ELSEVIER

Carbohydrate Polymers 46 (2001) 125–134

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Characterization of pectic polysaccharides having intestinal immune system modulating activity from rhizomes of *Atractylodes lancea* DC

K.-W. Yu[a,b], H. Kiyohara[a], T. Matsumoto[a], H.-C. Yang[b], H. Yamada[a,*]

[a]*Oriental Medicine Research Center, The Kitasato Institute, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8642, Japan*
[b]*Korea University, Graduate School of Biotechnology, #1, 5-ka, Anam-dong, Sungbuk-ku, Seoul 136-701, South Korea*

Received 21 December 1999; revised 26 June 2000; accepted 31 August 2000

Abstract

Two acidic polysaccharides (ALR-a and ALR-b, former names ALR-5IIb-2-2 and 5IIc-3-1, respectively; although ALR-5IIb-2-2 and 5IIc-3-1 were used as their abbreviations in a previous paper (Planta Med., 64 (1998) 714), here the polysaccharides have been abbreviated to ALR-a and ALR-b, respectively, in order to avoid complexity) have been purified from rhizomes of *Atractylodes lancea* DC. as intestinal immune system modulating polysaccharides (Planta Med., 64 (1998) 714). Endo-α-D-(1 → 4)-polygalacturonase digestion of ALR-b gave large proportions of a fragment (PG-1) eluted in the void volume, and the lowest-molecular-weight fraction (PG-3) in addition to a small proportion of intermediate fraction (PG-2). Component sugar and methylation analyses using base-catalyzed β-elimination indicated that PG-1 consisted of a rhamnogalacturonan core with side chains rich in neutral sugars and that PG-3 mainly contained (1 → 4)-linked galacturono-oligosaccharides. PG-2 comprised characteristic component sugars such as 2-Me-Fuc, 2-Me-Xyl, apiose (Api) and aceric acid (AceA), but PG-2 lacked some glycosidic linkages compared with those of the typical rhamnogalacturonan II (RG-II). PG-2 showed potent intestinal immune system modulating activity, but PG-1 and galacturono-oligosaccharides in PG-3 had no activity.

Further gel filtration and anion-exchange chromatography of ALR-a gave a potent intestinal immune system modulating polysaccharide (ALR-a-Bb). Component sugar and methylation analyses indicated that ALR-a-Bb also comprised unusual component sugars characteristic in RG-II as well as PG-2 derived from ALR-b.

ALR-a-Bb or PG-2 from ALR-b little affected directly the proliferation of bone marrow cells. PG-2 from ALR-b expressed similar significant intestinal immune system modulating activity to RG-II (GL-RI) isolated from leaves of *Panax ginseng* C.A. Meyer, but RG-II obtained from a pharmacologically active pectin (bupleuran 2IIb) of *Bupleurum falcatum* L. had no activity.

Keywords: *Atractylodes lancea*; Pectic polysaccharide; Intestinal immune system modulating activity; Active site

1. Introduction

The inner surface of the intestinal tract contains a large area of mucosal membrane, which is continuously exposed to various substances in the intestinal lumen. Gut-associated lymphoreticular tissues (GALT) exist on the intestinal mucosal sites, and play important roles in host defense such as IgA production in the intestinal immune system (Stephen & Martin, 1994). GALT are composed of lamina propria aggregates, Peyer's patches, mesenteric lymph nodes, which contain lymphocytes (Bienenstock & Befus, 1980). The part of GALT composed of Peyer's patches is, in particular, known to be an inductive site for IgA in the mucosal immune system. After lymphocytes in Peyer's patches interact with antigens, they differentiate and mature in the germinal center of the lymphoid follicles in the patches. The mature lymphocytes further migrate to reach systemic circulation through the mesenteric lymph nodes, and are delivered to peripheral lymph nodes, spleen and other mucosal sites. Therefore, it is thought that the intestinal immune system not only acts as the defense system in the mucosa but also regulates the systemic immune system through Peyer's patches, thus preventing allergic reactions and autoimmune diseases (Stephen & Martin, 1994). Since most traditional herbal medicines have been taken orally, there is a possibility that these medicines express their clinical effects through the intestinal immune system.

Hong, Matsumoto, Kiyohara and Yamada (1998) have found that one of the Japanese herbal (Kampo) medicines, Juzen-Taiho-To (TJ-48, Si-Quan-Da-Bu-Tang in Chinese) shows intestinal immune system modulating activity in vitro and in vivo through the activation of T cells in Peyer's patches to stimulate secretion of hematopoietic growth

* Corresponding author. Tel.: +81-3-5791-6121; fax: +81-3-3445-1351.
E-mail address: yamada-h@kitasato.or.jp (H. Yamada).

0144-8617/01/$ - see front matter
PII: S0144-8617(00)00292-7

factors. In the previous study, it has been found that one of the component herbs of TJ-48, rhizomes of *Atractylodes lancea* DC. mainly contribute to the expression of the intestinal immune system modulating activity of TJ-48 (Yu, Kiyohara, Matsumoto, Yang & Yamada, 1998). Three polysaccharides (ALR-5IIa-1-1, ALR-a and ALR-b) have been purified from rhizomes of *A. lancea* DC. as intestinal immune system modulating polysaccharides (Yu et al., 1998). One of the active polysaccharides (ALR-5IIa-1-1) has been characterized as arabino-3,6-galactan (Yu et al., 1998). However, other active polysaccharides (ALR-a and ALR-b) have not been characterized. The present paper deals with the characterization of ALR-a and ALR-b with intestinal immune system modulating activity from rhizomes of *A. lancea* DC.

2. Materials and methods

2.1. Materials

Rhizomes of *Atractylodes lancea* DC., which were cultivated at Huabei Province in China, were purchased commercially from Tochimoto-Tenkaido Co. Ltd (Osaka, Japan). Bio-gel P-30 was obtained from Bio-Rad, and Sep-pak C_{18} cartridges from Waters Associates. Mono-Q column (HR 5/5), was obtained from Pharmacia Biotech. (Sweden). RPMI 1640 medium and Hank's balanced salt solution (HBSS) were obtained from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Cell Culture Laboratories (Cleveland, OH) and Alamar Blue™ from Alamar Bio-Sciences Inc. (Sacramento, CA).

2.2. General method

Total carbohydrate and uronic acid were determined by phenol–sulfuric acid (Dubois, Gilles, Hamilton, Revers & Smith, 1956) and *m*-hydroxybiphenyl methods (Blumenkrantz & Asboe-Hansen, 1973), respectively, by using Gal and GalA as the respective standards. The contents of Kdo and Dha were determined colorimetrically by a modified thiobarbituric acid (TBA) method (Karkhanis, Zeltner, Jackson & Carlo, 1978). Component sugars of polysaccharides were analyzed as alditol acetates after hydrolysis of polysaccharides with 2 M TFA for 1.5 h at 121°C (Johnes & Albersheim, 1972) and analyzed by GLC as described previously (Zhao, Kiyohara, Yamada, Takemoto & Kawamura, 1991). The composition of Kdo and Dha were determined by GLC as alditol acetates according to the modified methods of Stevenson, Darvill and Albersheim (1988a), and York, Darvill, McNeil and Albersheim (1985). Briefly, the samples were hydrolyzed partially under a mild acid condition (0.1 M TFA, 100°C, 1 h), and the resulting hydrolyzates were reduced with $NaBD_4$. The samples were then treated with 2 M TFA for 1 h at 121°C, and the resulting lactones were reduced with $NaBD_4$ under neutral conditions. For Dha, the products were further treated with 2 M TFA 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). HPLC was performed on a Shimadzu LC-6A equipped with combined columns of Asahi-pak GS-510 + 320 or GS-320 + 220 (Asahi Chemical Industry Co. Ltd, Japan) and a 0.2 M NaCl was used as an eluent. Molecular weights of polysaccharides were estimated from a calibration curve constructed for standard pullulans (P-400, 200, 100, 50, 20 and 5, Showa Denko Co. Ltd, Japan). Single radial gel diffusion using the β-D-glucosyl-Yariv antigen was performed according to the method of Holst and Clarke (1985).

2.3. Purification of intestinal immune system modulating polysaccharides

A crude polysaccharide fraction (ALR-5) was prepared from rhizomes of *A. lancea* DC. by hot water extraction, precipitation with ethanol and dialysis (Yu et al., 1998). ALR-5IIb and 5IIc were purified as active fractions from ALR-5 by DEAE-Sepharose CL-6B (HCO_3^-) on a linear gradient mode using NH_4HCO_3 (Yu et al., 1998). ALR-5IIb gave the fraction (ALR-5IIb-2) eluted in the inner volume by gel filtration on Sepharose CL-6B, and was further fractionated by Sephacryl S-200 (0.2 M NaCl) to obtain the active fraction (ALR-a; yield from ALR-5, 0.36%). ALR-a was further fractionated on Bio-gel P-30 with 50mM acetate buffer (pH 5.2) and ALR-a-A and ALR-a-B were obtained as intermediate and lower-molecular-weight fractions, respectively (yield from ALR-a: ALR-a-A, 41.3%; ALR-a-B, 32.1%). ALR-a-B (12 mg) was fractionated by the FPLC system using the Mono-Q column (HR 5/5). The unabsorbed fraction was washed with H_2O, and then the bound polysaccharides were eluted with a linear gradient of 0–700 mM NaCl. Two absorbed fractions (ALR-a-Ba and ALR-a-Bb) were obtained as lyophilizates after dialysis using Spectra/Por (MWCO 1000, Spectram Medical Industries Inc., USA) (yields from ALR-a-B: ALR-a-Ba, 38.8%; ALR-a-Bb, 41.6%). ALR-5IIc was gradually fractionated on Sephacryl S-300 and S-200 to obtain an active polysaccharide (ALR-b) as described previously (Yu et al., 1998) (yield from ALR-5, 0.41%).

2.4. Endo-α-D-(1 → 4)-polygalacturonase digestion

(a) *Purification of the enzyme*: Endo-α-D-(1 → 4)-polygalacturonase was purified from the dried leaves of *Panax ginseng* C.A. Meyer, which were cultivated in Chang Bai mountain, Jilin, China. The crude enzyme preparation was prepared by precipitation with 80% $(NH_4)_2SO_4$ from 1 M NaCl extract of the leaves. The crude enzyme preparation was fractionated by affinity chromatography on Sepharose 6B, cation-exchange chromatography on Mono-S and gel filtration on Sephadex

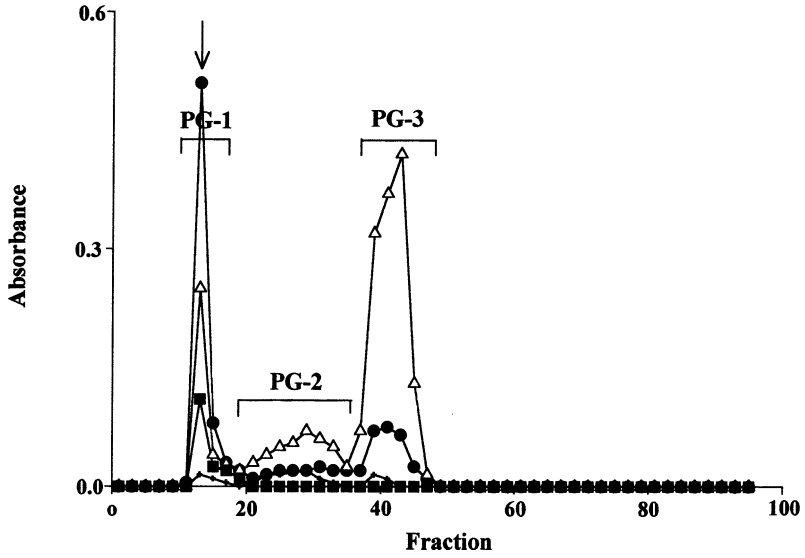

Fig. 1. Elution profile of digestion products of ALR-b with endo-α-D-(1 → 4)-polygalacturonase by gel filtration on Bio-gel P-30. The arrow in the figure indicates the elution positions of intact ALR-b. (●) Carbohydrate (490 nm); (△) uronic acid (520 nm); (■) UV absorption (280 nm); (+) thiobarbituric acid-positive material (Kdo/Dha, 548 nm).

G-75 to purify endo-α-D-(1 → 4)-polygalacturonase (details of purification and properties will be reported elsewhere).

(b) *Digestion*: After ALR-a-B (10 mg) and ALR-b (100 mg) each were de-esterified with 0.1 M NaOH (1 and 10 ml, respectively, room temperature, 2 h) and neutralized with acetic acid, the products were digested with endo-α-D-(1 → 4)-polygalacturonase from leaves of *P. ginseng* (0.3 and 1.5 nkat) in 20 mM acetate buffer (pH 4.34, 1 and 10 ml) for 3 days. The digestion products were desalted by using an electrophoretic dialyzer (Microacylizer, Asahi Chemical Industry Co. Ltd, Japan), and fractionated on the respective columns (2 × 50 and 2.6 × 90 cm) of Bio-gel P-30 with 50 mM acetate buffer (pH 5.2). The digestion product from ALR-a-B gave only the fraction eluted in the same position of ALR-a-B whereas that of ALR-b gave the fraction eluted in the void volume (PG-1), intermediate fraction (PG-2) and the lowest-molecular-weight fraction (PG-3) on Bio-gel P-30. PG-2 was further purified by rechromatography on Bio-gel P-30 (yield: PG-1, 20.4%; PG-2, 10.0%; and PG-3, 28.8%).

2.5. Methylation analysis

Methylation analysis was performed according to the method of Hakomori (1964), and the methylated products were recovered by the modified procedure of Waeghe, Darvill, McNeil and Albersheim (1983). Uronic acids in the methylated polysaccharides were reduced by $LiB(C_2H_5)_3D$ in THF (Super-Deuteride, Aldrich, 1 ml, room temperature, 1 h) (York, Darvill, McNeil, Stevenson & Albersheim, 1986). The resulting partially methylated alditol acetates were analyzed by GLC and GLC-EIMS using an SP-2380 capillary column as described previously (Zhao et al., 1991). They were identified by their fragment ions and relative retention times, and their molar ratios were estimated from the peak areas and response factors (Sweet, Shapiro & Albersheim, 1975). Base-catalyzed β-elimination of the methylated polysaccharides was performed according to the methods of Lindberg, Lönngren and Thompson (1973) and McNeil, Darvill and Albersheim (1982). To a solution in DMSO (1 ml) of dry methylated PG-1 (0.5 mg), which was obtained from ALR-b, was added methylsulfinyl carbanion, and the mixture was stirred for 24 h at room temperature. To one-third of the solution was added 10% AcOH, and the products were recovered by Sep-pak C_{18} cartridge. The products were derived into partially methylated alditol acetates, and analyzed by GLC and GLC-MS in order to know glycosidic linkages (R_1) attached to position 4 of 4-linked hexuronic acid. To the remaining solution an excess of C_2H_5I was added, and the mixture was stirred overnight at room temperature. After C_2H_5I was evaporated, the products were recovered by using a Sep-pak C_{18} cartridge as described above. The products were hydrolyzed and derived into alditol acetates to determine the glycosidic linkages (R_2) which were attached with 4-linked hexuronic acid.

2.6. Intestinal immune system modulating activity

The activity was measured according to the procedure of Hong et al. (1998). Briefly, 200 μl of aliquots of Peyer's patch cells suspension (2×10^6 cells/ml in RPMI 1640 medium supplemented with 5% FBS (RPMI-1640-FBS)), which was prepared from a small intestine of C3H/HeJ mice (6–8 weeks old, SLC, Shizuoka, Japan), was cultured with the test samples for 5–6 days at 37°C in a humidified atmosphere of 5% CO_2–95% air. The resulting culture supernatant (50 μl) was further incubated with bone marrow

Table 1
Chemical properties of ALR-a-Bb, ALR-b and fragments (PG-1, PG-2 and PG-3) obtained from ALR-b by endo-α-D-(1 → 4)-polygalacturonase digestion

	ALR-a-Bb	ALR-b	PG-1	PG-2	PG-3
Molecular weight	2,800	16,000	n.d.[a]	14,000 7800	n.d. n.d.
Carbohydrate content (%)	29.3	24.4	n.d.	n.d.	n.d.
Uronic acid content (%)	84.3	57.8	n.d.	n.d.	n.d.
Protein content (%)	6.8	13.4	n.d.	n.d.	n.d.
Component sugar (mol.%)					
Ara	6.8	26.5	45.1	10.5	0.3
Xyl	0.2	1.3	1.8	3.7	0.1
Rha	5.8	10.1	10.1	14.6	0.1
Fuc	2.7	0.3	0.1	5.0	–
Man	0.8	1.9	2.4	4.9	1.9
Gal	4.6	13.5	24.8	8.4	0.2
Glc	1.3	0.4	0.8	3.5	trace
GlcA	12.4	7.0	4.9	3.9	12.8
GalA	50.8	40.4	10.1	24.4	84.7
2-Me-Xyl	1.1	0.5	–	3.1	trace
2-Me-Fuc	2.1	0.6	–	4.1	trace
Api	4.0	1.3	–	3.8	trace
AceA	2.1	n.d.	–	4.3	trace
Kdo	2.1	n.d.	–	3.3	n.d.
Dha	3.2	n.d.	–	2.7	n.d.

[a] Not determined.

cells suspension (2.5×105 cells/ml in RPMI-1640-FBS, 100 μl) from C3H/HeJ mice for 6 days by the same culture condition used in the culture of Peyer's patch cells in order to evaluate ability to growth on bone marrow cells. After 20 μl of Alamar Blue™ solution was added to each well, to count cell numbers the fluorescence intensity was measured during incubation for over 5 h. A Fluoroskan II (Labsystems) at an excitation wavelength of 544 nm and emission wavelength of 590 nm was used. (Pagé, Pagé & Noël, 1993). The differences between the control (a cell number of bone marrow cells cultured with supernatant of Peyer's patch cells which were incubated with water) and the treatment in the experiments were tested for statistical significance using Student's *t*-test. A value of $p < 0.05$ was considered to show that the test sample have statistically significant intestinal immune system modulating activity.

In order to test a direct effect on the proliferation of bone marrow cells, bone marrow cell suspension (2.5×10^5 cells/ml in RPMI-1640-FBS) was incubated with or without the test samples for 6 days, and the proliferation was measured by using Alamar Blue as above.

3. Results

ALR-b was digested with endo-α-D-(1 → 4)-polygalacturonase from leaves of *P. ginseng* C.A. Meyer after de-esterification, and the products were fractionated on Biogel P-30. ALR-b gave a fraction (PG-1) eluted in the void volume and the lowest-molecular-weight fraction (PG-3) as major fractions, and an intermediate fraction (PG-2) as minor fraction (Fig. 1). PG-1 mainly contained Ara, Gal, Rha and GalA, whereas PG-3 mainly contained GalA (Table 1). Although PG-2 mainly contained Ara, Rha and GalA, it also contained 2-Me-Fuc, 2-Me-Xyl, Api, AceA, Kdo and Dha. PG-3 also consisted mainly of GlcA in addition to traces of 2-Me-Fuc, 2-Me-Xyl and Api (Table 1). Methylation analysis suggested that PG-1 comprised a relatively significant proportion of terminal Ara*f* and 4- or 5-linked and 3,4- or 3,5-branched Ara, in addition to 2-linked Rha, 2,4-linked Rha, 4-linked Gal and 4-linked GalA (Table 2). The methylated product of PG-1 was also subjected to base-catalyzed β-elimination using methylsulfinyl carbanion. After the elimination, 2-linked and 2,4-branched Rha significantly decreased in addition to terminal Gal (Table 2, $R_1 \rightarrow$ HexA). It was also shown that 2-mono-*O*-ethyl-3-tri-*O*-methylrhamnitol triacetate and 6-mono-*O*-ethyl-2,3,4-tri-*O*-methylgalactitol diacetate were detected after β-elimination followed by ethylation (Table 2, HexA $\rightarrow R_2$). These results indicate that parts of 2-linked Rha and 2,4-branched Rha are attached to position 4 of GalA in PG-1, and that 4-linked GalA was linked to position 2 of 2,4-branched Rha, suggesting that PG-1 is a rhamnogalacturon core with side chains ("ramified" region in pectic polysaccharide) consisting of a rhamnogalacturonan core with side chains. Methylation analysis indicated that PG-2 consisted of various glycosyl linkages such as terminal Ara*f*, 3,4- or 3,5-branched Ara, terminal and 2,4-branched Rha, terminal Gal, 3′-linked Api, and terminal, 4-linked and 3,4-branched GalA (Table 2). PG-3 mainly comprised terminal and 4-linked GalA, and HPAEC-PAD analysis suggested that it consisted of

Table 2
Methylation analysis of fragments (PG-1 and PG-2) from ALR-b by digestion with endo-α-D-(1 → 4)-polygalacturonase and linkage composition of PG-1 before and after base-catalyzed β-elimination of uronic acid

Glycosyl residue	Position of OEt group	Deduced glycosidic linkage	mol.%			
			PG-1			PG-2
			Before elimination	After elimination		
				$R_1 \rightarrow$ HexA	HexA $\rightarrow R_2$	
Ara		Terminal (*f*)	22.0	33.6	31.9	9.5
		4 or 5	11.3	18.7	15.7	2.0
		3,4 or 3,5	17.2	21.2	17.7	1.6[a]
Xyl		terminal (*p*)	1.1	–	1.0	4.6[b]
Rha		terminal	0.9	0.1	–	6.6
		3	0.7	0.2	0.8	2.8
		2	5.6	0.6	1.7	2.6
		2,4	6.3	0.7	1.5	6.4
	2	2,4	–	–	1.7	–
		2,3	–	–	–	1.2
Fuc		Terminal	1.8	3.7	1.9	3.3[c]
		3	0.6	0.9	1.0	–
		3,4	0.2	0.2	–	3.5
Man		3	2.1	1.9	1.6	1.1
Gal		Terminal	4.6	2.3	5.2	8.4
		6 (*p*)	2.7	1.9	1.7	–
	6	6	–	–	1.0	
		4	7.1	7.1	7.6	3.2
		2,4	–	–	–	3.0
		3,6	3.8	4.3	3.6	–
		4,6	–	0.5	0.8	–
Glc		Terminal (*f*)	1.0	1.4	2.6	3.6
		4	–	–	–	1.5
		2,6	0.1	0.1	0.3	1.7
		3,4,6	–	0.5	0.3	
Api		3′	–	–	–	6.7
GalA		terminal	–	n.d.[d]	n.d.	6.4
		4	7.7	n.d.	n.d.	11.7
		3,4	0.3	n.d.	n.d.	3.6
GlcA		2	0.2	0.2	0.5	2.0
		2,4	2.7	n.d.	n.d.	3.4

[a] This arabinosyl residue might be 2,3-branched.
[b] Parts of terminal Xyl might be terminal 2-Me-Xyl.
[c] Parts of terminal Fuc might be terminal 2-Me-Fuc.
[d] Not determined.

α-D-(1 → 4)-linked oligogalacturonides below d.p. 4 (data not shown). The molecular weight of PG-2 was estimated by HPLC, and gave two peaks having 14,000 and 7,800 Da. When the digestion products (PG-1, -2 and -3) from ALR-b were tested for intestinal immune system modulating activity, PG-2 and PG-3 showed more potent activity than ALR-b even at a concentration of 25 μg/ml whereas PG-1 had negligible activity (Fig. 2). PG-3 was further fractionated on Bio-gel P-6 and P-2, and several sizes of oligosaccharides were obtained (data not shown). Among them, the fraction eluted as the largest fraction had similar potent activity to PG-2 whereas other fractions related to α-D-(1 → 4)-linked oligogalacturonides showed no activity (data not shown). Component sugar analysis indicated that the active fraction obtained from PG-3 characteristically consisted of 2-Me-Fuc, 2-Me-Xyl, Api and AceA (data not shown), suggesting that the activity of PG-3 was shown by the contaminated PG-2.

In the previous study, ALR-a was seemed to be a homogenous polysaccharide by evaluation with HPLC (Yu et al., 1998); however, ALR-a gave two polysaccharides (ALR-a-A and ALR-a-B) having different molecular weight by further purification on Bio-gel P-30 (Fig. 3A). ALR-a-B showed similar potent intestinal immune system modulating activity to ALR-a whereas ALR-a-A gave weaker activity than ALR-a at a concentration of 100 μg/ml (Fig. 4A). The active fraction, ALR-a-B, was further fractionated on a Mono-Q column, and two absorbed fractions (ALR-a-Ba and ALR-a-Bb) were obtained (Fig. 3B). ALR-a-Bb showed potent activity, but ALR-a-Ba had weak activity at the high concentration (Fig. 4B). Molecular weight of ALR-a-Bb

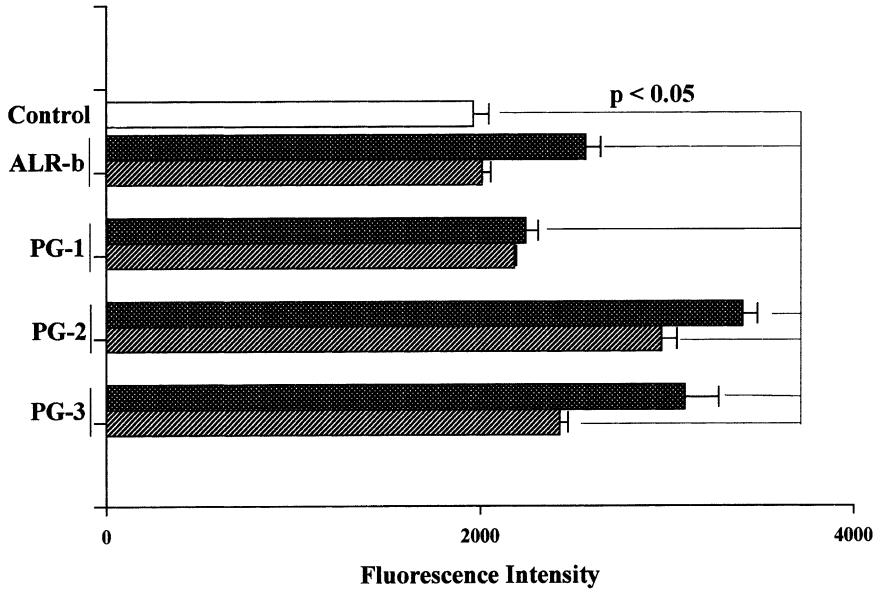

Fig. 2. Intestinal immune system modulating activity of digestion products from ALR-b with endo-α-D-(1 → 4)-polygalacturonase; (▨) 25 μg/ml; (▩) 100 μg/ml.

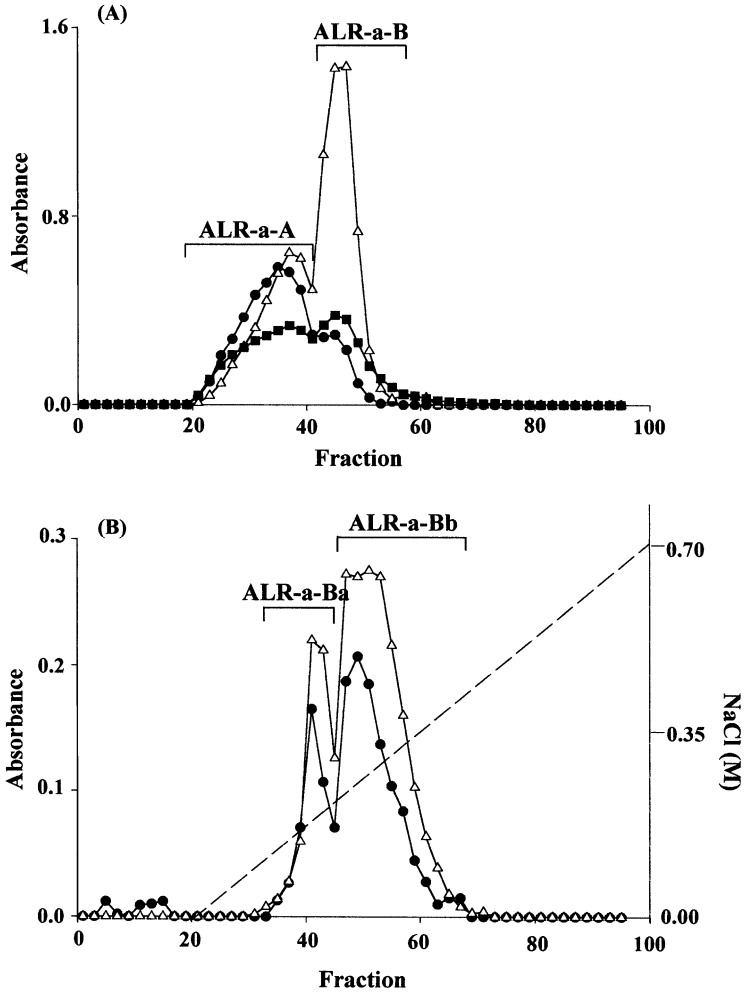

Fig. 3. (A) Elution profile of ALR-a by gel filtration on Bio-gel P-30. (B) Elution profile of ALR-a-B from Fig. 3A by FPLC on Mono-Q. (●) Carbohydrate (490 nm); (△) uronic acid (520 nm); (■) UV absorption (280 nm).

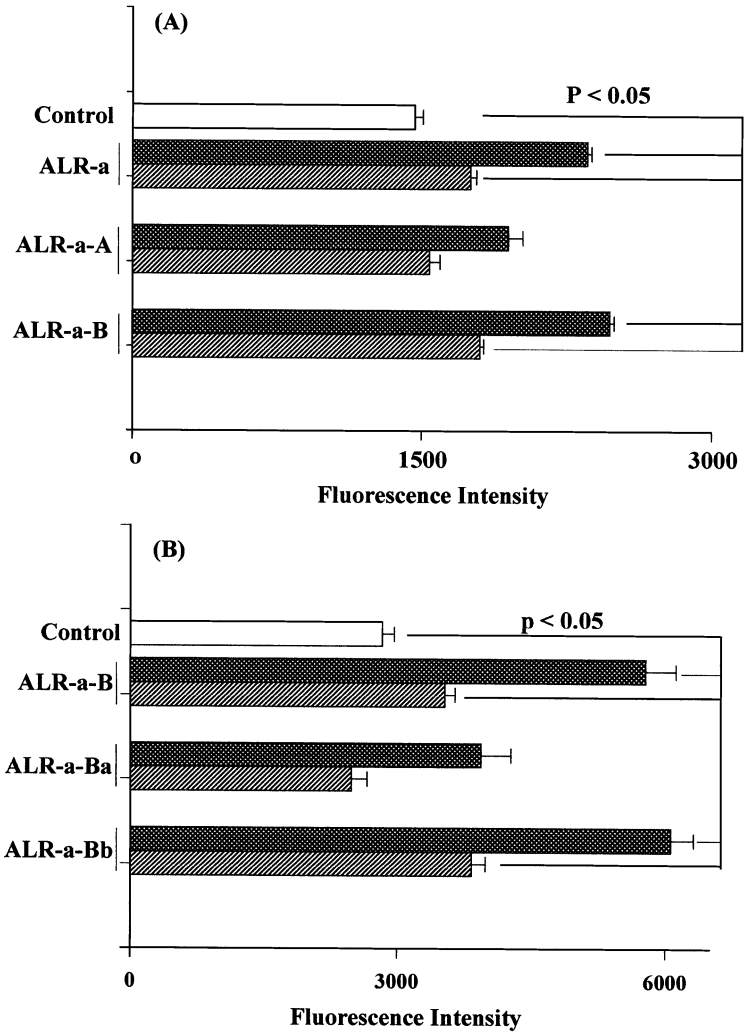

Fig. 4. (A) Intestinal immune system modulating activity of polysaccharides purified from ALR-a on Bio-gel P-30. (B) Intestinal immune system modulating activity of purified polysaccharides from ALR-a-B by FPLC on Mono-Q. (▨) 25 μg/ml; (▩) 100 μg/ml.

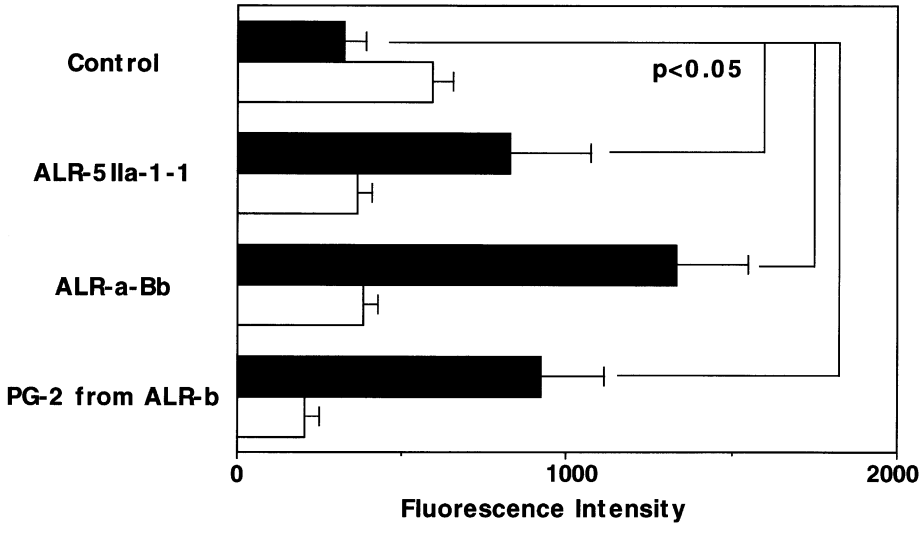

Fig. 5. Comparison of intestinal immune system modulating activity of ALR-a-Bb and PG-2 obtained from ALR-b with their direct effects on bone marrow cell proliferation. (■) intestinal immune system modulating activity; (□) direct effect on bone marrow cell proliferation.

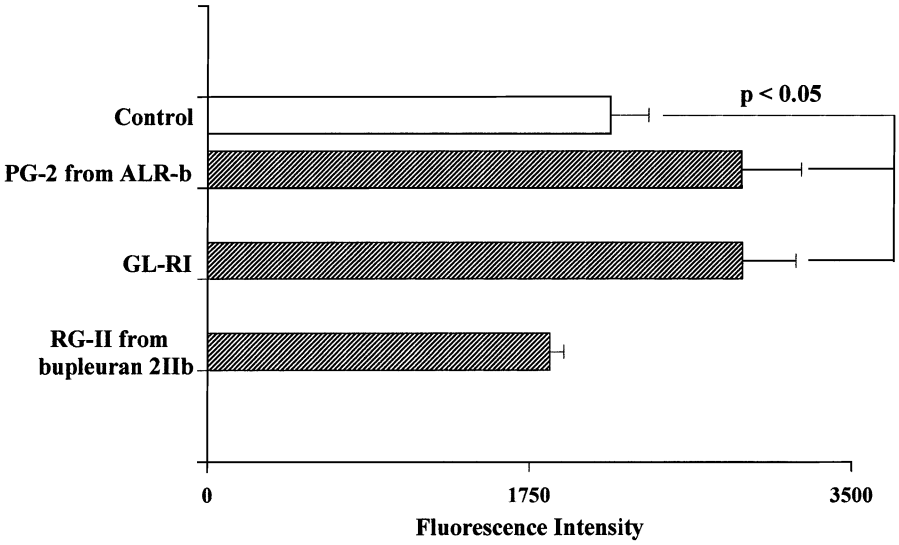

Fig. 6. Comparison of intestinal immune system modulating activity of RG-IIs from medicinal herbs and PG-2 from ALR-b (100 μg/ml).

was 2,800 by HPLC (Table 1). ALR-a-Bb comprised about 29% of neutral sugars, and 84% of uronic acid (Table 1). Component sugar analysis showed that ALR-a-Bb mainly comprised Ara, Rha, Gal, GlcA and GalA; in addition, it was also found to contain 2-Me-Fuc, 2-Me-Xyl, Api, AceA, Dha and Kdo, similar to PG-2 from ALR-b (Table 1). However, contents of GlcA and GalA were significantly higher than PG-2 from ALR-b.

Because the intestinal immune system modulating activity was assayed by the proliferation of bone marrow cells in the conditioned medium from Peyer's patch cells incubated with test samples (Hong et al., 1998), there is a possibility that the polysaccharides that are contained in the conditioned medium directly stimulate the proliferation of bone marrow cells. ALR-a-Bb and PG-2 from ALR-b each were incubated with Peyer's patch cells at a concentration of 100 μg/ml, and bone marrow cells were further incubated with the conditioned medium from Peyer's patch cells. The proliferation of bone marrow cells was significantly stimulated compared with a control in which Peyer's patch cells were incubated with water, to show intestinal immune system modulating activity (Fig. 5). However, they did not directly stimulate proliferation of bone marrow cells when bone marrow cells were cultured with ALR-a-Bb or PG-2 from ALR-b as above at a concentration of 25 μg/ml corresponding to the final concentration of the polysaccharides in the conditioned medium, which was used for the culture of bone marrow cells (Fig. 5). These results suggest that ALR-a-Bb and PG-2 from ALR-b show intestinal immune system modulating activity through Peyer's patch cells. Component sugar and methylation analyses indicated that the property of PG-2 from ALR-b resembled the rhamnogalacturonan-II (RG-II) of plant cell wall polysaccharides. Several kinds of RG-II have been isolated from plant cell walls, enzyme preparation and purified pectins (Darvill, McNeil & Albersheim, 1978; Doco & Brillouet, 1993; Hirano, Kiyohara & Yamada, 1994; Kaneko, Ishii & Matsunaga, 1997; Kobayashi, Matoh & Azuma, 1996; Pellerin, Doco, Vidal, Wiliams, Brillouet & O'Neill 1996; Whitcombe, O'Neill, Steffan, Albersheim & Darvill, 1995), therefore intestinal immune system modulating activities of some RG-IIs were tested. RG-II (GL-RI) from leaves of *Panax ginseng* (Shin, Kiyohara, Matsumoto & Yamada, 1997) had a significant activity similar to PG-2 from ALR-b whereas RG-II from pharmacologically active pectin (bupleuran 2IIb) from roots of *Bupleurum falcatum* (Hirano et al., 1994) showed no activity (Fig. 6).

4. Discussion

In the present paper, we studied structures and active sites of the intestinal immune system modulating polysaccharides from *A. lancea* DC. (Yu et al., 1998). Structural characterization using endo-α-D-(1 → 4)-polygalacturonase digestion suggests that ALR-b comprised a large proportion of "ramified" region (PG-1, rhamnogalacturonan core possessing side chains rich in neutral sugars) and a galacturonan region, therefore ALR-b can be classed as a pectic polysaccharide. When ALR-b was digested with endo-α-D-(1 → 4)-polygalacturonase, intestinal immune system modulating activity was observed only in PG-2. Several studies have been performed on clarification of active sites of pectic polysaccharides for the expressions of anti-complementary activity (Yamada & Kiyohara, 1999), mitogenic activity (Zhao et al., 1991), up-regulating activity of macrophage Fc receptors (Matsumoto et al., 1993), and anti-ulcer activity (Yamada, Sun, Matsumoto, Ra, Hirano & Kiyohara, 1991). For these activities, "ramified" regions are suggested to be important in the active sites. However, the present study indicates that PG-2 from ALR-b plays an important role in the expression of intestinal immune system

modulating activity, but the "ramified" region does not. PG-2 of ALR-b comprised unusual sugars such as 2-Me-Fuc, 2-Me-Xyl, Api, AceA, Kdo and Dha. These sugars have been found in RG-II as its characteristic component sugars (McNeil, Darvill, Fry & Albersheim, 1984). Hirano et al. (1994) have found that some pectins comprised the region, which consist of the same component sugars as RG-II, and these regions in the pectins were identified as RG-II by structural analysis. However, terminal Ara*p* and 2,4-branched GalA, which were known as characteristic glycosidic linkages of RG-II, were not detected in PG-2 from ALR-b by methylation analysis. Therefore it is not known whether the present PG-2 has a similar structure to RG-II. RG-II is known to associate to the dimeric form through borate ion interactions (Ishii, Matsunaga, Pellerin, O'Neill, Darvil & Albersheim, 1999). PG-2 from ALR-b also gave two peaks on HPLC owing to different molecular weights. It is reasonable to assume that one peak corresponds to a monomer and the other to a dimer. We do not know whether the dimer formation affects the intestinal immune system modulating activity.

In the present study, the other active polysaccharide (ALR-a-Bb) was purified from ALR-a, and characterized as a similar polysaccharide to PG-2. By comparison of component sugars, it was indicated that ALR-a-Bb comprised a significantly higher proportion of GalA and GlcA than sycamore RG-II (Stevenson, Darvill & Albersheim, 1988b). Shin et al. (1997), and Shin, Kiyohara, Matsumoto and Yamada (1998) have reported that the macrophage Fc receptor expression enhancing and IL-6 production enhancing polysaccharides from leaves of *Panax ginseng* are identified to be RG-II, which are free from other pectic components such as "ramified" and galacturonan regions. ALR-a-Bb also had relatively low molecular weight (MW 2,800), and could not be digested with endo-α-(1 → 4)-polygalacturonase (data not shown). We do not know the reason why ALR-a-Bb revealed such low molecular weight although it could be purified by the procedure using dialysis tube (molecular cut-off: 12,000–14,000). No other types of plant polysaccharide, which consist of characteristic component sugars such as 2-Me-Fuc, 2-Me-Xyl, AceA, Api, Kdo and Dha like RG-II, have been reported; however, we could not clarify in the present study whether ALR-a-Bb as well as PG-2 from ALR-b have a similar structure to RG-II.

Comparison of intestinal immune system modulating activity indicates that RG-II isolated from *P. ginseng* shows activity similar to PG-2 from ALR-b. Because RG-II from bupleuran 2IIb had no activity, it is assumed that there may be some structural differences among RG-IIs tested in the present study.

The present study indicates that at least unique carbohydrate molecules (ALR-a-Bb and PG-2 from ALR-b) are responsible for expression of the intestinal immune system modulating activity through Peyer's patch cells, but we must await further study to clarify whether these molecules contain a known RG-II structure or not.

References

Bienenstock, J., & Befus, A. D. (1980). Mucosal immunology. *Immunology*, *41*, 249–270.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acids. *Analytical Biochemistry*, *54*, 484–489.

Darvill, A. G., McNeil, M., & Albersheim, P. (1978). Structure of plant cell walls VIII. A new pectic polysaccharide. *Plant Physiology*, *62*, 418–422.

Doco, T., & Brillouet, J. -M. (1993). Isolation and characterization of a rhamnogalacturonan II from red wine. *Carbohydrate Research*, *243*, 333–343.

Dubois, M., Gilles, K. A., Hamilton, J. K., Revers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.

Hakomori, S. (1964). A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethylsulfoxide. *Journal of Biochemistry (Tokyo)*, *55*, 205–208.

Hirano, M., Kiyohara, H., & Yamada, H. (1994). Existence of a rhamnogalacturonan II-like region in bioactive pectins from medicinal herbs. *Planta Medica*, *60*, 450–454.

Holst, G. J., & Clarke, A. E. (1985). Quantification of arabinogalactan-protein in plant extracts by single radial gel diffusion. *Analytical Biochemistry*, *148*, 446–450.

Hong, T., Matsumoto, T., Kiyohara, H., & Yamada, H. (1998). Enhanced production of hematopoietic growth factors through T cell activation in Peyer's patches by oral administration of Kampo (Japanese herbal) medicine "Juzen-Taiho-To". *Phytomedicine*, *5*, 353–360.

Ishii, T., Matsunaga, T., Pellerin, P., O'Neill, H. A., Darvill, A., & Albersheim, P. (1999). The plant cell wall polysaccharide rhamnogalacturonan II self-assembles into a covalently cross-linked dimer. *Journal of Biological Chemistry*, *274*, 13,098–13,104.

Johnes, T. M., & Albersheim, P. (1972). A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharides. *Plant Physiology*, *49*, 926–936.

Kaneko, S., Ishii, T., & Matsunaga, T. (1997). A boron-rhamnogalacturonan-II complex from bamboo shoot cell walls. *Phytochemistry*, *44*, 243–248.

Karkhanis, Y. D., Zeltner, J. Y., Jackson, J. J., & Carlo, D. J. (1978). A new and improved microassay to determine 2-keto-3-deoxyoctonate in lipopolysaccharide of gram-negative. *Analytical Biochemistry*, *85*, 595–601.

Kobayashi, M., Matoh, T., & Azuma, J. (1996). Structural and glycosyl composition of the boron-polysaccharide complex of radish roots. *Plant Cell Physiology*, *36*, S139.

Lindberg, B., Lönngren, J., & Thompson, J. L. (1973). Degradation of polysaccharides containing uronic acid residues. *Carbohydrate Research*, *28*, 351–357.

Matsumoto, T., Cyong, J. -C., Kiyohara, H., Matsui, H., Abe, A., Hirano, M., Danbara, H., & Yamada, H. (1993). The pectic polysaccharide from *Bupleurum falcatum* L. enhances immune complexes binding to peritoneal macrophages through Fc receptor expression. *International Journal of Immunopharmacology*, *15*, 683–693.

McNeil, M., Darvill, A. G., & Albersheim, P. (1982). Structure of plant cell walls, XII. Identification of seven differentary linked glycosyl residues attached to O-4 of the 2,4-linked L-rhamnosyl residues of rhamnogalacturonan I. *Plant Physiology*, *70*, 1586–1591.

McNeil, M., Darvill, A. G., Fry, S. C., & Albersheim, P. (1984). Structure and function of the primary cell walls of plants. *Annual Review of Biochemistry*, *53*, 625–663.

Pagé, B., Pagé, M., & Noël, C. (1993). A new fluorometric assay for

cytotoxicity measurements in vitro. *International Journal of Oncology*, *3*, 473–476.

Pellerin, P., Doco, T., Vidal, S., Williams, P., Brillouet, J. -M., & O'Neill, M. -A. (1996). Structural characterization of red wine rhamnogalacturonan II. *Carbohydrate Research*, *290*, 183–197.

Shin, K. S., Kiyohara, H., Matsumoto, T., & Yamada, H. (1997). Rhamnogalacturoan II from the leaves of *Panax ginseng* C.A. Meyer as a macrophage Fc receptor expression-enhancing polysaccharide. *Carbohydrate Research*, *300*, 239–249.

Shin, K. S., Kiyohara, H., Matsumoto, T., & Yamada, H. (1998). Rhamnogalacturoan 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. *Carbohydrate Research*, *307*, 97–106.

Stephen, P. J., & Martin, Z. (1994). Human gastrointestinal mucosal T cells. In L. O. Pearay, M. Jiri, E. L. Michael, S. Warren, R. M. Jerry & B. John, *Handbook of Mucosal Immunology* (pp. 275–285). London: Academic Press.

Stevenson, T. T., Darvill, A. G., & Albersheim, P. (1988a). 3-deoxy-D-*lyxo*-2-heptulosaric acid, a component of the plant cell-wall polysaccharide rhamnogalacturonan II. *Carbohydrate Research*, *179*, 269–288.

Stevenson, T. T., Darvill, A. G., & Albersheim, P. (1988b). Structural features of the plant cell-wall polysaccharide rhamnogalacturonan II. *Carbohydrate Research*, *182*, 207–226.

Sweet, D. P., Shapiro, R. H., & Albersheim, P. (1975). Quantitative analysis by various G.L.C. response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydrate Research*, *40*, 217–225.

Waeghe, T. T., Darvill, A. G., McNeil, M., & Albersheim, P. (1983). Determination by methylation analysis of the glycosyl-linkage compositions of microgram quantities of complex carbohydrates. *Carbohydrate Research*, *123*, 281–304.

Whitcombe, A. T., O'Neill, M. A., Steffan, W., Albersheim, P., & Darvill, A. G. (1995). Structural characterization of the pectic polysaccharides, rhamnogalacturonan-II. *Carbohydrate Research*, *271*, 15–29.

Yamada, H., & Kiyohara, H. (1999). Complement activating polysaccharides from medicinal herbs. In H. Wagner, *Immunomodulating agents from plants* (pp. 161–202). Basel: Birkhäuser.

Yamada, H., Sun, X. -B., Matsumoto, T., Ra, K. -S., Hirano, M., & Kiyohara, H. (1991). Purification of anti-ulcer polysaccharides from the roots of *Bupleurum falcatum*. *Planta Medica*, *57*, 555–559.

York, W. S., Darvill, A. G., McNeil, M., & Albersheim, P. (1985). 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) is a component of rhamnogalacturonan II, a pectic polysaccharide in the primary cell walls of plants. *Carbohydrate Research*, *138*, 109–126.

York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T., & Albersheim, P. (1986). Isolation and characterization of plant cell walls and cell wall components. *Methods in Enzymology*, *118*, 3–40.

Yu, K. -W., Kiyohara, H., Matsumoto, T., Yang, H. -C., & Yamada, H. (1998). Intestinal immune system modulating polysaccharides from rhizomes of *Atractylodes lancea* DC. *Planta Medica*, *64*, 714–719.

Zhao, J. F., Kiyohara, H., Yamada, H., Takemoto, N., & Kawamura, H. (1991). Heterogeneity and characterization of mitogenic and anticomplementary pectic polysaccharides from roots of *Glycyrrhiza uralensis* Fisch et DC. *Carbohydrate Research*, *219*, 149–172.