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Full Length Research Paper

Chemical composition and immunological activities of polysaccharides isolated from the malian medicinal plant *Syzygium guineense*

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In Mali and other West African countries, the leaves of the tree *Syzygium guineense* are utilized in the traditional medicine against various ailments such as wound healing, ulcers, diarrhoea, rheumatism and infections. The immune modulating activity may in part be conferred by pectic polysaccharides, which is a major constituent of the plant cell wall. Previous studies on pectic polysaccharides from the leaves of Malian medicinal trees have shown that they posses several immunological activities. In the present study, we have isolated and characterised two immunologically active polysaccharide fractions from the leaves of *S. guineense*. One of the fractions contained an arabinogalactan type II polysaccharide, called Sg50A1, while the other polysaccharide fraction was a mixture of oligosaccharides of the pectic type, called Sg50A2. Both polysaccharides had high complement fixing ability, as well as the ability to stimulate nitric oxide release from macrophages, up-regulation of CD86 on dendritic cells, and proliferation of B cells. Sg50A1 and Sg50A2 also stimulated the secretion of pro-inflammatory and anti-inflammatory cytokines from both B cells and dendritic cells. Collectively, these results indicate that the presence of arabinogalactan structures within the polysaccharides is important for immunological activity and that plant-derived polysaccharides may be able to influence inflammatory processes.

Key words: Pectic polysaccharides, arabinogalactan, *Syzygium guineense*, immunomodulation.

INTRODUCTION

Plant polysaccharides (from mushrooms, algae and higher plants) have attracted attention due to their therapeutic potential as anti-tumour, wound healing, anti-ulcer, or anti-atherosclerotic agents (Schepetkin and Quinn, 2006). Most of these actions are thought to involve components of the innate immune system such as the complement system, and the release of reactive oxygen species (ROS), nitric oxide (NO), and cytokines by macrophages, dendritic cells, and granulocytes (Michaelsen et al., 2000; Schepetkin and Quinn, 2006). Diverse plant polysaccharides are shown to interact specifically with pattern recognition receptors on innate leukocytes such as lectin-like receptors, toll-like receptors, and scavenger receptors on leukocytes (Kim et al., 2007; Yamada and Kiyohara, 2007; Goodridge et al., 2009).

Pectic polysaccharides are major constituents of the plant cell wall, and 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 carboxy-methyl 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 arabinogalactan side chains that may be either type I or type II. Complexes of RG-I and arabinogalactan types I and II are often referred to as pectic "hairy regions"(Perez et al., 2003). Type II arabinogalactans are widespread in plants, found as separate molecules, as arabinogalactan proteins, or as attached to the rhamnose units of RG-I in pectic polysaccharides (Clarke et al., 1979; Paulsen and Barsett, 2005). Plant-derived arabinogalactans are acknowledged as potent inducers of biological activities, such as activation of the complement system (Yamada and Kiyohara, 2007), and many plants used in traditional medicine all over the world appear to be rich in immunologically active polysaccharides (Paulsen, 2001).

We have previously shown that pectic polysaccharides from several Malian medicinal plants like *Entada africana, Trichilia emetica, Vernonia kostchyana, Cochlospermum tinctorium, Biophytum petersianum,* and *Glinus oppositifolius* have immunomodulatory activities in biological screening assays, with effects varying from complement fixation to activation of macrophages and dendritic cells (Diallo et al., 2001; Diallo et al., 2002; Nergard et al., 2005; Inngjerdingen et al., 2005; Inngjerdingen et al., 2006; Kim et al., 2007; Yamada and Kiyohara, 2007; Inngjerdingen et al., 2007b; Inngjerdingen et al., 2008). The ability of pectic polysaccharides to modulate components of the immune system responses, may in part explain some of the beneficial effects of medicinal plants. Previously published data on the structure of bioactive pectins indicates that their observed biological activities are due to rhamnogalacturonan regions rich in neutral sugar side chains such as arabinan, galactan and arabinogalactan (Yamada and Kiyohara, 2007).

Syzygium guineense (Willd.) DC, Myrtaceae, is a flowering tree growing wild in sub-Sahara as well as in southern regions of Africa. In Mali, Senegal, and Sierra Leone a decoction of its leaves have been used in the traditional medicine for the treatment of wounds, ulcers, diarrhoea, rheumatism, and infections (Burkhill, 1997; Djoukeng et al., 2005). In this paper, we describe the isolation, chemical composition, and immunological activities of pectic polysaccharides from the leaves of *S. guineense*.

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Abbreviations: CFSE, carboxyfluoroscein succinimidyl ester; **LPS,** lipopolysaccharide; **MFI,** median fluorescence intensity; **NO,** nitric oxide; **RG,** rhamnogalacturonan; **TLR,** toll- like receptor.

MATERIALS AND METHODS

Plant material

The leaves of *Syzygium guineense* (Wild) DC, of the family Myrtaceae, were purchased from the Centre of herbalists at the Medina market in Bamako, Mali. The material was identified by professor Drissa Diallo, Department of Traditional Medicine, Bamako, Mali, and a voucher specimen is kept in the herbarium of the institute, file numbers, 1816, 1817 and 2498.

Extraction and purification of polysaccharides

In order to remove low molecular weight compounds, the leaves of *S. guineense* (200 g) were pre-extracted by Soxhlet-extraction with dichloromethane (DCM) and methanol. Exchange of extraction solvent was determined by the colour of the solution. Subsequently, the dried plant material was extracted twice with 50°C water (3 L) for 4 h and filtered through a fine nylon mesh, and concentrated by rotary evaporation under diminished pressure and 40°C. To remove ions and other low molecular weight compounds the extract was passed through a BIO-GEL P6-DG column and eluted with distilled water. The eluate was monitored with the phenol-sulphuric acid test (Dubois et al., 1956), and the high molecular weight material pooled. This extract was called SgC50. After extraction of the plant material at 50°C, the residue was extracted with water of 100°C. SgC50 and SgC100 were both separated by anion-exchange chromatography on DEAE Sepharose Fast Flow column, (5 x 30 cm) (Amersham Bioscience). The column was coupled to a Perimax pump (Spetec). The extract was filtered (5 µm) before application on the column. Neutral polysaccharides were eluted with distilled water (2 ml/min), while acidic polysaccharides were eluted with a NaCl gradient (0 - 2.0 M, 2 ml/min). Fractions of 10 ml were collected using a Superfrac fraction collector (GE Healthcare). The phenol-sulphuric acid assay was used to determine the carbohydrate content in the fractions (Dubois et al., 1956). The relevant acidic fractions were pooled, dialysed and freeze dried. These pools were denominated Sg50A and Sg100A respectively. Sg50A and Sg100A were both further separated on a Sephacryl S-400 HR column (GE Healthcare), (2.5 x 80 cm). The relevant fractions were collected into two parts, Sg50A1 and Sg50A2 for Sg50A, and into three parts for Sg100A termed Sg100A1, A2 and A3, followed by dialysis and freeze drying. Scheme 1 gives the summary of the fractionation. The fractions Sg50A1 and Sg50A2 were rechromatographed on the Sephacryl S-400HR column for final purification. The polysaccharides were routinely tested for lipopolysaccharide (LPS) contamination. The protein content of both fractions were determined by the Lowry test and found to be 0.3 and 2.3%, respectively (data not shown).

Chemical analysis of polysaccharides

Determination of carbohydrate composition

1 mg of samples was subjected to methanolysis with 4 M hydrochloric acid (HCl) in anhydrous methanol for 24 h at 80°C (Barsett and Paulsen, 1991; Chambers and Clamp, 1971). Mannitol was used as an internal standard. After the methanolysis the reagents were removed under a stream of N_2 and the methyl-

glycosides were dried in vacuum over phosphorus pentoxide (P2O5) for at least 1 h prior to conversion into the corresponding trimethyl silyl ethers (TMS)-derivates. The samples were subjected to capillary gas chromatography (Carlo Erba 6000 Vegas Series 2) as described by Barsett and Paulsen (1991).

Glycosidic linkages within the polymers

Prior to methylation the free uronic acids were reduced to their corresponding neutral sugars. The free uronic acids were activated with carbodiimide and reduced with sodium tetradeuteroborate ($NaBD_4$) as described by Sims and Bacic (1995). The reduced polymers were methylated with Ciucanu and Kerek's method (1984) modified by McConville et al. (1990). The methylated polysaccharides were hydrolysed with trifluoroacetic acid (TFA) and the monomers reduced with 1 M $NaBD_4$ in 2 M ammonium hydroxide (NH_4OH). The monomers were acetylated to partially methylated alditol acetates (PMAA) by adding 200 µl 1-methylimidazole and 2 ml acetic acid anhydride. The samples were mixed and dissolved by sonication and allowed to stand for 10 min. Excess of reagent was destroyed by adding 10 ml distilled water, mixed and allowed to stand for another 10 min. The PMAA were extracted over in 2 x 1 ml DCM. The DCM-phase was extracted with 2 x 5 ml distilled water before the DCM was evaporated under N_2. The PMAA were dissolved in 100 µl methanol prior to GC-MS analysis. The derived partially methylated alditol acetates were analyzed by GC-MS on Fisons GC 8065 using split injection and a Fisons fused silica column (30 m x 0.2 mm i.d.) with a film thickness of 0.20 mm as described by Inngjerdingen et al. (2006). The compounds at each peak were characterized by interpretation of the retention time and the characteristic mass spectra. The relative amounts of each linkage type were estimated from the total amount of each monosaccharide obtained from the methanolysis analysis.

Weak acid hydrolysis

In order to determine whether the arabinose units were present in furanose or pyranose form in the polymer, weak acid hydrolysis were performed using 0.05 M oxalic acid at 100°C for 2 h followed by neutralization with sodium carbonate. High and low molecular weight material was separated on a PD 10 (Amersham) column and the relevant fractions analyzed for monosaccharide composition by GC as above.

Polydispersity and molecular weight determination

Polydispersity and molecular weight determination of the acidic polysaccharides were determined by gel filtration on a Superdex 200 10/30 GL (GE Healthcare) coupled to a RI-detector. The samples were eluted with 10 mM NaCl at 0.5 ml/min (1.5 times the column volume), collecting 1 ml fractions.

Bioactivity studies

Animals

Eight to 12-week-old rats of the PVG strain were used. Animals were killed by CO_2 inhalation. The rats have been maintained at the Institute of Basic Medical Sciences for more than 20 generations. Rats were housed in compliance with guidelines set by the Experimental Animal board under the Ministry of Agriculture of Norway and The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes . The laboratory animal facilities are subject to a routine health-monitoring program and tested for infectious organisms according to a modification of Federation of European Laboratory Animal Science Associations (FELASA) recommendations.

Cells and cell culture

The mouse macrophage cell line RAW 264.7 was maintained in cRPMI (cRPMI: Roswell Park Memorial Institute (RPMI) 1640, 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1% sodium pyruvate, 1% non-essential amino acids, and 2-mercaptoethanol; all chemicals purchased from Invitrogen, Paisley, UK) and split every second day. Rat B cells were isolated from splenic mononuclear cells by positive selection using sheep anti-rat IgG Dynabeads (Invitrogen Dynal, Oslo, Norway) (70 µl beads/2 x 10^7 spleen cells). The cell/bead conjugates were resuspended in cRPMI, and incubated overnight at 37°C in humidified atmosphere with 5% of CO_2. Dendritic cells were generated from bone marrow cells. Bone marrow from rat femurs was passed through a 70 µm cell strainer to obtain a cell suspension. The resulting cell suspension was layered onto Nycoprep (Axis-Shield, Scotland) and spun at 1800 rpm for 20 min at room temperature. Cells were harvested and cultured for 7 days in 100 mm Petri dishes (Nunc, Roskilde, Denmark) at 1 x 10^6 cells/ml in cRPMI containing 50 ng/ml granulocyte macrophage colony-stimulating factor (GM-CSF) and 1 ng/ml interleukin (IL)-4 (R&D Systems, UK). Cells were fed every third day with fresh medium supplemented with GM-CSF and IL-4. The cells developed the phenotype of dendritic cells, and the purity was routinely 80 - 90% CD11c positive cells (using the antibody CD11c-FITC, BD Biosciences Europe).

Complement fixation assay

The complement fixation test is based on inhibition of haemolysis of antibody sensitized sheep red blood cells, SRBC, by human sera as described by Michaelsen et al. (2000) (Method A). PMII, a pectin fraction from the leaves of Plantago major, was used as a positive control (Samuelsen et al., 1996). Inhibition of lysis induced by the test samples were calculated by the formula ((Acontrol – Atest)/ Acontrol) x 100%. From these data a dose-response curve was created to calculate the concentration of test sample giving 50% inhibition of lysis (IC_{50}). A low IC_{50} value means a high complement fixing activity.

Nitric oxide release

RAW 264.7 macrophages were plated at a density of 5 x 10^5 cells/ml in 96-well flat bottomed plates (5 x 10^4 cells/well), and cultured in duplicates with the indicated concentrations of polysaccharides, LPS (derived from *Pseudomonas aeruginosa* 10, Sigma-Aldrich) or medium alone. Cells were incubated for 24 h and then centrifuged at 1300 rpm for 2 min. Cell-free supernatants were harvested, and the amount of nitric oxide was determined using a colorimetric method with sodium nitrite ($NaNO_2$) as a standard (Griess, 1879). Supernatants (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 addition of 50 µl 0.1% (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride in water (Griess reagent B), the absorbance was measured at 540 nm.

B-cell proliferation

B cells were stained with 5 µM carboxyfluoroscein succinimidyl ester (CFSE; Molecular Probes Invitrogen, Paisley, UK) in

phosphate buffered saline (PBS) supplemented with 2% FCS for 10 min/37˚C. After washes in cRPMI, cells were cultured in duplicates in 96-well plates (2 x 10^5 cells/well) with indicated concentrations of polysaccharides, LPS as positive control, or medium alone as negative control. Proliferation was assessed after 5 days, by analyzing the dilution of CFSE in the population by flow cytometry. Dead cells were excluded by staining the cells with propidium iodide (Molecular Probes Invitrogen, Paisley, UK).

Maturation of dendritic cells

Dendritic cells were isolated by gentle pipetting. Cells (1 x 10^6 cells/ml) were added to 96-well flat bottom plates, and polysaccharide extracts or LPS were added in the indicated concentrations. The cells were incubated for 24 h, and acquisition of a mature phenotype was analysed by co-staining the cells with CD11c-FITC and PE-conjugated rat CD86 (BD Biosciences Europe, Belgium). An acquisition gate was set for cells that were positive for CD11c. The samples were analysed on a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences Europe, Belgium).

Cytokine measurements

B cells or dendritic cells were stimulated overnight in duplicates in the presence of polysaccharide fractions, LPS, or medium as negative control in cRPMI (1 x 10^5 cells/100 µl). The supernatants were harvested and frozen at -80°C until assayed. Concentrations of released cytokines were measured using a multiplex cytokine immuno-assay (MilliplexTM MAP, rat cytokine/chemokine kit) in duplicates of 25 µL undiluted supernatants according to the protocol supplied by the manufacturer. The presence of IL-1α, IL-6, IL-10, IL-12p70, IL-18, tumor necrosis factor (TNF)-α, monocyte chemotactic protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and interferon-inducible protein (IP)-10 were measured simultaneously. The samples were read and analyzed using the Luminex xMAP platform (Bio-Rad, Hercules, CA). The cytokine concentrations were determined from a standard curve assayed at the same time with defined cytokine reference samples using Bio-Plex Manager 4.1 software.

Statistical analysis

Experimental values were expressed as mean ± SEM. The statistical significance of differences between two mean values (treated sample versus negative control) was evaluated by the two-tailed unpaired t-test, whereas the statistical differences between mean values of sample-treated groups were analyzed by the one-way analysis of variance (ANOVA) with Tukey's multiple comparison test. Values of $p < 0.05$ were considered to be statistically significant.

RESULTS

Purification and structural elucidation of *S. guineense* polysaccharides

Fractions of polysaccharides were obtained from the leaves of *S. guineense* after extractions with organic solvents followed by hot water extraction at 50 and 100°C (Scheme 1). The two main acidic polysaccharide fractions, Sg50A and Sg100A, were further purified by gel filtration yielding the sub fractions Sg50A1, Sg50A2, and Sg100A1-3. To confirm the pectic nature of the polysaccharides, the monosaccharide composition was determined for all five fractions. The major constituents of Sg50A were Arabinose (Ara, 23%) and Galactose (Gal, 33%), while Sg100A consisted primarily of Galacturonic acid (GalA, 69%). Thus arabinogalactans was mainly present in the Sg50A polymer, as well as in the two acidic sub-fractions, Sg50A1 and Sg50A2 (Table 1). However, Sg50A1 contained more Ara and Gal as compared to Sg50A2. The higher amount of GalA in Sg50A2 (20% vs 0%), indicates the presence of homogalacturonan backbones in this particular fraction. While Sg100A contained little Ara and Gal, we detected in the sub fraction Sg100A1 the presence of both Ara (18%) and Gal (33%) (Table 1). Thus, Sg100A1 bears certain similarities with Sg50A and its sub fractions.

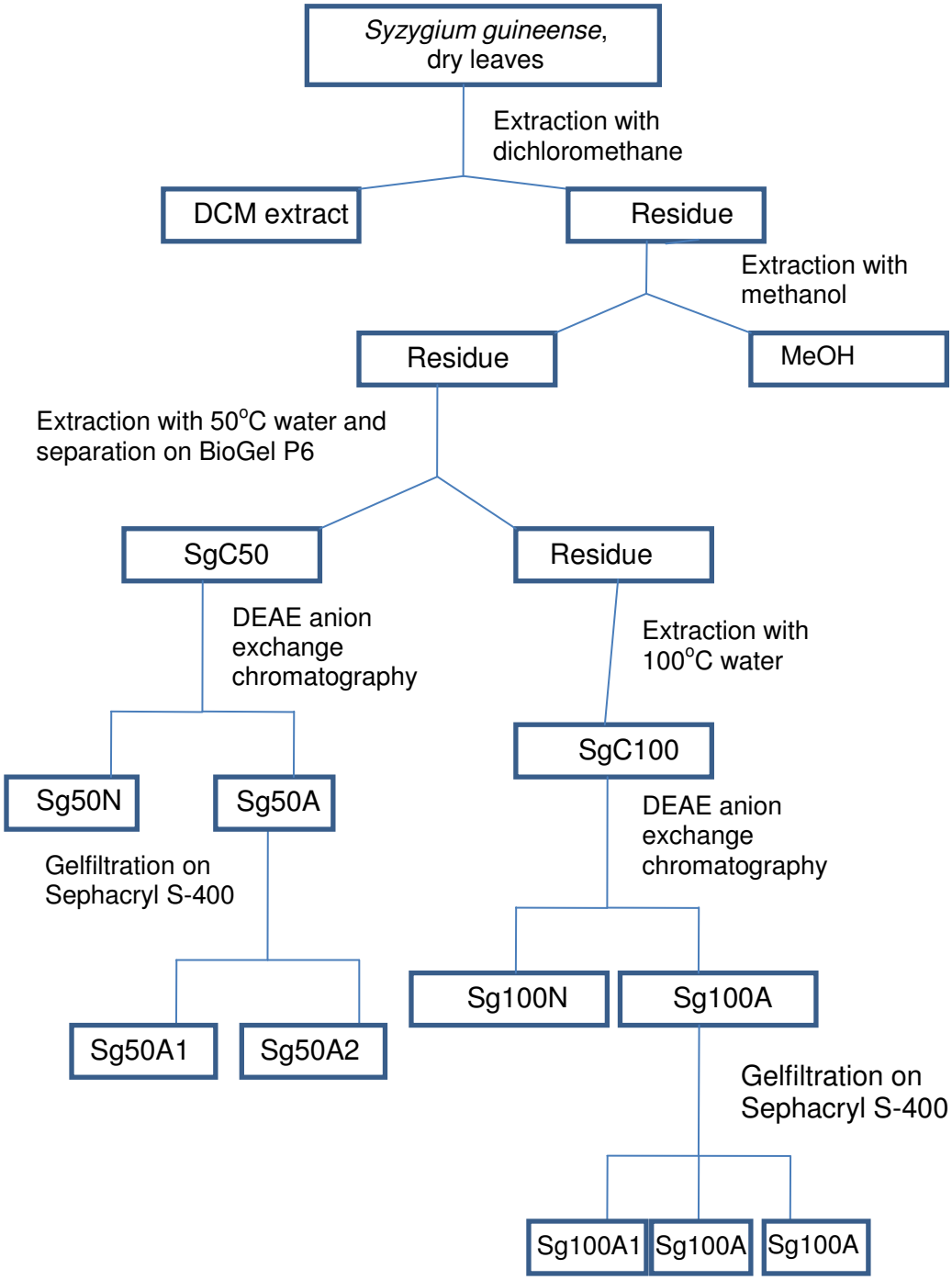
Structural elucidations revealed that Sg50A1 was of an almost pure, highly branched arabinogalactan type II polysaccharide (Table 2). All arabinose units were present in the furanose form. Negligible amounts of Rha and GalA may suggest a minor backbone of RG-I present in the molecule. Sg50A2 contained small amounts of terminal Rha (5.8%) and 1→2p Rha (2.0%) which is indicative of only a short backbone of rhamnogalac-turonan (Table 2). The presence of 1→4p GalA suggests presence of also small amounts of homogalacturonan chains, which is not present in Sg50A1. Determination of the molecular weight distribution of Sg50A1 and Sg50A2 showed a low degree of polydispersity, with a mean of app. 36 kDa for Sg50A1 and ap. 2.3 kDa for Sg50A2 (Table 2).

Complement fixation activity of Sg50A1 and Sg50A2

To determine the bioactivity of the isolated pectic polysaccharide fractions, we analysed their complement fixation ability. The purified sub fractions Sg50A1 and Sg50A2 both demonstrated high complement fixation activities, as compared to a pectic polysaccharide from *Plantago major* (termed PMII) with previously documented complement activity (Table 3) (Michaelsen et al., 2000). Similarly, we observed complement activity of the arabinogalactan-containing sub-fraction Sg100A1, while Sg100A2 and Sg100A3 were less active. Thus the presence arabinogalactan structures are important for complement fixation activity. Based on the activity of the fractions and amount of material available, only the two acidic sub-fractions from the 50°C extracts were studied further.

Sg50A1 and Sg50A2 activates macrophages, dendritic cells and B cells

We next determined the ability of Sg50A1 and Sg50A2 to activate macrophages. To assess macrophage activation, we measured the production of nitric oxide (NO) following



Scheme 1. Extraction and fractionation scheme of *S. guineense*. After ion-exchange chromatography two acidic fractions were isolated: Sg50A and Sg100A. Gel filtration of Sg50A yielded the two sub fractions Sg50A1 and Sg50A2, while gel filtration of Sg100A yielded Sg100A1-3.

treatment of the mouse macrophage cell line RAW 264.7 with Sg50A1 or Sg50A2 for 24 h. LPS, a constituent of the outer membrane of gram negative bacteria, was utilised as a positive control, as it potently stimulates cells of the monocytic lineage (Sweet and Hume, 1996). A dose-dependent release of NO was observed from macrophages stimulated with both Sg50A1 and Sg50A2 (Figure 1). The polysaccharides caused an almost four-fold increase in NO-release at 100 µg/ml compared to the negative control. There was no significant difference in NO-release between Sg50A1 and Sg50A2.

An important leukocyte population of the monocytic

Table 1. Monosaccharide compositions (mol%) of the acidic polysaccharide fractions obtained at 50 or 100ºC from the leaves of *S. guineense*.

Monosaccharide composition[1]	Sg50A	Sg50A1	Sg50A2	Sg100A	Sg100A1	Sg100A2	Sg100A3
Ara	23	54.1	30.8	8	18	8	5
Rha	7	1.5	8.3	8	6	10	8
Fuc	Traces	n.d.[2]	n.d.	Traces	Traces	1	Traces
Xyl	1	n.d.	n.d.	2	1	2	2
Man	7	n.d.	n.d.	1	5	1	1
Gal	33	37.4	27.5	10	33	12	11
Glc	5	1.3	2.4	3	7	3	4
4-O-methyl GlcA	4	n.d.	n.d.	n.d.	8	n.d.	n.d.
GlcA	9	5.7	10.9	n.d.	9	5	2
GalA	11	n.d.	20.1	69	14	59	66

[1] % of total carbohydrate content, [2] n.d.: not detected.

Table 2. The linkages (mol%) of the monosaccharides present in the polysaccharide fractions Sg50A1 and Sg50A2.

Monosaccharide	Type of linkage	Sg50A1	Sg50A2
Arabinose*f*	T	33	22
	1,3	2.7	1.6
	1,5	15.7	6.1
	1,3,5	2.2	0.6
	1,2,5	0.5	0.4
Rhamnose*p*	T	1.5	5.8
	1,2	n.d.[1]	2.0
	1,2,4	n.d.	0.4
Glucose*p*	T	n.d.	Traces
	1,4	n.d.	2.4
Galactose*p*	T	3.3	4.1
	1,3	2.2	2.5
	1,4	2.2	n.d.
	1,6	3.4	2.4
	1,3,6	13.1	12.5
	1,3,4,6	13.1	5.8
Galacturonic acid*p*	1,4	n.d.	20.1
Glucoronic acid*p*	T	n.d.	10.9
	1,4	5.7	8.0

Mw, average 36.1 kDa 2.3 kDa, [1]n.d.: Not detected.

lineage are dendritic cells, which like macrophages are specialized in recognizing foreign molecules. Activation of dendritic cells may lead to their maturation and up-regulation of many cell surface markers, including the co-stimulatory molecule CD86. Here, we stimulated rat bone marrow-derived dendritic cells for 24 h with either LPS as positive control (250 ng/ml) or 100 µg/ml of Sg50A1 and Sg50A2. The expression levels of CD86 were analysed by flow cytometry (Figure 2). The median fluorescence intensity (MFI) of CD86 expression by stimulated dendritic cells relative to the MFI of untreated cells was calculated. Both polysaccharides Sg50A1 and Sg50A2 increased CD86 expression on dendritic cells significantly (up to 1.9 ± 0.17) and comparably to LPS-stimulated

Table 3. Complement fixation assays showing the IC_{50} values (µg/ml) of *S. guineense* polysaccharide fractions compared to the IC_{50} values of the positive control PMII (a pectic polysaccharide from Plantago major).

Fractions	IC_{50}	IC_{50} PMII
Sg50A1	14,0	> 125
Sg50A2	82,8	> 125
Sg100A1	25,2	> 125
Sg100A2	> 125	> 125
Sg100A3	> 125	> 125

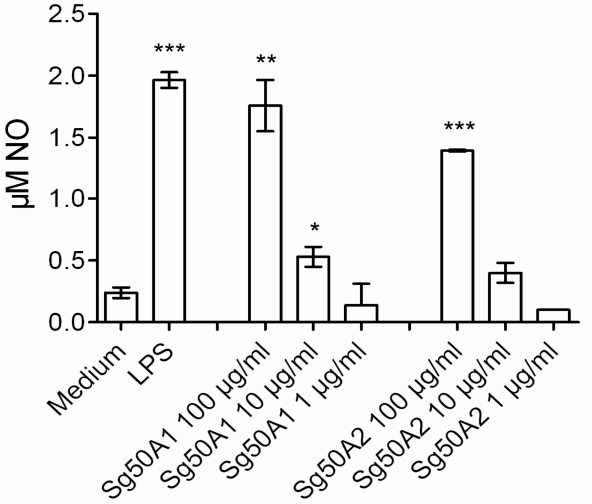


Figure 1. Measurement of nitric oxide release from Raw 264.7 macrophages after overnight stimulation with different concentrations of the *S. guineense* fractions Sg50A1 or Sg50A2, 250 ng/ml LPS as positive control, or medium alone. Data are presented as the mean of three independent experiments ± SEM. ***, $p < 0.001$; **, $p < 0.0095$; and *, $p < 0.045$ (versus negative control).

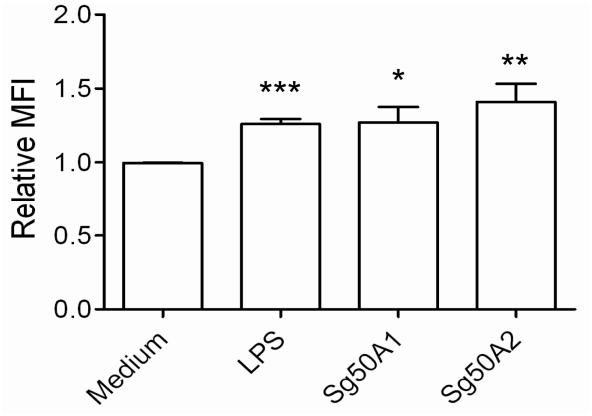


Figure 2. Maturation of immature dendritic cells with *S. guineense* polysaccharides. Bone marrow-derived dendritic cells were incubated for 24 h with 100 µg/ml of Sg50A1 or Sg50A2, 250 ng/ml LPS, or medium alone. The surface expression level of CD86 was evaluated by flow cytometry. The median fluorescence intensity of CD86 by untreated cells was set to 1, and the fluorescence of CD86 on the stimulated cells calculated relative to the untreated sample. Data are presented as the mean of three independent experiments ± SEM. ***, $p < 0.0002$; **, $p < 0.0096$; and *, $p < 0.013$ (versus negative control).

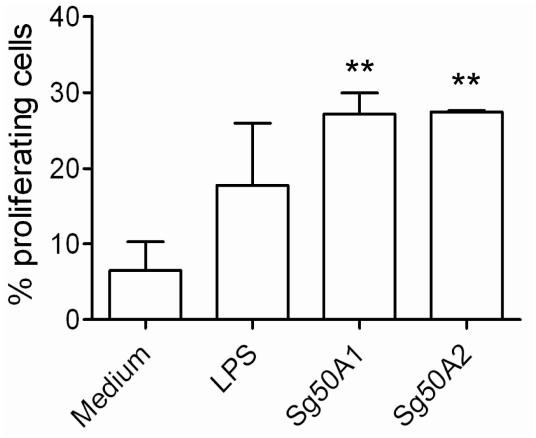


Figure 3. B-cell proliferation induced by *S. guineense* polysaccharides. CFSE-stained splenic B cells were cultured in the presence of 100 µg/ml Sg50A1 or Sg50A2, 250 ng/ml LPS, or medium alone for 5 days. Percent proliferated cells were calculated on the basis of the decrease in CFSE fluorescence intensity of B cells in the different samples. Data are presented as the mean of three independent experiments ± SEM. **, $p < 0.0075$ (versus negative control).

cells (1.27 ± 0.1).

We next tested whether the polysaccharides could induce B-cell proliferation. LPS, a known B cell mitogen, was included as positive control. Rat splenic B cells were labelled with the intracellular dye CFSE (see Materials and Methods), and cultured in the presence of Sg50A1, Sg50A2, or LPS for 5 days. Cell divisions will result in dilution of the CFSE fluorescence from the starting population, and we applied flow cytometric analysis to calculate the percent proliferating cells in untreated and treated samples. We observed a robust proliferative response of both Sg50A1 and Sg50A2 as compared to LPS (Figure 3).

Cytokine release by B cells and dendritic cells

The production and secretion of cytokines is an important event following activation of leukocytes. To further confirm the stimulatory properties of the polysaccharides from *S. guineense*, we measured their ability to induce cytokine release from both dendritic cells and B cells, as representatives of the innate and adaptive arms of

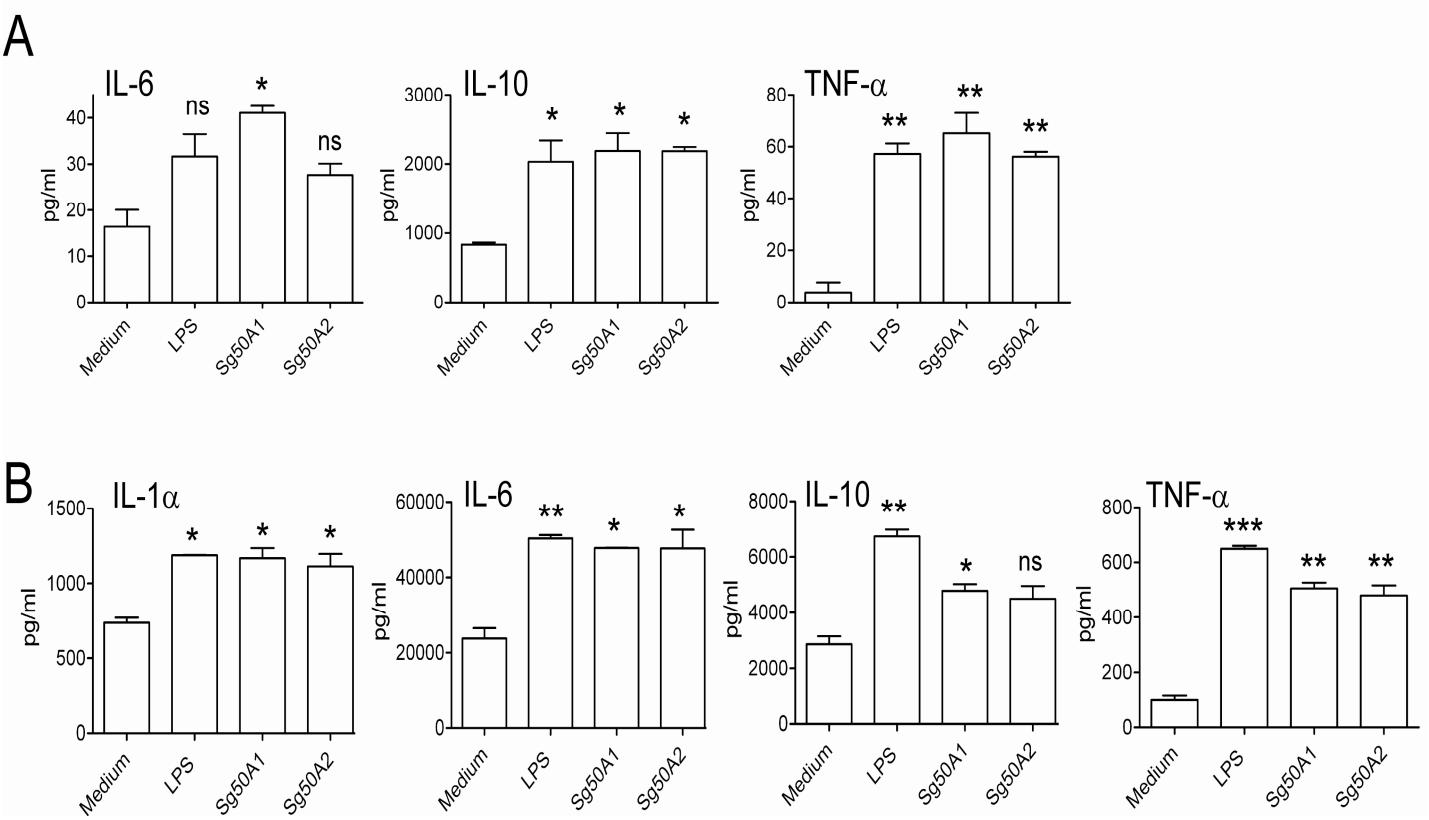


Figure 4. Induction of cytokine secretion from (A) B cells or (B) dendritic cells. Cells were stimulated for 20 h in duplicates with Sg50A1 or Sg50A2 at 100 µg/ml, 250 ng/ml LPS as positive control, or medium alone as negative control. Cell-free supernatants were harvested, and the concentrations of cytokines measured as described in Materials and Methods. The data presented represents the mean ± SEM from one out of two independent experiments. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$; ns, not significant. Data were analyzed by the one-way analysis of variance (ANOVA), with Tukey's multiple comparison test.

immunity. The cells were stimulated for 24 h in the presence of either medium alone, 250 ng/ml LPS, or 100 µg/ml of Sg50A1 or Sg50A2. Cell-free supernatants were tested using a multiplex cytokine assay, where we simultaneously measured the presence of IL-1α, IL-6, IL-10, IL-12p70, IL-18, TNF-α, MCP-1, MIP-1α, and IP-10. We found that B cells secreted IL-6, IL-10, and TNF-α in response to both Sg50A1 and Sg50A2, and none of the other cytokines tested (Figure 4A). Similarly, dendritic cells were induced to release IL-1α, IL-6, IL-10, and TNF-α (Figure 4B). There were no differences in the abilities of Sg50A1 or Sg50A2 to induce cytokine release from either B cell or dendritic cells, and we therefore conclude that both Sg50A1 and Sg50A2 are equally potent inducers of cytokines.

DISCUSSION

We have studied the chemical composition and immunological activities of polysaccharides isolated from the Malian medicinal plant *Syzygium guineense* that is used by traditional African healers for treatment of wounds, infections, and gastric ulcers. Based on both monosaccharide composition and linkage analysis we have concluded that the structure of the fraction Sg50A1 appears to be a typical arabinogalactan type II. This was also confirmed with the Yariv reagent test (data not shown). Also, long chains of 5-linked arabinofuranosides, slightly branched on position 2 and 3, as well as high amounts of terminal arabinofuranosides support the arabinogalactan type II structure (Paulsen and Barsett, 2005). The Sg50A2 fraction consists of small oligosaccharides of ap. 10-15 units with the typical pectin structures.

The ability to activate macrophages is the predominant finding from a number of studies investigating the immunomodulating effects of plant polysaccharides (Schepetkin and Quinn, 2006). These effects include increased phagocytic activity, increased reactive oxygen species and nitric oxide production, as well as enhanced cytokine production. In a number of previous studies, we have shown that pectic polysaccharide fractions from *Vernonia kotschyana* (Nergard et al., 2005), *Glinus oppositifolius* (Inngjerdingen et al., 2005), and *Trichilia emetica* (Diallo et al., 2003) have complement fixation

activity. We documented similar complement fixation activity of polysaccharides within the acidic fractions obtained from *S. Guineense* in our present study, in contrast to low activity of polysaccharides in the neutral fractions (data not shown). In addition, pectic polysaccharide fractions from *Biophytum petersianum* have shown complement fixing activities, as well as potent activation of nitric oxide release from macrophages (Inngjerdingen et al., 2006; Inngjerdingen et al., 2008). Pectic polysaccharides isolated from *Bupleurum falcatum*, *Glinus oppositifolius*, and *Opilia celtidifolia* (Grønhaug TE, submitted manuscript) have also been shown to promote B-cell proliferation and activation of dendritic cells (Sakurai et al., 1999; Inngjerdingen et al., 2007a). Also our analysis of cytokine secretion suggests that the pectic polysaccharides may influence inflammation, as we observe potent production of the pro-inflammatory cytokines IL-1α, IL-6, and TNF-α. However, as both B cells and dendritic cells also produce high amounts of the anti-inflammatory cytokine IL-10 in response to the pectic polysaccharides, the polysaccharides may also contribute to dampening or regulating the strength of the inflammatory processes. Similarly, macrophages have been shown to produce both pro-inflammatory and anti-inflammatory cytokines in response to plant polysaccharides (Schepetkin et al., 2005).

Sg50A1 and Sg50A2 differ in structure. While Sg50A1 is a pure arabinogalactan, Sg50A2 is a “classical” pectin with a backbone of rhamnogalacturonan. However, both samples induced a comparable response, indicating that the presence of arabinogalactans is a determining factor for the bioactivity of both polysaccharides. The precise molecular mechanism by which arabinogalactans interacts with components of the immune system remains to be determined, but we speculate that both polysaccharides may interact with a common structure present on the surface of antigen presenting cells. The immunomodulatory function of other plant-derived polysaccharides is thought to be mediated through pattern recognition receptors such as lectin-like receptors (mannose receptor and Dectin-1), Toll-like receptors (TLRs) and scavenger receptors on leukocytes (Schepetkin and Quinn, 2006). Specifically, TLR4 has been identified as a receptor for acidic plant-derived polysaccharides (Shao et al., 2004; Kim et al., 2007). TLR4 may therefore be a potential candidate receptor for *S. guineense* polysaccharides, as this receptor is expressed on macrophages, dendritic cells and B cells. Of note, TLR4, as well as other pattern recognition receptors, are the same receptors that are involved in the recognition of microbial polysaccharides. Plant-derived arabinogalactans may therefore mimic microbial arabinogalactans.

In conclusion, we have shown that polysaccharides from the leaves of the Malian medicinal plant *S. guineense* contain fractions rich in arabinogalactan structures. These arabinogalactan polysaccharides potently stimulate macrophages, dendritic cells, and B cells, and may alongside other arabinogalactan-containing polysaccharides be ideal candidates for therapeutic agents such as adjuvants.

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