Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 339 (2004) 763–770
Carbohydrate RESEARCH

Structure of oligosaccharide side chains of an intestinal immune system modulating arabinogalactan isolated from rhizomes of *Atractylodes lancea* DC

Ikue Taguchi,[a] Hiroaki Kiyohara,[a,b] Tsukasa Matsumoto[a,b] and Haruki Yamada[a,b,*]

[a]*Kitsato Institute for Life Sciences and Graduate school of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108-8642, Japan*
[b]*Oriental Medicine Research Center, The Kitasato Institute, Minato-ku, Tokyo 108-8641, Japan*

Received 2 October 2003; accepted 16 January 2004

Abstract—An intestinal immune system modulating arabino-3,6-galactan (ALR-5IIa-1-1) has been found in rhizomes of *Atractylodes lancea* DC. [*Planta Medica* **1998**, *64*, 714–719; *Carbohydr. Polyms.* **2001**, *46*, 147–156], however other arabino-3,6-galactans from *Larix* and *Acacia* failed to express the modulating activity. Degradation of the galactosyl side chains in Ara*f*-side chain-trimmed ALR-5IIa-1-1 (AF-ALR-5IIa-1-1) with an endo-β-D-(1→6)-galactanase remarkably decreased the activity of AF-ALR-5IIa-1-1. Structural analysis indicated that the major endo-β-D-(1→6)-galactanase-digestable side chains in ALR-5IIa-1-1 are composed of β-D-(1→6)-galactopyranosyl oligosaccharides having d.p. 1–8. Because degradation of the β-D-(1→3)-galactan backbone in AF-ALR-5IIa-1-1 also significantly reduced its activity, some of these galactosyl side chains attached to β-D-(1→3)-galactan backbone are suggested to be responsible for expression of the activity of ALR-5IIa-1-1.

Keywords: Arabino-3,6-galactan; β-D-(1→6)-Galactooligosaccharides; Peyer's patch cells

1. Introduction

Peyer's patches are unique islands of lymphoid tissue in gut-associated lymphoid tissue (GALT), and play important roles as an inductive site for IgA production.[1] Lymphocytes are rapidly eliminated from the patches, and migrate through the mesenteric lymph nodes into the thoracic duct to the systemic circulation, where they regulate the local mucosal and systemic immune systems.[2] The intestinal immune system, not only contributes to the defence system of the mucosa including IgA production, but also regulates the systemic immune system, resulting in suppression of allergic reactions and autoimmune diseases.[3] The regulative molecules of the intestinal immune system have potential as new immuno-modulators of both the mucosal and systemic immune systems.

Three kinds of polysaccharides (ALR-5IIa-1-1, 5IIb-2-2-Bb and 5IIc-3-1) have been isolated[4,5] from a hot water extract of rhizomes of *Atractylodes lancea* DC. (Compositae) as the modulating polysaccharides of Peyer's patch cells in intestinal immune system. ALR-5IIa-1-1 was shown to comprise mainly arabino-3,6-galactan (Type II arabinogalactan) whereas ALR-5IIb-2-2Bb and ALR-5IIc-3-1 also have rhamnogalacturonan II (RG-II) moieties that might express the activity.[4–6] The analysis of the structure–activity relationship of ALR-5IIa-1-1 by glycosidase digestions has suggested that the arabino-3,6-galactan moiety in the nonreducing terminal side of ALR-5IIa-1-1 mainly (~70% from total activity) contributes to expression of the activity.[6]

The present study describes the analysis of the fine carbohydrate structures, which are responsible for

* Corresponding author. Tel.: +81-3-5791-6364; fax: +81-3-3445-1351; e-mail: yamada@lisci.kitasato-u.ac.jp

0008-6215/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2004.01.003

expression of the intestinal immune system modulating activity of ALR-5IIa-1-1.

2. Experimental

2.1. Materials

Rhizomes of *A. lancea* DC., which were cultivated at Huabei Province in China, were purchased from Tochimoto-Tenkaido Co. Ltd. (Osaka, Japan). ALR-5IIa-1-1 was purified[4] from the rhizomes, and Ara*f*-side chain-trimmed ALR-5IIa-1-1 (AF-ALR-5IIa-1-1) was prepared by digestion with exo-α-L-arabinofuranosidase as described previously.[4,6] Larch wood arabinogalactan and *Acacia* arabinogalactan (gum arabic) were purchased from Sigma, and partially purified by EtOH precipitation and dialysis. Beta-D-glucosyl-Yariv antigen was obtained from Biosupplies (Australia). Exo-α-L-arabinofuranosidase and exo-β-D-galactosidase (Megazyme, Australia) from *Aspergillus niger* were purified by FPLC in according to the modified procedure of Lerouge et al.[7] Driselase (*Irpex lacteus*) was a kind gift from Kyowa-Hakko Co. Ltd. (Japan), and exo-β-D-(1 → 3)-galactanase[8] was purified from Driselase. Endo-β-D-(1 → 6)-galactanase (*Trichoderma viride*)[9] was a kind gift from Prof. Tsumuraya.

2.2. General methods

The carbohydrate content of column eluates were determined by phenol–H_2SO_4 method.[10] Component sugars of poly- and oligosaccharides were analysed as TMS methyl glycoside derivatives by GLC[11] or 1-phenyl-3-methyl-5-pyrazolone derivatives[12] by HPLC. HPLC was performed on a Shimadzu LC-6A equipped with Capcell pak ODS (4.6 × 150 mm, Shiseido Co. Ltd.) in according to the procedure of Honda et al.[12] Single radial gel diffusion by using the β-glucosyl-Yariv antigen was performed according to the procedure of Holst and Clarke.[13] The degree of enzymic hydrolysis of carbohydrate chains was analysed for reducing terminal by Park–Johnson method[14] using Gal as a standard. High performance anion-exchange chromatography equipped with pulsed amperometric detector (HPAEC-PAD) was performed on a Dionex Bio-LC system (Dionex Inc.) using a Carbopak PA-1 column (9 × 250 mm, Dionex Inc.), and 0.1 M NaOH (solvent A) and 1 M NaOAc (solvent B) at flow rate of 2.5 mL min^{-1}. The elution program for analytical experiments was: 0% of solvent B (1 min), 0 → 60% of solvent B (1 → 50 min), 100% of solvent B (50.1 → 60 min), and that for preparative experiments was: 0% of solvent B (1 min), 0 → 10% of solvent B (1 → 40 min), 10 → 40% of solvent B (40 → 50 min) and 100% of solvent B (50.1 → 60 min).

2.3. Purification of oligosaccharide side chains released from AF-ALR-5IIa-1-1

To a solution of AF-ALR-5IIa-1-1 (34.9 mg) in 50 mM acetate buffer (pH 4.2) containing 0.05% NaN_3 (10 mL) was added exo-α-L-arabinofuranosidase and exo-β-D-(1 → 3)-galactanase, and incubated at 37 °C for 3 days. The digestion products were fractionated on a column (2.2 × 100 cm) of Bio-gel P-6 with 50 mM acetate buffer (pH 5.2). The enzymic digestion-resistant fraction (AF-GN-1, yields; 19.0%) and oligosaccharide fractions (AF-GN-2 and 3, yields; 14.0% each) were obtained as the fractions eluted in the void volume, and in the intermediate and the lowest molecular weight fractions, respectively. Oligosaccharides in AF-GN-2 and 3 were further purified by HPAEC-PAD, and the collected oligosaccharides were desalted by passing through AG50WX8 (H^+) resin.

2.4. Enzymic digestions

After AF-GN-2 and 3 (10 μg each) and the purified oligosaccharides were digested with endo-β-D-(1 → 6)-galactanase at 37 °C for 24 h in 500 mM acetate buffer (pH 4.2), the resulting products were further digested with exo-β-D-galactosidase under the same conditions, and analysed by HPAEC-PAD.

AF-ALR-5IIa-1-1 (2 mg) was digested with endo-β-D-(1 → 6)-galactanase [50 mM acetate buffer (pH 4.2, 2 mL), 37 °C, 24 h], and the products were fractionated on a column (1.5 × 47 cm) of Bio-gel P-30 with 50 mM acetate buffer (pH 5.2) to obtain the resistant fraction (GN-AF-ALR-5IIa-1-1) in the void volume and the oligosaccharide fraction in the inner volume. AF-ALR-5IIa-1-1 and GN-AF-ALR-5IIa-1-1 (100 μg each) were digested with exo-β-D-(1 → 3)-galactanase at 37 °C for 2 days in the same buffer, and the resulting digestion products were analysed by HPAEC-PAD.

2.5. MALDI-TOF-MS analysis of ABOE- or 2-AB-labelled oligosaccharides

Oligosaccharides were labelled with *p*-aminobenzoate octylester (ABOE) or 2-aminobenzamide (2-AB) by reductive amination in according to the manual of Seikagaku Kogyo and the procedure of Ishii et al.[15] Solutions (0.5 μL each) of ABOE- or 2-AB-labelled oligosaccharides in 0.1% TFA was loaded on MALDI plates and added 0.5 μL of 2,5-dihydrobenzoic acid solution (10 mg mL^{-1}) in CH_3CN containing 0.1% TFA. The plate was gently dried in the normal atmosphere and analysed by KRATOS AXIMA-CFR MALDI-TOF mass spectrometer (Shimadzu Co. Ltd., S/W Version 2). Ions were accelerated by energy of 20 kV before

entering the TOF mass spectrophotometer. The spectra were measured in the linear mode, and PSD analyses were performed by reflector mode.

2.6. NMR spectroscopy

Oligo-1 was deuterium-exchanged by freeze-drying three times from D_2O, and then examined as a solution in 100% D_2O at 23 °C. Internal dioxane (δ_C 69.200 ppm) was used as a reference for ^{13}C NMR, and chemical shift of D_2O (δ_H 4.760 ppm) was used as a reference for ^1H NMR. Spectra were recorded on a Varian UNITY-400 spectrometer. 2D-NMR spectra were obtained using standard Varian software.

2.7. Measurement of intestinal immune system modulating activity

The intestinal immune system modulating activity was measured as an enhancement of production of bone marrow cells-proliferative cytokines from Peyer's patch cells in according to the procedure of Hong et al.[16] Suspensions (2×10^6 cells mL^{-1}, 200 μL) of Peyer's patch cells from a small intestine of C3H/HeJ mice were cultured with distilled water (control) or test samples (20 μL) in 96-well flat bottom microtitre plate for 5 days at 37 °C in a humidified atmosphere of 5% CO_2–95% air. The resulting culture supernatant (50 μL) was incubated with a bone marrow cell suspension (2.5×10^5 cells mL^{-1}) from C3H/HeJ mice for 6 days in a humidified atmosphere of 5% CO_2–95% air. The number of proliferated bone marrow cells were measured by Alamar Blue™ as described previously.[16] All results are expressed as the mean ± SE. The difference between the control and the treatment in these experiments was tested for statistical significance by Student's *t*-test. A value of $p < 0.05$ was considered to indicate statistical significance.

3. Results and discussion

3.1. Effect of various arabinogalactans on cytokine production of Peyer's patch cells

Arabinogalactans (AGs) and arabinogalactan-proteins (AGPs) are classified into Type I (arabino-4-galactan), Type II (arabino-3,6-galactan) and the other type (macromolecules containing arabinogalactan side chains such as pectins, pectic polysaccharides and plant glycoproteins having arabinogalactan side chains).[17] When the different arabinogalactans were compared for their effects on cytokine production from Peyer's patch cells (Fig. 1), ALR-5IIa-1-1 significantly enhanced the productions of the cytokines, however the other AGs even type II AGs (*Acacia* and Larch wood) did not show any

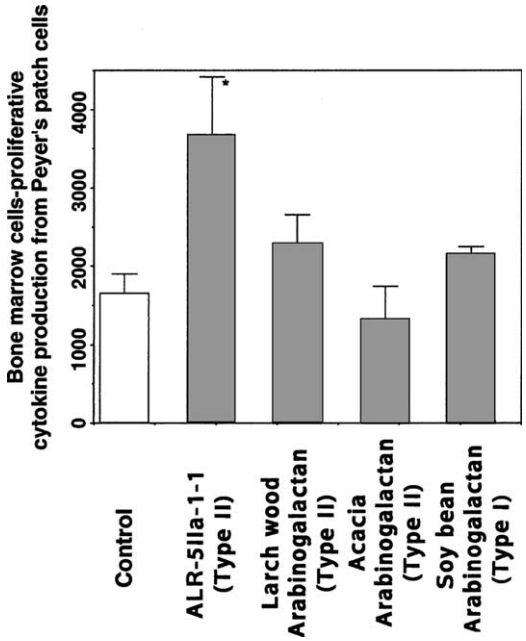


Figure 1. Comparison of the effect of various arabinogalactans on bone marrow cells proliferative cytokine production from Peyer's patch cells. Peyer's patch cells from C3H/HeJ mice were cultured with arabinogalactans at a concentration of 100 μg/mL. (*, $p < 0.05$).

stimulating effects. The results suggested that a certain fine carbohydrate structure of ALR-5IIa-1-1 is responsible for expression of its stimulating effects on cytokine production from Peyer's patch cells.

3.2. Effect of degradation of β-D-(1 → 6)-Gal-containing side chains of ALR-5IIa-1-1 on intestinal immune system modulating activity against Peyer's patch cells

Yu et al. have reported[6] that the degradation of β-D-(1 → 3)-galactan backbone using exo-β-D-(1 → 3)-galactanase significantly reduces the intestinal immune system modulating activity of Ara*f* side chain-trimmed ALR-5IIa-1-1 (AF-ALR-5IIa-1-1), and it has been postulated that the side chains, which are attached to a β-D-(1 → 3)-galactan backbone, may play an important role for expression of the activity of ALR-5IIa-1-1. It has also been reported[6] that the oligosaccharides, released from AF-ALR-5IIa-1-1 by exo-β-D-(1 → 3)-galactanase digestion, consist mainly of 6-linked Gal. Endo-β-D-(1 → 6)-galactanase digestion of AF-ALR-5IIa-1-1 significantly reduced its intestinal immune system modulating activity (Fig. 2). The digestion products gave the enzymic digestion resistant (GN-AF-ALR-5IIa-1-1) and oligosaccharide fractions by gel filtration on Bio-gel P-30 (Scheme 1) (data not shown). Methylation analysis indicated that 4 or 5-linked Ara and 6-linked Gal were decreased significantly in GN-AF-ALR-5IIa-1-1 by comparison with AF-ALR-5IIa-1-1 (data not shown). Single radial gel diffusion analysis with β-D-glucosyl-Yariv antigen[13]

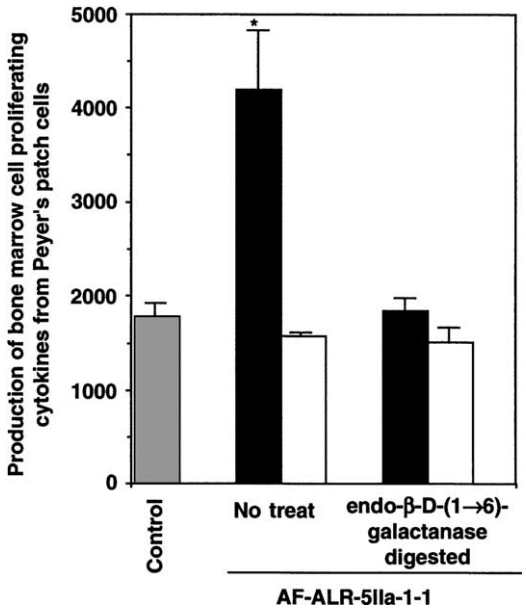
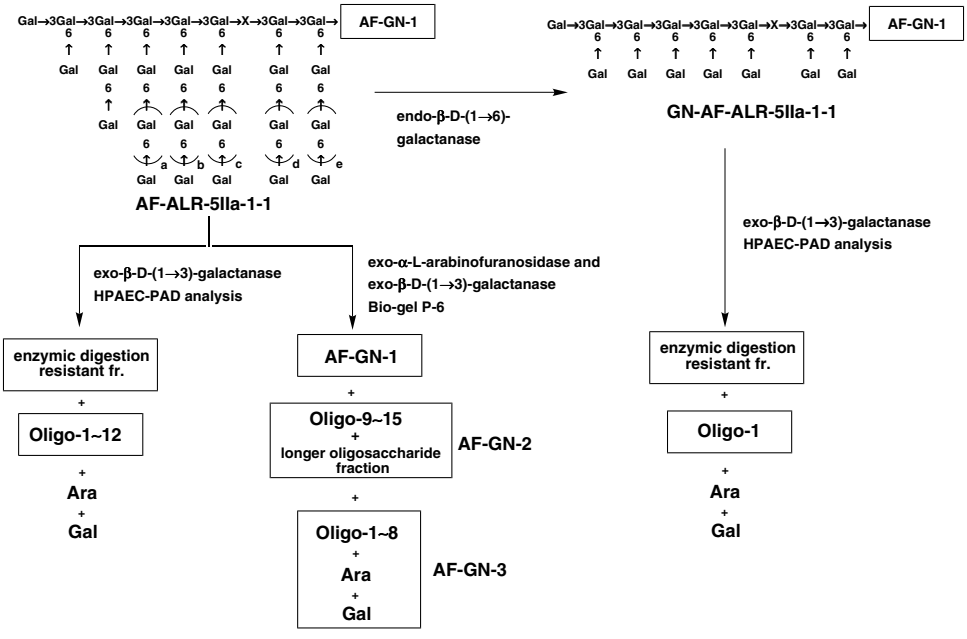


Figure 2. The effect of endo-β-D-(1 → 6)-galactanase digestion of Ara*f* side chains-trimmed ALR-5IIa-1-1 on enhancing activity of cytokine production from Peyer's patch cells. (■) 100 μg/mL; (□) 10 μg/mL; (*) $p < 0.01$.

indicated that GN-AF-ALR-5IIa-1-1 had the same reactivity with Yariv antigen as AF-ALR-5IIa-1-1 (data not shown), suggesting that GN-AF-ALR-5IIa-1-1 still contain a β-D-(1 → 3,6)-galactan structure. When AF-ALR-5IIa-1-1 was digested with exo-β-D-(1 → 3)-galactanase to release oligosaccharides due to the side chains attached to β-D-(1 → 3)-galactan backbone, several sizes of oligosaccharides (Oligo-1–12) in addition to the monosaccharides Ara and Gal were detected by HPAEC-PAD analysis (Scheme 1, Fig. 3A). However, GN-AF-ALR-5IIa-1-1 did not give oligosaccharides except Oligo-1 in addition to Ara and Gal (Scheme 1, Fig. 3B), strongly suggesting that the side chain oligosaccharides, Oligo-2–12, released by exo-β-D-(1 → 3)-galactanase digestion, are important for expression of the activity of AF-ALR-5IIa-1-1.

3.3. Purification and structural characterisation of galactosyl side chains

The combined enzymic digestions of AF-ALR-5IIa-1-1 with exo-α-L–arabinofuranosidase and exo-β-D-(1 → 3)-galactanase gave longer (AF-GN-2) and shorter oligosaccharide fractions (AF-GN-3) in addition to the enzymic digestion-resistant fraction (AF-GN-1) by gel filtration on Bio-gel P-6 (Scheme 1) (data not shown). AF-GN-3 mainly contained eight oligosaccharides (Oligo-1–8) in addition to the monosaccharides Ara and Gal by HPAEC-PAD (Fig. 4A). AF-GN-2 gave oligosaccharides (Oligo-9–15) in addition to small proportions of Oligo-3–8 (Fig. 4D). AF-GN-2 also gave significant amounts of the fraction of longer oligosaccharides (longer oligosaccharide fraction, Fig. 4D). When AF-GN-2 and 3 each were digested with endo-β-D-(1 → 6)-galactanase, most of the oligosaccharide peaks related to Oligo-2–15 disappeared after the digestion, and it was seemed that these oligosaccharides



Scheme 1. Sequential degradation of Ara*f*-trimmed ALR-5IIa-1-1 X; Yu et al. have reported that β-D-(1 → 3)-galactan backbone in ALR-5IIa-1-1 consists partly of unknown glycosyl moiety, which is cleaved by exo-α-L-arabinofuranosidase digestion.[5] Detail of this unknown glycosyl moiety is reported elsewhere.

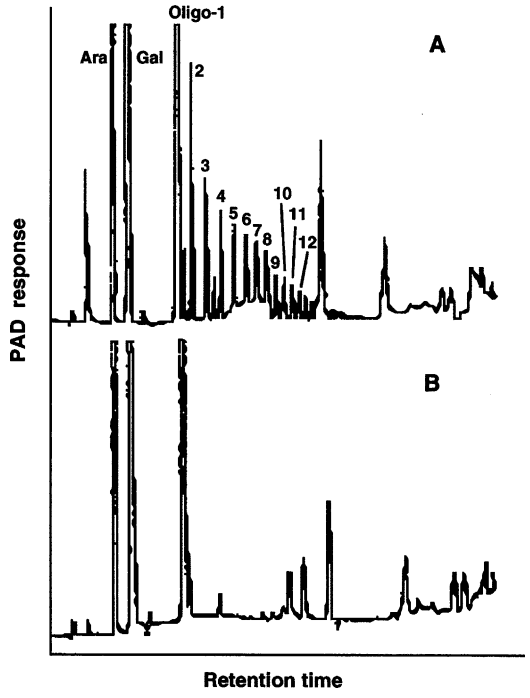


Figure 3. HPAEC patterns of oligosaccharides released from AF-ALR-5IIa-1-1 (A) and endo-β-D-(1→6)-galactanase-treated AF-ALR-5IIa-1-1 (GN-AF-ALR-5IIa-1-1) (see text) (B) with exo-β-D-(1→3)-galactanase digestion. Ara and Gal indicate the elution position of monosaccharide, Ara and Gal.

were changed to Oligo-1 and Gal (Fig. 4B and E). The products in Fig. 4B and E were further digested with exo-β-D-galactosidase giving the monosaccharide Gal, suggesting that Oligo-1 was β-D-galactopyranosyl oligosaccharide (Fig. 4C and F). In addition, the longer oligosaccharide fraction seemed to be digested by the galactanase and the galactosidase (Fig. 4E and F). In order to analyse the structure of the side chain oligosaccharides responsible for the activity of AF-ALR-5IIa-1-1, relatively major oligosaccharides, Oligo-1–Oligo-8 were analysed (Table 1). Analyses of component sugars and digestibility with exo-β-D-galactosidase indicated that Oligo-1 consisted only of β-D-Gal, and MALDI-TOF-MS of ABOE-derivative of Oligo-1 gave $(M+Na)^+$ of hexobiose at m/z 598 (Table 1). ^{1}H NMR analysis assigned all the protons of Oligo-1 by COSY, 1D-TOCSY and decoupling techniques as shown in Table 2. The H-6 of reducing terminal D-Gal (δ 3.802, 4.013, 3.833 and 4.028 ppm) were down field shifted by 0.1–0.3 ppm compared with H-6 of nonreducing terminal D-Gal, and the similar down field shifts were also observed between C-6 of the reducing terminal D-Gal (δ 72.004 and 71.761 ppm) and that of the nonreducing terminal D-Gal (δ 63.643 ppm). These results correlated well with the methylation analysis (Table 3). 1D-NOESY spectra indicated that H-1 of the nonreducing terminal β-D-Gal had a correlation with H-6 of reducing terminal β-D-Gal (data not shown). HMBC also showed that H-1 (δ 4.414 and 4.405 ppm) of nonreducing terminal D-Gal had cross peak with C-6 (δ 72.004 and 71.761 ppm) of reducing terminal D-Gal, and that H-6 (δ 3.802–4.028 ppm) of the reducing terminal D-Gal showed the cross peak with C-1 (δ 105.82 ppm) of the nonreducing terminal D-Gal (data not shown). Therefore, Oligo-1 was identified as β-D-Gal-(1→6)-D-Gal. Oligo-2 and Oligo-5 were mainly comprised of terminal

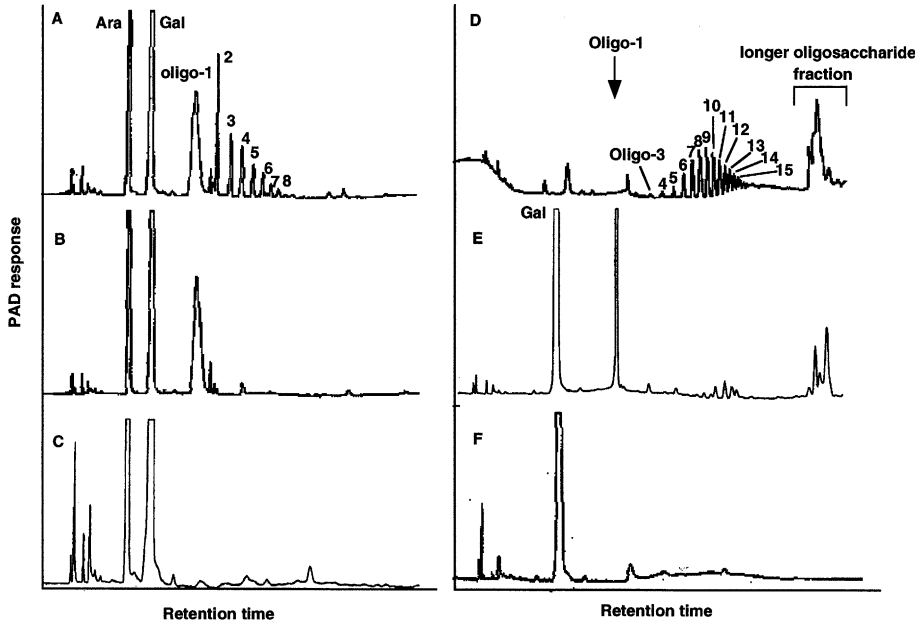


Figure 4. (A, D) HPAEC patterns of oligosaccharides in AF-GN-3 (A) and AF-GN-2 (D). (B, E) HPAEC patterns of digestion products from AF-GN-3 (B) and 2 (E) with endo-β-D-(1→6)-galactanase. (C, F) HPAEC patterns of digestion products from 4B (C) and 4E (F) with exo-β-D-galactosidase.

Table 1. Properties of Oligo 1–8 purified from AF-GN-3

	Component sugar[a] (Gal: Ara)	Peak in HPAEC-PAD[b]	Digestability against exo-β-D-galactosidase	D.p.[c] $(M+Na)^+$	D.p.[d] $(M+Na)^+$	Deduced structure[e]
Oligo-1	Gal only	Single peak	Completely hydrolysed	Hex_2 (m/z 598)	n.d.[f]	β-D-Gal*p*-(1→6)-D-Gal*p*
Oligo-2	Gal only	Single peak	Completely hydrolysed	n.d.	Hex_3 (m/z 649)	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-D-Gal*p*
Oligo-3	Gal only	Peak a (30)	Completely hydrolysed	Hex_4 (m/z 922)	n.d.	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*
		Peak b (1)	Not hydrolysed	n.d.	n.d.	n.d.
Oligo-4	Gal only	Peak a (26)	Completely hydrolysed	Hex_5 (m/z 1084)	Hex_5 (m/z 973)	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-D-Gal*p*
		Peak b (1)	Not hydrolysed	n.d.	n.d.	n.d.
Oligo-5	Gal only	Single peak	Completely hydrolysed	Hex_6 (m/z 1246)	n.d.	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-D-Gal*p*
Oligo-6	1:1	Single peak	Partially hydrolysed	n.d.	n.d.	n.d.
Oligo-7	2:1	Peak a (2)	Completely hydrolysed	n.d.	Hex_8 (m/z 1459)	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-D-Gal*p*
		Peak b (1)	Not hydrolysed	n.d.	n.d.	n.d.
Oligo-8	Gal only	Single peak	Completely hydrolysed	n.d.	Hex_9 (m/z 1621)	β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-D-Gal*p*

[a] Component sugars of oligosaccharides were analysed by PMP method as described in Experimental.
[b] Numbers in parenthesis indicate the ratio of oligosaccharides in each oligosaccharide fraction.
[c] D.p. of oligosaccharides were analysed as ABOE-derivatives from the data of $(M+Na)^+$ and of PSD analysis by MALDI-TOF-MS.
[d] D.p. of oligosaccharides were analysed as 2-AB-derivatives from the data of $(M+Na)^+$ and of PSD analysis by MALDI-TOF-MS.
[e] Structures of oligosaccharides were deduced from the results in this table and the results of endo-β-D-(1→6)-galactanase digestion (see Fig. 4).
[f] Not determined.

Table 2. Assignment of Oligo-1 on ^{1}H NMR

	Chemical shifts (ppm)					
	H-1	H-2	H-3	H-4	H-5	H-6[c]
→6)-α-D-Gal*p*	5.226	3.759	3.825	3.982	4.240	3.802 4.013
→6)-β-D-Gal*p*	4.554	3.452	3.613	3.922	3.867	3.833 4.028
β-D-Gal*p*-(1→[a]	4.414	3.495	3.613	3.880	3.660	3.708 3.749
β-D-Gal*p*-(1→[b]	4.405					

[a] Signal of nonreducing terminal β-D-Gal linked with β-D-Gal of reducing terminal.
[b] Signal of nonreducing terminal β-D-Gal linked with α-D-Gal of reducing terminal.
[c] *C*-6 of reducing terminal D-Gal was observed at 72.004 and 71.761 ppm in ^{13}C NMR, and *C*-6 of nonreducing terminal D-Gal was observed at 63.643 ppm.

Table 3. Methylation analysis of purified oligosaccharides from AF-GN-3

Glycosyl residue	Deduced glycosidic linkage	mol%					
		Oligo-1	Oligo-2	Oligo-3	Oligo-4	Oligo-5	Oligo-6
Gal	Terminal (p)	43.4	34.9	27.2	22.7	19.5	13.6
	Terminal (f)		0.3			2.2	
	2		0.7				
	3		0.7	1.4			
	4		0.7				
	6 (p)	48.5	40.1	52.0	55.7	52.2	23.8
	6 (f)	8.1[a]	11.4[a]	10.5[a]	11.7[a]	5.9[a]	21.3[a]
	2,6		1.0	1.8			
	3,6		1.8	2.1	2.3	2.8	4.0
	4,6		2.2		3.0	3.8	3.3
	3.4,6		1.0	1.3	2.2		
Ara	Terminal (f)		2.3	1.8	2.4	4.6	18.3
	4 or 5		1.8				15.7
Xyl	4		0.6				
Glc	4		0.5	2.0		6.1	

[a] 1,4,6-Tri-OAc-2,3,5-tri-OMe-galactitol was suggested to be derived from 6-linked Gal of reducing terminal.

Gal*p*, and 6-linked Gal*p*, MALDI-TOF-MS analysis and enzymic digestion with exo-β-D-galactosidase indicated that Oligo-2 and Oligo-5 were (1→6)-linked β-D-galactopyranosyltriose and hexaose, respectively (Table 1). Because Oligo-8 could be digested to β-D-(1→6)-galactobiose and Gal with endo-β-D-(1→6)-galactanase completely, and its 2-AB-derivative gave $(M+Na)^+$ at m/z 1621, it was β-D-(1→6)-galactopyranosylnonaose (Table 1). Oligo-3, 4 and 7 still contained two oligosaccharide peaks. However, major oligosaccharide peaks in these preparations were digested to only Gal by exo-β-D-galactosidase. Their ABOE- or 2-AB-derivatives gave $(M+Na)^+$ at m/z 922 (Oligo-3, ABOE), 1084 (Oligo-4, ABOE) and 1459 (Oligo-7, 2-AB) in MALDI-TOF-MS analysis (Table 1). By PSD analysis of MALDI-TOF-MS, major peaks in Oligo-3, 4 and 7 were suggested to be hexosyl tetra-, penta- and octa-saccharides, respectively (Table 1, detailed data was not shown). Because Oligo-3 and Oligo-4 were mainly comprised of terminal and 6-linked Gal*p*, they were identified as β-D-(1→6)-galactopyranosyl-tetra- and pentaose, respectively (Tables 1 and 3). The major peak in Oligo-7 was digested to β-D-(1→6)-galactobiose by endo-β-D-(1→6)-galactanase (Table 1 and Fig. 4), suggesting that it was β-D-(1→6)-galactopyranosyloctaose. Oligo-6 also gave a single peak on HPAEC-PAD analysis containing Gal and Ara (Table 1). Methylation analysis indicated that Oligo-6 was composed mainly of terminal and 6-linked Gal*p* and terminal Ara*f* in addition to 4- or 5-linked Ara (Table 3). The oligosaccharide was partially hydrolysed by exo-β-D-galactosidase, resulting in shorter oligosaccharide (data not shown), suggesting that Oligo-6 might be galactosyl oligosaccharide bearing arabinosyl side chains. The structure of Oligo-6 could not be deduced in the present study because of too small amount available.

Acknowledgements

A part of the present work was supported by a fund from Tsumura & Co., Tokyo. A part of the present work was also supported by The 21st Century COE Program, Ministry of Education, Culture, Sports,

Science and Technology, and Grants-in Aid for Scientific Research (C) from Ministry of Education, Culture, Sports, Science and Technology. We thank Prof. Y. Tsumuraya (Faculty of Science, Saitama University) for his kind gift of endo-β-D-(1→6)-galactanase and Ms. N. Sato for measurements of NMR.

References

1. Kato, T.; Owen, R. L. Mucosal immunology. In *Structure and Function of Intestinal Mucosal Epithelium*; Ogra, P. L., Lamm, M. E., Strober, W., Bienenstock, J., McGhee, J. R., Eds.; 2nd ed.; Academic: New York, 1999; pp 115–132.
2. Kelsall, B.; Strober, W. Mucosal immunology. In *Gut-Associated Lymphoid Tissue. Antigen Handling and T-lymphocyte Responses*; Ogra, P. L., Lamm, M. E., Strober, W., Bienenstock, J., McGhee, J. R., Eds.; 2nd ed.; Academic: New York, 1999; pp 293–317.
3. McGhee, J. R.; Lamm, M. E.; Strober, W. Mucosal immunology. In *Mucosal Immune Responses. An Overview*; Ogra, P. L., Lamm, M. E., Strober, W., Bienenstock, J., McGhee, J. R., Eds.; 2nd ed.; Academic: New York, 1999; pp 485–506.
4. Yu, K.-W.; Kiyohara, H.; Matsumoto, T.; Yang, H.-C.; Yamada, H. *Planta Med.* **1998**, *64*, 714–719.
5. Yu, K.-W.; Kiyohara, H.; Matsumoto, T.; Yang, H.-C.; Yamada, H. *Carbohydr. Polyms.* **2001**, *46*, 125–134.
6. Yu, K.-W.; Kiyohara, H.; Matsumoto, T.; Yang, H.-C.; Yamada, H. *Carbohydr. Polyms.* **2001**, *46*, 147–156.
7. Lerouge, P.; O'Neill, M. A.; Darvill, A. G.; Albersheim, P. *Carbohydr. Res.* **1993**, *243*, 373–378.
8. Tsumuraya, Y.; Mochizuki, N.; Hashimoto, Y.; Kovak, P. *J. Biol. Chem.* **1990**, *265*, 7207–7215.
9. Okemoto, K.; Uekita, T.; Tsumuraya, Y.; Hashimoto, Y.; Kosama, T. *Carbohydr. Res.* **2003**, *338*, 219–230.
10. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. *Anal. Chem.* **1956**, *28*, 350–356.
11. York, W. S.; Darvill, A. G.; McNeil, M.; Stevenson, T. T.; Albersheim, P. *Methods Enzymol.* **1986**, *118*, 3–40.
12. Honda, S.; Akao, E.; Suzuki, S.; Okuda, M.; Kakehi, K.; Nakamura, J. *Anal. Biochem.* **1989**, *180*, 351–357.
13. Holst, G. J.; Clarke, A. E. *Anal. Biochem.* **1985**, *148*, 446–450.
14. Park, J. T.; Johnson, M. J. *J. Biol. Chem.* **1949**, *81*, 149–151.
15. Ishii, T.; Ichita, J.; Matsue, H.; Ono, H.; Maeda, I. *Carbohydr. Res.* **2002**, *337*, 1023–1032.
16. Hong, T.; Matsumoto, T.; Kiyohara, H.; Yamada, H. *Phytomedicine* **1998**, *5*, 353–360.
17. Basic, A.; Currie, G.; Gilson, P.; Mau, S.-L.; Oxley, D.; Schultz, C. Cell and developmental biology of arabinogalactan-proteins. In *Structural Classes of Arabinogalactan-Proteins*; Nothnagel, E. A., Basic, A., Clarke, A. E., Eds.; Kluwer Academic/Plenum: New York, 2000; pp 11–23.