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PII: S0144-8617(97)00004-0

Carbohydrate Polymers 32 (1997) 249–253

0144-8617/97/$17.00 + .00

Effect of exo-β-D-(1→3)-galactanase digestion on complement activating activity of neutral arabinogalactan unit in a pectic arabinogalactan from roots of *Angelica acutiloba* Kitagawa[1]

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(Received 4 August 1995; revised version received 20 October 1995; accepted 26 October 1996)

A complement activating pectic arabinogalactan (AGIIb-1) has been isolated from roots of *Angelica acutiloba* Kitagawa, and a neutral arabinogalactan (N-I) unit, which was liberated from a side chain in AGIIb-1 by mild acid hydrolysis, has found to show the most potent complement activating activity among the arabinogalactan side chains of AGIIb-1. In order to clarify the essential carbohydrate side chains in N-I for the activity, neutral arabinogalactan (AF-N-I) unit, which was obtained from exo-α-L-arabinofuranosidase-digested AGIIb-1, was digested with exo-β-D-(1→3)-galactanase from *Irpex lacteus*. The galactanase digestion of the AF-N-I unit completely stopped its reactivity with β-D-glucosyl-Yariv antigen, and significantly reduced its complement activating activity. The digestion products gave high molecular weight fragments (GN-1A and -1B) and intermediate size fragments in addition to β-D-(1→6)-galactosyl mono to pentasaccharides as the side chains of the AF-N-I unit. Among the fragments, GN-1A, GN-1B and intermediate size fragments showed relatively potent complement activating activity, suggesting that these side chains in the N-I unit might be responsible for expression of the activity of the N-I unit. © 1997 Elsevier Science Ltd

The complement system is known to play an important role in host defence, inflammation and allergic reactions. Complement activation is associated with regulations of the immune system such as activation of macrophages and lymphocytes (Eginvarg *et al.*, 1984). Anti-complementary polysaccharides, which are classified into pectic polysaccharides, have been isolated and characterized from various kinds of medicinal herbs such as *Angelica acutiloba, Bupleurum falcatum, Glycyrrhiza uralensis, Panax ginseng, Artemisia princeps, Coix lacryma-jobi, Lithospermum euchromum, Zyzyphus jujuva*, and *Malva verticillata* (Yamada *et al.*, 1985, Shimizu & Tomoda, 1988; Yamada, 1994; Yamada & Kiyohara, 1989; Zhao *et al.*, 1993). Most of these anti-complementary polysaccharides have been shown to activate the complement system (Yamada & Kiyohara, 1989).

Of these polysaccharides some are found to be pectins which generally consist of galacturonan region [polymerized α(1→4)-GalA], 'ramified' region (rhamnogalacturonan core possessing side-chains rich in neutral sugars) (Yamada, 1994). It has been found that the active pectins also comprise rhamnogalacturonan-II (RGII) like units (Hirano *et al.*, 1994). We have investigated the structure–activity relationship of the active pectins, and found that the 'ramified' regions represent active sites for expression of their complement activating activity (Kiyohara *et al.*, 1989b; Yamada & Kiyohara, 1989). It has also been suggested that neutral oligosaccharide chains in the 'ramified' regions are responsible for expression of the anti-complementary activity and it has been proposed that the attachment of active neutral oligosaccharide chains to the rhamnogalacturonan core is necessary for amplification of the activity (Kiyohara *et al.*, 1989b; Yamada, 1994). Some of the complement activating polysaccharides are grouped into polysaccharides which are mainly comprised of arabinogalactans (Yamada & Kiyohara, 1989). Because these arabinogalactan moieties consist mainly of arabino-3,6-galactans, it has been assumed that this arabino-3,6-galactan moiety would be important for expression of the activity in the polysaccharides (Yamada & Kiyohara, 1989).

We have purified a complex pectic arabinogalactan (AGIIb-1) from *A. acutiloba* as a complement activating

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polysaccharide (Kiyohara *et al.*, 1986) and its detailed structure has been studied (Kiyohara & Yamada, 1989). AGIIb-1 comprises a rhamnogalacturonan core possessing at least four kinds of arabinogalactan side-chains in addition to the α-L-(1→3,5)-arabinan side-chain. Arabinogalactan side-chains also consist of arbino-3,6-galactan moieties. The neutral arabinogalactan unit (N-I) in particular, which is suggested to locate on the non-reducing end of an arabinogalactan side-chain, is composed of arabino-3,6-galactan. Among the carbohydrate chains derived from AGIIb-1, the N-I unit has been found to show the most potent complement activating activity, suggesting that N-I unit might be important for expression of the activity of AGIIb-1 (Kiyohara *et al.*, 1989a). Therefore, it is thought that clarification of essential carbohydrate chains in N-I for expression of complement activating activity may lead us to an understanding of the role of carbohydrate chains in complement activating polysaccharides containing arabinogalactan. Recently, Tsumuraya *et al.* have purified and characterized exo-β-D-(1→3)-galactanase from *Irpex lacteus* (Tsumuraya *et al.*, 1990). Although the galactanase is an exo-acting enzyme, the enzyme can cleave the β-D-(1→3)-galactan backbone with and without side-chains from the side of non-reducing terminal in the backbone, and the side-chain oligosaccharides are released as the digestion products. Therefore, the galactanase is useful for analyzing carbohydrate chains for expression of the activity of N-I unit having a highly branched 3,6-galactan structure.

The neutral arabinogalactan unit (AF-N-I) was prepared from arabinofuranosidase digested AGIIb-1 (AF-AGIIb-1) by mild acid hydrolysis (Yamada *et al.*, 1987). AF-N-I consisted mainly of Ara and Gal in a molar ratio of 0.2:1.0. Methylation analysis indicated that AF-N-I mainly comprised terminal Ara*f*, 6-1inked Gal and 3,6-disubstituted Gal (Table 1). When AF-N-I was digested with exo-β-D-(1→3)-galactanase from *I. lacteus*, the reactivity of AF-N-I with β-D-glucosyl-Yariv antigen completely disappeared (data not shown), suggesting that most of the β-D-(1→3)-galactan backbone was degraded by the digestion. Although AF-N-I showed potent anti-complementary activity, the activity was reduced significantly by the enzymic digestion (Fig. 1A) but the digest still had significant activity. The products were fractionated on Bio-gel P-30, and the fractions eluted in the void volume (GN-1), intermediate fraction and the lowest molecular weight fraction were obtained (Fig. 2A). All fractions mainly comprised Ara and Gal. When the anti-complementary activity of the fractions were measured, GN-1 and the intermediate fraction still showed similar potent activity, but the lowest molecular weight fraction had weak activity (Fig. 1B). Methylation analysis indicated that the intermediate fraction consisted mainly of 6-1inked Gal in addition to 4- or 5-1inked Ara, 4,6- and 3,6-disubstituted Gal (Table 1).

The lowest molecular weight fraction also comprised mainly of 6-1inked Gal (data not shown). After oligosaccharides in the lowest molecular weight fraction were

Table 1. Methylation analysis of products from AF-N-I by exo-β-D-(1→3)-galactanase digestion

Glycosyl residue	Deduced glycosidic linkage	Mol %			
		AF-N-I	GN-1A	GN-1B	Intermediate fraction
Rha	terminal	2.9	7.9	3.0	
	3	1.1		0.4	
	4				
Ara	terminal (furanosyl)	11.0	12.7	11.2	0.9
	terminal (pyranosyl)				
	3 (pyranosyl)	1.2			
	4 or 5		2.5	4.6	9.5
Gal	terminal	8.3	7.7	9.6	0.7
	3				
	4	1.3	1.6	1.1	
	6	37.5	29.6	40.8	77.4
	4,6	2.5	5.2		5.7
	3,6	10.8	11.9	11.7	5.7
	3,4,6				
Man	terminal	1.5	1.3		
	3	2.3			
	6	1.7	1.8		
Glc	terminal	5.6	1.7	0.6	
	6	4.7	3.3		
	3				
	4	6.1	1.6	0.8	
	4,6	1.4		8.2	

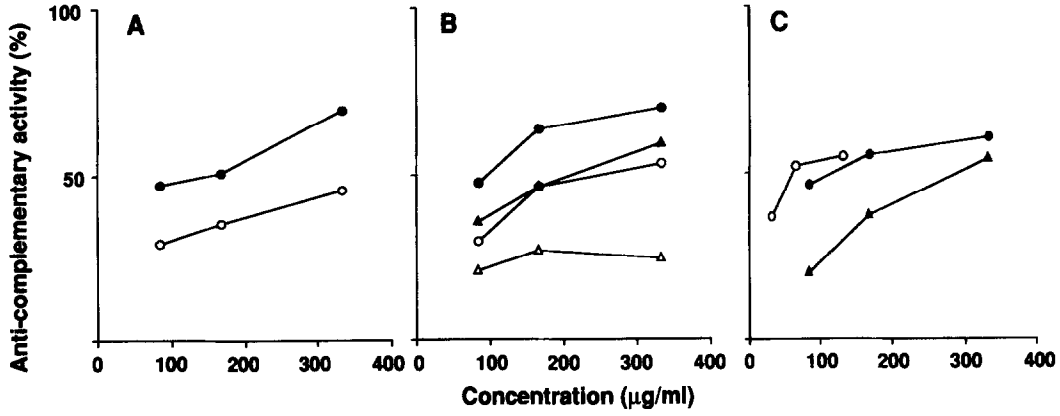

Fig. 1. Anti-complementary activity of products from AF-N-I by exo-β-D-(1→3)-galactanase digestion: (A) ●, AF-N-I; ○, mixture of digestion products. (B) ●, AF-N-I; ○, GN-1; ▲, intermediate fraction; △, lowest molecular weight fraction. (C) ●, AF-GN- 1; ○, GN-1A; ▲, GN-1B.

labelled with *p*-aminobenzoic acid butyl ester (ABBE), ABBE-oligosaccharides were fractionated on HPLC, and 19 oligosaccharide peaks were obtained. Among them five major peaks (oligo-9, -11, -13, -17, -19) were analyzed by FAB-MS (Table 2). When these peaks were further digested with exo-β-D-galactosidase, and the products were analyzed by HPLC, all the peaks were digested into Gal-ABBE (data not shown). From these results the peaks were suggested to be β-D-galactosyl mono- to pentasaccharide-ABBE as shown in Table 2. In the present study methylation analysis of the peaks could not be performed because of the small amount of sample available. Since the lowest molecular weight fraction consisted mainly of 6-linked Gal, their galacto-oligosaccharides were assumed to mainly comprise 6-linked Gal.

Since GN-1 was eluted in the void volume on Bio-gel P-30, GN-1 was further fractionated on Sepharose CL-6B, and two fractions (GN-1A and -1B) were obtained (Fig. 2B). Although both GN-1A and -1B had activity, GN-1A showed a similar activity to AF-N-I (Fig. 1C). GN-1A and -1B mainly comprised Ara and Gal (GN-1A, 10.8:72.5 and GN-1B, 10.9:80.5) in addition to Xyl, Man and Glc. Methylation analysis indicated that both fractions consisted mainly of terminal Ara*f* and terminal, 6-linked and 3,6-disubstituted Gal (Table 1).

The present β-D-(1→3)-galactanase digestion gave the fractions of the side-chains (GN-1A, -1B, intermediate and lowest molecular weight fraction) of AF-N-I in the weight ratios of 1.0:3.0:0.7:2.8, and the results indicated that these side-chains were attached to β-D-(1→3)-galactan because exo-β-D-(1→3)-galactanase cleaves β-D-(1→3)-galactan backbone with side-chains (Tsumuraya *et al.*, 1990). The degradation of the backbone decreased the activity of AF-N-I, however some of the side-chains (GN-1A, -1B, and intermediate fraction) were suggested to be active. These observations suggest that the side-chains were responsible for expression of complement activating activity of the N-I unit, and that the attachment of these active side-chains to the β-D-(1→3)-galactan backbone is necessary to express the potent activity. We have reported that oligosaccharide-alditol fractions from the 'ramified' region of complement activating pectin show the activity and that these fractions mainly comprised 6-linked Gal (Yamada, 1994). The active side-chains from AF-N-I also consisted mainly of 6-linked Gal. These observations suggested that 6-linked galactosyl chains contributed to the expression of the complement activating activity. However, the lowest molecular weight fraction from AF-N-I, which consisted mainly of 6-linked galacto-oligosaccharides, did not have potent anti-complementary activity. Since the fraction mainly contained mono- to trigalacto-oligosaccharides, it was assumed that the chain length of the galacto-oligosaccharide might be necessary for expression of the potent activity.

EXPERIMENTAL

Exo-β-D-(1→3)-galactanase digestion of AF-N-I

AF-N-I from α-L-arabinofuranosidase digested AGIIb-1 was purified as described previously (Kiyohara *et al.*, 1987). AF-N-I (10 mg) was digested with exo-β-D-(1→3)-galactanase (0.1 U) in 50 mM acetate buffer (pH 4.5) at 37°C for 3 days, and the products were desalted by an electrodialyzer (Mioroacylizer, Asahi Chemical Industry). The digests were analysed for reactivity with β-D-glucosyl-Yariv antigen by single radial gel diffusion in according with the method of Holst & Clarke (1985). The products were fractionated on Bio-gel P-30 in H_2O, and the fractions eluted in the void volume (GN-1), intermediate fraction and the lowest molecular weight fraction were obtained. GN-1 was further fractionated on Sepharose CL-6B in 0.2 M NaCl, and the carbohydrate fractions eluted in higher (GN-1A) and lower fractions (GN-1B) were obtained.

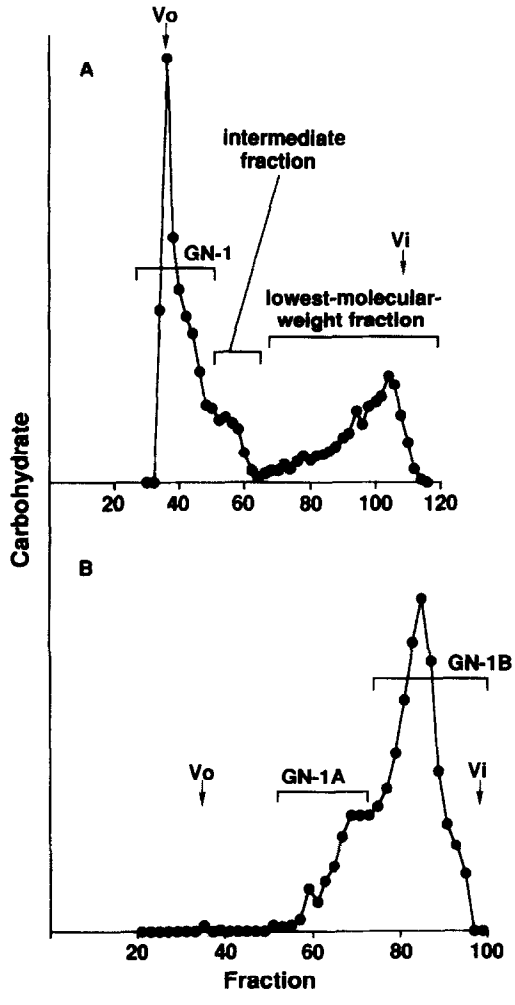

Fig. 2. (A) Gel filtration pattern of products from AF-N-I by exo-β-D-(1→3)-galactanase digestion on Bio-gel P-30. (B) Gel filtration pattern of GN-1 from (A) on Sepharose CL-6B in 0.2 M NaCl. Carbohydrate was monitored by the phenol–H_2SO_4 method (Dubois *et al.*, 1956). Vo, void volume. Vi, inner volume.

Table 2. ABBE-labelled oligosaccharides in the lowest molecular weight fraction derived from AF-N-I by exo-β-D-(1→3)-galactanase digestion

Peaks	Pseudo-molecular ion in FAB-MS $[M+H]^+$	$[M-H]^-$	Proposed structure
Oligo 9	1006	1004	$(\beta\text{-D-Gal})_4 \rightarrow \beta\text{-D-Gal-ABBE}$
11	844	842	$(\beta\text{-D-Gal})_3 \rightarrow \beta\text{-D-Gal-ABBE}$
13	682	680	$(\beta\text{-D-Gal})_2 \rightarrow \beta\text{-D-Gal-ABBE}$
17	520	518	$\beta\text{-D-Gal} \rightarrow \beta\text{-D-Gal-ABBE}$
19	358	356	β-D-Gal-ABBE

Methylation analysis

Each sample was methylated by the method of Hakomori (1964), and the product was recovered using a Sep-Pak C_{18} cartridge (Waters Assoc.) (Waeghe *et al.*, 1983). The methylated products were hydrolyzed with 2 M TFA at 121°C for 1 h, and the products were converted conventionally into the partially methylated alditol acetates, and analysed by GLC and GLC-MS on a SP-2380 capillary column as described (Zhao *et al.*, 1991).

Analysis of oligosaccharides

Oligosaccharides in the lowest-molecular weight fraction, which was derived from AF-N-I by exo-β-(1→3)-galactanase digestion, were labelled with *p*-aminobenzoic acid butyl ester (ABBE) in accordance with the method of Wang *et al.* (1984). Oligosaccharide fraction (500 μg) was dissolved in 10 μl distilled water and 40 μl reagent mixture (made by mixing with 1 mmol ABBE, 35 mg $NaBCNH_3$, 41 μl AcOH and 350 μl MeOH), and heated at 80°C for 30 min. The reaction mixture was evaporated and partitioned between water and $CHCl_3$, and labelled oligosaccharides were recovered in the aqueous layer. The labelled oligosaccharides were fractionated on HPLC equipped with a ODS-column (Capcell pak C_{18}, 4.6×150 mm, Shiseido Co. Ltd, Japan) by a linear gradient programm [40%→100% MeOH (60 min), flow rate at 1 ml/min], and detected at 300 nm. ABBE-oligosaccharides were digested with exo-β-D-galactosidase from *Aspergillus niger* (Megazyme, Australia), which was further purified by FPLC, in 50 mM acetate buffer (pH 5.2) at 37°C for 4 h, and the digestion products were analyzed by HPLC as above. FAB-MS was performed on a Jeol JMS AX505 HA mass spectrometer in positive and negative modes. A solution of each sample in water was mixed with glycerol and thioglycerol (1:1) as matrix, and loaded on a silver plate.

Anti-complementary activity

Anti-complementary substances express their activity by either inhibition or activation of complement system. AF-N-I has been found to express the anti-complementary activity only by activation of the complement system (Kiyohara *et al.*, 1989b). Therefore, complement activating activity of samples obtained from AF-N-I were expressed as anti-complementary activity in the present study.

Various dilutions of the sample in water (50 μl) were mixed with 50 μl each of normal human serum (NHS) and gelatin-veronal-buffered saline (pH 7.4) containing 500 μM $MgCl_2$ and 150 μM $CaCl_2$ (GVB^{2+}), and the mixtures were preincubated at 37°C for 30 min. During this preincubation complements are consumed by the activation. The residual hemolytic complement titer (TCH_{50}) was determined using IgM-sensitized sheep erythrocytes. NHS was also incubated with only distilled water to provide a control. The degree of consumption of complement by samples was expressed as the per cent reduction from TCH_{50} of the control (Kiyohara *et al.*, 1986).

ACKNOWLEDGEMENTS

The authors thank Dr Y. Hashimoto and Dr Y. Tsumuraya (Department of Biochemistry, Faculty of Science, Saitama University) for their kind gift of standard exo-β-D-(1→3)-galactanase.

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