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Structural studies of endopolygalacturonase-resistant fragments of an antiulcer pectin from the roots of *Bupleurum falcatum* L. †,*

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ABSTRACT

The endo-α-(1 → 4)-polygalacturonase-resistant fractions (PG-1, PG-2, and PG-3) from an antiulcer pectin (Bupleuran 2IIc), isolated from the roots of *Bupleurum falcatum* L., were further analysed by lithium degradation. The results indicated that PG-1 contained a small proportion of long, branched arabinosyl chains and a large proportion of short, neutral oligosaccharide chains. GLC–MS analysis showed that, after methylation the short, neutral oligosaccharide fraction consisted of at least 22 kinds of di- to tetra-saccharide alditols, such as Gal-(1 → 4)-Rha-ol (a major component), Ara-(1 → 4)-Rha-ol, Glc-(1 → 4)-Rha-ol, Ara → Ara → Ara-ol, and Ara → Ara → Ara → Ara-ol (minor components) in addition to heteroglycosyl alditols. After deesterification, PG-2 and PG-3 were digested with endo-α-(1 → 4)-polygalacturonase again, and the enzyme-resistant intermediate size fraction (PG-2′) was purified. Component sugar analysis indicated that PG-2′ contained 2-Me-Fuc, 2-Me-Xyl, apiose (Api), aceric acid (AceA), 3-deoxy-D-*lyxo*-heptulosaric acid (Dha), and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) in addition to Rha, Fuc, Ara, Xyl, Man, Gal, Glc, GalA, and GlcA. Lithium degradation of PG-2′ gave mainly a pentosyl → 6-deoxyhexosyl → 6-deoxyhexosyl → pentitol fragment, with some neutral di- and tri-saccharide alditols, including a pentosyl → deoxyhexitol. Methylation analysis of these degradation products indicated that they contained terminal Rha, Ara*f*, Fuc, Xyl, and Gal, 4-linked Rha, 3-linked Fuc, 3-linked Ara, and 3′-linked Api. Bupleuran 2IIc was eluted as essentially a single peak on gel filtration on Sepharose CL-6B. The neutral sugar content of the successive fractions increased with increasing molecular weight, but each fraction also contained, in addition to Rha, Ara, and Gal, 2-Me-Fuc, 2-Me-Xyl, and Api.

INTRODUCTION

An antiulcer polysaccharide, Bupleuran 2IIc, isolated[2,3] from the roots of *Bupleurum falcatum* L. (Japanese name, Saiko) has been characterized[1] as a pectin, primarily (~ 86%) galacturonan in character and so composed mainly of (1 → 4)-linked α-D-GalA residues. Some (1 → 4)-linked α-galacturono-tetra- and

† Dedicated to Professor C.E. Ballou.

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-penta-saccharide units possessed one and three methyl-ester groups, respectively, and some (1 → 4)-linked α-galactosyluronic units had GalA side chains at position 2 or 3. Endo-α-(1 → 4)-polygalacturonase digestion indicated that Bupleuran 2IIc gave small proportions of three enzyme-resistant fractions (PG-1, PG-2 and PG-3; yield 8.6, 2.3, and 3.3%, respectively) together with a large proportion of oligogalacturonides (PG-4). Base-catalysed β-elimination and partial acid hydrolysis suggested that PG-1 consisted of a rhamnogalacturonan core with neutral carbohydrate chains. PG-2 and PG-3 contained 2-*O*-methylfucose, 2-*O*-methylxylose, apiose (Api), aceric acid (AceA), and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) in addition to rhamnose, arabinose, galactose, galacturonic acid, and glucuronic acid as their component sugars. Since endopolygalacturonase digestion of Bupleuran 2IIc reduced[2] the antiulcer activity, it was suggested that the galacturonan region participates in the expression of this activity. Orange polygalacturonic acid, however, showed[2] only moderate activity, and some pectins showed no activity. Therefore, other structural features of Bupleuran 2IIc may contribute to its antiulcer properties.

We now report further on the structures of the endo-α-(1 → 4)-polygalacturonase-resistant regions (PG-1–PG-3) in Bupleuran 2IIc.

EXPERIMENTAL

Materials.—The roots of *B. falcatum* L. were purchased from Uchida Wakanyaku Co. Ltd. Bio-Gel P-10 (200–400 mesh) and P-30 (200–400 mesh) were obtained from Bio-Rad, Sepharose CL-6B from Pharmacia, and Sep-Pak C_{18} cartridges from Waters Associates. Pectinase from *Aspergillus niger* was purchased from Sigma, and endo-α-(1 → 4)-polygalacturonase [(1 → 4)-α-D-galacturonan glycanohydrolase; EC 3.2.1.15] was purified using the procedure of Thibault and Mercier[4]. The acidic polysaccharide fraction (BR-2) was prepared[3] from the roots of *B. falactum* L. by a hot-water extraction and precipitations with EtOH and Cetavlon (cetyltrimethylammonium bromide), and Bupleuran 2IIc was purified from BR-2 by anion-exchange chromatography on DEAE-Sepharose CL-6B as described[2,3].

General.—Carbohydrate and uronic acid in column eluates were assayed by the phenol–H_2SO_4 (ref. 5) and *m*-hydroxybiphenyl methods[6], respectively. Polysaccharides were hydrolysed with 2 M CF_3CO_2H for 1.5 h at 121°C, and TLC of hydrolysates was performed on cellulose (Merck) with 5:5:1:3 EtOAc–pyridine–AcOH–water. Reducing sugars were detected with alkaline silver nitrate[7]. Neutral sugars and uronic acids in hydrolysates were converted[8] into the corresponding alditol acetates, and analysed by GLC in a Hewlett–Packard model 5890 series II gas chromatograph equipped with an SP-2380 capillary column (0.2-μm film thickness, 0.25 mm i.d. × 30 m, Supelco), and temperature-programmed as follows: 60°C for 1 min, 60 → 215°C (30°C/min), 215–250°C (8°C/min), and 250°C for 5 min. Molar ratios were calculated from the peak areas and response factors

determined for the flame-ionization detector. 3-Deoxy-D-*lyxo*-heptulosaric acid (Dha) and Kdo were each converted to carboxyl-reduced alditol acetates according to the method of Stevenson et al.[9] For the determination of Dha and Kdo polysaccharides were hydrolysed with 0.1 M CF_3CO_2H for 1 h at 100°C, and the hydrolysates were reduced with $NaBD_4$. The products were subjected to second acid treatment (2 M CF_3CO_2H) for lactonization, and a second reduction with $NaBD_4$. For the analysis of Dha, the products were treated again with 2 M CF_3CO_2H, and reduced with $NaBD_4$. Acetylation gave the alditol acetates of Kdo and Dha, which were analysed by GLC–MS using an SP-2380 capillary column as described[9]. HPLC was performed on a Waters Model ALC/GPC244 equipped with columns (0.76 × 50 cm each) of Asahi-Pak GS-510 + GS-320 (Asahi Chemical Industry Co. Ltd.) and developed with 0.2 M NaCl. Molecular weights of polysaccharides were estimated from a calibration curve of the elution volumes of standard pullulans (P-400, 200, 100, 50, 20, and 5, Showa Denko Co. Ltd.).

Preparation of PG-1 and PG-2'.—Bupleuran 2IIc was digested[1] with endo-α-(1 → 4)-polygalacturonase for 4 days at 37°C in 50 mM acetate buffer, pH 4.2. The products were fractionated[1] on a column of Sephadex G-50, to give PG-1, PG-2, PG-3, and PG-4. After the mixture of PG-2 and -3 was deesterified, the products were digested with the polygalacturonase again, and refractionated on a column of Bio-Gel P-10 in 50 mM acetate buffer, pH 5.5, to give PG-2,3-1 and PG-2,3-2 in the void volume and near the void volume, respectively. PG-2,3-1 and PG-2,3-2 were each rechromatographed on the same column to give PG-1' (from PG-2,3-1) and PG-2' (from PG-2,3-2).

Treatment of PG-1 and PG-2' with lithium in ethylenediamine.—The procedure was performed according to the method of Lau et al.[10] To a solution of PG-1 (20 mg) or PG-2' (1 mg) in ethylendiamine (4 or 1 mL) was added lithium wire, the mixture was stirred for 1 h at room temperature, and the reaction was stopped by the addition of water. The solvent was evaporated, the residue was dissolved and desalted with AG 50W-X8 (H^+) resin, and then the products were reduced with $NaBH_4$.

Methylation analysis.—Samples (500 μg) were methylated once (Hakomori)[11] in order to prevent[12] β-elimination, but methylsulphinylcarbanion was added two or three times until conversion of the polysaccharide into a polyalkoxide was complete as checked[13] by using triphenylmethane. The methylated polysaccharides were recovered by a Sep-pak C_{18} cartridge by the procedure of Waeghe et al.[14], except that samples were eluted with EtOH. Uronic acid in methylated polysaccharides was reduced[14,15] with $NaBD_4$ in 7:3 tetrahydrofuran (THF)–EtOH at room temperature for 18 h followed by incubation at 80°C for 1 h. Each methylated polysaccharide was hydrolyzed with 2 M CF_3CO_2H for 1.5 h at 121°C, and the products were reduced with $NaBD_4$ then acetylated. The resulting methylated alditol acetates were analysed by GLC and GLC–EIMS with an SP-2380 capillary column. GLC and EIMS were 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 → 150°C (30°C/min), 150–250°C (1.5°C/min), and 250°C for 5 min. 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[16] determined for the flame-ionization detector.

GLC–EI- and CI-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) operated under a temperature program of 100°C for 1 min, 100 → 150°C (30°C/min), and 150 → 270°C (2°C/min). CIMS (isobutane) was performed on a Jeol DX-300 mass spectrometer, and EIMS was carried out on a Hewlett–Packard model 5970B mass-selective detector. CIMS[17,18] and EIMS fragment ions [A, J, and alditol (ald)][19] were used to determine the structures of the methylated oligosaccharide alditols.

RESULTS

Analysis of neutral carbohydrate chains in PG-1.—The endo-α-(1 → 4)-polygalacturonase-resistant fractions PG-1 from Bupleuran 2IIc has been suggested[1] to consist of a rhamnogalacturonan core, and it was also rich in neutral sugars. In order to analyse the structures of the neutral carbohydrate chains, PG-1 was subjected to degradation mediated by lithium in ethylenediamine. After treatment with $NaBH_4$ to complete the reduction, the resulting oligosaccharide alditols were separated by gel filtration on Bio-Gel P-10 into a fraction (NS-1) eluting in the void volume, an intermediate fraction (NS-2), and a fraction (NS-3) of low molecular weight (Fig. 1). NS-1 still contained glycosyluronic acid residues even after lithium degradation, whereas NS-2 and NS-3 lacked uronic acid. Therefore, NS-2 and NS-3 were further studied. Methylation analysis showed that

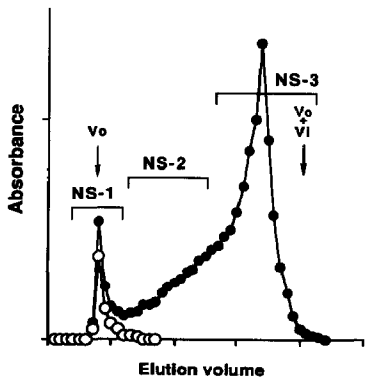

Fig. 1. Gel filtration on Bio-Gel P-10 of products from PG-1 by lithium degradation: •, carbohydrate (490 nm); ○, uronic acid (520 nm); V_o, void volume; V_i, internal volume.

TABLE I

Methylation analysis of the products obtained from PG-1 by lithium degradation

Residue	Positions of OMe groups	Linkages	Composition (mol%)	
			NS-2	NS-3
Rha	1,2,3,5	4(reducing terminal)		1.6
	2,3,4	terminal		3.0
	2,3	4	0.4	2.8
Ara	2,3,5	terminal(furanosyl)	30.9	16.1
	2,4	3	4.8	4.7
	2,3	4 or 5	25.1	17.2
	2	3,4 or 3,5	19.6	5.9
	3	2,4	1.1	
Xyl	2,4	3	0.8	
Gal	2,3,4,6	terminal	1.2	29.4
	2,4,6	3	2.2	3.1
	2,3,6	4	1.8	5.9
	2,3,5	4	0.5	
	2,3,4	6	0.4	1.8
	2,6	3,4	1.6	1.6
	2,3	4,6	0.8	0.2
	2,4	3,6	1.1	0.3
	2	3,4,6	1.6	
Glc	2,3,4,6	terminal	0.8	3.1
	2,4,6	3	0.7	1.1
	2,3,6	4	2.2	1.7
	2,6	3,4	0.6	
	2,3	4,6	0.4	

NS-3 mainly contained terminal Ara*f*, 4- or 5-linked Ara, and terminal Gal (Table I). NS-3 also gave traces of 4-*O*-acetyl-1,2,3,5-tetra-*O*-methyl rhamnitol (reducing terminal Rha), in addition to alditols from Rha, Ara, Gal, and Glc in various linkages. The long, neutral carbohydrate chain fraction (NS-2) contained mainly terminal Ara*f*, and 4- or 5-linked and 3,4- or 3,5-disubstituted Ara, suggesting that NS-2 consisted mainly of branched arabinosyl chains.

The methylated oligosaccharide alditols derived from NS-3 were analysed by GLC–EI- and CI-MS. EIMS indicated that NS-3 contained at least 22 kinds of di- to tetra-saccharide alditols (1N–22N, Fig. 2), and 7 oligosaccharide alditols (5N, 12N–16N, and 19N) could also be identified by CIMS (Table II). Peaks 1N–9N were eluted in the region for disaccharide alditols, 10N–16N in the region for trisaccharide alditols, and 17N–22N in the region for tetrasaccharide alditols. The most prominent peak, 5N, gave fragment ions at *m/z* 441 $[(M + H)^+]$, 219 (bA_1), and 205 (aJ_2) in CIMS (Table II), suggesting the structure hexosyl → deoxyhexitol, and also fragment ions of the ald series at *m/z* 307, 275, and 133 in EIMS (Table III), suggesting the linkage hexosyl-(1 → 4)-6-deoxyhexitol. The result of methylation analysis also suggested that 5N was Gal-(1 → 4)-Rha-ol. Peaks 12N–16N are

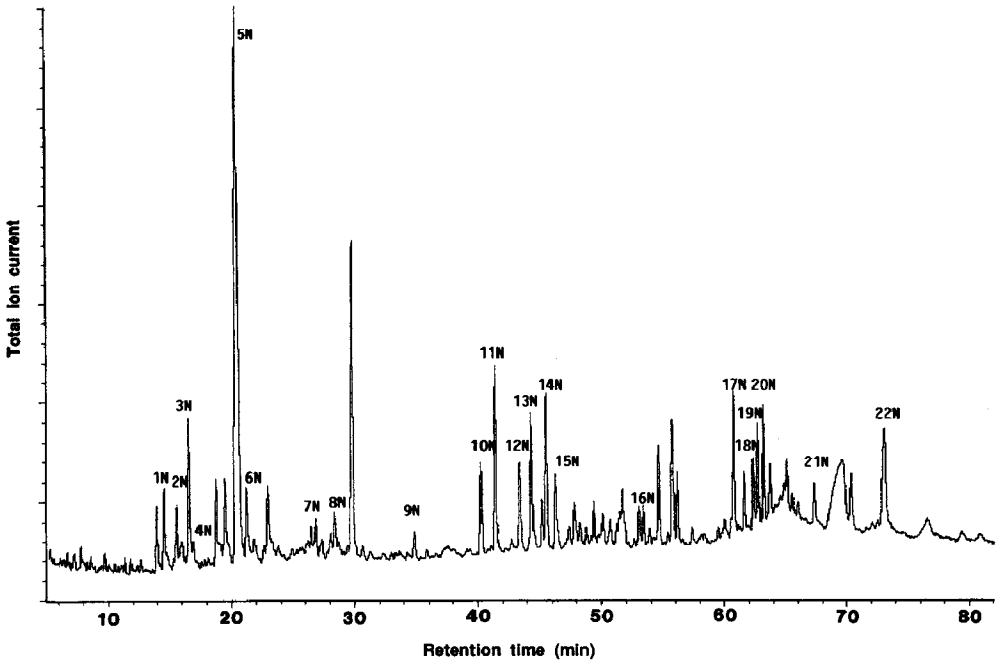

Fig. 2. Gas–liquid chromatogram of methylated oligosaccharide alditols derived from PG-1 by lithium degradation, monitored by EIMS.

proposed to represent Pen → Hex → deoxyHex-ol, Pen → Pen → Pen-ol, Hex → Hex → deoxyHex-ol, Pen → Pen → Pen-ol, and Hex → Hex → Hex-ol structures, respectively, from the protonated molecular ions and the fragment ions in CI- and EI-MS (Tables II and IV). Since NS-3 contained Ara, 13N and 15N are suggested to be Ara → Ara → Ara-ol isomers having different retention times. NS-3 contained of Gal and Glc as hexoses and Ara as its pentose component, therefore 12N and 14N are assumed to be Ara → Hex → deoxyHex-ol and Hex → Hex → deoxyHex-ol, respectively. CIMS and EIMS indicated that 19N had a Pen → Pen → Pen → Pen-ol structure, based on $(M + H)^+$ and fragment ions of the aJ_2, abJ_2, $abcJ_2$, and dA_1 types (Tables II and IV); thus it must be an Ara → Ara → Ara → Ara-ol tetrasaccharide alditol. EIMS (Table III) indicated that 1N had a 6-deoxyHex-(1 → 3)-deoxyHex-ol structure, and since NS-3 contained terminal Rha 1N is suggested to be Rha-(1 → 3)-deoxyHex-ol. Peaks 2N and 3N are proposed to possess Ara → deoxyHex-ol structures from the results of EIMS and methylation analysis. The deoxyhexitol of 2N is assumed to be 4-linked 6-deoxyHex-ol on the basis of the ald fragment ions (m/z 263 and 133); therefore, 2N is suggested to be Ara-(1 → 4)-Rha-ol. Peak 4N is proposed to be Rha-(1 → 4)-Rha-ol from the results of EIMS and methylation analysis. Peak 6N, like 5N, had a Hex-(1 → 4)-6-deoxyHex-ol structure; however, 6N was eluted later. Since Glc is eluted later than Gal in an SP-2380 capillary column, 6N is assumed to be Glc-(1 → 4)-Rha-ol.

TABLE II

Diagnostic CIMS fragment ions of di- to tetra-saccharide alditols derived from PG-1 by lithium degradation

Peak	Fragment ions [m/z (relative abundance)]									Structure inferred
	$(M+H)^+$	$(M+H)^+ - MeOH$	aJ_1	aJ_2	$aJ_2OH_2^+$	bA_1	bA_2			
Disaccharide alditol										
5N	441 (10.3)	409 (100)		205 (24.1)	223 (20.7)	219 (94.8)	187 (100)			Hex → deoxyHex-ol
			aJ_2	$aJ_2OH_2^+$	cA_1	cA_2	bcA_1	bcA_2		
Trisaccharide alditol										
12N	601 (3.4)	569 (17.2)	205 (24.1)	223 (13.8)	175 (51.7)	143 (38.8)	379 (100)	347 (13.8)		Pen → Hex → deoxyHex-ol
13N	543 (7.9)	511 (5.5)	191 (13.8)	209 (12.9)	175 (29.3)	143 (100)				Pen → Pen → Pen-ol
14N		613 (5.2)	205 (13.8)	223 (10.3)	219 (100)	187 (69.0)				Hex → Hex → deoxyHex-ol
15N	543 (3.4)	511 (1.7)	191 (11.2)	209 (15.5)	175 (100)	143 (20.7)				Pen → Pen → Pen-ol
16N	675 (6.9)	643 (1.7)	235 (12.9)	253 (44.8)	219 (100)	187 (88.8)				Hex → Hex → Hex-ol
			aJ_2	abJ_2	$abcJ_2$	dA_1	dA_2	dcA_1	dcA_2	
Tetrasaccharide alditol										
19N	703 (1.7)	671 (3.4)		369 (48.3)	529 (15.5)	175 (60.3)	143 (100)	335 (27.6)	303 (6.0)	Pen → Pen → Pen → Pen-ol

TABLE III

Diagnostic EIMS fragment ions of disaccharide alditols derived from PG-1 by lithium degradation

Peak	Fragment ions [m/z (relative abundance)]										Structure inferred
	aJ_1	aJ_2	bA_1	bA_2	ald						
1N	265 (34.0)	205 (5.8)	189 (33.3)	157 (18.4)	275 (1.2)	289 (0.8)	307 (1.0)	351 (0.4)			Rha-(1 → 3)-deoxyHex-ol
2N	265 (14.0)	205 (17.8)	175 (23.7)	143 (30.7)	133 (2.6)	263 (13.6)					Ara-(1 → 4)-Rha-ol
3N		205 (1.2)	175 (10.1)	143 (14.4)	305 (0.1)	337 (0.2)					Ara → deoxyHex-ol
4N	265 (2.1)	205 (36.6)	189 (35.7)	157 (9.9)	245 (1.9)	277 (1.3)	321 (0.4)				Rha-(1 → 4)-Rha-ol
5N	265 (16.1)	205 (47.3)	219 (12.4)	187 (32.6)	133 (10.3)	275 (4.8)	307 (1.4)	351 (0.1)	381 (0.1)	395 (0.1)	Gal-(1 → 4)-Rha-ol
6N	265 (3.2)	205 (67.9)	219 (13.1)	187 (40.1)	133 (9.9)	275 (2.1)	307 (0.7)				Glc-(1 → 4)-Rha-ol
7N	295 (14.0)	235 (47.9)	219 (16.1)	187 (32.0)	349 (12.0)	381 (2.7)					Hex → Hex-ol
8N	295 (13.7)	235 (51.8)	219 (14.2)	187 (32.0)	337 (1.2)	349 (2.7)	381 (1.3)				Hex → Hex-ol
9N	295 (2.1)	235 (58.6)	219 (7.5)	187 (24.3)	337 (3.4)						Hex → Hex-ol

TABLE IV

Diagnostic EIMS fragment ions of tri- and tetra-saccharide alditols derived from PG-1 by lithium degradation

Peak	Fragment ions [m/z (relative abundance)]								Structure inferred
	aJ_1	aJ_2	cA_1	cA_2	abJ_1	abJ_2	cbA_1	cbA_2	
Trisaccharide alditol									
10N	265 (2.9)	205 (8.7)	175 (2.4)	143 (37.3)		365 (0.5)	335 (1.2)	303 (0.3)	Ara → Ara → deoxyHex-ol
11N		205 (4.2)	175 (10.8)	143 (17.2)		365 (0.1)		303 (0.1)	Ara → Ara → deoxyHex-ol
12N		205 (23.0)	175 (50.2)	143 (77.3)		409 (0.9)	379 (9.5)	347 (0.9)	Ara → Hex → deoxyHex-ol
13N	251 (0.2)	191 (4.9)	175 (34.7)	143 (90.8)	411 (1.2)	351 (0.5)	335 (2.5)	303 (2.0)	Ara → Ara → Ara-ol
14N	265 (20.8)	205 (69.6)	219 (22.6)	187 (70.6)	469 (2.4)	409 (1.3)	423 (1.1)	391 (0.6)	Hex → Hex → deoxyHex-ol
15N	251 (1.6)	191 (6.6)	175 (12.6)	143 (17.2)	411 (0.4)	351 (0.3)	335 (1.2)	303 (0.2)	Ara → Ara → Ara-ol
16N	295 (1.6)	235 (30.7)	219 (42.0)	187 (99.5)			423 (3.7)	391 (2.1)	Hex → Hex → Hex-ol
	aJ_1	aJ_2	dA_1	dA_2	abJ_1	abJ_2	dcA_1	dcA_2	
Tetrasaccharide alditol									
17N		205 (3.2)	175 (21.9)	143 (61.1)		365 (1.5)	335 (2.8)	303 (1.1)	Ara → Ara → Ara → deoxyHex-ol
18N		205 (4.8)	175 (14.2)	143 (20.1)	412 (0.4)	365 (0.8)	335 (0.8)	303 (0.2)	Ara → Ara → Ara → deoxyHex-ol
19N		191 (4.4)	175 (25.5)	143 (32.0)	412 (0.3)	365 (0.8)	335 (0.3)	303 (1.4)	Ara → Ara → Ara → Ara-ol
20N	265 (0.8)	205 (19.7)	175 (44.3)	143 (51.6)		365 (0.8)	335 (7.8)		Ara → Ara → Ara → deoxyHex-ol
21N	251 (0.9)	191 (8.0)	175 (13.5)	143 (68.1)		365 (5.0)	335 (4.2)	303 (1.0)	Ara → Ara → Ara → Ara-ol
22N	265 (14.3)	205 (59.0)	219 (35.2)	187 (100)	469 (4.25)		423 (2.7)		Hex → Hex → Hex → deoxyHex-ol

Peaks 7N, 8N, and 9N are proposed to have Hex → Hex-ol structures; however their component sugars and linkages could not be deduced. From the results of EIMS (Table IV) and methylation analysis data peaks 10N and 11N are proposed as Ara → Ara → deoxyHex-ols having different retention times. EIMS suggested that 17N, 18N, and 20N possessed Pen → Pen → Pen → deoxyHex-ol structures having different retention times (Table IV), and they are assumed to be Ara → Ara → Ara → deoxyHex-ol from the results of methylation analysis. Peak 21N gave fragment ions in EIMS similar to those from 19N, therefore 19N is assumed to be an Ara → Ara → Ara → Ara-ol different from 21N. Peak 22N is suggested to be Hex → Hex → Hex → deoxyHex-ol from the results of EIMS.

Redigestion of PG-2 and PG-3 by endo-α-(1 → 4)-polygalacturonase.—It was found that the two endo-α-(1 → 4)-polygalacturonase-resistant fractions (PG-2 and PG-3) contained similar unusual component sugars, such as 2-Me-Fuc, 2-Me-Xyl, AceA, and Kdo. Accordingly, PG-2 and PG-3 were remixed and redigested with endo-α-(1 → 4)-polygalacturonase after deesterification. The products when chromatographed on Bio-Gel P-10 gave two fractions (PG-2,3-1 and PG-2,3-2), which were eluted in the void volume and near the void volume, respectively (Fig. 3A). When PG-2,3-1 and PG-2,3-2 were each rechromatographed on the same column, PG-1′ and PG-2′ were obtained as single peaks (Figs. 3B and C). PG-1′ had about the same component sugars (Rha, Ara, Glc, Gal, and GalA, with small amounts of Fuc, Xyl, and Man) as PG-1, whereas PG-2′ contained 2-Me-Fuc, 2-Me-Xyl, and Api in addition to Rha, Fuc, Ara, Xyl, and Gal, and so were similar to PG-2 and PG-3 (Table V). GLC–EIMS showed that the deuterium-labelled alditol acetates derived from PG-2′ contained peaks due to derivatives of Kdo (*m/z* 124, 128, 132, 204, and 234) and Dha (*m/z* 132, 156, 204, 234, and 276). Therefore, it is suggested that Bupleuran 2IIc is composed of 2 kinds of regions (PG-1 and PG-2′) resistant to endo-α-(1 → 4)-polygalacturonase, in addition to a partially methylesterified and branched (1 → 4)-α-galacturonan region.

Analysis of PG-2′.—Methylation analysis indicated that PG-2′ mainly contained 4-linked GalA in addition to 3,4- and 2,4-disubstituted GalA, and Rha, Ara, Gal, Glc, and Man in various linkages (data not shown). PG-2′ was then degraded by lithium treatment.

Methylation analysis of the resulting neutral oligosaccharide alditols showed that 1,5-di-OAc-2,3,4-tri-OMe-rhamnitol, 1,5-di-OAc-2,3,5-tri-OMe-arabinitol, and 1,5-di-OAc-2,3,4,6-tetra-OMe-galactitol were the major alditol acetates, suggesting that PG-2′ mainly contained terminal Rha, Ara*f*, and Gal as neutral sugars (Table VI). PG-2′ is also suggested to contain Ara*p*, Xyl, and Fuc as nonreducing terminal residues, and 4-linked Rha, 3-linked Rha, 3-linked Fuc, 3,4-disubstituted Fuc, 3′-linked Api, 3-linked Gal, and 2,4-disubstituted Gal. When the methylated neutral oligosaccharide alditols were analysed by GLC–EIMS, five kinds of peaks (PG-2′a–PG-2′e) due to oligosaccharide alditols were detected (Fig. 4), but the structural unit of the oligosaccharide alditols could not be identified by GLC–CIMS, because the sensitivity of the method was lower than that of EIMS.

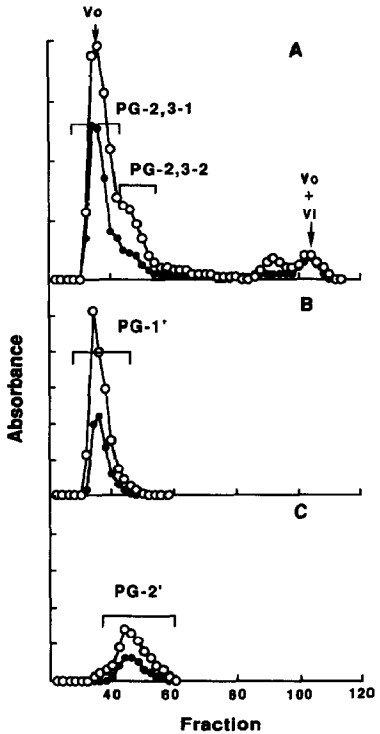

Fig. 3. Gel filtration on Bio-Gel P-10 of (A) products from deesterified PG-2 and -3 by digestion with endo-α-(1 → 4)-polygalacturonase, (B) PG-2,3-1 from (A), and (C) PG-2,3-2 from (A): •, carbohydrate (490 nm); ○, uronic acid (520 nm); V_o, void volume; V_i, internal volume.

PG-2′a–PG-2′c were eluted in the region for disaccharide alditols, PG-2′d in the region for trisaccharide alditols, and PG-2′e in the region for tetrasaccharide alditols. EIMS indicated that the most abundant oligosaccharide alditol (PG-2′e) gave intense ions at m/z 143 (dA_2), 175 (dA_1), 191 (aJ_2), 251 (aJ_1), 317 (cdA_2), 349 (cdA_1), and 411 (abJ_0), suggesting that it had a Pen → 6-deoxyHex → 6-

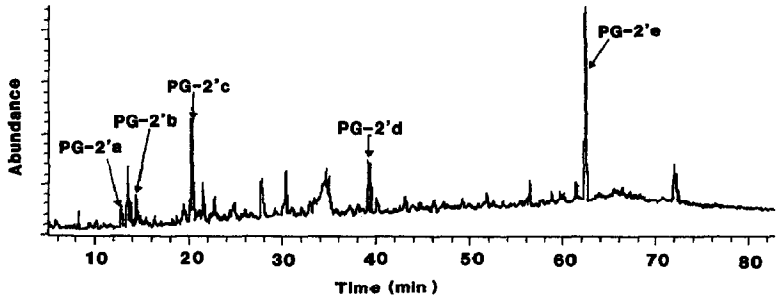

Fig. 4. Total ion chromatogram (EIMS) of permethylated oligosaccharide alditols derived from PG-2′ by lithium degradation.

TABLE V

Component sugars of PG-2′ from Bupleuran 2IIc

Sugar [a]	Molar ratios		
	PG-2′	PG-2 [b]	PG-3 [b]
Rha	1.8	2.7	3.1
Fuc	0.4	0.5	0.9
Ara	1.4	1.1	2.8
Xyl	0.2	0.1	0.2
Man	0.1	0.1	0.1
Gal	1.0	1.0	1.0
Glc	0.2	0.4	0.2
2-Me-Fuc	0.3	0.3	0.3
2-Me-Xyl	0.2	0.1	0.4
Api	0.3	0.2	0.4
GalA	n.d. [c]	4.3	5.9
GlcA	n.d.	1.0	1.0
AceA	n.d.	0.2	0.1
Kdo	+ [d]	+	+
Dha	+	n.d.	n.d.

[a] Neutral sugar, uronic acid (GalA, GlcA, and AceA), and ketose (Dha and Kdo) were analysed separately, therefore, the respective molar ratios could not be compared. [b] Reported[1] by Yamada et al. [c] Not determined. [d] Molar ratios of Kdo and Dha were not calculated; + indicates detected.

TABLE VI

Methylation analysis of the products obtained from PG-2′ by lithium degradation

Residue	Positions of OMe groups	Linkages	Composition (mol%)
Rha	2,3,4	terminal	20.3
	3,4	2	4.2
	2,4	3	3.0
	2,3	4	6.7
	2	3,4	1.7
	4	2,3	1.1
	3	2,4	3.7
Fuc	2,3,4	terminal	6.0
	2,4	3	6.6
	2	3,4	1.6
	4	2,3	0.9
Ara	2,3,5	terminal(furanosyl)	13.1
	2,3,4	terminal(pyranosyl)	6.4
Xyl	2,3,4	terminal	4.2
	2,3	4 or 5	1.3
Api	2,3	3′	6.0
Gal	2,3,4,6	terminal	8.3
	2,4,6	3	1.9
	2,6	3,4	0.5
	3,6	2,4	2.6

TABLE VII

Diagnostic EIMS fragment ions of di- to tetra-saccharide alditols derived from PG-2′ by lithium degradation

Peak	Fragment ions [m/z (relative abundance)]								Structure inferred
	aJ_1	aJ_2	bA_1	bA_2	ald				
Disaccharide alditol									
PG-2′a	265 (9.4)	205 (4.9)	175 (22.8)	143 (31.1)	337 (2.0)				Pen → deoxyHex-ol
PG-2′b	265 (15.8)	205 (4.3)	189 (21.7)	157 (13.2)					6-deoxyHex → deoxyHex-ol
PG-2′c	265 (7.6)	205 (28.0)	219 (7.6)	187 (22.3)	305 (0.7) 307 (0.7)	115 (18.0) 133 (9.0)			Hex-(1 → 2)-6-deoxyHex-ol and Hex-(1 → 4)-6-deoxyHex-ol
	aJ_1	aJ_2	cA_1	cA_2	abJ_1	abJ_2	bcA_1	bcA_2	
Trisaccharide alditol									
PG-2′d	265 (1.0)	205 (7.2)	175 (48.5)	143 (100)		379 (2.0)	349 (2.4)	317 (2.5)	Pen → 6-deoxyHex → deoxyHex-ol
	aJ_1	aJ_2	dA_1	dA_2	abJ_0	abJ_2	cdA_1	cdA_2	
Tetrasaccharide alditol									
PG-2′e	251 (3.5)	191 (32.2)	175 (61.9)	143 (100)	411 (10.1)	365 (3.0)	349 (1.7)	317 (2.7)	Pen → 6-deoxyHex → 6-deoxyHex → Pen-ol

deoxyHex → Pen-ol structure (Table VII). It was also shown that PG-2′ contained four kinds of neutral disaccharide units and one neutral trisaccharide unit, on the basis of the finding that PG-2′c was apparently a mixture of Hex-(1 → 2)-6-deoxyHex-ol and Hex-(1 → 4)-6-deoxyHex-ol (Table VII).

Distribution of unusual component sugars such as 2-Me-Fuc, 2-Me-Xyl, and Api in Bupleuran 2IIc.—Bupleuran 2IIc was eluted as essentially a single peak on gel filtration on Sepharose CL-6B, and the neutral sugars co-eluted with the uronic acid (Fig. 5A). When the fractions from the gel filtration were analysed by HPLC

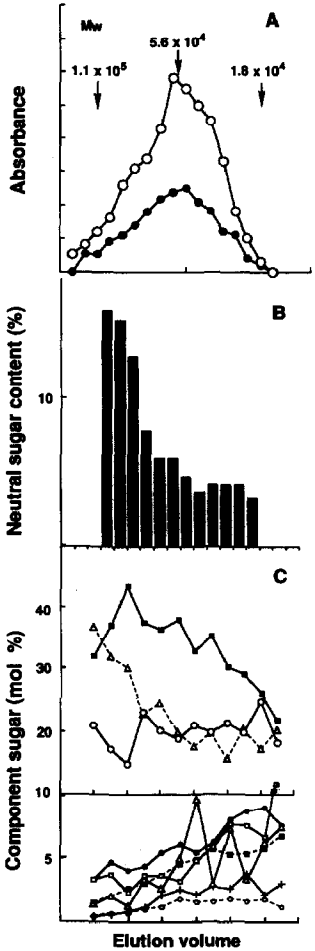

Fig. 5. A, Gel filtration of Bupleuran 2IIc on Sepharose CL-6B: ●, carbohydrate (490 nm); ○, uronic acid (520 nm). B, Neutral sugar content of each fraction from A. C, Mol% of component sugars in each fraction from A. The fractions were lyophilised, then 1 mg of each sample was hydrolysed, and derivatised to alditol acetates. The component sugars were analysed by GLC–MS as described in the Experimental. Xyl and Api were not well separated in this analysis: ■———■, Rha; △------△, Ara; ○———○, Gal; □———□, Glc; ●———●, Xyl (Api); ■------■, Fuc; △———△, Man; +———+, 2-Me-Xyl; ○------○, 2-Me-Fuc.

on Asahi-pak GS-510 + GS-320, each had a different molecular weight (1.1×10^5–1.8×10^4). These results indicate that Bupleuran 2IIc has a wide distribution of molecular weights (data not shown). The neutral sugar content of each fraction increased with increasing molecular weight (Fig. 5B). Component sugar analysis of each fraction showed that all contained not only Rha, Ara, Gal, and GalA, but also 2-Me-Fuc, 2-Me-Xyl, and Api (Fig. 5C).

DISCUSSION

An acidic polysaccharide fraction (BR-2) from *B. falcatum*, which contains the two antiulcer pectins[2,3] Bupleuran 2IIb and 2IIc, significantly protected[20] against a wide variety of experimental gastric lesions such as HCl–EtOH-, EtOH-, and water immersion stress-induced gastric lesions in mice and pylorus-ligated ulcer in rats. Although polygalacturonic acid from orange moderately inhibited the formation of HCl–EtOH-induced gastric lesions in ICR mice, methyl-esterified polygalacturonic acid did not show antiulcer activity (Table VIII). However, methyl-esterified BR-2, which contains Bupleuran 2IIb and 2IIc, still retained significant antiulcer activity (Table VIII). Therefore, it was strongly suggested that the overall structure of Bupleuran 2IIc, including the endo-α-(1 → 4)-polygalacturonase-resistant parts, is important for the expression of antiulcer activity. The present study concluded that Bupleuran 2IIc consists of two endopolygalacturonase-resistant regions (PG-1 and PG-2′) and a polygalacturonan region. PG-1 has been suggested[1] to have a rhamnogalacturonan core in which some of the Rha is substituted with neutral sugars or with 4-linked GalA at the position 4, and was proposed[1] to be a "ramified" region.

The present lithium-mediated degradation of PG-1 suggested that PG-1 contains small proportions of long, branched arabinofuranosyl chains (NS-2) and large

TABLE VIII

Effect of methyl esterification on antiulcer activity of BR-2 and polygalacturonic acid

Treatment	Dose	Number of animals	Lesion index [a]	Percent inhibition [a]
Control		10	24.7 ± 3.61	
BR-2 [b]	100	8	9.8 ± 1.98 [c]	60
Methyl-esterified [d] BR-2	100	8	12.4 ± 2.16 [e]	50
Polygalacturonic acid [f]	100	8	13.5 ± 3.22 [e]	45
Methyl-esterified [d] polygalacturonic acid	100	8	20.8 ± 5.62	16

[a] Antiulcer activity was measured using the HCl-EtOH induced gastric lesion model on ICR mice as described[2] previously. [b] Acidic polysaccharide fraction from *B. falcatum* containing Bupleuran 2IIb and 2IIc. [c] $p < 0.01$. [d] GalA residues in the polysaccharides were esterified with diazomethane as described previously[21]. [e] $p < 0.05$. [f] From oranges.

TABLE IX

Proposed structures of neutral carbohydrate side chains in the "ramified" region of Bupleuran 2IIc

4-GalA-(1 → 2)-Rha-(1 → 4)-GalA-(1 → 2)-Rha-(1 → 4)-GalA-(1 → 2)-Rha-(1 →
4 4
↑ ↑
R^1 R^2 → 4)-GalA

	Neutral carbohydrate side chains	Oligosaccharide alditols [a]
R^1	Ara	2N
	Rha	4N
	Gal	5N
	Glc	6N
	Ara → Ara	10N, 11N
	Ara → Hex	12N
	Hex → Hex	14N
	Ara → Ara → Ara	17N, 18N, 20N
	Hex → Hex → Hex	22N
R^2	Ara	Ara-ol-1-*d* [b]
	Gal	Gal-ol-1-*d* [b]
	Glc	Glc-ol-1-*d* [b]
	Hex → Hex	7N, 8N, 9N
	Ara → Ara → Ara	13N, 15N
	Hex → Hex → Hex	16N
	Ara → Ara → Ara → Ara	19N, 21N

[a] Neutral oligosaccharide alditols derived from PG-1 by lithium degradation (Tables II–IV). [b] Alditols liberated from PG-1 by base-catalysed β-elimination in the presence of $NaBD_4$.

proportions of short, neutral oligosaccharide chains (NS-3) consisting of at least 22 kinds of arabinosyl, hexosyl, and heteroglycosyl units of dp < 5. Lithium-mediated degradation[10] is useful for the release of neutral carbohydrate chains attached to position 4 or 2 of GalA in the polysaccharide, but 3-linked GalA cannot cleave, and some of it is converted to 2-deoxyhexitols. The previous results[1] suggested that PG-1 contained 3,4-disubstituted GalA (4.1 mol%) in addition to terminal, 4-linked, and 2,4-disubstituted GalA (3.3, 19.0, and 2.3 mol%, respectively), therefore a small portion of the deoxyhexitols in the released oligosaccharide alditols might have originated from 3-linked GalA during the lithium degradation. The content of 3,4-disubstituted GalA and the results of EIMS analysis, however, suggest that most of the deoxyHex-ol in the neutral oligosaccharide alditols is due to Rha. It was also assumed that many of the side chains in NS-3 are directly attached to position 4 of 2,4-disubstituted Rha in the rhamnogalacturonan core (Table IX, R^1). However, eight kinds of neutral oligosaccharide alditols (7N–9N, 13N, 15N, 16N, 19N, and 21N, Tables III and IV) do not have deoxyHex-ol at their reducing terminals. It has been reported[1] that much of Rha in the rhamnogalacturonan core is also substituted with 4-linked GalA, therefore these oligosaccharides may be assumed to attach to position 4 of GalA in the side chains (Table IX, R^2). During the present study we also analysed neutral carbohydrate chains in PG-1 by base-catalysed β-elimination[22] in the presence of $NaBD_4$, and Ara-ol-1-*d*, Gal-ol-

1-*d*, and Glc-ol-1-*d* were detected in addition to neutral oligosaccharide alditols similar to those liberated by the lithium degradation (data not shown). This result suggests that PG-1 contains monosaccharides such as Ara, Gal, and Glc attached to position 4 of GalA in the side chains (Table IX, R^2).

Purified PG-2′ contains several unusual component sugars, such as 2-Me-Fuc, 2-Me-Xyl, Api, AceA, and Kdo; Dha was also detected in the present analyses. These unusual sugars have previously been found in a plant polysaccharide. RG-II, which has been isolated from the walls of suspension-cultured sycamore cells[23], rice cell walls[24], and Douglas fir[25] by digestion with endopolygalacturonase, and from Pectinol AC[26]. Stevenson et al. reported[9] that lithium degradation of RG-II from sycamore cells gave α-Xyl-(1 → 3)-α-Fuc-(1 → 4)-β-Rha-(1 → 3′)-Api-ol as a major product. The results of lithium degradation and methylation analysis suggest that PG-2′ also contains the same tetrasaccharide fragment.

HPLC analysis indicated that Bupleuran 2IIc is microheterogeneous in terms of molecular weight. However, component sugar analysis indicated that 2-Me-Fuc, 2-Me-Xyl, and Api, which were identified as component sugars of RG-II, were contained in all fractions obtained by gel filtration, a result which strongly suggests that the RG-II-like region is distributed uniformly in all the molecules, in spite of the microheterogeneity of Bupleuran 2IIc. Stevenson et al. proposed[9] that the backbone of RG-II is composed of at least seven 4-linked α-D-GalA units, and that a variety of oligosaccharide chains are attached to the backbone. The fact that PG-2′ also consists mainly of 4-linked GalA in addition to 2,4- or 3,4-disubstituted GalA strongly suggests that the "ramified" region and the RG-II-like region may be combined through 4-linked α-galacturonan in Bupleuran 2IIc.

We have recently reported[27] the presence of an RG-II-like region in the purified mitogenic and anticomplementary pectins isolated from the roots of *Glycyrrhiza uralensis* Fisch *et* D.C. This finding and the present observation suggest that most pectins, regardless of source, may contain a region having an RG-II-like structure in addition to a "ramified" region and a polygalacturonan region. However, conclusions regarding the generality of the presence of RG-II-like regions must await further analyses of biologically active and inactive pectins. RG-II fractions isolated[23–36] from several different plant cell walls and the present PG-2′ are composed of similar sugars[25], but their molar ratios differ[26] from each other. RG-II from Douglas Fir[26] and PG-2′ from Bupleuran 2IIc contained Man as one of the component sugars, however RG-II from sycamore culture cells did not contain[23] this sugar. These facts suggest that the detailed structures of the RG-II-like regions may differ in pectins from different sources.

Further structural analysis and studies on the biological activity of PG-2′ are now in progress.

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