

Pectic polysaccharides from *Biophytum petersianum* Klotzsch, and their activation of macrophages and dendritic cells

Marit Inngjerdingen^{1,2,3}, Kari T Inngjerdingen²,
Trushar R Patel^{4,9}, Stephanie Allen⁵, Xinyong Chen⁵,
Bent Rolstad⁶, Gordon A Morris⁴, Stephen E Harding⁴,
Terje E Michaelsen^{2,7}, Drissa Diallo⁸, and Berit S Paulsen²

²Department of Pharmaceutical Chemistry, School of Pharmacy, P.O. Box 1068, Blindern, N-0316 Oslo; ³Institute of Immunology, Rikshospitalet-Radiumhospitalet Medical Centre, University of Oslo, N-0027 Oslo, Norway; ⁴National Centre for Macromolecular Hydrodynamics, School of Biosciences, Sutton Bonington Campus, Sutton Bonington, LE12 5RD; ⁵Laboratory of Biophysics and Surface Analysis, School of Pharmacy, University of Nottingham, NG7 2RD, UK; ⁶Department of Anatomy, Institute of Basic Medical Science, University of Oslo, P.O. Box 1105, Blindern, N-0317 Oslo; ⁷The Norwegian Institute of Public Health, P.O. Box 4404, Nydalen, N-0403 Oslo, Norway; ⁸Department of Traditional Medicine, B.P. 1746, Bamako, Mali; and ⁹Department of Chemistry, University of Manitoba, Winnipeg, Manitoba, R3T 2N2, Canada

Received on May 2, 2008; revised on August 14, 2008; accepted on September 16, 2008

The Malian medicinal plant *Biophytum petersianum* Klotzsch (Oxalidaceae) is used as a treatment against various types of illnesses related to the immune system, such as joint pains, inflammations, fever, malaria, and wounds. A pectic polysaccharide obtained from a hot water extract of the aerial parts of *B. petersianum* has previously been reported to consist of arabinogalactans types I and II (AG-I and AG-II), probably linked to a rhamnogalacturonan backbone. We describe here further structural characteristics of the main polysaccharide fraction (BP1002) and fractions obtained by enzymatic degradations using *endo- α -D-(1 \rightarrow 4)-polygalacturonase* (BP1002-I to IV). The results indicate that in addition to previously reported structures, rhamnogalacturan type II and xylogalacturonan areas appear to be present in the pectic polymer isolated from the plant. Atomic force microscopy confirmed the presence of branched structures, as well as a polydisperse nature. We further tested whether the BP1002 main fraction or the enzymatically degraded products could induce immunomodulating activity through stimulation of subsets of leukocytes. We found that macrophages and dendritic cells were activated by BP1002 fractions, while there was little response of T cells, B cells, and NK cells. The enzymatic treatment of the BP1002 main fraction gave important information on the structure–activity relations. It seems that the presence of rhamnogalacturonan type I is important for the bioactivity, as the bioactivity decreases with the decreased amounts of rhamnose, galactose, and arabinose. The demonstration of bioactivity by the plant extracts might indicate the mechanisms behind the traditional medical use of the plant.

Keywords: *Biophytum petersianum* Klotzsch/dendritic cells/macrophages/pectin/rhamnogalacturonan

Introduction

Several recent papers have described the characterization of immune modulating pectic polysaccharides isolated from various medicinal plants, with effects varying from complement fixation to B-cell proliferative activities (Samuelsen et al. 1996; Yamada and Kiyohara 1999; Michaelsen et al. 2000; Wagner and Kraus 2000; Dourado et al. 2004; Nergard et al. 2004; Han et al. 2006). The ability of pectic polysaccharides to boost certain immune responses may, in part, explain some of the beneficial effects of medicinal plants, in particular, since the polysaccharides are easily obtained by extracting the plant material with water at 100°C. *Biophytum petersianum* Klotzsch (syn. *Biophytum sensitivum* (L.) DC) (Oxalidaceae) is a medicinal plant used in the traditional medicine in Mali and other African as well as Asian countries (Inngjerdingen et al. 2004, 2006; Guruvayoorappan and Kuttan 2007). In Malian traditional medicine, *B. petersianum* is used in the treatment of wounds, inflammations, gastric ulcers, malaria, and fever (Diallo et al. 2001). It is commonly prepared as a decoction of a fine powder of the aerial parts.

Pectic polysaccharides are one of the major constituents of the plant cell wall, and are one of the most complex classes of polysaccharides. Native pectins are composed of a backbone of linear homogalacturonan (HG) chains. The HG chain consists of 1,4-linked galacturonosyl (GalA) residues, some of which are carboxymethyl esterified. This chain is interrupted by ramified rhamnogalacturonan I (RG-I) regions, which consist of a backbone of alternating 1,4-linked GalA and 1,2-linked rhamnopyranosyl (Rha) units. To the rhamnose units at position 4 are attached neutral (1,3- and 1,3,5-linked arabinan and 1,4- and 1,3,4-linked and/or 1,3-, 1,6- and 1,3,6-linked (arabino)galactans) and acidic oligosaccharides. The arabinogalactan (AG) side chains may either be of the AG type I or AG type II. AG type I is usually composed of 4-linked galactose units, normally with substitutions of arabinose at position 3 of some of the galactose units. AG type II is composed of 3,6-linked galactose with chains of both 3- and 6-linked galactans that are decorated with arabinose units. Complexes of RG-I and arabinogalactans are often referred to as pectic “hairy regions,” in which AG type I and arabinan are the “hairs.” It is still unclear whether AG type II is part of the pectin complex, but co-extraction and linkage analyses suggest that they are covalently linked. Also substituted galacturonans, such as rhamnogalacturonan II (RG-II) and xylogalacturonan (XG), may be attached to the RG-I backbone. RG-II has an extremely complex structure containing a homogalacturonan backbone of 1,4-linked GalA substituted with four

¹To whom correspondence should be addressed: Tel: +47-23-07-30-12; Fax: +47-23-07-35-10; e-mail: marit.inngjerdingen@rr-research.no

different oligosaccharide side chains (Perez et al. 2003) and xylogalacturonan consisting of a homogalacturonan backbone to which residues of xylose are attached through position 3 of the GalA units (Vincken, Schols, Oomen, Beldman, et al. 2003).

Compounds that are capable of interacting with the immune system to upregulate or downregulate specific parts of the host response can be classified as immune modulators. A wide range of polysaccharides have been reported to exhibit a variety of immunological activities, affecting both the adaptive and innate immune systems. Polysaccharides such as β -glucans, inulin, and pectins from various plants are frequently reported to have effects on the immune system (Yamada and Kiyohara 2007). We have previously isolated a pectic polysaccharide from *B. petersianum*, which was degraded by *endo*-polygalacturonase leading to the isolation of a RG-I structure ("hairy" region) (Inngjerdingen et al. 2006). The isolated hairy region and the native polymer were tested for complement fixing activity. We found that the hairy region possessed the highest activity. There are several reports that point to a more potent complement-activating activity of the hairy region than of the corresponding original pectin (Yamada and Kiyohara 1999; Paulsen and Barsett 2005). Previously published data on the structure of bioactive pectins indicate that the observed biological activities are due to rhamnogalacturonan chains rich in neutral sugar side chains such as arabinan, galactan, and arabinogalactan (Samuelsen et al. 1996; Yamada and Kiyohara 1999). Reported activities seem to correlate with the presence of specific linkages such as 1,4-, 1,6-, and 1,3,6,- linked galactosyl and branched 3,5-arabinan.

We recently described the partial characterization of a pectic polysaccharide isolated from *B. petersianum*, called BP100 III (Inngjerdingen et al. 2006). The aim of this study was to elucidate the immune modulating properties of pectic polysaccharides isolated from *B. petersianum* and to correlate structural data with biological activity. In order to obtain more material of BP100 III, we prepared a new batch of pectic polysaccharides from the same plant using the same isolation procedure as previously described (Inngjerdingen et al. 2006). The new main fraction was called BP1002 in order to distinguish it from the previous preparation. Enzymatic degradations of BP1002 were performed to isolate the hairy regions, and the fractions were compared with respect to both their structure and immunological activity.

Results

Carbohydrate composition of the BP1002 main fraction

The pectic polymer BP100 III isolated from *B. petersianum* was previously described by us (Inngjerdingen et al. 2006). The monosaccharide composition of the native polymer BP100 III was shown to be one typical for pectins, consisting mainly of galacturonic acid (64.1 mol%), and almost equal amounts of rhamnose, galactose, arabinose, and xylose (~8 mol%). In order to obtain more material of the polysaccharide, the same isolation procedure was employed (Inngjerdingen et al. 2006). Compared to BP100 III, the new batch (BP1002) contains less galacturonic acid (45.8 mol%) and slightly higher amounts of rhamnose (9.8 mol%), galactose (10.5 mol%), arabinose (15.6 mol%), and xylose (10.8 mol%) (Table I). There are also minor amounts of fucose, 2-*O*-Me-Fuc, 2-*O*-Me-Xyl, mannose, glucuronic acid,

Table I. Monosaccharide compositions (mol%) of the native pectic polymer BP1002, and the fractions BP1002-I, BP1002-II, BP1002-III, and BP1002-IV, obtained after treatment of BP1002 with *endo*-polygalacturonase

Composition	BP1002	BP1002-I	BP1002-II	BP1002-III	BP1002-IV
Ara	15.6	30.2	15.4	13.0	3.6
Rha	9.8	21.5	14.0	9.6	0.4
2- <i>O</i> -Me-Fuc	0.9	0	2.9	2.5	Trace
Fuc	0.9	0.4	1.3	2.1	0.2
2- <i>O</i> -Me-Xyl	1.2	0	2.9	2.8	Traces
Xyl	10.8	2	11.0	13.0	19.6
Man	0.4	0.9	0.4	0.1	Traces
Gal	10.5	23.8	8.1	6.3	2.7
Glc	2.9	2.6	2.2	1.9	1.5
GlcA	1	2.6	2.1	1.8	0
4- <i>O</i> -Me-GlcA	0	0	0	0	0
GalA	45.8	15.7	39.7	47.1	71.8
Apiose	0	0	0	0	0
Molecular weight (M_w), kDa	64 \pm 10	98 \pm 4	11 \pm 1	5 \pm 1	2 \pm 1
Sed coefficient, $s_{20,w}$ Svedbergs	2.0 \pm 0.1	2.4 \pm 0.1	1.6 \pm 0.1	1.0 \pm 0.1	nd

nd, not determined (due to lack of material).

^aMol% of total carbohydrate content.

Molecular weights and sedimentation coefficients are weight averages.

and glucose present. The plant material for the two studies was collected from the same site, but in different years. The observed differences in monosaccharide composition may be caused by collection of the plants at different stages of growth, and there may also be slightly different growing conditions (e.g., amount of water available).

The total carbohydrate content of BP1002 was determined to be 47.6% (Table I). The fraction was shown to contain less than 0.05% phenolic compounds, expressed as ferulic acid equivalents. The phenolic content may thus be regarded as trace amounts only. The moisture content was approximately 10%. The remaining mass is unaccounted for, but some could arise from proteins, inorganic material, or as a consequence of the inaccuracies of the colorimetric assays (Bui et al. 2006). In this paper, only the carbohydrate part of the fractions is studied in detail.

Enzymatic degradation of Bp1002 by *endo*-polygalacturonase

In order to further elucidate the structure and structure-activity relationships of BP1002, an enzymatic degradation of the pectic polysaccharide was performed. The enzyme *endo*-polygalacturonase is used for the hydrolysis of the homogalacturonan (HG) regions of deesterified GalA 1,4-linkages. Treating high-molecular-weight pectin with this enzyme usually generates rhamnogalacturonan I (RG-I) (100 kDa), RG-II (5–10 kDa), and oligogalacturonides with degrees of polymerization between 1 and 5 (O'Neill and York 2003). These components can be separated by size-exclusion chromatography (SEC). Degradation of BP1002 by *endo*-polygalacturonase in order to isolate the different regions of the pectin polymer was performed as previously described (Inngjerdingen et al. 2006). The isolation procedure was repeated in order to obtain more material of the enzymatically degraded fractions. This resulted in the isolation of four fractions: BP1002-I, a high-molecular-weight fraction appearing in the void volume; BP1002-II and BP1002-III having molecular weights in decreasing order; and finally BP1002-IV containing oligogalacturonides. BP1002-I

Table II. The linkages of the pectic arabinogalactan BP1002 and the enzymatic resistant fractions obtained after enzymatic degradation of BP1002 (mol%) determined by reduction, methylation, and GC-MS.

Monosaccharide	Type of linkage	BP1002		BP1002-I		BP1002-II		BP1002-III		BP1002-IV	
		Mol (%)	(%)	Mol (%)	(%)	Mol (%)	(%)	Mol (%)	(%)	Mol (%)	(%)
Ara	Tf	8.5	54.5	10.4	34.4	12.9	83.8	8.9	68.5	2.9	80.6
	1,2f	0.2	1.3	0.2	0.7	Traces		Traces		–	
	1,3f	0.4	2.6	0.5	1.7	Traces		Traces		–	
	1,5	3.6	23.1	16.7	55.3	0.5	3.2	3.1	23.8	0.7	19.4
	1,3,5	0.6	3.8	2.1	7.0	–		–		–	
	1,2,3	2.3	14.7	0.3	1.0	2.0	13.0	1.0	7.7	–	
		15.6		30.2		15.4		13.0		3.6	
Rha	T	4.5	45.9	5.3	24.7	9.7	69.3	5.4	56.3	0.4	~100
	1,2	3.3	33.7	8.5	39.5	2.4	17.1	3.1	32.3	Traces	
	1,2,4	1.2	12.2	7.7	35.8	0.1	0.7	0.2	2.1		
	1,3	0.8	8.2	–		1.8	12.9	0.9	9.4		
		9.8		21.5		14.0		9.6		0.4	
Xyl	T	9.8	90.7	2.0	100	10.8	98.2	12.1	93.1	18.7	95.4
	1,4	1.0	9.3	–		0.2	1.8	0.9	6.9	0.9	4.6
		10.8		2.0		11.0		13.0		19.6	
2-O-Me Xyl	l.n.d.	1.2	–		2.9		2.8		–		
Man	l.n.d.	0.4	–	0.9		0.4		0.1		–	
Fuc	T	0.4	44.4	0.4	100.0	0.8	61.5	1.1	52.4	Traces	
	1,3,4	0.5	55.6	–		0.5	38.5	1.0	47.6	–	
		0.9		0.4		1.3		2.1		0.2	
2-O-Me Fuc	l.n.d.	0.9	–		2.9		2.5		–		
Glc	T	0.5	17.2	1.1	42.3	0.5	22.7	1.6	84.2	1.0	66.7
	1,4	2.2	75.9	1.5	57.7	1.7	77.3	0.3	15.8	0.5	33.3
	1,4,6	0.2	6.9	–		–		–		–	
		2.9		2.6		2.2		1.9		1.5	
Gal	Tf										
	Tp	2.1	20.0	6.8	28.6	3.6	44.4	3.4	54.0	0.1	3.7
	1,3p	1.4	13.3	4.6	19.3	0.5	6.2	1.1	17.5	–	
	1,4p	3.2	30.5	3.2	13.4	0.6	7.4	0.6	9.5	0.2	7.4
	1,6	1.1	10.5	3.1	13.0	0.3	3.7	–	–	–	
	1,3,4	–		0.4	1.7	–		–		2.4	88.9
	1,3,6	2.1	20.0	5.7	23.9	0.4	4.9	–	–	–	
	1,2,4	0.6	5.7	–		2.7	33.3	1.2	19.0	–	
	10.5		23.8		8.1		6.3		2.7		
Api	1,3'	a.n.d.		–		a.n.d.		a.n.d.			
GlcA	T	0.2	20.0	0.5	19.2	0.2	18.2	–			
	1,4	0.4	40.0	2.1	80.8	0.2	6.1	–			
	1,2	0.4	40.0	–		1.7	75.8	1.8	100		
		1.0		2.6		2.1		1.8			
GalA	T	0.9	2.0	0.9	5.7	3.0	7.6	10.6	22.5	34.1	47.5
	1,4	39.1	85.4	14.6	93.0	14.4	36.3	11.5	24.4	37.7	52.5
	1,3,4	5.2	11.4	0.2	1.3	21.6	54.4	23.9	50.7	–	
	1,2,4	0.6	1.3	–		0.7	1.8	1.1	2.3	–	
		45.8		15.7		39.7		47.1		71.8	

a.n.d. = amount not detected.

was shown to consist of mainly rhamnose (21.5 mol%), galactose (23.8 mol%), arabinose (30.2 mol%), and galacturonic acid (15.7 mol%) (Table I). Compared to the high-molecular fraction BP100 III.1 isolated previously (Inngjerdingen et al. 2006), the high-molecular-weight fraction BP1002-I differs in the amount of galacturonic acid (38.5 versus 15.7% mol) and arabinose (7.9 versus 30.2% mol) (Inngjerdingen et al. 2006). As expected, the BP1002-I has a higher molecular weight than BP1002-II and BP1002-III and contains higher amounts of arabinose, rhamnose, and galactose and lower amounts of galacturonic acid and xylose. Interestingly, this is the same pattern observed with our previous batches of BP100 III.2 and BP100 III.3 (Inngjerdingen et al. 2006). In addition, the intermediate fractions BP1002-II and BP1002-III also contain 2-O-Me-xylose and 2-O-Me-fucose. Finally, the BP1002-IV fraction was analyzed by high-performance anion exchange chromatography-pulsed am-

perometric detection (HPAEC-PAD) confirming the presence of mono- and oligomers of galacturonic acid (data not shown), which represents the breakdown products of the HG-chain by the enzyme.

Linkage analysis of BP1002 fractions

Linkage analysis was further performed to elucidate the structure of the enzymatically degraded fractions. We found that BP1002 contains glycosidic linkages characteristic for HG, RG-I, and RG-II, as well as XG (Table II). However, the distribution of sugar residues and their linkages suggests that a RG-I-like structure is the predominant feature of BP1002-I (Table II). The BP1002-I fraction contains 1,2-linked Rha and 1,4-linked GalA in an approximately 1:1 ratio. Side chains of about 35% of the rhamnose units in BP1002-I are attached through position 4.

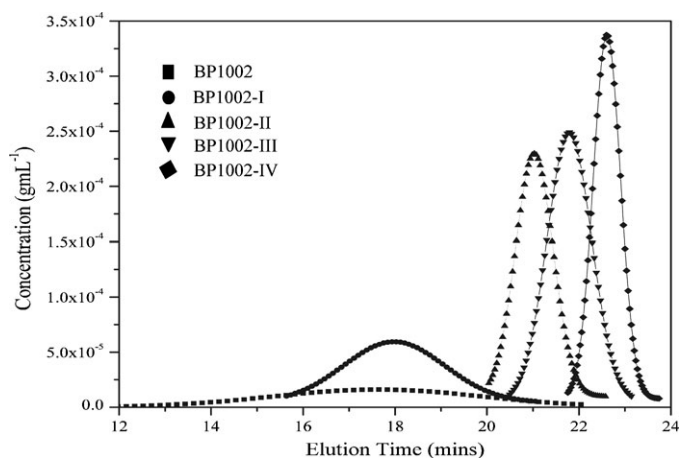


Fig. 1. Concentration profiles from size-exclusion chromatography coupled to multiangle laser light scattering of BP1002 and the fractions BP1002-I, BP1002-II, BP1002-III, and BP1002-IV, obtained after treatment of BP1002 with *endo*-polygalacturonase.

Arabinofuranosyl residues in BP1002-I were mainly present as terminal (34.4%) and 1,5-linked (55.3%) units. The galactans were shown to be highly branched, 23.9% of the residues being present as 1,3,6-linkages. The glycosidic linkages for the rest of the galactopyranosyl units were terminal, 1,3-, 1,4-, 1,6-, and 1,3,6-linked (Table II). The minor monosaccharides are present as mainly terminally or 1,4-linked residues.

The intermediate fractions, BP1002-II and BP1002-III, contain similar types and amounts of glycosidic linkages (Table II). According to the linkage analysis, all these fractions contain glycosidic linkages characteristic for RG-II type structures, such as terminal- and 1,2,3-linked arabinose, terminal-, 1,2- and 1,3-linked rhamnose, terminal-linked galactose, 1,3,4-linked fucose, 1,3-linked apiose, 1,2-linked glucuronic acid, and 1,4-linked galacturonic acid with branch points in positions 2 and 3. Additionally, the presence of 2-*O*-Me-Fuc and 2-*O*-Me-Xyl, characteristics of RG-II, were shown to be present by monosaccharide analysis. Moreover, in addition to the presence of a RG-II-type structure, BP1002-II and BP1002-III also seem to contain a xylogalacturonan region. This is indicated by the high amount of 1,3,4-linked GalA units and terminally linked xylose residues. The branched galacturonic acid residues may be substituted with T-Xylp units, forming the xylogalacturonan part of the pectin (Perez et al. 2003).

Analysis of homogeneity and molecular weight

It is difficult to measure the molecular weight of pectic polysaccharides accurately because of the presence of heterogeneous groups along with hairy and smooth regions (Daas et al. 2001). The weightaverage molecular weight for these samples was determined as previously described (Inngjerdigen et al. 2007) using SEC coupled with multiangle laser light scattering (MALLS) which has been widely used to determine the molecular weight of various pectins (Harding 1994; Hokputsa et al. 2004). The elution profiles (concentration versus elution time) are shown in Figure 1 for BP1002, BP1002-I, BP1002-II, BP1002-III, and BP1002-IV. As can be seen from the figure, all the polysaccharide fractions are polydisperse. In the case of the native pectin BP1002, we find a weight average molecular weight of $64 \pm$

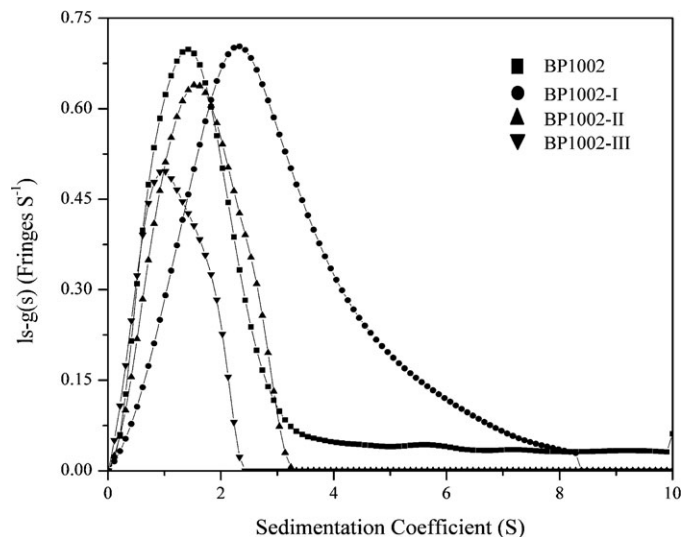


Fig. 2. Sedimentation coefficient distributions of BP1002 and the fractions BP1002-I, BP1002-II, BP1002-III, and BP1002-IV, obtained after treatment of BP1002 with *endo*-polygalacturonase. Rotor speed 40,000 rpm; temperature 20.0°C and sedimentation distributions were produced using the least-squares $g(s^*)$ procedure of Schuck and Rossmannith (2000).

10 kDa. The wide, main peak in BP1002 decreases after enzymatic degradation to give four peaks that were separated by gelfiltration. The first compound, BP1002-I, has an elution time of 18 min (Figure 1) with a weight average molecular weight of 98 ± 4 kDa. The second peak, BP1002-II, is evident at about 21 min, the third, BP1002-III, at 22 min and the last, BP100-IV, elutes at 22.5 min and has a weight average molecular weight of 2.1 ± 0.7 kDa. BP1002-I appears in this system to have a molecular weight (M_w) higher than that of the parent molecule. This is most probably due to the aggregation of this fraction, the hairy region of the polymer, as we previously have shown to take place for pectins from plants. The aggregation of pectin molecules in solution is highly possible. Molecular weights (weight averages), M_w , for all the fractions are given in Table I.

Another complimentary approach to study the molecular weights of pectins is to study their sedimentation during ultracentrifugation. Previous experiments have shown that there is a general decrease in sedimentation coefficient with a decrease in molecular weight (Hokputsa et al. 2003). For the purpose of comparison, the sedimentation coefficient for all the samples was measured at 1 mg/mL and results are shown in Table I and Figure 2. Results are in general agreement with SEC-MALLS insofar as each polysaccharide gives a wide polydisperse peak and that BP1002-I appears to be of larger size than the native polysaccharide (BP1002) possibly due to aggregation. The solubility could also be the driving force for this as BP1002 is less soluble than the fractions obtained after enzymatic degradation. Due to its low molecular weight, we were unable to get a meaningful sedimentation coefficient for BP1002-IV.

Atomic force microscopy

Pectin fractions BP1002, BP1002-I, BP1002-II, and BP1002-III were shown by SEC-MALLS to consist of polydisperse polymers with a small amount of aggregated material. In order to visualize the individual pectic polysaccharides, we further

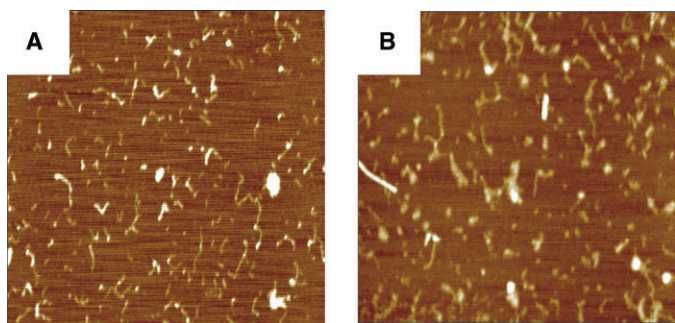


Fig. 3. AFM images of BP1002 (A) and BP1002-I (B). The scan sizes are $1 \mu\text{m} \times 1 \mu\text{m}$; z-scale for A is 1 nm and for B 3 nm.

employed atomic force microscopy (AFM). Images of BP1002 and BP1002-I are presented in Figure 3. As can be seen from the images, both fractions comprise a hierarchy of separate molecules and aggregated species. Based on their histograms, evaluated values of the contour lengths for BP1002 and BP1002-I range from about 5 to about 100 nm (data not shown). The majority of the molecules are calculated to have smaller contour lengths than 10 nm. As pectin molecules often tangle with each other, it is difficult to give exact statistical data on their chain lengths (Harding 1994; Hokputsa et al. 2004; Yang et al. 2006). From Figure 3, it is evident that some of the molecules are branched, which has also been previously indicated by AFM images of pectins (Round et al. 2001; Ovodova et al. 2006) and which confirms our linkage analysis results.

Modulation of lymphocyte activity

Having generated data on the detailed structure of BP1002 and its degraded fractions, we set up experiments to test their activity on subsets of leukocytes. We first tested the potential ability of the pectic polysaccharides to activate T cells, B cells, or natural killer (NK) cells. T- and B-cell proliferative activities in response to BP1002 were tested *in vitro*. The cells were isolated from rat spleen cell suspensions by positive selection using Dynabeads and treated for 5 days with either medium or increasing concentrations of the pectin fractions. While B cells showed proliferative responses toward the positive control lipopolysaccharide (LPS) and T cells toward Concanavalin A, we observed no significant responses of BP1002 or its derivatives (data not shown). We have also tested whether the pectic polysaccharide previously isolated, BP100 III (Inngjerdingen et al. 2006), could induce NK-cell activity (cytotoxicity or chemotaxis), but no such activity was observed (data not shown).

Activation of antigen-presenting cells

We continued to study the effect of the BP1002 extracts on macrophages and dendritic cells. Macrophages function as phagocytes and produce nitric oxide (NO), growth factors, and cytokines upon activation. These effector functions are important in the immune response against an infection which may often accompany a wound. We measured the production of NO after treatment of macrophages with the BP1002 fractions. We utilized monocyte-derived macrophages from rat spleen, as well as the rat macrophage cell line R2. As shown in Figure 4, the main pectic fraction BP1002 and the BP1002-I fraction induced NO release from macrophages, both from the spleen-derived

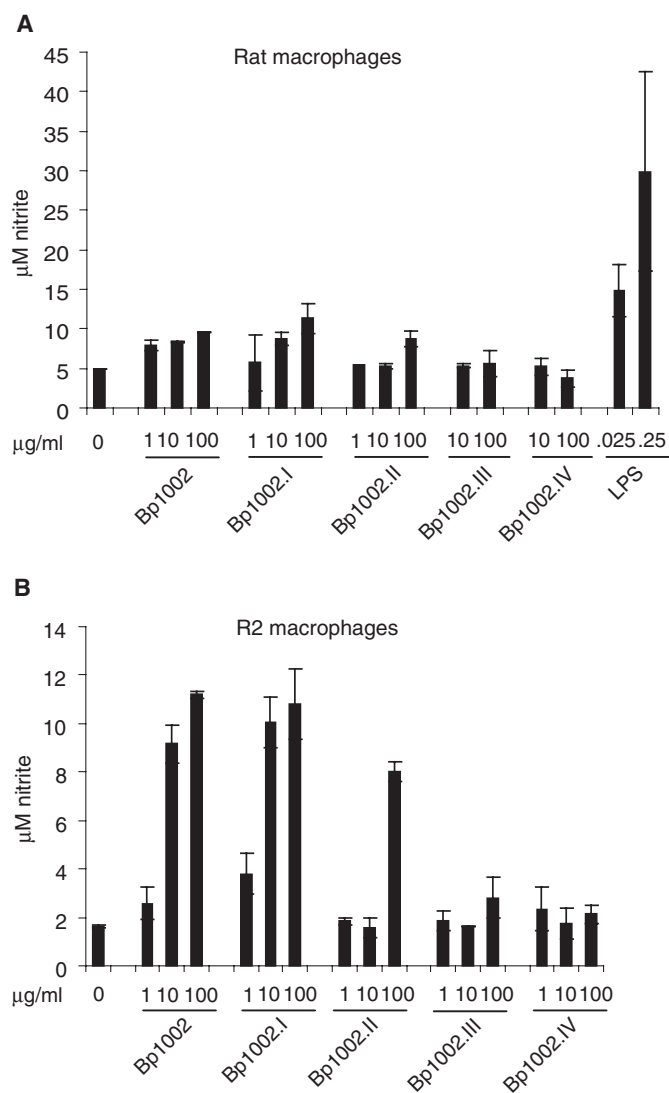


Fig. 4. Stimulation of macrophages with BP1002 fractions. Measurement of nitric oxide release from macrophages after overnight stimulation with BP1002 fractions, or LPS as a positive control. (A) Spleen-derived macrophages. (B) The rat macrophage cell line R2. Data are presented as the mean of three independent experiments \pm SD. *** $P \leq 0.0001$; ** $P \leq 0.008$; and * $P \leq 0.03$.

rat macrophages (Figure 4A) and from the rat R2 macrophage cell line (Figure 4B). The NO release was most prominent in the cell line. The BP1002-II fraction induced NO release at $100 \mu\text{g/mL}$, but not at the lower concentrations, indicating that it is not as potent as BP1002 and BP1002-I. The BP1002-III and BP1002-IV fractions did not induce NO release, suggesting that the presence of arabinogalactan side chains is part of the structural requirements for the induction of the macrophage response.

Dendritic cells are the most potent antigen-presenting cells of the immune system, orchestrating the initiation of the adaptive immune responses. Immature dendritic cells are located throughout the body, but migrate to lymphoid compartments upon their activation into a mature phenotype. Maturation typically leads to upregulation of class II MHC and of the

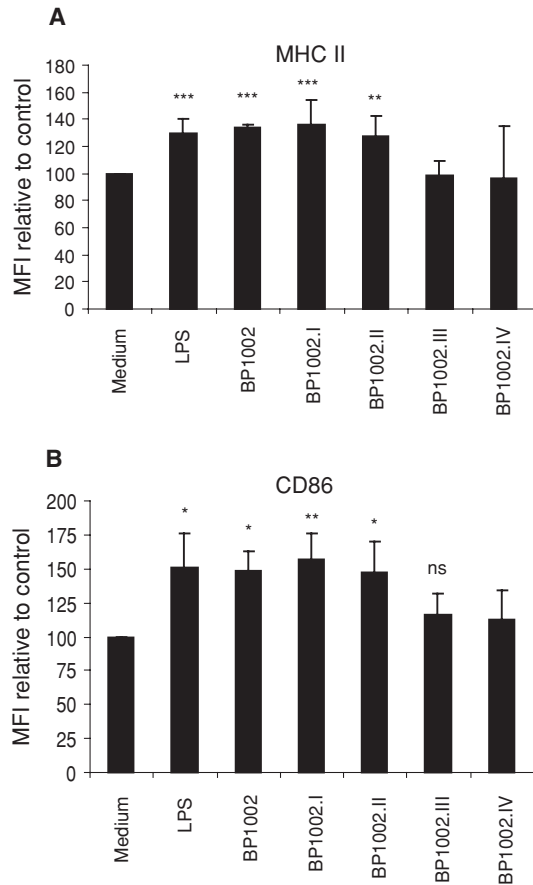


Fig. 5. Maturation of immature dendritic cells with BP1002 fractions. Spleen-derived dendritic cells were incubated for 24 h with the indicated BP1002 fractions, and the expression level of (A) MHC class II and (B) CD86 evaluated by flow cytometry. The mean fluorescence of CD86 and MHC class II on untreated cells was set to 100, and the fluorescence of the markers on treated cells calculated relative to the untreated sample. Data are presented as the mean of three independent experiments \pm SD. *** $P \leq 0.0008$; ** $P \leq 0.008$; and * $P \leq 0.03$; ns, not significant.

costimulatory molecules CD83 and CD86. We generated immature dendritic cells from spleen-derived monocytes. The cells were then harvested and stimulated for 24 h with either LPS as a positive control (500 ng/mL) or 50 μ g/mL of BP1002 fractions. The coexpression of MHC class II (using the mAb OX6) and the maturation marker CD86 were analyzed by flow cytometry. We normalized the mean fluorescence intensity (MFI) values of the stimulated cells relative to the MFI of unstimulated cells. We observed a small but significant increase in MHC class II expression on cells stimulated with BP1002, BP1002-I, and BP1002-II, which was comparable to cells stimulated with the positive control LPS (1.3-fold increase) (Figure 5A). We also observed a 1.5-fold increase in the expression of CD86 in the same samples (Figure 5B).

Taken together, it seems that the native polysaccharide and the RG-I regions of BP1002-I are the most potent immune activators, compared to the intermediate fractions containing RG-II-type structures. This suggests that the hairy regions are the active sites for the immune activity of BP1002.

Discussion

The presence of immunomodulating polysaccharides in medicinal plants may be related to their medical uses. Hot water extracts of different Malian medicinal plants are a commonly used preparation, and it is therefore relevant to study bioactive high-molecular-weight compounds in these extracts. There is no clear information on the optimal structure for polysaccharides for inducing a potent stimulatory effect on leukocytes. In some cases, minor changes in structure, molecular weight, or confirmation of the polysaccharide may have dramatic effects on biological activity. Both the three-dimensional structure of exposed, flexible side chains and specific types of monosaccharides may be important for their activity. Here we report the detailed chemical characterization of a pectic polysaccharide isolated from *B. petersianum*, called BP1002, along with data that indicate its ability to activate leukocyte subsets.

Structural features of BP1002

The main pectic polysaccharide, BP1002, was treated with *endo*- α -D-(1 \rightarrow 4)-polygalacturonase, yielding the fractions BP1002-I, BP1002-II, BP1002-III, and BP1002-IV. The enzymatically degraded fractions can be considered as the branched regions of the main pectic polysaccharide.

BP1002 appears to contain homogalacturonan (HG), rhamnogalacturonan (RG), and arabinogalactan structures, which are similar to the structures we have seen in other pectic polysaccharides isolated in our lab (Nergard et al. 2005; Inngjerdigen et al. 2007). A highly methyl esterified linear homogalacturonan region seems to alternate with ramified regions comprising highly branched rhamnogalacturonans. The rhamnogalacturonan in BP1002 seems to contain both AG type I and AG type II according to linkage analysis, while the previous study on pectin from *B. petersianum* showed predominantly the presence of AG type II in the side chains. According to linkage analysis, about 10% of the galacturonic acid residues in BP1002 are branched through position 3. In addition, the high xylose content (10.8 mol%) and the presence of substantial amounts of terminally linked xylose indicate that BP1002 also contains xylogalacturonan (XGA) structures. These linkages are not present in BP1002-I, suggesting that xylogalacturonan either coelute or is part of the other fractions obtained after enzymatic degradation. This means that XGA is not closely linked to the hairy region part or rhamnogalacturonan region of the mother polymer. Previous studies have reported the release of xylosyl galacturonic acid dimers from pectic polysaccharides (Huisman et al. 2001). This is in consistency with our own findings (Inngjerdigen et al. 2006). The position of XGA relative to the backbone of BP1002 is not clear. It has been suggested by Vincken and co-workers that the XGA is not an integral part of the pectic backbone (Vincken, Schols, Oomen, McCann, et al. 2003). It has also been suggested that XGA might be a side chain of RG-I (Oechslin et al. 2003), while Perez et al. (2003) indicate that it is a part of the main chain of the pectins.

BP1002-I contains a RG-I-like structure, which was evident from the relative high amount of 1,2,4-linked Rha_p (35.8%) as compared to other fractions. RG-I is normally found in the range of 20–80% in pectins (O'Neill and York 2003). We assume that RG-I is the predominant rhamnogalacturonan structure in BP1002, as RG-I is more abundant than RG-II in pectic polysaccharides from most dicot cell walls (Carpita and McCann 2002).

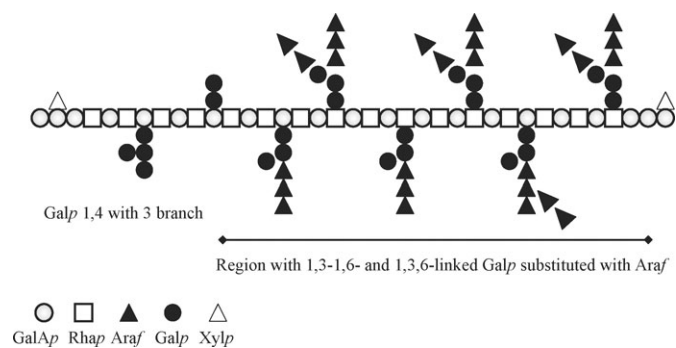


Fig. 6. Proposed structure of the enzymatically degraded polysaccharide fraction BP1002-I.

The proportion of GalA in the rhamnogalacturonan of BP1002-I was low, as compared to a similar pectic polysaccharide fraction, GOA2-I, which was recently published (Inngjerdengen et al. 2007). However, BP1002-I seems to correspond to a true rhamnogalacturonan with a strict alternance of Rha and GalA, with a GalA/Rha ratio of approximately 1:1. The distribution of side chains in the RG backbone is not established, they might be randomly distributed, arranged in a cluster-like fashion with hairs of the same kind, or of clusters of different kinds of hairs. The differently linked glycosyl residues can combine to form a large number of side chain structures that may be arranged in many different ways along the backbone. The length of the RG sections in the pectin backbone is not known, but it has been estimated that the RG-I backbone could be a few hundred residues in length (Renard et al. 1999).

In BP1002-I, more than 50% of the arabinose units were 1,5-linked, indicating a rather linear arabinan. Fucose and xylose, which were only present in low amount, occurred mainly terminally linked. The high amount of terminal Ara1f residues in BP1002-I cannot be explained by the low amount of branched Ara1f residues. This indicates that some of the terminal Ara1f residues could be linked to other branched glycosyl residues. The presence of 1,3-, 1,4-, and 1,6-linked galactopyranosyl units indicates that both AG type I and AG type II are present in the RG-I side chains. The AG side chains present in BP1002-I seem to be more complex than suggested in our previous paper (Inngjerdengen et al. 2006). The arabinans have been shown to have a 1,5-linked backbone, to which single arabinose residues or small oligoarabinans are attached at position 3 and to a lower extent at position 2. The arabinans may be a part of the arabinogalactan structures or indirectly linked to the pectic backbone attached through small galactans (Strasser and Amadò 2001). This has also been reported for RG-I-type pectins isolated from *Glinus oppositifolius* (Inngjerdengen et al. 2007). In Figure 6, we have proposed a structural model of BP1002-I based on the data presented here.

Treatment of primary cell walls of higher plants by *endo*-polygalacturonase usually releases the complex polymer rhamnogalacturonan II (RG-II) with a molecular weight of 5–10 kDa. The successful degradation by *endo*-polygalacturonase, thus indicates the presence of a HG chain. The structure of RG-II has been shown to be virtually the same in every plant analyzed hitherto (Perez et al. 2003). A typical primary wall will contain on average one RG-II molecule per 50 GalA residues

in a HG chain. Some of the GalA residues in the backbone are in addition methyl esterified. The monosaccharides apiose (Api), 2-*O*-methyl L-fucose, and 2-*O*-methyl xylose were detected in BP1002, indicating the presence of RG-II. The presence of the glycosidic linkages Ara 1,2,3, Rha 1,3 and 1,2, Fuc 1,3,4, Gal 1,2,4, GalA 1,4, 1,2,4 and 1,3,4, Apiose 1,3, and GlcA 1,2 in BP1002-II further indicates the presence of RG-II structures (Perez et al. 2003; O'Neill et al. 2004). In addition, according to the monosaccharide analysis 2-*O*-Me-Fuc and 2-*O*-Me-Xyl are present in the fractions. TGalp and 1,2,4-Galp are typical residues of RG-II and represent about 80% of the galactose in BP1002-II. Another characteristic constituent of RG-II is 1,2-linked glucuronic acid, which was also identified in the BP1002-II (75.8%). The high content of galacturonic acid in the fractions is due to the backbone of RG-II which contains exclusively galacturonic acid. Besides the linear 1,4-linked GalAp, the branched residues 1,3,4-GalAp have been shown to be present in considerable amounts in the backbone. According to the structural model of RG-II, two terminally linked galacturonic acid residues are present in the uronic acid rich side chains. The method for methylation analysis used in this work is known to destroy the monosaccharides 3-deoxy-D-manno-2-octulosonic acid (KDO) and 3-deoxy-D-lyxo-2-heptulosaric acid (Dha). It was therefore not possible to identify them together with other constituents. The native pectins from *G. oppositifolius* and *B. petersianum* were previously analyzed for the presence of rhamnogalacturonan type II (RG-II) in the thiobarbituric acid assay (Inngjerdengen et al. 2005, 2006). They gave a negative reaction, indicating that KDO and Dha, and thereby RG-II, were not present in those fractions.

BP1002 has a high degree of heterogeneity, which has also been reported in pectic polysaccharides isolated from *G. oppositifolius* (Inngjerdengen et al. 2005, 2007). In the case of BP 1002-IV, the error in molecular weight is high because of low-molecular weight as the amount of light scattered depends on both molecular weight and concentration.

Structure–activity relations

We have previously reported that pectic polysaccharides isolated from *B. petersianum* (BP100 III) have potent complement fixing activities (Nergard et al. 2005; Inngjerdengen et al. 2006, 2007). We have also reported that there appears to be a positive correlation between a high complement fixing activity and B-cell proliferating activity. We did not detect B-cell proliferative activity for pectic polysaccharides of *B. petersianum* (BP1002 fractions), but did find activity toward macrophages and dendritic cells. BP1002 and BP1002-I, which are the most active fractions, correspond to the previously reported BP100 III and BP100 III.1 polysaccharides with potent complement fixing activity (Inngjerdengen et al. 2006). These fractions are all represented by branched regions of arabinogalactan side chains, which have been regarded as the possible structural unit for the recognition of carbohydrate receptors on leukocytes (Sakurai et al. 1999). Arabinogalactan-containing compounds are known to be potent immune modulators, and arabinogalactan side chains are thought to be the site of biological activity in pectic polysaccharides.

We also observed a modest activity of BP1002-II. This fraction contains mainly RG-II-like structures. Pectic polysaccharide fractions isolated from *G. oppositifolius* containing

RG-II-like structures were recently shown by us to contain no activity in either the complement fixation test, the B-cell proliferation assay, or for macrophage activation, as opposed to fractions containing RG-I-like structures (Inngjerdingen et al. 2007). However, pectic fractions containing RG-II structures isolated from other plants have been reported to have biological activity. Antiulcer polysaccharides from the roots of *B. falcatum* have been related to the occurrence of an RG-II-containing pectic fraction (Hirano et al. 1994). RG-II has also been identified as the bioactive components in leaves of *Panax ginseng* (Shin et al. 1997). The differences in terms of bioactivity could be explained by some slight structural variations within the RG-II structure (Perez et al. 2003).

We have previously observed a modest chemotactic response of IL-2-activated human NK cells toward the pectic extract GOA2 isolated from *G. oppositifolius* (Inngjerdingen et al. 2005), but no effect on the cytolytic behavior of NK cells was observed (data not shown). None of the fractions obtained from *B. petersianum* had any chemotactic effect on NK cells or any modulation of their cytolytic activity (data not shown). The absence of activation of B- and T-cell proliferation, as well as NK-cell activation, led us to conclude that BP1002 and its enzymatically degraded fractions are not potent immunomodulators of lymphocytes. However, increased activities of macrophages and dendritic cells were observed. This could mean that BP1002 possibly recognizes receptors that are preferentially expressed by antigen-presenting cells.

DCs are responsive to inflammatory cytokines or bacterial products, such as LPS. These factors induce phenotypic and functional changes in the DC, which undergoes a transition from an immature phenotype to a mature phenotype. The phenotypical change is typically characterized by upregulation of MHC class II, CD83 and CD86. In addition to cytokines and bacterial products, β -glucans and other polysaccharides have been shown to induce the maturation of DCs (Kim et al. 2007). Similarly, we observed that the main fraction BP1002, as well as BP1002-I and BP1002-II, could induce the maturation of immature DCs. We assume that the observed effects on macrophages and DCs are induced by the binding of the pectic polysaccharides to receptors expressed by these cells. Angolan, a pectic polysaccharide from *Angelica gigas*, has been shown to interact with Toll-like receptor 4 (TLR4), a pattern recognition receptor belonging to the TLR family of receptors (Kim et al. 2007). This receptor is also the receptor for LPS. We are currently testing whether TLR4, or other carbohydrate recognizing receptors, may bind our pectic polysaccharides.

This study illustrates that the pectic polysaccharide BP1002 isolated from *B. petersianum* has many characteristics in common with pectins of other sources, consisting of HG and branched regions, and that the bioactivities are expressed by the rhamnogalacturonan I (RG-I) region, and possibly also of the RG-II region. However, further studies are essential in order to determine the structure–activity relations of the branched region of BP1002.

Material and methods

Materials

The aerial parts of *B. petersianum* Klotzsch (Oxalidaceae) were collected in Blendio, Mali, in 2003. The plant was identified by

Professor Drissa Diallo, Department of Traditional Medicine (DMT), Bamako, Mali, and voucher specimens were deposited in the herbarium at DMT. BP1002 (= BP100 III), a pectic polysaccharide, was isolated from the aerial parts using the same isolation procedure as described previously (Inngjerdingen et al. 2006), followed by degradation by *endo*- α -D-(1 \rightarrow 4)-polygalacturonase as previously described (Inngjerdingen et al. 2006).

Animals

Eight- to twelve-week-old rats of the PVG.7B strain (which possesses a “non-immunogenic” CD45 allotype, RT7^b, but 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 under compliance with guidelines set by the Experimental Animal Board under the Ministry of Agriculture in Norway.

Analysis of carbohydrate content and composition

Methyl glycosides of neutral sugars and uronic acids were obtained after methanolysis, converted into trimethylsilyl glycoside derivatives and analyzed by capillary gas chromatography on a Carlo Erba 6000 Vega Series 2 chromatograph (Milan, Italy) 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.

Monosaccharide linkage analysis

Linkage elucidation was performed by methylation studies. Prior to methylation, the uronic acids of the polymer fractions were reduced to primary alcohols. To distinguish between reduced uronic acids and the corresponding neutral sugars in gas chromatography and mass spectroscopy (GC–MS), sodium borodeuteride was used. Carboxyl esters were first reduced with sodium borodeuteride in an imidazole buffer, pH 8, 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 employing or using 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, San Carlos, CA) on a SPB-1 fused silica capillary column (30 m \times 0.20 mm i.d.) with film thickness of 0.20 μ m. The E.I. mass spectra were obtained using a Hewlett-Packard Mass Selective Detector 5970 (Palo Alto, CA) with a Hewlett-Packard GC (Palo Alto, CA). The injector temperature was 250°C, the detector temperature 300°C, and the column temperature 80°C when injected; the latter was 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).

Determination of phenolic content

The quantitative determination of total phenols was performed with the Folin-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 to the same amount of Folin-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 Na₂CO₃ was added; the tubes were mixed and allowed to stand for 1 h. The absorbance was measured at 750 nm in a Helios Epsilon Spectrophotometer (Thermo Spectronic, Waltham, MA). The standard curve was plotted using ferulic acid, and the total phenolic content was determined as ferulic acid equivalents (FA/sample) × 100%.

Degradation by *endo-α-D-(1→4)-polygalacturonase*

The BP1002 polymer (650 mg) was dissolved in 65 mL of 0.05 M NaOH for deesterification and left for 24 h at 0°C. The solution was neutralized by adding a few drops of acetic acid. The deesterified sample (5 mg/mL) in 50 mM acetate buffer (pH 5.0) was treated with *endo-α-D-(1→4)-polygalacturonase* from *Aspergillus japonicus* (430 units/mg protein, EC 3.2.1.15) (Sigma, St. Louis, MO) at 30°C. One unit of the enzymatic solution liberates 1.0 μmol of galacturonic acid from polygalacturonic acid per minute. The hydrolysis proceeded until the increase in reducing end groups stopped (26 h) and was determined in a reaction mixture using dinitrosalicylic acid (DNS) (Miller 1959), modified by Knutsen (1991). The reactions were terminated by heating at 100°C. The deesterified and partially hydrolyzed materials were fractionated by SEC on a BioGel P30 column (2.5 × 90 cm, Bio-Rad Laboratories, Hercules, CA). The column was coupled to a Peristaltic pump P-3 (Pharmacia, Uppsala, Sweden) and a Pharmacia LKB FRAC 100 fraction collector (Pharmacia) and eluted with a 50 mM acetate buffer (pH 5.0) at 30 mL/h. The carbohydrate profile obtained was determined using the phenol-sulphuric acid assay, and the relevant fractions were pooled (Dubois et al. 1956).

Homogeneity and molecular weight determination

Homogeneity and molecular weights of the acidic polysaccharide fractions were determined by size-exclusion chromatography coupled to a multiangle laser light scattering (SEC-MALLS) detector and by analytical ultracentrifugation using the sedimentation velocity method (Harding 1994). Purified samples were dissolved in Dulbecco's phosphate buffered saline (PBS) (pH 7.0, I = 0.1 M) in screw-capped tubes with constant stirring at low speed. During this period the temperature was raised to 80.0°C for 10 min to obtain maximum solubility. Stirring continued at room temperature (20.0°C) overnight at low speed. The weight average molecular weight was determined using SEC-MALLS which has widely been used to determine the molecular weight of various pectins (Harding et al. 1991; Daas et al. 2001; Hokputsa et al. 2004). The chromatography assembly consisted of a HPLC pump (Model PU-1580, Jasco Corporation, Tokyo, Japan), a Rheodyne injection valve (Model 7125, Rheodyne, St Louis, MO) fitted with a 100 μL loop with Phenomenex guard column (Phenomenex, Macclesfield, UK), and TSK (Tosoh Biosciences, Tokyo, Japan) Gel G 4000 connected in series with TSK Gel G 3000. The intensity of scattered light

was detected using a Dawn DSP multiangle laser light scattering photometer, and the concentration was determined using an Optilab 903 interferometric refractometer (both instruments from Wyatt Technology, Santa Barbara, CA) with PBS at pH 7.0 as mobile phase. The SEC-MALLS system was calibrated overnight at a flow rate of 0.8 mL/min at 20°C. Samples (100 μL) with accurately known concentration were filtered through 0.45 μm filters (Whatman, Maidstone, UK) and injected at the same flow rate. Repeated injections were made for each sample for reproducibility in the measurements. Signals from the light scattering photometer and the refractometer were captured and analyzed (Debye model) considering the refractive index increment (dn/dc) of 0.146 mL/g (Chapman et al. 1987; Theisen 2000) on a PC using the ASTRA™ (for Windows XP) software supplied by the manufacturer. Sedimentation velocity experiments were performed using an Optima XL-I analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA). Reference solvent (400 μL) and sample solution (380 μL) were injected into the solvent, and sample channels of 12 mm carbon filled centerpieces and loaded into a 8-hole titanium rotor. Samples were centrifuged at 40,000 rpm and 20.0°C. Data were analyzed using the least-squares $g^*(s)$ method in SEDFIT (Schuck and Rossmann 2000). Sedimentation coefficients, $s_{20,b}$, were then corrected to standard solvent conditions (density and viscosity of water at 20°C) to yield $s_{20,w}$ (S) using computer algorithm SEDENTERP (Laue 1992).

Atomic force microscopy

For imaging, the pectin fractions were dispersed in distilled water to a concentration of 1 mg/mL and further diluted to 10 μg/mL. Aliquots (10 μL) of the diluted samples were deposited onto sheets of mica and allowed to dry under ambient conditions before imaging by AFM in air. AFM imaging was performed using a Multi-Mode Atomic Force Microscope (Veeco Instruments, Rochester, NY) with a Nanoscope IIIa controller, operated in tapping mode. Silicon cantilevers (Olympus, Tokyo, Japan) with spring constants of about 40 N/m were employed for all images. Most images were acquired at a scan speed of 5000 nm/s (2.5 Hz over a 1000 nm × 1000 nm area) although some smaller scans were obtained at 2500 nm/s (2.5 Hz over a 500 nm × 500 nm area). Analysis of feature areas and lengths was made using the software SPIP (Image Metrology, Hørsholm, Denmark).

Cells and cell culture

Leukocytes were isolated from rat spleen cell suspensions by layering the suspension onto Lymphoprep (Nycomed, Norway) and spinning for 20 min at 650 g. Dendritic cells were generated from spleen monocytes, isolated by adherence of the spleen cells to plastic for 2 h at 37°C in cRPMI (5 × 10⁶ cells/mL). The nonadherent cells were washed away and the adherent cells cultured for 6 days in the presence of 10 ng/mL rat IL-4 (Peprotech, London, UK) and 50 ng/mL rat GM-CSF (R&D Systems, City, UK). The cells developed the phenotype of dendritic cells, being positive for CD11c, CD86, OX62, and MHC-II (using the OX6-FITC antibody) (data not shown). The purity was routinely 80–90% CD11c positive cells. Macrophages were generated from monocytes isolated by adherence as described above, but cultured with 40 ng/mL M-CSF for 7 days prior to use. The cells

developed the phenotype of macrophages. The rat macrophage cell line R2 was cultured in cRPMI and split every second day.

Measurement of NO release

Macrophages were plated at a density of 1×10^6 cells/mL in a 96-well flat bottomed plate (a total of 5×10^4 cells/well) and incubated in a medium alone, or medium containing various concentrations of the polysaccharide fractions or LPS as a positive control. Cells were incubated overnight at 37°C in humidified atmosphere containing 5% of CO₂. The test solutions were then centrifuged at 1300 rpm for 2 min, and the amount of NO in the culture medium was determined using a colorimetric method with NaNO₂ as a standard. The culture supernatant (50 µL) was mixed with an equal volume of Griess reagent A (1% (w/v) sulphanilamide in 5% (V/V) phosphoric acid) and incubated at room temperature in the dark for 10 min. After the addition of 50 µL 0.1% (w/v) *N*-(1-naphthyl) ethylenediamine dihydrochloride in water, the absorbance was measured at 540 nm.

Maturation of dendritic cells

Dendritic cells were isolated by gentle pipetting and resuspended in cRPMI at 1×10^6 cells/mL. Cells were added to 96-well flat bottom plates, at 100 µL per well. BP1002 extracts were added at a final concentration of 50 µg/mL, and LPS as a positive control at a final concentration of 200 ng/mL. The cells were incubated for 24 h at 37°C in humidified atmosphere containing 5% of CO₂. Acquisition of a mature phenotype was analyzed by staining the cells with an antibody for MHC class II (mAb OX6 directly coupled to FITC, kind gift from the MRC Cellular Immunology Unit, Oxford, UK) and PE-conjugated rat CD86 (BD Biosciences Europe, Belgium). To control for cytotoxic effects of the BP1002 extracts, the dendritic cells were also separately stained with propidium iodide in order to quantify dead cells. The samples were analyzed on a Becton–Dickinson FACSCalibur flow cytometer.

Statistical analysis

Experimental values were expressed as mean \pm SD. The statistical significance of differences between two mean values was evaluated by the two-tailed unpaired *t*-test, where values of $P \leq 0.05$ were considered to be statistically significant.

Funding

The Global Health Programme under the Norwegian Research Council and the National Program for Development, Research, and Education (NUFU project PRO 22/2002).

Acknowledgements

The authors are indebted to Finn Tønnesen, School of Pharmacy, University of Oslo, for performing the GC–MS analysis.

Conflict of interest statement

None declared.

Abbreviations

AFM, atomic force microscopy; AG, arabinogalactan; Api, apiose; Ara, arabinose; DC, dendritic cell; Dha, 3-deoxy-D-lyxo-2-heptulosaric acid; DNS, dinitrosalicylic acid; FCS, fetal calf serum; Fuc, fucose; Gal, galactose; GalA, galacturonic acid; GC, gas chromatography; Glc, glucose; GlcA, glucuronic acid; HG, homogalacturonan; KDO, 3-deoxy-D-manno-2-octulosonic acid; LPS, lipopolysaccharide; MALLS, multiangle laser light scattering; MFI, mean fluorescence intensity; MS, mass spectroscopy; NK, natural killer; PBS, Dulbecco's phosphate buffered saline; RG-I, rhamnogalacturonan I; RG-II, rhamnogalacturonan II; Rha, rhamnose; SEC, size-exclusion chromatography; Xyl, xylose.

References

- Barsett H, Paulsen BS, Habte Y. 1992. Further characterization of polysaccharides in seeds from *Ulmus glabra* Huds. *Carbohydr Polym.* 18:125–130.
- Bui AK, Bacic A, Pettolino F. 2006. Polysaccharide composition of the fruit juice of *Morinda citrifolia* (Noni). *Phytochemistry.* 67:1271–1275.
- Carpita NC, McCann MC. 2002. The functions of cell wall polysaccharides in composition and architecture revealed through mutations. *Plant Soil.* 247:71–80.
- Chambers RE, Clamp JR. 1971. An assessment of methanolysis and other factors used in the analysis of carbohydrate-containing materials. *Biochem J.* 125:1009–1018.
- Chapman HD, Morris VJ, Selvendran RR, O'Neill MA. 1987. Static and dynamic light-scattering studies of pectic polysaccharides from the middle lamellae and primary cell walls of cider apples. *Carbohydr Res.* 165:53–68.
- Ciucanu I, Kerek F. 1984. A simple and rapid method for the permethylation of carbohydrates. *Carbohydr Res.* 131:209–217.
- Daas PJ, Voragen AG, Schols HA. 2001. Study of the methyl ester distribution in pectin with endo-polygalacturonase and high-performance size-exclusion chromatography. *Biopolymers.* 58:195–203.
- Diallo D, Marston A, Terreaux C, Toure Y, Paulsen BS, Hostettmann K. 2001. Screening of Malian medicinal plants for antifungal, larvicidal, molluscicidal, antioxidant and radical scavenging activities. *Phytother Res.* 15:401–406.
- Dourado F, Madureira P, Carvalho V, Coelho R, Coimbra MA, Vilanova M, Mota M, Gama FM. 2004. Purification, structure and immunobiological activity of an arabinan-rich pectic polysaccharide from the cell walls of *Prunus dulcis* seeds. *Carbohydr Res.* 339:2555–2566.
- Dubois M, Giles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method of determination of sugars and related substances. *Anal Chem.* 28:350–356.
- Guruvayoorappan C, Kuttan G. 2007. Effect of *Biophytum sensitivum* on cell-mediated immune response in mice. *Immunopharmacol Immunotoxicol.* 29:337–350.
- Han SB, Lee CW, Kang MR, Yoon YD, Kang JS, Lee KH, Yoon WK, Lee K, Park SK, Kim HM. 2006. Pectic polysaccharide isolated from *Angelica gigas* Nakai inhibits melanoma cell metastasis and growth by directly preventing cell adhesion and activating host immune functions. *Cancer Lett.* 243:264–273.
- Harding SE. 1994. Determination of absolute molecular weights using sedimentation equilibrium analytical ultracentrifugation. *Methods Mol Biol.* 22:75–84.
- Harding SE, Vårum KM, Stokke BT, Smidsrød O. 1991. Molecular weight determination of polysaccharides. In: White CA, ed. *Advances in carbohydrate analysis.* 1:63–144.
- Hirano M, Kiyohara H, Matsumoto T, Yamada H. 1994. Structural studies of endopolygalacturonase-resistant fragments of an antiulcer pectin from the roots of *Bupleurum falcatum* L. *Carbohydr Res.* 251:145–162.
- Hokputsa S, Harding SE, Inngjerdigen K, Jumel K, Michaelsen TE, Heinze T, Koschella A, Paulsen BS. 2004. Bioactive polysaccharides from the stems of the Thai medicinal plant *Acanthus ebracteatus*: Their chemical and physical features. *Carbohydr Res.* 339:753–762.
- Hokputsa S, Jumel K, Alexander C, Harding SE. 2003. A comparison of molecular mass determination of hyaluronic acid using SEC/MALLS and sedimentation equilibrium. *Eur Biophys J.* 32:450–456.

- Huisman MM, Brul LP, Thomas-Oates JE, Haverkamp J, Schols HA, Voragen AG. 2001. The occurrence of internal (1→5)-linked arabinofuranose and arabinopyranose residues in arabinogalactan side chains from soybean pectic substances. *Carbohydr Res.* 330:103–114.
- Inngjerdingen K, Nergard CS, Diallo D, Mounkoro PP, Paulsen BS. 2004. An ethnopharmacological survey of plants used for wound healing in Dogonland, Mali, West Africa. *J Ethnopharmacol.* 92:233–244.
- Inngjerdingen KT, Coulibaly A, Diallo D, Michaelsen TE, Paulsen BS. 2006. A complement fixing polysaccharide from *Biophytum petersianum* Klotzsch, a medicinal plant from Mali, West Africa. *Biomacromolecules.* 7:48–53.
- Inngjerdingen KT, Debes SC, Inngjerdingen M, Hokputsa S, Harding SE, Rolstad B, Michaelsen TE, Diallo D, Paulsen BS. 2005. Bioactive pectic polysaccharides from *Glinus oppositifolius* (L.) Aug. DC., a Malian medicinal plant, isolation and partial characterization. *J Ethnopharmacol.* 101:204–214.
- Inngjerdingen KT, Patel TR, Chen X, Kenne L, Allen S, Morris GA, Harding SE, Matsumoto T, Diallo D, Yamada H, et al. 2007. Immunological and structural properties of a pectic polymer from *Glinus oppositifolius*. *Glycobiology.* 17:1299–1310.
- Kim J-B, Carpita NC. 1992. Changes in esterification of the uronic acid groups of cell wall polysaccharides during elongation of maize coleoptiles. *Plant Physiol.* 98:646–653.
- Kim JY, Yoon YD, Ahn JM, Kang JS, Park SK, Lee K, Song KB, Kim HM, Han SB. 2007. Angelan isolated from *Angelica gigas* Nakai induces dendritic cell maturation through toll-like receptor 4. *Int Immunopharmacol.* 7:78–87.
- Knutsen SH. 1991. Carrageenase production in a culture of *Pseudomonas carrageenovora* growing on kappa-carrageenan. In: Garcia-Reina G, Pedersen M, editors. *Proceedings of a COST-48 Workshop*. Universidad de Las Palmas de Gran Canaria, Las Palmas, Spain. Grand Canaria (Spain).
- Laue TM, Shah BD, Ridgeway TM, Pelletier SL. 1992. Computer-aided interpretation of analytical sedimentation data for proteins. In: Analytical ultracentrifugation in biochemistry and polymer science—Harding SE, Rowe AJ, Horton JC, eds. Cambridge (UK): The Royal Society of Chemistry. 90–125.
- Michaelsen TE, Gilje A, Samuelsen AB, Hogasen K, Paulsen BS. 2000. Interaction between human complement and a pectin type polysaccharide fraction, PMII, from the leaves of *Plantago major* L. *Scand J Immunol.* 52:483–490.
- Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem.* 31:426–428.
- Nergard CS, Diallo D, Michaelsen TE, Malterud KE, Kiyohara H, Matsumoto T, Yamada H, Paulsen BS. 2004. Isolation, partial characterisation and immunomodulating activities of polysaccharides from *Vernonia kotschyana* Sch. Bip. ex Walp. *J Ethnopharmacol.* 91:141–152.
- Nergard CS, Matsumoto T, Inngjerdingen M, Inngjerdingen K, Hokputsa S, Harding SE, Michaelsen TE, Diallo D, Kiyohara H, Paulsen BS, et al. 2005. Structural and immunological studies of a pectin and a pectic arabinogalactan from *Vernonia kotschyana* Sch. Bip. ex Walp. (Asteraceae). *Carbohydr Res.* 340:115–130.
- Oechslein R, Lutz MV, Amado R. 2003. Pectic substances isolated from apple cellulosic residue: Structural characterisation of a new type of rhamnogalacturonan I. *Carbohydr Polym.* 51:301–310.
- O'Neill MA, Ishii T, Albersheim P, Darvill AG. 2004. Rhamnogalacturonan II: Structure and function of a borate cross-linked cell wall pectic polysaccharide. *Annu Rev Plant Biol.* 55:109–139.
- O'Neill MA, York WS. 2003. The composition and structure of plant primary cell walls. In: Rose JKC, editor. *The Plant Cell Wall*. Oxford (UK): Blackwell. p. 1–54.
- Ovodova RG, Popov SV, Bushneva OA, Golovchenko VV, Chizhov AO, Klinov DV, Ovodov YS. 2006. Branching of the galacturonan backbone of comaruman, a pectin from the marsh cinquefoil *Comarum palustre* L. *Biochemistry (Moscow)*. 71:538–542.
- Paulsen BS, Barsett H. 2005. Bioactive pectic polysaccharides. *Adv Polym Sci.* 186:69–101.
- Perez S, Rodríguez-Carvajal MA, Doco T. 2003. A complex plant cell wall polysaccharide: Rhamnogalacturonan II. A structure in quest of a function. *Biochimie.* 85:109–121.
- Renard CM, Crepeau MJ, Thibault JF. 1999. Glucuronic acid directly linked to galacturonic acid in the rhamnogalacturonan backbone of beet pectins. *Eur J Biochem.* 266:566–574.
- Round AN, Rigby NM, MacDougall AJ, Ring SG, Morris VJ. 2001. Investigating the nature of branching in pectin by atomic force microscopy and carbohydrate analysis. *Carbohydr Res.* 331:337–342.
- Sakurai MH, Matsumoto T, Kiyohara H, Yamada H. 1999. B-cell proliferation activity of pectic polysaccharide from a medicinal herb, the roots of *Bupleurum falcatum* L. and its structural requirement. *Immunology.* 97:540–547.
- Samuelsen AB, Paulsen BS, Wold JK, Otsuka H, Kiyohara H, Yamada H, Knutsen SH. 1996. Characterization of a biologically active pectin from *Plantago major* L. *Carbohydr Polym.* 30:37–44.
- Schuck P, Rossmannith P. 2000. Determination of the sedimentation coefficient distribution by least-squares boundary modeling. *Biopolymers.* 54:328–341.
- Shin KS, Kiyohara H, Matsumoto T, Yamada H. 1997. Rhamnogalacturonan II from the leaves of *Panax ginseng* C.A. Meyer as a macrophage Fc receptor expression-enhancing polysaccharide. *Carbohydr Res.* 300:239–249.
- Singleton VL, Rossi JAJ. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am J Enol Viticult.* 37:144–158.
- Strasser GR, Amadó R. 2001. Pectic substances of red beet (*Beta vulgaris conditiva*): Part I. Structural analysis of rhamnogalacturonan I using enzymatic degradation and methylation analysis. *Carbohydr Polym.* 44:63–70.
- Sweet DP, Shapiro RH, Albersheim P. 1975. Quantitative analysis by various g.l.c. response-factor theories for partially methylated and partially ethylated alditol acetates. *Carbohydr Res.* 40:217–225.
- Theisen A, Johann C, Deacon MP, Harding SE. 2000. Refractive increment data-book for polymer and biomolecular scientists. Nottingham (UK): Nottingham University Press
- Vincken JJP, Schols HA, Oomen RJ, Beldman G, Visser RG, Voragen AG. 2003. Pectin – The hairy thing. In: Voragen AGJ, Schols H, Visser R, editors. *Advances in Pectin and Pectinase Research*. Dordrecht (Germany): Kluwer. p. 47–59.
- Vincken JP, Schols HA, Oomen RJ, McCann MC, Ulvskov P, Voragen AG, Visser RG. 2003. If homogalacturonan were a side chain of rhamnogalacturonan I. Implications for cell wall architecture. *Plant Physiol.* 132:1781–1789.
- Wagner H, Kraus S. 2000. News on immunologically active plant polysaccharides. In: Paulsen BS, editor. *Bioactive Carbohydrate Polymers*. Dordrecht (Germany): Kluwer. p. 1–14.
- Yamada H, Kiyohara H. 1999. Complement-activating polysaccharides from medicinal herbs. In: Wagner, editor. *Immunomodulatory Agents from Plants*. Basel (Switzerland): Birkhäuser. p. 161–202.
- Yamada H, Kiyohara H. 2007. Immunomodulating activity of plant polysaccharide structures. In: Kamerling JP, editor. *Comprehensive Glycoscience: From Chemistry to Systems Biology*. Amsterdam: Elsevier. p. 663–694.
- Yang H, An H, Li Y. 2006. Manipulate and stretch single pectin molecules with modified molecular combing and fluid fixation techniques. *Eur Food Res Technol.* 223:78–82.