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Structure-immunomodulating activity relationships of a pectic arabinogalactan from *Vernonia kotschyana* Sch. Bip. ex Walp.

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Abstract—Structure and immunological characteristics of the pectic arabinogalactan Vk2a (previously reported as Vk100A2a) from the roots of *Vernonia kotschyana* Sch. Bip. ex Walp. were investigated after enzymatic digestion of the galacturonan moiety and the side chains of the rhamnogalacturonan structure of Vk2a. *endo*-α-D-(1→4)-Polygalacturonase digestion released the high molecular weight 'hairy region' (Vk2a-HR) and oligogalacturonides. Vk2a-HR consisted of GalA (4-linked) and Rha (2- or 2,4-linked) in a 1:1 ratio, with 60% of Rha branched at C-4. The Rha located in the rhamnogalacturonan core was branched randomly by Gal units. Vk2a-HR was rich in neutral sugars such as Ara*f* 5- (12.2%) and 3,5-substituted (12.8%) and terminally- (14.1%) linked and Gal 4- (13.0%), 3- (0.9%), 6- (2.2%) and 3,6- (1.1%) substituted. Arabinans with chain lengths up to 11 units were identified. Ara*f* residues were attached to C-3 of α-L-(1→5)-Ara*f* chains and to C-4 of Gal residues. Single Gal units and chains of β-D-(1→6)-linked galacto di- to penta-saccharides were attached to a β-D-(1→3)-galactan core. All the enzyme resistant fractions expressed potent complement fixation and induction of B-cell mitogenic activity, and the present study indicates that there may be several and possibly structurally different active sites involved in the bioactivity of Vk2a. The bioactive sites may be located both in the more peripheral parts of the molecule but also in the inner core of the 'hairy region' or in larger enzyme-resistant chains.

Keywords: Pectic arabinogalactan; *Vernonia kotschyana*; Mitogenic activity; Complement fixation; Structure–activity relationship

1. Introduction

The roots of *Vernonia kotschyana* Sch. Bip. ex Walp. are widely used in the treatment of ulcers and other gastrointestinal disorders and to promote wound healing in traditional medicine in Africa.[1,2] Immunomodulating polysaccharides have been isolated from several medicinal plants and are suggested to contribute to the wound healing capacity[3–7] or anti-ulcer activity[8–11] of these plants. In a previous study a potent immunomodulating pectic arabinogalactan, Vk100A2a, was isolated from the hot water extract of *V. kotschyana* roots.[12] A pectic arabinogalactan is generally considered to comprise a smaller quantity of the α-(1→4)-linked galacturonan region and a larger amount of the ramified region or 'hairy region' (a rhamnogalacturonan core substituted with side chains rich in neutral sugars such as Ara and Gal) compared to other pectins.[13,14] Vk100A2a was subjected to several in vitro bioassays and showed a high dose-dependent complement fixation activity (human) and a

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T cell independent induction of B-cell proliferation (C3H/HeJ mice), in addition to the promotion of chemotaxis by human macrophages, T cells and NK cells.[12]

A wide variety of plant polysaccharides have been reported for their effect on the complement system, several of these belonging to the group of pectic polysaccharides.[15] Studies of *endo*-α-D-1,4-polygalacturonase digested plant-pectins from *Glycyrrhiza uralensis*,[16] *Bupleurum falcatum*,[10] *Angelica acutiloba*[17] and *Plantago major*[18] indicate that the complement fixation activity of these pectins is mainly expressed by the ramified regions. However, there are differences in the reported activities, some of the 'hairy regions' expressing slightly lower activity[16–19] than the native polysaccharides while others demonstrate the opposite[10,16,17] This discrepancy is not fully understood and more research in this field is needed. Since, many of the complement fixing pectic arabinogalactans possess major amounts of 3,6-galactan chains, these structures have been assumed to be one of the typical units that are important for the activity expressed.[15] Zhang et al. suggested that the complement activating ability of a pectic arabinogalactan from *Angelica acutiloba* was expressed by inner galactan chains rather than by outer chains,[20] and Kiyohara et al. identified complement activating 6-linked galactosyl chains attached to a β-D-(1→3)-galactan backbone as active components.[21] In the 'hairy regions' of *Glycyrrhiza uralensis*, neutral carbohydrate chains are also suggested to be responsible for the expression of the complement activation.[22] Ramified regions have also been considered to be important for expression of the mitogenic activities expressed by certain pectic polysaccharides.[10,23] In the ramified region of the pectin BR2IIc from *B. falcatum* 6-linked galactosyl chains containing terminal GlcA or 4-O-Me-GlcA substituted to (1→3)-β-D-galactosyl chains have been suggested as active sites for the expression of B-cell mitogenic activity.[9]

In the present study, we elucidate further the fine carbohydrate structures of Vk100A2a and discuss the relationship between the immunological activity and structure. Vk100A2a will hereafter be referred to as Vk2a.

2. Results and discussion

2.1. Structural elucidation

The pectic arabinogalactan, Vk2a, mainly composed of GalA substituted on C-4 (25.0%), C-2 (5.2%) and Rha on C-2,4 (5.5%), and rich in the neutral sugars Ara*f* substituted on C-5 (11.2%), C-3,5 (11.8%) and terminally linked (8.0%) and on C-4 (15.7%), C-3 (0.6%), C-6 (1.7%) and C-3,6 (2.9%) of Gal (Table 1), was subjected to *endo*-α-D-1,4-polygalacturonase digestion after de-esterification. A high molecular weight fraction, Vk2a-HR and oligogalacturonides were obtained by gel filtration on Bio-gel P-30 (Fig. 1A). 40% reduction in GalA was obtained, and the ratio of GalA to Rha in Vk2a-HR was approximately 1:1 (Table 1). This indicated the presence of a typical 'hairy region' backbone with alternating GalA 1,4-linked and Rha 1,2-linked with a high proportion (about 60%) of the Rha units branched in C-4 by side chains composed of the neutral sugars Ara and Gal. The digested products eluted from Bio-gel P-30 were tested for the presence of KDO and Dha typically present in rhamnogalacturonan type II (RGII) regions, but was not detected (Fig. 1A).

Vk2a-HR was digested with *exo*-α-L-arabinofuranosidase and the enzyme resistant fraction AF-HR, was isolated by gel filtration on Bio-gel P-30 (Fig. 1B). Glycosyl-linkage analysis of AF-HR indicated that terminal Ara*f* and 3,5-branched Ara*f* were decreased with an increase in 5-linked Ara*f*, which indicates that Ara in the original polymer is present as 5-linked regions with about one out of every two Ara residues branched at C-3 possibly by a terminal Ara*f* (Table 1). A reduction in 4-linked Gal with a concomitant increase in terminally linked Gal and decrease in terminally linked Ara*f* also indicated that some Ara*f* residues were attached to C-4 of Gal residues (Table 1).

In order to analyse in more detail the oligosaccharide sequences in Vk2a, AF-HR was subjected to further digestion with an enzyme preparation from Driselase. This purified enzyme preparation has previously been employed in order to study galactan chains in arabinogalactans due to its *exo*-β-D-(1→3)-galactanase activity, and has been designated *exo*-β-D-(1→3)-galactanase.[20,21,24–26] The products formed were fractionated on Bio-gel P-30, resulting in eight fractions (AF-GN-1–AF-GN-8, Fig. 1C) (yield ratios: 20:13:13:9:18:12:12:3). The *m*-hydroxybiphenyl assay on the column eluate indicated that all fractions formed contained variable amounts of uronic acids (Fig. 1C), which was confirmed by determination of component sugar and linkage analyses (Table 1). Although this *exo*-β-D-(1→3)-galactanase preparation has previously been used on the pectic polysaccharide bupleuran 2IIc without encountering problems with contaminating enzyme activities,[27] the present results indicated that several different types of glycosidic linkage in AF-HR had been cleaved. The *exo*-β-D-(1→3)-galactanase preparation was therefore investigated for additional enzyme activities. Both *endo*-α-L-(1→5)-arabinanase and rhamnogalacturonidase activities were detected (Kiyohara, unpublished data). Structural differences between bupleuran 2IIc and AF-HR may be the reason for AF-HR being susceptible to the additional enzyme activities since bupleuran 2IIc was not. In this study the enzyme preparation is referred to as an *exo*-β-D-(1→3)-galactanase-rich fraction in order to include the presence of additional enzyme activities.

Table 1. Methylation analysis of the native polysaccharide, Vk2a, and the products obtained after sequential enzymatic degradation of Vk2a with *endo*-α-D-(1→4)-polygalacturonase (Vk2a-HR) followed by *exo*-α-L-arabinofuranosidase (AF-HR) and finally by the *exo*-β-D-(1→3)-galactanase-rich fraction (AF-GN-1, AF-GN-2, AF-GN-3, AF-GN-4, AF-GN-5, AF-GN-6, AF-GN-7 and AF-GN-8)

Glycosyl residue	Deduced linkage	mol. %										
		Vk2a	Vk2a-HR	AF-HR	AF-GN-1	AF-GN-2	AF-GN-3	AF-GN-4	AF-GN-5	AF-GN-6	AF-GN-7	AF-GN-8
Ara	Terminal *f*	8.0	14.1	6.1	2.5	10.0	7.7	8.1	6.2	5.2	36.6	49.6
	Terminal *p*	Trace	Trace	Trace	Trace	1.4	1.3	0.4	Trace	Trace	Trace	21.5
	1,2 *p*	—	—	—	—	—	—	—	—	—	2.7	Trace
	1,3 *f*	0.2	—	0.4	0.3	1.7	0.8	0.9	0.4	—	1.4	3.1
	1,5 *f*	11.2	12.2	23.5	10.3	33.5	34.9	20.5	11.9	8.6	11.8	5.3
	1,3,5 *f*	11.8	12.8	4.0	0.2	1.6	1.1	0.6	0.3	—	—	—
Rha	Terminal	0.3	0.5	0.6	3.5	0.5	1.0	0.6	5.6	17.8	8.0	0.9
	1,2	5.2	6.2	6.5	9.7	1.5	3.1	8.0	4.1	8.8	4.6	1.1
	1,4	—	—	—	1.0	4.5	4.6	3.8	7.3	8.8	2.2	—
	1,2,4	5.5	9.9	12.0	3.9	3.9	7.5	8.5	14.3	6.5	2.3	0.3
Gal	Terminal *p*	3.2	4.8	6.2 (26.3)	3.6 (11.7)	5.4	5.1	8.9	11.7	10.9	5.4	1.9
	Terminal *f*	—	—	—	—	—	—	—	—	—	1.2	1.6
	1,3	0.6	0.9	1.1 (4.7)	3.0 (9.7)	0.8	0.6	0.6	0.5	0.3	0.3	0.2
	1,4	15.7	13.0	11.5 (48.3)	20.1 (65.3)	18.0	12.6	10.0	6.7	3.7	2.2	0.5
	1,6 *p*	1.7	2.2	3.6 (15.0)	2.9 (9.4)	6.8	6.4	6.5	4.2	1.9	1.4	0.1
	1,6 *f*	—	—	—	—	—	—	—	—	—	1.0	0.4
	1,3,6	2.9	1.1	1.3 (5.6)	1.2 (3.9)	—	—	—	—	—	—	—
	1,3,4	0.4	—	—	—	—	—	—	—	—	—	—
Xyl	Terminal *p*	0.7	0.7	0.9	5.1	—	—	—	—	—	—	—
Man[a]		0.3	0.2	0.4	3.8	—	—	—	—	—	—	—
Glc	Terminal	1.2	0.5	0.4	2.7	—	—	—	Trace	—	2.2	1.7
	1,4	4.5	4.4	3.8	4.6	—	—	—	Trace	—	1.2	0.9
Fuc	Terminal	Trace	Trace	Trace	3.6	—	—	Trace	—		Trace	—
GlcA[b]	Terminal	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
GalA[b]	Terminal *p*	—	—	—	2.3	ND	ND	Trace	Trace	Trace	Trace	5.3
	Terminal *f*	—	—	—	—	ND	ND	—	—	—	1.3	0.6
	1,3	—	—	—	—	ND	ND	—	—	—	—	1.6
	1,4	25.0	15.3	16.3	11.1	ND	ND	22.5	26.3	27.4	9.5	3.5
	1,2,4	0.6	0.3	0.6	1.9	ND	ND	—	—	—	—	—
	1,3,4	0.8	0.6	0.9	2.7	ND	ND	—	—	—	—	—

Values in parentheses represent molar percentage of linkages calculated from Gal residues only.
ND: Relevant fragments could not be detected by GC–MS, although methanolysis showed the presence of galacturonic acid in fraction AF-GN-2 (10.1 %, mol. %) and in fraction AF-GN-3 (13.3%, mol. %). Reduction could not be repeated due to lack of material.
[a] The linkage was not deduced.
[b] The uronic acids were reduced with sodium borodeuteride prior to methylation and GC–MS.

The fraction (AF-GN-1) eluted in the void volume comprises the high molecular weight enzyme resistant fraction, which was a highly complex polymer (Table 1). The relative amount of 3,6-branched Gal in AF-GN-1 was decreased but that of the 3-linkage was increased, which may be the result of the loss of Ara (about 60%) which has previously been shown to be linked to Gal in C-6.[12] The remaining 3-linked β-D-galactosyl side chains in AF-GN-1 may be due to the inability of the enzyme to cleave the inner region of AF-GN-1 probably caused by steric hindrance by other side chains.

Both Rha-(1→4)-GalA and GalA-(1→2)-Rha linkages were cleaved by the *exo*-β-D-(1→3)-galactanase-rich preparation. Typical rhamnogalacturonan type I (RG-I) oligomers composed of alternating disaccharide units of (→2)-α-L-Rha-(1→4)-α-D-GalA- (1→) were released and identified by MALDI- and ES-qoToF-MS. The RG-I oligomers were branched with terminal Gal only (Fig. 2), and the removal of neutral side chains may have contributed to an increase in the susceptibility of the RG-I backbone to enzymatic breakdown. Partial degradation of the RG-I, arabinosyl- and galactosyl-side chains left a complex core polysaccharide, AF-GN-1, with a relative increase in the amount of branched GalA at C-3 and terminally linked Xyl (Table 1), which may originate from the presence of small quantities of xylogalacturonan fragments. Such structural features have previously been reported in some pectic 'hairy regions' with terminal Xyl attached to pos C-3 of 1,4-linked GalA.[28–31] The accumulation of Man and terminally linked Fuc and 2,4-branched GalA in

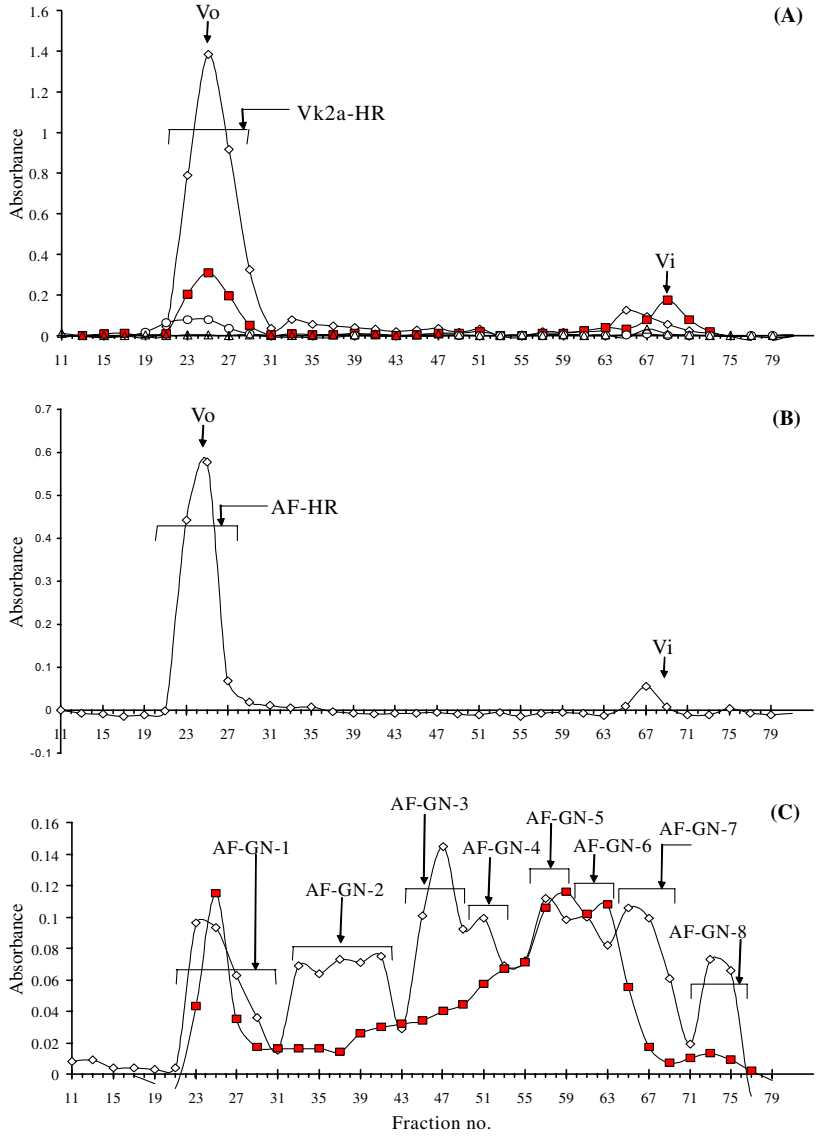

Figure 1. Gel filtration chromatograms on Bio-gel P-30 of digestion products from Vk2a by *endo*-α-(1→4)-polygalacturonase (A), of Vk2a-HR by *exo*-α-L-arabinofuranosidase (B) and of AF-HR by the *exo*-β-D-(1→3)-galactanase-rich fraction (C). (◊) carbohydrate (490 nm); (○) UV absorbance (280 nm); (■) uronic acid (520 nm); (△) RGII-type sugars (548 nm).

AF-GN-1 indicates that these units are also located in the 'hairy region' of Vk2a.

AF-GN-2 and AF-GN-3 were similar in carbohydrate composition and linkages (Table 1), and seem to consist of 'hairy region' segments with 5-linked Ara and 4- and 6-linked Gal as the dominant side chains. Initial MALDI-qoToF-MS combined with glycosyl-linkage analyses indicated the presence of both hexose and pentose chains up to six units in addition to arabinofuranosyl oligomers up to six units, attached to one or two Gal units (data not shown). AF-GN-4 contained more of the rhamnogalacturonan region and a higher ratio of 2-linked versus 2,4-branched Rha (1:1) compared to AF-GN-2 (1:2) and AF-GN-3 (1:2.5) (Table 1), which indicates a lower degree of branching of the AF-GN-4 polymers compared to AF-GN-2 and AF-GN-3. AF-GN-4 contained less 5-linked Ara (about 50%) but no change in terminal Ara, which may suggest that shorter 5-linked Ara chains are present in AF-GN-4. AF-GN-5 also contained a larger number of side chains terminated by Gal (about 70%) compared to AF-GN-2 and AF-GN-3.

MALDI- and ES-qoToF-MS analyses were performed on native and peracetylated oligosaccharide fractions (AF-GN-5–AF-GN-7), each comprising complex mixtures of several oligomers, and only the main structural characteristics will be commented on here (Fig. 1). The main fragments detected are given in Table 2, and the mono-saccharide compositions and linkages are given in Table 1. Methanolysis and glycosyl-linkage composition analyses give an indication of the total content of the different mono-saccharides present and their linkage pattern, but as each fraction is a mixture of sev-

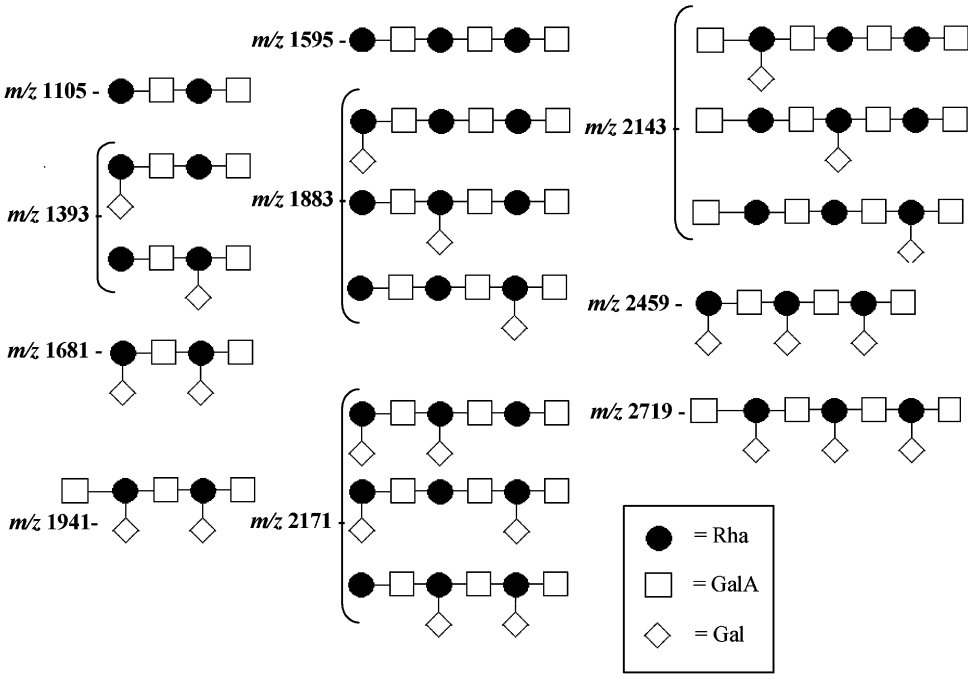

Figure 2. Proposed oligosaccharide structures present in fractions AF-GN-5, AF-GN-6 and AF-GN-7 as determined by MS/MS as described in Section 3.

eral different oligomers, these give a very complex picture. Using the MALDI- and ES-qoToF-MS and MS/MS analyses some of the individual fragments were characterised. Methanolysis and glycosyl-linkage composition analyses suggest that the pentose, hexose, deoxyhexose and hexuronic acid units identified by the MALDI- and ES-qoToF-MS analyses can be assigned to Ara, Gal, Rha and GalA.

Tandem mass spectrometry using electrospray ionisation (ES) of the ion at *m/z* 1595.4 from AF-GN-5 with composition Rha_3GalA_3 showed that the structure is composed of alternating Rha and GalA residues (Fig. 3). The ions at *m/z* 1883.3, 2171.5 and 2459.5 correspond to this structure with 1, 2 and 3 additional Gal residues branching from the Rha residues. The tandem spectra of *m/z* 1883.3 and 2171.5 show that all the possible different isomers of these ions are present. The ions at *m/z* 1941.4 and 2719.6 correspond to $Rha_2GalA_3Gal_2$ and $Rha_3GalA_4Gal_3$. The proposed structures of these ions are shown in Figure 2.

The peracetylated sample, AF-GN-6, yielded an ion at *m/z* 1105.2 with composition Rha_2GalA_2 which tandem MS showed to correspond to alternating Rha and GalA residues. In addition, the ions at *m/z* 1393.3 and 1681.4 correspond to the presence of one and two Gal units respectively on the Rha residues—again these data contain evidence for the presence of both the different isomers of the monohexosylated species (Fig. 4). The ion for Rha_3GalA_3 and the monohexosylated species are observed in the spectrum of the native oligomers as a minor series yielding ions at *m/z* 1007.2 and 1169.2 with a digalactosylated species also present at *m/z* 1331.2 (Fig. 5). Arabinan chains were also observed, consisting of 5–11 Ara units (Fig. 5). The ion at *m/z* 1941.4 has the same structure as was described for AF-GN-5. AF-GN-7 gave similar data to AF-GN-6 on peracetylation.

The ES-qoToF-MS(/MS) data thus reveal deoxyhexose-containing structures in fractions AF-GN-5, AF-GN-6 and AF-GN-7, which is consistent with the mono-saccharide analysis data. The structures determined from tandem MS are consistent with pectic rhamnogalacturono-tetra to hepta-saccharides, with a high degree of rhamnose branches, which seem to be randomly distributed. The relatively large amounts of 4-linked rhamnose identified by glycosyl-linkage composition analysis (Table 1), are consistent with the proposed oligosaccharide structures determined from the ES-qoToF-MS/MS analysis (Fig. 2), and indicate that some of the rhamnose units ending the rhamnogalacturonan oligosaccharide chain are branched at C-4 by one Gal unit, only. Previous attempts to remove Gal from native Vk2a showed that terminally linked Gal was resistant to enzymatic degradation by galactanase enzymes.[12] The present study confirms our speculations from the previous study that Gal seems to be attached to Rha in the rhamnogalacturonan region.

The terminally- and 6-linked Gal and terminal GalA in furanose forms detected in fractions AF-GN-7 and AF-GN-8 are formed after enzymatic degradation of the polymer representing the reducing residues of the oligomers or monomers formed.

Table 2. Oligosaccharide ions observed in the MALDI- and ES-qoToF mass spectra from the native and peracetylated fractions AF-GN-5, AF-GN-6 and AF-GN-7

Fractions	Native oligosaccharides		Peracetylated oligosaccharides	
	Ions *m/z*	Proposed assignment	Ions *m/z*	Proposed assignment
AF-GN-5	689.1	Hex_4	557.1	$Pent_2$
	833.2	$Pent_6$	701.1	Hex_2
	965.2	$Pent_7$	773.2	$Pent_3$
	1097.2	$Pent_8$	989.2	$Hex_3/Pent_4$
	1229.2	$Pent_9$	1277.3	Hex_4
	1361.3	$Pent_{10}$	1595.4	$dHex_3HexA_3$
	1493.3	$Pent_{11}$	1883.4	$dHex_3HexA_3Hex$
			1941.4	$dHex_2HexA_3Hex_2$
			2143.5	$dHex_3HexA_4Hex$
			2171.5	$dHex_3HexA_3Hex_2$
			2459.5	$dHex_3HexA_3Hex_3$
			2719.6	$dHex_3HexA_4Hex_3$
AF-GN-6	689.1	Hex_4	701.1	Hex_2
	701.1	$Pent_5$	773.1	$Pent_3$
	833.2	$Pent_6$	1105.2	$dHex_2HexA_2$
	965.2	$Pent_7$	1393.3	$dHex_2HexA_2Hex$
	1007.2	$dHex_3HexA_3$	1681.4	$dHex_2HexA_2Hex_2$
	1097.2	$Pent_8$	1941.4	$dHex_2HexA_3Hex_2$
	1169.2	$dHex_3HexA_3Hex$		
	1229.2	$Pent_9$		
	1331.2	$dHex_3HexA_3Hex_2$		
	1361.3	$Pent_{10}$		
	1493.3	$Pent_{11}$		
AF-GN-7	527.1	Hex_3	557.1	$Pent_2$
	689.1	Hex_4	701.1	Hex_2
	701.1	$Pent_5$	773.1	$Pent_3$
	833.2	$Pent_6$	989.2	$Hex_3/Pent_4$
	851.2	Hex_5	1105.2	$dHex_2HexA_2$
	965.2	$Pent_7$	1277.3	Hex_4
	1097.2	$Pent_8$	1393.3	$dHex_2HexA_2Hex$
			1681.4	$dHex_2HexA_2Hex_2$
			1941.4	$dHex_2HexA_3Hex_2$

In order to investigate further the hexosyloligosaccharides detected by MALDI-TOF-MS, the native polymer Vk2a was digested with the *exo*-β-D-(1→3)-galactanase-rich fraction from Driselase together with *exo*-α-L-arabinofuranosidase in order to increase the digestion efficacy of the galactanase by removing arabinofuranosyl side chains from the type II arabinogalactan moiety. The digestion products were analysed by HPAEC-PAD to show the degradation efficacy of the galactanase digestion. The HPAEC-PAD analysis of the digestion products of Vk2a gave small proportions of oligosaccharides (labelled peak with * in Fig. 6) having similar retention times as β-D-(1→6)-galacto di- to hexa-saccharides. In order to confirm whether the oligosaccharides eluting with retention times of 11–21 min were related to β-D-(1→6)-galactooligosaccharides, the digestion products of Vk2a were further digested with *endo*-β-D-(1→6)-galactanase from *Trichoderma viride*. From the HPAEC-PAD analysis it could be observed that the amount of β-D-(1→6)-galacto di- and mono-saccharides increased compared to the peaks around the retention times of 13–21 min (Fig. 6). These results suggest that Vk2a contains a β-D-(1→3)-linked galactan core bearing side chains. Some side chains are single Gal and/or β-D-(1→6)-galacto di- to penta-saccharides. The reducing terminal Gal of the β-D-(1→6)-galactooligosaccharides are derived on cleavage from the β-D-(1→3)-galactan core.

The current structure elucidation of Vk2a by enzymatic digestion with *endo*-α-(1→4)-polygalacturonase, *exo*-α-L-arabinofuranosidase and the *exo*-β-D-(1→3)-galactanase-rich fraction, revealed that Vk2a is composed of both α-(1→4)-polygalacturonan- (smooth region) and rhamnogalacturonan-I regions ('hairy region'), with about 40% of the GalA located in the smooth region. About 60% of the rhamnose units are branched in a highly random fashion by Gal units. Some of the neutral side chains are related to β-D-(1→6)-galacto di- to penta-saccharides attached to a β-D-(1→3)-galactan core, and some arabinofuranosyl residues are attached to C-3 of α-L-(1→5)-arabinofuranosyl chains and to C-4 of galactosyl residues. Some of the

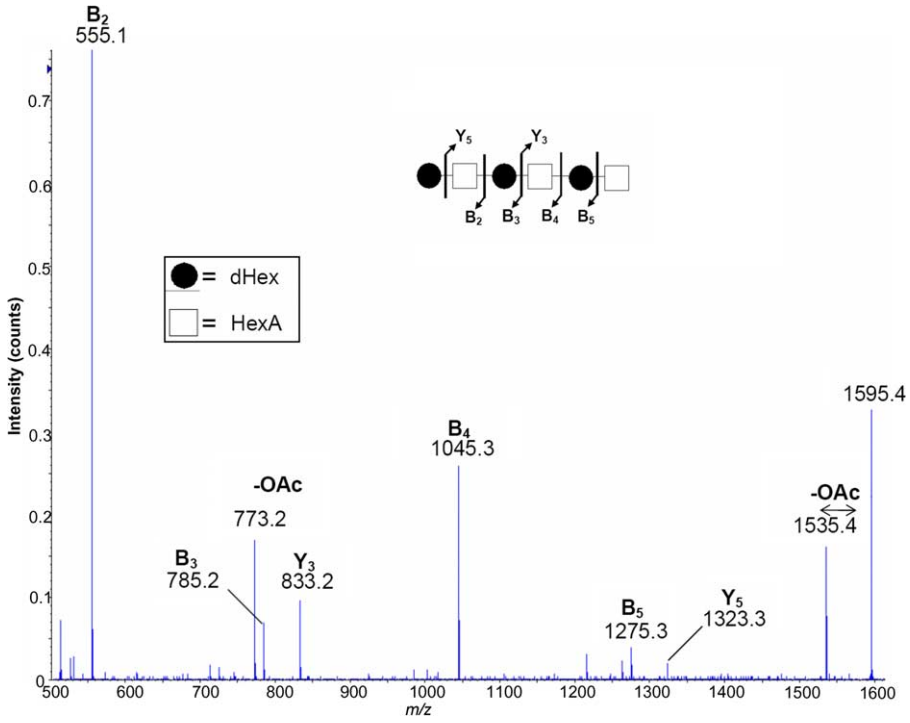

Figure 3. Product ion spectrum of the *m*/*z* 1595.4 ion from peracetylated AF-GN-5.

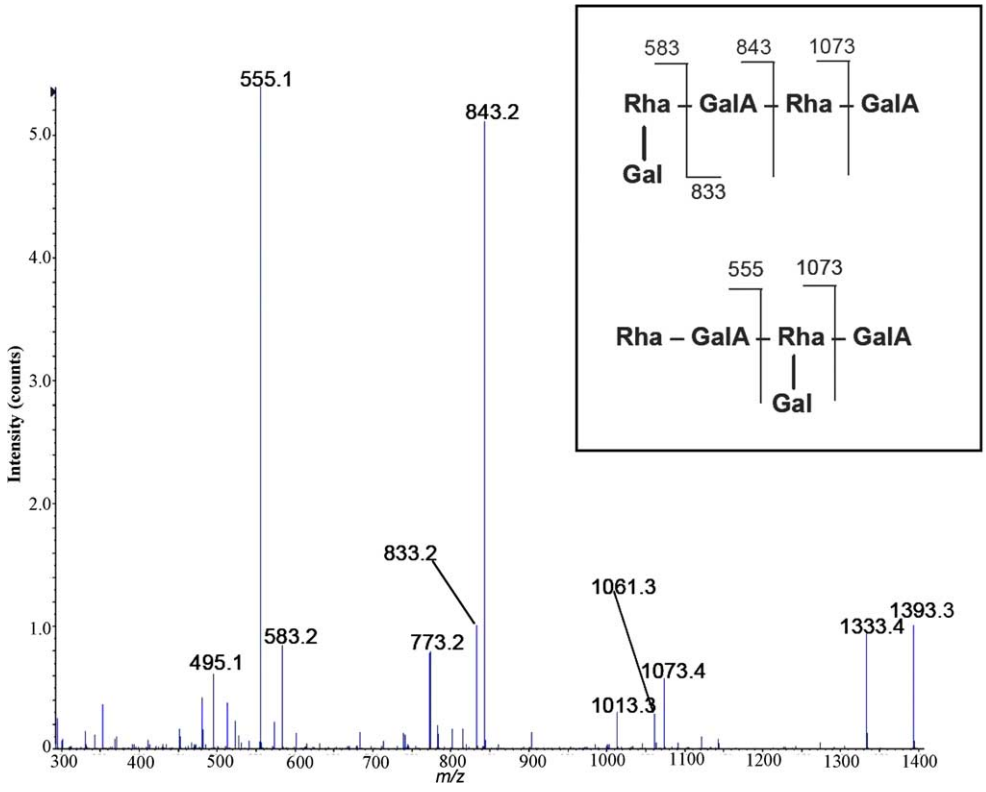

Figure 4. Product ion spectrum of the *m*/*z* 1393.3 ion from peracetylated AF-GN-6. The ion corresponds to the composition $GalRha_2GalA_2$ with structure of alternating Rha and GalA residues containing one additional Gal residue branching one of the Rha residues. The spectrum shows that both isomers are present.

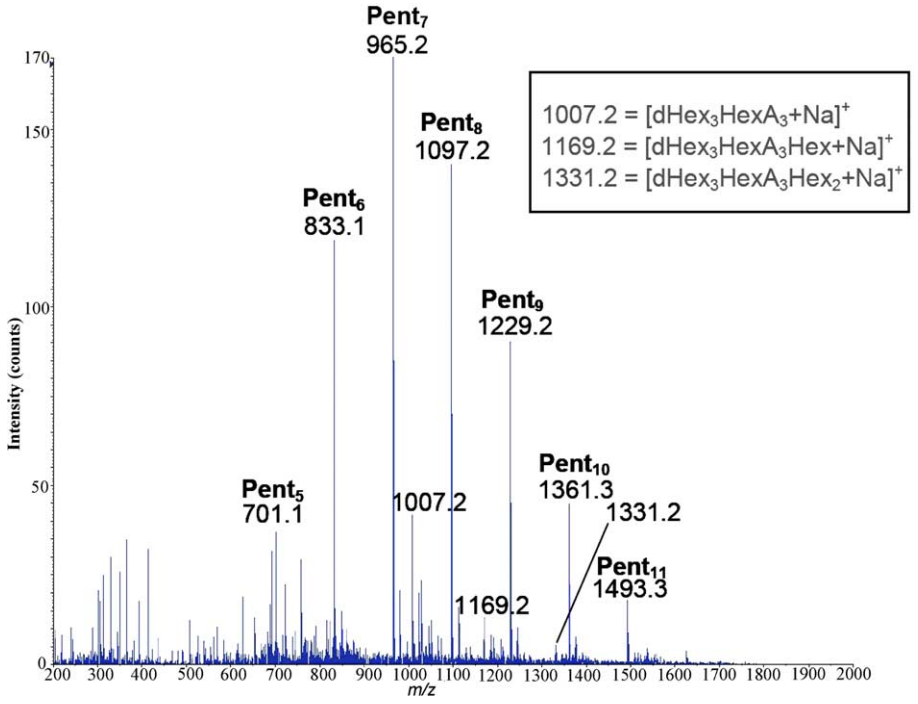

Figure 5. MALDI-qoToF-MS spectrum of native fraction AF-GN-6.

arabinofuranosyl chains are composed of as many as 11 consecutive residues.

2.2. Structure and immunological activity

2.2.1. Complement fixation. All the high molecular weight enzyme resistant fractions, Vk2a-HR, AF-HR and AF-GN-1, retained potent dose-dependent complement fixation activities (Fig. 7). The activity of the 'hairy region', Vk2a-HR, seemed to be lower than that of the native polysaccharide, which may indicate that the polygalacturonan moiety of the pectic arabinogalactan Vk2a contributes to the expression of complement fixation. The removal of 4-linked GalA may also induce a change in the configuration of Vk2a, making the active sites less available for interaction with complement factors. Partial removal of arabinose subunits (AF-HR) did not seem to change the activity of 'hairy region' (Fig. 7).

The highest molecular weight fraction (AF-GN-1), obtained after digestion of AF-HR with the *exo*-β-D-(1→3)-galactanase-rich fraction, seemed to possess the most potent complement fixation activity of all polymers. The oligosaccharides formed after degradation, indicate loss of arabinan- , galactan- and arabinogalactan- side chains in addition to smaller rhamnogalacturonan regions of four to seven residues substituted with terminally linked Gal (Table 2, Fig. 2). This may lead to a more open structure of the remaining polymer and possibly an increase in the expression of bioactive sites, which may be present in the inner core. Compared with the enzyme resistant fractions the activity of the oligosaccharide fractions tested (AF-GN-2–AF-GN-5) was low, although they seem to be dose dependent. The lack of bioactivity may be due to the simplified structures of the oligosaccharides comprising simple rhamnogalacturonan oligosaccharides with only one Gal in the branching point in addition to individual Ara*f*- (5–11 residues), Gal- (4–5 residues) (Table 2) and arabinogalactan- (six Ara units with one or two terminal Gal units) oligomers. These results may be in accordance with the proposal that the attachment of active neutral oligosaccharide chains to the rhamnogalaturonan core is necessary for amplification of complement fixation activity.[14,32] The results suggest that a more complex structure with a certain three-dimensional configuration may be important for the activities observed, and that the polygalacturonan regions are involved. More than one binding site in each molecule giving an overall shape and surface may be important for binding to factors involved in the biological system studied.

Whether the inner-galactan chains of Vk2a are responsible for the complement fixation effects observed cannot be concluded from the results obtained. The relative amounts of galactosyl residues 3- and 6-linked (3.0% and 2.9%, respectively) or 3,6-branched (1.2%) of AF-GN-1 are relatively small, and other structural features may therefore also be important for the activities expressed. After partial removal of arabinan-, galactan- and arabinogalactan- side chains in addition to rhamnogalacturonan oligomers, a relative increase in Xyl, Man, Fuc and branched GalA was observed, however the contribution of the activity by such moieties is

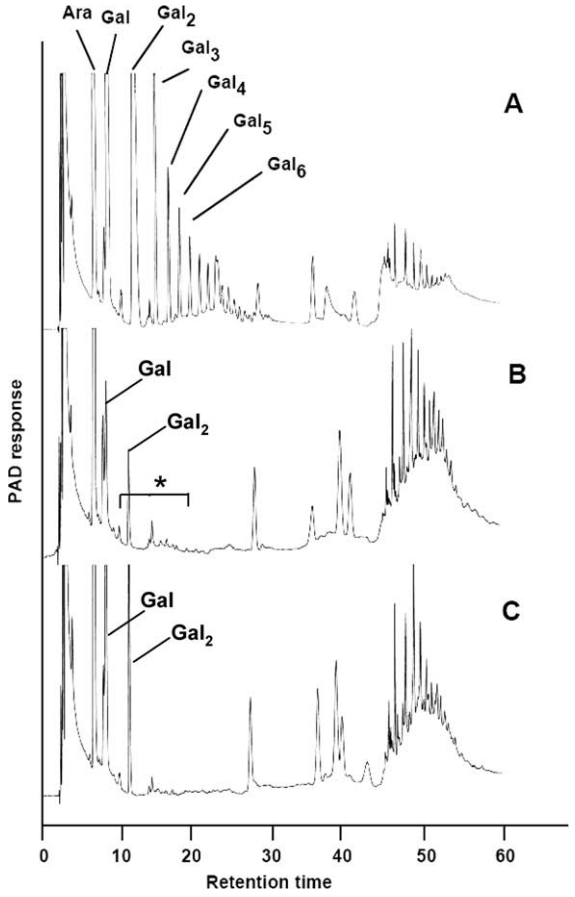

Figure 6. HPAEC-PAD chromatogram of the *exo*-β-D-1,3-galactanase-rich fraction digestion products from ALR-5IIa-1-1 from *Atractylodes lancea* DC (A). HPAEC-PAD chromatogram of the *exo*-β-D-1,3-galactanase-rich fraction digestion products from Vk2a (B). HPAEC-PAD pattern of the *exo*-β-D-1,3-galactanase-rich fraction digestion products from Vk2a after digestion with *endo*-β-D-1,6-galactanase. Gal2-Gal6; elution position of β-D-1,6-galacto di- to hexa-saccharides. *Oligosaccharides liberated from Vk2a having the same retention times as β-D-1,6-galacto di- to hexa-saccharides.

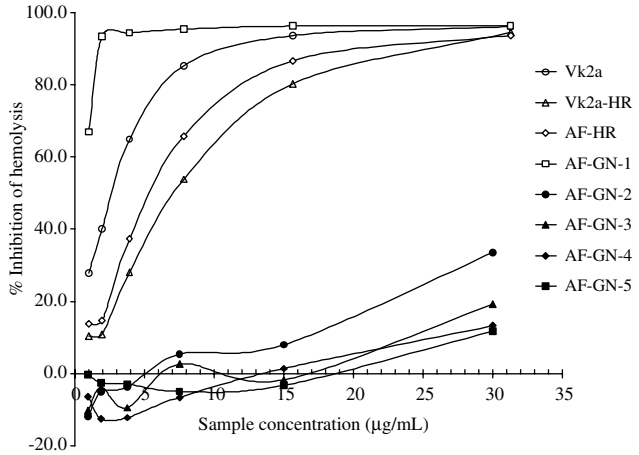

Figure 7. Complement fixation activity, expressed as % inhibition of lysis of sensitised sheep erythrocytes, of the original polysaccharide (Vk2a) and the products obtained after treatment of Vk2a with *endo*-α-D-1,4-polygalacturonase (Vk2a-HR, 'hairy region'), *exo*-α-L-arabinofuranosidase (AF-HR), and the *exo*-β-D-1,3-galactanase-rich fraction (AF-GN-1, AF-GN-2, AF-GN-3, AF-GN-4 and AF-GN-5). A complement fixing polysaccharide (PMII) from *Plantago major* L. was used as positive control, and showed 50% inhibition of haemolysis at a concentration of 100 µg/mL.

not known. The frequency of branching of GalA in the homogalacturonan region has previously been suggested to affect complement fixation,[15] although a possible effect of branching of GalA in the 'hairy region' has not been investigated.

2.2.2. Mitogenic activity. Vk2a has previously shown potent B-cell mitogenic activity,[12] therefore we attempted to investigate structure–activity relationships of this bioactive polymer as regards to mitogenic activities.

All fractions investigated after enzymatic digestion with *endo*-α-(1→4)-polygalacturonase, *exo*-α-L-arabinofuranosidase and the *exo*-β-D-(1→3)-galactanase-rich fraction showed direct mitogenic activity on B cells when compared with the control (ANOVA, $p < 0.01$) (Fig. 8A). The 'hairy region' (Vk2a-HR) showed significantly higher activity than the original polysaccharide ($p < 0.0001$), which is in accordance with results obtained by Sakurai et al. on the 'hairy region' from the *B. falcatum* pectin, BR2IIc,[9] indicating that structures in the 'hairy region' are important for B-cell mitogenic activity. Partial removal of L-α-arabinofuranosyl units (AF-HR) did not change the activity ($p > 0.05$), indicating that these arabinose-subunits located on the side chains are not important for the mitogenic activity of Vk2a.

After digestion of AF-HR with the *exo*-β-D-(1→3)-galactanase-rich fraction, the mitogenic activity of the mixture of the digested polymer and the oligosaccharides formed (AF-HR-GN-mix), the enzymatic resistant fraction (AF-GN-1), and the highest molecular weight oligosaccharide fraction (AF-GN-2) was investigated (Fig. 8A). The activity of the AF-HR-GN-mix showed a decrease in the activity compared with Vk2a-HR and AF-HR ($p < 0.0001$), and the oligosaccharide chains (AF-GN-2) showed low mitogenic activity compared with the other fractions investigated. From this it may be concluded that the combination of the backbone and the side chains of the 'hairy region' seems to be important for the B-cell mitogenic activity.

The high molecular weight enzyme resistant fraction (AF-GN-1) expressed high B-cell mitogenic activity. As for the complement fixation activity, the removal of the outer part of the molecule seems to expose structures in the inner core of the molecule that express B-cell mitogenic activity. These structures may not be exposed in the original polysaccharide. Since, the highest molecular weight oligosaccharide fraction (AF-GN-2) after enzyme digestion also expressed significant B-cell

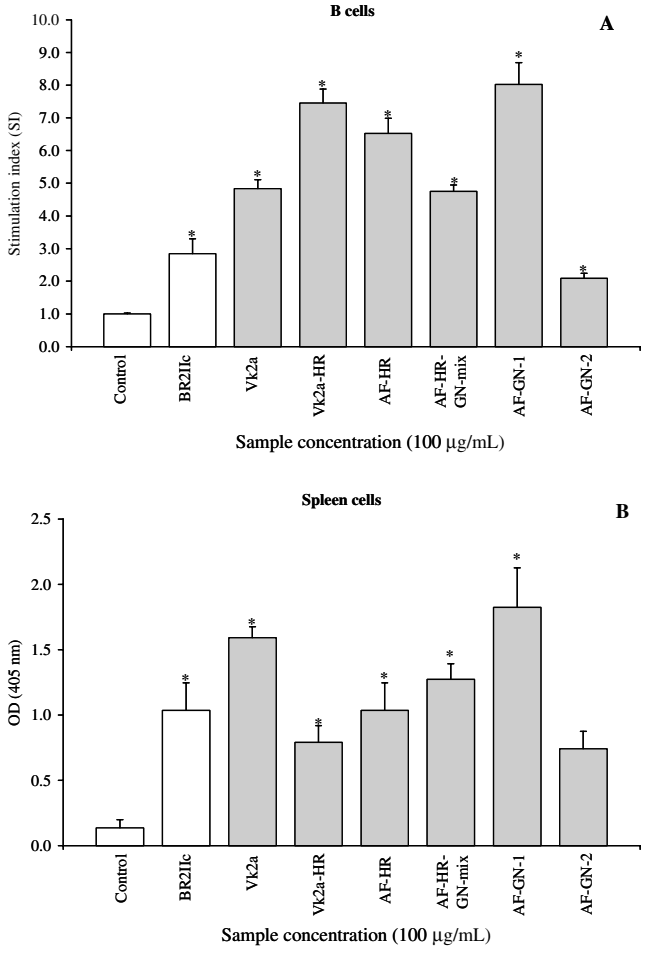

Figure 8. Mitogenic activities of the original polysaccharide (Vk2a) and the products obtained after treatment of Vk2a with *endo*-α-D-1,4-polygalacturonase (Vk2a-HR, 'hairy region'), *exo*-α-L-arabinofuranosidase (AF-HR), *exo*-β-D-1,3-galactanase-rich fraction (AF-GN-1, AF-GN-2) and a mixture of all the products from AF-HR after digestion with the *exo*-β-D-1,3-galactanase-rich fraction (AF-HR-GN-mix) on purified B cells (A) and on B cells in the presence of other spleen immune cells (B). The cells (2 million cells/well, in a 96-well plate) isolated from spleens from C3H/HeJ mice were cultured with samples, water (unstimulated control) or bupleuran 2IIc (BR2IIc, positive control) for 3 days, and the proliferative responses were assessed either by the AlamarBlue™ reduction assay (A) measuring the relative fluorescence intensity with an excitation wavelength at 544 nm and emission wavelength at 590 nm, with the stimulation index (SI) calculated as described in Section 3, or the alkaline phosphatase assay (B) measuring the absorbance at 405 nm as described in Section 3. Each value is presented as mean ± SD. *Indicates response (*$P < 0.001$) compared to unstimulated control.

mitogenic activity, Vk2a may possess multiple sites that are important for bioactivity, which may partly explain the very potent activity of this polysaccharide. Active sites for mitogenic activity of the ramified region of the pectin BR2IIc from *B. falcatum* have previously been assigned 6-linked galactosyl chains with terminal GlcA and/or 4-O-Me-GlcA residues attached to β-D-(1→3)-galactosyl chains.[9] Although Vk2a-HR contained all the relevant mono-saccharide residues and their respective linkages, no Me-HexA or fragments containing Hex with terminally linked HexA were detected among the oligosaccharides analysed by ES-qo-ToF-MS(/MS). Although we cannot exclude that such carbohydrate sequences may exist in the native Vk2a and in the high molecular weight enzyme resistant fractions, the results indicate that other structural features are likely be involved in the B-cell mitogenic activity of the Vk2a polysaccharide.

The sample fractions were also tested for mitogenic activity on B cells in the presence of other spleen cells by determination of alkaline phosphatase (APase) activity (Fig. 8B). Since, T cells and macrophages lack APase activity, it is possible to detect proliferation of B cells in the presence of a large number of such cells.[33] The results of mitogenic activity assays on the whole spleen cell suspension (Fig. 8B) did not correspond exactly to the results from the mitogenic activity on purified B cells (Fig. 8A). This may indicate that the partially degraded polysaccharides interact not only with B cells but may also have effects on other immune spleen cells. Interaction with other cells may lead to consumption of the 'hairy region', leaving a smaller amount of the active principle available for activation of B cells. The 'hairy region' could also possibly stimulate cytokine production in other cells that may affect the activity of the B cells. The α-D-(1→4)-galacturonan backbone or the galacturonan oligosaccharides in the original polysaccharide may also contribute to the regulation of biological activity in the spleen cell suspension. In a previous study it has been established that polygalacturonic acid alone, does not express mitogenic activity.[34]

Both the enzyme resistant high molecular weight fraction (AF-GN-1) and the highest molecular weight oligosaccharide fraction (AF-GN-2) formed after digestion by the *exo*-β-D-(1→3)-galactanase-rich fraction, expressed mitogenic activity, although the enzyme resistant fraction expressed the highest activity ($p < 0.0001$). From this study we found that the oligosaccharides alone also expressed B-cell mitogenic activity, but we cannot conclude whether they also affect other cells. To the authors knowledge activities of oligosaccharide side chains from pectins on other immune-cells have not yet been investigated.

Among the structural moieties of Vk2a, ramified region seems to be involved in both the expression of B-cell mitogenic activities and in complement fixation. Degradation of the native polysaccharide to the 'hairy region' seems to also influence other immune spleen cells. Vk2a is a highly complex pectic arabinogalactan and the bioactive sites seem to be located both in the more periferal parts of the molecule, but also in the inner core of the 'hairy region' or in larger enzyme-resistant chains. The exact structure of a specific active site for the complement fixation and B-cell mitogenic activity remains to be deduced, but the present study indicate

that there may be several and possibly structurally different active sites involved in the bioactivity of Vk2a.

3. Experimental

3.1. Materials

The roots of *V. kotschyana* were collected near the Baoulé river in the Kolokani area, Mali, in February 1998 and identified by the Department of Traditional Medicine, Bamako, Mali, where a voucher specimen is deposited. The pectic arabinogalactan, Vk2a, was isolated as described previously.[12,35] Briefly, powdered roots were extracted with ethanol, the residue was extracted with water at 50 °C and further with water at 100 °C. The 100 °C extract (Vk100) was separated by ion-exchange chromatography (DEAE-Sepharose fast flow) and the fraction Vk100A2 was separated further by gel filtration (Sephacryl S-400) to yield the pectic arabinogalactan, Vk2a.

Pectinase from *Aspergillus niger* was purchased from Sigma, and *endo*-α-(1→4)-polygalacturonase was purified according to the method of Thibault and Mercier.[36] *exo*-α-L-Arabinofuranosidase (Megazyme, Ireland) from *A. niger* was further purified by FPLC according to the procedure of Lerouge et al.[37] Driselase (*Irpex lacteus*) was kindly supplied from Kyowa-Hakko Co. Ltd. (Japan), and fractionated by DE-Cellulose and CM-Toyopearl by the procedure of Tsumuraya et al.[26] to obtain the enriched fraction of *exo*-β-D-(1→3)-galactanase. *endo*-β-D-(1→6)-Galactanase from *Trichoderma viride* was a kind gift from Prof. Y. Tsumuraya (Saitama University).

3.2. General methods

The relative carbohydrate, uronic acid and protein contents in the column eluates were assayed by the phenol–sulfuric acid,[38] *m*-hydroxybiphenyl[39] methods and absorbance at 280 nm, respectively. The presence of mono-saccharides (KDO and Dha) typically present in rhamnogalacturonan type II (RGII) regions was investigated by the thiobarbituric acid (TBA) assay[37] on the column eluate of Vk2a after polygalacturonase digestion. Neutral mono-saccharides and uronic acids were methanolysed and converted into trimethylsilyl glycoside derivatives[40] and analysed by gas chromatography.[4]

3.3. Preparation of 'hairy region' (Vk2a-HR) from Vk2a

After de-esterification of Vk2a (50 mg) in 0.1 M NaOH (5 mL) for 2 h at room temperature, the products were digested with *endo*-α-(1→4)-polygalacturonase[41] (0.3 U) in 50 mM acetate buffer (pH 4.2) at 37 °C for 3 days, then fractionated on Bio-gel P-30. The 'hairy region' Vk2a-HR eluted in the void volume. About 20% of the digest was lyophilised and the remaining subjected to sequential enzymatic digestion.

3.4. Sequential enzymatic digestion

Vk2a-HR was digested with *exo*-α-L-arabinofuranosidase (5 μL, 0.6 U) in 5 mL 50 mM acetate buffer (pH 4.2) at 37 °C for 7 h. The digestion product was fractionated on a column (2 × 50 cm) of Bio-gel P-30 in 50 mM acetate buffer (pH 5.2), and the fraction eluted in the void volume (AF-HR) was desalted by dialysis (Spectra Pore 6, MWCO 1000) for 4 days, and about 20% was lyophilised. The remaining product was incubated with the *exo*-β-D-(1→3)-galactanase-rich fraction (50 μL, 0.07 U as the galactanase activity) in 5 mL 50 mM acetate buffer (pH 4.2) at 37 °C for 3 days followed by fractionation on Bio-gel P-30 to obtain eight fractions (AF-GN-1–AF-GN-8). Each fraction was desalted by passing through cation-exchange resin with AG®50W-X8 (H^+, Bio-Rad) and evaporated to dryness.

3.5. Analysis of released oligosaccharides from VK2a with the *exo*-β-D-(1→3)-galactanase-rich fraction from Driselase by HPAEC-PAD

Vk2a (50 μg) was digested with *exo*-β-D-(1→3)-galactanase-rich fraction (5 μL, 0.007 U) in 500 mM acetate buffer (pH 4.2, 100 μL, 37 °C, 8 h) in the presence of *exo*-α-L-arabinofuranosidase (5 μL, 0.6 U). The digestion mixture was further digested with *endo*-β-D-(1→6)-galactanase (5 μL, 0.1 U, 37 °C, 8 h). The digestion products were analysed by HPAEC in according to the condition described by Taguchi et al.[24]

3.6. Glycosyl-linkage composition analysis

Prior to methylation, samples containing uronic acids were reduced to primary alcohols at the polymer level. To distinguish between reduced uronic acids and the corresponding neutral sugars in GC–MS, sodium borodeuteride was used. Uronic acids in oligosaccharides were reduced with sodium borodeuteride after methyl esterification.[42,43] In the methylation procedure, free hydroxyl groups in the carbohydrates were de-protonated and methylated, then the glycosidic linkages were hydrolysed and the partially methylated mono-saccharides were reduced to alditols and acetylated as described by Kim and Carpita.[44] The partially methylated alditol acetates were analysed by GC using a Fisons GC 8065 with an SPB-1 fused silica capillary column (30 m × 0.20 mm i.d.) with film thickness 0.20 μm. The injector temperature was 250 °C, the detector temperature was 300 °C and the column temperature was 80 °C when injected, then increased at 20 °C/min to 170 °C, followed by 0.5 °C/min to 200 °C and then to 30 °C/min to 300 °C. Helium was the carrier

gas with a flow rate of 0.9 mL/min. EI mass spectra were obtained using a Hewlett–Packard Mass Selective Detector 5970 with a Hewlett–Packard GC. The compound in each peak was characterised by interpretation of the characteristic mass spectra and retention times in relation to authentic standard derivatives. Estimation of the relative amounts of each linkage type was related to the total amount of each mono-saccharide type determined by methanolysis.

3.7. Mass spectrometry

Lyophilised polysaccharide fractions were reconstituted in HPLC grade water and analysed directly, or peracetylated using the acid catalysed acetylation method.[45] prior to mass spectrometric analysis. All MS experiments were performed on an Applied Biosystems QStar pulsar *i* quadrupole orthogonal time-of-flight (qoToF) mass spectrometer. Samples analysed with MALDI-qoToF-MS(/MS) were ablated from a 2,5-dihydroxybenzoic acid (DHB) matrix. The spots were prepared by mixing 0.7 μL of DHB (5 mg/mL^{-1}) and 0.7 μL of sample solution on the target and allowing the spots to air dry before being taken for MALDI-MS(/MS) analysis. For electrospray (ES) qoToF-MS(/MS) experiments both the native and peracetylated samples were solvated in methanol–water–formic acid (50:50:1, v/v/v) and infused into the source of the mass spectrometer at 0.3 μL/min^{-1} (spray voltage 5.5 kV). Nitrogen was used as the collision gas in all product ion experiments.

3.8. Complement fixation assay[1,46]

The effect on human complement was measured by complement consumption and degree of red blood cell lysis by the residual complement using pectic polysaccharides, PMII, from *Plantago major* L. as positive control.[4] The % inhibition of lysis is given by the formula $100 \times (A_{\text{control}} - A_{\text{sample}})/A_{\text{control}})$. The results were plotted as initial sample concentration when added to the wells versus % inhibition of lysis. An influence of contaminating LPS (lipopolysaccharide) on complement activity was disregarded as a previous study indicated that the amount of LPS present in the polysaccharide sample did not influence the activities observed.[12]

3.9. Mitogenic activity

Mitogenic activity was measured as described previously.[12] Specific-pathogen-free C3H/HeJ female mice (LPS low sensitive) were purchased from SLC (Shizouka, Japan) and used at 6–8 weeks of age for the spleen cell cultures. B cells were isolated and purified from the spleen cell suspension by a magnetic cell sorting (MACS) system as described by Guo et al.[47] The efficiency of the separation was determined by flow cytometry (FCM), and the cell fraction was shown to be 95% CD45R/B220 and CD19 double positive B cells (data not shown).

Two methods were employed for measurement of proliferation: (a) Cell growth was measured by means of a fluorometric assay, the Alamar Blue™ reduction assay.[9,48] Purified B cells (2 million cells/mL) were cultured with samples (100 μg/mL) or bupleuran 2IIc[8,10] (BR2IIc, 100 μg/mL). Four hours before culture termination 20 μL Alamar Blue™ solution (Alamar Bio-Science, Inc., USA) was added to each well. The fluorescence intensity was measured using a Fluoroscan II (Labosystems Oy, Finland) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The delta soft II (Version 4.13 FL, BioMetallics, Inc., USA) was used for data management. The stimulation index (SI) was calculated as follows: SI = (relative fluorescence with stimuli)/(relative fluorescence without stimuli). (b) Selective measurement of B-cell proliferation in the spleen cell suspension was measured by the alkaline phosphatase assay (APase assay).[33,49] Spleen cells (2 million cells/mL) were cultured with samples (100 μg/mL) or bupleuran 2IIc (BR2IIc, 100 μg/mL). Cell cultures were terminated by spinning the plates at 400*g* for 5 min. The culture medium was removed by aspiration. Thereafter, 50 μL 1% Triton X-100 and 150 μL 1 mg/mL *p*-nitrophenyl phosphate disodium salt in 0.1 M diethanolamin buffer (pH 9.5) was added to each well. The reaction was terminated by adding 50 μL 3 M NaOH to each well and the optical density at 405 nm was measured using a microplate reader (Bio-Rad, Model 250, Nippon Bio-Rad, Japan).

3.10. Statistics

Data are presented as the mean ± SD. Statistically significant differences between the treatments were tested by analysis of variance (one-way-ANOVA) followed by Fisher's PLSD. *p*-Values of 0.05 or less were considered statistically significant.

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