Structures and Structure−Activity Relationships of Three Mitogenic and Complement Fixing Pectic Arabinogalactans from the Malian Antiulcer Plants *Cochlospermum tinctorium* A. Rich and *Vernonia kotschyana* Sch. Bip. ex Walp

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Structures of three pectic arabinogalactans, one from *Vernonia kotschyana* (Vk2a) and two from *Cochlospermum tinctorium* (Ct50A1 and Ct50A2), and their complement fixation and induction of B cell proliferation in vitro were compared. The polysaccharide Vk2a expressed potent biological activity in both assays compared with Ct50A1 and Ct50A2. Vk2a possessed a very high molecular weight (1150 ± 20 kDa) compared with Ct50A1 and Ct50A2 which both showed a polydisperse nature with the highest molecular weight polymers in each fraction estimated at ∼105 kDa (Ct1a) and 640 ± 100 kDa (Ct2a), respectively. The HMW polymers showed complement fixation in the same range as the native fractions. The arabinogalactan II content was low in Vk2a (2%) compared with that in Ct50A1 (23%) and Ct50A2 (12%). The high molecular weight polymers were subjected to digestion with a β-D-(1, 3)-galactanase-rich fraction from Driselase, oligomers were isolated by HPAEC, and their finer structures were determined by MALDI- and ES-qoToF-MS, linkage, and monosaccharide composition analyses. Vk2a consists of both a galacturonan core and a rhamnogalacturonan core rich in neutral side chains. The backbones of both Ct-polysaccharides consist mainly of RG-I regions with numerous neutral side chains dominated by galactosyl residues, whereas the homogalacturonan regions seem to be small. Differences in the chain lengths of the 6-linked galacto-oligosaccharides attached to the 3-linked galactan core could not be related to the differences in the potencies of the biological activities observed.

1. Introduction

Three immunomodulating polysaccharides of the pectic arabinogalactan type have previously been reported from the roots of the medicinal plants *Vernonia kotschyana* (Vk2a)[1,2] and *Cochlospermum* tinctorium (Ct50A1 and Ct50A2)[3]. The polysaccharides are highly complex and show complement fixation activities and induction of B cell proliferation in vitro. They are all composed of typical monosaccharides (Ara*f*, Gal, GalA, Rha, GlcA, Xyl, Man, Fuc, and Glc) possessing similar types of linkages. However, the relative amounts and linkage patterns differ. Arabinogalactan type II structures (AG-II) were identified in all polysaccharide fractions. Since several pectic arabinogalactans with β-3,6-galactan moieties as neutral sugar side chains have been reported for their potent effect on the complement system, it is speculated that the β-3,6-galactan part may be essential for expression of the activities observed.[4] Potent mitogenic activities have also been associated with polysaccharides containing a rhamnogalacturonan core rich in neutral sugar chains such as 6-linked galactosyl chains containing terminal GlcA or 4-O-Me-GlcA, which are substituted on β-D-(1→3)-galactosyl chains.[5] Therefore, we wanted to compare the biological activities of the polysaccharides and focus on potential differences in structures, among these the AG-II type structures as these have been shown to be coupled to the bioactivity in other polymers.[4,6−8] In this study the amount of AG- II was measured, and the oligosaccharide structures liberated by digestion of the high molecular weight polymers with an exo-β-D-(1,3)-galactanase-enriched fraction from Driselase were investigated. As chains of AG-II-types may be associated with a small amount of protein with a typical amino acid composition, we also estimated the amounts of protein and the amino acid compositions of Vk2a, Ct50A1, and Ct50A2. Finally, since the immunomodulating ability of polysaccharides may also be

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dependent on their molecular weights, we also compared the molecular weights of the isolated polymers.

2. Experimental Section

2.1. Plant Materials. The roots from *Vernonia kotschyana* were collected near the Baoulé river in the Kolokani area, Mali, in February 1998. The pectic arabinogalactan, Vk2a, was isolated as described previously.[1,2] Powdered roots from *Cochlospermum tinctorium* were bought locally at the medical herbal market, Hérboristerie du Marché d'Hamdallayé commune IV, in Bamako in February 1999. The roots were collected 30 km northeast of Bamako, near the river in the Bagineda area in Mali (1999). The pectic arabinogalactans, Ct50A1 and Ct50A2, were isolated as previously described.[3] The plant parts were identified by the Department of Traditional Medicine, Bamako, Mali, where voucher specimens are deposited.

2.2. General Procedures. To determine the monosaccharide compositions of the poly- and oligosaccharides, samples were methanolysed by 4 M HCl in anhydrous methanol at 80 °C for 24 h followed by trimethylsilylation and GC as described by Chambers and Clamp,[9] with the modifications described by Samuelsen et al.[10] Mannitol as internal standard was included throughout the total procedure.

For determination of protein content and amino acid composition, the samples were subjected to hydrolysis under vacuum in 6 M HCl at 110 °C for 24 h. After removal of HCl under reduced pressure, the amino acid composition was determined using a Applied Biosystems 421 Amino Acid Analyzer. The analysis was performed by Ola Rumohr Blingsmo at the Biotechnology Centre of Oslo.

The amount of arabinogalactan protein in the polysaccharide fractions was quantified using β-glucosyl-Yariv reagent (prepared by one of the authors, BSP) by single radial gel diffusion as described by van Holst and Clarke.[11] A standard curve was constructed for each plate using a serial dilution of standard gum arabic solution.

The polysaccharides were subjected to size exclusion chromatography/multi-angle laser light scattering (SEC/MALLS) for the determination of weight-average molecular weight with the same instrumentation as described by Hokputsa et al.,[12] or by SEC (weight-average molecular weight) comparing their elution pattern with that of dextran polymers (2000, 475, 223, 98, 16, 12, and 6 kDa, Pharmacia) as described previously.[3] Only the molecular weights from the SEC/MALLS analyses are given with standard deviations.

After de-esterification of the Ct polysaccharides (200 mg) in 0.1 M NaOH (20 mL) for 2 h at room temperature and neutralized with acetic acid, the products were digested with pectinase (250 μL, Pectinase, Sigma (P2736)) in 50 mM sodium acetate (pH 4.2) at 37 °C for 3 days. Toluene was added to prevent microbial growth. Native or pectinase-digested Ct polysaccharides were fractionated on Biogel P-30 (3 cm × 80 cm) with 50 mM sodium acetate buffer (pH 5.2) as eluent, and 5 mL fractions were collected using a Pharmacia LBK−RediFrac. The carbohydrate elution pattern was determined using the phenol-sulfuric acid assay.

The 2D-NMR ROESY spectra of Ct2a were dissolved in D_2O (Aldrich) at 70 °C and were acquired on a Bruker Avance AV 600 spectrometer at 70 °C with a 5 mm TXI Triple Resonance Inverse probe equipped with Z-gradient coil. Chemical shift of D_2O (δ_H 4.70) was used as a reference for ^{1}H NMR. The data were processed using Bruker XWIN NMR (version 3.5) software.

2.3. Biological Assays. *Complement Fixation.*[13,14] 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 a positive control.[10] The % inhibition of lysis is given by the formula $100 \times (A_{control} - A_{sample})/A_{control}$. The results were plotted as initial sample concentration when added to the wells vs % inhibition of lysis.

B Cell Proliferation. Induction of B cell proliferation was measured as described previously.[2] 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.[15] 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). Cell growth was measured by means of a fluorometric assay, the Alamar Blue reduction assay.[5,16] B cells (2 million of cells/mL) were cultured with samples (100 μg/mL), bupleuran 2IIc[9,17] (BR2IIc, 100 μg/mL) or medium (control). Four hours before culture termination 20 μL of Alamar Blue (Alamar Bio-Science, Inc., USA) was added to each well. The fluorescence intensity was measured by Fluoroscan II (Labosystems Oy, Finland) at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The DeltaSoft 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). 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. The P-values of 0.001 or less were considered statistically significant.

2.4. Enzymatic Hydrolysis and HPAEC−PAD Fractionation of the Resulting Mono- or Oligosaccharides. Driselase (*Irpex lacteus*) was kindly supplied from Kyowa-Hakko Co. Ltd. (Japan) and fractionated by DEAE-cellulose and CM-Toyopearl using the procedure of Tsumuraya et al.[18] to obtain the exo-β-D-(1→3)-galactanase enriched fraction. This enzyme preparation was used to compare products obtained from the three different polysaccharides after enzyme digest in order to detect possible differences in the products formed.

The polysaccharide samples (5 mg) were incubated with the Driselase fraction (5 μL, 0.007 U) in 5 mL of 50 mM acetate buffer (pH 4.2) at 37 °C for 3 days with the addition of toluene to prevent microbial growth. This process was followed by fractionation of the liberated oligo- or monosaccharides using high performance anion-exchange chromatography combined with pulsed amperometric detection (HPAEC−PAD). HPAEC−PAD was performed on a Dionex Bio LC instrument equipped with a CarboPac PA-1 (Dionex) column (9 × 250 mm). Carbohydrates were eluted at 1.0 mL/min with a gradient using the following program: 0−5 min 20 mM sodium acetate (NaOAc) in 100 mM NaOH, 5−60 min 20−200 mM NaOAc in 100 mM NaOH, 60−120 min 200−400 mM NaOAc in 100 mM NaOH. The carbohydrates were desalted on-line using a Carbohydrate Membrane Desalted (CMD, Dionex) which was regenerated with 75 mM sulfuric acid at 7 mL/min with the SRC-1 power supply (Dionex) at the highest setting (500 mA), collected manually and lyophilised.

2.5. Monosaccharide Linkage Analysis. Prior to methylation, samples containing uronic acids were reduced to primary alcohols on the polymer level.[19] 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.[20,21] In the methylation procedure, free hydroxyl groups in the carbohydrates were de-protonated and methylated, then the glycosidic linkages were hydrolyzed, and the partially methylated monosaccharides were reduced to alditols and acetylated as described by Kim and Carpita.[19] The partially methylated alditol acetates were analyzed by GC-MS on a Fison fused silica capillary column (30 m × 0.20 mm i.d.) with film thickness of 0.20 μm and E. I. mass spectra were obtained using a Hewlett-Packard Mass Selective Detector 5970 coupled with a Fisons GC 8065. Conditions for GC-MS were as described previously.[1] The compound at each peak was characterized by an interpretation of the characteristic mass spectra and retention times in relation to standard sugar derivatives.

2.6. Mass Spectrometry. Lyophilized polysaccharide fractions were reconstituted in HPLC-grade water and analyzed directly or peracetylated using the acid catalyzed acetylation method[22] 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 analyzed with MALDI-QoToF-

Table 1. Monosaccharide Compositions (mol. %) and Molecular Weights of Vk2A, Ct50A1, Ct50A2 and the High Molecular Weight Pectinase Treated Ct50A1 and Ct50A2 (Ct1a-PN and Ct2a-PN, Respectively)[a]

		glycosyl residues (mol. %)								
	MW (kDa)	Ara	Rha	Gal	GalA	GlcA	Glc	Xyl	Fuc	Man
Ct50A1	polydisperse	6.7	16.7	53.7	8.4	12.7	1.2		trace	trace
Ct1a-PN	60 ± 4[b]	11.2	19.2	46.4	10.1	9.9	2.8			0.4
Ct1a	~105[c]	22.8	13.6	44.6	8.0	7.4	2.3			
Ct1b	~31[c]	4.3	18.5	58.4	5.9	11.2	0.5	1.3		
Ct1c	~12[c]	1.8	19.7	57.9	3.5	16.1	0.5	0.6		
Ct1d	~6[c]	1.7	21.5	53.8	4.4	15.1	1.6	1.9		
Ct50A2	polydisperse	4.4	29.0	25.5	30.8	8.3	1.8		trace	trace
Ct2a-PN	338 ± 4[b]	2.6	27.3	25.0	32.5	10.0	1.1			
Ct2a	640 ± 100[b]	5.0	36.6	22.7	27.6	7.7	0.9			
Ct2b	~100[c]	2.2	28.2	41.9	16.4	10.3	1.1			
Ct2c	~16[c]	4.7	25.3	38.3	16.4	13.5	1.7			
Vk2a	1150 ± 20	31.3	11.0	24.4	26.4	trace	5.7	0.7	trace	0.3

[a] Ct1a−d and Ct2a−c are fractions obtained from gel filtration of native Ct50A1 and Ct50A2, respectively, with Ct1a and Ct2a as the highest molecular weight fractions. [b] MW estimated by SEC/MALLS. [c] Average MW estimated by gel filtration on Superose 6 compared with dextran standards.

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 were infused into the source of the mass spectrometer at 0.3 μL/min (spray voltage 5.5 kV). Nitrogen was used as the collision gas in all product ion experiments at a setting value of '6', and the collision energy setting was varied between 30 and 60 eV. First, the HPAEC oligosaccharide fractions were analyzed directly, and if the mass spectrometric results obtained in this way were poor, the HPAEC fractions were peracetylated prior to mass spectrometric analysis in order to facilitate removal of residual salt and to increase the mass spectrometric response.[23] Some of the oligosaccharide components were also subjected to tandem mass spectrometry to define sequence/branching patterns of the oligosaccharides.

3. Results and Discussion

Vk2a showed significantly higher mitogenic activity on B cells compared to the Ct polysaccharides ($P < 0.0001$) (Figure 1), which showed only moderate induction of B cell proliferation. Vk2a also possessed very high complement fixation activity compared to both Ct polysaccharides (Figure 2). There appears to be a positive correlation between a high mitogenic activity and a high complement fixation activity. Pectins from *Bupleurum falcatum*[5,24] and *Glycyrrhiza uralensis*,[25] both containing arabinogalactan side chains, have also been reported to be highly active both in the complement and mitogenic assays. Previously, we have reported an inactive pectin, Vk100A2b, which predominantly consisted of a smooth region (almost 80% 1,4-linked GalA) with only minor amounts of a ramified region and neutral monosaccharides. The fact that Vk100A2b expressed neither mitogenic nor complement fixation activities[1] may be due to lack of appropriate neutral side chains attached to an RG-I core.[25,26] The most immunogenic polysaccharide Vk2a consists of about 40% of the GalA located in the smooth region and a hairy region (alternating Rha and GalA, ratio 1:1) with neutral side chains containing AG-I and AG-II type structures. According to previous findings the smooth region of Vk2a is not essential for the potent bioactivities observed.[27]

Both Ct50A1 and Ct50A2 contained a high Rha to GalA ratio (2:1 and 1:1 respectively) (Table 1), indicating that the Ct polysaccharides' backbones consist mainly of RG-I type structures. Ct50A1 and Ct50A2 were both demethylesterified and

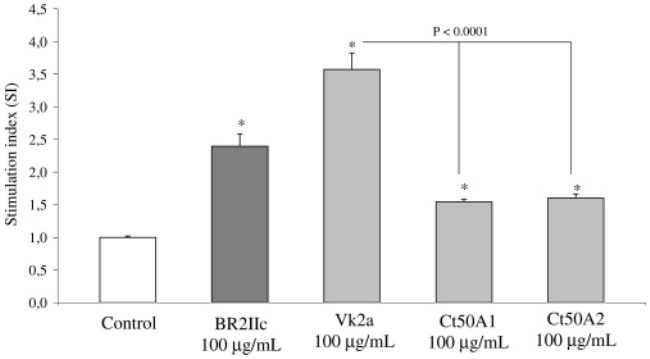

Figure 1. Comparison of B cell mitogenic activities of the polysaccharides Vk2a, Ct50A1, and Ct50A2. The cells (2 millions of cells/well, in a 96 well plate) isolated from spleens from C3H/HeJ mice were cultured with samples, water (unstimulated control) or bupleuran 2IIc (BR2IIc, positive control) for 3 days, and the proliferative responses were assessed by the AlamarBlueTM reduction assay 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 the Experimental Section. Each value is presented as mean ±S.D. Asterisks indicate significant (*$P < 0.001$) compared to unstimulated control.

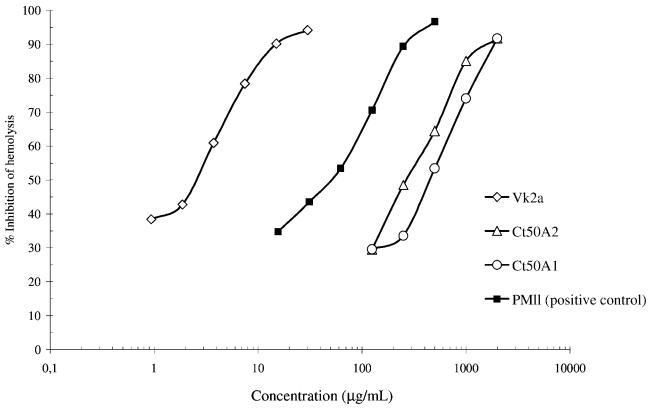

Figure 2. Complement fixation activity, expressed as % inhibition of lysis of sensitized sheep erythrocytes, of the polysaccharides Vk2a, Ct50A1 and Ct50A2. A complement fixing polysaccharide (PMII) from *Plantago major* L. was used as positive control.

subjected to pectinase (*Aspergillus niger*, Sigma) treatment, and the highest molecular weight fractions formed (Ct1a-PN and Ct2a-PN) were isolated by gel filtration on a Bio-gel P-30 column. Comparing the native high molecular weight fractions (Ct1a and Ct2a) with the high molecular weight pectinase-treated

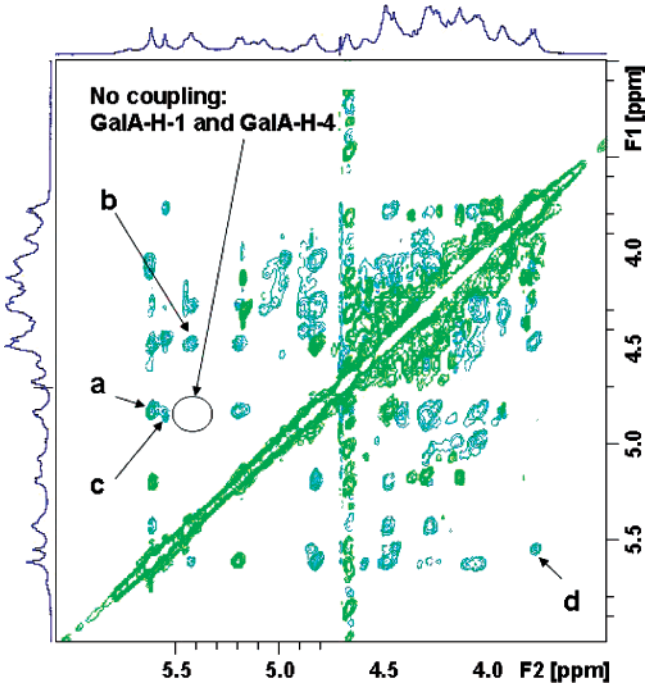

Figure 3. ROESY spectrum of Ct2a. Positive and negative cross-peaks are not distinguished. Peaks a−d (all negative) are identified in Table 2.

Table 2. Interresidue Cross-Peaks Identified in the ROESY Spectrum (Figure 3) of Ct2a[a]

cross-peak label (ppm)	connected protons	
a (5.61, 4.82)	H-1 α-Rha*p* (group 1)	H-4 α-GalA
b (5.41, 4.49)	H-1 α-GalA	H-2 α-Rha*p*
c (5.56, 4.82)	H-1 α-Rha*p* (group 2)	H-4 α-GalA
d (5.56, 3.78)	H-1 α-Rha*p* (group 2)	H-4 α-Rha*p*

[a] The group 1 units are the 2-/2,4-linked Rha residues, and the group 2 units are terminally linked Rha residues.

fractions (Ct1a-PN and Ct2a-PN), a slight increase in the Rha/GalA ratio was observed only for Ct1a-PN and not for Ct2a-PN (Table 1). There was, however, observed a decrease in the molecular weights of both polymers after pectinase treatment from ~105 kDa (Ct1a) to 60 ± 4 kDa (Ct1a-PN) and from 640 ± 100 kDa (Ct2a) to 338 ± 4 kDa (Ct2a-PN), which suggests that small regions of homogalacturonan may be present in both Ct polysaccharides. Even though the Rha/GalA ratio in Ct2a-PN did not increase nor did the total GalA content decrease compared to Ct2a, the relative amounts of the different GalA linkages were altered after pectinase digestion. A decrease in 4-linked GalA with a concomitant increase in terminally linked GalA was observed, which indicated that digestion of some 4-linked GalA had occurred. No coupling between GalA-H-1 and GalA-H-4 was observable by 2D-NMR ROESY of Ct2a (Figure 3), which indicates a negligible/small amount of homogalacturonan regions present in Ct2a. The ROESY spectrum also gave cross-peaks due to couplings between GalA-H-4 and Rha-H-1, and GalA-H-1 and Rha-H-2 (Figure 3 and Table 2). The results indicate an alternating structure of Rha and GalA units in Ct2a, namely, α-GalA-(1→2)-α-Rha*p*-(1→4)-α-GalA-(1→2)-α-Rha*p*, which is a typical backbone for pectin "hairy-regions".[28] A weak coupling was observed between GalA-H-4 and terminal Rha-H-1 (cross-peak c, Figure 3) and between Rha-H-4 and Rha-H-1 (group 2) (cross-peak d, Figure 3). These results suggest that the rhamnogalacturonan backbone is terminated by Rha linked top position 4 of GalA and that some of the Rha units also are terminally linked to position 4 of 1,2-linked Rha. The most evident differences between Ct1a and Ct1a-PN was the small amount of Ara subunits in Ct1a-PN compared to Ct1a (11.2% versus 22.8%), which may be due to breakdown of arabinofuranosyl residues during sample handling in the pectinase treatment process. Within each fraction the monosaccharide compositions of the lower molecular weight polysaccharides were very similar. They all contained smaller amounts of Ara and GalA and larger amounts of GlcA, Rha and Gal (Table 3), indicating not only differences in molecular weights compared to the higher molecular weight fractions, but also differences in the finer structures.

Table 3. Linkage Analyses of the Glycosyl Residues of Vk2a, Ct50A1, and Ct50A2, the High Molecular Weight Polymers of Ct50A1 and Ct50A2 (Ct1a and Ct2a), and the High Molecular Weight Fraction from Pectinase Treatment of Ct50A2 (Ct2a-PN)[a]

glycosyl residue	deduced linkage	mol. %					
		Vk2a	Ct50A1	Ct1a	Ct50A2	Ct2a	Ct2a-PN[b]
Ara	terminal *f*	8.0	4.2	13.6	2.9	2.8	1.5
	terminal *p*	trace					
	1,3	0.2	0.7	4.4	0.3	1.8	1.0
	1,5	11.2	1.8	4.8	1.2	0.3	0.1
	1,3,5	11.8					
Rha	terminal	0.3	13.3	11.9	11.2	11.8	4.5
	1,2	5.2	2.3	1.5	10.7	15.6	10.3
	1,2,4	5.5	1.1	0.3	7.1	9.0	12.5
Gal	terminal	3.2	2.3	2.4	2.3	2.7	8.4
	1,4	15.7	1.7	0.9	9.2	10.0	9.0
	1,3	0.6	4.7	3.2	1.8	1.6	2.1
	1,6	1.7	29.0	28.3	7.3	3.9	1.8
	1,3,6	2.9	16.1	9.8	4.9	4.5	3.7
	1,3,4	0.4					
Fuc	terminal	trace	trace		trace		
Man[c]		0.3	trace		trace		
Xyl	terminal *p*	0.7					
GalA	terminal		0.7	0.8	2.6	3.1	18.9
	1,4	25.2	7.7	7.2	22.3	20.5	7.6
	1,2,4	0.6					
	1,3,4	0.8			5.9	4.1	6.0
GlcA	terminal	trace	0.3	0.3	3.9	4.7	9.0
	1,4		12.4	7.1	4.1	2.5	0.6
	1,2,4				0.3	0.5	0.1
Glc	terminal	1.2	trace	0.2	0.6	0.4	0.6
	1,4	4.6	1.2	2.1	1.2	0.5	0.5

[a] The partially methylated alditol acetates were analyzed by GC-MS. [b] Ct2a-PN is the high molecular weight fraction isolated from pectinase treated Ct50A2. [c] Linkage not deduced.

The complement fixation activities of Ct1a, Ct2a, and Ct2a-PN were all in the same range as the fractions they originated from (Figure 4). However, the complement fixation dose−response curve for Ct1a-PN seemed to be steeper than those of Ct50A1 and Ct1a. The increased activity may be due to removal of arabinofuranose residues.[24,29−31] The lower molecular weight fractions (Ct1b−d and Ct2b−c, Table 1) showed modest or no activity on the complement system (data not shown) and the fractions were not investigated further. These results indicate that the complement fixation activities were mainly assigned to the high molecular weight polymers of Ct50A1 and Ct50A2. For polysaccharides in vaccines (bacterial capsular polysaccharides) it has been shown that immunogenicity is related to their molecular size and that in general the larger the molecule the more immunogenic it is likely to be. However, there is no absolute size above which a substance is immunogenic.[32,33] A correlation between immunogenicity and molecular weight of plant polysaccharides may therefore also exist. Conformational epitopes are suggested to be better expressed in larger saccharides, and larger molecules may also contain more than one recognition site which may increase the immunogenicity of the substance.[34]

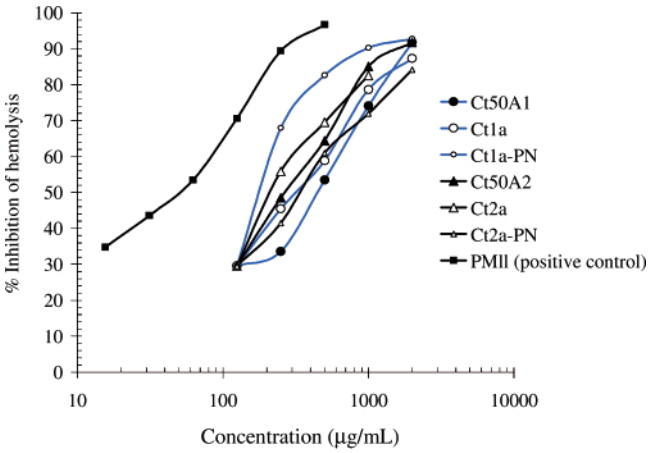

Figure 4. Complement fixation activity, expressed as % inhibition of lysis of sensitized sheep erythrocytes, of the polysaccharides Ct50A1, Ct50A2 and the high molecular weight fractions obtained after gelfiltration of native Ct50A1 (Ct1a) and Ct50A2 (Ct2a) or pectinase treated Ct50A1 (Ct1a-PN) and Ct50A2 (Ct2a-PN). A complement fixing polysaccharide (PMII) from *Plantago major* L. was used as positive control.

Table 4. Amino Acid Compositions, AG-II and Protein Content of the Polysaccharides Vk2a, Ct50A1, and Ct50A2

	Vk2a	Ct50A1	Ct50A2
AG-II (%, w/w)[a]	2%	23% (70%)[b]	12% (17%)[b]
amino acid (rel. mol %)			
serine	17.1	4.7	7.7
alanine	14.9	3.1	4.8
glutamic acid	13.4	2.9	10.7
glycine	12.1	3.7	9.0
aspartic acid	8.6	1.7	9.4
hydroxy proline	7.4	23.8	17.1
valine	4.7	5.4	3.9
threonine	4.7	8.1	8.6
leucine	3.7	20.2	10.3
proline	3.1	22.5	11.8
isoleucine	2.2	1.5	1.4
lysine	2.1	0.6	0.9
arginine	2.0	0.8	3
phenylalanine	1.5	0.3	0.3
methionine	0.9	0.4	0.1
tyrosine	0.9	0.1	0.2
histidine	0.8	0.3	0.9
protein content % (w/w)	0.7	3.1	0.9

[a] AG-II was quantified by single radial diffusion with Yariv reagent with acacia gum as a reference. [b] Values in parentheses represent the highest molecular weight polymers (Ct1a and Ct2a) which were obtained by gel filtration (Bio-gel P-30) of the respective fractions. Ct1a constituted 25% of Ct50A1, whereas Ct2a constituted 75% of Ct50A2.

Since AG-II linked to RG-I structures has been associated with potent biological activities,[4,35] we investigated the AG-II characteristics of the polymers. Quantification of arabinogalactan type II structures was performed by the single radial gel diffusion method with the β-glucosyl-Yariv reagent, and a standard curve made with acacia gum. Vk2a contained only 2% AG-II-type structures, while Ct50A1 and Ct50A2 contained 23% and 12% respectively. This is in accordance with the linkage analysis, which showed the amounts of 3,6-linked Gal to be lowest in Vk2a (2.9%) followed by Ct50A2 (4.9%) and finally Ct50A1 (16.1%) (Table 3). An accumulation of AG-II type structures was observed in the high molecular weight Ct-fractions (Table 4). The lower molecular weight fractions of Ct50A1 showed minor interaction with the Yariv reagent. It does, however, not appear that larger amounts/numbers of AG-II binding sites increase the potency of the polysaccharides in the complement fixation or mitogenic assays, since polysaccharide Vk2a with the most potent activity showed the smallest number of binding sites/amount of AG-II. All fractions showed low protein content (Table 4) which is typical of AG-II type structures associated with proteins.[36] Heterogeneity was observed in the amino acid compositions (Table 4), which indicates differences in the structures related to the arabinogalactans of type II. Hyp, Pro, Ala, and Ser are all typical components of the protein part which is generally linked via Hyp to Ara or Gal residues of AG-II structures.[37] The relatively larger amounts of AG-II in Ct50A1 and Ct50A2 compared to Vk2a are therefore in accordance with the corresponding larger amounts of Hyp in the Ct-polysaccharide fractions (Table 4). Heterogeneity in protein components is well-known,[37] but how this is related to structural differences in the arrangements of the carbohydrates associated with the proteins has not been established. To compare and investigate the carbohydrates associated with the AG-II structures of the polysaccharides, Vk2a, Ct1a-PN, and Ct2a-PN were subjected to enzymatic degradation by an exo-β-D-(1,3)-galactanase-enriched fraction from Driselase. The oligosaccharides liberated were separated by HPAEC (high performance anion exchange chromatography) and detected by PAD (pulsed amperometric detection). The fine structures of the major oligosaccharides released were determined by combining the results from component sugar-, glycosyl linkage-, and MALDI- and ES-qoToF-MS(/MS) analyses (Table 5).

Five major oligosaccharide fractions were liberated from Ct1a-PN (Table 5), in addition to Gal monomers which arise from exo-β-D-(1,3)-galactanase (present in Driselase) digestion of unbranched 3-linked galactan backbones. The *m*/*z* ions observed in fractions 1, 2, and 3 correspond to the $[M+Na]^+$ for oligosaccharides composed of di-, tri-, and tetra-6-linked galactan chains. In fraction 4, 6-linked galactosyl tetramers with one Ara*f* branched to one of the Gal in position 3 were identified. The MS data support the presence of a mixture of several branched structures. The highest *m*/*z* ions present in fraction 5 correspond to $[M+Na]^+$ for a tetramer $Hex_2HexAdHex$. The collision induced dissociation (CID) product ion data obtained from the $Hex_2HexAdHex$ ion suggest that the species is linear with the dHex residue in the reducing terminal position in the sequence Hex-Hex-HexA-dHex. Combining these results with those of component sugar and linkage analyses the proposed oligosaccharide structure is Gal*p*-(1→6)-Gal*p*-(1→4)-GalA-(1→2)-Rha. The 6-linked di- to tetra-galactosyl chains isolated after exo-β-D-(1,3)-galactanase digestion of Ct1a-PN by HPAEC correspond to mono-, di-, and tri-galactans attached to a 3-linked galactopyranosyl backbone via position 6. The formation of Gal*f* (Table 5) is the result of the equilibrium between the furanose and pyranose forms of Gal when present as the reducing terminal residue. No Gal*f* was detected in the original samples. Some of the 6-linked galactan side chains are branched with arabinose residues in position 3. Whether these are the starting point for larger arabinan chains in the native polymer is not known at this point, as only monomers were attached to the oligomers investigated.

The combined results of the sugar compositions, linkage-, and MALDI- and ES-qoToF-MS(/MS) analyses indicated that the Ct2a-PN and Vk2a polysaccharides were subjected to a more elaborate enzymatic or chemical degradation than Ct1a-PN (Table 5). An investigation of the exo-β-D-(1,3)-galactanase-enriched preparation indicated additional minor rhamnogalacturonidase and endo-α-L-(1,5)-arabinofuranosidase activities

Table 5. Composition of the Major Oligosaccharides from HPAEC Fractions Obtained from Vk2a, Ct1a-PN, and Ct2a-PN by Digestion with an Exo-β-D-(1,3)-galactanase Enriched Fraction Observed in the MALDI and ES-qoToF Mass Spectra[a]

HPAEC fraction	*m/z* native oligosaccharides	*m/z* peracetylated oligosaccharides	proposed structures
			Ct1a-PN
#1	365.1		Gal*p*-(1→6)-Gal*f*/*p*
#2	527.1		Gal*p*-(1→6)-Gal*p*-(1→6)-Gal*f*/*p*
#3	689.2	1277.3	Gal*p*-(1→6)-Gal*p*-(1→6)-Gal*p*-(1→6)-Gal*f*/*p*
#4	821.2	1493.4	Gal*p*-(1→6)-Gal*p*-(1→6)-Gal*p*-(1→6)-Gal*f*/*p* CID product ion data indicate a mixture of several branched structures. Ara*f* is linked to either of the internal Gal residues in position 3.
#5	687.2	1191.3	Gal*p*-(1→6)-Gal*p*-(1→4)-GalA-(1→2)-Rha
			Ct2a-PN
#1	365.2	701.2	Gal*p*-(1→6)-Gal*f*/*p*
#2	527.2	989.4	Gal*p*-(1→6)-Gal*p*-(1→6)-Gal*f*/*p*
#3		637.2 tandem data	GalA-(1→2)-Rha and Rha-(1→4)-GalA (Na salt)
#4	749.0[b]		GlcA-(1→4-GlcA-(1→4)-GlcA-(1→4)-GlcA (Na salt)
#5		925.4	Gal*p*(1→4)Rha(1→4)GalA*f*/*p*(Na salt)
#6		1393.5 tandem data	Rha-(1→4)-GalA-(1→2)-Rha-(1→4)-GalA with t-Gal*p* linked to either of the Rha in position 4. Both isomers present.
#7		843.3*	Gal*p*-(1→4)-Rha-(1→4)-GalA
#8		1365.5 tandem data	GalA-(1→2)-Rha-(1→4)-GalA-(1→2)-Rha-(1→4)-GalA
			Vk2a
#1		701.2	Gal*p*-(1→6)-Gal*f*/*p*
#2	365.1	701.2	Gal*p*-(1→6)-Gal*f*/*p*
	527.2		Gal*p*-(1→6)-Gal*p*-(1→6)-Gal*f*/*p*
	689.2		Gal*p*-(1→6)-Gal*p*-(1→6)-Gal*p*-(1→6)-Gal*f*/*p*
#3	833.3		Ara*f*-(1→5)-Ara*f*-(1→5)-Ara*f*-(1→5)-Ara*f*-(1→5)-Ara*f*-(1→5)-Ara*f*
#4	685.3 tandem data	1105.4 tandem data	Rha-(1→4)-GalA-(1→2)-Rha-(1→4)-GalA
#5		1393.5	Gal*p*-(1→4)-Rha-(1→4)-GalA-(1→2)-Rha-(1→4)-GalA
#6		1393.5	Rha-(1→4)-GalA-(1→2)-Rha-(1→4)-GalA with t-Gal*p*branched to either of Rha in position 4
#7	1007.3 tandem data	1595.5 tandem data	Rha-(1→4)-GalA-(1→2)-Rha-(1→4)-GalA-(1→2)-Rha-(1→4)-GalA
#8		815.3[b]	GalA-(1→2)-Rha-(1→4)-GalA
		843.3[b]	GalA-(1→2)-Rha with t-Gal*p* branched to Rha in position 4.

[a] The proposed structures and linkages of the major oligomers are determined by using linkage and monosaccharide analyses combined with the mass spectrometric analyses. [b] The ion is either a chemical or enzymatic dehydration product or a mass spectrometric B type fragment ion. All ions are formed with Na^+ as the charge bearing species (M^+Na^+).

(Kiyohara, unpublished data). These activities explain the presence of oligomers liberated containing rhamnogalacturonan regions and/or arabinofuranosyl chains. Structural differences may account for Vk2a and Ct2a-PN being more susceptible to degradation by the additional enzyme activities than Ct1a-PN. The fact that the Gal content in Ct1a-PN was relatively high compared to that in Vk2a and Ct2a-PN, 50% versus approximately 25%, may be of importance.

Eight major oligosaccharide fractions in addition to Gal monomers were liberated from Ct2a-PN (Table 5) after digestion with the exo-β-D-(1,3)-galactanase-enriched fraction. From Ct2a-PN, 6-linked galactosyl chains composed of up to three units were liberated. However the majority of the galactan-oligomers liberated could be assigned to the disaccharide Gal*p*-(1→6)-Gal*f*/*p* which corresponds to single Gal units attached to position 6 of a 3-linked galactosyl backbone. Fraction 5 contained ions at *m*/*z* 397, 573, and 749 assigned to dehydro-$HexA_2$, dehydro-$HexA_3$, and dehydro-$HexA_4$, which according to the monosaccharide composition and linkage analyses consist of chains of 4-linked GlcA residues with up to 4 units. Tetramers of 4-linked homoglucuronic acids were liberated from Ct2a-PN. Ct50A1 and Ct1a contained larger amounts of 4-linked GlcA than Ct2a, but the release of similar fragments was not observed after enzymatic digestion of Ct1a-PN, which may indicate that their location may differ. The importance of the structure and the location of the homoglucuronan chains in relation to the function of the Ct polysaccharides are not known. Although GlcA units are common in pectins, their locations have been reported mainly as terminal residues. The majority of the glucuronans described in the literature are extracellular β-D-(1→4)-glucuronans originating from the *Sinorhizobium* (*Rhizobium*) *meliloti* bacterium,[38] moulds[39] or as cell wall polysaccharides in certain green algae.[40] The remaining peaks eluted contained variable amounts of rhamnogalacturonan (RG-I) oligomers with substitution of certain Rha residues with one Gal unit. The highest *m*/*z* ion was observed at 1393.5, which corresponds to $[M+Na]^+$ for a pentamer, and was assigned Rha-(1→4)-GalA-(1→2)-Rha-

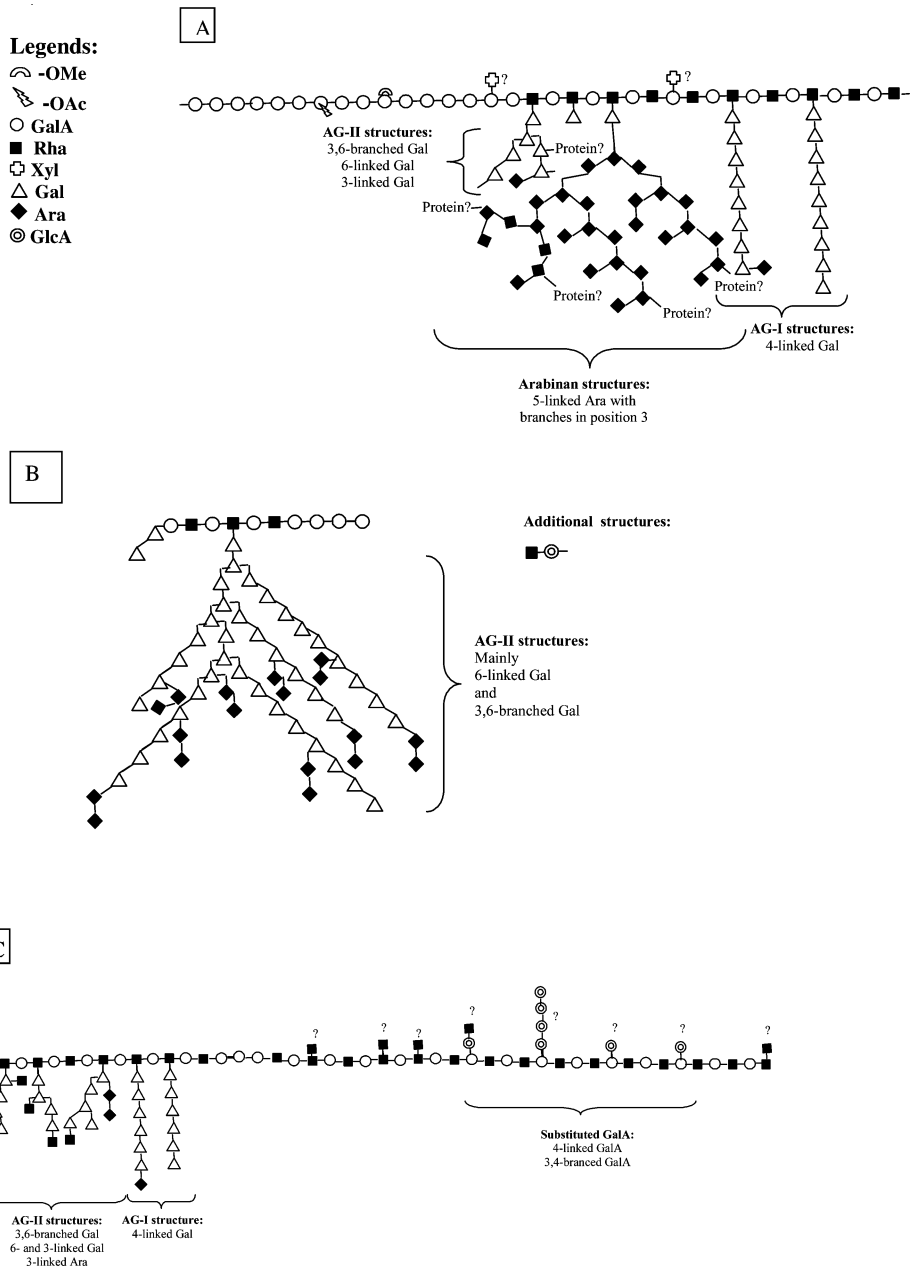

Figure 5. Proposed average structures of the polysaccharides Vk2a (A), Ct1a (B) and Ct2a (C).

(1→4)-GalA with a t-Gal*p* unit linked to either of the Rha in position 4. Both isomers were identified by tandem MS.

After exo-β-D-(1,3)-galactanase digestion of Vk2a, di- and tetra 6-linked galactosyl oligomers were identified (Table 5). In addition, 5-linked Ara*f* oligomers up to 6 units were obtained (Tables 5). Oligosaccharides mainly composed of alternating 2-linked Rha and 4-linked GalA residues with intermittent substitution of the Rha residues in position 4 by one Gal unit were also identified. In fraction 8 small amounts of the *m*/*z* ions 569.3, 701.3, and 833.3 corresponding to $[M+Na]^+$ for arabinan oligomers of 4, 5, and 6 units were identified in addition to oligomers of branched rhamnogalacturonan regions (Table 5). The arabinans may originally be attached to the RG-I region via the Gal residue. The rhamnogalacturonan region of Vk2a seems to be more susceptible to degradation than that of the Ct-polysaccharides, which may be due to different arrangements of the structures present in the respective polysaccharides. A proposed overall structure of the 3 polysaccharides studied can be found in Figure 5.

Investigations of immunomodulating polysaccharides from *Bupleurum falcatum*, *Atractylodes lancea* and *Angelica acutiloba* suggest that galactan moieties such as β-D-(1→6)-galactosyl chains attached to a β-D-(1→3)-galactan backbone, are responsible for expression of the activities observed.[5,6,8,41,42] Chain lengths of more than 4 units have been proposed as important features for the expression of potent bioactivity.[8,41] Enzymatic digestion with the exo-β-D-(1,3)-galactanase-enriched fraction from Driselase indicated that all the polysaccharides investigated contained a β-D-(1→3)-linked galactan core bearing side chains, and that some side chains consist of Gal and/or β-D-(1→6)-galactosyl chains. Both Ct1a-PN and Vk2a contained 6-linked galactans with chain lengths up to 3 units. For the 3-linked galactan core of Ct2a-PN, branching in position 6 by one single Gal unit dominated. From the results obtained, the high bioactivity of Vk2a compared to that of the Ct-polysaccharides does not seem to be correlated to differences in the lengths of the 6-linked galactan side chains of the β-D-(1→3)-linked galactan core. Other structural features may therefore be

important for expression of the immunomodulating activities of Vk2a and the Ct-polysaccharides.

Although important structural features for biological activities have mainly been assigned to AG-II type structures, there are studies which indicate that 4-linked galactan chains (AG-I) attached to a rhamnogalacturonan core also may be involved.[43−47] Vk2a contains a large amount of 4-linked Gal compared to the Ct-polysaccharides (Table 3), indicating that AG-I type and not AG-II type structures dominate in Vk2a. The AG-I structures may therefore be involved in the biological activities observed.

4. Conclusion

The three pectic arabinogalactan fractions Vk2a, Ct50A1, and Ct50A2 all show complement fixation and B cell mitogenic activities; however, Vk2a was the most potent polysaccharide. The polysaccharides differ substantially in molecular weight (Table 1), with Vk2a the largest molecule. The highest molecular weight polymers of Ct50A1 and Ct50A2 fractions retained complement fixation activities, whereas the lower molecular weight fractions showed modest activities. The differences in molecular weight of the polymers may be important for the differences in expressions of the biological activities reported. However, as seen in Figure 5, the overall structures of the 3 polysaccharides are also different. The amount of AG-II type structures reacting with the β-glucosyl-Yariv reagent was not important for the potencies of the activities observed, as Vk2a contained the lowest amount of the AG-II type. The amino acid compositions of Vk2a, Ct50A1, and Ct50A2 differed, which may indicate differences in the structures related to the arabinogalactan type II of the respective polysaccharides. Differences in the chains lengths of the 6-linked galactans attached to the β-D-(1→3)-linked galactan core could not be related to the differences in the expression of the immunomodulating activities. One of the most evident differences between Vk2a and the Ct polysaccharides was the minor amount of homogalacturonan region in Ct compared to Vk2a. However, in a previous study, removal of the homogalacturonan region from Vk2a only slightly reduced the potency of Vk2a;[27] therefore, the lack or low amount of a smooth region does not seem to be a possible explanation for the lower activities of the Ct-polysaccharides. Finally, the Ct polysaccharides contain substantial amounts of GlcA compared to Vk2a (Tables 1 and 3), and sequences of 4-linked homoglucuronans were identified (Table 5). When compared to the rigid homogalacturonans, homoglucuronans are semirigid polymers,[48] and may contribute to the overall structure for the Ct-polysaccharides being less favorable than that of Vk2a for expression of the immunomodulating activities.

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