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Characterization of Bone Marrow Cell Proliferating Arabinogalactan through Peyer's Patch Cells from Rhizomes of *Atractylodes lancea* DC

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Abstract

Bone marrow cell proliferating arabinogalactan-like polysaccharide (ALR-3IIa-1-1) has been purified from rhizomes of *Atractylodes lancea* DC. In order to characterize the essential structure of ALR-3IIa-1-1 for expression of the activity, sequential enzymatic digestion using exo-α-L-arabinofuranosidase (AFase) and exo-β-D- (1→3)-galactanase (GNase) was employed. After ALR-3IIa-1-1 was digested with the AFase, the GNase digestion cleaved only 10% and 23% of 3-linked and 3,6-branched galactose, respectively, from arabinose-trimmed ALR-3IIa-1-1 (AT-ALR- 3IIa-1-1), and gave small amounts of intermediate size (AT-G-2) and shorter oligosaccharides (AT-G-3) fractions in addition to a large amount of the GNase resistant fraction (AT-G-1). When AT-G-1 was redigested gradually with the AFase and GNase, it released trace amounts of oligosaccharides in addition to a large amount of the resistant fraction. When the final enzyme-resistant fraction from AT-G-1 was digested simultaneously with both AFase and GNase, the resistant fraction was significantly degraded into two long fragments (3AT-3G-1 and 2). The mixture of digestion products from the first GNase digestion of AT-ALR-3IIa-1-1 showed a significantly decreased bone marrow cell proliferation activity to about 30% of the activity of ALR-3IIa-1-1, but the GNase resistant fraction (AT-G-1) still had significant activity. Although the second gradual enzymatic digestion of AT-G-1 showed a marginal decrease in activity, the resulting fragments (3AT-3G-1 and 2) by the final simultaneous enzymatic digestion lost most of the activity. Component sugar, methylation and FAB-MS analyses indicated that the digestion products (AT-G-2, AT-G-3, 2AT-2G-2 and 2AT-2G-3) released from AT-ALR-3IIa-1-1 by the sequential enzymatic digestion contained galactose-containing oligosaccharides mainly comprising 6-linked galactose, that some of which were partially arabinosylated, and these oligosaccharides were attached to β-D-(1→3)-galactan backbone in its non-reducing terminal side as side chains.

Key words: *Atractylodes lancea*, arabino-3,6-galactan, bone marrow cell proliferation activity, essential structure

INTRODUCTION

The term "arabinogalactan" specifies certain structural characteristics of the carbohydrate component (1). The "galactan" portion of the name specifies a polysaccharide backbone or framework in which galactosyl residues are important components. The "arabino" portion of the name specifies that arabinosyl residues must also be important components, either as substituents on the galactan framework or as residues within the framework itself.

Aspinall (2) classified plant arabinogalactan as either type 1 or type II. Type I arabinogalactans have a linear (1→4)-β-D-galactan backbone with arabinose oligosaccharide side chains. Type II arabinogalactan have a highly branched framework consisting in a (1→3)-β-D-galactan backbone with (1→6)-β-D-galactan side chains attached at carbon atom 6 of some of the galactosyl residues in the backbone. Most of the arabinosyl residues in type II arabinogalactans are attached at carbon atom 3 of some of the galactosyl residues in the side chains.

In aspects of plant physiological functions, arabinogalactan-proteins have a role in cell identity and/or cell-to-cell signalling, and contribute to cell proliferation, somatic embryogenesis, and pollen tube growth (3,4). Although the most popular arabinogalactan, gum arabic from *Acacia* sp., has been used for food additives, other arabinogalactans have been evaluated for their pharmacological activity and medicinal uses. Yamada et al. (5) have reported that an arabino-3,6-galactan, which is isolated from a medicinal herb, the roots of *Angelica acutiloba*, shows a complementary activity through classical and alternative pathways. Several arabino-galactan and arabinogalactan-containing polysaccharides further have been found as complement activators, and structures of these galactan portions were suggested to contribute to expression of their activity (6). It also has been reported that some arabinogalactans such as larch wood arabinogalactan can stimulate natural killer (NK) cell cytotoxicity, TNF production and tumor cytotoxicity of macrophages, and are applicable as drug carriers for hepatic drug delivery (7,8).

In previous studies (9), we have described the purification of polysaccharides from rhizomes of *Atractylodes lancea* DC. having potent bone marrow cell proliferation activity. ALR-3IIa-1-1 of these polysaccharides is comprised mainly of arabinose and galactose, and consisted mainly of

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terminal, 4- or 4-linked, 3,4- or 3,5-branched arabinose, 3-, 4-linked and 3,6-branched galactose. ALR-3IIa-1-1 also showed a strong reactivity with β-D-glucosyl-Yariv antigen. Since these properties are characteristics of arabino-3,6-galactan (10), ALR-3IIa-1-1 has been assumed to contain arabino-3,6-galactan moiety as bone marrow cell proliferating polysaccharide.

In the present paper, we describe the structural characterization of galactan portion of ALR-3IIa-1-1 using specific enzymes such as exo- α-L-arabinofuranosidase (AFase) and exo-β-D-(1→3)-galactanase (GNase), and its essential structure for expression of the bone marrow cell proliferation activity.

MATERIALS AND METHODS

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-6 and P-30 were obtained from Bio-Rad (USA), β-D-glucosyl-Yariv antigen form Biosupplies (Australia), and Sep-pak C_{18} cartridges from Waters Associate (USA). Exo- α-L-arabinofuranosidase (Megazyme, Australia) from *Aspergillus niger* was purified by FPLC (11). Driselase (*Irpex lacteus*) was purchased from Kyowa-Hakko Co. Ltd., and exo-β-D-(1→3)-galactanase was purified from Driselase according to the procedure of Tsumuraya et al. (12).

General methods

Total carbohydrate, pentose, uronic acid and protein contents in column eluates were measured by the phenol-H_2SO_4 (13), phloroglucino-AcOH (14), *m*-hydroxydiphenyl (15) and Bradford's methods (16) with Bio-Rad dye (Bio-Rad) by using galactose, arabinose, galacturonic acid and IgG as the respective standards. Sugars were converted into TMS methyl glycoside derivatives (17) and analyzed by GLC using a DB-1 capillary column (0.20 μm film, 0.25 mm i.d. × 30 m, Supelco). GLC was performed on an HP-5890 Series II gas chromatography (Hewlett-Packard, USA) and the program was : 60°C for 1 min, 60→170°C (30°C/min), 170→190°C (1°C/min), and 190→300°C (30°C/min). Single radial gel diffusion by using the β-D-glucosyl-Yariv antigen was performed according to the procedure of Holst and Clarke (18).

Purification of ALR-3IIa-1-1

A crude polysaccharide fraction (ALR-3) was prepared from rhizomes of *Atractylodes lancea* DC. by hot-water extraction, EtOH precipitation, and dialysis (19). ALR-3 was fractionated by anion-exchange chromatography on DEAE-Sepharose CL-6B (HCO_3^- form) to obtain the bone marrow cell proliferating fraction (ALR-3IIa). ALR-3IIa was further fractionated by gel filtration on Sephacryl S-200 and S-300 columns in 0.2 M NaCl to obtain the active polysaccharide, ALR-3IIa-1-1 (yield from ALR-3, 0.14%) (9).

Sequential enzymatic digestion of ALR-3IIa-1-1

Procedure 1

ALR-3IIa-1-1 (17 mg) was digested with exo- α-L-arabinofuranosidase (AFase, 0.01 U) in 4 mL of 50 mM acetate buffer (pH 4.5) at 40°C for 7 hr. 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 fractions eluted in the void volume (AT-ALR-3IIa-1-1) and in the inner volume were obtained. After AT-ALR-3IIa-1-1 was desalted by electric dialyzer and lyophilized, AT-ALR-3IIa-1-1 (10 mg) was further digested with exo-β-D-(1→3)-galactanase (GNase, 0.1 U) in 50 mM acetate buffer (pH 4.5, 5 mL) at 37°C for 48 hr. 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 (AT-G-1, 8 mg) eluted in the void volume, intermediate fraction (AT-G-2) and the lowest-molecular-weight fraction (AT-G-3) were obtained.

Procedure 2

AT-G-1 (8 mg) was re-digested with AFase, and the resulting 2AT-G-1 (7 mg) was further digested with GNase. The digestion products were fractionated on Bio-gel P-6 to obtain the fraction eluted in the void volume (2AT-2G-1, 5 mg), intermediate fraction and the lowest-molecular-weight fraction.

Procedure 3

2AT-2G-1 was simultaneously digested with AFase and GNase at 37°C for 48 hr. The digestion products were fractionated on a column (2 × 50 cm) of Bio-gel P-6, and the resistant fraction eluted in the void volume (3AT-3G-1) and the digested fraction (3AT-3G-2) were obtained.

Methylation analysis

Each sample was methylated according to the Hakomori method (20) and the methylated products were recovered using Sep-pak C_{18} cartridge by the procedure of Waeghe et al. (21) except that samples were eluted only by EtOH. Carboxymethyl groups in methylated products were reduced with $LiB(C_2H_5)_3D$ in THF (Super-Deuteride®, 1 mL, Aldrich) at room temperature for 1 hr (17), and the reduced products were recovered by a Sep-pak C_{18} cartridge. The methylated products were hydrolyzed with 2 M TFA at 121°C for 1.5 hr 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 chromatography equipped with a SP-2380 capillary column, and GLC-EIMS was done on a Hewlett- Packard model 5970B mass spectrometer. Conditions of GLC were as the procedure of Zhao et al. (22). Methylated alditol acetates were identified by their fragments ions in GLC-EIMS and relative retention times in GLC. Their mole percentages were estimated from the peak areas and response factors in FID (23).

FAB-MS analysis of methylated oligosaccharides

A JEOL JMS-AX 505 HA mass spectrometer interfaced

with an OA-5000 computer was used. Xenon was used as the bombarding gas, and the atom gun was operated at 3 kV, 10 mA. The instrument was scanned at *m/z* 0-1500 with a scan rate of 20 s/decade, and the accelerating voltage was 3 kV. A mixture of 1 : 1 glycerol-mono-thiglycerol was used as a matrix. One μL of a MeOH solution of methylated oligosaccharides was placed on the target of the probe, and was mixed with 1 μL matrix. B/E (daughter ions) linked scans were performed by using a linked scan unit at a scan rate of 120 s/decade using He as a collisional gas.

Bone marrow cell proliferation activity through Peyer's patch cells

The bone marrow cell proliferation activity was measured according to the procedure of Hong et al. (24).

Preparation of Peyer's patch cells suspension

C3H/HeJ mice (5~7 weeks old, female, Daehan-Biolink Co., Chungbuk) were sacrificed and their small intestines were exposed on sheets of clean paper. Peyer's patches were carefully dissected out using fine scissors from wall of the small intestines, and placed in ice cold RPMI 1640 medium supplemented with 5% FBS (RPMI 1640-FBS). The Peyer's patch cells were dispersed by tapping gently with a rubber rod on a 100-gauge sterile stainless sieve. The cell suspensions were passed through a 200-gauge sterile stainless sieve and washed 2 times with HBSS supplemented with 5% FBS (HBSS-FBS), and then resuspended in RPMI 1640-FBS. The cells were suspended at a density of 2×10^6 cells/mL in RPMI 1640-FBS. 180 μL of aliquots of cell suspension were dispensed into 96-well plates and cultured with 20 μL of the appropriate diluted test samples for 5 days at 37°C in a humidified atmosphere of 5% CO_2-95% air. The resulting culture supernatant (conditioned medium) was used for stimulation of bone marrow cells.

Preparation of bone marrow cells suspension

Bone marrow cells were obtained from the femora of C3H/HeJ mice. After the mice were sacrificed, the femora were excised and flushed of bone marrow cells using 23-gauge needle. Bone marrow cells were suspended in RPMI 1640-FBS. and the cells were washed 2 times with HBSS-FBS and resuspended in RPMI 1640-FBS at a density of 2.5×10^5 cells/mL in RPMI 1640-FBS.

Measurement of the proliferation of bone marrow cells

Cell growth was measured by means of a fluorometric assay, Alamar Blue™ reduction assay (25). One hundred μL of bone marrow cell suspensions was dispensed into 96-well plate, and then 50 μL of RPMI 1640-FBS and 50 μL of conditioned medium were added to each well of the plate. The cells were cultured for 6 days in a humidified atmosphere of 5% CO_2-95% air. After 20 μL of Alamar Blue solution was added to each well, the cells were then continuously cultured for 5~24 hr. The fluorescence intensity was measured to count cell numbers by Fluoroskan II (Labsystems, Finland) at an excitation wavelength of 544 nm and emission wavelength of 590 nm during cultivation. The bone marrow cell proliferation activity was expressed as stimulation of cell growth of bone marrow cells compared with that of control in which Peyer's patch cells were incubated with saline instead of test samples.

RESULTS AND DISCUSSION

Characterization of ALR-3IIa-1-1 by sequential enzymatic digestion with AFase and GNase

The previous study suggested that bone marrow cell proliferation activity of ALR-3IIa-1-1 was expressed by arabino-3, 6-galactan moiety in ALR-3IIa-1-1 (9). When bone marrow cell proliferation activity was compared between ALR-3IIa-1-1 and other commercially available arabino-3,6-galactans, ALR-3IIa-1-1 showed potent and significant activity, whereas commercially available arabino-3,6-galactan from larch wood and acacia had no activity (Fig. 1). Therefore, it is assumed that the fine structure of ALR-3IIa-1-1 plays an important role for expression of bone marrow cell proliferation activity.

In order to analyze essential structure for expression of the activity of ALR-3IIa-1-1, after ALR-3IIa-1-1 was digested with exo-α-L-arabinofuranosidase (AFase), the digestion products were fractionated on Bio-gel P-30 to obtain the resistant fraction (AT-ALR-3IIa-1-1) and the fraction eluted in the inner fraction (Fig. 2A). The fraction eluted in the inner volume consisted mainly of arabinose (93%) and arabinose of ALR-3IIa-1-1 was degraded about 60% in AT-ALR-3IIa-1-1 (Table 1). Methylation analysis of AT-ALR-3IIa-1-1 indicated that terminal arabinose, 3,4- or 3,5-branched arabinose

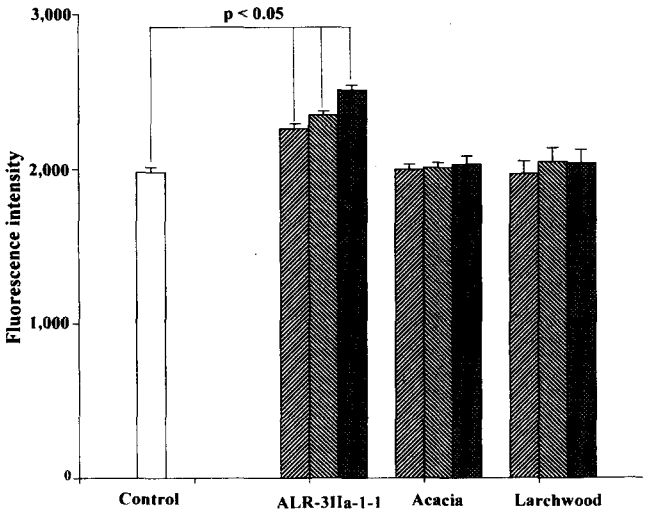

Fig. 1. Comparison of bone marrow cell proliferation activity between ALR-3IIa-1-1 and other arabino-3,6-galactan. After Peyer's patch cells obtained from C3H/HeJ mouse (5~7 weeks old, female) and cultured with test samples for 5 days, the resulting cell-free supernatant was subjected to stimulation of bone marrow cells. The proliferation of bone marrow cells was measured by a fluorometric method using Alamar Blue™ reduction assay. $p < 0.05$: Significant difference between control and samples. □, Control (saline); ▨, Sample (50 μg/mL); ▧, Sample (100 μg/mL); ▩, Sample (200 μg/mL).

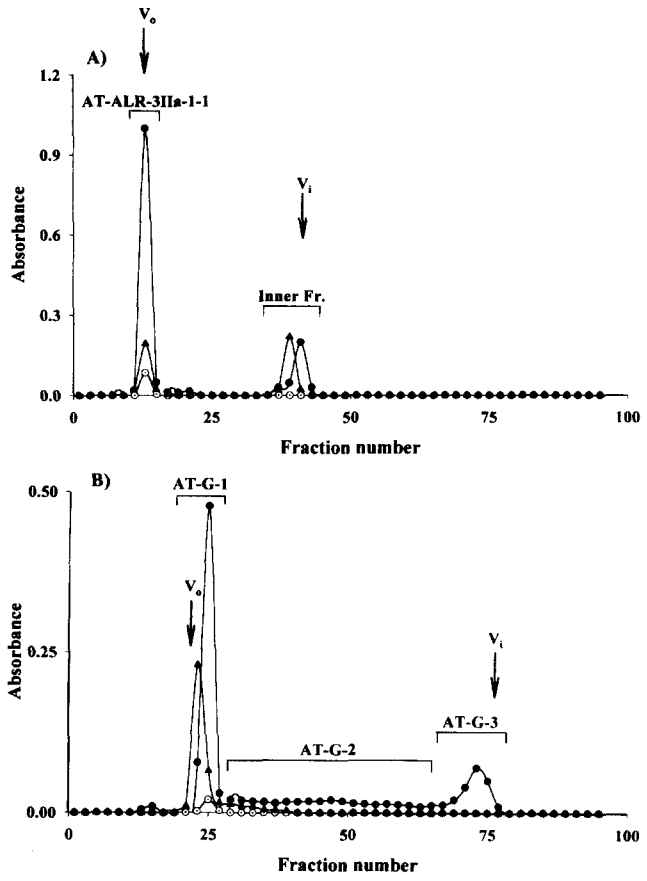

Fig. 2. Gel filtration pattern of A) the products digested from ALR-3IIa-1-1 with exo-α-L-arabinofuranosidase (AFase) on Bio-gel P-30 and B) the products digested from AT-ALR-3IIa-1-1 with exo-β-D-(1→3)-galactanase (GNase) on Bio-gel P-6. The columns of Bio-gel P-30 (2×50 cm) and Bio-gel P-6 (1.5×90 cm) were eluted with 50 mM acetate buffer (pH 5.2) at flow rate of 0.2 mL/min. The symbol is V_o, void volume; V_i, inner volume. ●, Carbohydrate (490 nm); ○, Uronic acid (520 nm); ▲, UV absorbance (280 nm).

and 3,6-branched galactose were decreased along with increasing of 4- or 5-linked arabinose and 6-linked galactose (Table 2). AT-ALR-3IIa-1-1 was digested with exo-β-D-(1→3)-galactanase (GNase), and the digestion products were fractionated on Bio-gel P-6. The products gave a large amount of the fraction eluted in the void volume (AT-G-1), and small amount of an intermediate fraction (AT-G-2) and a fraction eluted in the inner volume (AT-G-3) (Fig. 2B). Component sugar and methylation analysis of AT-G-1 suggested that the digestion ratio of galactose in AT-ALR-3IIa-1-1 was very low, about 3.6% (Table 1), and a little amount (3.4% and 17.4%) of linkages corresponding to 3-linked and 3,6-branched galactose of AT-ALR-3IIa-1-1 could be cleaved by the GNase digestion (Table 2). However, when AT-G-1 was reacted with β-D-glucosyl-Yariv antigen on a single radial gel diffusion, this resistant fraction did not react with the antigen (data not shown). These results indicated that the digestion of AT-ALR-3IIa-1-1 with GNase might be imperfect. Therefore, AT-G-1 was re-digested with AFase followed by GNase using the above sequential procedure, and

Table 1. Component sugar of the products digested from ALR-3IIa-1-1 with exo-α-L-arabinofuranosidase (AFase) and exo-β-D-(1→3)-galactanase (GNase)

Component sugar (mol. %)	ALR-3IIa-1-1	AT-ALR-3IIa-1-1	AT-G-1	AT-G-2	3AT-3G-1	3AT-3G-2
Arabinose	32.7	12.9	5.2	27.8	6.4	19.6
Xylose	1.0	1.4	2.0	-	1.6	-
Rhamnose	0.4	0.6	1.4	-	2.2	1.7
Fucose	trace	trace	0.8	-	1.9	-
Mannose	2.5	3.7	7.5	-	8.7	7.4
Galalctose	60.5	76.8	74.0	71.2	56.6	51.9
Glucose	2.9	4.6	9.0	0.6	22.6	19.4
Glucuronic acid	trace	trace	trace	-	n.d.[1)]	n.d.
Galacturonic acid	trace	trace	trace	0.4	n.d.	n.d.

[1)]not determined.

Table 2. Methylation analysis of the products derived from ALR-3IIa-1-1 with AFase and GNase

Glycosyl residue	Deduced linkage	mol. %				
		ALR-3IIa-1-1	AT-ALR-3IIa-1-1	AT-G-1	3AT-3G-1	3AT-3G-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	7.9	7.8	24.5
	3	7.2	5.8	5.6	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	18.3	12.1	9.6
	2,6	0.3	0.5	1.1	1.0	-
	3,6	15.2	6.9	5.7	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

fractionated on Bio-gel P-6 to obtain a large amount fraction of the void volume (2AT-2G-1), intermediate fraction (2AT-2G-2) and a small amount of the inner volume (2AT-2G-3) (Fig. 3). In spite of re-digestion, only a small amount of oligosaccharide fraction was produced from AT-G-1. To extend the effect of the enzyme action and to supplement inefficient enzyme action, the resistant fraction (2AT-2G-1) was re-digested with AFase and GNase by simultaneous treatment. By this treatment, 2AT-2G-1 was fully degraded and produced

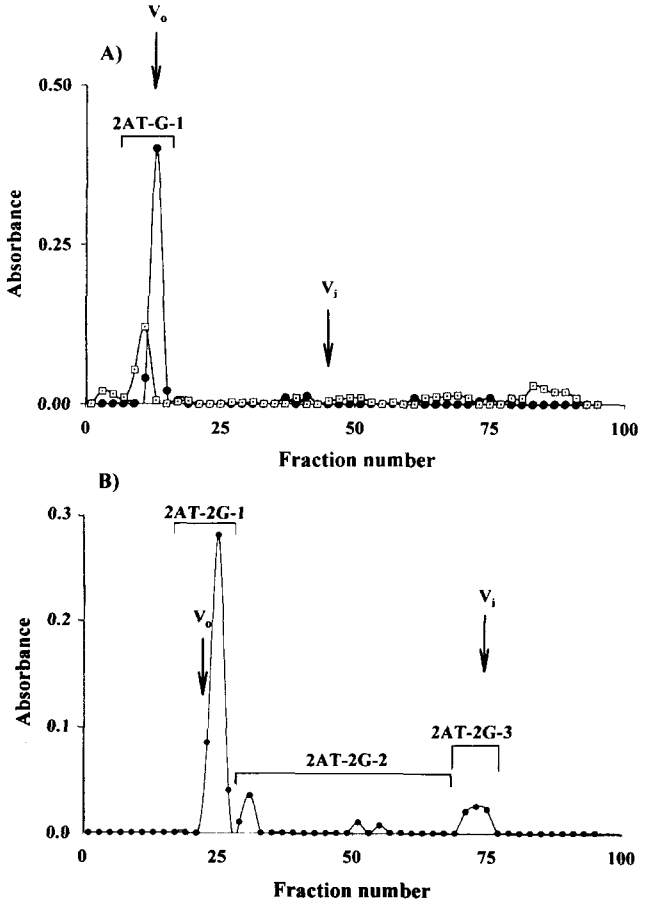

Fig. 3. Gel filtration patterns of A) the products digested from AT-G-1 with AFase on Bio-gel P-30 and B) the products digested from 2AT-G-1 with GNase on Bio-gel P-6. The columns of Bio-gel P-30 (2×50 cm) and Bio-gel P-6 (1.5×90 cm) were eluted with 50 mM acetate buffer (pH 5.2) at flow rate of 0.2 mL/min. The symbol is V_o, void volume; V_i, inner volume. ●, Carbohydrate (490 nm); ⊡, Pentose (552-510 nm).

the resistant fraction (3AT-3G-1) and the digested fraction (3AT-3G-2) (Fig. 4). Component sugar analysis of 3AT-3G-1 showed that galactose of AT-G-1 was digested about 24%, though this fraction consisted mainly of mannose, glucose and galactose, and 3AT-3G-2 was composed of about 52% of galactose (Table 1), suggesting that galactose quite digested by simultaneous enzyme treatment of AFase and GNase. Methylation analysis of 3AT-3G-1 indicated that 3-linked and 3,6-branched galactose of AT-ALR-3IIa-1-1 were degraded about 29% and 55% by this enzymatic treatment, and also had terminal galactose, 4-linked and 6-linked galactose, terminal glucose, 4-linked and 6-linked glucose, and terminal arabinose (Table 2). In contrast, 3AT-3G-2 was mainly composed of terminal arabinose, 4- or 5-linked arabinose, terminal galactose, 3-linked and 6-linked galactose in addition to terminal mannose, terminal glucose and 4-linked glucose (Table 2). These results suggested that 3-linked and 3,6-branched galactose of ALR-3IIa-1-1 were fully degraded by AFase and GNase.

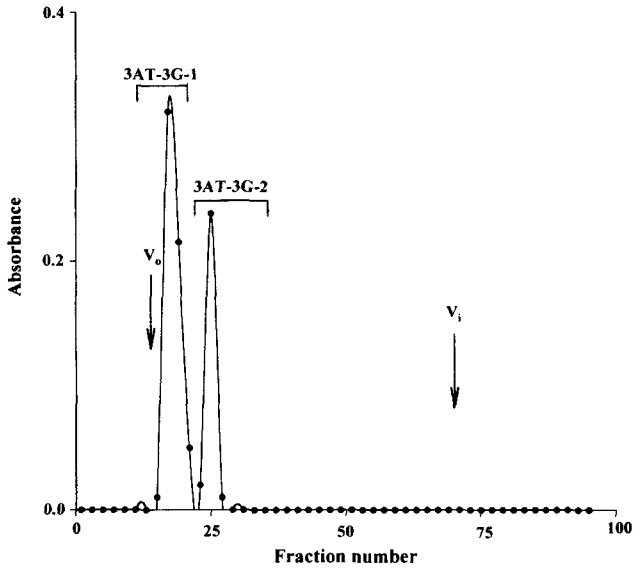

Fig. 4. Gel filtration pattern of the products digested from 2AT-2G-1 with AFase and GNase on Bio-gel P-6. The column of Bio-gel P-6 (1.5×90 cm) was eluted with 50 mM acetate buffer (pH 5.2) at flow rate of 0.2 mL/min. The symbol is V_o, void volume; V_i, inner volume. ●, Carbohydrate (490 nm).

Structure of oligosaccharide fraction

The sequential enzymatic digestion of ALR-3IIa-1-1 with AFase and GNase produced oligosaccharide fractions I (AT-G-2 and 2AT-2G-2) and II (AT-G-3 and 2AT-2G-3), which might be present in the non-reducing terminal of ALR-3IIa-1-1. Component sugar and methylation analyses indicated that oligosaccharide fraction I consisted mainly of arabinose (28%) and galactose (71%) in addition to a trace of glucose and galacturonic acid (Table 3), and terminal galactose, 6-linked, 3,6-branched galactose, terminal arabinose and 4- or 5-linked arabinose were the main glycosidic linkages of fraction I (Table 4). Oligosaccharide fraction II was galactose-containing oligosaccharides (93% of galactose) in addition to arabinose, glucose, glucuronic acid and galacturonic acid (Table 3), and this fraction was composed of terminal and 6-linked galactose, in addition a large amount of 6-linked galactose (furanose form) (Table 4). The presence of a galactose-containing oligosaccharides of fraction I and

Table 3. Component sugar of oligosaccharide fractions derived from ALR-3IIa-1-1 with AFase and GNase digestion

	AT-G-2	AT-G-3
Component sugar (mol. %)		
Arabinose	27.8	3.2
Xylose	trace	-
Fucose	trace	-
Rhamnose	-	-
Mannose	-	-
Glucose	0.6	1.6
Galactose	71.2	93.3
Glucuronic acid	-	1.5
Galacturonic acid	0.4	0.4

Table 4. Methylation analysis of oligosaccharide fractions derived from ALR-3IIa-1-1 with AFase and GNase digestion

Glycosyl residue	Deduced linkage	mol. %	
		AT-G-2	AT-G-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	-
Man	2	0.4	-
Gal	terminal	18.9	48.3
	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	-

II was confirmed by FAB-MS. The positive-ion spectrum of oligosaccharide fraction I showed pseudomolecular ions ($[M+Na]^+$) due to methylated tri-, tetra-, penta- hexa- and heptasaccharide of hexose, and fraction II had methylated di-, tri-, tetra-, pentasaccharide of hexose (Table 5), suggesting that fraction I and II mainly contained galactooligosaccharides.

Bone marrow cell proliferation activity of fragments

The bone marrow cell proliferation activity of these fractions obtained by the simultaneous enzymatic digestion was tested. AT-G-1 obtained from ALR-3II-1-1 with AFase and GNase still had significant potent activity (about 24% decrease), even though the mixture of digestion products (no fractionation) decreased the activity by 65% (Fig. 5). 2AT-2G-1 re-digested from AT-G-1 with both enzymes decreased the activity by about 40% compared to ALR-3IIa-1-1 (Fig. 5). The final resistant product (3AT-3G-1) significantly reduced the activity (about 80%) (Fig. 5). These results suggest that arabino-3,6-galactan moiety of ALR-3IIa-1-1 plays an important role in the bone marrow cell proliferation activity of *Atractylodes lancea* DC. rhizomes.

The present study clarified by the enzymatic digestion of

Table 5. Oligosaccharides detected in methylated fragment of products digested from ALR-3IIa-1-1 with AFase and GNase by FAB-MS

Fraction	*m/z* ($[M+Na]^+$)	Deduced oligosaccharide
Oligosaccharide fraction I[1)]	681	Hex_3Na^+
	886	Hex_4Na^+
	1090	Hex_5Na^+
	1294	Hex_6Na^+
	1497	Hex_7Na^+
Oligosaccharide fraction II[2)]	477	Hex_2Na^+
	681	Hex_3Na^+
	886	Hex_4Na^+
	1090	Hex_5Na^+

[1)]Oligosaccharide fraction I : the mixture of AT-G-2 and 2AT-2G-2.
[2)]Oligosaccharide fraction II : the mixture of AT-G-3 and 2AT-2G-3.

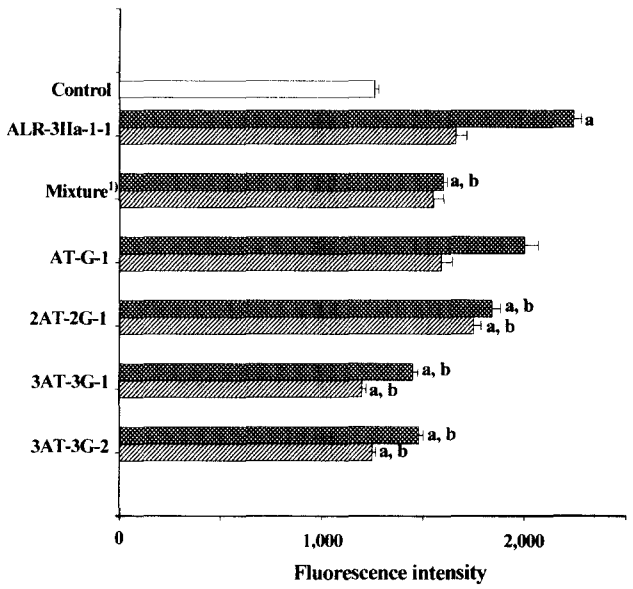

Fig. 5. Bone marrow cell proliferation activity of the products digested from ALR-3IIa-1-1 with AFase and GNase.
After Peyer's patch cells obtained from C3H/HeJ mouse and cultured with test samples for 5 days, the resulting cell-free supernatant was subjected to stimulation of bone marrow cells. The proliferation of bone marrow cells was measured by a fluorometric method using Alamar Blue™ reduction assay.
[1)]Mixture : no fractionation of the products digested from ALR-3IIa-1-1 with AFase and GNase. a, $p<0.05$: Significant difference between control and samples. b, $p<0.05$: Significant difference between ALR-3IIa-1-1 and other samples. □, Control (saline); ▨, Sample (25 μg/mL); ▩, Sample (100 μg/mL).

ALR-3IIa-1-1 strongly suggests that ALR-3IIa-1-1 consisted mainly of a (1→3)-linked galactan backbone. In addition, (1→3)-linked galactan backbone was regularly or randomly interrupted by α-L-arabinose. Since the galacto-oligosaccharides (oligosaccharide fraction I and II) derived from the resistant arabinogalactan side chains (AT-ALR-3IIa-1-1 and AT-G-1) were composed mainly of 6-linked galactose, suggesting that (1→6)-linked galactosyl chains were attached to position 6 of the backbone as the side chains. GNase digestion of AT-ALR-5IIa-1-1, which was obtained from ALR-3IIa-1-1 with AFase, did not affect bone marrow cell proliferation activity of ALR-3IIa-1-1. This fact indicates that the first (AT-G-2 and 3) and second digested β-D-(1→3)-galactan chains (2AT-2G-2 and 3) in ALR-3IIa-1-1 containing the short neutral side-chains weakly contribute to expression of the activity of ALR-3IIa-1-1 (each about 20% contribution to activity). The results of sequential enzymatic digestion indicated that the third digested galactan chains (3AT-3G-2) in ALR-3IIa-1-1 also consisted mainly of a β-D-(1→3)-galactan backbone which carried some side-chains at position 4 or 6 of the backbone. Therefore, these results propose that bone marrow cell proliferation activity of ALR-3IIa-1-1 is expressed by the third digested galactan chains rather than by the first digested galactan chains in ALR-3IIa-1-1.

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