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Characterization of antigenic epitopes in anti-ulcer pectic polysaccharides from *Bupleurum falcatum* L. using several carbohydrases

Masumi H. Sakurai [a], Hiroaki Kiyohara [a], Tsukasa Matsumoto [a], Yoichi Tsumuraya [b], Yohichi Hashimoto [b], Haruki Yamada [a,*]

[a] *Oriental Medicine Research Center, The Kitasato Institute, 5-9-1, Shirokane, Minato-ku, Tokyo 108-8642, Japan*

[b] *Department of Biochemistry and Molecular Biology, Faculty of Science, Saitama University, 255 Shimo-okubo, Urawa 338, Japan*

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Abstract

A polyclonal antibody (anti-bupleuran 2IIc/PG-1-IgG) against the ''ramified'' region (PG-1) of an anti-ulcer pectic polysaccharide was prepared and its antigenic epitopes were analyzed by using several carbohydrases. Enzymatic removal of arabinosyl residues from PG-1 by endo-(1→5)-α-L-arabinanase (from *Aspergillus niger*) did not reduce the binding ability of anti-bupleuran 2IIc/PG-1-IgG to PG-1. When the endo-(1→5)-α-L-arabinanase-resistant fraction (EA-1) was digested with rhamnogalacturonase A (rRGase A from *A. aculeatus*), a high-molecular-mass fragment fraction (RA-1) and an oligosaccharide fraction (RA-3) were obtained. RA-3 contained at least four kinds of oligosaccharides liberated from the rhamnogalacturonan core. This partial removal of the rhamnogalacturonan core in EA-1 also did not reduce the binding of the antibody to the polysaccharide. Further digestion of RA-1 with exo-(1→3)-β-D-galactanase (from *Irpex lacteus*), gave a high-molecular-mass fragment (EXG-1) and a trace of oligosaccharides (EXG-3). Methylation and FABMS analyses indicated that EXG-3 contained mono- and di-galactosyl oligosaccharides possessing terminal GlcA or GlcA4Me. Removal of the EXG-3 fraction from RA-1 by exo-(1→3)-β-D-galactanase significantly reduced the ability of the binding of the antibody to the polysaccharide. When PG-1 was digested with endo-(1→6)-β-D-galactanase (from *Trichoderma viride*) or β-D-glucuronidase (from *A. niger*), the reactivities of both enzyme-resistant fractions to the antibody were decreased in comparison with that of PG-1. Both radish arabinogalactan (containing GlcA4Me) and β-D-Glc*p*A-(1→6)-β-D-Gal*p*-(1→6)-D-Gal*p* were shown to inhibit the reactivity of PG-1 to the antibody by competitive ELISA. These results suggest that 6-linked galactosyl chains containing terminal GlcA or GlcA4Me attached to (1→3)-β-D-galactosyl chains, are important sugar residues in the antigenic epitopes of the ''ramified'' region of bupleuran 2IIc.

Keywords: *Bupleurum falcatum* L.; Pectic polysaccharides; Carbohydrases; Antigenic epitopes

* Corresponding author. Fax: +81-3-3445-1351.

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1. Introduction

The roots of *Bupleurum falcatum* L. (Japanese name = Saiko) have been used in Chinese and Japanese herbal medicines for the treatment of chronic hepatitis, inflammatory diseases, and ulcer of digestive organs. The pharmacologically active polysaccharides, bupleuran 2IIb and 2IIc [1–4], which have anti-complementary [2], macrophage Fc receptor up-regulating [3] and anti-ulcer activities [4,5], were isolated from the hot water extract of the roots of *B. falcatum*, and characterized as pectic polysaccharides [6,7]. Both polysaccharides consist of galacturonan regions, ''ramified'' regions which contain a rhamnogalacturonan core having side chains rich in neutral sugars, and rhamnogalacturonan II-like regions [7]. The ''ramified'' region is considered to be responsible for these activities [3,4]. Previously, a polyclonal antibody (anti-bupleuran 2IIc/PG-1-IgG) against the ''ramified'' region (PG-1) of an anti-ulcer pectic polysaccharide (bupleuran 2IIc) from *B. falcatum* was prepared and used for the analysis of the distribution of this polysaccharide in vivo [8]. Although anti-bupleuran 2IIc/PG-1-IgG recognized the ''ramified'' regions [8], detailed antigenic epitopes have not been clarified yet.

In order to elucidate the antigenic epitopes, several carbohydrases, which are able to specifically hydrolyze certain carbohydrate structures, are useful analytical tools. Tsumuraya et al. [9] have reported the purification and characterization of an exo-(1→3)-β-D-galactanase from Driselase (*Irpex lacteus*), which is able to hydrolyze β-(1→3)-galactan with and without side chains in an exo-fashion. Endo-(1→6)-β-D-galactanase and β-D-glucuronidase (EC 3.2.1.31) have been purified from *Trichoderma viride* and *Aspergillus niger*, respectively, by Okemoto et al. [10]. Schols et al. [11] were the first to report on rhamnogalacturonase (RGase) from *A. aculeatus*, an enzyme able to cleave Gal*p*A-Rha*p* linkages in pectin. Kofod et al. [12] cloned and characterized two recombinant rhamnogalacturonases (rRAase A and rRGase B) from the full-length cDNA of *A. aculeatus*. Azadi et al. [13] further purified rRGase A and rRGase B, and characterized rRGases A and B as an endohydrolase and an endolyase, respectively, by analysis of the structure of the oligosaccharide fragments generated from partially debranched sycamore rhamnogalacturonan I when treated with the purified enzymes. The enzymes characterized are useful tools for analyzing the fine structure of the ''ramified'' region and for specific trimming of certain carbohydrate chains.

In the present paper, we describe the characterization of the antigenic epitopes in the ''ramified'' region of bupleuran 2IIc using these enzymes.

2. Experimental

Materials.—The roots of *B. falcatum* L. were purchased from Uchida Wakanyaku Co. Ltd. (Tokyo, Japan). A voucher specimen was deposited at the herbarium of the Oriental Medicine Research Center of the Kitasato Institute. Bupleurans 2IIb and 2IIc were purified from the acidic polysaccharide fraction (BR-2) of the roots of *B. falcatum* by anion-exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Uppsala, Sweden) as described previously [5]. ''Ramified'' region, (PG-1, a rhamnogalacturonan possessing side chains rich in neutral sugars) was prepared from bupleuran 2IIc by endo-(1→4)-α-D-polygalacturonase digestion as reported previously [5]. Radish root arabinogalactan-protein (radish AGP) and β-D-Glc*p*A-(1→6)-β-D-Gal*p*-(1→6)-D-Gal*p* and β-D-Glc*p*A-(1→6)-D-Gal*p* from Acacia gum were prepared as reported in ref. [9,10]. (1→3)-β-D-Galactan was isolated from Acacia gum by Smith degradation [9]. (1→6)-β-D-Galactopyranosyl di-, tri- and tetrasaccharides were isolated from the partial acid hydrolysate of larch wood arabinogalactan and gum ghatti [9].

Enzymes.—Endo-(1→5)-α-L-arabinanase (from *A. niger*; EC 3.2.1.99), obtained from Megazyme (Australia), was purified on a Mono-Q column as described in ref. [14]. Recombinant rhamnogalacturonase A (rRGase A), provided by Novo Nordisk A, was purified on a Mono-S column as described in ref. [13]. Exo-(1→3)-β-D-galactanase from Driselase was purified by the procedure of Tsumuraya et al. [9]. Endo-(1→6)-β-D-galactanase and β-D-glucuronidase were purified from *T. viride* and *A. niger*, respectively, by the method of Okemoto et al. [10] (details of purifications will be reported elsewhere). The presence of contaminating enzyme activities in the purified enzymes was assayed by using *p*-nitrophenyl derivatives of α-L-Ara*f*, α-D-Glc, β-D-Glc, β-D-Gal, α-L-Rha, β-D-GlcA, and α-L-Man as substrates. No such enzyme activities could be detected.

Antibody.—The polyclonal antibody (anti-bupleuran 2IIc/PG-1-IgG) against the "ramified" region (PG-1) of bupleuran 2IIc was generated and purified as described previously [8]. Two rabbits were immunized with PG-1 according to the method of Vreeland [15] and Moore et al. [16] with some modifications. Anti-bupleuran 2IIc/PG-1-IgG was purified to give an antibody-I by Protein G-Sepharose, and antibody I was further purified to give an antibody-II by bupleuran 2IIc/PG-1 immobilized EAH-Sepharose [8]. Antibody-II was conjugated to NHS-LC-biotin (Pierce, Rockford, USA) according to the manufacturer's instruction.

General methods.—Total carbohydrate and uronic acid were determined by the phenol–H_2SO_4 [17] and the *m*-hydroxybiphenyl method [18], respectively, by using Gal and GalA as the respective standards. Sugars were converted into Me_3Si derivatives [19] and analyzed by GLC using a DB-1 capillary column (0.20 μm film, 0.25 mm $\times$30 m, Supelco). GLC was performed on a HP-5890 Series II gas chromatograph (Hewlett–Packard, USA) set at the following temperature gradient: 60 °C for 1 min, 60→170 °C (30 °C/min), 170→190 °C (1 °C/min), and 190→300 °C (30 °C/min).

Enzyme-linked immunosorbent assay (ELISA).—Two-site sandwich ELISAs, which are able to measure the binding of antibody to polysaccharides, were carried out as follows. A solution (10 μg/100 μL) of antibody-I in phosphate buffered saline (PBS) was added to microtiter plates (96 wells, MS-3596F/H, Sumitomo, Japan), and incubated at room temperature overnight. Unbound antibody-I was removed by washing the plate with PBS containing 0.05% Tween 20 (PBS-Tween, 250 μL/well) four times. The plate was further incubated with 0.8% gelatin in PBS (250 μL/well) at 37 °C for 1 h, and washed with PBS-Tween four times. Test samples (100 μL/well) were added to the wells, and incubated at room temperature for 1 h. The plate was washed five times with PBS-Tween containing 0.5% BSA (PBS-Tween-BSA). Then biotinylated antibody-II was added to the wells (100 μL/well), and incubated at room temperature for 1 h. The plate was washed four times with PBS-Tween-BSA. Alkaline phosphatase-labelled streptavidin (Gibco, BRL, USA), diluted with PBS-Tween-BSA (1:2000), was added to each well (100 μL/well) and the plates were then incubated at room temperature for 1 h. After washing the wells with PBS-Tween-BSA five times, each well was incubated with 150 μL of a chromogenic substrate solution (1 mg of *p*-nitrophenylphosphate disodium salt in 1 mL 1 M diethanolamine buffer, pH 9.8). The reaction was stopped by the addition of 3 M NaOH (50 μL), and subsequently the absorbance at 405 nm was measured using a microplate reader (Bio-Rad Model 250).

In order to measure the ability of oligosaccharides to inhibit the binding of anti-bupleuran 2IIc/PG-1-IgG to immobilized PG-1, competitive ELISAs were carried out as follows. PG-1 (500 ng/well in PBS) was immobilized to 96 well-microtiter plates by incubation overnight at 37 °C. Various amounts of oligosaccharides and biotinylated anti-bupleuran 2IIc/PG-1-IgG were pre-incubated in the test tubes at 37 °C for 1 h. After washing and blocking of the PG-1-immobilized plate, the pre-incubated solutions were added to the wells, and incubated at room temperature for 1 h.

Enzymatic digestion.—PG-1 (20 mg) was incubated with endo-(1→5)-α-L-arabinanase (0.1 U) in 50 mM acetate buffer (pH 4.6) at 37 °C for 5 h. The digestion mixtures were then fractionated on a Bio-Gel P-30 column (2.6×50 cm) with 50 mM acetate buffer (pH 5.2) to obtain EA-1, EA-2 and EA-3. The resulting products were desalted by using an electrophoretic dialyzer (Microacylizer, Asahi Chemical Industry Co. Ltd., Japan). EA-1 (8 mg) was incubated with rhamnogalacturonase A (14 μg) in 50 mM acetate buffer (pH 4.5) at 37 °C for 20 h and fractionated as above to obtain RA-1, RA-2 and RA-3. RA-1 (5 mg) or PG-1 (2 mg) was incubated with exo-(1→3)-β-D-galactanase (0.1 U) in 50 mM acetate buffer (pH 4.6, 3 mL) at 37 °C for 96 h and fractionated as above. PG-1 (2 mg) was also incubated with endo-(1→6)-β-D-galactanase (0.2 U) in 50 mM acetate buffer (pH 4.3, 2 mL) at 37 °C for 24 h and another sample with β-D-glucuronidase (0.1 U) in 50 mM acetate buffer (pH 4.6, 2 mL) at 37 °C for 24 h. The digests were fractionated as described above.

The time courses of the digestions were monitored by analyzing liberated oligo- or mono-saccharides using high performance anion-exchange chromatography combined with pulsed electrochemical detection (HPAEC-PED). HPAEC-PED was performed on a Dionex Bio LC instrument equipped with a CarboPac PA-1 column (4×250 mm). Carbohydrates were eluted at 1 mL/min with 100 mM NaOH for 5 min, then with a 40-min linear gradient to 600 mM NaOAc in 100 mM NaOH.

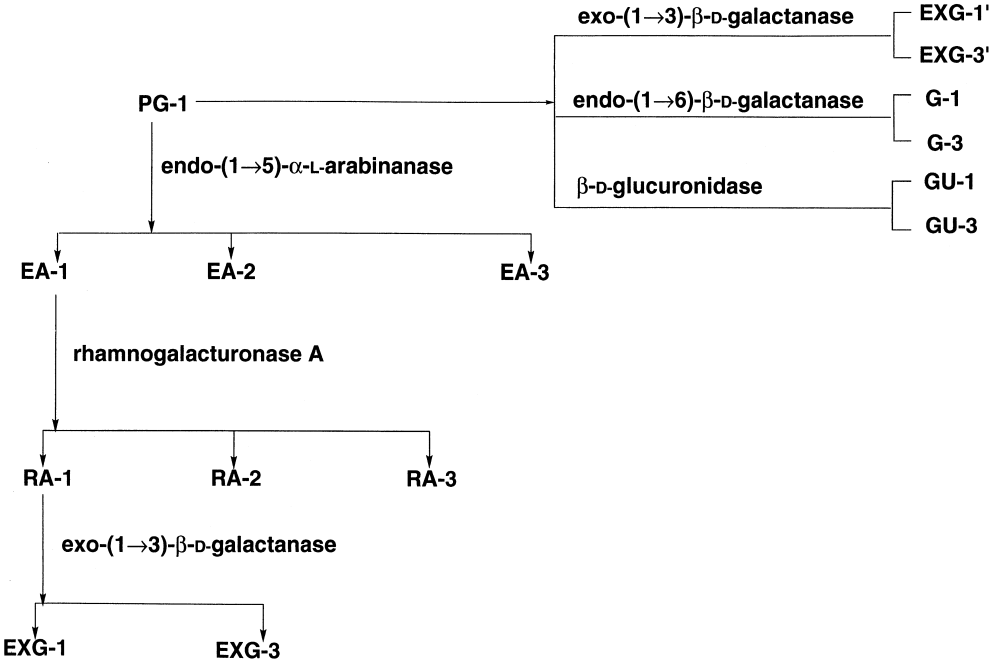
Methylation analysis.—Poly- and oligosaccharides were methylated according to the Hakomori method [20] and the methylated products were recovered using a Sep-pak C_{18} cartridge by the procedure of Waeghe et al. [21], except that samples were eluted with only EtOH. Carboxy–methyl groups of uronic acids in the methylated products were reduced with $LiB(C_2H_5)_3D$ in THF (Super-Deuteride®, 1 mL, room temperature, 1 h, Sigma) [19], and the reduced products were recovered by using a Sep-pak C_{18} cartridge. The methylated products were hydrolyzed with 1 M CF_3CO_2H at 100 °C for 1.5 h, and converted into partially methylated alditol acetates. The presence of terminal GlcA4Me residues was confirmed by using C_2H_5I instead of CH_3I. The alditol acetates were analyzed by GLC and GLC-EIMS [22]. 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. The temperature gradient was set as described previously [7].

GLC-EIMS of methylated oligosaccharides.—Solutions of methylated oligosaccharides in acetone were analyzed [23] on an SP-2380 capillary column (0.2 μm film thickness, 0.25 mm×15 m, Supelco) making use of an on-column injector and a temperature program as follows: 100 °C for 1 min, 100→150 °C (30 °C/min) and 150→270 °C (2 °C/min). EIMS was carried out on a Hewlett–Packard model 5970B mass selective detector.

FABMS analysis of methylated oligosaccharides.—A Jeol JMS-AX505 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. The accelerating voltage was 3 kV. A mixture of 1:1 glycerol–monothioglycerol was used as a matrix. One μL of a MeOH soln of oligosaccharide was placed on the target of the probe and was mixed with 1 μL matrix containing 5% HOAc. 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; the registration was carried out with an UV oscillograph.

3. Results

Sequential enzymatic digestion of the "ramified" region of bupleuran 2IIc.—The "ramified" region (PG-1) of bupleuran 2IIc was hydrolyzed step by step with specific carbohydrases in order to fragment arabinosyl and galactosyl side chains and to depolymerise the rhamnogalacturonan core in PG-1 (Scheme 1). The resulting products were tested for the binding to anti-bupleuran 2IIc/PG-1-IgG by two-site sandwich and competitive ELISAs.



Scheme 1. Enzymatic digestions of the "ramified" region (PG-1) of bupleuran 2IIc.

Firstly, PG-1 was digested with endo-(1→5)-α-L-arabinanase, and the products were separated into a void fraction (EA-1), an intermediate fraction (EA-2), and a fraction (EA-3) of low-molecular-mass fragments by gel filtration on Bio-Gel P-30 (weight ratios; EA-1:EA-2:EA-3 = 35.1:0.1:13.8) (Fig. 1A). The binding ability of anti-bupleuran 2IIc/PG-1-IgG to EA-1 was similar to that of PG-1 (Fig. 2). By competitive ELISA it was shown that EA-3 had a weak inhibitory activity on the binding of the antibody to PG-1 (data not shown). Sugar composition and methylation analyses indicated that the branched (1→5)-linked arabinosyl chains in PG-1 were removed by the digestion and that EA-3 consisted mainly of 5- and 3,5-linked Ara (Tables 1 and 2). EA-3 gave neutral (65%) and acidic fractions (35%) upon fractionation on a DEAE-Sepharose CL-6B column (data not shown). The neutral fraction was composed mainly of Ara whereas the acidic fraction consisted mainly of GlcA and GalA with a trace of Ara and Gal. Only the acidic fraction of EA-3 had the inhibitory activity (data not shown). These results

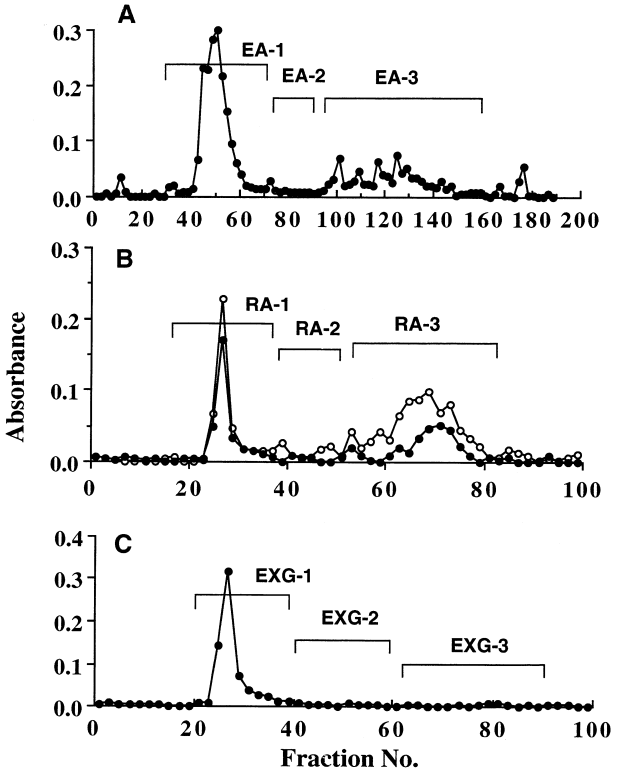


Fig. 1. Gel filtration on Bio-Gel P-30 of digestion products derived from (A) PG-1 by endo-(1→5)-α-L-arabinanase, (B) EA-1 in A by rhamnogalacturonase A, and (C) RA-1 in B by exo-(1→3)-β-D-galactanase. ●, carbohydrate (490 nm); ○, uronic acid (520 nm).

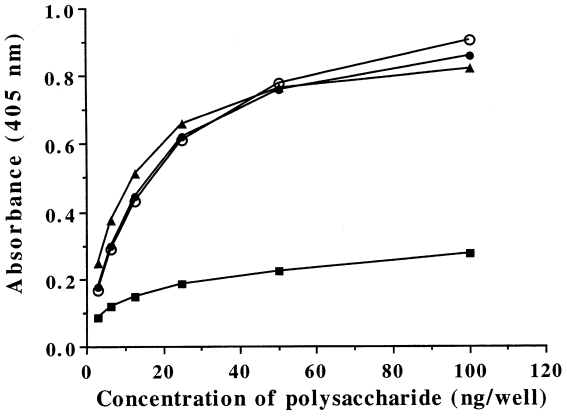


Fig. 2. Reactivity of enzyme-resistant fractions to anti-bupleuran 2IIc/PG-1-IgG on two-site sandwich ELISA. ○, PG-1; ●, EA-1; ▲, RA-1; ■, EXG-1.

suggest that PG-1 contains branched (1→5)-α-L-arabinofuranosyl chains which are partially substituted with uronic acids, and that the neutral arabinofuranosyl chains are not related to the epitope.

In order to investigate the contribution of the rhamnogalacturonan core to the reactivity of EA-1 against the antibody, EA-1 was digested with rhamnogalacturonase A, which specifically hydrolyzes the linkage of α-D-GalA-(1→2)-L-Rha in the rhamnogalacturonan backbone of the "ramified" region to leave Rha at the non-reducing end of the reaction products. The digested products gave fraction RA-1 (void), RA-2 (intermediate fraction), and a fraction RA-3 containing oligosaccharides upon gel filtration on Bio-Gel P-30 (weight ratio; RA-1:RA-2:RA-3 = 39:5:30) (Fig. 1B). RA-1 had the same reactivity to anti-bupleuran 2IIc/PG-1-IgG as PG-1 (Fig. 2), whereas RA-3 weakly inhibited the binding of the antibody to PG-1 by

Table 1
Component sugars of the products obtained from the "ramified" region of bupleuran 211c (PG-1) by sequential enzymatic digestions

Glycosyl residue	mol%						
	PG-1	EA-1	RA-1	EXG-1	EA-3	RA-3	EXG-3
Ara	17.0	6.1	4.9	14.1	84.2	3.1	3.4
Rha	14.1	17.3	6.1	15.2		32.5	
Fuc	1.3	1.9	2.9	1.3			
Xyl	1.9	2.2	3.0	2.1			
Man	3.2	3.9	6.6	3.6			
Gal	27.7	28.5	34.6	22.5	4.7	17.5	53.5
Glc	8.4	10.2	18.5	9.6	1.9		3.1
GalA	17.2	16.7	13.9	17.8	7.1	46.9	11.5
GlcA4Me	Trace	Trace	Trace				13.4
GlcA	9.1	12.8	9.5	13.7	2.0		15.0

Table 2
Methylation analysis of the products obtained from the "ramified" region of bupleuran 2IIc (PG-1) by sequential enzymatic digestions

Glycosyl residue	Deduced glycisidic linkage	mol%						
		PG-1	EA-1	RA-1	EXG-1	EA-3	RA-3	EXG-3
Ara	Terminal (*f*)[a]	4.3	3.4	2.4	2.8	48.8	2.2	
	4 or5	11.9	2.3			21.4		
	3,4 or 3,5	5.6				16.0		
Xyl	Terminal (*p*)[b]	4.3	3.8	5.1	4.8	5.5		
Rha	Terminal	1.0	1.1	1.6	1.3		7.1	
	4			1.0	1.2		6.9	
	2	17.6	17.8	9.9	6.9		25.2	
	2,4	10.4	12.6	6.7	7.0		9.9	
	2,3	1.1	1.1	3.0	0.8			
Gal	Terminal	9.1	7.8	5.5	4.5	6.1	15.1	15.1
	6(*f*)					2.3		22.8
	4	2.9	3.3	4.1	4.3	4.9	3.5	
	6 (*p*)							23.2
	3	2.8	2.7	5.5	7.0		1.1	2.9
	3,6	1.3	1.1	4.5	6.7			5.4
Glc	4	1.2	1.5	4.2	7.5			
Man	Terminal			2.2	2.2			
	3							
	3,4		1.3					
GlcA	Terminal			4.5	5.4	n.d.[c]		29.0[d]
	4	1.6	2.4	7.6	10.5	n.d.		
	3,4					n.d.		
GalA	Terminal	1.7	2.1	3.5	3.0	n.d.	1.3	
	4	20.0	30.8	17.2	15.2	n.d.	27.6	1.6
	2					n.d.		
	3,4	1.8	3.4	6.2	6.4	n.d.		
	2,4	1.3	2.9	5.2	2.4	n.d.		

[a]Furanosyl.
[b]Pyranosyl.
[c]Not determined.
[d]This derivative was derived from terminal GlcA and GlcA4Me which was confirmed by using C_2H_5I instead of CH_3I in methylation analysis.

competitive ELISA (data not shown). Methylation analysis showed that RA-1 contained lower proportions of 4-linked GalA and 2- and 2,4-substituted Rha in comparison to EA-1 (Table 2). Sugar analysis indicated that RA-3 mainly consisted of Rha, GalA and Gal (Table 1). Methylation analysis indicated that RA-3 was composed mainly of 2- and 2,4-substituted Rha, terminal Gal and 4-linked GalA (Table 2). Positive-ion FABMS of permethylated oligosaccharides from RA-3 gave characteristic peaks at *m*/*z* 853, 1057, 1245, and 1449 (Table 3). In order to confirm the structure of these ions, collisional activated dissociation (CAD) mass spectrometry using B/E-linked scan was performed. The CAD spectrum of the ion at *m*/*z* 853 showed daughter ions at *m*/*z* 664, 447 and 272 which arose from the successive elimination of 6-deoxyhexose, hexuronic acid, and 6-deoxyhexose from the non-reducing terminal of the ion at *m*/*z* 853 (data not shown). Positive-ion FABMS of permethylated oligosaccharides from RA-3 also gave a peak at *m*/*z* 839. The CAD spectrum indicated that the ion at *m*/*z* 839 was produced from the ion at m/z 853 by elimination of a fragment with a mass of 14. Positive-ion FABMS of the permethylated trigalacturonide gave characteristic ions at *m*/*z* 723 and 709 which corresponded to

Table 3
Deduced structures of oligosaccharides in RA-3 by FABMS

Observed characteristic ions $[M+Na]^+$	$[M+Na-14]^+$	Deduced structures
853	839	Rha→GalA→Rha→GalA
1057	1043	Rha→GalA→(Gal→)Rha→GalA
1245	1231	Rha→GalA→Rha→GalA→Rha→GalA
1449	1435	Rha→GalA→Rha→GalA→(Gal→)Rha→GalA

$[M+Na]^+$ and $[M+Na\text{-}14]^+$ although permethylated glucosyloligosaccharides gave only characteristic ions which corresponded to $[M+Na]^+$, suggesting that the fragment ion $[M+Na\text{-}14]^+$ in the trigalacturonide might be the result of the elimination of one methyl-ester group. Permethylated trigalacturonide contains two other carboxyl methyl groups, however the fragment ions such as $[M+Na\text{-}28]^+$ and $[M+Na\text{-}42]^+$ due to further elimination of other methyl-ester groups could not be observed. This result suggests the possibility that the fragment ions due to elimination of over two methyl-ester groups might be unstable. This indicates that the ion at *m/z* 839 is formed by elimination of one methyl-ester group of a permethylated oligosaccharide containing uronic acid residues. These results suggest that the ion at *m/z* 853 correlates with the sequence Rha→GalA→Rha→GalA (Table 3). The other oligosaccharides were analyzed in the same manner and the results indicate structures of rhamnogalacturono- tetra to hepta-saccharides with one galactosyl side chain attached to Rha as shown in Table 3. The results suggest that the rhamnogalacturonan core is not part of the antigenic epitopes for anti-bupleuran 2IIc/PG-1-IgG since RA-1 still contained the major antigenic epitopes.

Methylation analysis indicates that RA-1 contained (1→3)-galactosyl chains (Table 2). In order to investigate the contribution of (1→3)-galactosyl chains in the reactivity of the antibody to PG-1, RA-1 was further digested with exo-(1→3)-β-D-galactanase, which is able to cleave (1→3)-β-D-galactan structures with or without side chains from the non-reducing terminal [9]. The digestion products were fractionated into fraction EXG-1 (void), in addition to trace amounts of intermediate (EXG-2) and low-molecular-mass (EXG-3) fractions by gel filtration on Bio-Gel P-30 (weight ratios; EXG-1:EXG-2:EXG-3 = 10:0.1:0.1) (Fig. 1C). EXG-1 showed a significantly lower reactivity with anti-bupleuran 2IIc/PG-1-IgG than PG-1 (Fig. 2), while in competitive ELISA, EXG-3 showed low inhibitory activity on the reactivity of the antibody to PG-1 (data not shown). Linear (1→3)-β-D-galactan and (1→6)-β-D-galactosyl di-, tri-, and tetra-saccharides were also poor inhibitors (data not shown). Because EXG-2 and 3 were only formed in trace amounts, the methylation analysis of EXG-1 did not significantly differ from RA-1. Sugar analysis indicated that EXG-3 consisted mainly of Gal in addition to GalA, GlcA and GlcA4Me. Methylation analysis using ethyl iodide indicated that EXG-3 mainly comprised terminal Gal, 6-linked Gal*f* and Gal*p*, terminal GlcA and GlcA4Me (Table 2). Positive-ion FABMS of permethylated oligosaccharides derived from EXG-3 gave major characteristic ions at *m/z* 477, 491, and 695 (Fig. 3). Methylation analysis and CAD spectra of ions at *m/z* 491 and 695 suggested that these ions could be assigned to GlcA-(1→6)-Gal and GlcA-(1→6)-Gal-(1→6)-Gal (data not shown). The CAD spectrum also indicated that the ion at *m/z* 477 was derived from that at *m/z* 491 by demethylation (minus 14 mass) of carboxymethyl groups of methylated uronic acids as above (data not shown). However, the possibility also remained that the ion at *m/z* 477 was due to Gal-(1→6)-Gal because methylation analysis showed a remarkable proportion of terminal Gal (Table 2). Therefore, the ion at *m/z* 477 was assumed to contain Gal-(1→6)-Gal as well as GlcA-(1→6)-Gal. EXG-3 also gave ions corresponding to GlcA-(1→6)-Gal-(1→6)-Gal-(1→6)-Gal (*m/z* 899) and GlcA-(1→6)-Gal-(1→6)-Gal that were substituted with one (*m/z* 855) and two (*m/z* 1015) arabinosyl residues,

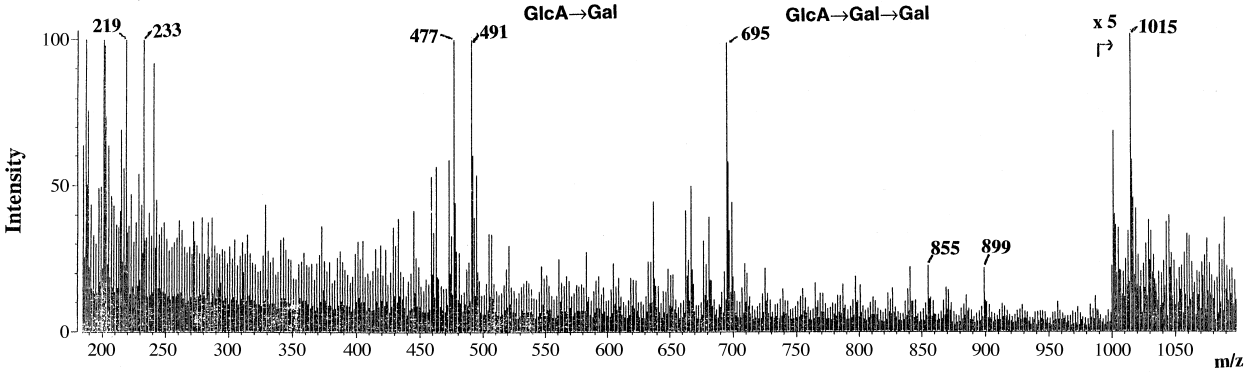


Fig. 3. Positive FABMS spectrum of methylated oligosaccharide derivatives from EXG-3.

respectively. Since EXG-3 comprised terminal GlcA4Me as well as terminal GlcA as uronic acid, some of the above proposed oligosaccharides might contain GlcA4Me instead of GlcA. These results suggest that 6-linked galactosyl chains containing terminal GlcA or GlcA4Me and which are attached to (1→3)-β-D-galactosyl chains may be involved as major antigenic epitopes in the "ramified" region (PG-1) of bupleuran 2IIc.

Contribution of 6-linked galactosyl chains containing terminal GlcA or GlcA4Me to the antigenic epitope of anti-bupleuran 2IIc/PG-1-IgG.—In order to investigate the contribution of 6-linked galactosyl chains containing terminal GlcA or GlcA4Me to the reactivity of the antibody with PG-1, PG-1 was separately digested with exo-(1→3)-β-D-galactanase from *Irpex lacteus*, endo-(1→6)-β-D-galactanase from *T. viride* or β-D-glucuronidase, which is able to hydrolyze both GlcA and GlcA4Me, from *A. niger*. The respective digests were fractionated by gel filtration on Bio-Gel P-30 into the fraction (EXG-1′, G-1 and GU-1) eluting respectively in the void fraction, an intermediate fraction (EXG-2′, G-2 and GU-2) and a low-molecular-mass fraction (EXG-3′, G-3 and GU-3) (data not shown). Sugar analysis indicated that EXG-3′, G-3 and GU-3, the fractions liberated from PG-1, also contained GlcA and GlcA4Me in addition to GalA. By GLC-EIMS of the permethylated oligosaccharides derived from G-3 using trideuteriomethyl iodide two major peaks were detected which could be attributed to disaccharides containing uronic acid (data not shown). EIMS of the peaks gave fragment ions at *m/z* 207 (aA_2, GlcA4Me), 231 and 162 (bA_1 and bA_2, respectively, Gal), and *m/z* 210 (aA_2, GlcA) and 231 (bA_1, Gal). Based on these data, the peaks could be assigned to be GlcA4Me→Gal and GlcA→Gal. The results also suggest that endo-(1→6)-β-D-galactanase splits of a part of 6-linked galactosyl chains containing terminal GlcA or GlcA4Me in PG-1.

EXG-1′, G-1 as well as GU-1 decreased the reactivity of anti-bupleuran 2IIc/PG-1-IgG to PG-1 compared with PG-1. The exo-(1→3)-β-D-galactanase digestion was most effective in removal of the antigenic epitope from PG-1 (Fig. 4). These results strongly suggest that side chains containing galactosyl chains with a non-reducing GlcA or GlcA4Me residue attached to a (1→3)-β-D-galacto backbone form the major antigenic epitopes in the "ramified" region (PG-1) of bupleuran 2IIc.

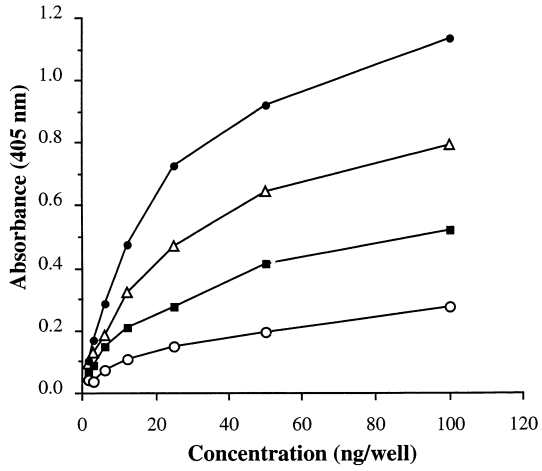


Fig. 4. Reactivity of enzyme-resistant fractions to anti-bupleuran 2IIc/PG-1-IgG on two-site sandwich ELISA. ●, PG-1; △, G-1; ■, GU-1; ○, EXG-1.

Radish arabinogalactan-protein (radish AGP) has been reported to consist of only GlcA4Me as uronic acid in the non-reducing terminal of the side chains (Gal:Ara:GlcA4Me = 72:17:10) [9,10]. Radish AGP and β-D-GlcA-(1→6)-β-D-Gal-(1→6)-D-Gal and β-D-GlcA-(1→6)-D-Gal prepared from acacia gum were tested for their ability to inhibit the binding of anti-bupleuran 2IIc/PG-1-IgG to PG-1 by using competitive ELISA. As shown in Fig. 5, radish AGP and β-D-GlcA-(1→6)-β-D-Gal-(1→6)-D-Gal showed notable inhibitory activities, but the activity of β-D-GlcA-(1→6)-D-Gal was negligible. These results also confirmed that anti-bupleuran 2IIc/PG-1-IgG recognized galactosyl chains containing both GlcA and GlcA4Me in the non-reducing terminals.

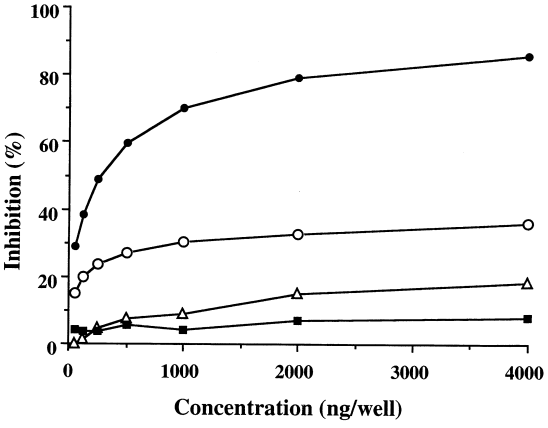


Fig. 5. Inhibitory activity of radish arabinogalactan and GlcA-containing galacto-oligosaccharides on binding of anti-bupleuran 2IIc/PG-1-IgG to PG-1. ●, PG-1; ○, radish AGP; △, GlcA-(1→6)-Gal-(1→6)-Gal; ■, GlcA-(1→6)-Gal.

4. Discussion

It has been proposed that the "ramified" region of bupleuran 2IIc (PG-1) is a rhamnogalacturonan core substituted with carbohydrate side chains directly at O-4 of rhamnose residues or through GalA also attached at O-4 of rhamnose residues (Fig. 6) [7]. PG-1 contains small proportions of long, highly branched arabinan side chains and large proportions of short neutral oligosaccharide side chains mainly consisting of arabinosyl or galactosyl residues [7]. Therefore, the major antigenic epitopes in PG-1 for anti-bupleuran 2IIc/PG-1-IgG were identified and characterized by using several carbohydrases having different substrate specificities. The present results suggest that PG-1 possesses a rhamnogalacturonan core substituted with one galactosyl side chain, branched (1→5)-α-L-arabinofuranosyl chains and (1→3)-β-D-galactosyl chains to which 6-linked galactosyl chains having terminal GlcA or GlcA4Me are attached.

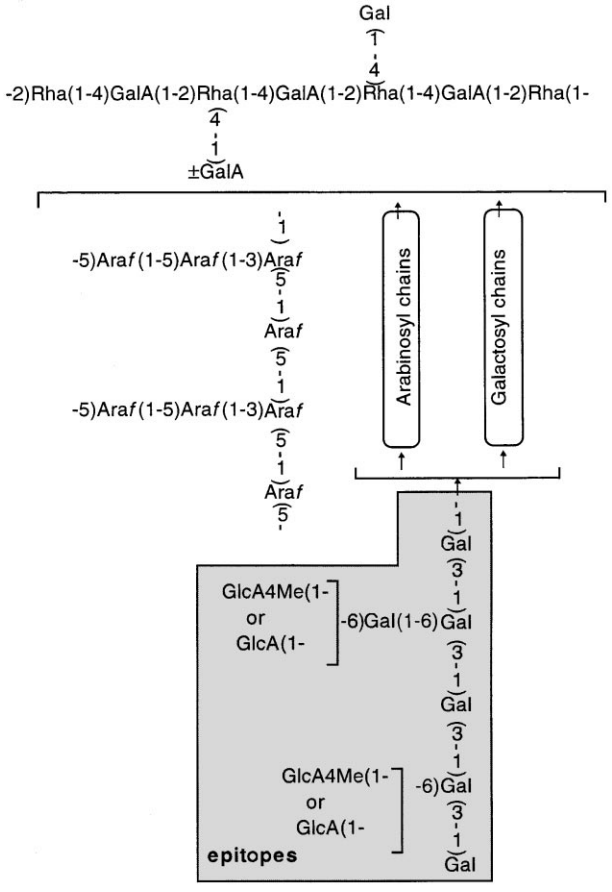


Fig. 6. Proposed structure of the antigenic epitopes in the "ramified" region of bupleuran 2IIc for anti-bupleuran 2IIc/PG-1-IgG.

We propose that the (1→6)-linked galactosyl residues carrying a terminal GlcA or GlcA4Me, which are attached to (1→3)-β-D-galactosyl chains are responsible for the major antigenic epitopes in PG-1 for anti-bupleuran 2IIc/PG-1-IgG (Fig. 6). The evidences supporting this conclusion are summarized as follows.

Firstly, (1→ 3,6)-β-D-galactosyl chains in PG-1 are essential for the binding of anti-bupleuran 2IIc/PG-1-IgG to PG-1, because exo-(1→3)-β-D-galactanase and endo-(1→6)-β-D-galactanase digestion of PG-1 resulted in significant loss of the binding ability of anti-bupleuran 2IIc/PG-1-IgG to PG-1. It seems that exo-(1→3)-β-D-galactanase can digest the (1→3)-β-D-galactosyl side chains from the outer region of PG-1 but can not remove all the galactosyl side chains in the inner region of PG-1 because of steric hindrance by other side chains. This suggests that the antigenic (1→3,6)-β-D-galactosyl chains in PG-1 are present as the outer portion which is recognized by the antibody. Exo-(1→3)-β-D-galactanase digestion of radish AGP also resulted in significant loss of the binding ability of anti-bupleuran 2IIc/PG-1-IgG to the polysaccharides (data not shown). The oligosaccharide fraction, EXG-3, which was liberated by exo-(1→3)-β-D-galactanase digestion of PG-1, contained 6-linked Gal*f* in addition to 6-linked Gal*p*. Mild acid treatment of EA-1 (10 mM HCl, 100 °C, 1 h), causing hydrolysis of furanosyl linkages, led to a decrease of 40% of the binding ability of EA-1 to the antibody (data not shown). Therefore, it is assumed that also 6-linked Gal*f* may play an important role in the antigenic epitopes.

Secondly, terminal GlcA as well as GlcA4Me are also important for the binding of bupleuran 2IIc/PG-1-IgG to the polysaccharide because elimination of GlcA and GlcA4Me by β-D-glucuronidase digestion significantly reduced the binding ability of the antibody to PG-1. Further evidence can be derived from the observation that the antibody reacted with a 6-linked galactodisaccharide possessing terminal GlcA and with radish AGP, which consists of terminal GlcA4Me. When carboxyl groups of uronic acids in the polysaccharide fraction (BR-2) containing bupleuran 2IIb and 2IIc were reduced by a modified method of Taylor and Conrad [24], the binding ability of anti-bupleuran 2IIc/PG-1-IgG to BR-2 was significantly decreased (data not shown), suggesting that the acidic moiety of bupleuran 2IIc was related to the antigenic epitopes.

GlcA and GlcA4Me residues have been found to be present in various plant polysaccharides including the arabinogalactan-type gum exudates [25], glycanorhamnogalactan-type mucilages [25], and rhamnogalacturonan I type polysaccharide such as the ''ramified'' region [26]. Rhamnogalacturonan I from sycamore comprises galactan chains to which terminal GlcA and GlcA4Me residues are attached at the 6-position of the galactan chains [26]. Anti-bupleuran 2IIc/PG-1-IgG also showed weak cross-reactivity with xylan (data not shown) which also carry single units of GlcA4Me residues [25]. These results suggest that also GlcA4Me may be an essential glycosyl residue for antigenic epitopes of anti-bupleuran 2IIc/PG-1-IgG.

It can also be concluded that the acidic fragment in EA-3 released from PG-1 by the arabinanase digestion also contained antigenic epitopes for anti-bupleuran 2IIc/PG-1-IgG. However, exo-(1→3)-β-D-galactanase digestion of the acidic fragment also reduced its binding ability to the antibody, assuming that the similar antigenic epitope shown in Fig. 6 may be present in the arabinanase-sensitive side chains of PG-1.

Several antibodies against plant polysaccharides have been developed and characterized for their antigenic epitopes. Steffan et al. [27] have reported that the epitope recognized by the monoclonal antibody CCRC-M7 to sycamore rhamnogalacturonan I consists of a (1→6)-β-D-galactan containing at least three galactosyl residues with one or more arabinosyl residues.

Miskiel and Pazur [28] showed that polyclonal antibodies against Gum Arabic and Gum mesquite recognized α-Ara-(1→4)-GlcA, β-D-GlcA-(1→6)-Gal and β-D-GlcA4Me-(1→6)-Gal. These antigenic epitopes containing GlcA or GlcA4Me and/or (1→6)-linked β-D-Gal were similar to those of anti-bupleuran 2IIc/PG-1-IgG although their structures were different.

Several pharmacologically active pectic polysaccharides are present in plants used as foods and medicinal herbs, but their absorption and tissue distribution in the body after oral administration have not been well clarified. Previously, anti-bupleuran 2IIc/PG-1-IgG has been applied for analysis of absorption and tissue distribution of the polysaccharide [8], and it was found that a part of bupleuran 2IIc was absorbed in the body on oral administration, and that the polysaccharide was also taken into Peyer's patches of the mucosa-associated lymphoid tissue (MALT) on intestine. It is still not known how the active pectic polysaccharide interacts with the cells in the body. Therefore the antibody may be useful as tool in these studies.

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