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An immunomodulating pectic polymer from *Glinus oppositifolius*

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Abstract

An immunomodulating pectic polymer, GOA1, obtained from the aerial parts of the Malian medicinal plant *Glinus oppositifolius* (L.) Aug. DC. (Aizoaceae) has previously been reported to consist of arabinogalactans type I and II, probably linked to a rhamnogalacturonan backbone. To further elucidate the structure of the polymer GOA1, enzymatic degradation studies and weak acid hydrolysis were performed. Five different glycosidases were used, *endo*-α-D-(1 → 4)-polygalacturonase, *exo*-α-L-arabinofuranosidase, *endo*-α-L-(1 → 5)-arabinanase, *endo*-β-D-(1 → 4)-galactanase and *exo*-β-D-galactosidase. It appears that GOA1 may contain a structural moiety consisting of a 1,3-linked galactopyranosyl (Gal*p*) main chain with 1,6-linked Gal*p* side chains attached to position 6 of the main chain. The 1,6-linked Gal*p* side chain may be branched in position 3 with arabinofuranosyl (Ara*f*) side chains. A 1,4-linked Gal*p* backbone which might carry side chains or glycosyl units attached to position 3 is also a structural element in the polymer. We further show that GOA1 induce proliferation of B cells and the secretion of IL-1β by macrophages, in addition to a marked increase of mRNA for IFN-γ in NK-cells. To elucidate structure–activity relations the native polymer and the digested fractions were tested for complement fixing activity and intestinal immune stimulating activity. The partial removal of Ara*f* residues after enzymatic degradations did not affect the bioactivities, while the acid hydrolysed fraction showed reduced complement fixing activity. A decrease in Ara*f* units, 1,3,6-linked Gal*p* units and a partial hydrolysed rhamnogalacturonan backbone, in addition to a reduction in molecular weight are factors that might have contributed to reduced bioactivity.

Keywords: *Glinus oppositifolius*; Aizoaceae; Immunomodulating; Pectic polysaccharides; Arabinogalactans

1. Introduction

A range of polysaccharides isolated from higher plants are considered to be biological response modifiers and have been reported to enhance various immune responses, like complement activation, proliferation of lymphocytes and stimulation of macrophages. The use of immunomodulators derived from plants is attractive because it allows for enhanced host-derived mechanisms while not involving the use of microorganism-specific therapeutics such as antibiotics.

In the class of biologically active carbohydrates derived from the cell wall of medicinal plants, pectic polysaccharides with arabinogalactan side chains or pure arabinogalactans are frequently found (Yamada and Kiyohara, 1999; Paulsen, 2002). Plants with well established

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immune-enhancing properties, such as *Plantago major* (Samuelsen et al., 1998), *Echinacea purpurea*, *Larix occidentalis* (Kim et al., 2002), *Atractylodes lancea* (Taguchi et al., 2004), *Vernonia kotschyana* (Nergard et al., 2005) and *Phleum pretense* (Brecker et al., 2005) all contain significant amounts of arabinogalactans. Because of their potent immunomodulating activities, and being unique dietary fibers, arabinogalactans are receiving increased attention.

The arabinogalactans can be subdivided into two main structural types. Arabinogalactan type I (AG-I) are arabinosyl-substituted derivatives of linear 1,4-linked β-D-galactopyranosyl units. Arabinosyl and galactosyl units can be linked via position 3 along the main chain. AG-I is found as ramified regions of rhamnogalacturonan backbones in pectin complexes. The second group, arabinogalactan type II (AG-II), comprise a highly branched polysaccharide with ramified chains of 1,3-linked and 1,6-linked β-D-Gal*p* units, the former predominantly in the interior and the latter in the exterior chains. The arabinosyl units might be attached through position 3 of the 1,6-linked galactosyl side chains. In addition to arabinose and galactose, AG-II contains a range of other monosaccharides, including glucuronic acid and its 4-*O*-methyl ether (Huisman et al., 2001). AG-II may occur in a complex family of proteoglycans known as arabinogalactan-proteins (AGP) (Willats et al., 2001) or be covalently linked to a rhamnogalacturonan type I backbone (Vincken et al., 2003). It is mainly AG-II that has been reported as immunomodulating activators (Yamada and Kiyohara, 1999).

Glinus oppositifolius (L.) Aug. DC. (Aizoaceae) is used in Malian traditional medicine in the treatment of various types of ailments related to the immune response, like joint pains, inflammations, malaria, fever, and wounds. Immunomodulating pectic polysaccharides, denominated GOA1 and GOA2, have previously been isolated from a hot water extract of the aerial parts of *G. oppositifolius*, and their structures partially characterized. They were shown to exhibit potent dose-dependent complement fixing activities, and chemotactic properties towards human macrophages, T cells and NK cells (Inngjerdingen et al., 2005).

Galactose and arabinose comprised 70 mol% of the monosaccharide constituents in the pectic polymer GOA1, and glycosidic linkages characteristic for both AG-I and AG-II regions were found. It was suggested that the AG-I and AG-II structural elements were attached to a rhamnogalacturonan backbone present in GOA1, probably through position 4 of the rhamnose units. The weight average molecular weight was determined to be 70 kDa by SEC/MALLS (Inngjerdingen et al., 2005). In the present study further structural characterisation and immunomodulating properties of GOA1 is elucidated, as well as a discussion of structure–activity relations.

2. Results and discussion

2.1. Structural characterization of GOA1

The isolation and partial structural characterization of the bioactive pectic polymer GOA1 isolated from the aerial parts of *G. oppositifolius* have been previously described (Inngjerdingen et al., 2005). The monosaccharide composition and the different glycosidic linkages of the polymer are shown in Tables 1 and 2, respectively.

According to linkage analysis the pectic polymer GOA1 contains relatively branched galactosyl regions, 36.9% of the galactopyranosyl (Gal*p*) units are either 1,3,4-, 1,3,6- or 1,3,4,6-linked. Arabinofuranosides (Ara*f*) are found mainly as terminally linked units (41.1%), or as linear chains of 1,3- or 1,5-linked units (Table 2). The high amount of terminal Ara*f* residues does not correlate with the low amount of branched Ara*f* residues, only 13.2%. This indicates that some terminal Ara*f* residues could be linked to other branched glycosyl residues, most probably to the Gal*p* units. The presence of both 1,4-linked and 1,3- and 1,6-linked Gal*p* units indicates that both AG-I and AG-II regions are present in the polymer. The arabinogalactan regions are most

Table 1
Characterization of the pectic polymer GOA1, and the fractions obtained after enzymatic degradation and weak acid hydrolysis of GOA1

	GOA1	GOA1-1	GOA1-2	GOA1-3	GOA1-AH
Composition					
Total carbohydrate content (%, w/w)	57.6	56	78.5	66.6	n.d.
Protein content (%, w/w)	10	n.d.	n.d.	n.d.	n.d.
Phenolic esters (% w/w)	0.2	n.d.	n.d.	n.d.	n.d.
Monosaccharide composition[a]					
Ara	26.5	12.1	13.6	14.3	1.3
Rha	6.3	5.2	6.4	7	6.5
Xyl	3.3	2.3	2.6	2.3	2.9
Man	4.1	4.3	4.9	5	5.6
Gal	39	42.5	47.2	47.1	48.8
Glc	2.9	6.1	4.1	3.5	3.5
GlcA	2.2	2.3	2.2	–	3.5
4-*O*-Me-GlcA	3.4	3.4	3.5	2.6	5.1
GalA	12.5	21.9	15.4	18.2	22.7

n.d., not determined (due to lack of material).
[a] mol% of total carbohydrate content.

Table 2
The linkages of the pectic polymer GOA1, and the fractions obtained after enzymatic degradation and weak acid hydrolysis of GOA1 (mol%)

	Type of linkage	GOA1		GOA1-1		GOA1-2		GOA1-3		GOA1-AH	
		mol%	%	mol%	%	mol%	%	mol%	%	mol%	%
Ara	T*f*	10.9	41.1	6.6	54.5	7.1	52.2	8.0	55.9	0.7	53.8
	1,2*f*	1.1	4.2	0.9	7.4	0.6	4.4	1.0	7.0	Traces	
	1,3*f*	3.4	12.8	2.5	20.7	2.6	19.1	3.2	22.4	0.3	23.7
	1,5	7.6	28.7	2.1	17.4	3.3	24.3	2.1	14.7	0.3	23.7
	1,3,5	2.0	7.5	Traces		Traces		Traces		Traces	
	1,2,5	1.5	5.7	Traces		Traces		Traces		Traces	
		26.5		12.1		13.6		14.3		1.3	
Rha	T	2.7	42.9	3.0	57.7	2.9	45.3	2.6	37.1	4.3	66.2
	1,2	1.6	25.4	1.0	19.2	1.5	23.4	1.9	27.1	1.0	15.4
	1,2,4	2.0	31.7	1.2	23.1	2.0	31.3	2.5	35.7	1.2	18.5
		6.3		5.2		6.4		7.0		6.5	
Xyl	T	2.1	63.6	1.2	52.2	1.8	69.2	0.4	17.4	1.0	34.5
	1,4	1.2	36.4	1.1	47.8	0.8	30.8	1.9	82.6	1.9	65.5
		3.3		2.3		2.6		2.3		2.9	
Man	1,2	1.7	41.5	1.5	34.9	2.2	44.9	1.8	36.0	Traces	
	1,2,3	2.4	58.5	2.8	65.1	2.7	55.1	3.2	64.0	Traces	
		4.1		4.3		4.9		5.0		5.6	
Glc	T					2.0	48.8			0.8	22.9
	1,4	1.8	62.1	4.2	68.9	2.1	51.2	2.9	82.9	2.7	77.1
	1,4,6	1.1	37.9	1.9	31.1			0.6	17.1	Traces	
		2.9		6.1		4.1		3.5		3.5	
Gal	T*f*	0.5	1.3	0.5	1.2	0.5	1.0	0.4	0.8	Traces	
	T*p*	5.4	13.8	7.1	16.7	8.9	18.9	7.8	16.6	7.9	16.2
	1,3*p*	5.0	12.8	5.6	13.2	5.7	12.1	5.8	12.3	6.8	13.9
	1,4*p*	5.7	14.6	5.7	13.4	5.7	12.1	3.9	8.3	5.3	10.9
	1,6	8.0	20.5	11.4	26.8	12.8	27.1	15.1	32.1	17.6	36.1
	1,3,4	0.6	1.5	0.4	0.9	0.5	1.0	1.1	2.3	Traces	
	1,3,6	11.4	29.2	9.4	22.1	10.8	22.9	11.0	23.4	11.2	23.0
	1,3,4,6	2.4	6.2	2.4	5.6	2.3	4.9	2.0	4.2	Traces	
		39		42.5		47.2		47.1		48.8	
GlcA/4-*O*-Me-GlcA	T	4.5	80.4	4.2	73.7		1.8	69.2	6.5	75.6	
	1,4	1.1	19.6	1.5	26.3	2.9	50.9	0.8	30.8	2.1	24.4
		5.6		5.7		5.7		2.6		8.6	
GalA	1,4	12.1	96.8	20.9	95.4	14.9	96.8	17.2	94.5	22.7	~100
	1,3,4	0.4	3.2	1.0	4.6	0.5	3.2	1.0	5.5	Traces	
		12.5		21.9		15.4		18.2		22.7	

Determined by reduction, methylation and GC–MS.

probably attached to a pectic rhamnogalacturonan type I backbone identified by the presence of 1,2- and 1,2,4-linked rhamnose (Rha), and 1,4-linked galacturonic acid (GalA), as the structural regions co-eluted. The presence of terminally linked units of Rha in addition to 1,4-linked glucuronic acid (GlcA) suggests terminal disaccharides consisting of T-Rha linked to GlcA in position 4, as has previously been described to be present in pectic polymers containing AG-II (Strasser and Amadò, 2001). There may be variations in the structural features along the polymer, and whether the molecule consists of repeating structures remains to be investigated. Due to the presence of relatively small amounts of rhamnogalacturonan backbone monomers compared to side chain monomers we suggest the neutral side chains to be quite complex.

2.1.1. Degree of esterification

Methyl-esterification is widespread in native pectins, contrary to acetyl-esterification, which is usually low (Levigne et al., 2002). The degree of methylation (DM) in GOA1 was determined to be 32.7%, telling that on the average every four unit of galacturonic acid in the polymer is methyl-esterified. Regarding the degree of acetylation (DAc), defined as the percentage of monosaccharide residues esterified with one acetyl group, was determined to be 4.3%.

2.1.2. Determination of total phenolic content

The primary cell walls of dicotyledons typically contain 0–3% of phenolic esters (O'Neill and York, 2003). The quantitative determinations of total phenolic compounds

in the crude extract GO and in the purified GOA1 after ion exchange chromatography were analysed. The fractions were shown to contain 0.5% and 0.2% phenolic compounds, respectively, expressed as ferulic acid equivalents.

2.1.3. NMR

The HSQC spectrum revealing the ^{13}C–^{1}H shift-correlations confirmed the polymers complex structure with many signals in the anomeric regions (spectrum not shown). The signals in the spectrum were assigned as completely as possible according to sugar composition and literature data (Colquhoun et al., 1990; Dong and Fang, 2001; Bushneva et al., 2002; Tischer et al., 2002; Brecker et al., 2005; Ha et al., 2005). The $^{1}H/^{13}C$ cross-peaks in the HSQC spectrum at δ 5.07/110.5 ppm and at δ 5.10/110.5 ppm were assigned to the anomeric carbons and protons of terminal Ara*f*. Correlation peaks at δ 5.14/110 ppm, δ 5.20/109.5 ppm and δ 5.25/111.5 ppm might arise from 1,3, 1,5 and 1,3,5-linked α-Ara*f*, respectively. The signals revealing cross-peaks at δ 4.46/106 ppm, δ 4.60/107 ppm, δ 4.62/107 ppm and δ 4.69/106.5 ppm probably belong to the anomeric carbons and protons of β-Gal*p*. The pectin component of GOA1 is manifested by the $^{1}H/^{13}C$ cross-peaks at δ 4.81/103.5 ppm attributed to α-D-Gal*p*A, and the cross-peaks at δ 5.03/102.5 ppm assigned to α-L-Rha*p*. The presence of CH_3-6 of the rhamnosyl units was shown by a $^{1}H/^{13}C$ correlation peak at δ 1.25/19.5 ppm. The ^{13}C region between δ 62–87 ppm was heavily crowded and many correlation peaks between ^{1}H and ^{13}C could be detected, but it was not possible to assign the spectrum completely because of the complexity and signal overlap. The COSY and TOCSY spectra (spectra not shown) show at least 4 and 6 coupling networks that could be attributed to the α-Ara*f* and the β-Gal*p* residues, respectively, reflecting the complexity of the polysaccharide.

2.1.4. Enzymatic degradation

In order to further elucidate the structure and structure–activity relationships of GOA1 enzymatic degradation of the pectic polymer was performed.

Treatment with *endo*-α-D-(1 → 4)-polygalacturonase after de-esterification of the polymer did not lead to any release of galacturonic residues from GOA1 as shown by HPAEC–PAD and the use of standards of $GalA_2$ and $GalA_3$ (data not shown). This may indicate that the polymer does not contain long homogalacturonan chains, or the amount of homogalacturonan is too low and similarly the amount of possibly released galacturonan fragments. Recently, it has been postulated that galacturonans are side chains attached to the RG-I backbone (Vincken et al., 2003), and if this is true for GOA1, other side chains masking the galacturonan might be another explanation why the polygalacturoanse did not lead to any release of galacturonic acid units. This has previously been reported by Huisman et al. (2001) where CDTA-extractable pectins from soybean meal could not be degraded by polygalacturonase. They suggested the network of side chains might be too complex or too dense to be penetrated by the applied enzyme.

The use of side chain degrading enzymes was further applied to GOA1 in order to obtain more information on the polymer. Treatment with *exo*-α-L-arabinofuranosidase led to the release of arabinose as determined by HPAEC–PAD (data not shown), in accord with the low Ara content in the enzyme resistant fraction denominated GOA1-1. The monosaccharide composition of the enzyme resistant product GOA1-1 and the sugar linkages as determined by methylation are given in Tables 1 and 2, respectively. As can be seen from Table 2 the treatment with *exo*-α-L-arabinofuranosidase led to a reduction of terminal-, 1,5-, 1,3,5- and 1,2,5-linked Ara*f* units. This might result from the release of single arabinose residues or small oligoarabinans linked to position 2 and 3 in a 1,5-linked backbone, and a concomitant breakdown of the backbone by the enzyme. A slight increase in 1,6-linked Gal*p* and a simultaneous decrease in 1,3,6-branched Gal*p* units indicate that at least some of the arabinosyl residues are linked to position 3 of 1,6-linked Gal*p*. The detection of a slight increase in terminally linked galactose indicates that arabinosyl residues or side chains may be linked to the non-reducing end of the 1,3- or 1,6-linked galactosyl chains.

The combined enzymatic digestion of GOA1 with *exo*-α-L-arabinofuranosidase and *endo*-α-L-(1 → 5)-arabinanase was used in order to degrade the arabinosyl units in the polymer further. The α-(1 → 5)-arabinanase is specific for splitting of α-1,5 linkages between arabinose residues. As the action of the *endo*-enzyme can be strongly hindered by the presence of arabinofuranosyl side chains the arabinofuranosidase was used in combination with the *endo*-arabinanase (Beldman et al., 1993; Huisman et al., 1999). The treatment did not lead to the release of any oligosaccharides, but monomers only, as determined by HPAEC–PAD (data not shown). The linkage pattern of the resistant fraction GOA1-2 is similar to that of the enzyme resistant fraction GOA1-1 after degradation with only *exo*-α-L-arabinofuranosidase (Table 2). The combined treatment did not lead to any further decrease of the arabinosyl units in the polymer compared to *exo*-α-L-arabinofuranosidase working on its own. The fact that the *endo*-arabinanase do not seem to completely degrade the 1,5-linked arabinan has also been reported by Huisman et al. (1999), and they postulated that a release of larger arabinan oligomers may have taken place, but that the arabinofuranosidase present in the incubation mixture is able to degrade these oligomers. Øbro et al. (2004) suggested that the arabinosyl side chains may carry decorations that hinder the degradation of the arabinan backbone with *endo*-arabinanase. These decorations might be galactosyl side chains with a degree of polymerization above four.

endo-β-D-(1 → 4)-Galactanase is specific for β-1,4 linkages between galactose residues, and in combination with *exo*-α-L-arabinofuranosidase it led to the release of arabinose monomers, and monosaccharide- and disaccharide units of galactose (data not shown). The linkage analysis

of GOA1-3 shows that the enzymatic treatment led to a reduction of 1,4-Gal*p* residues as compared to the native polymer GOA1 (Table 2), indicating the presence of linear 1,4-linked galactans, and hence arabinogalactan type I, in the polymer. Short β-1,4-linked galactan chains have been shown to occur in red beet RG-I as well, most of them directly attached to the rhamnogalacturonan backbone (Strasser and Amadò, 2001). The incomplete degradation of 1,4-linkages might suggest that the polymer does not consist of homogenous β-1,4-galactans. Terminal Ara*f* can be linked to galactose through position 3 of the 1,4-linked backbone, and somehow the arabinofuranosidase not being able to cleave this linkage. Furthermore the galactan chains may be inaccessible to the enzyme for steric reasons. When the enzymatic resistant fraction GOA1-3 was further digested with *exo*-β-D-galactosidase, the peak for di-Gal as shown by HPAEC–PAD was reduced, indicating that the oligomers formed by *endo*-galactanase are probably further degraded by the *exo*-galactosidase activity.

As can be seen from Table 2, during the different enzymatic degradation steps the ratio of the backbone sugars 1,2-linked rhamnose and 1,2,4-linked rhamnose remained nearly unchanged in all samples, indicating the sugars attached to the O-4-position of rhamnose were not removed.

2.1.5. Weak acid hydrolysis

It appears that the arabinosyl units in the side chains of the polymeric structure in GOA1 could not be degraded completely by the purified enzymes tested here. The use of weak acid hydrolysis, which is less specific, was therefore undertaken. Glycoside linkages of arabinosyl residues in furanose form are generally more easily hydrolysed under mild acidic conditions compared to residues in pyranose form. The arabinose content in GOA1 decreased drastically (Table 1) after the treatment with oxalic acid, yielding fraction GOA1-AH.

The position of Ara*f* in GOA1 can be determined by linkage analysis of the remaining polymer after acid hydrolysis, GOA1-AH, and comparison with the linkage distribution of the original polymer. According to linkage analysis (Table 2) there has been a relative decrease of 1,3- and 1,3,6-linked Gal*p* residues compared to 1,6-linked Gal*p* after the acidic hydrolysis. These are the same findings as for the enzymatic treated fractions, and further supports that arabinosyl units are attached to position 3 of 1,6-linked galactose. A slight increase in terminally linked galactose again indicates that some arabinosyl residues are attached to the non-reducing end of the 1,6-linked galactan chains. The mild acid seems in addition to have hydrolysed parts of the rhamnogalacturonan backbone in GOA1-AH, giving hydrolysed fragments with backbones terminating with rhamnose. This is suggested by the slight decrease seen in the amount of backbone rhamnose residues in GOA1-AH compared to the native polymer GOA1, and a concomitant increase in terminal rhamnose (Table 2). This has also been reported after acid hydrolysis of an acidic arabinogalactan (A-I) isolated from the roots of *Angelica acutiloba* Kitagawa (Kiyohara and Yamada, 1989).

2.2. Immunomodulating properties

2.2.1. Complement fixing activity

Several naturally occurring polysaccharides from higher plants, algae, fungi and lichens are known to exhibit complement modulating activities, and due the important physiological role of the complement system it is an interesting target for drug development. The majority of the plant polysaccharides reported to modulate the complement are pectic polysaccharides containing 1,3,6-branched and/or 6-linked galactosyl residues, and the effects are thought to be due to a combination of the rhamnogalacturonan core and the neutral sugar side chains. There is however exceptions, as arabinans and other heteroglycans, e.g. glucuronoarabinoxylan have also shown to affect the complement system (Yamada and Kiyohara, 1999). β-(1 → 3)-D-Glucans, ubiquitous glucose polymers found in the cell walls of plants, lichens, fungi, and bacteria, have been also been shown to exhibit effects on complement system. A β-1,3-linked D-glucan isolated from the fungus *Grifola frondosa* has been shown to activate the alternative complement pathway (Suzuki et al., 1989), a branched β-(1 → 3)-D-glucan isolated from the lichen *Thamnolia vermicularis* is reported to reduce complement-induced hemolysis (Olafsdottir et al., 2003), while sulphated polysaccharides, fucoidans, from brown algae are complement inhibitors (Tissot et al., 2003).

The native pectic polymer GOA1 has previously shown to be highly active in the complement fixing assay, possessing the same level of activity as the positive control PMII, a pectin fraction from the leaves of *Plantago major* L. (Inngjerdingen et al., 2005). In order to study structure–activity relations a comparison of the complement fixing activities of GOA1 and the fractions obtained after enzymatic degradations and weak acid hydrolysis was performed.

The enzyme resistant fractions GOA1-1, GOA1-2 and GOA1-3 retained potent dose-dependent activities (Fig. 1a), and no significant change in bioactivity was observed compared to the native polymer GOA1. Thus, the arabinosyl units in the outer part of the molecule do not seem to play an important role for the bioactivity. However, after weak acid hydrolysis there was a 10-fold decrease in bioactivity (Fig. 1b). The loss of arabinofuranosyl units in combination with a decrease in 1,3,6-linked Gal*p* may have contributed to the reduced activity. A partially hydrolysed rhamnogalacturonan backbone in GOA1-AH, splitting the polymer into smaller fragments, is another factor that may affect the bioactivity. The possible presence of several binding sites for a complement factor on GOA1 may influence the effect on the complement system. The reduced side chain complexity in GOA1-AH might therefore have a negative effect on activity. Another

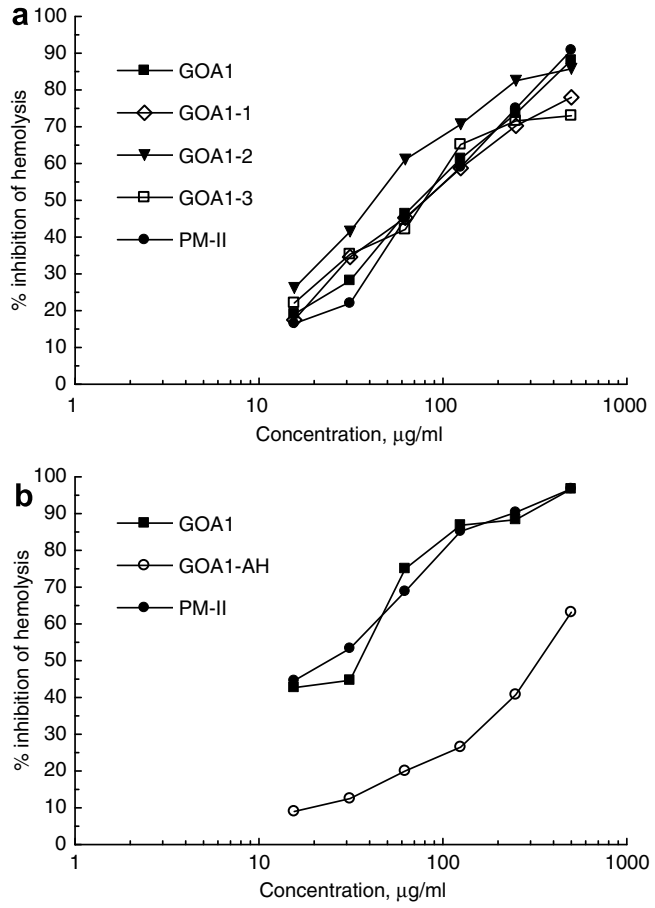

Fig. 1. Concentration-dependent effect on the inhibition of hemolysis. (a) The native pectic polymer, GOA1, and the fractions GOA1-1, GOA1-2 and GOA1-3 obtained after enzymatic degradations; (b) GOA1, and the fraction GOA1-AH obtained after weak acid hydrolysis. PMII from *Plantago major* L. was used as a positive control.

possibility is whether the bioactivity was decreased as a result of a reduction in molecular weight after weak acid hydrolysis. Kweon et al. (2003) have reported the size and the degree of branching of β-glucans to be important for complement fixing activity, the largest molecule expressing the most potent activity. The same has been seen for chitosan particles, complement activation increasing in a chain length dependent manner (Suzuki et al., 2003). Whether this is also valid for pectic polysaccharides is not fully investigated, although accumulated results indicate that size may be of importance (Yamada and Kiyohara, 1999).

On the basis of these findings a high degree of branch points in pectic polymer giving a three dimensional structure of exposed, flexible side chains may be important for the complement fixing activity observed. The importance of the rhamnogalacturonan core for complement fixing pectic polymers has also previously been reported (Yamada and Kiyohara, 1999). Neutral oligosaccharides originating from side chains of pectins from *A. acutiloba* showed reduced activity, and it was proposed that 1,6-linked galacto-oligosaccharides attached to a 1,3-galactan backbone again being linked to a rhamnogalacturonan core was the minimal essential structure for activity (Kiyohara et al., 1989). And, oligosaccharide fractions obtained after enzymatic treatment of an arabinogalactan pectic polymer isolated from the roots of *Vernonina kotschyana* lacked bioactivity, probably due to simplified structures comprising simple side chain oligosaccharides with only galactose in the branching point in addition to individual arabinofuranosyl, galactosyl and arabinogalactan oligomers (Nergard et al., 2005).

2.2.2. Measurement of lymphocyte proliferation

The relationship between complement modulating activities and other biological activities have not been fully elucidated, but several of the pectins with potent effects on the complement system have also shown potent mitogenic activities. We tested whether GOA1 could induce proliferation of B cells, as studies having addressed the mitogenic potential of pectic plant-derived polysaccharides have concluded that they might be potent B-cell mitogens (Chintalwar et al., 1999; Sakurai et al., 1999; Han et al., 2003; Nergard et al., 2005). B cells were purified from rat spleen cell suspensions by positive selection using rat anti-IgG-coated Dynabeads. The purity of the cells was consistently above 90% CD19 positive. The cells were labeled with the fluorescent dye CFSE, which allows for the quantifications of cell divisions within a cell population by flow cytometry. Upon cell division, the daughter cells will contain half the amount of CFSE compared with the mother cell. Thus fluorescence of the cell population will decrease over time in an actively dividing cell population. Cells were treated with either medium or increasing concentrations of GOA1 for 5 days. B cells showed a high proliferative response towards the positive control LPS (Fig. 2). The

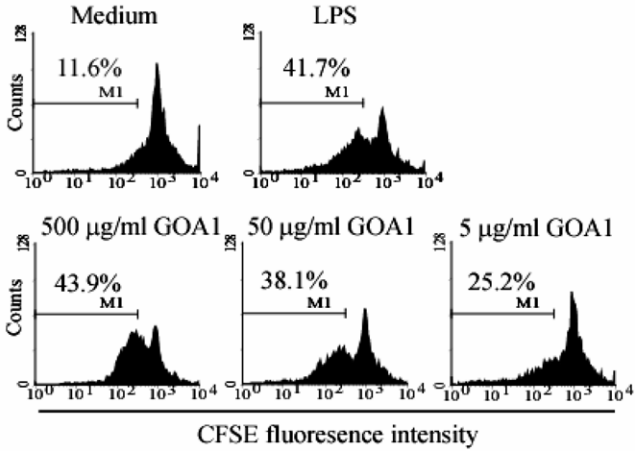

Fig. 2. GOA1 induces proliferation of B cells. Purified B cells were prestained with the fluorescent dye CFSE and incubated with either medium alone, 250 ng/ml LPS or increasing concentrations of GOA1 for 5 days in flat bottom 96-wells. Proliferative activity of the cells was analysed by flow cytometry. Decreasing amounts of CFSE fluorescence in samples was indicative of proliferation, and the numbers indicate the percentage of cells undergoing proliferation. Panels shown are representative of three independent experiments.

percentage of cells that had undergone divisions, were based on a gate in the histogram set to define cells that had divided at least once. We found a dose-dependent induction of B-cell proliferation in response to GOA1 (Fig. 2). As proliferation of the cells was induced by GOA1 extract alone, we propose that pectins might function as thymus-independent mitogens in a similar fashion as LPS, as reported by for other acidic polysaccharides isolated from plant extracts (Chintalwar et al., 1999; Sakurai et al., 1999; Han et al., 2003). However, we have used quite high concentrations of pectic extracts in order to induce proliferation, which might indicate that additional activation through the B-cell receptor may be required for optimal proliferation.

Recently Han et al. (2003) reported that CD19 of the B-cell co-receptor was directly involved in the induction of selective B-cell proliferation by an acidic plant polysaccharide, AK, isolated from the roots of *Acanthopanax koreanum*. It was also suggested that AK interacts with Toll-like receptor 4 (TLR4), as the use of TLR4-deficient B cells resulted in a decreased response to the AK polysaccharide. TLR4 have also been shown to be required for activation of B lymphocytes by a neutral polysaccharide fraction, rich in glucose and fructose, isolated from the fungus *Ganoderma lucidum* (Shao et al., 2004). It will be interesting in the future to determine whether GOA1 acts through the same receptors.

2.2.3. Measurements of cytokine mRNA of macrophages and NK cells

We next examined whether GOA1 might induce secretion of proinflammatory cytokines from cells of the innate immune system. Cytokine secretion by macrophages and NK cells can influence the outcome of wound healing or early infections. The rat macrophage cell line R2-MΦ or the rat NK cell line RNK-16 (resembling the phenotype of resting NK cells) were stimulated with GOA1 for 3 days, after which RNA was harvested from the cells and subjected to RT-PCR. There was a marked upregulation (~100-fold) in mRNA for the proinflammatory cytokine IL-1β in R2-MΦ after GOA1 stimulation (Fig. 3a). We also observed a weak increase in mRNA for the chemokine CXCL10 (IP-10, inflammatory protein 10) (Fig. 3a). No differences were observed for CCL4, IL-6, IL-12, or TNF-α. IL-1β is mainly produced by blood monocytes, and mediates the generation acute phase proteins important in the early host reactions to infections. Thus GOA1 might be able to boost the early responses by macrophages, by the secretion of IL-1β. However, we have not studied the actual secretion of cytokine protein, which may have a different pattern than mRNA generation.

Macrophages have for a long time been considered as one of the main targets for polysaccharides. A polysaccharide isolated from *Chlorella pyrenoidosa* containing 30% arabinose and 25% galactose has shown to be a potent activator of human monocytes increasing mRNA levels of IL-1β and TNF-α (Pugh et al., 2001). Sonoda et al. (1998) reported a polysaccharide isolated from the root of *Panax ginseng* inducing the production of significant amounts of IL-1 with subsequent IL-8 production by human monocytes. The group suggested that the regulation of IL-8 production was primarily at the transcriptional level. Increased levels of IL-1β and TNF-α has also been reported after treatment of macrophage cultures with a polysaccharide, consisting of a branched β-1,3-linked D-glucan, from the fungus *G. lucidum* (Wang et al., 1997).

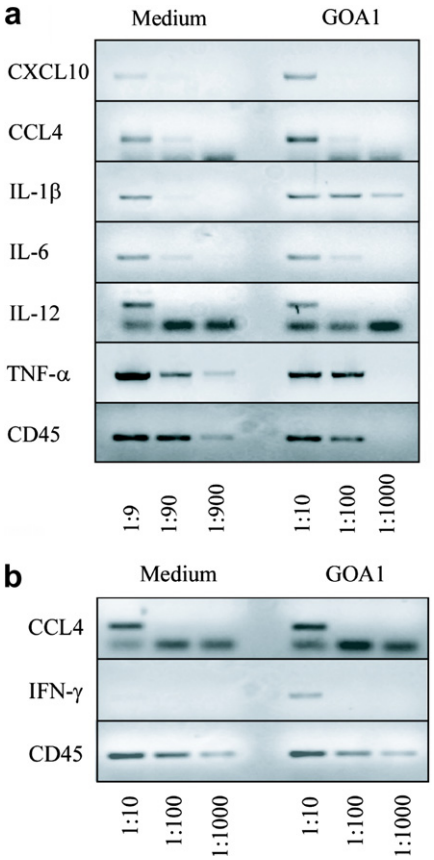

Fig. 3. Analysis of cytokine mRNA upregulation by GOA1. The rat macrophage cell line R2-MO (a) or the rat NK cell line RNK-16 (b), were stimulated with either medium alone or 100 μg/ml GOA1 for three days. mRNA was harvested and subjected to semiquantitative RT-PCR as described in Section 4. PCR was performed with primers specific for various cytokines or chemokines. A 10-fold dilution series of cDNA was analyzed, as indicated below the panels, with CD45 used as a control of equal amounts of cDNA between the samples. PCR products were fractionated by agarose gels followed by ethidium bromide staining.

In NK cells we found a marked increase of mRNA for IFN-γ after GOA1 treatment (Fig. 3b). No mRNA was detected for CCL4. IFN-γ is the principal cytokine released by activated NK cells, normally in response to IL-12 released from macrophages or dendritic cells, and is important for initiating a proper T cell response. GOA1 has previously been shown to induce modest chemotaxis of IL-2-activated human NK cells (Inngjerdingen et al., 2005), and we have also determined that is has no effect on the cytolytic behavior of NK cells (data not shown). Collectively, these results might indicate that GOA1

principally plays a role for cytokine production in NK cells. However, the detection of released cytokines remains to be examined.

2.2.4. Measurement of intestinal immune system modulating activity

Active substances from herbal medicines taken orally may be absorbed from the intestine, or there is a possibility that they may express their clinical effects through the intestinal immune system. Peyer's patches are unique islands of lymphoid tissue in gut-associated lymphoid tissue (GALT), and is the primary site for antigen processing in the intestine. Lymphocytes are rapidly drained from the patches, and migrate through the mesenteric lymph nodes into the thoracic duct to the systemic circulation, where they participate in the local mucosal and systemic immune systems. Molecules that regulate the intestinal immune system may therefore have potential as new immunomodulators of both the mucosal and systemic immune systems (Taguchi et al., 2004). Sakurai et al. (1996) detected a pharmacologically active pectin from *Bupleurum falcatum* in the T cell area of follicles of Peyer's patches after its oral administration in mice, and Kiyohara et al. (2002) has suggested the proliferation of bone marrow cells by polysaccharides isolated from the Japanese traditional herbal prescription Juzen-Taiho-To (TJ-48) to be caused by stimulation of the production of cytokines such as IL-6 and GM-CSF from immune cells in Peyer's patches.

Peyer's patch cells from mice were cultured in the presence of GOA1 and the different enzyme resistant fractions at concentrations of 10 and 100 μg/ml for 5 days *in vitro*. The resulting cell-free supernatant (conditioned medium) of Peyer's patch cells was used to stimulate bone marrow cells. As can be seen from Fig. 4 the polymer fractions showed potent and statistical significant activities on proliferation of bone marrow cells at concentrations of 100 μg/ml. The intestinal immune stimulating activities of the enzyme treated fractions were at the same level as that of the native polymer, which was also seen for the complement fixing activity.

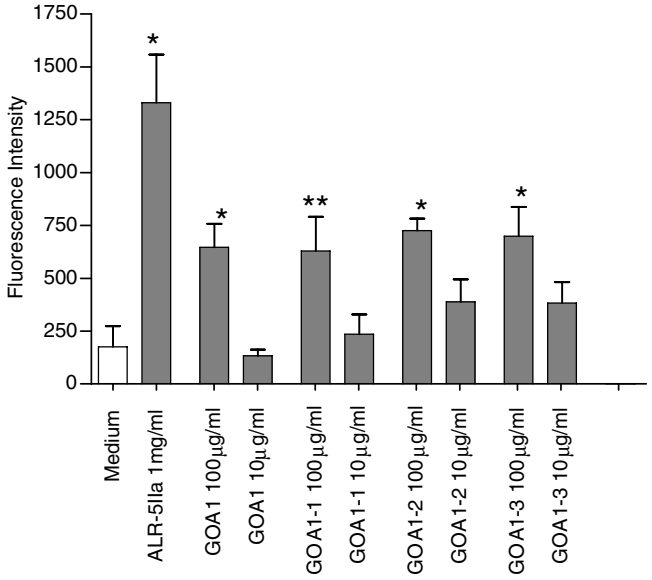

Fig. 4. Intestinal immune system modulating activity of the native pectic polymer GOA1 and the fractions GOA1-1, GOA1-2 and GOA1-3 obtained after different enzymatic treatments. ALR-5IIa (1 mg/ml) from the rhizome of *Atractylodes lancea* DC. was used as positive control. Asterisks indicate significancy ($^{*}P < 0.005$, $^{**}P < 0.05$).

Previously an arabinogalactan type II polymer, ALR-5IIa-1-1, isolated from the rhizomes of *Atractylodes lancea* has been shown to act through the intestinal immune system to increase proliferation of bone marrow cells. It was suggested that it was the arabino-3,6-galactan moiety in the non-reducing terminal side of the polymer that mainly contributed to the expression of activity (Taguchi et al., 2004). The length of the side chains in arabinogalactans have been proposed to be important for the bioactivity, as arabinogalactans with short side chains from *Larix occidentalis* were not active (Yu et al., 2001).

3. Conclusion

In conclusion, the water extract of *G. oppositifolius* contains a pectic polymer, GOA1, consisting of highly branched arabinogalactan regions. These arabinogalactans are most probably linked to a rhamnogalacturonan backbone being a minor component of GOA1. The immunomodulating properties of the pectic polymer GOA1 were demonstrated by its potent complement fixing activity, immune stimulating activity, the ability to proliferate of B cells, and the secretion of IL-1β by macrophages, in addition to a marked increase of mRNA for IFN-γ in NK-cells. Structure–activity relations were studied by comparing the complement fixing activities and the intestinal immune stimulating activities of the native polymer and the fractions obtained by enzymatic degradations and weak acid hydrolysis. These results showed the importance of the branched moieties of the arabinogalactans, as a decrease in 1,3,6-linked galactosyl and a loss of arabinosyl units led to a significant lower complement fixing activity. So far there is no clear information available on the structural requirements of polysaccharides in order to have an optimal inducing effect on different immune cells, but the three-dimensional structure of exposed, flexible side chains in the polymer, in addition to molecular weight seems to be of importance.

4. Experimental

4.1. Materials

The aerial parts of *G. oppositifolius* (L.) Aug. DC. (Aizoaceae) were collected in Diré, Mali, in April 1996, and identified by the Department of Traditional Medicine (DMT), Bamako, Mali. A voucher specimen is deposited in the

herbarium at DMT. The pectic polysaccharide fraction GOA1 was isolated from the aerial parts of *G. oppositifolius* by hot water extraction and ion exchange chromatography as previously described (Inngjerdingen et al., 2005).

endo-α-D-(1 → 4)-Polygalacturonase [poly(1,4-α-D-galacturonide) glycanohydrolase; EC 3.2.1.15] was purified from Pectinase (*Aspergillus niger*, Sigma) according to the method of Thibault and Mercier (1977). *exo*-α-L-arabinofuranosidase, *endo*-α-L-(1 → 5)-arabinanase, and *exo*-β-D-galactosidase (Megazyme, Australia) from *Aspergillus niger* were all purified by FPLC according to the modified procedure of Lerouge et al. (1993). *endo*-β-D-(1 → 4)-galactanase from *Aspergillus niger* was purchased from Megazyme, Australia.

4.2. Structural characterization

4.2.1. General methods

The polysaccharide samples (1 mg) were methanolysed and converted into trimethylsilyl glycoside derivatives and analysed by capillary gas chromatography on a Carlo Erba 6000 Vega Series 2 chromatograph with an ICU 600 programmer (Chambers and Clamp, 1971; Barsett et al., 1992), in order to determine the composition and content of carbohydrate in the different polymer fractions. Mannitol as internal standard was included throughout the total procedure.

Linkage elucidation was performed by methylation studies. Prior to methylation the uronic acids of the polymer fractions were reduced to primary alcohols on the polymer level. To distinguish between reduced uronic acids and the corresponding neutral sugars in GC–MS, sodium bordeuteride was used. Carboxyl esters were first reduced with sodium borodeuteride in imidazole buffer to generate 6,6-dideuteriosugars. The free uronic acids were activated with a carbodiimide and reduced with sodium borodeuteride (Kim and Carpita, 1992). After reduction of the polymers methylation was carried out after the method of Ciucanu and Kerek (1984). The methylation procedure was followed by GC–MS analysis of the derived partially methylated alditol acetates using a Fisons GC 8065 (Fisons Instruments) on a SPB-1 fused silica capillary column (30 m × 0.20 mm i.d.) with film thickness 0.20 μm. E.I. mass spectra were obtained using a Hewlett–Packard Mass Selective Detector 5970 with a Hewlett–Packard GC. The injector temperature was 250 °C, the detector temperature 300 °C and the column temperature 80 °C when injected, then increased with 30 °C/min to 170 °C, followed by 0.5 °C/min to 200 °C and then 30 °C/min to 300 °C. Data were processed with Fisons Masslab software. The compound at each peak was characterized by an interpretation of the characteristic mass spectra and retention times in relation to standard sugar derivatives. Effective carbon-response factors were applied for quantification (Sweet et al., 1975).

The quantitative determination of total phenols was performed with the Fiolin–Ciocalteu reagent (Singleton and Rossi, 1965) with ferulic acid as standard reference. Four hundred microliters of lyophilized samples dissolved in water (three replicates) was added the same amount of Fiolin–Ciocalteu's phenol reagent (1:2 in water, Merck/Kebo), mixed and left for 3 min at room temperature. Four hundred microliters of 1 M Na_2CO_3 was added, the tubes were mixed and allowed to stand for 1 h. The absorbance was measured at 750 nm in a Helios Epsilon Spectrophotmeter (Thermo Spectronic). The standard curve was plotted using ferulic acid. The total phenolic content was determined as ferulic acid equivalents (FA/sample) × 100%.

4.2.2. Degree of esterification

The degree of esterification with methanol and/or acetic acid was determined by saponification of GOA1 followed by methanol and acetic acid separation by HPLC on a C18 column and quantification by refractometry (Levigne et al., 2002). The polymer (5 mg) was saponified by suspension in 0.5 ml of a solution containing 10 mM $CuSO_4$ and 25 mM isopropanol as internal standard; 0.5 ml of 1 M NaOH was added to achieve saponification. The reaction mixture was left at 4 °C for 1 h before centrifugation at room temperature. The supernatant was purified by the aid of a 1 ml syringe equipped with a Maxi-clean IC–H device (Alltech) prior to injection on a C18 endcapped column (Superspher 100RP-18, 250 × 4 mm). Elution was carried out with 4 mM H_2SO_4 at 0.7 ml/min, with refractometric detection. The alkaline saponification has to be performed in a heterogeneous system and copper ions being able to insolubilize pectins were used for this purpose.

4.2.3. NMR

NMR spectra were obtained from a solution of GOA1 (10 mg) in about 1 ml D_2O (99.9 atom% D, Sigma–Aldrich). The spectra were acquired on a Bruker Avance DRX 500 MHz spectrometer with a 5 mm TXI ($^1H/^{13}C$, ^{15}N–^2H) Triple Resonance Inverse probe equipped with Z-gradient coil. The data were processed using Bruker XWIN–NMR (version 3.5) software. NMR assignments were obtained from examination of ^1H, ^1H water-suppressed, gs-COSY, preset-TOCSY and gs-HSQC NMR spectral data. Chemical shifts, determined at 353 K are reported relative to internal 3-trimethylsilylpropionic acid sodium salt d4 (98% D, Fluorochem Ltd., UK) at 0.00 ppm, which placed the D*H*O/H_2O residual water signal in the ^1H spectrum at 4.215 ppm.

4.2.4. Enzymatic degradations

4.2.4.1. Degradation with endo-α-D-(1 → 4)-polygalacturonase. One hundred microliters GOA1 (1 mg/ml) was de-esterified by treatment with 100 μl 0.1 M NaOH before digestion with *endo*-α-D-(1 → 4)-polygalacturonase in 1 M acetate buffer (pH 4.2) at 37 °C overnight. The resulting enzyme hydrolysate was analysed by HPAEC–PAD (High performance anion-exchange chromatography equipped with pulsed amperometric detector) performed on a Dio-

nex Bio-CL system (Dionex Inc.) using a Carbopak PA-1 column (9 × 250 MM, Dionex Inc.), and 0.1 M NaOH (solvent A) and 1 M NaOAc (solvent B) at flow rate of 2.5 ml/min. The elution program was: 0% of solvent B (1 min), 0 → 100% of solvent B (1 → 100 min). $GalA_2$ and $GalA_3$ were used as standards (10 μl, 1 mg/ml).

4.2.4.2. Degradation with exo-α-L-arabinofuranosidase. GOA1 (2 mg) was digested with 10 μl *exo*-α-L-arabinofuranosidase (1576 nKatal/mg protein) in 2 ml of 50 mM acetate buffer (pH 4.2) at 37 °C for 3 days. The resulting enzyme hydrolysate was analysed by HPAEC–PAD as described above in order to determine the release of Ara*f* and oligosaccharides. The elution program was: 0% of solvent B (1 min), 0 → 60% of solvent B (1 → 48 min). The enzyme digest was dialyzed against distilled water in SpectraPor 6 dialysis tubes (MWCO 1000) for 2 days, and the non-dialysable parts lyophilized, giving the enzyme resistant fraction denominated GOA1-1.

4.2.4.3. Degradation with a combination of exo-α-L-arabinofuranosidase and endo-α-L-(1 → 5)-arabinanase. GOA1 (2 mg) was simultaneously digested with 10 μl *exo*-α-L-arabinofuranosidase (1576 nKatal/mg protein) and 10 μl *endo*-α-L-(1 → 5)-arabinanase (255 nKatal/mg protein) in 2 ml of 50 mM acetate buffer (pH 4.2) at 37 °C for 3 days. The resulting enzyme hydrolysate was analysed by HPAEC–PAD as described above in order to determine the release of Ara*f* and oligosaccharides. The elution program was: 0% of solvent B (1 min), 0 → 60% of solvent B (1 → 48 min). The enzyme digest was dialyzed against distilled water in SpectraPor 6 dialysis tubes (MWCO 1000) for 2 days, and the non-dialysable parts lyophilized, giving the enzyme resistant fraction denominated GOA1-2.

4.2.4.4. Sequential degradation with a combination of exo-α-L-arabinofuranosidase and endo-β-D-(1 → 4)-galactanase and exo-β-D-galactosidase. GOA1 (2 mg) was simultaneously digested with 10 μl *exo*-α-L-arabinofuranosidase (1576 nKatal/mg protein) and 5 μl *endo*-β-D-(1 → 4)-galactanase (403 U/ml) in 2 ml of 50 mM acetate buffer (pH 4.2) at 37 °C for 3 days. Fifty microliters of the digested sample was further digested with 5 μl *exo*-β-D-galactosidase (704 nKatal/mg protein) at 37 °C for 24 h. The resulting enzyme hydrolysate was analysed by HPAEC–PAD as described above in order to determine the release of mono- and oligosaccharides. The elution program was: 0% of solvent B (1 min), 0 → 60% of solvent B (1 → 48 min). The enzyme digest was dialyzed against distilled water in SpectraPor 6 dialysis tubes (MWCO 1000) for 2 days, and the non-dialysable parts lyophilized, giving the enzyme resistant fraction denominated GOA1-3.

4.2.5. Weak acid hydrolysis

Arabinose was hydrolysed off the native polymer GOA1 with 50 mM oxalic acid at 100 °C for 2 h, and the resulting hydrolysate was dialysed (MW cut-off 1000) and the non-dialysable parts lyophilized (Cartier et al., 1987), resulting in the hydrolysed fraction denominated GOA1-AH.

4.3. Bioassays

4.3.1. Complement fixing assay

The polymers effect on human complement was measured as previously described by complement consumption and degree of lysis of antibody sensitized sheep red blood cells by the residual complement using a pectic polysaccharide, PMII, from *Plantago major* L. as a positive control (Michaelsen et al., 2000). Inhibition of lysis induced by the test sample was calculated by the formula: $[(A_{control} - A_{test})/A_{control}] \times 100\%$. A dose response curve was constructed to calculate the concentration of test sample able to give 50% inhibition of lysis (ICH_{50}). Low ICH_{50} means high complement fixing activity. An influence of contaminating lipopolysaccharide (LPS) on complement activity was disregarded as a previous study indicated that the amount of LPS present in the polymer sample did not influence the activities observed (Inngjerdingen et al., 2005).

4.3.2. Mitogenic activity

Eight to 12-week-old rats of the PVG.7B strain (which possesses a 'non-immunogenic' CD45 allotype, $RT7^b$, but is otherwise interchangeable with the standard PVG strain $RT7^a$) have been maintained at the Institute of Basic Medical Sciences for more than 20 generations. Rats were maintained under conventional conditions and regularly screened for common pathogens. The animals were housed in compliance with guidelines set by the Experimental Animal Board under the Ministry of Agriculture of Norway.

4.3.2.1. Isolation of leukocyte subsets. Peripheral blood mononuclear cells were isolated from rat spleen cell suspensions by layering onto Lymphoprep (Nycomed, Norway) and spinning for 20 min at 1800 rpm. B cells were isolated by positive selection using sheep anti-rat IgG Dynabeads. Cells were harvested and incubated overnight in complete RPMI (cRPMI; RPMI 1640, 10% FCS, 5×10^{-5} M 2-ME, L-glutamine, and antibiotics) in order to induce release of the Dynabeads prior to functional analysis. The cells were more than 90% CD19 positive, as analysed by flow cytometry.

4.3.2.2. Proliferation assay. B cells were suspended in 2%FCS in PBS at 1×10^7 cells/ml. Cells were preheated for 1 min at 37 °C, then stained with 5 μM CFSE (Molecular Probes, OR) for 10 min at 37 °C. The cells were washed twice in PBS, and then resuspended at 2×10^6 cells/ml in cRPMI. One hundred microliters

(2×10^5 cells) were seeded into flat bottom 96-wells, and added 100 μl of medium or samples. The cells were harvested after 5 days, and analysed by flow cytometry.

4.3.3. Measurement of cytokine mRNA by RT-PCR

RNK-16 cells (a rat leukemic NK cell line) or R2-MΦ (a rat macrophage cell line), were suspended in cRPMI and stimulated overnight at 37 °C with 100 μg/ml of pectic extracts or medium alone. Total RNA was isolated with TriReagent (Sigma–Aldrich), and cDNA was generated with Moloney murine leukemia virus reverse transcriptase (Promega) according to manufacturers protocol. PCRs were performed on a GeneAmp PCR thermocycler (Applied Biosystems) using hot start for 3 min at 94 °C. *Dynazyme* polymerase (Finnzymes, Finland) was added at 80 °C. The following upper and lower primers, respectively, were used: *IL*-1β, 5′-TGAAAGCTCTCCACCTCAATGGAC-3′ and 5′-TGCAGCCATCTTTAGGAAGACACG-3′(Tm 58 °C, 40 cycles); *IL-6*, 5′-TCTGGAGTTCCGTTTCTACCTG G-3′ and 5′-CATAGCACACTAGGTTTGCCGAG-3′ (Tm 55 °C, 40 cycles); *TNF*-α, 5′-AGCACAGAAAGCA TGATCCGAG-3′ and 5′-CCTGGTATGAAGTGGCAAATCG-3′(Tm 55 °C, 39 cycles); *CXCL10*, 5′-AAGCACCATGAACCCAAGTG-3′ and 5′-TGCATGTCTAGGT TCCTGTG-3′(Tm 55 °C, 40 cycles); *CCL4*, 5′-ATGAA GCTCTGCGTGTCTGCCTTCT-3′ and 5′-TCAGTT CAACTCCAAGTCATTCACAT-3′(Tm 55 °C, 40 cycles); *IFN*-γ, 5′-GTTACTGCCAAGGCACACTCATTGAAA GCC-3′ and 5′-TCAGCACCGACTCCTTT TCCGC TTCCTTAGGC-3′(Tm 53 °C, 40 cycles); *IL-12*, 5′-CCGATGCCCCTGGAGAAAC-3′ and 5′-CCTTCTT GTGGAGCAGCAG-3′(Tm 55 °C, 40 cycles); *CD45*, 5′-CGGGGTTGTTCTGTGCTCTGTTC-3′ and 5′-CTTTG CTGTCTTCCTGGGCTTTGT-3′(Tm 67 °C, 30 cycles). PCR products were resolved by agarose gel electrophoresis (1% Tris-borate-EDTA), and visualized by ethidium bromide staining.

4.3.4. Measurement of intestinal immune system modulating activity

Specific-pathogen-free C3H/HeJ female mice were purchased from SLC (Shizuoka, Japan) and used at 6–8 weeks of age. The mice were maintained under specific pathogen-free conditions and given free access to standard laboratory chow (CE-2), CLEA, Inc., Japan) and water. The procedure from the Prime Minister's Office of Japan (No. 6 of March 27, 1980) for the care and use of laboratory animals was followed. The experiments were conducted in accordance with the Guidelines for Animal use and Experimentation of the Kitasato Institute, Tokyo.

The intestinal immune system modulating activity was measured as proliferation of bone marrow cells as stimulated by the conditioned medium of Peyer's patch cells (Hong et al., 1998). Briefly, C3H/HeJ mice were sacrificed by cervical dislocation and their small intestines were exposed on sheets of clean paper. The Peyer's patches were carefully dissected out using fine scissors from the wall of the small intestine, and placed in ice-cold RPMI-1640 medium in a flat-bottomed Petri dish. The Peyer's patch cells were dispersed tapping gently with a rubber rod on a 150-gauge sterile stainless sieve. The cell suspensions were passed through a 200-gauge sterile stainless sieve, washed 3 times with RPMI-1640, and then resuspended in the same medium at a density of 2×10^6 cells/ml. Two hundred microliters of the cell suspension was cultured with 20 μl of test samples (final concentration of 10 and 100 μg/ml) in a 96-well flat bottom microtiter plate for 5 days at 37 °C in a humified atmosphere of 5% CO_2–95% air. ALR-5IIa (1 mg/ml) from the rhizome of *Atractylodes lancea* DC. was used as positive control and RPMI-1640 as blank. The experiment was performed in triplicates. The resulting culture supernatants (conditioned medium) were used for stimulation of bone marrow cells. Bone marrow cells were obtained from the femora of C3H/HeJ mice. The mice were sacrificed by cervical dislocation, the femora were excised and flushed of bone marrow cells using a 23-gauge needle and then suspended in RPMI 1640 supplemented with 5% FBS (RPMI-FBS). The cells were washed and resuspended in RPMI-FBS at a density of 2.5×10^5 cells/ml. The resulting culture supernatant (10 μl) of Peyer's patch cells and 90 μl of medium (RPMI-FBS) was incubated with 100 μl of bone marrow cell suspension (2.5×10^5 cells/ml) for 6 days in a humified atmosphere of 5% CO_2–95% air in order to evaluate the ability for the growth of bone marrow cells. Proliferation of bone marrow cells was measured by the Alamar Blue™ reduction assay. At 8 h prior to culture termination 20 μl of Alamar Blue™ solution (Alamar Bio-Sciences Inc., Sacramento, CA) was added to each well, and the cells were then continuously cultured. To count cell numbers the fluorescence intensity was measured by Fluoroskan II (Labosystems) at an excitation wavelength of 544 nm and emission wavelength of 590 nm. The delta soft II (Ver 4.13 FL, BioMetallics, Inc.) was used for data management. The results are expressed as the mean ± SE. The difference between the control and the treatment in these experiments was tested for statistical significance by Student's *t*-test. A value of $p < 0.05$ was considered to indicate statistical significance for immune system modulating activity.

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