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Isolation, characterization and structural determination of a unique type of arabinogalactan from an immunostimulatory extract of *Chlorella pyrenoidosa*

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Abstract—An arabinogalactan was isolated from a hot water extract of freeze-dried cells of the green microalga, *Chlorella pyrenoidosa*. This hot water extract is a proprietary immunomodulator, with the trademark Respondin™ (ONC-107). The arabinogalactan was recovered from the ethanol-soluble fraction of the supernatant resulting from a process that involved controlled ethanol precipitation followed by size exclusion chromatography on Sephadex G-100, then Cetavlon precipitation. Sugar analyses, GC–MS data for (*S*)-2-octyl glycosides, and 1D and 2D NMR experiments established unambiguously that the repeating unit was →2)-α-L-Ara*f*-(1→3)-[α-L-Ara*f*-(1→4)]-β-D-Gal*p*-(1→. This structure does not fit into any of the known classes of arabinogalactans. SEC/MALS experiments gave a molecular mass for the arabinogalactan isolated as 47 ± 4 kDa but the original structure was probably larger.

Keywords: *Chlorella pyrenoidosa*; Immunomodulators; NMR spectroscopy; Polysaccharide structure determination; Arabinogalactans

1. Introduction

Chlorella, one of the prime targets of the algae industry is a unicellular green alga found in both fresh and marine water. Its popularity in the Far East has been based on the belief, and more recently, on scientific evidence that it can have health benefits.[1] There is considerable evidence that *Chlorella* cells contain immunostimulatory principles, many of them of polysaccharide nature[2–11] but detailed studies of the structures of these polysaccharides are lacking. In most cases, only monosaccharide composition has been reported.

The first structural information about polysaccharides from *Chlorella* was reported by White and Barber in 1972.[3] An acidic polysaccharide was isolated from *Chlorella pyrenoidosa* containing mostly rhamnose (52%) with both arabinose and galactose in about equal amounts (12% and 13%, respectively). One reticuloendothelial system activating polysaccharide has been isolated from *Chlorella ellipsoidea* and two others were isolated from *C. pyrenoidosa* that all contained glucose as the major component.[4,11] The first of these contained both arabinose and galactose in minor quantities[4] while the latter two contained fucose or rhamnose with lesser amounts of galactose and other sugars but not arabinose.[11] Two neutral glycans were isolated from a *Chlorella* extract, an α-D-glucan and an α-D-arabino-α-L-rhamno-α-D-galactan.[5] Matsubayashi reported a high-viscosity hetero-polysaccharide from *Chlorella* sp. K-4035 containing rhamnose, arabinose, mannose and uronic acids.[8] Noda et al. isolated a mainly galactose-containing (80%) extracellular polysaccharide from

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Chlorella that contained 80% galactose.[9] Yalcin et al. reported the isolation of another extracellular polysaccharide from *Chlorella* sp. containing glucuronic acid and arabinose as major components (38.3% and 32.5%, respectively).[7] Recently, three different mono-*O*-methylated sugars, 3-*O*-methyl-D-galactose and 2- and 3-*O*-methyl-L-rhamnose, have been reported as minor components in polysaccharide preparations from *Chlorella vulgaris* K-22 by Ogawa et al.[12,13] Partial hydrolysis of an acidic polysaccharide from this species yielded α-D-Glc*p*A(1→3)-L-Rha[14] and α-D-Glc*p*A(1→3)-α-L-Rha*p*(1→2)-L-Rha[15] and this latter structure has been confirmed by synthesis.[16] In 2001, Pugh et al. reported the isolation of a polysaccharide from *C. pyrenoidosa* that contained both arabinose (31.6%) and galactose (26.3%) plus traces of many other sugars.[2] This polysaccharide was a potent activator of human monocytes/phagocytes.

Polysaccharides are also responsible for the bulk of the immunostimulatory activity of Respondin™, a proprietary immune builder that Ocean Nutrition Canada Ltd has developed recently from extracts of *C. pyrenoidosa* cells.[17] Its stimulatory activity has been proven in a human clinical trial[18] and some evidence as to how the immunomodulation is induced has been provided by studies in mice.[19]

Analyses of the immune response of some fractions of Respondin™ isolated by chromatography suggested that a number of polysaccharides or polysaccharide–protein complexes are responsible for the immunoactivity.[17,20] We have now started a detailed study of these components to better understand the immune response. Further fractionation resulted in isolation of several distinctive polysaccharides and here we report on isolation and complete structure of a unique arabinogalactan.

2. Experimental

2.1. Materials

Lyophilized *C. pyrenoidosa* (CP) cells were obtained from Taiwan Chlorella Manufacturers Ltd (Taipei, Taiwan). Chemicals (analytical grade) and dialysis membranes with a molecular mass cutoff of 12 kDa were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO), unless otherwise specified. Alditol acetate standards for GLC–MS carbohydrate analyses were obtained from Supelco, Inc. (St. Louis, MO).

2.2. Extraction of *C. pyrenoidosa* cells and isolation of the polysaccharide

C. pyrenoidosa freeze-dried cells (200 g) were suspended in 1 L of distilled water and extracted for 1 h at 80 °C. The supernatant was separated by centrifugation at 3829*g* for 20 min and the residual cells were re-extracted with 500 mL of distilled water under identical conditions. After centrifugation both supernatants were combined and the volume 10-fold reduced in vacuo yielding the crude extract (CE).

The crude extract (CE) was fractionally precipitated with ethanol. One and one-half volumes of 95% ethanol were added to CE and the suspension kept overnight at 4 °C. The sediment (A, 13.2 g) was centrifuged off and the supernatant re-precipitated using a 3:1 ethanol–water ratio (v/v). After centrifugation, the precipitate (B) was stored for further purification and the supernatant treated with more 95% ethanol (5:1 ratio) yielding a precipitate (C) and a soluble fraction (D). The precipitate B was decolourized by stirring with 2:1 (v/v) $CHCl_3$–CH_3OH mixtures (2×100 mL) for 30 min. Decolourized B was dissolved in water, dialyzed and freeze dried to yield fraction E (1.4 g).

Fraction E was further fractionated by size exclusion chromatography (SEC). The separation was performed under normal pressure in an AKTA FPLC system (Amersham Pharmacia Biotech, Uppsala, Sweden), UV monitor: UPC-900, pump: P-920, control system: UNICORN software version 3.0 using a HR 10/30 column of Sephadex G-100 (Amersham Biosciences, PQ, Canada) and scaled up using larger preparative runs. The sample was dissolved in 0.2 M sodium chloride, filtered through a 0.45 μm filter and chromatographed in the same mobile phase at 0.1 mL/min flow rate. The elution was automatically monitored by continuously measuring the optical density at 280 nm for protein detection purposes. Fractions (1 mL) were collected and the presence of carbohydrates determined by the phenol–sulfuric acid method.[21] For preparative purposes, an XK 50/100 column (Amersham Biosciences) was employed with the flow rate of the mobile phase adjusted to 1.5 mL/min. Fractions (18 mL) were collected. One fraction (E1, 388 mg) was in the excluded volume and was studied further. Two others were included (E2, 244 mg and E3, 82 mg).

Aqueous Cetavlon (hexadecyltrimethylammonium bromide) solution (3 mL, 10%) was slowly added to 30 mL of a 1% aqueous solution of fraction E1. The mixture was kept overnight at 4 °C and centrifuged to yield a precipitate (F, 204 mg) and a supernatant (G). The former was kept for further studies and the latter precipitated with 5 vol of 95% ethanol. The precipitate (H, 96 mg) isolated by centrifugation was kept and the supernatant was concentrated in vacuo, dialyzed and freeze dried to yield the fraction of interest (I, 20 mg).

Fraction I was isolated a second time using exactly the same procedure as above, then was further purified by anion exchange chromatography on a Q-Sepharose-Fast-Flow column using a flow rate of 0.6 mL/min. Elution with water, followed by dialysis and freeze-drying yielded the neutral polysaccharide, fraction J.

2.3. Sugar and protein analysis

Gas liquid chromatography–mass spectrometry (GLC–MS) was carried out with a ThermoFinnigan Trace 2000 gas chromatograph coupled to a PolarisQ ion-trap mass spectrometer (Finnigan, Austin, TX) in the electro-impact ionization (EI) mode with an ionization potential of 70 eV. The operating conditions were as follows: column BPX-70 (30 m × 0.25 mm internal diameter × 0.25 μm film thickness); carrier gas, helium at 1.5 mL/min constant flow; PTV injector temperature 250 °C; PTV split ratio, 10:1; GC oven temperature profile: 1 min at 190 °C, raised to 240 °C at 5 °C/min, hold for 3 min, 7 °C/min to 260 °C and hold for 1.14 min.

Fraction I (~1 mg) was hydrolyzed with 1 M TFA (0.5 mL, 100 °C, 1.5 h). The released monosaccharides were reduced with 0.5 M $NaBH_4$ in 1 M NH_4OH (0.6 mL) and acetylated with 3 mL Ac_2O after the excess of $NaBH_4$ had been quenched by successive washing with 10% AcOH in MeOH. The resulting products were subjected to GLC–MS analysis.[22,23]

The absolute configurations of the monosaccharides that were obtained by hydrolysis of fraction I with 1 M TFA at 100 °C for 1.5 h were determined by preparation of glycosides with (*S*)-2-octanol, peracetylation and comparison with authentic standards using GLC as described.[24,25]

Protein determination was performed using the bicinchoninic acid (BCA) kit purchased from Pierce (Rockford, IL) using the method of Smith et al.[26] Amino acid analysis was performed in the Alberta Peptide Institute, Heritage Medical Research Centre, University of Alberta, Edmonton, Alberta, Canada. The procedure was: 1.1 mg of the sample was weighed, dissolved in 6 M HCl and split into two, then kept for 1 h at 160 °C. The samples were concentrated under vacuum, dissolved in 1000 μL of running buffer and then 50 μL of each was injected into a Beckman System 6300 Amino acid Analyzer; System Gold v 8.1.

2.4. Nuclear magnetic resonance (NMR) spectroscopy

All NMR spectra were recorded at 27 °C on a Bruker–Avance 500 MHz spectrometer equipped with an Hewlett–Packard XW-5000 computer workstation, where data were acquired and processed using the standard Bruker XWINNMR version 3.5 software. Samples were dissolved in 99.97% D_2O. All proton decoupled ^{13}C NMR spectra were recorded using a Bruker 5 mm broadband observe probe at 125.76 MHz with a spectral width of 32,679 Hz and 64k complex data points. Chemical shifts are expressed in parts per million (ppm) relative to the methyl signal of internal acetone (30.89 ppm).[27]

The 1D–^1H and 2D NMR spectra (^1H detected mode) for fraction I were measured with a Bruker-5 mm inverse probe equipped with a z-gradient coil. The ^1H NMR spectrum was recorded at 500.13 MHz with a spectral width of 5482 Hz and the data collected in 64k data points. The chemical shifts in ppm were referenced to the residual HOD signal (4.79 ppm).[27] The 2D homonuclear chemical shift correlated spectroscopy (COSY) spectra were recorded over a spectral width of 5482 Hz using data sets ($t_1 \times t_2$) of 256 × 2048 data points using gradient pulses for coherence selection. The double quantum filtered 2D homonuclear chemical shift correlated spectroscopy (DQF-COSY) spectra were recorded over a spectral width of 1383 Hz using data sets ($t_1 \times t_2$) of 256 × 1024 data points and 16 scans using a magnitude double-quantum filtered COSY pulse sequence. The heteronuclear 2D ^{13}C–^1H chemical shift correlations were measured via single quantum coherence (HSQC) with ^{13}C-decoupling during acquisition of the FID using the standard pulse sequence and spectral widths of 32,679 Hz for t_1 and 5482 Hz for t_2. Data sets of 256 × 2048 points were used and for each t_1 value 16 scans were acquired. An HSQC experiment without ^{13}C-decoupling was acquired for the determination of the ^{13}C–^1H coupling ($^1J_{C,H}$) values, with the digital resolution in F_2 increased to 1.25 Hz/point. The 2D heteronuclear multiple bond-correlated (HMBC) experiment was carried out using the standard pulse sequence with a 62.5 ms delay for the evolution of the long-range couplings. For inter residue correlations, a two dimensional nuclear Overhauser enhancement spectroscopy (NOESY) experiment was performed in the phase-sensitive manner with a mixing time of 300 ms. Total correlation spectroscopy (TOCSY) 2D-spectra were recorded with a spin-lock time of 80 ms.

2.5. Homogeneity and molecular weight determination of fraction I

Homogeneity and molecular weight determination of fraction I were accomplished by SEC coupled to refractive index (RI) and multi-angle light-scattering (MALS) detectors.[28,29] Fraction I was dissolved in 88 mM sodium acetate buffer, pH 4.5 at a concentration of 4.17 mg/mL and chromatographed in the same mobile phase at 0.6 mL/min. The chromatography system consisted of a HPLC pump (Model HP 1100 iso-pump), an injection valve (Model HP 1100 ALS) fitted with a 100 μL loop and a column system comprising G3000 PW × 1 and G2500 PW × 1 columns (both 7.8 × 300 mm) connected in series. The eluent was monitored through an OptiLab DSP interferometric refractometer and then into a DAWN DSP MALS photometer placed at angles between 60° and 132°.

2.6. Biological activity

2.6.1. RAW 264.7 cell culture. Raw 264.7 cells (murine macrophage line) were obtained from the American

Tissue Culture Collection (Rockville, MD). Cells were cultured in Dulbecco's Modified Essential Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1 mM pyruvic acid, 4 mM glutamine, and 1% penicillin–streptomycin (100 U/mL each) at 37 °C in 5% CO_2. For nitric oxide measurements, cells were seeded in 48-well plates at a density of 2.5×10^5 cells per well in 500 μL culture medium (without phenol red) and grown overnight to 80–90% confluence. Cells were then treated with various concentrations of the samples to be tested dissolved in 100 μL of culture medium (final volume 600 μL). Control wells (Basal) received 100 μL of culture medium alone. LPS (10 ng/mL) from *Escherichia coli* (Sigma, Illinois) was routinely run as a positive control. Experiments were performed in duplicate or triplicate and, after 24 h treatment, culture media from replicate wells were pooled and assayed for nitrite concentration. This method was adapted from the procedure described by Rininger et al.[30]

2.6.2. Measurement of nitric oxide production. Nitric oxide production was assessed by measuring nitrite concentration in 50 μL of cell culture medium. Samples were incubated with 50 μL of Griess reagent (1.0% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride and 2.5% phosphoric acid) for 5 min at room temperature and absorbance was measured at 550 nm. Sodium nitrite dissolved in culture medium was used as the standard.

3. Results and discussion

3.1. Fractionation of CE and isolation of an arabinogalactan

The hot aqueous extraction of CP cells under the conditions described in the experimental (Fig. 1) yielded approximately 15% of the total wet cell mass, the crude extract (CE). The addition of 1.5 vol of 95% ethanol to CE precipitated approximately 45% of the amount extracted. The subsequent addition of another 1.5 vol of 95% ethanol to the supernatant (3:1 ratio) provided an additional 1.4 g of precipitate (4.7%, fraction E). Fraction E was fractionated by size exclusion chromatography using Sephadex G-100 into three different fractions (Fig. 2). The first fraction (E1) that eluted in the void volume, retained the bioactivity of E and was studied further. The other two fractions (E2) and (E3) were included and had no bioactivity. Fraction E2 also consisted mainly of polysaccharides as shown by the presence of intense broad peaks both in the anomeric and ring carbon regions in the ^{13}C NMR spectrum. The prominent signals in the anomeric region of the ^{13}C NMR spectrum of E1 were not present in that of E2 indicating that the structures of the polysaccharides in fraction E2 were not related to those in fraction E1.

Cetavlon precipitation of E1 yielded fraction F containing 58% of the total mass of E1 that was stored for further analysis. A neutral fraction (H, 27%) was recovered from the supernatant by adding 5 vol of 95% ethanol. The supernatant from the latter yielded a second neutral fraction, the fraction of interest (I, 20 mg, 7%). In the ^{13}C NMR spectrum of fraction H, the signals attributed to fraction I had about the same intensity as signals from other sugars. In the anomeric region, signals appeared at 107.0 (tallest), 105.0, 104.2, 107.7 and 108.2 ppm (shortest) in addition to the signals attributed to fraction I.

3.2. Determination of the structure of the arabinogalactan repeating unit

Monosaccharide composition analysis by GLC–MS of the alditol acetates derived after full acid hydrolysis showed that fraction I was composed of arabinose and galactose in a 2:1 ratio, respectively. The absolute configurations of arabinose and galactose were determined to be L and D, respectively, by GLC of the acetylated (*S*)-2-octyl glycosides.

The ^{13}C NMR spectrum of fraction I contained three anomeric signals at 109.1, 108.7 and 102.8 ppm, and the number of other signals expected for a trisaccharide repeating unit containing two arabinose units and one galactose unit. In agreement, the ^1H NMR spectrum displayed signals for three anomeric protons at 5.47 and 5.44 ppm (2H, broadened singlets) labelled A and B, respectively, and 4.68 ppm (d, $^3J_{1,2} = 8$ Hz) labelled C. As judged by the position of the deshielded anomeric signals (108.7 and 109.1 ppm), two out of three sugar residues are present as α-furanosides.[31,32] The ring sizes were confirmed by the positions of two nonanomeric signals at 84.9 and 84.5 ppm, chemical shifts typical of C-4 of furanosides,[31,33] and the α-configurations were confirmed by the values of $^1J_{C,H}$ for the C-1 signals (both 177 Hz) in the undecoupled HSQC spectrum.[34] The presence of only one highly deshielded nonanomeric signal from a secondary carbon atom at 89.6 ppm suggested that there was only one site of substitution on a furanoside ring, leaving the other furanose ring as a terminal residue.

In addition to the signals mentioned above, the ^1H NMR spectrum of fraction I contained smaller signals in the anomeric region at 5.52, 5.28 and 5.06 ppm. In order to determine whether these signals were due to impurities or to sugars linked covalently to the trisaccharide repeating unit, a second sample of fraction I was purified further by anion exchange chromatography to give fraction J, a neutral polysaccharide. The intensities of these minor signals were much reduced in the ^1H NMR spectrum of fraction J, indicating that they were due to impurities.

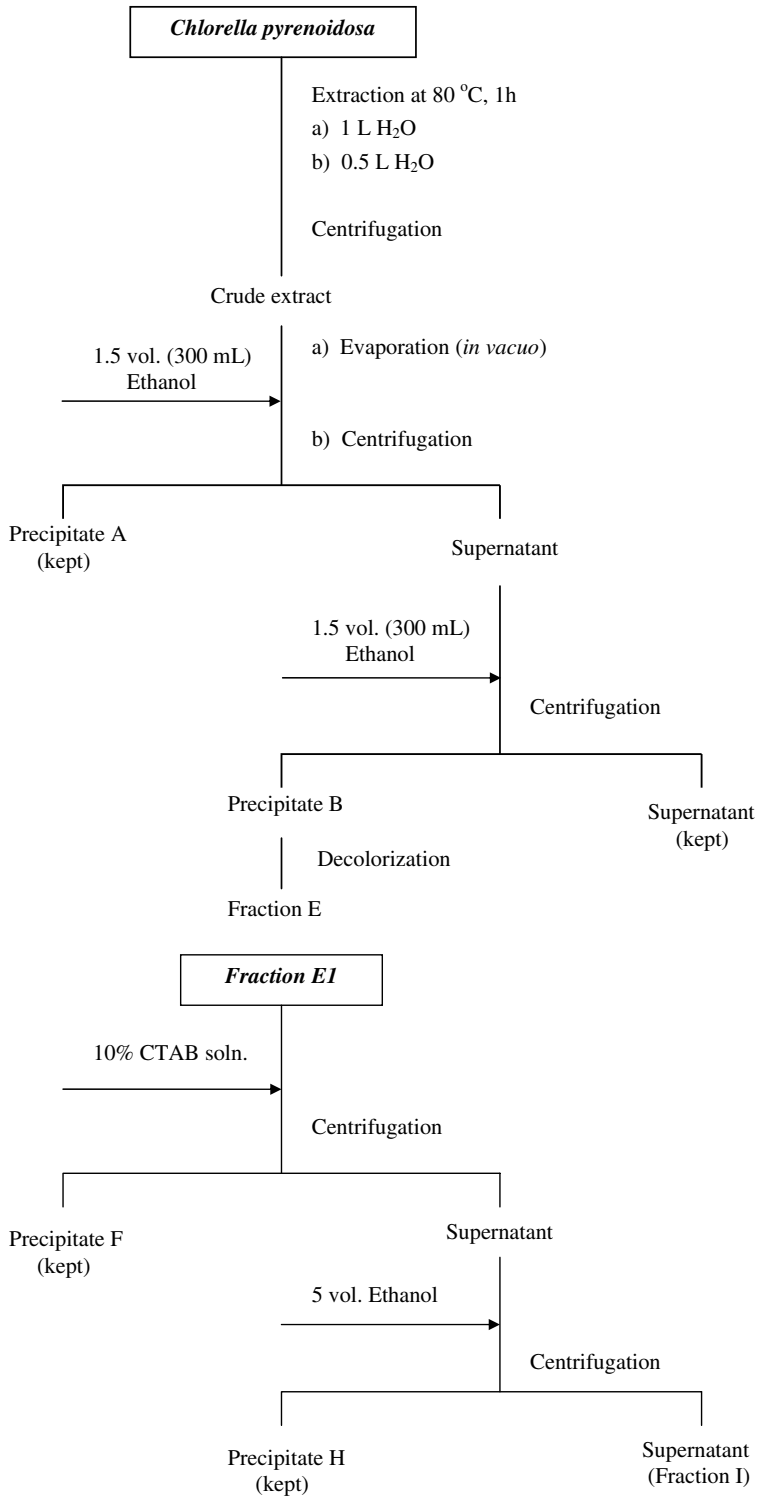


Figure 1. Description of the process used to obtain fraction I. Part 1: isolation of E. Part 2: fractionation of E1.

All ^{1}H and ^{13}C NMR signals were assigned to the three residues unequivocally (Tables 1 and 2) using DQF COSY, HSQC, DEPT and TOCSY experiments. The absence of ^{13}C NMR signals in the region 63–68 ppm, characteristic[31] of C-5 for both anomers of methyl arabinopyranoside suggested that both arabinose residues are furanosides. This conclusion requires that the galactose residue have a pyranose ring form, confirmed by the absence of signals for C-6 between 63.0–64.5 ppm, characteristic of both anomers of

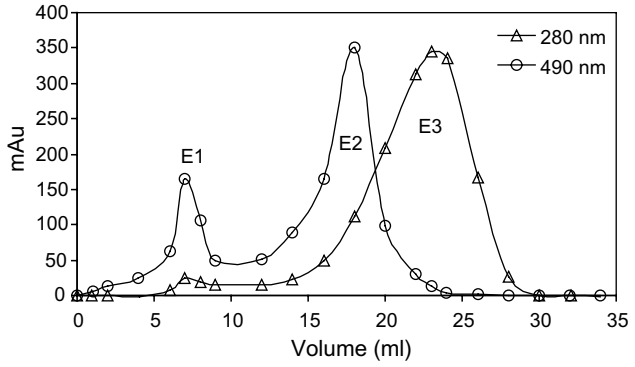


Figure 2. Size exclusion chromatography of fraction E on Sephadex G-100.

galactofuranosides[31] and confirmed by the chemical shifts of the three signals for CH_2OH groups at 62.0, 61.6 and 61.8 ppm.

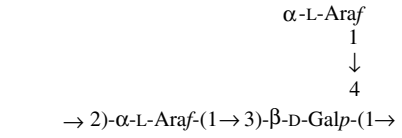
The broadened anomeric singlet at 5.47 ppm ($^3J_{1,2} < 3$Hz) from the α-L-arabinofuranoside residue A correlated in the DQF COSY experiment with the signal at 4.41 ppm (H-2) that was in turn correlated in the HSQC spectrum with a signal at 89.6 ppm. The deshielding of this C-2 signal requires that this interior arabinofuranoside unit be substituted at O-2. The chemical shift of C-3 in this residue is 75.9 ppm, similar to the value observed for C-3 of methyl α-arabinofuranoside, indicating that O-3 was unsubstituted, and as noted above, O-5 is unsubstituted.

The anomeric configuration for the D-galactopyranoside unit, residue C, was assigned as β from the $^3J_{1,2}$ value (8 Hz) spectrum measured from the signal for H-1 (4.68 ppm) and confirmed by the relatively small $^1J_{C,H}$ value (163 Hz)[34] from the correlated C-1 signal at 102.8 ppm. In the undecoupled HSQC spectrum that yielded the $^1J_{C,H}$ value, the C-1 signal appeared as a dd pattern also coupled by $^2J_{C,H}$ of 7.5 Hz to H-2. This observation is also reliable evidence for the β-configuration; $^2J_{C,H}$ values for C-1 are 5.6 Hz for β-D-glucopyranose[35] and 5.7 Hz for β-D-galactopyranose[36] but are too small to be observed for α-pyranoses and also for α-arabinofuranosides.[33]

The HMBC spectrum (Fig. 3) was used to determine the connectivity in this trisaccharide repeating unit. The presence of inter residue crosspeaks between C-1 (102.8 ppm) of residue C and H-2 of residue A (4.41 ppm) and between C-3 of residue C (80.5 ppm) with the anomeric proton signal (5.47 ppm) of residue A showed that the backbone of I has the →3)-β-D-Gal*p*(1→2)-α-L-Ara*f*-(1→ linear structure. Because residue A is only substituted at one position, O-2 (see earlier), the terminal arabinofuranoside (residue B) must be connected to the galactopyranoside unit. The ^{13}C NMR chemical shifts of this terminal residue B were almost identical (±0.4 ppm) to the corresponding signals of methyl α-L-arabinofuranoside,[31] in agreement with its assignment as a terminal unit. Residue B was shown to be attached to O-4 of the β-D-Gal*p* residue (unit C) by an HMBC correlation between H-1 of B (5.44 ppm) and the signal at 74.9 ppm, assigned to C-4 of C. This value is deshielded by 5.2 ppm from the value for C-4 in methyl β-D-galactopyranoside,[31] consistent with substitution.

Further confirmation of the linkage sequence was the observation of correlations between the anomeric protons and protons at the linked carbons in the NOESY spectrum (Fig. 4) of fraction I: Gal*p* H-1, Ara*f* (A) H-2 at 4.68 and 4.41 ppm, respectively; Ara*f* (A) H-1, Gal*p* H-3 at 5.47 and 3.87 ppm, respectively, and Ara*f* (B) H-1, Gal*p* H-4 at 5.44 and 4.17 ppm, respectively.

Based on the above mentioned data, the structure of the branched trisaccharide repeating unit of the arabinogalactan isolated from the green microalgae *C. pyrenoidosa* is as shown below.



3.3. Homogeneity and molecular weight of the arabinogalactan

Figure 5 shows the SEC/RI/MALS chromatogram for fraction I. Based on the RI profile the main component of fraction I eluted at a volume of ~12.0 mL and comprises 89% of the fraction as estimated from the area under the RI peak. The weight average molecular weight (M_w) over the whole peak was 47 ± 4 kDa and the

Table 1. ^1H NMR chemical shifts (ppm) of fraction I

	Residue	H-1	H-2	H-3	H-4	H-5 (a/b)	H-6 (a/b)
A	2)-α-L-Ara*f*-(1→	5.47	4.41	4.165	4.171	3.86/3.73	—
B	α-L-Ara*f*-(1→	5.44	4.19	3.94	4.09	3.85/3.75	—
C	3,4)-β-D-Gal*p*-(1→	4.68	3.73	3.87	4.174	3.81	3.69/3.64

Table 2. ^{13}C NMR chemical shifts (ppm) of fraction I

	Residue	C-1	C-2	C-3	C-4	C-5	C-6
A	2)-α-L-Ara*f*-(1→	108.7	89.6	75.9	84.5	61.6	—
B	α-L-Ara*f*-(1→	109.1	82.0	77.4	84.9	62.0	—
C	3,4)-β-D-Gal*p*-(1→	102.8	71.1	80.5	74.9	75.6	61.8

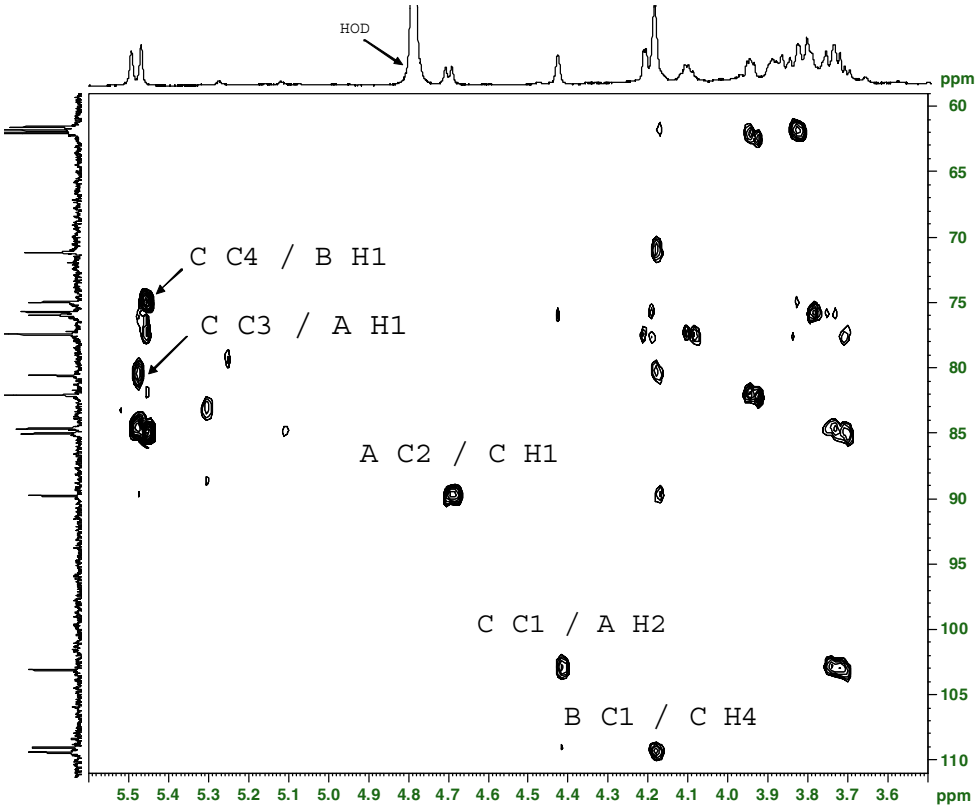


Figure 3. 500.1 MHz ^{1}H, ^{13}C HMBC spectrum of fraction I. Correlations involving glycosidic linkages are annotated.

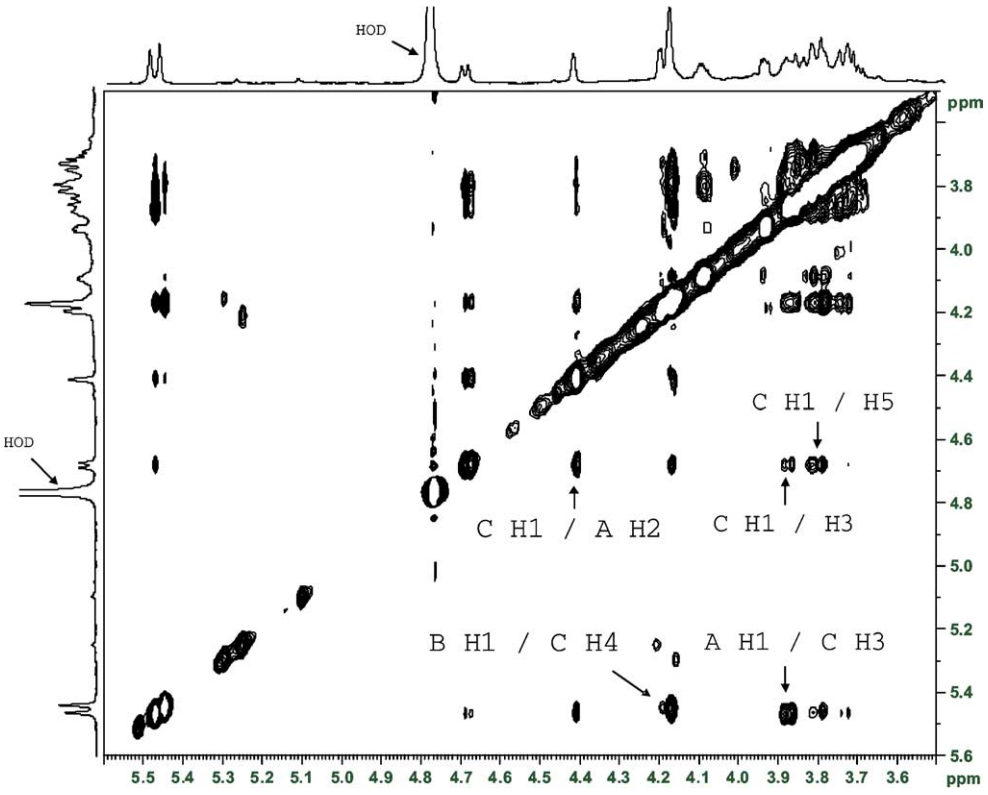


Figure 4. 500.1 MHz ^{1}H, ^{1}H NOESY spectrum of fraction I with the more relevant correlations annotated.

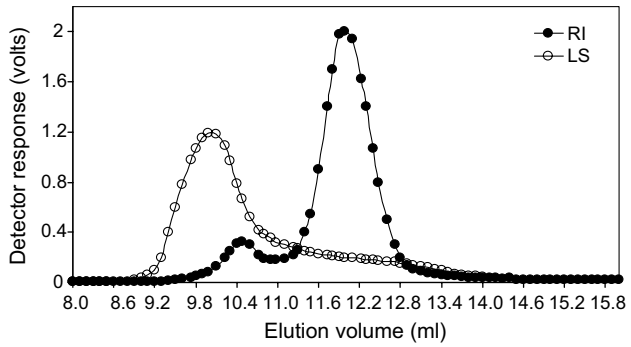


Figure 5. Light scattering and refractive index profiles from SEC-MALS of fraction I.

polydispersity value (M_w/M_n) was 2.7. Although originally precipitated from an aqueous solution by addition of three volumes of ethanol, in the final separation, fraction I was soluble when five volumes of ethanol was added to an aqueous solution. However, fraction H, the precipitate from 5 vol of ethanol, still appears to contain about 50% of fraction I, based on its ^{13}C NMR spectrum. In view of the much greater acid lability of furanosides than pyranosides,[37] it seems likely that fraction I is a fraction obtained from the original polymer by cleavage of some of the labile backbone furanoside linkages during isolation.

Additionally, fraction I contained a relatively small amount of a high molecular weight component (M_w value of 300 ± 19 kDa) eluting at a volume of ~10.5 mL as shown by the overlapping of LS and RI peaks between 9.9 and 10.8 mL. This minor component accounts for 11% of fraction I. Protein analysis of fraction I indicated that 13% protein was present and the amino acid composition of fraction I is shown in Table 3. The absence of minor peaks in fraction J, obtained by additional anion exchange chromatography of fraction I, suggests that the minor high molecular weight fraction contained most of the amino acid content.

3.4. Biological activity

The results of biological testing on the later fractions are shown in Figure 6. The activity of fraction E, as measured by its stimulation of NO synthesis, increased in the high molecular weight fraction E1. Figure 6 shows that the fraction precipitated by Cetavlon (F) had some activity but more was present in the supernatant. All of the latter activity was present in the fraction (H) precipitated by ethanol and none was present in the supernatant, fraction I, that consists mainly of the unique arabinogalactan, whose structure has been determined here. This change may result from the lower molecular weight of the polysaccharide in fraction I.

3.5. General discussion

Arabinogalactans (AGs) are present in the tissues and exudates of plants as free polysaccharides and as glycoproteins with a wide range of degree of glycosylation.[38–43]

Table 3. Amino acid composition of fraction I

	Amino acid							
	Glu	Ala	Asp	Gly	Ser	Thr	Val	Leu
% of fraction I	0.66	0.58	0.38	0.25	0.25	0.25	0.25	0.25
% of protein fraction	5.8	5.1	3.4	2.2	2.2	2.2	2.2	2.2

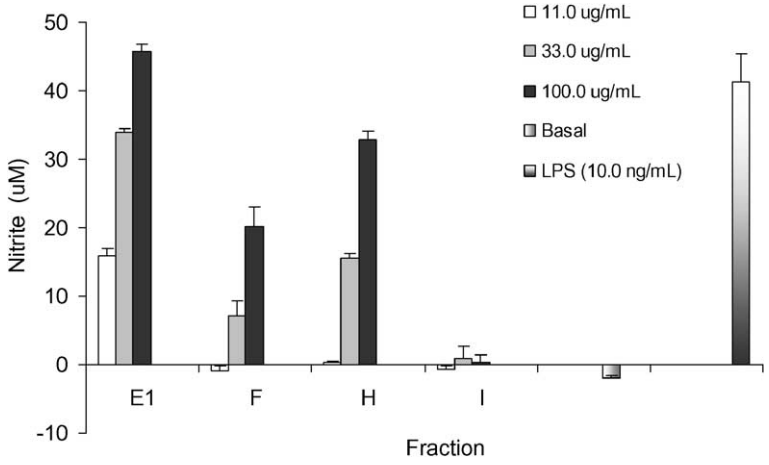


Figure 6. Stimulation of NO synthesis in RAW 264.7 cells culture supernatants treated with fractions derived from Cetavlon precipitation of fraction I.

Three types of arabinogalactans have been identified.[38] Aspinall termed arabino-4-galactans, type I AGs and arabino-3,6-galactans, type II AGs.[44,45] Type I AGs have a linear β-D-Gal*p*-(1→4) backbone with single L-Ara*f* units or 1→3, 1→5 linked L-arabinofuranosyl disaccharide side chains and in some cases D-Gal*p* attached at O-3. Other monosaccharides such as rhamnose, xylose and uronic acids are often present but no association with protein has been reported for this group. Type II AGs contain frameworks of 3-, 6-, and 3,6- linked β-D-Gal*p* residues that are substituted with α-L-Ara*f* and other sugars to varying extents, including uronic acids and other neutral monosaccharides.[38,40,41,46] Type II AGs attached to protein (AGPs) have attracted considerable attention and are thought to participate in several aspects of the life cycle of plants such as vegetative, reproductive and cell growth and development, molecular interactions and signalling, among others.[40,47] A third type contains arabinogalactan side chains attached to other polysaccharides.[38]

The structure of the AG we have described here does not resemble any of the types of AGs previously reported in the literature, mainly originating from plants. All previous arabinogalactans have backbones consisting of galactose only, as far as we are aware. We suggest that polysaccharides with linear backbones containing alternate D-galactopyranosides and L-arabinofuranosides be called type IV arabinogalactans. Polysaccharides from microalga have not been the subjects of as many detailed studies as those from plants and it seems likely that many more unique structures will be uncovered as more studies are performed.

An immunomodulating AG affecting the intestinal immune system was isolated from Juzen-Taiho-To, Japanese herbal (Kampo) medicine.[48] As with Respondin™, the complex polysaccharide composition includes AGs as one component, in that case, an arabino-3,6-galactan. Another intestinal system modulating AG, with the arabino-3,6-galactan structure, was isolated by the same group from *Atractyodoles lancea* DC.[49] Although fraction I does not stimulate directly proliferation of B cells or activity of macrophages (Fig. 6), it is our perception that the original larger polysaccharide plays a role in overall immunostimulatory activity of Respondin™. Since the bulk of the immunoactivity of Respondin™ is associated with molecular mass greater than 100 kDa, it is likely that its immunostimulatory activity in vivo is triggered (similarly to the polysaccharides composing Juzen-Taiho-To) at intestinal immunocyte sites.[20]

The immunoactivity of Respondin™, a proprietary and clinically proven immunoadjuvant, is thought to be due to the polysaccharides present in it. Further resolution of some of the fractions has already been achieved and studies in progress to elucidate the structures of these biopolymers will be reported shortly.

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