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First isolation and structural determination of cyclic β-(1→2)-glucans from an alga, *Chlorella pyrenoidosa*

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ABSTRACT

The aqueous extract of the edible green microalgae *Chlorella pyrenoidosa* is of interest because of its immunostimulatory activity. Some components in the extract have been identified previously, namely a unique type of arabinogalactan and a galactofuran. Further fractionation of this extract was accomplished by treating the aqueous solution of the fraction precipitated by addition of 1.5 vol of 95% ethanol with cetyltrimethylammonium bromide. The residue obtained by concentration of the supernatant was fractionated further by anion-exchange chromatography and size-exclusion chromatography on Sephadex G-100. Two fractions from the latter column were retained, of which one was a starch-like α-(1→4)-linked D-glucan with some α-(1→6) branches, and the other contained a starch plus a mixture of β-(1→2)-D-glucans. ESI mass spectrometry was used to show that the mixture contained both cyclic and linear β-(1→2)-D-glucans in a cyclic:linear ratio of 64:36, based on intensities of mass spectral peaks. For the cyclic β-(1→2)-D-glucans, ring sizes ranged from 18 to 35 monosaccharides with the ring containing 21 glucose units (54% of the cyclic glucans) being greater than three times more abundant than the next most abundant component, the ring containing 22 glucose units (15%). No rings containing 20 glucose units were present. This is the first observation of cyclic β-(1→2)-D-glucans in algae, as far as we are aware. For the linear β-(1→2)-D-glucans, the component containing 20 glucoses was most abundant (35% of the linear glucans), while the component containing 21 glucose units was the next most abundant (17%). These relatively low-molecular-weight glucans had low immunostimulatory activity.

1. Introduction

There is evidence that consumption of the green microalgae *Chlorella* can have health benefits,[1,2] and Ocean Nutrition Canada Ltd has developed a proprietary immune builder, Respondin™, from aqueous extracts of *Chlorella pyrenoidosa* cells.[3,4] Its stimulatory activity has been demonstrated in a human clinical trial,[5] and some evidence as to how the immunomodulation is induced has been provided by studies in mice[6] and in human blood cells.[7] It is thought that the immunostimulatory activity is due to the main constituents of the extract, the polysaccharides, and we have initiated a program to fractionate this extract, to determine the structures of the polysaccharides therein, and to identify the most active immunostimulatory components. We have previously isolated an arabinogalactan having a type of structure different from any isolated previously, one with both arabinose and galactose present in the backbone, as well as a galactofuranan.[8,9] In this publication, we describe the isolation and identification of two glucan fractions from a neutral portion of the aqueous extract, one of which contains the first cyclic β-(1→2)-D-glucans found in a source other than proteobacteria.

Although cyclic oligosaccharides and polysaccharides are comparatively rare, many examples are known,[10–12] of which the cyclomaltooligosaccharides (cyclodextrins)[13,14] are most common. Cyclic β-(1→2)-D-glucans are abundant in the periplasmic space of the α-2 subdivision of proteobacteria, which includes the mammalian pathogen *Brucella*, the plant pathogen *Agrobacterium*, and the endosymbiotic plant *Sinorhizobium*.[15–18] Rings have been found that contained between 15 and 40 glucose units, and other proteobacteria also contain cyclic D-glucans that are β-(1→2)-linked with one α-(1→6)-linkage.[19–22] These compounds appear to help the bacteria suppress plant or mammalian defenses,[23–25] while in some bacteria, they are also involved in osmoregulation.[15] Linear β-(1→2)-D-glucans have only been identified in a few bacteria, such as *Pseudomonas syringae*,[26] *Pseudomonas aeruginosa*,[27] and *Escherichia coli*,[28] and the bacteria producing linear glucans do not appear to produce cyclic glucans.

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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 specified otherwise. Alditol acetate standards for GLC–MS carbohydrate analysis were obtained from Supelco, Inc. (St. Louis, MO).

2.2. Extraction of *C. pyrenoidosa* cells and fractionation of the crude extract

C. pyrenoidosa freeze-dried cells (1000 g) were extracted as described previously[8,9] to produce an aqueous crude extract. The crude extract was sequentially precipitated with 1.5 vol, then 3 vol, and then 5 vol of 95% C_2H_5OH, yielding three precipitates (A, B, and C) as previously described. Precipitate A was decolorized by stirring it with 2:1 (v/v) $CHCl_3$–CH_3OH mixtures (3 × 500 mL) for 30 min. Decolorized A was dissolved in water, dialyzed and freeze-dried to yield fraction D (10 g).

Fraction D was fractionated further by treatment with cetyltrimethylammonium bromide (CTAB) (Fig. 1). An aqueous CTAB solution [100 mL, 10% (w/v)] was slowly added to 1 L of a 1% (w/v) aqueous solution of D and kept for 16 h at 4 °C, then centrifuged. The precipitate (E; 3.8 g) was kept, and the supernatant was dialyzed and freeze-dried to yield fraction F (4.0 g). Fraction F was separated further by anion-exchange chromatography on a Q-Sepharose Fast Flow column (XK 50/35; 620 mL bed volume). The sample was dissolved in 500 mL of 0.02% (w/v) aq sodium azide, loaded onto the column and washed with eight bed volumes of the same solution at 1.5 mL/min flow rate to elute the non-bound components (fraction G after dialysis and freeze-drying; 1.12 g). The bound components were eluted with eight volumes of a 2 M aq NaCl at the same flow rate to yield, after dialysis and freeze-drying, fraction H (818 mg; kept) (Fig. 1).

The fraction containing the bound components G was separated by size-exclusion chromatography on a Sephadex G-100 (column XK 50/100, Amersham Biosciences, PQ, Canada) using a 0.2 M aq NaCl as the mobile phase adjusted to 1.1 mL/min flow rate, with collection of 14.0-mL fractions. The carbohydrate elution profile was built as described.[9] The chromatographic separation yielded three fractions, I (488.0 mg; kept) that was excluded, and both J (275.0 mg) and K (195.0 mg) that were retained (see Fig. 2). Fractions J and K were separated further.

2.3. Purification of fraction J to obtain fraction J-P3

The residue from the concentration of J was dissolved in 1.75 mL of distilled water and treated with enough 95% C_2H_5OH to induce cloudiness (0.3 vol). Centrifugation, followed by dialysis and freeze-drying, allowed the recovery of the C_2H_5OH-precipitated portion (fraction J-P1). The process was repeated six more times yielding the same number of C_2H_5OH-precipitated fractions, which were recovered after the addition of 1.1, 1.4, 1.6, 1.9, 2.4, and 3.6 vol of 95% C_2H_5OH (fractions J-P2 to J-P7, respectively). After inspection of the ^{13}C NMR spectrum of each fraction, the fraction recovered by addition of 1.4 vol of 95% C_2H_5OH (fraction J-P3) was chosen for characterization.

2.4. Purification of fraction K to obtain fraction L-P2

Fraction K was treated with an α-amylase preparation (Sigma–Aldrich Chemical Company; St. Louis, MO) (Fig. 1). An aqueous

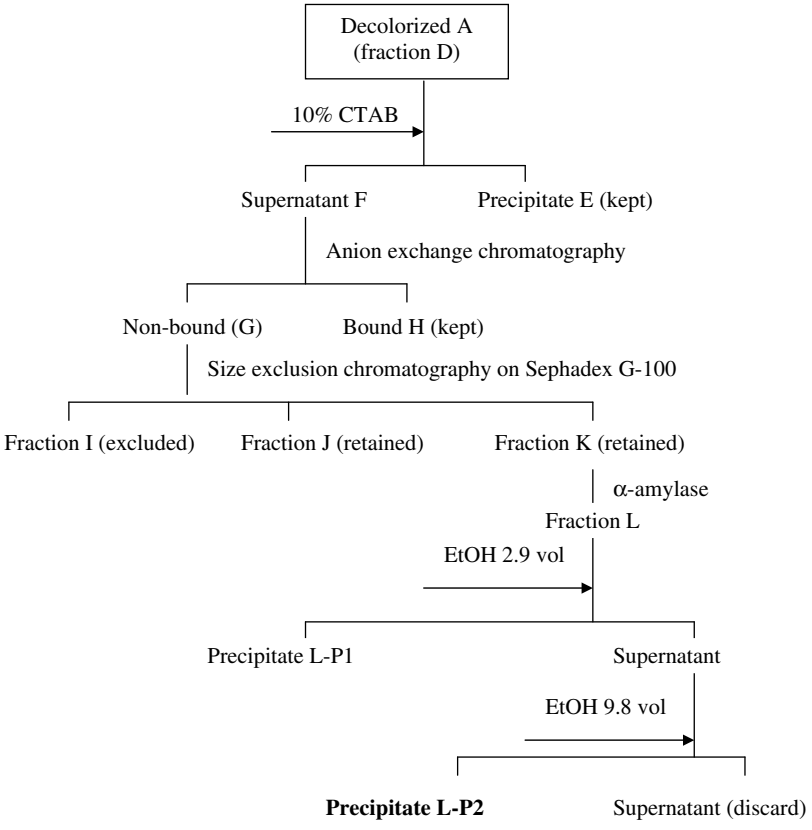

Figure 1. Description of the process to obtain precipitate L-P2.

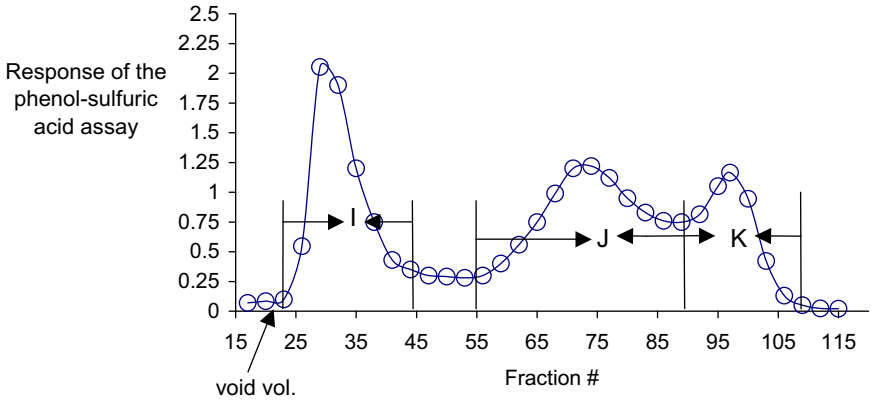

Figure 2. Size-exclusion chromatography of fraction G on Sephadex G-100.

solution of the enzyme (5 mg) in 7.5 mL of 0.05 M Tris buffer, pH 7.4, was added to 7.5 mL of fraction K in the same buffer (final enzyme concentration 0.33 mg/mL), and the mixture was incubated for 16 h at 37 °C. The enzyme was inactivated by heating at 80 °C for 30 min, followed by centrifugation. The supernatant was dialyzed and freeze-dried to yield fraction L (15.0 mg). Fraction L was finally purified by precipitation with 2.9 and 9.8 vol of 95% C_2H_5OH (Fig. 1), yielding fractions L-P1 (2.0 mg) and L-P2 (10.0 mg), respectively, the latter being examined further.

2.5. Monosaccharide composition, absolute configuration, methylation, and periodate oxidation analyses

Identification of the monosaccharide constituents of fractions J-P3 and L-P2 was accomplished by acid-catalyzed hydrolysis, followed by conversion into alditol acetates and GLC–MS analysis.[8] Assignment of the absolute configuration of the glucose present in fractions J-P3 and L-P2 was accomplished by preparation of the peracetylated (*S*)-2-octyl glycoside derivatives, followed by GLC–MS analysis and comparison with authentic standards.[8,29] For methylation analysis, 2 mg of fraction J-P3 was dissolved in 4 Å molecular sieve-dried DMSO (0.4 mL) and methylated according to the method of Ciucanu and Kerek.[30] The permethylated polysaccharide was dissolved in 0.8 mL of distilled water, extracted with 4 mL of chloroform (3×), and the combined extracts were dried and concentrated before GLC–MS analysis. Hydrolysis, reduction with $NaBD_4$, acetylation, and GLC–MS analysis were performed as described.[8]

For periodate oxidation, 4.1 mg of fraction J-P3 was dissolved in 2 mL of 0.1 M $NaIO_4$ and kept in the darkness at 4 °C for 48 h. The excess of $NaIO_4$ was decomposed by addition of ethylene glycol (0.2 mL), and the resulting mixture was dialyzed and freeze-dried. The oxidized material was reduced with $NaBH_4$ (40 mg), dialyzed, and freeze-dried to yield the polyhydroxylated derivative (2.2 mg), which was fully hydrolyzed, reduced, acetylated, and analyzed by GLC–MS as described.[8]

2.6. NMR spectroscopy, mass spectrometry, and biological activity

NMR methods have been described previously.[8,9] High-resolution electrospray-ionization mass spectra (HRESIMS) were recorded with samples dissolved in CH_3OH on a microTOF LC-Bruker Daltonics mass spectrometer using the Tunemix from Agilent as reference. Immunostimulatory activity was evaluated by measurement of nitric oxide production from murine macrophage cells as described previously.[8] LPS (10 ng/mL) from *E. coli* (Sigma–Aldrich Chemical Co., St. Louis, MO) was run as a positive control.

3. Results and discussion

3.1. Fractionation of the *C. pyrenoidosa* crude extract and isolation of the fractions of interest

In previous reports,[8,9] we described the isolation and characterization of a non-immunologically active galactofuranan ($M_w \sim 16$ kDa) and variants of an arabinogalactan with a unique repeating unit, having molecular weights of 27, 50, 188, and 1020 kDa, all obtained by precipitation of the aqueous extract of *C. pyrenoidosa* with 3 vol of 95% C_2H_5OH. The M_w values were obtained by combination of SEC-MALS and DOSY-NMR measurements. An immunostimulation assay based on NO production by activated macrophages showed that only the higher M_w variants of the arabinogalactan were active.[9]

In this report, the compositions, structures, and immunostimulatory activities of two neutral polysaccharides obtained by precipitation of the crude extract of *C. pyrenoidosa* with 1.5 vol of 95% C_2H_5OH are described. When the crude extract was treated with 1.5 vol of 95% ethanol, 30.1 g of precipitate A was recovered after dialysis and freeze-drying. Decolorization of A by successive washes with $CHCl_3$–CH_3OH mixtures yielded fraction D (10.0 g) after dialysis and freeze-drying. Precipitation of an aqueous solution of D with the surfactant CTAB, yielded a precipitate E (3.8 g) that accounted for 25% of the total mass of fraction D and was stored (Fig. 1). The soluble fraction F (4.0 g) was studied further.

The first step in the fractionation of F consisted of anion-exchange chromatography on a Q-Sepharose Fast Flow column. The non-bound components were eluted with eight bed (column) volumes of aqueous sodium azide to produce fraction G (1.12 g), whereas the bound components were removed from the column by washing with the same amount of a 2 M aqueous NaCl solution (fraction H; 818 mg). Fraction H was kept, whereas fraction G was studied further.

Fraction G was then separated according to size on a Sephadex G-100 column (Fig. 2), resulting in three fractions. One fraction was eluted in the void volume (I; 488 mg) and was excluded, whereas the two retained fractions (J; 275 mg and K; 195 mg) were eluted as two partially overlapping peaks and were included for further studies. As judged by its ^{13}C NMR spectrum (not shown), fraction I is mostly composed of polysaccharides having both the galactofuranan and arabinogalactan-like repeating units previously described by us[8,9] in an approximately 4:1 molar ratio. On the other hand, the ^{13}C NMR spectrum of fraction J (not shown) showed major ^{13}C resonances corresponding to a galactofuranan-like repeating unit plus other major resonances arising from a not previously identified polysaccharide(s) having broad C-1 resonances at 100.6 and 100.4 ppm. Eighteen other minor C-1 signals,

including those from an arabinogalactan-like repeating unit (109.1, 108.7, and 102.8 ppm), were also present.

The ^{13}C NMR spectrum of fraction K (not shown) showed major broad C-1 resonances at 100.6 and 100.4 ppm and a second set of broad resonances with C-1s located at 102.9 and 102.8 ppm, the latter representing a different polysaccharide. In addition to the polysaccharide signals, minor signals were observed attributed to protein (carbonyl, aromatic, and aliphatic signals). In order to remove starch-like polysaccharides from fraction K, the fraction was treated with an α-amylase preparation (Fig. 1). After 16 h of exposure, a significant decrease in the proportion of the C-1 signals at 100.6 and 100.4 ppm in the ^{13}C NMR spectrum was observed (spectrum not shown). The isolation procedure was completed by two steps of precipitation with 95% C_2H_5OH (2.9 and 9.8 vol), which yielded fractions L-P1 (2.0 mg; kept) and L-P2 (10.0 mg), respectively, the latter being examined in more detail.

Fraction J was purified by sequential C_2H_5OH precipitation from an aqueous solution. This process produced six fractions. Of these, the precipitate recovered after addition of 1.4 vol of 95% C_2H_5OH, fraction J-P3, contained the least amount of the galactofuranan-like repeating unit and was chosen for characterization.

3.2. Determination of the structure of the polysaccharide of fraction L-P2

Monosaccharide composition analysis of fraction L-P2 showed that it is composed of glucose along with traces of galactose attributed to residual galactofuranan (see below). The absolute configuration of the glucose residue was determined to be D by GLC of the acetylated (*S*)-2-octyl glycosides.

The ^{13}C DEPTQ-135 NMR spectrum (Fig. 3) displayed major signals for a hexopyranose residue of a glucan (C-1 at 102.8 ppm), a set of minor signals arising from a galactofuranan-like repeating unit[9] and other minor signals, likely arising from other D-glucose units. The ^{1}H NMR spectrum displayed a major resonance for the glucan at 5.10 ppm (d, $^{3}J_{H\text{-}1,H\text{-}2}$ 8.1 Hz). The anomeric configuration in this glucan was assigned as β from the large $^{3}J_{H\text{-}1,H\text{-}2}$ coupling value of 8.1 Hz. This conclusion is supported[31] by the relatively small $^{1}J_{C\text{-}1,H\text{-}1}$ coupling value of 165 Hz, measured from the signal at 102.8 ppm in a coupled HSQC experiment. In the latter experiment, the C-1 signal appeared as a dd pattern coupled by a $^{2}J_{C,H}$ of 6.2 Hz to H-2, which also constitutes reliable evidence for the β-anomeric configuration, since $^{2}J_{C,H}$ for C-1 is 5.6 Hz for β-D-glucopyranose but is too small to be observed for the α anomer.[32] No signals attributable to protein were present in fraction L-P2.

Table 1
NMR chemical shifts of the β-(1,2) glucan of fraction L-P2

Nucleus	H/C-1	H/C-2	H/C-3	H/C-4	H/C-5	H/C-6
^{1}H	5.10	3.80	4.02	3.72	3.72	4.17/4.00
^{13}C	102.8	83.4	76.2	69.5	77.0	61.5

The remaining ^{13}C and ^{1}H resonances were assigned using HSQC, DQF-COSY, TOCSY, and HSQC-TOCSY experiments (Table 1). A TOCSY experiment recorded with a 60-ms spin-lock time displayed connectivities along the H-1 (5.10 ppm) axis with the signals at 4.02, 3.72, and 3.80 ppm, the latter assigned to H-2 based on the observation of DQF-COSY correlation with H-1. In an HSQC experiment (Fig. 4), the H-2 signal at 3.80 ppm showed a cross peak with the highly deshielded ^{13}C NMR signal at 83.4 ppm. The downfield displacement (+9.3 ppm) of the C-2 signal with respect to that from methyl β-glucopyranoside[33] is consistent with substitution at this position and reveals the substitution pattern of the polysaccharide. Additional evidence was supplied by an HMBC experiment (Supplementary data-S1) optimized for a $J_{C,H}$ value of 10 Hz (a 50-ms mixing time), which displayed correlations between H-1 (5.10 ppm) and C-2 (83.4 ppm) and between H-2 (3.80 ppm) and C-1 (102.8 ppm), and also by a NOESY spectrum (Supplementary data-S2) recorded at an 80-ms mixing time, which showed a strong through-space correlation between the antiperiplanar H-1 (5.10 ppm), and H-2 (3.80 ppm) signals, most likely if the two β-D-glucose units in the $^{4}C_1$ conformation are glycosidically linked through O-2. Observation of NOE cross peaks between the three axially oriented protons H-1 (5.10 ppm), H-3 (4.02 ppm), and H-5 (3.72 ppm) along the glucopyranose ring in the NOESY spectrum is also consistent with a β-anomeric configuration for the D-glucose unit.

The evidence gathered above indicates that the glucan isolated in fraction L-P2 after fractionation of the crude extract of *Chlorella pyrenoidosa* has the following repeating unit: →2)-β-D-Glc*p*-(1→.

3.3. Molecular weight and molecular weights distribution analysis

We reported previously[9] that for neutral polysaccharides, molecular weights obtained by SEC–MALS measurements and through measured self-diffusion coefficients (D_o) at very dilute regime using DOSY NMR are very similar. That work[9] and work of others[34] extended the scaling relationship proposed by Viel et al.,[35] obtained by calibration of self-diffusion coefficients against

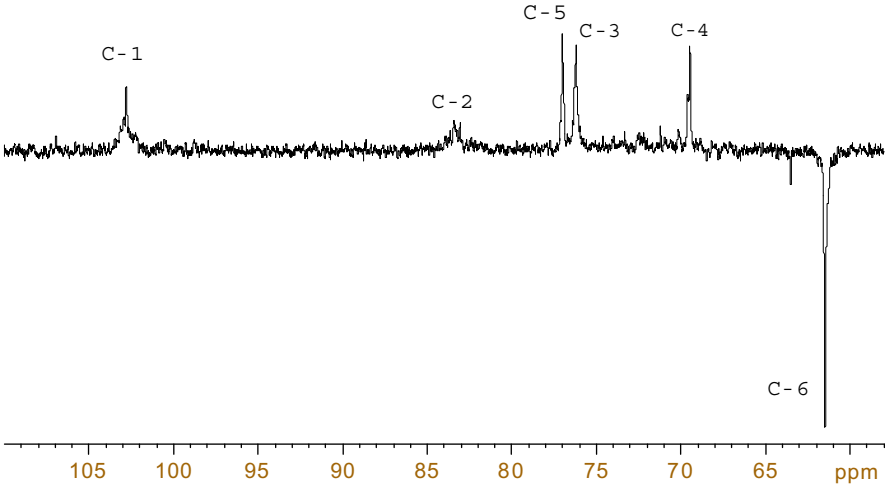

Figure 3. 125-MHz ^{13}C DEPTQ-135 NMR spectrum of fraction L-P2 recorded at 50 °C.

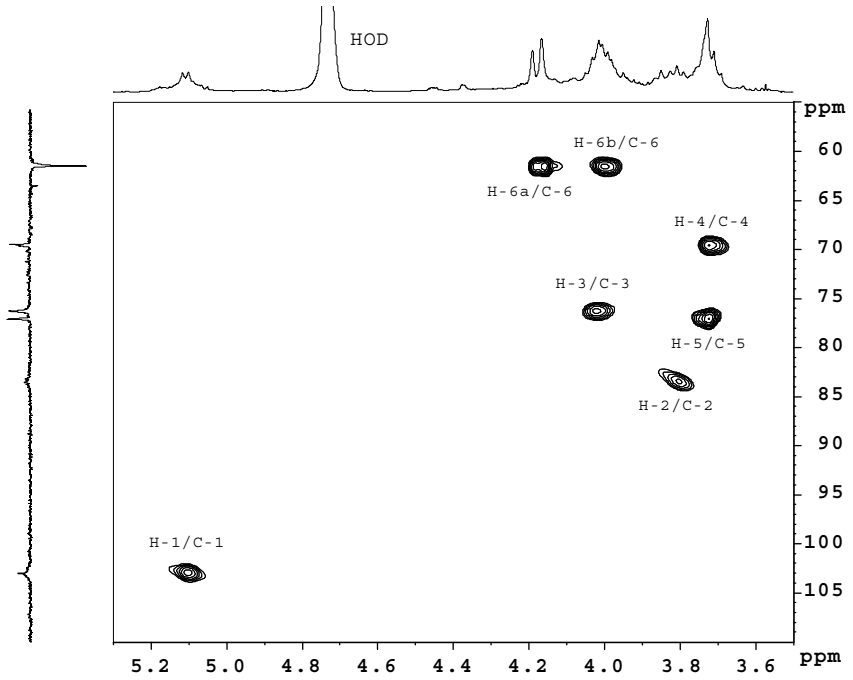

Figure 4. ^{1}H, ^{13}C HSQC spectrum of fraction L-P2.

known molecular weights of pullulan fractions with narrow molecular weight distributions, to other naturally occurring neutral polysaccharides. In order to estimate the weight-average molecular weight and the molecular weight distribution of the glucan of fraction L-P2, we employed an approach similar to that described previously.[9] Using a dilute sample (1.43 mg/mL) to avoid aggregation, the intensities for the two components of the H-1 doublet centered at 5.10 ppm were obtained as the pulsed-field gradient (PFG) amplitudes were incremented. Then the observed intensity as a function of the PFG amplitude was fitted to a mono-exponential decay (assumes one component with single D and M_w values) as defined by the modified Stejskal–Tanner relationship,[36,37]

$$I = I_0 e^{(-kD)}, \quad (1)$$

where I_o is the initial intensity, k is $g^2\gamma^2\delta^2(\Delta - \delta/3)$, g and δ are the gradient pulse amplitude and duration, respectively, γ is the magnetogyric ratio, and Δ is the time between the leading edges of the gradient pulses. Figure 5 shows the results of the fitting for one component of the doublet. Both the experimental and predicted curves are in close agreement at lower PFG amplitudes. However, it is noticeable that some substantial deviation from the linear

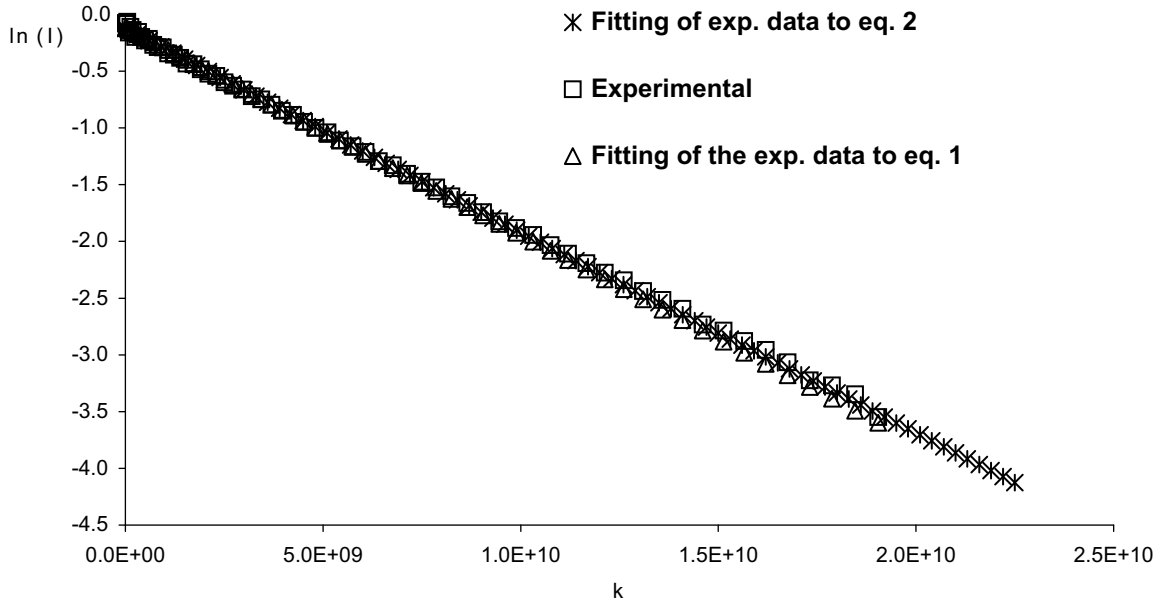

Figure 5. Plots of ln$I$ versus k showing the fits to experimental data of the two fitting procedures described in the text for one of the components of the anomeric doublet of fraction L-P2.

behavior predicted for a monodisperse system occurs at higher PFG amplitudes, indicating a moderate polydisperse system.

To evaluate the magnitude of the polydispersity, the experimental signal decay was instead fitted using a non-linear squared fitting procedure to Eq. 2, which inherently considers a continuous distribution of self-diffusion coefficients (DSDC) and molecular weights, represented in the probability function $P(D)$. A normalized Gaussian distribution function was employed as the functional form of $P(D)$:

$$I = \int_0^\infty P(D)\mathrm{e}^{(-kD)}\mathrm{d}D. \quad (2)$$

This fitting result is also shown in Figure 5. As in the previous case, both the experimental and the calculated curves coincide at lower PFG amplitudes. However, unlike for the monodisperse model, the calculated curve in this case (assuming a DSDC) is in excellent agreement with the experimental curve at higher PFG amplitudes.

To calculate the weight-average molecular weight of the polymer, the self-diffusion coefficient at the center of the DSDC was substituted in Eq. 3, using the Viel group values of the scaling parameters ($K = 8.2 \times 10^{-9}\ \mathrm{m^2\ s^{-1}}$; $\alpha = -0.49$),[35] yielding a value of 2.4 kDa.

$$D = K \times M_w^{\alpha}\ (\mathrm{m^2\ s^{-1}}). \quad (3)$$

The range of self-diffusion coefficients can be expressed as a function of the half-width of the DSDC (standard deviation of the distribution; $\sigma = 2.3 \times 10^{-11}\ \mathrm{m^2\ s^{-1}}$) as $D_o \pm 1/2\sigma$, which leads to a weight-average molecular weight range of (2.4 ± 0.3) kDa that corresponds to a polysaccharide having 15 ± 2 repeating glucose units, but the structure could not be assigned as cyclic or linear.

A mass spectrum of a solution of L-P2 in CH_3OH was recorded using electrospray-ionization mass spectrometry. The most intense peaks were due to clusters of peaks having m/z values of $[(n \times 162) + (2 \times 23)]/2$ and $[(n \times 162) + (3 \times 23)]/3$ corresponding to doubly and triply charged ions containing two and three sodium ions, respectively (Fig. 6). The doubly and triply charged ions are easily identified from the mass differences between isotopic peaks (see Supplementary data S-3 and S-4 for expansions of these peaks). Although the triply charged peaks had peak heights that were more than double the heights of the doubly charged peaks, no quadruple-charged ions were observed. This particular set of masses is consistent with cyclic structures; linear polysaccharides would have given peaks with m/z values 18 Da greater. Although the ratios of peak heights were different for the 2+ and 3+ series, in both series, the $n = 21$ peak had an intensity more than three times greater the intensity of the next most intense peaks, the $n = 22$ peak and the $n = 23$ peak. In the 2+ series, peaks were observed from $n = 19$ to $n = 29$, with the exception of $n = 20$ and in the 3+ series, peaks were observed from $n = 18$ to $n = 35$, with the exceptions of $n = 20$ and $n = 33$. Peak heights for the M+1 isotopic peak at each step in the clusters have been converted into mole fractions in Table 2, and the observations from the 2+ and 3+ series were combined by assuming that the peak intensities

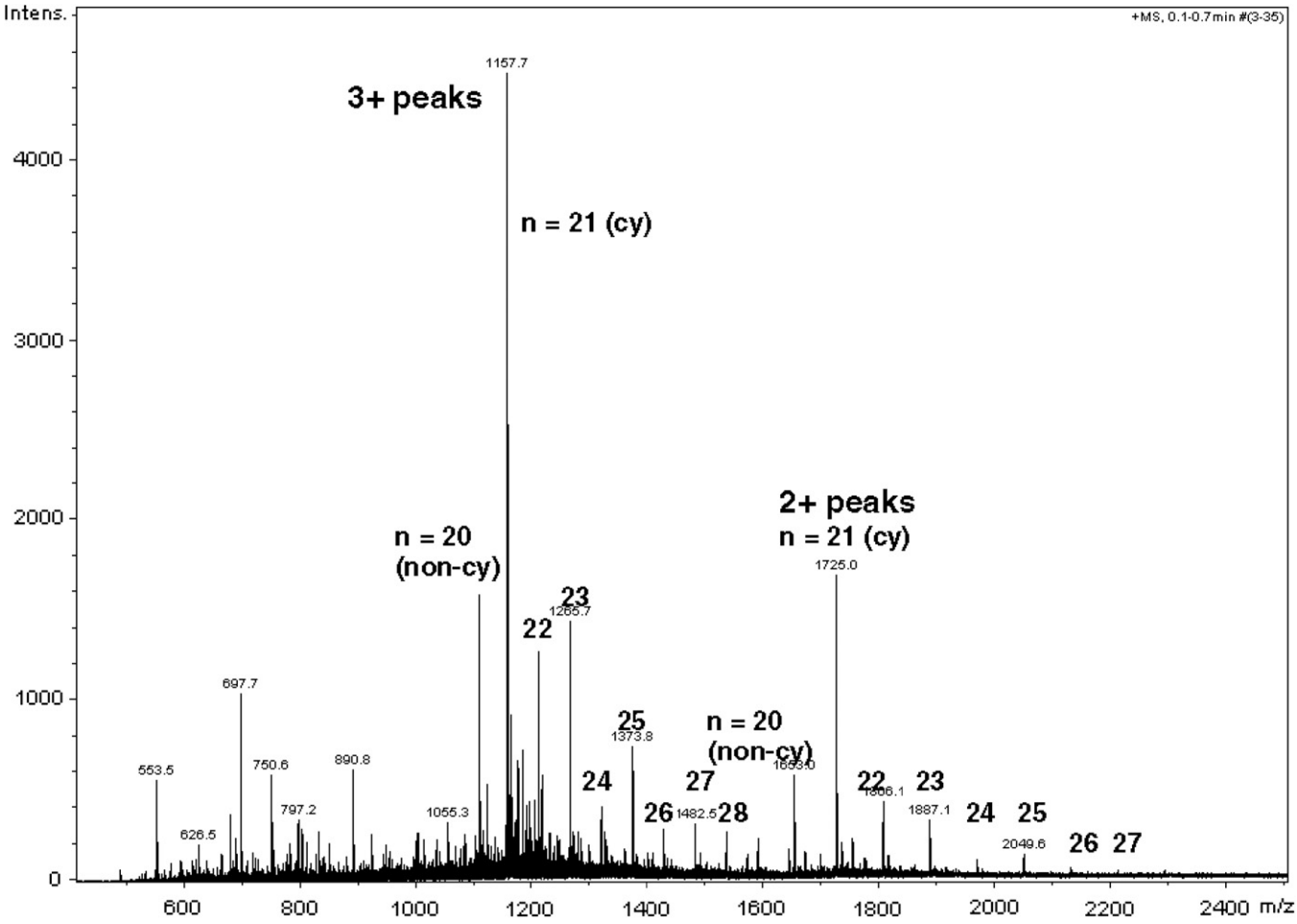

Figure 6. HRESI mass spectrum of fraction L-P2 recorded in CH_3OH. The cluster of peaks centered around m/z 1157.7 are triply charged peaks, whereas the cluster centered around m/z 1725.0 are doubly charged peaks. The labels on peaks indicate the number of glucose residues present. Most labeled peaks are due to cyclic structures.

Table 2
Peak heights of mass spectral peaks of cyclic β-(1→2)-D-glucans expressed as mole fractions

n	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
2 + peaks	0	.017	0	.61	.14	.131	.027	.041	.013	.009	.009	.004	–	–	–	–	–	–
3 + peaks	.006	.007	0	.46	.13	.15	.041	.078	.025	.027	.023	.016	.013	.012	.010	0	.005	.002
Com.[a]	.003	.010	0	.54	.14	.13	.032	.055	.018	.037	.014	.009	.005	.005	.004	0	.002	.001
M_W (Da)	2917	3079	3241	3403	3565	3727	3889	4051	4213	4375	4537	4700	4862	5024	5186	5348	5510	5672

[a] The combined mole fractions were calculated on the basis that peak intensity was proportional to charge.

from individual ions were proportional to the energy of the ion, which is in turn proportional to charge. On this basis, the $n = 21$ compound constituted 54% of the total, while the $n = 22$ compound and the $n = 23$ compound represented 14% and 13% of the total, respectively.

Also present in the mass spectrum were less intense clusters of peaks having m/z values of $[(n \times 162) + (2 \times 23) + 18]/2$ and $[(n \times 162) + (3 \times 23) + 18]/3$ corresponding to doubly and triply charged ions containing two and three sodium ions, respectively (Fig. 6). These masses correspond to linear polysaccharides. The possibility that these ions were caused by cyclic structures complexed to one molecule of water was considered but rejected on the basis that there were no peaks corresponding to complexes with CH_3OH, the solvent used. For these series of peaks, the $n = 20$ peak was most intense in both series (40% overall), followed by the $n = 21$ peak (14%). In the 2+ series, peaks were observed from $n = 17$ to $n = 24$ and in the 3+ series, peaks were observed from $n = 14$ to $n = 27$. Peak heights of the M+1 isotopic peaks at each step in the clusters have been converted into mole fractions in Table 3. It is interesting that no cyclic structures were found with $n = 20$, but this was the most abundant component for the linear β-(1→2)-D-glucans.

For comparison with the DOSY results, mole fractions were also calculated for the combined linear and cyclic β-(1→2)-D-glucans. These are shown in a table in the Supplementary data. From the mass spectral data, 62% of the β-(1→2)-D-glucans were cyclic and 38% were linear. The number-average and weight-average molecular weights calculated from Tables 1 and 2 are M_N= 3621, M_W = 3651 for cyclic β-(1→2)-D-glucans, and M_N = 3292, M_W = 3324 for linear β-(1→2)-D-glucans. These values are considerably larger than those obtained by DOSY NMR as might be expected for a cyclic molecule since the translational diffusion coefficient (D) of a particle at infinite dilution is inversely related to its hydrodynamic radius (r) by the Stokes–Einstein relationship,

$$D = (k_b T)/(6\pi\eta r)\ (\mathrm{m^2\ s^{-1}}), \tag{4}$$

where k_b is Boltzmann's constant, T is the temperature, and η is the solution viscosity. Viel et al.[35] developed the parameters relating D for neutral polysaccharides to molecular weight by using pullulans (linear polysaccharides) of known molecular weight for calibration. Since pullulans have random-coil conformations,[38] the β-(1→2)-D-glucans obtained here have smaller hydrodynamic radii relative to their molecular weights than do the pullulans and hence yield lower than the real molecular weights when these are obtained from self-diffusion constants.

In the ^{1}H NMR spectrum of fraction K (before α-amylase treatment), the ratio of the integrals of the anomeric signals over the range from 4.87 and 5.01 ppm to those over the range from 5.30 and 5.50 ppm was about 3:1. These former signals were correlated in an HSQC experiment to the ^{13}C NMR signals between 102 and 104 ppm, the anomeric carbon signals of the β-(1→2)-glucans, while the latter were correlated to ^{13}C NMR signals near 100 ppm, attributed to α-(1→4)-D-glucans, the starch. DOSY experiments on a dilute sample with analysis using the monodisperse model gave values of M_w of 2100 and 2900 for anomeric protons at 4.94 and 5.00 ppm, respectively similar to the values obtained for the β-(1→2)-D-glucans present in fraction L-P2, suggesting that this component was not attached to other components in fraction K (before α-amylase treatment). Confirmation of this tentative conclusion was obtained from the ESI mass spectrum (not shown) where the most intense peaks were the clusters of 2+ and 3+ ions with the two most intense peaks at the masses corresponding to the $n = 21$ ring. In the DOSY experiment, ^{1}H NMR signals at 5.33, 5.40, and 5.43 ppm yielded M_w values of 3400, 2600, and 1700,

Table 3
Peak heights of mass spectral peaks of linear β-(1→2)-D-glucans expressed as mole fractions

n	14	15	16	17	18	19	20	21	22	23	24	25	26	27
2 + peaks	0	0	0	.10	.092	.10	.44	.13	.078	.035	.021	0	0	0
3 + peaks	.007	.022	.025	.039	.050	.059	.34	.15	.12	.059	.059	.035	.021	.015
comb.[a]	.003	.009	.010	.078	.075	.084	.40	.14	.095	.044	.036	.014	.008	.006
M_W (Da)	2755	2917	3079	3241	3403	3565	3727	3889	4051	4213	4375	4537	4700	4862

[a] The combined mole fractions were calculated on the basis that intensity was proportional to charge.

respectively. These are assigned to the starch removed by the α-amylase treatment.

Since the first demonstration that the β-(1→2)-D-glucans in proteobacteria often had cyclic structures,[39] these compounds have attracted considerable attention. The proteobacteria in which these compounds are present are all pathogenic or symbiotic, and it appears that these compounds help the bacteria suppress plant or mammalian defenses.[23–25] In some bacteria, they are also involved in osmoregulation.[15] The question of the conformations available for rings containing β-(1→2)-glucoses has also attracted attention,[40–45] and it appears that these large rings also have a large number of energetically accessible conformations available to them. Nevertheless, the absence of the 20-membered ring in the mixtures obtained here does raise questions about the conformations available to it.

The homogeneous cyclic β-(1→2)-glucans described here have structures very similar to those obtained from some species of the *Rhizobaceae* family,[18] termed family II,[15] part of the α-subdivision of proteobacteria, although careful studies[39,46–55] have shown that the cyclic glucans in these bacteria are not accompanied by linear β-linear β-(1→2)-D-glucans as they are here. It should be noted that the bacteria *Xanthomonas campestris* and *Ralstonia solanacerum*, that produce smaller cylic glucans[19–21,56] (16 and 13 members, respectively) containing one α-(1→6)-linkage in rings where all other linkages are β-(1→2) do produce linear glucans along with the cyclic glucans.[19,20] In the bacteria producing homogeneous β-(1→2)-D-glucans, the cyclic glucans are often unsubstituted, but both the same and other bacteria from the same subdivision of proteobacteria produce cyclic β-(1→2)-glucans that are substituted with the anionic groups *sn*-1-phosphoglycerol, succinate, or methylmalonate.[48,49,57] Often their synthesis is osmoregulated,[15] but the same compounds are produced in *Brucellae* where their synthesis is not osmoregulated.[16] It is thought that a single enzyme, cyclic β-(1→2)-D-glucan synthase, controls the assembly and ring-closure of these bacterially derived homogeneous cyclic β-(1→2)-D-glucans.[57]

We ruled out the possibility that these compounds were produced by bacteria contaminating the *C. pyrenoidosa* cells for the following reasons. The immunostimulatory activity provided by the extract is evaluated at each stage against that provided by LPS from *E. coli*, a Gram-negative bacterium as are all of these bacterial cyclic glucan producers. The LPS produces comparable activity as the whole extract at a concentration level of about 0.001 that of the extract.[4,8] Neither fractionation of the extract nor washing with $CHCl_3$–CH_3OH reduces the activity of fractions of the extract, indicating that it was not contamination by bacterial LPS that caused the activity, and therefore that Gram-negative bacteria were not major contaminants. Supporting evidence is that bacteria have never been shown to produce mixtures of linear and homogeneous cyclic β-(1→2)-D-glucans,[47] as was observed here. In addition, the pattern of ring sizes produced by *C. pyrenoidosa* cells is very different than that produced by the bacterial enzyme(s) distribution. The bacteria produce ranges of ring sizes typically containing from 17 to 28 glucoses with no ring sizes missing. In these mixtures, the most prevalent ring sizes contain 19,[49,50,54] 20,[47,53] 21,[39,47] or 22[52] glucoses, and in all cases, 2–5 other ring sizes have abundances within 50% of that of the maximum. The pattern observed here was somewhat similar in that a range of ring sizes was produced with a maximum at 21. However, it was different in three respects: the intensity of the maximum observed here for the $n = 21$ peak was much greater with respect to those of the other members of the series, greater than three times that of the next most intense peak; the series here did not contain an $n = 20$ member; and linear β-(1→2)-glucans were also present, constituting about 38% of the glucan mixture.

3.4. Determination of the structure of the polysaccharide of fraction J-P3

Monosaccharide composition analysis of fraction J-P3 indicated that it is composed of glucose and traces of galactose, attributed to the remnants of the galactofuranan, evident as minor signals in the ^{1}H and ^{13}C NMR spectra.[9] The absolute configuration of the glucose residue was determined to be D by GLC of the acetylated (*S*)-2-octyl glycosides.

Methylation analysis of fraction J-P3 displayed the pattern characteristic of a starch-like structure consisting of repeating (1→4)-linked α-D-glucopyranosyl units with some units having O-6 side chains. Upon periodate oxidation and reduction, the resulting polyhydroxylated derivative released only erythritol (arises from terminal glucopyranosyl units) and glycerol (arises from 1,4-disubstituted- and 1,4,6-trisubstituted glucopyranosyl units) after hydrolysis, indicating that all the glucose residues had been completely removed, in agreement with the (1→4)-linkage pattern shown by methylation analysis.

The ^{13}C NMR spectrum of fraction J-P3 (Fig. 7) contained the major broad C-1 resonances originally present in fraction J at 100.7, 100.5, 100.2 ppm, plus a signal at 99.3 ppm, all arising from α-D-glucopyranosyl units.[58–60] On the other hand, the ^{1}H NMR spectrum (not shown) contained a band of H-1 resonances from 5.36 to 5.50 ppm arising from α-D-glucopyranosyl units attached to the O-4 of the next glucose,[58–60] and a signal at 5.02 ppm assigned to H-1 of α-D-glucopyranosyl units attached at O-6 of a glucose unit.[58–60] The ratio of integrals of the signals at 5.36–5.50 ppm to that at 5.02 ppm was approximately 5:1, which is indicative of the presence of five α-(1→4) glycosidic linkages per every α-(1→6) glycosidic linkage.

The length of the O-6 side chains that can take the form of terminal α-D-glucopyranosyl units and/or oligosaccharide chains of α-(1→4)-linked glucopyranosides could not be assigned

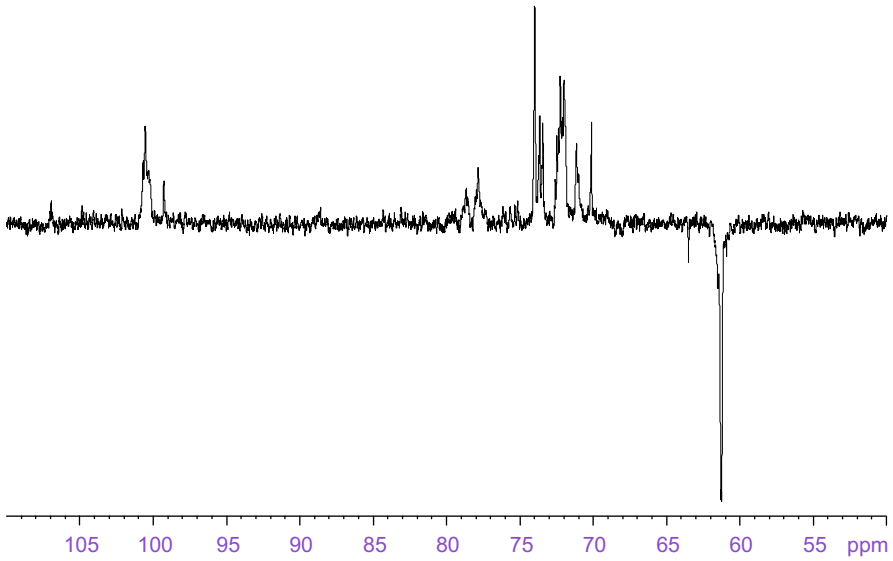

Figure 7. 125-MHz ^{13}C DEPTQ-135 NMR spectrum of fraction J-P3 recorded at 50 °C.

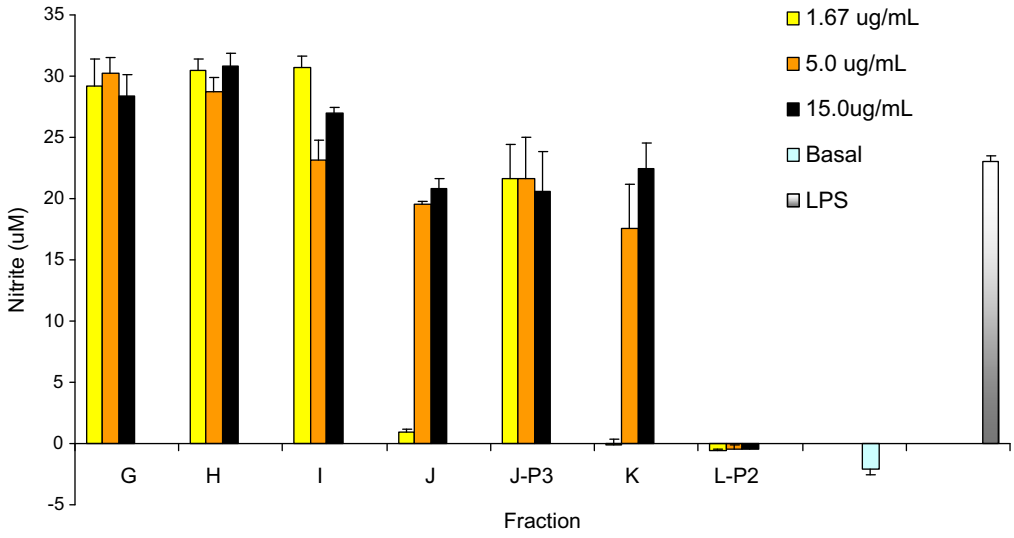

Figure 8. Stimulation of NO synthesis in RAW 264.7 cells cultures treated with some fractions derived from fractionation of a *Chlorella pyrenoidosa* crude extract. LPS (10 ng/mL) from *E. coli* was run as positive control.

unambiguously from analysis of the 2D NMR data; nonetheless, the appearance of the ^{13}C NMR spectrum (Fig. 7) suggests a regular structure for the polysaccharide. On the basis of the NMR results, structures with repeating units that have variable length O-6 side chains are proposed as being most likely for the glucan of fraction J-P3, the general formula is shown below

$$\alpha\text{-D-Glc}p\text{-}(1\big(\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\big)_y$$
$$\downarrow$$
$$6$$
$$\Big[\big(\rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\big)_x \rightarrow 4)\text{-}\alpha\text{-D-Glc}p\text{-}(1\Big]_n \rightarrow$$

where $x = 1–4$; $y = 0–3$ (when $y = 0$ the terminal glucopyranosyl unit is linked directly to position O-6) and $n = 6–9$. The values of n were estimated from a DOSY NMR experiment on a dilute sample of fraction J-P3 (with analysis using the monodisperse model) that resulted in M_w values of 6500, 5900, and 6400 Da for anomeric protons at 5.02, 5.41, and 5.44 ppm, respectively. These molecular weights suggest that the polymer chains contain between 36 and 51 glucose units.

3.5. Immunostimulatory activity

The results of biological testing on the latter fractions are shown in Figure 8. The activity of fraction G, as measured by its stimulation of NO synthesis, was mostly located in the high-molecular-weight fraction I, the mixture of galactofuranan and the arabinogalactan, the latter previously shown to have considerable activity if present in the high-molecular-weight form.[9] The lower molecular weight fractions, J and K, and the components obtained from them, had lower activity, although the activity of J was enriched in fraction J-P3, the starch. Finally, fraction L-P2, the mixture of cyclic and linear β-(1→2)-D-glucans, with molecular weights ranging from about 2 to 6 kDa (refer to Tables 2 and 3), had no activity. From this observation, the low immunostimulatory activity in fraction K (mostly a mixture of β-(1→2)-D-glucans and low-molecular-weight starch) probably comes from the starch, but a contribution from the protein present at low concentration cannot be excluded.

4. Conclusions

The aqueous extract of the microalgae *C. pyrenoidosa* has now been shown to contain a mixture of cyclic and linear β-(1→2)-D-glucans, and a starch-like α-(1→4)-D-glucan with α-(1→6)-branches. For the cyclic β-(1→2)-D-glucans, ring sizes ranged from 18 to 35 monosaccharides with the ring containing 21 glucose units (54% of the cyclic glucans) being more than three times more abundant than the next most abundant component, the ring containing 22 glucose units (15%). No rings containing 20 glucose units were present. This is the first observation of cyclic β-(1→2)-D-glucans in algae, as far as we are aware. For the linear β-(1→2)-D-glucans, the component containing 20 glucoses was most abundant (35% of the linear glucans), while the component containing 21 glucose units was the next most abundant (17%). These compounds do not provide immunostimulation.

The fraction containing starch-like compounds did exhibit immunostimulatory effects. The immunomodulating effects of plant-derived resistant starches (that resist digestion by pancreatic amylase in the small intestine and reach the colon) are documented[61–67] and appear to be associated with the production of a number of short-chain fatty acids (SCFA), in particular butyrate, via fermentation by the colonic microbiota (microbiota-dependent mechanisms).[61,62,66,67] Butyrate is known to be the preferential energy source for human colonial tissue and has been recognized to play a vital role in the maintenance of gut health and reduction of risk factors associated with the development of inflammatory bowel diseases and colorectal cancer.[61,62,66]

Microbiota-independent mechanisms of immunomodulation (indirect effects) that involve binding to receptors on cells of the immune system have been discussed from animal models and in vitro experiments for some non-digestible carbohydrates (NDC).[62] However, potential indirect effects on the immune system by resistant starches and other groups of NDC have received little attention.[62]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.07.009.

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