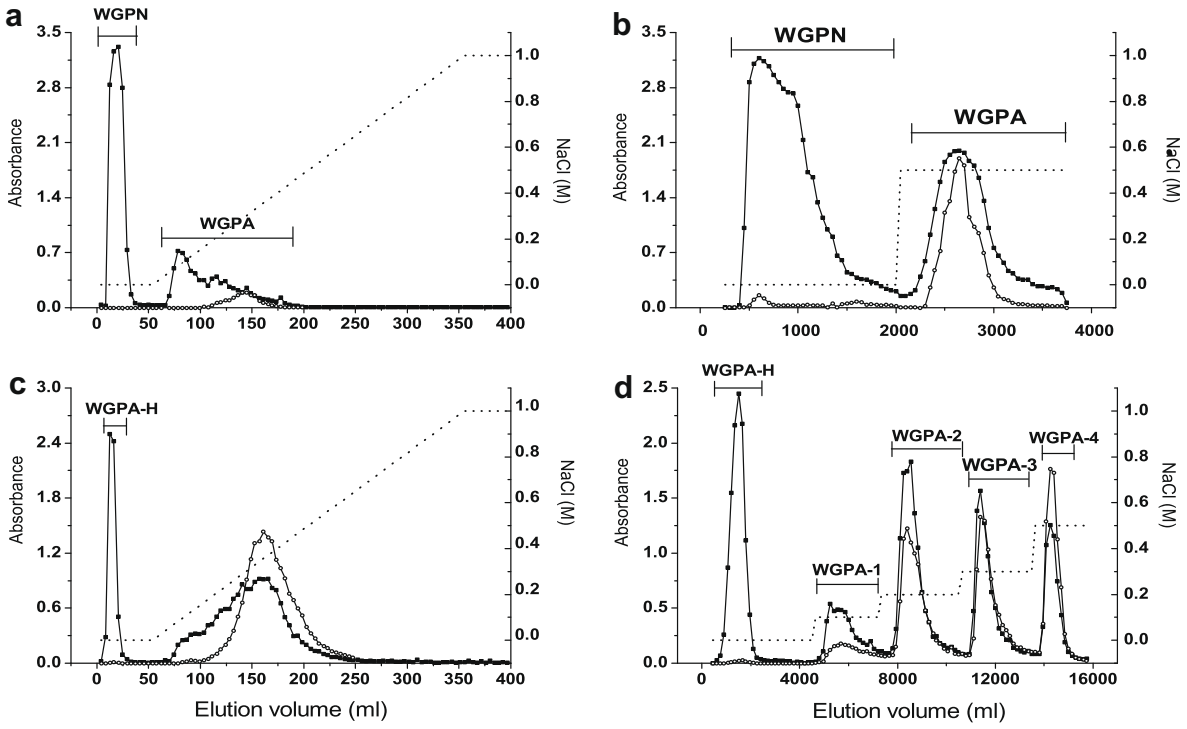

Fig. 2. Elution profiles of WGP and WGPA on DEAE-Cellulose column, eluted by (a and c) a linear gradient and (b and d) a stepwise gradient of NaCl, respectively (total sugars, -■-; uronic acid, -○-).

Table 1
Yield and structural feature of collected fractions.

Fraction	Yield (g%)[a]	Monosaccharide composition (%)							Structure features
		Gal	Glc	Ara	Rha	Man	GalA	GlcA	
WGPN	60.5	3.3	95.3	1.3	–	–	–	–	Starch-like glucans
WGPA-N	25.6	18.0	66.3	15.7	–	–	–	–	Starch-like glucans and arabinogalactans
WGPA	17.1	18.0	18.5	15.5	2.5	–	44.2	1.3	
WGPA-1	9.5	39.1	10.0	24.2	2.4	1.2	20.0	3.2	
WGPA-2	26.8	23.3	3.4	25.2	2.3	–	41.3	4.5	
WGPA-3	20.2	17.1	1.9	21.8	–	0.2	54.5	4.5	
WGPA-4	12.7	14.9	4.3	21.0	–	1.5	52.2	5.9	
WGPA-1-RG	21.6	56.2	3.5	34.0	0.2	2.5	1.8	1.9	Arabinogalactans containing RG-I domains
WGPA-2-RG	20.0	44.4	2.9	40.9	4.1	0.4	5.3	2.0	Arabinogalactans containing RG-I domains
WGPA-1-HG	43.1	15.2	7.6	7.1	1.6	3.6	62.4	2.6	α-(1-4)-GalA backbone (HG)
WGPA-2-HG	53.8	5.1	1.9	4.6	3.0	0.2	83.6	1.6	α-(1-4)-GalA backbone (HG)
WGPA-3-HG	62.8	3.5	1.3	2.2	1.5	–	90.9	0.5	α-(1-4)-GalA backbone (HG)
WGPA-4-HG	31.8	5.9	2.0	–	–	–	92.1	–	α-(1-4)-GalA backbone (HG)

[a] Yield in relation to fraction applied into column.

improved compared to the original WGP fraction. After the second separation step, the water eluate WGPA-N had a similar distribution to that of the precursor (WGPA), showing a polydispersed profile from chromatography on Sepharose CL-6B. However, the acidic fractions WGPA-1, WGPA-2, WGPA-3 and WGPA-4 showed relatively narrow distributions. Thus, the two neutral fractions, WGPN and WGPA-N, were very difficult to further fractionate by gel permeation chromatography, while the four acidic fractions could be further fractionated on Sepharose CL-6B.

Both WGPA-1 and WGPA-2 presented two major molecular populations, one rich in neutral sugars was eluted first and the other rich in uronic acid was eluted later (Fig. 3a). Therefore, WGPA-1 was further fractionated on Sepharose CL-6B to give WGPA-1-RG and WGPA-1-HG with recoveries of 21.6% and 43.1%, respectively. Similarly, WGPA-2 was separated into WGPA-2-RG and WGPA-2-HG with recoveries of 20.0% and 53.8%, respectively. Both WGPA-3 and WGPA-4 contained one main peak highly enriched in uronic acid and were further purified on a Sepharose CL-6B column to give WGPA-3-HG and WGPA-4-HG, respectively.

3.5. Sugar composition of the final fractions

The recoveries and sugar compositions of the collected fractions from the Sepharose CL-6B column are listed in Table 1. Both WGPA-1-RG and WGPA-2-RG were mainly composed of Gal and Ara. In addition, they contained Glc, Rha, Man, GalA and GlcA as minor components. The ratio of Gal to Ara was 1.7:1.0 in WGPA-1-RG and 1.1:1.0 in WGPA-2-RG. The total amount of Gal and Ara accounted for 90.2% of WGPA-1-RG and 84.9% of WGPA-2-RG. The amounts of uronic acid in WGPA-1-RG and WGPA-2-RG were 3.7% and 7.3%, respectively. This slight difference in the level of uronic acid may account for, as one of the factors, the elution order from the DEAE-Cellulose column. WGPA-1-HG, WGPA-2-HG, WGPA-3-HG and WGPA-4-HG were mainly composed of GalA. The order of GalA content was WGPA-1-HG (62.4%) < WGPA-2-HG (83.6%) < WGPA-3-HG (90.9%) < WGPA-4-HG (92.1%), which was in agreement with the elution sequence from the DEAE-Cellulose column.

3.6. Homogeneity and molecular weight

The homogeneity of each fraction purified by the combinatorial procedure was analyzed by Sepharose CL-6B chromatography and HPGPC on a TSK column. On the Sepharose CL-6B column, WGPA-1-RG, WGPA-2-RG, WGPA-1-HG, WGPA-2-HG, WGPA-3-HG and WGPA-4-HG each showed a single and symmetrical narrow peak, and the distribution of total sugars was consistent with that of uronic acid (data not shown). The elution profiles from the TSK column showed a single and symmetrical narrow peak for each fraction (Fig. 3b). These results indicated that WGPA-1-RG, WGPA-1-HG, WGPA-2-RG, WGPA-2-HG, WGPA-3-HG and WGPA-4-HG were homogeneous fractions related to molecular weight. The molecular weights were approximately deduced from the calibration curve of dextran standards by HPGPC to be 3.5×10^3 (WGPA-1-HG), 6.5×10^3 (WGPA-2-HG), 1.6×10^4 (WGPA-3-HG), 4.5×10^4 (WGPA-4-HG), 1.0×10^5 (WGPA-1-RG) and 1.1×10^5 (WGPA-2-RG). HPGPC on the TSK column separated molecules on the basis of their hydrodynamic volume and not on the basis of molar mass. As each of the fractions contained different HG, RG-I and arabinogalactan domains, they have different conformations to which the elution volume is related. Therefore, the molecular weights above are not comparable.

3.7. Features of collected fractions

To get more information about macromolecular features, the obtained fractions were tested with iodine to check for the presence of starch-like glucans and were also subjected to two hydrolytic treatments: hydrolysis with α-amylase, which preferentially cleaves the starch-type polysaccharides, and hydrolysis with pectinase, which cleaves pectin molecules. Moreover, NMR was also used to characterize the structural features of these homogeneous fractions.

3.7.1. Macromolecular features of WGPN and WGPA-N

WGPN and WGPA-N were two neutral fractions and generated a blue color when tested with iodine, which indicated the presence of starch-like glucans. By hydrolysis with α-amylase, WGPN was totally hydrolyzed into small molecules that appeared in the total volume on the Sepharose CL-6B column. However, about 30% of the polysaccharides from WGPA-N remained and mainly consisted of Gal and Ara in a ratio of 1.0:0.9, which was similar to that of WGPA-N. This suggested that the remaining polysaccharides might be the free arabinogalactans from WGPA-N. Based on these results, we deduced that WGPN mainly contained starch-like glucans and WGPA-N contained starch-like glucans and arabinogalactans.

3.7.2. Macromolecular features of WGPA-1-RG and WGPA-2-RG

WGPA-1-RG and WGPA-2-RG contained Gal and Ara as the main sugars detected and Glc, Man, Rha, GalA and GlcA as minor components, suggesting that these fractions might be composed of arabinans, galactans and/or arabinogalactans. The ratios of

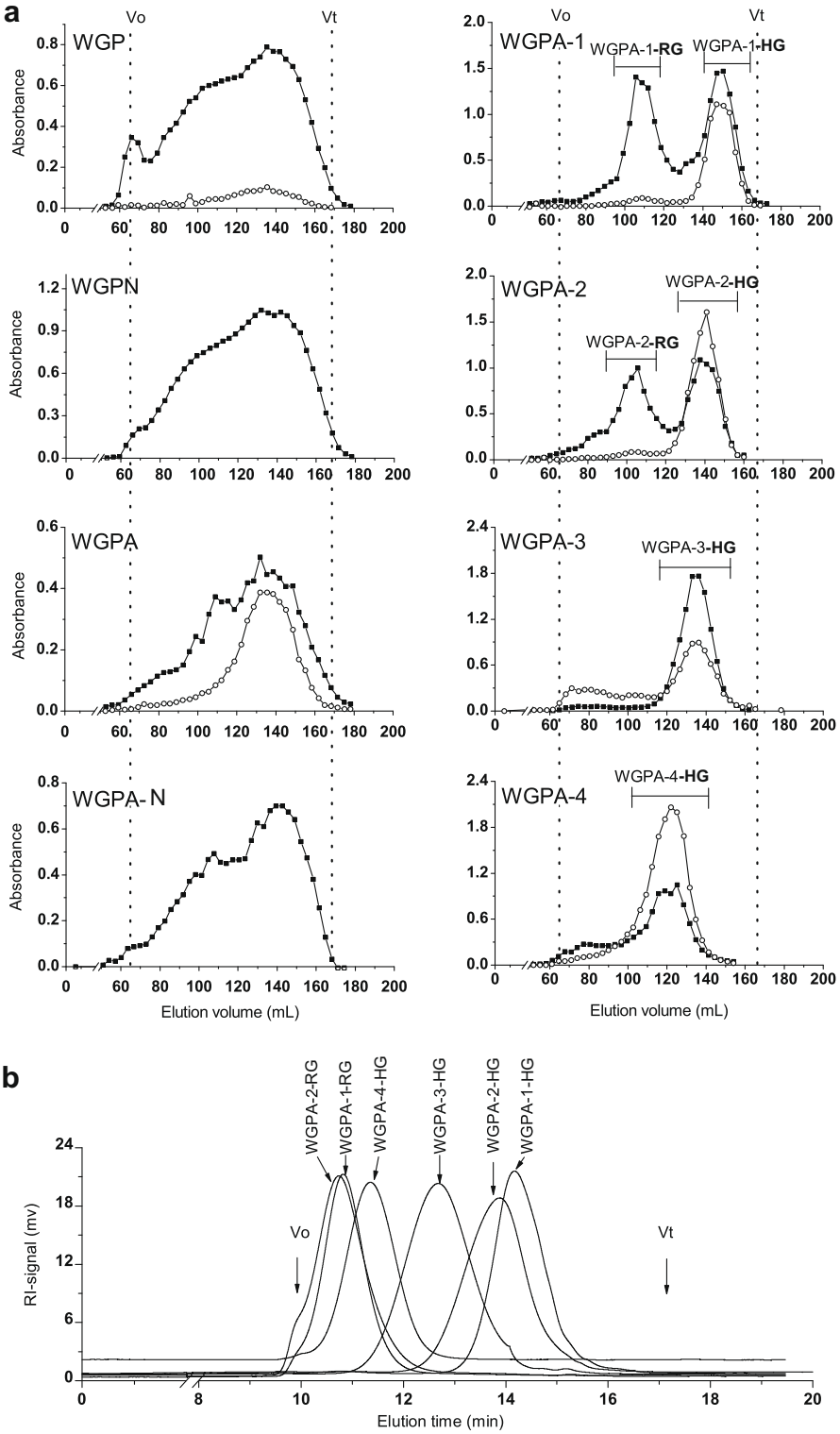

Fig. 3. Molecular weight distributions of ginseng polysaccharide fractions. (a) Sepharose CL-6B profiles of collected fractions from DEAE-Cellulose chromatography (total sugars, -■-; uronic acid, -○-). (b) HPGPC elution profiles of the purified fractions.

Rha/GalA determined for WGPA-1-RG and WGPA-2-RG were 0.11 and 0.77, respectively, which are among the RG-I range from 0.05 to 1.0 defined by Schols and Voragen (1996). This suggested that they might contain RG-I domains. RG-I has been reported to be composed of α-(1,4)-linked D-galacturonic acid and α-(1,2)-linked L-rhamnose, which are alternatively combined with each other in the backbone; and some of the rhamnose residues contained side chains, such as arabinan, galactan and arabinogalactan at 4-O-rhamnose (McNeil, Darvill, & Albersheim, 1980). Low contents of GalA in WGPA-1-RG and WGPA-2-RG indicated that the two fractions contained a very low proportion of rhamnogalacturonan regions. The arabinans, galactans and/or arabinogalactans might be associated with RG-I domains in non-covalent form or as side chains of RG-I.

The ^{13}C NMR spectra of WGPA-1-RG and WGPA-2-RG were quite similar (Fig. 4) and resembled those of arabinogalactans reported in the literature (Gane et al., 1995; Wang, Liu, & Fang, 2005). In the anomeric region, the signals at 110.4, 108.6 and 108.3 ppm were assigned to α-Ara*f*, and the signals at 105.6–103.8 ppm were β-Gal*p*. Although the content of Ara is less than that of Gal in WGPA-1-RG and WGPA-2-RG, the signals for carbons of α-Ara*f* were obviously stronger than those of β-Gal*p*, which suggested that Ara residues were in flexible side chains. Three anomeric carbon signals from Ara residues of WGPA-1-RG and WGPA-2-RG indicated that Ara in these two fractions might be present in three linkage forms, which usually exist in pectic arabinogalactan, including α-(1-5)-Ara*f*, α-(1-3)-Ara*f* and non-reducing terminal α-Ara*f* (Polle, Ovodova, Shashkov, & Ovodov, 2002). Based on the integration of the three signals for C-1 of Ara, the ratios of the three glycosidic linkages were 1.0:0.3:0.2 for WGPA-1-RG and 1.0:0.9:0.7 for WGPA-2-RG, respectively. As seen in Fig. 4, the multiple signals for C-1 of β-Gal*p* indicated that Gal had more linkage forms. These results implied that both WGPA-1-RG and WGPA-2-RG had very complex structures similarly to other pectins. Ara and Gal residues constituted hair regions containing both type I and type II arabinogalactan (Pérez, Rodríguez-Carvajal, & Doco, 2003), in which Gal residues were in the backbone and Ara residues were side chains or linked at non-reducing ends. The signal at 24.5 ppm indicated the presence of O-acetyl groups. The degree of acetylation in WGPA-2-RG was higher than WGPA-1-RG. Trace resonances at 17.8 and 176.9 ppm were assigned to C-6 in Rha residues and C-6 in uronic acids, which was consistent with the detected sugar compositions.

3.7.3. Macromolecular features of WGPA-1-HG, WGPA-2-HG, WGPA-3-HG and WGPA-4-HG

WGPA-1-HG, WGPA-2-HG, WGPA-3-HG and WGPA-4-HG contained high amounts of GalA and were totally hydrolyzed by pec-

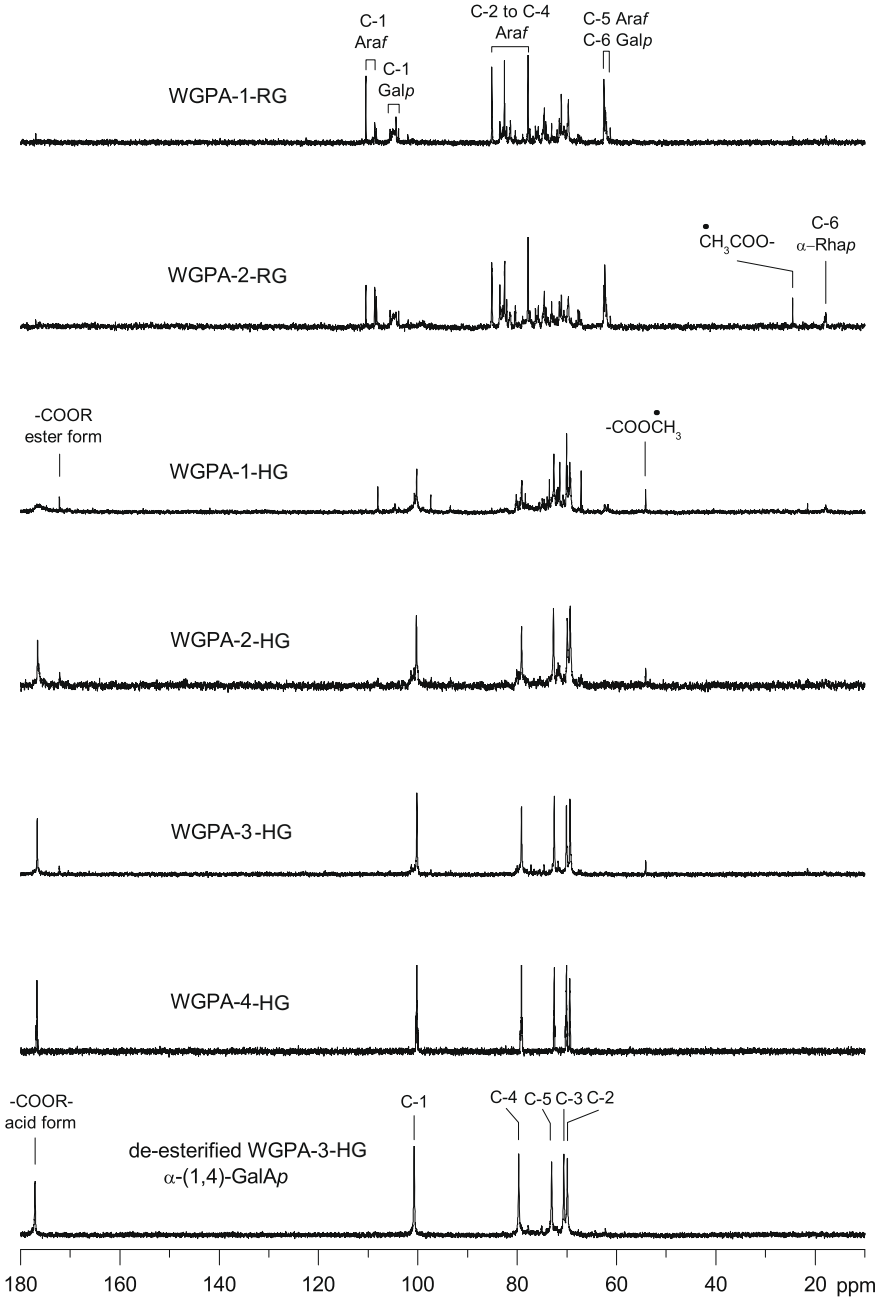

Fig. 4. ^{13}C NMR spectra of the homogeneous fractions.

tinase. The pectinase used is a pectolytic cocktail that has many pectinase activities on the different pectic domains, such as HG-rich and RG-I-rich regions. This result indicated that fractions WGPA-1-HG to WGPA-4-HG are composed of pectic domains.

The NMR spectra (Fig. 4) of these four fractions with high amounts of GalA showed predominant resonances from carbons in free and methyl-esterified galactopyranosyl uronic acid residues in accordance with those previously observed in HG-rich pectins (Catoire, Goldberg, Pierron, Morvan, & Hervé Du Penhoat, 1998). The signals for C-1–C-6 of unesterified GalA residues appeared at 100.7 (C-1), 69.9 (C-2), 70.6 (C-3), 79.7 (C-4), 73.1 (C-5) and 177.1 (C-6) ppm. C-1–C-5 from esterified GalA residues did not give obvious signals because of the low degree of esterification, while the methyl group and methyl ester carbonyl carbons showed the diagnostic signals at 54.1 and 172.2 ppm, respectively. After de-esterification, these two signals disappeared in the NMR spectrum of de-esterified WGPA-3-HG (Fig. 4). Based on the integration of the signals for the carboxyl and ester carbonyl carbons, the degree of esterification (DE) was estimated to be ~30%, ~20%, ~10% and <5% for WGPA-1-HG, WGPA-2-HG, WGPA-3-HG and WGPA-4-HG, respectively. The NMR spectrum of WGPA-1-HG was more complex than the other three fractions because of its relatively high esterification and more neutral sugars. The signal from the carboxyl carbons of free galacturonic acid residues in WGPA-1-HG was a broad peak that was also observed in other pectins (Catoire et al., 1998). This may be due to the influence of neighboring free and esterified galacturonic acid residues. In addition, a weak signal from C-6 in rhamnopyranosyl residues and some other trace signals from neutral sugar residues were observed in the spectrum of WGPA-1-HG, while these signals were not observed in the spectra of WGPA-2-HG, WGPA-3-HG and WGPA-4-HG, which is consistent with the sugar composition analysis.

Combining the pectolytic hydrolysis and NMR analysis, we deduced that the acidic fractions, WGPA-1-HG–WGPA-4-HG, are HG-rich pectins with different degrees of methyl-esterification, i.e., a linear α-(1,4)-linked D-galacturonic acid. A small amount of neutral sugars that existed in these fractions might constitute RG-I domains linked to HG domains in covalent or non-covalent forms. The amount of HG domains increased from WGPA-1-HG to WGPA-4-HG, while the amount of RG-I domains decreased from WGPA-1-HG to WGPA-4-HG. Pectin structure generally encompasses HG, RG-I and RG-II domains (Vidal, Williams, Doco, Moutounet, & Pellerin, 2003; Yapo, Lerouge, Thibault, & Ralet, 2007). Here, HG-rich pectins and RG-I-rich pectins were found in ginseng polysaccharides. However, RG-II domains were not detected by determining their diagnostic sugars (unpublished result). Perhaps the amount of RG-II domains was too low to be detected by the method used.

3.8. In vivo splenic lymphocyte proliferation

In this paper, the native polysaccharide fraction WGP, the fractionated neutral fraction WGPN and the acidic fraction WGPA were tested for ConA- and LPS-induced mitogen activity of lymphocytes in vivo. WGP significantly enhanced T lymphocyte proliferation at 10 mg/kg and significantly enhanced both T and B lymphocyte proliferation at 50 mg/kg. After separation, both WGPN and WGPA induced T and B cell proliferation. However, WGPN seemed to have better T and B lymphocyte proliferation-enhancing activity than WGPA did (Table 2). A dose-dependent induction of T and B cell proliferation in response to WGP, WGPN and WGPA in the tested dose range could be observed (Table 2).

Lymphocytes are important immune cells and play a pivotal role in immune response. These cells are able to produce many kinds of cytokines after differentiation and activation. Stimulating proliferation of lymphocytes results in releasing more cytokines, which may account for the anti-tumor activity of ginseng polysaccharides. The results that the neutral polysaccharides were more active as mitogens than the acidic polysaccharides is consistent with the finding reported in the literature that the neutral ginseng polysaccharides had better anti-tumor activity than the acidic polysaccharides (Fu et al., 1994).

Table 2
Effects of WGP, WGPA and WGPN on ConA- or LPS-induced lymphocyte proliferation in mouse splenocytes in vivo[a].

	Dose (mg/kg)	Lymphocyte	
		T cell (A570)	B cell (A570)
Control	NaCl	0.289 ± 0.02	0.362 ± 0.07
WGP	1	0.359 ± 0.02	0.467 ± 0.03
	5	0.389 ± 0.02	0.554 ± 0.02
	10	0.484 ± 0.02[b]	0.576 ± 0.01
	50	0.588 ± 0.02[c]	0.601 ± 0.02[b]
WGPA	1	0.367 ± 0.02	0.499 ± 0.02
	5	0.393 ± 0.02	0.566 ± 0.02
	10	0.491 ± 0.03[b]	0.593 ± 0.01
	50	0.599 ± 0.03[c]	0.652 ± 0.02[b]
WGPN	1	0.381 ± 0.01	0.520 ± 0.02
	5	0.407 ± 0.02	0.589 ± 0.02
	10	0.579 ± 0.02[c]	0.772 ± 0.02[c]
	50	0.597 ± 0.01[c]	0.783 ± 0.01[c]

[a] Each value represents the mean ± S.D. from five mice in each group.
[b] $P < 0.05$, significantly different from the control.
[c] $P < 0.01$, significantly different from the control.

WGPA contained RG-I-rich pectins and HG-rich pectins. Regarding proliferation enhancement of T and B cells, there are several reports that RG-I-rich pectins have more potent activities compared to the HG-rich pectins (Inngjerdingen et al., 2007; Nergard et al., 2006; Paulsen & Barsett, 2005). Therefore, we deduced that RG-I-rich pectins (WGPA-1-RG and WGPA2N) from P. ginseng would be more active in stimulating proliferation of T and B cells than HG-rich pectins (WGPA-1-HG, WGPA-2-HG, WGPA-3-HG and WGPA-4-HG). The RG-I-rich pectins of WGPA might be the main cause of the stimulation of T and B cell proliferation. Until now, no clear information has been obtained on the structure-activity relationship of polysaccharides. However, high molecular weight pectins appear to have more bioactivities, and side chain branches are needed for optimal activity (Inngjerdingen et al., 2007; Lin, 2005). The molecular weights of P. ginseng RG-I-rich pectins, WGPA-1-RG and WGPA2N, were estimated to be $1.0–1.1 \times 10^5$, which is similar to those of the pectins that showed significant proliferation stimulating activities on T and B cells (Duan, Wang, Dong, Fang, & Li, 2003; Inngjerdingen et al., 2007; Zheng, Wang, & Fang, 2006). Studies on the structure-activity relationship of P. ginseng polysaccharides are in progress.

4. Conclusion

In the present work, ginseng polysaccharides were completely fractionated into eight fractions by the established combinatory procedure. Among the eight fractions, there are two neutral fractions, WGPN and WGPA-N, and six acidic fractions, WGPA-1-RG, WGPA-2-RG, WGPA-1-HG, WGPA-2-HG, WGPA-3-HG and WGPA-4-HG. Investigation of the macromolecular features revealed that the water-soluble polysaccharides isolated from P. ginseng contained starch-like polysaccharides, pectic arabinogalactans and RG-I-rich and HG-rich pectins.

The initial bioassay indicated that ginseng polysaccharides stimulated proliferation of both T and B lymphocytes. The neutral polysaccharides may be more potent stimulators than the acidic polysaccharides. More work is needed to further fractionate the

neutral polysaccharides into homogeneous fractions. In addition, further investigation should be done to better characterize each of the collected fractions and evaluate their bioactivities so that the structure-activity relationships can be discussed. Such research studies are in progress in our research group.

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