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Note

Characterisation of the endo-polygalacturonase-resistant region of the pectin from *Bupleurum falcatum* L.—a polysaccharide with an active function in clearance of immune complexes

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The elevated level of circulating immune complexes (IC) has been shown to relate to the progress of autoimmune diseases such as systemic lupus erythematosus and other chronic and systemic diseases, and mononuclear phagocytic cells (e.g. macrophages) have an important function to remove IC from the circulation through the Fc receptor [1,2]. The roots of *Bupleurum falcatum* L. (Japanese name Saiko) have been used in Chinese and Japanese herbal medicine (Kampo medicine) for treatment of autoimmune diseases in addition to chronic hepatitis and nephrosis syndrome. We have purified [3] two pectins (bupleuran 2IIb and 2IIc) from the hot-water extract of the roots of *B. falcatum*, and found [4] that bupleuran 2IIb enhanced IC clearance of macrophages by up-regulation of Fc receptor. Bupleuran 2IIb has been characterised [3,5] as a pectin composed of a large proportion of α-D-(1 → 4)-galacturonan in addition to small proportions of a rhamnogalacturonan II-like region and an endo-polygalacturonase-resistant region (PG-1). PG-1 was considered to act as an active site for the IC clearance-enhancing activity of bupleuran 2IIb. We have previously reported [3] that PG-1 consisted of an acidic moiety such as → 2)-Rha-(1 → 4)-GalA-(1 → , → 4)-[→

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2)-Rha-(1] → 4)-GalA-(1 → , and → 4)-GalA-(1 → 2)-[→ 4)-Rha-(1] → by analysis with base-catalysed β-elimination. We now report further on the structural characterisation of the endo-polygalacturonase-resistant region (PG-1) in bupleuran 2IIb.

1. Experimental

Materials.—The roots of *B. falcatum* L. were purchased from Uchida Wakan-yaku Co. Ltd. Sepharose CL-6B, DEAE-Sepharose CL-6B and DEAE-Sephadex A-25 were obtained from Pharmacia and LKB. Bio-Gel P-30 (200–400 mesh) was obtained from Bio-Rad, and Sep-Pak C_{18} cartridge was from Waters Associates. Pectinase from *Aspergillus niger* was purchased from Sigma and endo-α-(1 → 4)-polygalacturonase [poly(1,4-α-D-galacturonide) glycohydrolase; EC 3.2.1.15] was purified using the procedure of Thibault and Mercier [6]. Endo-arabinanase (EC 3.2.1.99) from *A. niger* was purchased from Megazyme. An acidic polysaccharide fraction (BR-2) was prepared by hot-water extraction, and EtOH and cetavlon precipitations [7]. Bupleuran 2IIb was purified from BR-2 as described previously [3,8].

General.—Carbohydrate, uronic acid, and protein in column eluates were assayed by the phenol–H_2SO_4 [9], *m*-hydroxybiphenyl [10], and Lowry [11] methods, respectively. Polysaccharides were hydrolysed with 2 M TFA for 1.5 h at 121°C. Neutral sugars and uronic acids in hydrolysates were converted [12] into corresponding alditol acetates and analysed by GLC on an SP-2380 capillary column (0.2 μm film, 0.25 mm i.d. × 30m, SUPELCO). The molar ratios were calculated from the peak areas, and response factors.

Preparation of PG-1.—Bupleuran 2IIb (70 mg) was digested with endo-α-(1 → 4)-polygalacturonase (1750 nKat) for 4 days at 37°C in 50 mM acetate buffer (pH 4.2, 30 mL). After neutralisation, the products were fractionated on a column of Sephadex G-50, and PG-1 was obtained as the fraction eluted in the void volume. PG-1 used in the present study consisted of Rha, Fuc, Ara, Xyl, Man, Gal, Glc and GalA in the molar ratios of 0.8: 0.1: 0.9: 0.1: 0.2: 1.0: 0.5: 1.8.

Partial acid hydrolysis of PG-1.—PG-1 (5 mg) was hydrolysed with 100 mM TFA at 121°C for 1 h. The hydrolysates were eluted from DEAE-Sephadex A-25 ($HCOO^-$) with water and 5 M HCOOH, to give neutral and acidic fractions, respectively.

Degradation of PG-1 with lithium in ethylenediamine.—The procedure was performed according to the method of Lau et al. [13]. PG-1 (20 mg) was suspended in ethylenediamine (4 mL), and the solution was treated with lithium for 1 h at room temperature, and then the reaction was terminated by addition of water. The products were desalted with AG-50W X8 (H^+ form) resin, reduced with $NaBH_4$, and fractionated on DEAE-Sephadex A-25 ($HCOO^-$). Neutral and acidic fractions were eluted with H_2O and 5 M HCOOH, respectively, and the neutral fraction was further fractionated on Bio-Gel P-30.

HPLC of neutral oligosaccharide alditols.—*(a) Peracetylation.* Oligosaccharide-alditols in NS-3 were peracetylated by heating at 121°C for 3 h with Ac_2O and pyridine. Peracetylated products were extracted with $CHCl_3$.

(b) HPLC. HPLC was performed on a Shimadzu LC-6A system equipped with a Capcell-Pak C_{18} column (5 μm, 4.6 × 150 mm, SHISEIDO), and peracetylated oligosaccharide-alditols were eluted using a linear gradient from 40 to 100% CH_3CN in

30 min at a flow rate of 1 mL/min. Peracetylated oligosaccharide-alditols were detected by measurement of absorbance at 230 nm.

Methylation analysis.—Samples (500 μg) were methylated according to the method of Hakomori [14], and formation of a polyalkoxide was checked by using triphenylmethane [15]. The methylated products were recovered using a Sep-Pak C_{18} cartridge by the procedure of Waeghe et al. [16], except that samples were eluted with EtOH. Acidic oligosaccharides obtained by partial acid hydrolysis were reduced with $NaBD_4$ in 2 M NH_4OH prior to methylation. Methylated uronic acid in the products were carboxyl-reduced [16,17] with $NaBD_4$ in THF–EtOH (7:3) at room temperature for 18 h followed by incubation at 80°C for 1 h, and then re-methylated to obtain permethylated, carboxyl-reduced oligosaccharide-alditols. Each methylated product was hydrolysed with TFA, and reduced with $NaBH_4$ followed by acetylation. The resulting methylated alditol acetates were analysed by GLC and GLC-MS on an SP-2380 capillary column (0.2 mm film thickness, 0.20 mm i.d. × 25 m, SUPELCO). GLC and EI-MS was performed on a Hewlett–Packard model 5890A gas chromatograph and 5970B mass selective detector, respectively. The carrier gas was He (0.9 mL/min in GLC and 0.5 mL/min in GLC-MS), and the temperature programs were 60°C for 1 min, 60–180°C (30°C/min), 180–250°C (1.5°C/min), and 250°C for 5 min (for GLC); and 60°C for 1 min, 60 – 150°C (30°C/min), 150 – 250°C (1.5°C/min) (for GLC-MS). Methylated alditol acetates were identified by their fragment ions and relative retention times in GLC, and their molar ratios were estimated from the peak areas and response factors [18] on FID.

GLC-MS of methylated oligosaccharide-alditols.—Solutions of methylated oligosaccharide-alditols in acetone were injected directly into an SP-2380 capillary column (0.2 μm film thickness, 0.25 mm i.d. × 15 m, SUPELCO). The temperature program was 100°C (1 min), 100 → 150°C (30°C/min), 150 → 270°C (2°C/min), and 270°C (20 min). CI-MS (isobutane) was performed on a JEOL DX-300 mass spectrometer, and EI-MS was carried out on a Hewlett–Packard model 5970B mass-selective detector. The CI- and EI-MS fragment ions [A, J, and alditol (ald)] were used to determine the structures of the methylated oligosaccharide-alditols [19–21].

FAB-MS of methylated oligosaccharide-alditols.—FAB-MS was performed on a JEOL AX-505 HA mass spectrometer equipped with a DA-7000 computer, and a mixture of *m*-nitrobenzyl alcohol and NaI were used as a matrix.

NMR spectrometry.—^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) spectra of peracetylated oligosaccharide-alditols were recorded with a Varian XL-400 spectrometer at room temperature.

Endo-arabinanase digestion.—PG-1 (4.5 mg) was digested with endo-α-L-(1 → 5)-arabinanase (0.15U) from *A. niger* for 48 h at 40°C in 200 mM acetate buffer (pH 4.0, 3 mL). After neutralization, the products were fractionated on a column of Bio-Gel P-30 to obtain the enzyme resistant and oligosaccharide fractions.

2. Results and discussion

PG-1 was subjected to partial acid hydrolysis (0.1M TFA, 121°C, 1 h), and neutral and acidic fractions were obtained. The neutral fraction contained mainly Ara and Gal in

Table 1
Methylation analysis of acidic fraction derived from PG-1 by partial acid hydrolysis [a]

Glycosyl residue	Deduced glycosidic linkages	mol. %
Rha	2 (reducing terminal)	trace [a]
	terminal	6.5
	2	13.5
Gal	terminal	16.4
	4	1.8
Glc	terminal	12.4
	4	9.6
GalA	terminal	32.7
	4	5.3

[a] Acidic oligosaccharides in the acidic fraction were reduced with $NaBD_4$, methylated, carboxyl-reduced, and methylated for conversion to 6,6-di-deuteriated, permethylated oligosaccharide-alditols-1-d.The products were hydrolysed with 2 M TFA at 121°C for 1 h, and converted to partially methylated alditol acetates.

Table 2
CI- and EI-MS fragment ions of permethylated disaccharide-alditol in acidic fraction derived from PG-1 by partial acid hydrolysis

Structure of oligosaccharide-alditol	Fragment ions [m/z (relative abundance)]								
	CI-MS	EI-MS							
	$(M+H)^+$	aJ_1	aJ_2	bA_1	bA_2	bA_3	ald		
GalA-(1 → 2 and 4)-Rha-ol-1-d	444	266	206	221	189	157	308		
	(93)	(2)	(100)	(8)	(32)	(7)	(1)		
							134	277	309
							(30)	(2)	(1)

addition to a small amount of Fuc, Rha, Glc, Man and Xyl. The acidic fraction was composed mainly of Rha, Gal, Glc and GalA. Methylation analysis indicated that the acidic fraction contained 2-substituted Rha, terminal Gal and Glc, and terminal GalA (Table 1). The methylated oligosaccharide-alditols, derived from the acidic fraction,

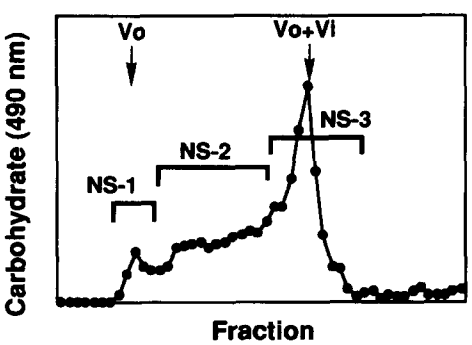

Fig. 1. Gel filtration on Bio-Gel P-30 of products from PG-1 by lithium degradation: Vo, void volume; Vi, internal volume.

were analysed by GLC-MS, and one major peak was detected. CI-MS gave a protonated molecular ion at m/z 444, and EI-MS gave fragment ions due to bA_1 (m/z 221) and aJ_2 (m/z 206), indicating the peak to possess HexA → 6-deoxyHex-ol-1-*d* unit (Table

Table 3
Methylation analysis of neutral oligosaccharide-alditol fractions (NS-1–NS-3) derived from PG-1 of bupleuran 2IIB by lithium degradation

Glycosyl residue	Deduced glycosidic linkage	Composition (mol %)		
		NS-1 [b]	NS-2 [b]	NS-3 [c]
6-deoxyHex [a]	4 (reducing terminal)			1.6
Rha	terminal	1.3	0.7	1.4
	3	2.0	0.8	
	4	4.8	2.8	0.7
Fuc	4	0.8	0.6	
Ara	terminal (furanosyl)	7.2	14.3	11.4
	terminal (pyranosyl)	1.1	2.8	9.6
	2 (furanosyl)			1.2
	4 or 5	6.0	16.6	8.5
	3 (pyranosyl)			0.8
	3,4 or 3,5	3.4	12.2	5.1
	2,4 or 2,5			0.3
Xyl	3 (pyranosyl)	1.3	2.3	3.4
Man	terminal			1.8
	3	2.0	1.6	0.5
	4	3.0	2.3	0.5
	4,6	0.7	0.7	
	3,6	0.5	0.2	
Gal	terminal (furanosyl)	0.5	0.3	
	terminal (pyranosyl)	7.6	6.0	16.0
	3	13.4	8.9	4.3
	4	2.1	2.2	4.3
	6	3.7	3.1	3.7
	4,6			0.8
	3,6	5.1	4.4	2.1
	3,4,6	1.0	0.5	0.7
Glc	terminal	4.8	3.5	1.8
	3	4.7	2.9	0.6
	6	4.4		
	4	15.3	8.4	1.6
	3,6	1.4	0.8	
	4,6	2.0	1.1	
Hex [a]	3 or 4 (reducing terminal)			1.3
	3 or 4 (reducing terminal)			8.7
	6 (reducing terminal)			0.6
	2			0.4
	6 (furanosyl)			0.4
	3,4			2.6
	3,4			3.4

[a] Glycosyl compositions could not be deduced.
[b] NS-1 and 2 were hydrolysed by 2 M TFA at 121°C for 1 h after methylation.
[c] NS-3 was hydrolysed by 1M TFA at 100°C for 1 h after methylation.

Table 4
FAB-MS analysis of NS-2

Detected molecular ion peak $(M + Na)^+$	Proposed oligosaccharide-alditol [a]
1334	$[deoxyHex]_6-[Hex]_1$
1494	$[deoxyHex]_6-[Pen]_1-[Hex]_1$
1554	$[deoxyHex]_2-[Pen]_7$
1672	$[deoxyHex]_1-[Pen]_5-[Hex]_3$
1786	$[deoxyHex]_4-[Pen]_5-[Hex]_1$
1802	$[deoxyHex]_2-[Pen]_6-[Hex]_2$
1816	$[deoxyHex]_3-[Pen]_5-[Hex]_2$
1846	$[deoxyHex]_2-[Pen]_5-[Hex]_3$
1862	$[Pen]_6-[Hex]_4$
1902	$[deoxyHex]_4-[Pen]_7$
1932	$[deoxyHex]_3-[Pen]_7-[Hex]_1$
1946	$[deoxyHex]_4-[Pen]_6-[Hex]_1$

[a] From monosaccharide analysis deoxyHex and Pen are Rha and Ara, respectively.

2). The peak also gave fragment ions due to ald series at 134, 277 and 309, suggesting the presence of 4-linked Rha-ol-1-*d*. In addition, the peak gave the fragment ion at *m/z* 308, which was produced by elimination of MeOH from the fragment ion of ald series at *m/z* 340, suggesting the presence of 2-linked Rha-ol-1-*d*. From the analysis of component sugars of the acidic fraction and comparison of retention times with the standard oligosaccharide-alditols, the peak was suggested to be a mixture of GalA-(1 → 2)-Rha-ol-1-*d* and GalA-(1 → 4)-Rha-ol-1-*d*. The presence of 4-substituted GalA attached to position 4 of Rha in the rhamnogalacturonan core has been proposed in the endo-polygalacturonase-resistant region of the pectin (bupleuran 2IIc) [22] and pectic arabinogalactan (AGIIb-1) [23].

The neutral carbohydrate chains in PG-1 were analysed after lithium degradation and $NaBH_4$-reduction. The products were fractionated on DEAE-Sephadex A-25 ($HCOO^-$) and a large amount of neutral fraction and a trace amount of acidic fraction were obtained (data not shown). The neutral fraction was further fractionated on Bio-gel P-30 and gave the fractions eluted in the void volume (NS-1), intermediate fraction (NS-2) and lower-molecular-weight fraction (NS-3) (Fig. 1). NS-1 contained mainly Ara, Man, Gal, and Glc, whereas NS-2 and NS-3 was mainly Ara, Gal, and Glc. When aliquots of the oligosaccharide-alditols were analysed by GC-CI-MS, rhamnitol, arabinitol, mannitol, and galactitol were detected in NS-1, NS-2 and NS-3. In addition, NS-1 and NS-3 contained glucitol. Methylation analysis indicated that NS-1 consisted mainly of 3-substituted Gal and 4-substituted Glc, and that NS-2 mainly comprised terminal Ara*f*, and 4- or 5-substituted and 3,4- or 3,5-di-substituted Ara (Table 3).

Methylated products from NS-2 analysed by positive FAB-MS using *m*-NBA showed 12 kinds of pseudo-molecular ions due to oligosaccharide-alditols (Table 4). Because the oligosaccharide-alditols consisted mainly of pentose, it was concluded that most of oligosaccharide-alditols in NS-2 comprised oligoarabinosyl units. Endo-α-L-(1 → 5)-arabinanase digestion of PG-1 gave the oligosaccharide fraction in addition to the enzyme-resistant fraction. The enzyme-resistant fraction showed a decreased proportion of Ara in proportion to Rha and Gal whereas oligosaccharide fraction consisted mainly

Table 5
Major oligosaccharide-alditols detected in NS-3 by GC-EI-MS [a]

Peak	Retention time (min)	Characteristic fragment ions (m/z)												Oligosaccharide-alditol
		bA_1	bA_2	aJ_1	aJ_2	ald								
C	18.08	219	187	265	205	147	261	293	305					β-D-Gal-(1 → 4)-Rha-ol [b]
						349	381	395						and
						133	275	307	319					β-D-Gal-(1 → 2)-Rha-ol [b]
						349	381	395						
		cA_1	cA_2	aJ_1	aJ_2	aJ_0	cbA_1	cbA_2	abJ_1	abJ_2	abJ_2-MeOH			
R	42.19	219	187	265	205		423	391	469	409	377			Hex → Hex → Rha-ol [c]
		dA_1	dA_2	aJ_1	aJ_2	aJ_0	dcA_1	dcA_2	abJ_1	abJ_2	$dcbA_1$	$dcbA_2$	$dcbJ_1$	$abcJ_2$
X	52.28	175	143		205	251	335			379	509	477		539	Ara → Ara → Rha → Rha-ol [d]
Y	67.13	219	187	265	205		423	391	469	409				613	Hex → Hex → Hex → Rha-ol [d]

[a] Analysed as permethylated NS-3.
[b] Details of structural analysis described in the text.
[c] Peak R was the predominant trisaccharide-alditol, and NS-3 consisted mainly of Rha-ol as deoxyHex-ol, therefore reducing terminal of peak R was proposed to be Rha-ol.
[d] Peak X and Y were the predominant oligosaccharide-alditols and NS-3 mainly comprised Ara and Rha-ol as pentose and deoxyHex-ol, therefore pentose units and reducing terminal of peaks X and Y suggested to be Ara and Rha-ol, respectively.

of Ara, suggesting that PG-1 consisted of α-L-(1 → 5)-oligoarabinofuranosyl chain. Arabinosyl chain sequences have also been found in rhamnogalacturonan I (RGI) from sycamore cell wall [24].

Methylation analysis indicated that NS-3 contained terminal Ara*f*, Ara*p* and Gal and 4- or 5-substituted Ara, in addition to various linkages of Rha, Fuc, Ara, Xyl, Man, Gal and Glc (Table 3). NS-3 also consisted of 4-substituted 6-deoxyhexitol, 3 or 4-substituted and 6-substituted hexitol as reducing terminal. A previous study using β-elimination [3] suggested that a part of terminal Gal was attached to 4 of GalA in PG-1. In the present study, arabinitol, galactitol, and glucitol were detected as neutral oligosaccharide-alditols in NS-3. GLC-EI-MS of methylated oligosaccharide-alditols, derived from NS-3 gave 26 kinds of di- to tetra-saccharides (peaks A–Z). Peaks A – N eluted in the region of disaccharide-alditols, peaks O – W in the region of trisaccharide-alditols, and peaks X – Z in the region of tetrasaccharide-alditols. EI-MS suggested the most predominant peak C possess Hex-(1 → 2)-6-deoxyHex-ol and Hex-(1 → 4)-6-deoxyHex-ol unit (Table 5), which by sugar analysis and NMR was identified as β-D-Gal-(1 → 4)-Rha-ol (data not shown). EI-MS of permethylated oligosaccharide-alditols from NS-3 also indicated that the predominant peaks R, X, and Y had Hex → Hex → deoxyHex-ol, Pen → Pen → deoxyHex → deoxyHex-ol, and Hex → $(Hex)_2$ → deoxyHex-ol units, respectively (Table 5). Component sugar analysis of NS-3 suggested that deoxyHex-ol of peaks R, X and Y and Pen units of peak X might be Rha-ol and Ara, respectively. In addition, NS-3 also contained small proportions of various oligosaccharide-alditols such as 6-deoxyHex-(1 → 5)-Pen-ol (peak A), Pen → Pen-ol (peak B), Hex-(1 → 2) and 4)-6-deoxyHex-ol (peak D), Hex-(1 → ?)-Hex-ol (peaks E–M), Hex-(1 → 6)-Hex-ol (peaks G–N), Pen → 6-deoxyHex → deoxyHex-ol (peaks O and P), Hex → Hex → Hex-ol (peaks Q–W) and Hex → Hex → Hex → Hex → Hex-ol (peak Z) (data not shown).

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