ELSEVIER

Carbohydrate Polymers 46 (2001) 147–156

Carbohydrate Polymers

www.elsevier.com/locate/carbpol

Structural characterization of intestinal immune system modulating new arabino-3, 6-galactan 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]*Graduate School of Biotechnology, Korea University, #1, 5-ka, Anam-dong, Sungbuk-ku, Seoul 136-701, South Korea*

Received 21 December 1999; revised 26 June 2000; accepted 4 September 2000

Abstract

Intestinal immune system modulating arabino-3,6-galactan (ALR-5IIa-1-1) has been purified from rhizomes of *Atractylodes lancea* DC. In order to characterize the structure of ALR-5IIa-1-1 sequential enzymic digestion using exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase was employed. After ALR-5IIa-1-1 was digested with the arabinofuranosidase to give AF-ALR-5IIa-1-1, the galactanase digestion cleaved only 10% and 23% of 3-linked and 3,6-branched Gal in AF-ALR-5Iia-1-1, respectively, and gave small amounts of intermediate size (AF-GN-2) and shorter oligosaccharide (AF-GN-3) fractions in addition to a large amount of the galactanase-resistant fraction (AF-GN-1). When AF-GN-1 was re-digested gradually with the arabinofuranosidase and galactanase, it released trace amounts of oligosaccharides in addition to a large amount of the enzyme-resistant fraction. When the final enzyme-resistant fraction from AF-GN-1 was digested simultaneously with both arabinofuranosidase and galactanase, the resistant fraction was significantly degraded into two long fragments (AF_3-GN_3-1 and 2). Mixture of digestion products from the first galactanase digestion of AF-ALR-5IIa-1-1 showed decreased intestinal immune system modulating activity which was correspond to about 30% of the activity of ALR-5IIa-1-1, but the galactanase resistant fraction (AF-GN-1) still had significant activity. Although the second gradual enzymic digestion of AF-GN-1 caused little decrease in activity, the resulting fragments (AF_3-GN_3-1 and 2) from the final enzymic digestion lost most of the activity.

Component sugar, methylation and MALDI-TOF-MS analyses indicated that the oligosaccharides released from AF-ALR-5IIa-1-1 by the first galactanase digestion consisted of galactosyl di- to hexadecasaccharides mainly comprising 6-linked Gal*p* and Gal*f* some of which were partially mono- or diarabinosylated. These oligosaccharides were attached to the non-reducing terminal side of β-D-(1 → 3)-galactan backbone as side chains. The final fragments (AF_3-GN_3-1 and 2) were located in the inner core of ALR-5IIa-1-1, and AF_3-GN_3-2 was composed mainly of terminal, 3-linked and 6-linked Gal whereas AF_3-GN_3-1 was suggested to have a complex structure

Keywords: *Atractylodes lancea*; Arabino-3,6-galactan; Intestinal immune system modulating activity; Structural requirement

1. Introduction

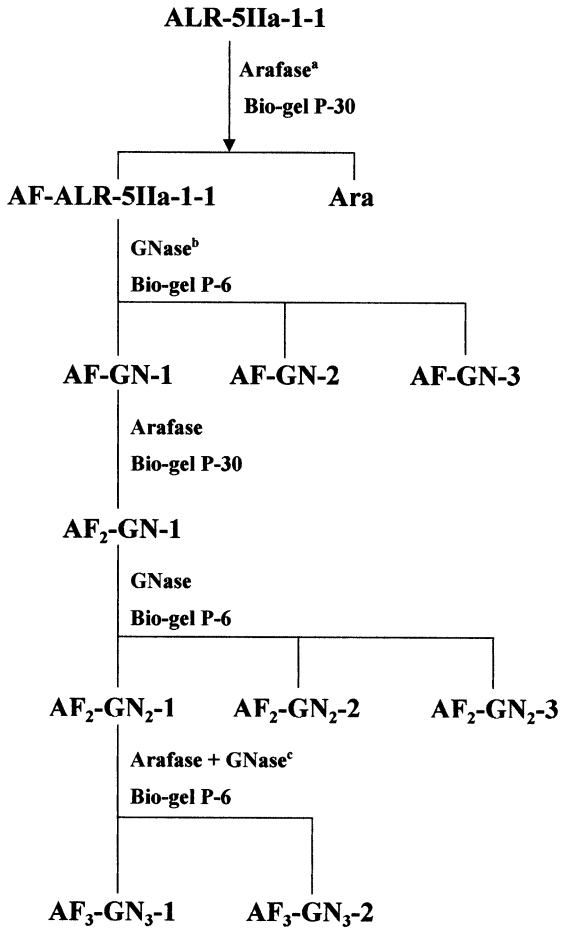
Arabinogalactans are polysaccharides rich in galactosyl and arabinosyl residues, and in some situations they occur in covalent association with protein as proteoglycans (arabinogalactan-proteins). They are found in higher plants and in liverworts, and occur on cell membranes, in extracellular matrices and in gum exudates (Clarke, Anderson & Stone, 1979).

Aspinall (1973) and Clarke et al. (1979) classified plant arabinogalactans and arabinogalactan-proteins into type I, type II and another type according to the structure of arabinogalactan portion. Type I arabinogalactans are arabino-4-galactan which have a (1 → 4)-β-D-galactan backbone with arabinosyl oligosaccharide side chains. Type II arabinogalactans are arabino-3,6-galactans which comprise a highly branched (1 → 3)-β-D-galactan backbone possessing (1 → 6)-β-D-galactosyl and/or arabinosyl side chains attached at position 6 to some of the galactosyl residues in the backbone. Other arabinogalactan types are polysaccharides with arabinogalactan side chains such as a pectic arabinogalactan from *Angelica acutiloba* Kitagawa (Kiyohara & Yamada, 1989).

In aspects of plant physiological functions, arabinogalactan-proteins have a role in cell identity and/or cell-to-cell signaling, and contribute to cell proliferation, somatic embryogenesis, and pollen tube growth (Bacic, Du, Stone & Clarke, 1996; Gao & Showalter, 1999; Kreuger & Holst, 1996; Nothnagel, 1997; Roy, Jauh, Hepler & Lord, 1998). Although the most popular arabinogalactan, gum arabic from *Acacia* sp. has been used for food additives, other

* 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)00294-0



Scheme 1. Sequential enzymic degradation procedure of ALR-5IIa-1-1 — (a) Arafase: exo-α-L-arabinofuranosidase digestion; (b) GNase: exo-β-D-(1 → 3)-galactanase digestion; (c) Arafase + GNase: simultaneous digestion with exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase.

arabinogalactans have been evaluated for their pharmacological activity and medicinal uses. Yamada, Kiyohara, Cyong and Otsuka (1985) have reported that an arabino-3,6-galactan, which is isolated from a medicinal herb, roots of *A. acutiloba*, shows complement activating activity through classical and alternative pathways. Several arabinogalactans and arabinogalactan-containing polysaccharides have been found as complement activators, and their galactan portions were suggested to contribute to the expression of their activity (Yamada & Kiyohara, 1999). It also has been reported that some arabinogalactans such as larch wood arabinogalactan stimulate natural killer (NK) cell cytotoxicity, TNF production of macrophages and tumor cytotoxicity of macrophages, and are applicable as drug carrier for hepatic drug delivery (Groman, Enriquez, Jung & Josephson, 1994; Hauer & Anderer, 1993; Kelly, 1999).

In the previous studies, we have purified an arabinogalactan (ALR-5IIa-1-1) having potent intestinal immune system modulating activity from rhizomes of *Atractylodes lancea* DC., and its β-D-(1 → 3,6)-galactan moiety has been suggested to play an important role for expression of the activity.

In the present paper, we describe the structural characterization of galactan portion of ALR-5IIa-1-1 using specific carbohydrases such as exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase, and its structural requirement for expression of the activity.

2. Materials and methods

2.1. Materials

Rhizomes of *A. lancea* DC., which were cultivated at Huabei Province in China, were purchased commercially from Tochimoto-Tenkaido Co. Ltd. (Osaka). Bio-gel P-6 and P-30 were obtained from Bio-Rad (USA), β-D-glucosyl-Yariv antigen from Biosupplies (Australia), and Sep-pak C_{18} cartridges from Waters Assoc. (USA). Exo-α-L-arabinofuranosidase (Megazyme, Australia) from *Aspergillus niger* was purified by FPLC (Lerouge, O'Neill, Darvill & Albersheim, 1993). Driselase (*Irpex lacteus*) was purchased from Kyowa-Hakko Co. Ltd. (Japan), and exo-β-D-(1 → 3)-galactanase was purified from Driselase according to the procedure of Tsumuraya, Mochizuki, Hashimoto and Kovác (1990).

2.2. General method

Total carbohydrate, pentose, uronic acid and protein contents in column eluates were measured by the phenol-sulfuric acid (Dubois, Gilles, Hamilton, Rebers & Smith, 1956), phloroglucinol-AcOH (Dische & Borenfreund, 1957), *m*-hydroxybiphenyl (Blumenkrantz & Asboe-Hansen, 1973) and Bradford's method (Bradford, 1976) with Bio-Rad dye (Bio-Rad) using Gal, Ara, GalA and bovine IgG as the respective standards. Sugars were converted into TMS methyl glycoside derivatives and analyzed by GLC using a DB-1 capillary column (0.20 μm film, 0.25 mm i. d. × 30 m, Supelco) (York, Darvill, McNeil, Stevenson & Albersheim, 1986). GLC was performed on an HP-5890 Series II gas chromatograph (Hewlett–Packard, USA), and the program was: 60°C for 1 min, 60 → 170 (30°C/min), 170 → 190 (1°C/min), and 190 → 300 (30°C/min). Single radial gel diffusion by using β-D-glucosyl-Yariv antigen was performed according to the procedure of Holst and Clarke (1985).

2.3. Purification of ALR-5IIa-1-1

A crude polysaccharide fraction (ALR-5) was prepared from rhizomes of *A. lancea* DC. by hot water extraction, EtOH precipitation, and dialysis (Yu, Kiyohara, Matsumoto, Yang & Yamada, 1998). ALR-5IIa-1-1 as an intestinal immune system modulating arabinogalactan was purified from ALR-5 on DEAE-Sepharose CL-6B (HCO_3^-), Sephacryl S-200 and S-300 as described previously (yield from ALR-5, 0.14%) (Yu et al., 1998).

2.4. Sequential enzymic digestion of ALR-5IIa-1-1 (Scheme 1)

(a) Procedure 1: ALR-5IIa-1-1 (17 mg) was digested with exo-α-L-arabinofuranosidase (0.01 U) in 4 ml of 50 mM acetate buffer (pH 4.5) at 40°C for 7 h. The digestion products were fractionated on a column (2 × 50 cm) of Bio-gel P-30 in 50 mM acetate buffer (pH 5.2), and the fractions eluted in the void volume (AF-ALR-5IIa-1-1) and in the inner volume were obtained. AF-ALR-5IIa-1-1 was desalted by electrophoretic dialyzer (Microacylizer, Asahi Chemical Industry, Japan). AF-ALR-5IIa-1-1 (10 mg) was further digested with exo-β-D-(1 → 3)-galactanase (0.1U) in 50 mM acetate buffer (pH 4.5, 5 μl) at 37°C for 48 h. The digestion products were fractionated on a column (1.5 × 90 cm) of Bio-gel P-6 with 50 mM acetate buffer (pH 5.2), and the fraction (AF-GN-1, 8 mg) eluted in the void volume, intermediate fraction (AF-GN-2) and the lowest-molecular-weight fraction (AF-GN-3) were obtained.

(b) Procedure 2: AF-GN-1 (8 mg) was re-digested with exo-α-L-arabinofuranosidase as above, and the resulting enzyme-resistant fraction, AF_2-GN-1 (7 mg) was further digested with exo-β-D-(1 → 3)-galactanase. The digestion products were fractionated on Bio-gel P-6 to obtain the fraction eluted in the void volume (AF_2-GN_2-1, 5 mg), intermediate fraction (AF_2-GN_2-2) and the lowest-molecular-weight fraction (AF_2-GN_2-3).

(c) Procedure 3: AF_2-GN_2-1 was simultaneously digested with both exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase at 37°C for 48 h in 50 mM acetate buffer (pH 4.5). The digestion products were fractionated on Bio-gel P-6, and the fraction eluted in the void volume (AF_3-GN_3-1) (yield from ALR-5IIa-1-1, 12.9%) and lower-molecular-weight fraction (AF_3-GN_3-2) (yield from ALR-5IIa-1-1, 7.6%) were obtained.

2.5. Methylation analysis

Each sample was methylated according to the Hakomori method (Hakomori, 1964), and the methylated products were recovered using a Sep-pak C_{18} cartridge following the procedure of Waeghe, Darvill, McNeil and Albersheim (1983) except that samples were eluted only with EtOH. Carboxymethyl groups in methylated products were reduced with $Li(Et)_3BD_3$ in THF (Super-Deuteride®, Aldrich) at room temperature for 1 h (York et al., 1986), and the reduced products were recovered on a Sep-pak C_{18} cartridge. The methylated products were hydrolyzed with 2M TFA at 121°C for 1.5 h and converted into partially methylated alditol acetates. The resulting partially methylated alditol acetates were analyzed by GLC and GLC-EIMS. GLC was performed on a Hewlett–Packard model 5890 gas chromatograph equipped with an SP-2380 capillary column, and EIMS was done on a Hewlett–Packard model 5970B mass spectrometer. Conditions of GLC were as described previously (Zhao, Kiyohara, Yamada, Takemoto & Kawamura, 1991). Methylated alditol acetates were identified by their fragment ions in EIMS and their relative retention times in GLC. Their molar ratios were estimated from the peak areas and the response factors (Sweet, Shapiro & Albersheim, 1975).

2.6. MALDI-TOF-MS analysis of methylated oligosaccharides

Methylated oligosaccharides derived from AF-GN-2 and AF-GN-3 were analyzed by MALDI-TOF-MS. For analysis 1 μl of sample solution in MeOH was added with 10 μl of 2,5-dihydrobenzoic acid solution (10 mg/ml H_2O), and 1 μl of the aliquot on the MALDI plate was dried in a gentle stream of air at room temperature. Samples were analyzed with a Voyger model RE-DP (PerSeptive Biosystems) with nitrogen laser of 337-nm wavelength and 3 ns-pulse width. The mass spectrometer was operating in the positive ion mode with a delayed extraction time of 100 ns. Ions were accelerated to an energy of 20 kV before entering the TOF mass spectrometer. The minimum laser power required to obtain a good spectrum was used and about 30 spectra were accumulated.

2.7. Intestinal immune system modulating activity

The activity was measured according to the procedure of Hong, Matsumoto, Kiyohara and Yamada (1998). Suspension of Peyer's patch cells in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) (RPMI 1640-FBS) was prepared from a small intestine of C3H/HeJ mice (6–8 weeks old, SLC, Shizuoka, Japan). About 200 μl of the cell suspension (2×10^6 cells/ml in RPMI 1640-FBS) was cultured with test sample in a 96-well flat bottom microtiter plate for 5–6 days at 37°C in a humidified atmosphere of 5% CO_2-95% air. The resulting culture supernatant (50 μl) was incubated with 100 μl of bone marrow cell suspension (2.5×10^5 cells/ml) from C3H/HeJ mice for 6 days in a humidified atmosphere of 5% CO_2-95% air in order to evaluate the ability for the growth of bone marrow cells. After 20 μl of Alamar Blue™ solution (Alamar Bio-Sciences Inc., Sacramento, CA) was added to each well, the cells were then continuously cultured for over 5 h to estimate the cell numbers (Pagé, Pagé & Noël, 1993). To count cell numbers the fluorescence intensity was measured during cultivation using a Fluoroskan II (Labsystems) at an excitation wavelength of 544 nm and emission wavelength of 590 nm. The differences between the control (a cell number of bone marrow cells cultured with supernant of Peyer's patch cells which were incubated with water) and the treatment in the experiments were tested for statistical significance by the student's *t*-test. A value of $p < 0.05$ was considered to show that the test sample had statistically significant intestinal immune system modulating activity.

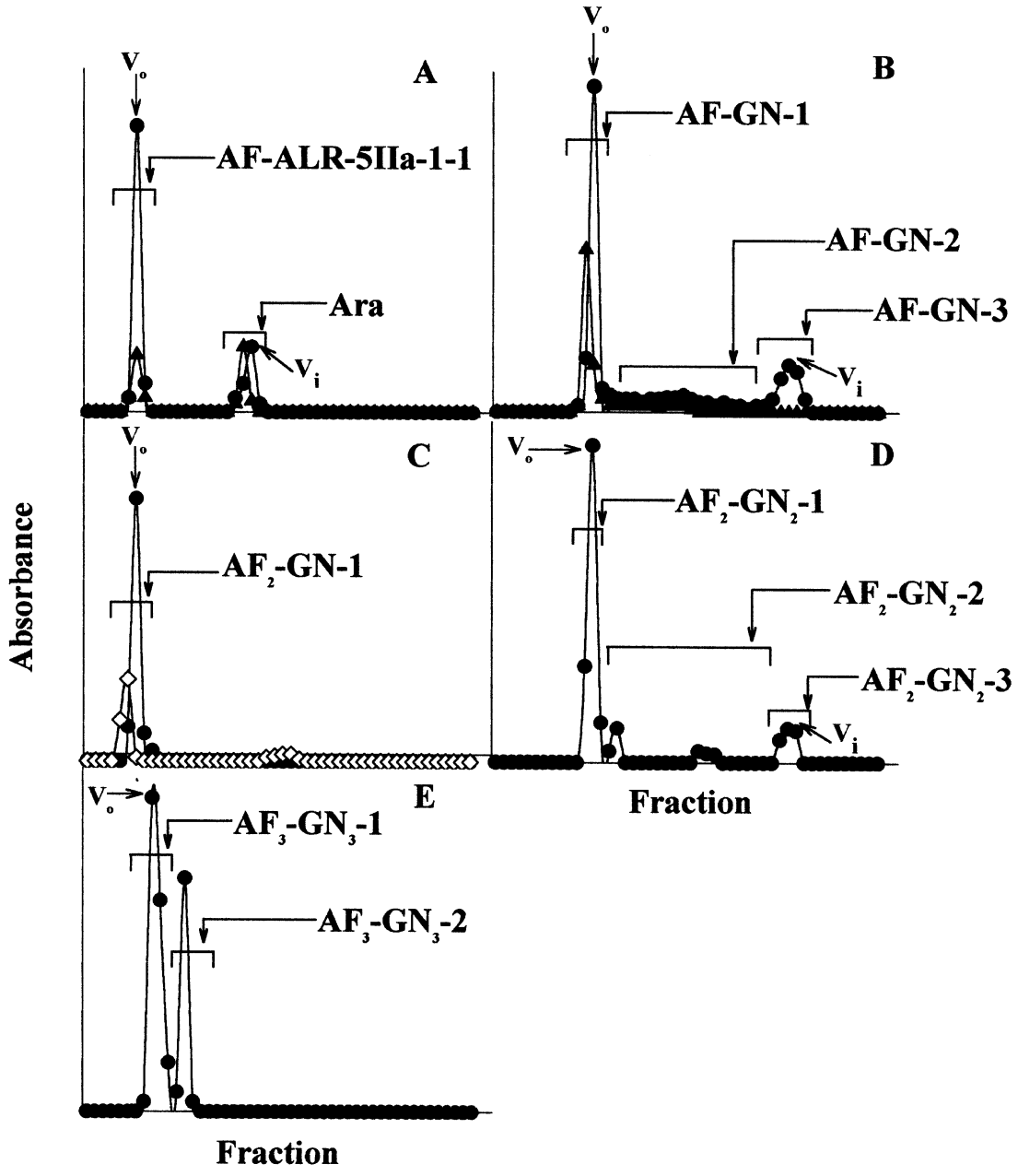


Fig. 1. Gel filtration patterns on Bio-gel P-30 (A, C) and P-6 (B, D, E) of digestion products from ALR-5IIa-1-1 by exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase. (A) exo-α-L-arabinofuranosidase digestion products of ALR-5IIa-1-1. (B) exo-β-D-(1 → 3)-galactanase digestion products of AF-ALR-5IIa-1-1 from A. (C) exo-α-L-arabinofuranosidase digestion products of AF-GN-1 from B. (D) exo-β-D-(1 → 3)-galactanase digestion products of AF_2-GN-1 from C. (E) products by simultaneous exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase digestion of AF_2-GN_2-1 from D. ●, Hexose (490 nm); ▲, UV absorbance (280 nm); ◇, pentose (552 nm).

3. Results

3.1. Sequential enzymic digestion of ALR-5IIa-1-1

The previous study have indicated that about 45% of galactosyl linkages in ALR-5IIa-1-1 were effectively cleaved by the simultaneously enzymic digestion with both exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase, and the intestinal immune system modulating activity was significantly decreased (Yu et al., 1998). Therefore, these enzymic digestions are useful for a structural analysis of ALR-5IIa-1-1, and we attempted to degrade ALR-5IIa-1-1 by these enzymes as shown in Scheme 1. After ALR-5IIa-1-1 was digested with exo-α-L-arabinofuranosidase, the digestion products were fractionated on Bio-gel P-30 to obtain the resistant fraction, AF-ALR-5IIa-1-1 and the fraction eluted in the inner volume (Fig. 1A). The fraction eluted in the inner volume consisted mainly of Ara, and about 60% of Ara in ALR-5IIa-1-1 was decreased (Table 1). Methylation analysis of AF-ALR-5IIa-1-1 indicated

Table 1
Component sugars of digestion products from ALR-5IIa-1-1 by exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase (n. d. — not determined)

	ALR-5IIa-1-1	AF-ALR-5IIa-1-1	AF-GN-1	AF-GN-2	AF-GN-3	AF_3-GN_3-1	AF_3-GN_3-2
Ara	32.7	12.9	5.2	27.8	3.2	6.4	19.6
Xyl	1.0	1.4	2.0	–	–	1.6	–
Rha	0.4	0.6	1.4	–	–	2.2	1.7
Fuc	Trace	Trace	0.8	–	–	1.9	–
Man	2.5	3.7	7.5	–	–	8.7	7.4
Gal	60.5	76.8	74.0	71.2	93.3	56.6	51.9
Glc	2.9	4.6	9.0	0.6	1.6	22.6	19.4
GlcA	Trace	Trace	Trace	–	1.5	n. d.	n. d.
GalA	Trace	Trace	Trace	0.4	0.4	n. d.	n. d.

that terminal Ara*f*, 3,4- or 3,5-branched Ara and 3,6-branched Gal were decreased with increasing of 4- or 5-linked Ara and 6-linked Gal (Table 2). AF-ALR-5IIa-1-1 was further digested with exo-β-D-(1 → 3)-galactanase, and the digestion products were fractionated on Bio-gel P-6. However, the products still gave a large amount of the fraction eluted in the void volume (AF-GN-1) in addition to small amounts of an intermediate fraction (AF-GN-2) and a fraction eluted in the inner volume (AF-GN-3) (Fig. 1B). Methylation analysis of AF-GN-1 suggested that the only 10% and 23% of 3-linked Gal and 3,6-branched Gal, respectively, in AF-ALR-5IIa-1-1 were released by the present galactanase digestion (Table 2). AF-GN-1 was tested for a reactivity with β-D-glucosyl-Yariv antigen by a single radial gel diffusion but AF-GN-1 showed no reactivity (data not shown). AF-GN-1 was re-digested with exo-α-L-arabinofuranosidase followed by exo-β-D-(1 → 3)-galactanase as the above same procedure, and fractionated on Bio-gel p-6. However, the digest gave very small amounts of an intermediate fraction (AF_2-GN_2-2) and the inner volume fraction

Table 2
Methylation analysis of digestion products from ALR-5IIa-1-1 by exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase (values in parenthesis represent molar percentage of linkages calculated from only Gal residues)

Glycosyl residue	Deduced linkage	Mol. %				
		ALR-5IIa-1-1	AF-ALR-5IIa-1-1	AF-GN-1	AF_3-GN_3-1	AF_3-GN_3-2
Ara	Terminal *(f)*	30.0	8.6	5.1	5.5	18.1
	Terminal *(p)*	1.0	1.3	–	–	–
	4 or 5	9.4	12.4	2.4	2.1	5.2
	3,4 or 3,5	6.7	–	–	–	–
Xyl	Terminal *(p)*	1.0	0.7	0.4	0.4	–
	2	0.3	0.3	0.6	0.7	–
	4 or 5	0.6	0.9	1.2	1.4	–
Rha	Terminal	1.4	0.6	1.0	1.1	0.6
	2	0.5	0.6	0.9	1.0	–
	3	0.2	0.3	0.6	0.6	–
Fuc	3	0.6	0.7	0.9	1.3	–
Man	Terminal	0.3	0.6	1.1	1.7	5.4
	2	0.9	1.3	2.6	3.5	–
	4	–	3.0	–	–	–
Gal	Terminal	1.3	7.5 (13.6)	7.9 (13.3)	7.8	24.5
	3	7.2	5.8 (10.5)	5.6 (9.4)	4.1	17.5
	4	7.6	11.3	13.0	16.3	–
	6 (f)	1.5	2.1	3.3	3.2	–
	6 (p)	2.6	17.4 (31.6)	18.3 (30.7)	12.1	9.6
	2,6	0.3	0.5	1.1	1.0	–
	3,6	15.2	6.9 (12.5)	5.7 (9.6)	3.1	4.0
	4,6	2.2	3.6	4.6	4.4	–
Glc	Terminal *(f)*	3.8	5.7	8.4	8.8	–
	Terminal	0.9	1.4	2.1	2.3	6.5
	3	1.7	2.2	3.4	4.8	3.3
	4	2.5	2.9	4.8	5.3	4.3
	6	–	3.3	4.6	6.9	–
	3,4,6	1.0	1.3	0.8	0.9	1.1

Table 3
Intestinal immune system modulating activity of products from ALR-5IIa-1-1 by exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase digestions

	Product	Concentration (μg/ml)	Fluorescence intensity[a] (Mean ± S.E.)	Remaining activity (%)[b]
Exp. 1	Control	–	1740 ± 55	
	ALR-5IIa-1-1	100	2510 ± 80[c]	100
	Mixture[d]	100	2000 ± 55[e]	33.8
		25	1990 ± 89	
	AF-GN-1	100	2330 ± 55	76.6
		25	2180 ± 89	
Exp. 2	Control	–	1260 ± 21	
	ALR-5IIa-1-1	100	2240 ± 38[c]	100
		25	1660 ± 45	
	AF-GN-1	100	2120 ± 53	87.8
		25	1750 ± 36	
	AF_2-GN_2-1	100	1850 ± 52[e]	60.2
		25	1850 ± 18[e]	
Exp. 3	Control	–	1641 ± 45	
	ALR-5IIa-1-1	100	3112 ± 92[c]	100
		25	2113 ± 55	
	AF_3-GN_3-1	100	1932 ± 44[e]	19.8
		25	1652 ± 42[e]	
	AF_3-GN_3-2	100	1902 ± 53[e]	17.7
		25	1328 ± 55	

[a] Experiments 1–3 could not be compared mutually because the reaction time with Alamar blue in each experiment was changed depending on the growth of cells.
[b] The values were calculated from [(mean of sample − mean of control)/(mean of ALR-5IIa-1-1 − mean of control)] × 100.
[c] $p < 0.005$; between control and sample.
[d] Mixture of digestion products from ALR-5IIa-1-1 with exo-α-L-arabinofuranosidase followed by exo-β-D-(1 → 3)-galactanase.
[e] $p < 0.05$; between ALR-5IIa-1-1 and samples.

Table 4
Methylation analysis of oligosaccharide fractions derived from ALR-5IIa-1-1 by exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase digestions

Glycosyl residue	Deduced linkage	Mol. %	
		AF-GN-2	AF-GN-3
Ara	Terminal *(f)*	5.3	2.0
	Terminal *(p)*	1.2	–
	2 *(f)*	–	0.3
	3 *(p)*	0.6	–
	4 or 5	6.8	–
Gal	Terminal	18.9	48.3
	3	–	–
	4	–	0.6
	6 *(f)*	3.4	20.3
	6 *(p)*	45.8	26.6
	3,6	7.0	0.5
	4,6	1.7	0.5
	3,4,6	2.3	–
Glc	Terminal	0.4	0.7
	3	3.4	0.2
GlcA	4	2.9	–

(AF_2-G_2-3) in addition to a large amount of a resistant fraction (AF_2-GN_2-1) (Fig. 1C and D). AF_2-GN_2-1 was further digested simultaneously with both exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase as the previous study (Yu et al., 1998). The resulting digest gave significant amounts of an intermediate size fraction (AF_3-GN_3-2) and a fraction, (AF_3-GN_3-1) eluted in the void volume (Fig. 1E). Component sugar analysis indicated that AF_3-GN_3-1 and 2 mainly comprised Gal and Glc, and Ara, Gal and Glc, respectively (Table 1), suggesting that AF_2-GN_2-1 was degraded significantly by the simultaneous enzymic digestion with exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase.

3.2. Intestinal immune system modulating activities of fragments

Intestinal immune system modulating activities of the fractions obtained by the enzymatic digestions were tested. Mixture of digestion products from AF-ALR-5IIa-1-1 with exo-β-D-(1 → 3)-galactanase significantly decreased the activity, but AF-GN-1 still had the significant activity at a concentration of 100 μg/ml (remaining activity was about 80% compared with ALR-5IIa-1-1) (Table 3). AF_2-GN_2-1 showed about 20%-decreased activity compared with AF-GN-1, and AF_3-GN_3-1 and AF_3-GN_3-2 showed the

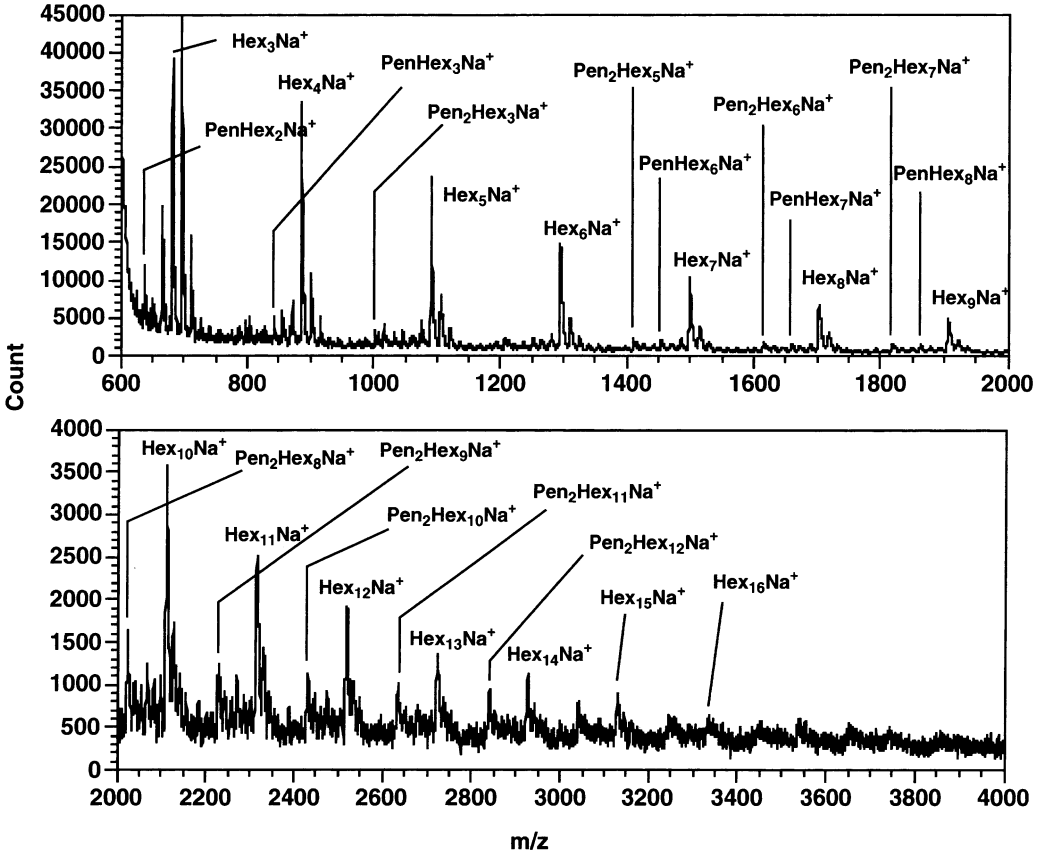


Fig. 2. MALDI-TOF-MS spectrum of methylated oligosaccharides derived from AF-GN-2.

significantly reduced activity from AF_2-GN_2-1 (Table 3). These results strongly suggest that oligosaccharide chains in AF-GN-2 and 3 mainly contributed (about 70% of total activity) to expression of the intestinal immune system modulating activity of ALR-5IIa-1-1. Although participation of oligosaccharides obtained as AF_2-GN_2-2 and 3 seemed to be little, carbohydrate chains in AF_3-GN_3-1 and 2 were suggested to contribute most of the remaining activity.

3.3. Analysis of fragments

Component sugar and methylation analyses indicated that AF-GN-2 consisted mainly of Ara and Gal (Table 1), and that it mainly comprised terminal and 6-linked and 3,6-branched Gal*p*, although it was seemed that terminal Ara*f* and 4- or 5-linked Ara were lost during the of methylation analysis procedure (Table 4). It was also postulated that AF-GN-3 consisted mainly of galactosyl-oligosaccahrides comprising terminal and 6-linked Gal*p*, and 6-linked Gal*f* (Tables 1 and 4). When the methylated products derived from AF-GN-2 were analyzed by MALDI-TOF-MS, it mainly gave peaks having pseudomolecular ions, $[M + Na]^+$ due to hexosyl tri- to hexadecasaccharides (Fig. 2). It was also suggested that AF-GN-2 contained hexosyl oligosaccharides having one or two pentosyl residues. Because AF-GN-2 consisted mainly of terminal and 6-linked Gal*p* and 6-linked Gal*f* as the hexosyl residue, and terminal Ara*f* and 4- or 5-linked Ara as the pentosyl residues, most of these oligosaccharides were considered to comprise partially mono- or di-arabinosylated 6-linked galactosyl oligosaccharides. MALDI-TOF-MS analysis also indicated that AF-GN-3 contained hexosyl di- to heptasaccharides (data not shown). Structural analyses by the same procedures indicated that AF_2-GN_2-2 and 3 also contained the similar partially arabinosylated 6-linked galactosyl oligosaccharides as AF-GN-2 and 3, but their amounts were suggested to be little (data not shown). Component sugar analysis showed that AF_3-GN_3-1 and 2 mainly comprised Gal and Glc, in addition AF_3-GN_3-2 also consisted mainly of Ara (Table 1). Methylation analysis suggested that AF_3-GN_3-2 was composed mainly of terminal Ara*f*, terminal, 3-linked and 6-linked Gal and terminal Glc, whereas AF_3-GN_3-1 contained more complex glycosidic linkages such as 4-linked and 6-linked Gal, and terminal Glc*f* (Table 2).

4. Discussion

Gut-associated lymphoreticular tissues (GALT) exist on the intestinal lumen and play important roles in host defense

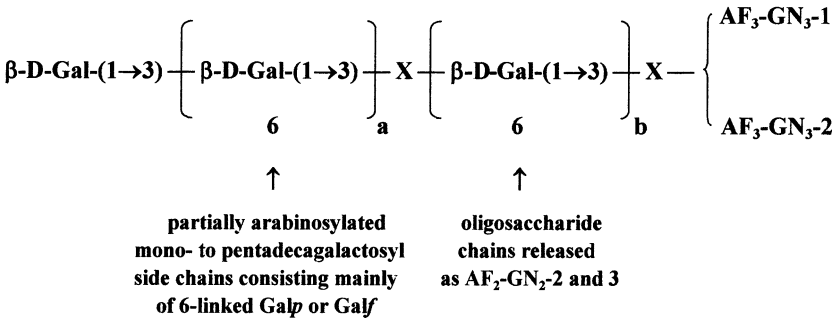


Fig. 3. Partial structural feature of galactan portion in ALR-5IIa-1-1 a, b: Degrees of polymerization of β-D-(1 → 3)-galactosyl backbone are unknown. X: exo-α-L-arabinofuranosidase-sensitive glycosyl residue.

such as IgA production in the intestine (Stephen & Martin, 1994). Because lymphocytes in Peyer's patches of GALT migrate through mesenteric lymph nodes, and reach systemic circulation to deliver into peripharal lymph nodes, spleen, and other mucosal sites, intestinal immune system regulates systemic immune system as well as mucosal immune system. Since most traditional herbal medicines have been used for treatments of diseases by oral administration, there is a possibility that these medicines express their clinical effects through the intestinal immune system.

In a previous study, the hot water extract of rhizomes of *A. lancea* DC. has been found to modulate the intestinal immune system through Peyer's patches, and that macromolecules rather than relatively low molecular-weight substances in *A. lancea* contribute to the activity (Yu et al., 1998). Three potently active polysaccharides (ALR-5IIa-1-1, 5IIb-2-2 and 5IIc-3-1) have been purified from rhizomes of *A. lancea* DC. ALR-5IIa-1-1 is an arabinogalactan (type II arabinogalactan). It has been suggested that β-D-(1 → 3,6)-galactan moiety contribute to the activity because a trimming of arabinosyl side chains did not affect the activity whereas hydrolysis of β-D-(1 → 3)-galactosyl linkages did (Yu et al., 1998). Previous study also indicated that the simultaneous digestion with exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase cleaved about 45% of galactosyl linkages in ALR-5IIa-1-1, and resulted a significant reduction of its intestinal immune system modulating activity (Yu et al., 1998), therefore in the present study these enzymes were considered to be useful for structural characterization of the galactan moiety in ALR-5IIa-1-1. Tsumuraya et al. (1990) have shown that exo-β-D-(1 → 3)-galactanase from *I. lacteus* can cleave β-D-(1 → 3)-galactosyl linkages of the galactan backbone in type II arabinogalactan from the non-reducing terminal of the backbone with or without side chains. The present results suggested that the first galactanase digestion of Ara*f*-trimmed ALR-5IIa-1-1 (AF-ALR-5IIa-1-1) cleaved parts of β-D-(1 → 3)-galactosyl linkages to release the small amounts of partially arabinosylated di- to hexadecasaccharides from the side chains which consisted mainly of 6-linked Gal*p* and Gal*f*, and that these side chains were located on the non-reducing terminal side of ALR-5IIa-1-1. However, this galactanase digestion could not degrade all of the β-D-(1 → 3)-galactosyl linkages sensitive to the galactanase. After the second arabinofuranosidase digestion of the resistant fraction (AF-GN-1 from AF-ALR-5IIa-1-1), the second galactanase digestion released only small amounts of side chains suggesting that the side chains were located on the inner portion of ALR-5IIa-1-1 whereas the side chains released by the first galactanase digestion were on the outer portion. These results suggest that the glycosyl residues such as Ara*f* residue might be inserted into the β-D-(1 → 3)-galactan backbone of ALR-5IIa-1-1 preventing a complete degradation of the galactan backbone. Ponder and Richards (1997) have reported that larch arabinogalactan comprises arabinosyl residues at the non-reducing terminal of the galactan main chain. Simultaneous digestion with both arabinofuranosidase and galactanase digestions could degrade the galactanase-resistant fraction (AF_2-GN_2-1) although it was not cleaved when only the galactanase digestion was performed. These result suggest that: (1) pieces of β-D-(1 → 3)-galactosyl backbones were connected to each other through unknown glycosyl residue in the backbone, which was sensitive against exo-α-L-arabinofuranosidase digestion; (2) these galactosyl backbones mainly possess partially arabinofuranosylated mono- to pentadecagalactosyl side chains in a non-reducing terminal side; (3) these partially arabinosylated galactosyl oligosaccharide side chains might be attached to position 6 of the backbone, because half of 3,6-branched Gal in AF-ALR-5IIa-1-1 was reduced by the repeated galactanase digestions (Table 2); (4) ALR-5IIa-1-1 consisted of galactan chain having the galactanase-resistant complex structure which mainly comprised 4-linked and 6-linked Gal and terminal Glc*f* and Gal in addition to small proportions of 3-linked and 3,6-branched Gal (Fig. 3).

Comparison of intestinal immune system modulating activities of digestion products from the enzyme-resistant fragments strongly suggests that outer galactosyl chains in ALR-5IIa-1-1 at the non-reducing terminal side play an important role for expression of the activity, and that the β-D-(1 → 3)-galactosyl outer backbone attached with partially arabinosylated galacto-mono- to pentadecasaccharide side chains may contribute to expression of the

activity. The present result also indicates that the resistant fraction (AF-GN-1) against the first galactanase digestion still keeps the structure for the activity, and that the complex galactosyl chains (AF_3-GN_3-1 and 2) located on the side of the reducing terminal of AF-ALR-5IIa-1-1 are more responsible rather than the branched β-D-(1 → 3)-galactosyl chains which were liberated by the second galactanase digestion. When the intestinal immune system modulating activity was compared between ALR-5IIa-1-1 and an arabinogalactan from larch wood (*Larix occidentalis*), the arabinogalactan from larch wood did not show activity (Yu et al., unpublished data). It has been reported that the larch wood arabinogalactan comprises β-D-(1 → 3)-galactan backbone possessing arabinofuranosylated β-D-(1 → 6)-galactooligosaccharide side chains less than trisaccharide in addition to mono- to diarabinosylated side chains (Bouveng & Lindberg, 1961; Ponder & Richards, 1997; White, 1942). Present results suggested that the galactosyl side chains of ALR-5IIa-1-1 consisted of not only 6-linked Gal*p* but also 6-linked Gal*f*, which was not found in larch wood arabinogalactan. Therefore, it is proposed that the galactosyl side chains consisting of 6-linked Gal*f* and Gal*p* over tetrasaccharide in ALR-5IIa-1-1 may be important for expression of the potent intestinal immune system modulating activity.

Acknowledgements

The authors thank to Dr Naohito Ohno (School of Pharmacy, Tokyo University of Pharmacy and Life Science) for his helpful advice in measurement of MALDI-TOF-MS.

References

Aspinall, G. O. (1973). Carbohydrate polymers of plant cell walls. In F. Loewus, *Biogenesis of plant cell wall polysaccharides* (pp. 95–115). New York: Academic Press.

Bacic, A., Du, H., Stone, B. A., & Clarke, A. E. (1996). Arabinogalactan proteins: a family of cell-surface and extracellular matrix plant proteoglycans. In D. K. Apps, *Essays in biochemistry* (pp. 91–101). , Vol. 31. London: Portland Press.

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

Bouveng, H., & Lindberg, B. (1961). Studies on arabinogalactans. V. Barry degradation of the arabinogalactans from western larch. A kinetic study of the mild acid hydrolysis of arabinogalactan A. *Acta Chemica Scandinavica*, *15*, 78–86.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*, 248–254.

Clarke, A. E., Anderson, R. L., & Stone, B. A. (1979). Form and function of arabinogalactans and arabinogalactan-proteins. *Phytochemistry*, *18*, 521–540.

Dische, D., & Borenfreund, E. (1957). A new color reaction for the determination of aldopentose in presence of other saccharides. *Biochimica et Biophysica Acta*, *23*, 639–642.

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

Gao, M., & Showalter, A. M. (1999). Yariv reagent treatment induces programmed cell death in *Arabidopsis* cell cultures and implicates arabinogalactan protein involvement. *Plant Journal*, *19*, 321–331.

Groman, E. V., Enriquez, P. M., Jung, C., & Josephson, L. (1994). Arabinogalactan for hepatic drug delivery. *Bioconjugate Chemistry*, *5*, 547–556.

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

Hauer, J., & Anderer, F. A. (1993). Mechanism of stimulation of human natural killer cytotoxicity by arabinogalactan from *Larix occidentalis*. *Cancer Immunology, Immunotherapy*, *36*, 237–244.

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.

Kelly, G. S. (1999). Larch arabinogalactan: clinical relevance of a novel immune-enhancing polysaccharide. *Alternative Medicine Review*, *4*, 96–103.

Kiyohara, H., & Yamada, H. (1989). Structure of an anti-complementary arabinogalactan from the root of *Angelica acutiloba* Kitagawa. *Carbohydrate Research*, *193*, 173–192.

Kreuger, M., & Holst, G. -J. (1996). Arabinogalactan proteins and plant differentiation. *Plant Molecular Biology*, *30*, 1077–1086.

Lerouge, P., O'Neill, M. A., Darvill, A. G., & Albersheim, P. (1993). The purification of commercially available endo-α-L-arabinanases and α-L-arabinosidase for use in the structural analysis of pectic polysaccharides. *Carbohydrate Research*, *243*, 373–378.

Nothnagel, E. A. (1997). Proteoglycans and related components in plant cells. In K. K. Jeon (Ed.), *International Review of Cytology*, Vol. 174 (pp. 195–291). New York: Academic Press.

Pagé, B., Pagé, M., & Noël, C. (1993). A new fluorometric assay for cytotoxicity measurements in vitro. *International Journal of Oncology*, *3*, 473–476.

Ponder, G. P., & Richards, G. N. (1997). Arabinogalactan from western larch, Part III: alkaline degradation revisited, with novel conclusion on molecular structure. *Carbohydrate Polymers*, *34*, 251–261.

Roy, S., Jauh, G. Y., Hepler, P. K., & Lord, E. M. (1998). Effects of Yariv phenylglycoside on cell wall assembly in the lily pollen tube. *Planta*, *204*, 450–458.

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.

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.

Tsumuraya, Y., Mochizuki, N., Hashimoto, Y., & Kovác, P. (1990). Purification of an exo-β-D-(1 → (3)-galactanase of *Irpex lacteus* (*Polyporus tulipiferae*) and its action on arabinogalactan-proteins. *Journal of Biological Chemistry*, *265*, 7207–7215.

Waeghe, T. J., 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.

White, E. V. (1942). The constitution of arabo-galactan. IV. The structure of the repeating unit. *Journal of the American Chemical Society*, *64*, 2838–2842.

Yamada, H., Kiyohara, H., Cyong, J. -C., & Otsuka, Y. (1985). Studies on polysaccharides from *Angelica acutiloba* IV. Characterization of

anti-complementary arabinogalactan from *Angelica acutiloba* Kitagawa. *Molecular Immunology*, *22*, 295–304.

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

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 anti-complementary pectic polysaccharides from the roots of *Glycyrrhiza uralensis* Fisch *et* DC. *Carbohydrate Research*, *219*, 149–172.