Immunostimulatory Polysaccharides from *Chlorella pyrenoidosa*. A New Galactofuranan. Measurement of Molecular Weight and Molecular Weight Dispersion by DOSY NMR

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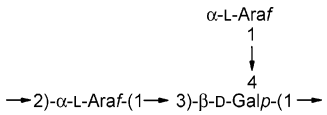
Fractionation of the hot water extract of *Chlorella pyrenoidosa* was performed using a combination of ethanol precipitation, size exclusion chromatography, and anion exchange chromatography. One fraction contained a new polysaccharide, and this compound was shown to be a 1→2-linked β-D-galactofuranan from its 1D and 2D ^{1}H and ^{13}C NMR spectra, with a molecular weight of 15 kDa from DOSY NMR measurements. A number of other fractions were shown to have the same repeating unit as the previously identified arabinogalactan. However, arabinogalactans from different fractions were shown by DOSY NMR to have different molecular weights, which ranged from 27 to 1020 kDa. Agreement with molecular weights measured for some of these fractions by SEC-MALS was very good, further confirming the relationship established by Viel et al. between molecular weights of neutral polysaccharides and self-diffusion coefficients. The smaller molecular weight polysaccharides, the galactofuranan and the 27 and 50 kDa arabinogalactans, were shown to be close to monodisperse by analysis of the distributions of the self-diffusion coefficients for the polymers. The larger arabinogalactans had considerable variation in their molecular weights (188 ± 109 kDa and 1020 ± 370 kDa). Only the two larger arabinogalactans showed immunostimulatory activity.

Introduction

Polysaccharides are responsible for the bulk of the immunostimulatory activity of hot aqueous extracts of *Chlorella pyrenoidosa* cells.[1] We have undertaken a detailed study of these polysaccharides to better understand the immune response and recently described an arabinogalactan having a unique structure, in which arabinofuranosyl and galactopyranosyl units alternate in the main chain.[2] This type of arabinogalactan, whose structure is shown below, was termed a type IV arabinogalactan.[2]



Here, we report the isolation and structure of a new polysaccharide present in the mixture. We have also found that variants of the arabinogalactan that we had previously characterized[2] were present in a number of fractions resulting from a more elaborate but very mild isolation procedure. To our surprise, these different fractions contained arabinogalactans having very different molecular weights. The higher molecular weight arabinogalactans showed immunostimulatory activity, but the lower molecular weight fractions did not.

Although there have been numerous publications linking biological activities of polysaccharides to molecular size,[3−7] the relationships between activities and structure have not been clearly identified. The antitumor activities of the branched 1,3-glucans, scleroglucan, lentinan, and schizophyllan depend on their molecular weight, with masses lower than 10 kDa being ineffective.[8−13] Some results suggest that this activity is related to the ability of higher molecular weight polysaccharides to form triple helix conformations;[14,15] others suggest that low branching ratios are important.[16] Alginates also exhibit this behavior, with polymannuronic acid and alginic acid containing a high concentration of mannuronic acid requiring molecular weights of 50 and 200 kDa, respectively, to induce human monocytes to give maximum production of tumor necrosis factor alpha.[17] Interestingly, alginic acid high in guluronic acid does not exhibit this behavior.

Advances in NMR spectral methods over the last 10 years have now made possible the unambiguous determination of the structures of the repeating units of pure isolated polysaccharides by spectral methods alone, once the identity and configurations of the constituent monosaccharides have been established.[18,19] Diffusion ordered spectroscopy (DOSY)[20−22] offers the possibility of performing structural studies on components of mixtures by separating the component signals along a diffusion axis in 2D or 3D experiments, the DOSY versions of standard 1D and 2D experiments.[23−27] In addition, as shown by Viel and co-workers[26,28] and confirmed by Jiménez-Barbero and co-workers,[27] DOSY measurements can be used to estimate the molecular weights of neutral polysaccharides. Accurate DOSY measurements also offer the possibility of obtaining information about dispersion of molecular weights.[29] Little data is available

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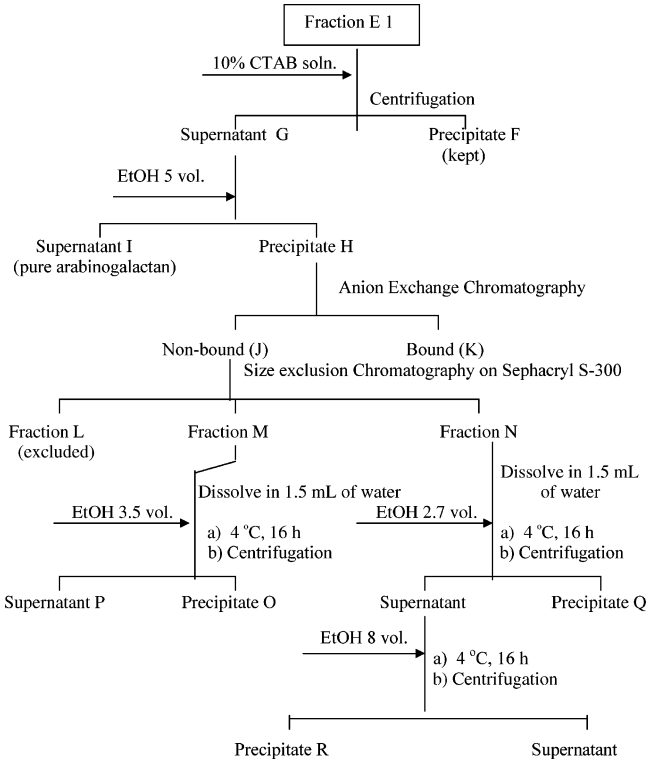
Chart 1. Flowchart Describing the Process to Obtain Fraction R Starting from Fraction E1, the Sephadex G-100 Excluded Fraction[2]



on molecular weight dispersion of natural polysaccharides and its significance for biological activity.

In this publication, we measure molecular weights of the arabinogalactans by both size exclusion chromatography−multiangle light scattering (SEC-MALS) and by DOSY experiments, extending the relationship between diffusion coefficients and molecular weights to different types of neutral polysaccharides. DOSY experiments were also used to determine molecular weight dispersions of the new polysaccharide and the various arabinogalactans. We now report that higher molecular weight versions of this arabinogalactan are active as immunostimulants, while lower molecular weight versions are inactive.

Experimental Section

Extraction of *Chlorella pyrenoidosa* Cells and Isolation of Fractions. Lyophilized *Chlorella pyrenoidosa* cells were obtained from Taiwan Chlorella Manufacturers Ltd. (Taipei, Taiwan). Chemicals (analytical-grade) and dialysis membranes with molecular weight cutoff of 12 kDa were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO), unless specified otherwise. Alditol acetate standards for GLC-MS carbohydrate analyses were obtained from Supelco, Inc. (St. Louis, MO). Chromatography columns, media, and supplies were purchased from Amersham Biosciences (PQ, Canada).

Chlorella pyrenoidosa (CP) freeze-dried cells (200 g) were extracted as described elsewhere to produce a crude extract (CE).[1,2] The crude extract was sequentially precipitated with 1.5 vol, then 3 vol, then 5 vol of 95% ethanol yielding three precipitates (A, B, and C) as previously described.[2] Size exclusion chromatography on Sephadex G-100 of decolorized B yielded two included fractions (E2 and E3) and an excluded fraction (E1) that retained the bulk of the immunostimulant activity originally present in fraction B.[2] Fraction E1 was fractionated further by treatment with cetyltrimethylammonium bromide (CTAB) to yield a precipitate (F) that was kept and a supernatant (G).[2] The latter supernatant (G) was treated with 5 vol of 95% ethanol (Chart 1) to yield a supernatant (I) that was found to consist mainly of an arabinogalactan with a unique structure and a precipitate (H) that was kept.[2]

In this publication, fraction H was fractionated further by anion exchange chromatography on a Q-Sepharose Fast Flow column (XK 16/40, 70 mL bed volume). The sample was dissolved in 5 mL of 0.02% aqueous sodium azide solution, loaded onto the column, and washed with eight bed volumes of the same solution at 0.6 mL/min flow rate to elute the nonbound components (fraction J after dialysis and freeze-drying). The bound components were eluted with eight bed volumes of a 2 M NaCl solution at the same flow rate to yield fraction K after dialysis and freeze-drying (Chart 1).

The fraction containing the neutral components (J) was separated using size exclusion chromatography on a Sephacryl S-300 column (XK 16/70, 140 mL bed volume). The sample was dissolved in 0.2 M NaCl aqueous solution, passed through a 0.45 μm filter, and chromatographed in the same mobile phase at 0.5 mL/min flow rate with collection of 1.6 mL fractions. The carbohydrate elution profile was built up using optical density measurements at 490 nm obtained from the implementation of the phenol−sulfuric acid method[30] to 40 μL aliquots taken from the collecting tubes at every third tube. The separation yielded three fractions, L (kept) that was excluded, and M and N that were both included. Fractions M and N were separated further. The residue from concentration of fraction M was dissolved in 1.5 mL of distilled water (Chart 1) and treated with enough 95% ethanol to observe cloudiness (3.5 vol). The precipitate recovered by centrifugation was dialyzed and freeze-dried to yield fraction O, whereas the supernatant was evaporated (ethanol removal) to yield fraction P after dialysis and freeze-drying.

Fraction N was subjected to a similar procedure. The residue from concentration of fraction N was dissolved in 1.5 mL of distilled water and treated with enough 95% ethanol to observe cloudiness (2.7 volumes). The precipitate (fraction Q) recovered by centrifugation was dialyzed and freeze-dried, while the supernatant was treated with enough 95% ethanol to cause cloudiness (8 vol). The suspension was kept overnight at 4 °C and centrifuged to recover the precipitate that yielded the fraction of interest (R) after dialysis and freeze-drying (Chart 1).

Sugar Analyses, Nuclear Magnetic Resonance (NMR) Spectroscopy, and Biological Activity. Sugar analyses and assignment of configuration to galactose from the galactofuranan in fraction R were performed as outlined previously.[2] Immunostimulation was measured by stimulation of macrophage activity to NO synthesis as described previously.[2] 1D and 2D NMR spectra were recorded as described previously.[2] The chemical shifts are expressed in parts per million (ppm) relative to the methyl signal of internal acetone (30.89 ppm) and to the residual HOD signal (4.79 ppm) for ^{13}C and ^{1}H, respectively.[31]

DOSY spectra were acquired using a stimulated-echo sequence incorporating bipolar gradients with a longitudinal eddy current delay (Bruker stebpg1s pulse sequence). The strength of the linear pulse gradient was incremented in 64 steps (8 scans for each linear gradient step) from 5% up to 95% of the maximum gradient amplitude (5.35 G cm^{-1}). The diffusion delay (Δ) was held constant at 0.2 s, and the gradient pulse duration (δ) was optimized for each experiment in order to achieve a 95% decrease in the resonance intensity at the largest gradient strength. Gradient pulse duration ranged between 7.0 and 11.9 ms. After processing and automatic zero-order baseline correction of the 1D ^{1}H spectra (F2 dimension), the diffusion dimension (F1) in the 2D-DOSY spectra was processed using a two-parameter monoexponential fit supplied in the *Xwinnmr* software package (version 3.5). Accurate calculation of diffusion coefficients was either performed using the integration and curve-fitting routine supplied with the *Xwinnmr* software package or using the formalism outlined[29] using a Gaussian curve for the distribution of self-diffusion coefficients (DSDC). Careful monitoring of temperature is necessary for obtaining accurate diffusion coefficients, and thus, the airflow rate, heater power, and inlet air temperature were optimized to achieve a stable temperature of 300 ± 0.1 K throughout data acquisition.

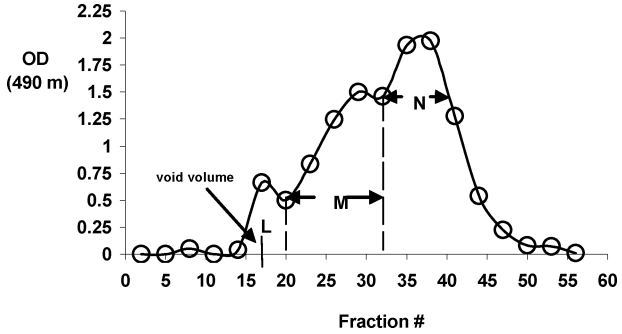


Figure 1. Size exclusion chromatography of fraction J on Sephacryl S-300.

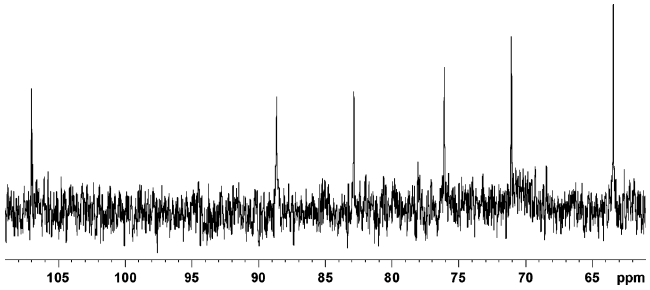


Figure 2. The 125 MHz ^{13}C NMR spectrum of fraction R.

Results and Discussion

Extraction of *Chlorella pyrenoidosa* Cells and Isolation of Fraction R. We previously reported that fractionation of a hot water extract of *Chlorella pyrenoidosa* by a process involving controlled ethanol precipitation, size exclusion chromatography on Sephadex G-100, followed by precipitation with 5 vol of 95% ethanol of the supernatant resulting from CTAB treatment resulted in the isolation of a unique arabinogalactan from the final soluble fraction (I).[2] None of the biological activity of the original fraction E1 (excluded on Sephadex G-100) was present in the latter supernatant, but the precipitate (fraction H) was active.[2]

In the current work, the first step in the isolation of the bioactive portion of fraction H was based on the separation of neutral and acidic components by anion exchange chromatography on a Q-Sepharose Fast Flow column (Chart 1). Neutral components were eluted with aqueous sodium azide to produce fraction J (36.5 mg), whereas the acidic components were removed by washing with a 2 M NaCl solution to produce fraction K (50.0 mg). Fraction J was separated according to size on a Sephacryl S-300 column (Figure 1), resulting in three fractions. One fraction eluted in the void volume (L, 11.2 mg), and as judged by the ^{1}H NMR spectrum (three anomeric protons at 5.47 and 5.44 ppm (2H, broadened singlets) and 4.68 ppm (*d*, $^3J_{1,2} = 8$ Hz); not shown), was composed of an arabinogalactan with the same repeating unit as previously described.[2] The two other fractions (M and N) eluted as two partially overlapped peaks that were included (15.3 mg and 18.1 mg, respectively). The ^{13}C NMR spectrum of fraction M (not shown) displayed the set of signals for the repeating unit of the arabinogalactan described previously[2] (C-1 signals at 109.1, 108.7, and 102.8 ppm) plus a number of signals attributable to a second polysaccharidic component (C-1 at 105.1 ppm). These polysaccharides were separated by sequential ethanol precipitation from a 1.5 mL aqueous solution of fraction M. The polysaccharide giving a ^{13}C NMR spectrum in which C-1 absorbed at 105.1 ppm was recovered from the precipitate (fraction O, 10.6 mg) obtained at an ethanol/water ratio of 3.5:1, whereas the arabinogalactan was recovered from the soluble fraction after dialysis and freeze-drying (fraction P, 3.5 mg).

The ^{13}C NMR spectrum of fraction N (not shown) suggested that it is mostly composed of a homopolymer, as judged by the observation of major signals expected for one hexose unit located at 107.0, 88.6, 82.9, 76.1, 71.1, and 63.4 ppm, plus other polysaccharide component(s) present in minor quantities (one anomeric signal at 103.7 plus a number of signals in the ring region and both the hydroxymethyl region between 60 and 62 ppm and the methyl region of 6-deoxysugars at 16.3, 17.3, and 17.5 ppm). Controlled precipitation by addition of ethanol to an aqueous solution of fraction N initially yielded a mixture (fraction Q), then at high ethanol concentration, the pure major polysaccharide (fraction R) (Chart 1). The ^{13}C NMR spectrum of fraction R (Figure 2) contained only the signals of the major constituent in fraction N. Monomer analysis by GLC-MS of the alditol acetates obtained after full acid hydrolysis showed that fraction R was uniquely composed of galactose. The ^{1}H NMR spectrum (not shown) displayed a signal for one anomeric proton at 5.35 ppm, which confirmed that the polysaccharide contain only one sugar residue (galactan). The absolute configuration of the galactose residue was determined to be D by GLC of the acetylated (*S*)-2-octylglycoside.[32,33] The chemical shift of the anomeric carbon (107.0 ppm) (Figure 2) indicates that a D-galactofuranoside residue is present, confirmed by the presence of nonanomeric signals at 82.9 ppm, a chemical shift typical of C-4 of furanosides, and at 88.6 pm (Figure 2), only possible in furanosides.[34,35]

The anomeric configuration could not be assigned from the chemical shift of C-1 of 107.0 ppm, since this value is midway between those of the two anomers of methyl D-galactofuranoside (differences −3.2 and +2.9 ppm with respect to the α- and β-anomers, respectively).[34] However, the anomeric proton of the galactofuranosyl residue appeared as a singlet ($^3J_{H1,H2} < 2$ Hz) at 5.32 ppm, typical of the β-anomeric form of galactofuranosides.[36−38] Note that $^3J_{H1,H2}$ is expected to be ∼4−5 Hz in the α-anomeric form.[39,40]

The remaining ^{1}H and ^{13}C signals were assigned using DQF−COSY, HSQC, and TOCSY spectra. The anomeric signal at 5.32 ppm correlates in the DQF−COSY spectrum (Figure 3, left) with the signal at 4.18 ppm (H-2), which in turn shows a cross-peak with the highly deshielded nonanomeric signal at 88.6 ppm in the HSQC spectrum (Figure 3, right), the latter being assigned to C-2. The deshielding of this C-2 signal (+7.3 ppm) with respect to that from methyl β-D-galactofuranoside[34] requires a substitution at this position and establishes the structure of the polysaccharide. Supporting evidence was obtained from a HMBC experiment optimized for a $^3J_{C,H}$ value of 8 Hz (Figure 4, left), which contained correlations between the signals of H-1 (5.32 ppm) and C-2 (88.6 ppm) and between those of C-1 (107.0 ppm) and H-2 (4.18 ppm) and by the observation of an nOe cross-peak between H-1 (5.32 ppm) and H-2 (4.18 ppm) in the NOESY spectrum (Figure 4, right). Although both of these correlations could have arisen by intra-ring pathways as well as inter-ring pathways, the presence of these correlations and the absence of others confirm the proposed structure.

The O-2 substituent shields the C-1 and C-3 nuclei (γ effect), explaining the low chemical shift value observed for the C-1 resonance (107.0 ppm) in the galactofuranan, compared to the value for C-1 of methyl β-D-galactofuranoside (109.9 ppm)[34] and in polysaccharides containing β-D-galactofuranosyl units without linkages to O-2.[41] Lower values of C-1 chemical shifts at 106.1 and 107.6 ppm were also observed recently for 2)-β-D-Gal*f*-(1→ residues in two exopolysaccharides isolated from

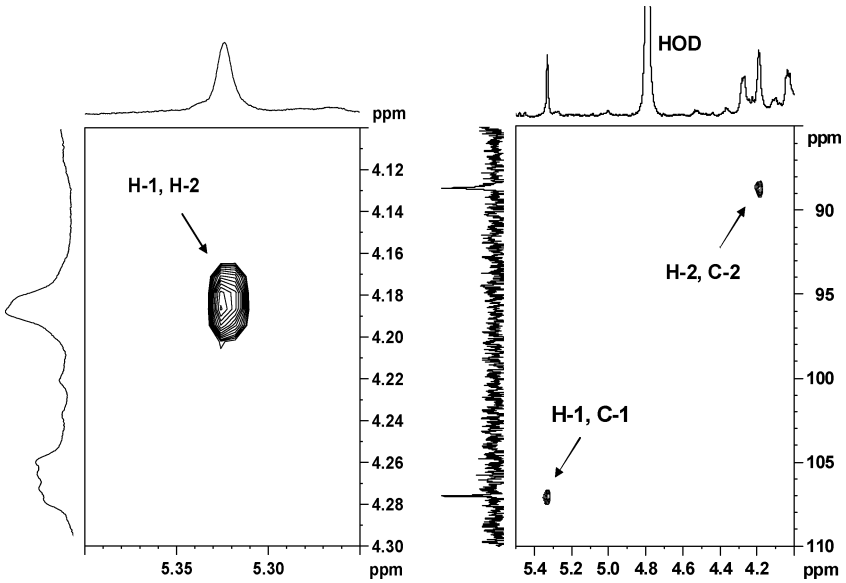


Figure 3. Portions of spectra of fraction R: on the left, the 500.1 MHz ^{1}H, ^{1}H DQF−COSY spectrum; on the right the ^{1}H, ^{13}C HSQC spectrum. The relevant correlations are annotated.

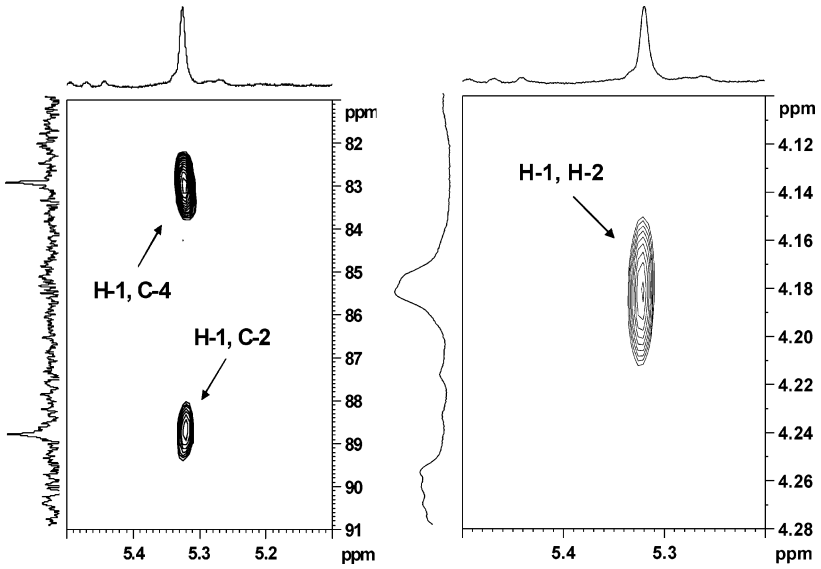


Figure 4. Portions of 500.1 MHz spectra of fraction R: on the left, the ^{1}H, ^{13}C HMBC spectrum; on the right, the ^{1}H, ^{1}H NOESY spectrum. The relevant correlations are annotated.

Table 1. NMR Chemical Shifts of the Galactofuranan (ppm)

nucleus	H/C-1	H/C-2	H/C-3	H/C-4	H/C-5	H/C-6
^{1}H	107.0	88.6	76.1	82.9	71.1	63.4
^{13}C	5.32	4.18	4.27	4.026	3.89	3.758/3.713

two strains of *Lactobacillus rhamnosus* by Lipiński et al.[37] and Vanhaverbeke et al.,[38] respectively. The remainder of the assignments are given in Table 1. The evidence shown above indicates that the galactofuranan isolated in fraction R has the following repeating unit

$$\rightarrow 2)\text{-}\beta\text{-D-Gal}f\text{-}(1\rightarrow$$

Molecular Weight and Molecular Weight Distribution. The weight average molecular weight of the galactofuranan of fraction R was estimated using the scaling relationship

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

where K and α are the scaling parameters and D is the self-diffusion coefficient.[20,28,42−44] This relationship has been shown to be valid only at sufficiently low concentrations (up to 1 wt %) that the molecules diffuse independently of each other. Under these conditions, D is referred to as the self-diffusion coefficient (SDC) at infinite dilution (D_0),[28,29,42,43,45,46] which is usually obtained by extrapolation of D to zero concentration. For saccharidic systems consisting of linear and slightly branched water-soluble uncharged oligo- and polysaccharides, the magnitude of the scaling parameters ($K = 8.2 \times 10^{-9}$ m s^{-1}, and $\alpha = -0.49$) were determined by Viel et al. by calibration of measured self-diffusion coefficients against known molecular weights of pullulan fractions with narrow molecular weight distributions.[28] The self-diffusion coefficients were obtained using DOSY spectroscopy.[20−22]

A typical DOSY experiment provides the information required to obtain the D values, based on the attenuation of the signal intensity at incremented pulsed-field gradient (PFG) amplitudes. Thus, the ^{1}H-detected DOSY spectra of a set of solutions of fraction R at concentrations of 1.43 (Figure 5, right), 7.28, and 14.57 mg/mL were recorded and the data obtained

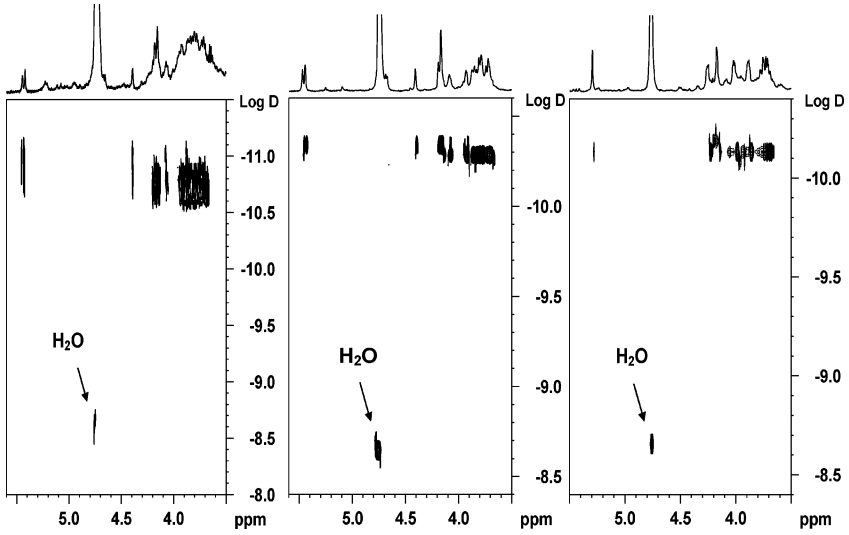


Figure 5. 500.1 MHz ^{1}H-DOSY spectra of the following: left, fraction L; center, fraction P; and right, fraction R; all at a concentration of 1.43 mg/mL.

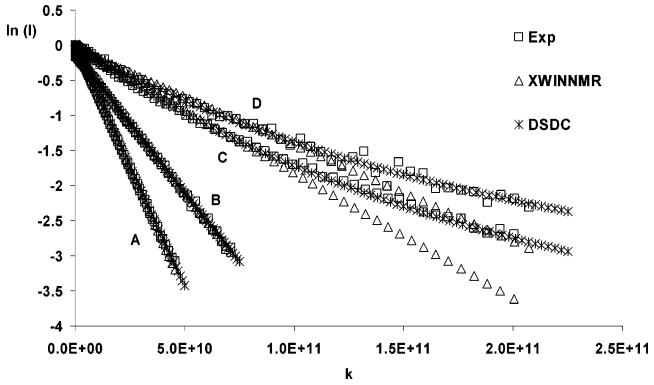


Figure 6. Plots of ln I versus k showing the fits to experimental data using the two fitting procedures described in the text: A, fraction R, galactofuranan; B, fraction P, an arabinogalactan; C, fraction U, an arabinogalactan; D, fraction L, an arabinogalactan.

during acquisition for the anomeric signal at 5.32 ppm was evaluated using the *Xwinnmr* T1/T2 fitting routine to obtain the *D* values more accurately.

The observed intensity, a function of the PFG amplitude, was fitted to a monoexponential decay (one component with single *D* and M_w values) defined by the modified Stejskal−Tanner relationship[47,48]

$$I = I_o e^{(-kD)} \quad (2)$$

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, while γ is the magnetogyric ratio and Δ is the time between the leading edges of the gradient pulses.

Figure 6, set A, shows the agreement between the experimental data and the signal intensities values predicted by the monoexponential fitting routine used to describe the attenuation of the anomeric signal at 5.32 ppm ($c = 1.43$ mg/mL). A slight deviation from linearity occurs only at greater attenuations, a behavior that was also reported by Håkansson et al.,[29] who described a slight but noticeable curvature in the ln *I* versus *k* plot of an artificial polymer with M_w of 1000 kDa and a polydispersity index of 1.05. These results are consistent with a low polydispersity index for the galactofuranan.

The concentration dependence of the self-diffusion coefficients for the galactofuranan is shown in Figure 7, top. The

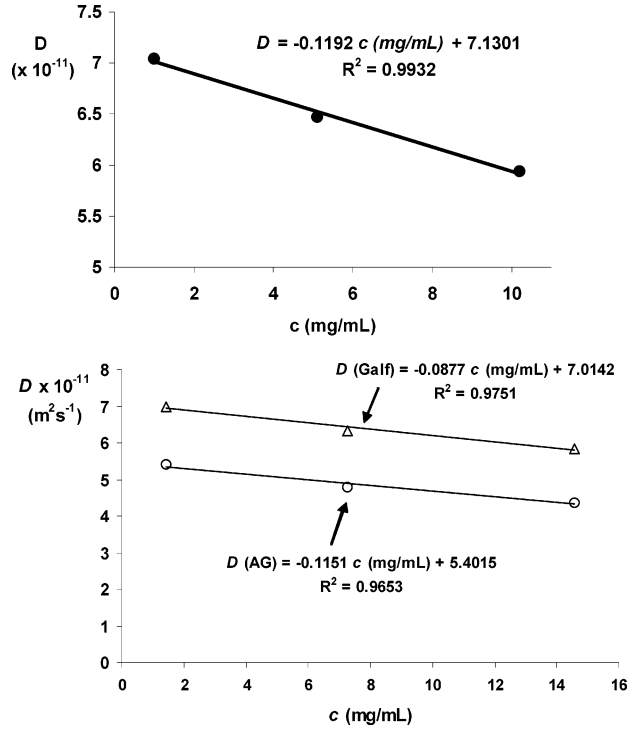


Figure 7. Concentration dependence of the self-diffusion coefficients of top, fraction R; bottom, the polysaccharides of fraction X (△ = galactofuranan, ○ = arabinogalactan).

dependence is linear along the concentration range in the study, and as expected, the *D* values decrease as the concentration increases.[42−45] Extrapolation of the individual curves to zero concentration using a least-squares linear regression yielded a D_o value of 7.13×10^{-11} ($m^2\ s^{-1}$). Using eq 1 with the scaling parameters reported by the Viel group,[28] the weight average molecular weight for the galactofuranan was estimated as 15.3 kDa.

To obtain the molecular weight dispersion for the galactofuranan, a mathematic formalism similar to that reported by Håkansson et al.[29] was employed. The polymer system is considered polydisperse, which implies that eq 2 is no longer valid. The observed signal becomes the sum of signals arising from polymers of different molecular weights and, in consequence, differing in self-diffusion coefficients. Hence, eq 3

applies. In theory, some account should be taken of T_2 variation in weighting signals from different molecular weight polymers. However, Heatley has shown that T_2 is molar-mass independent for polymers in the semidilute regime.[49]

$$I = \sum_i I_{oi}\, e^{(-kDi)} \tag{3}$$

Considering a continuous distribution of molecular weights and, therefore, a distribution of self-diffusion coefficients (DSDC), eq 3 can be rewritten as[29]

$$I = \int_0^\infty P(D)\, e^{(-kD)}\, dD \tag{4}$$

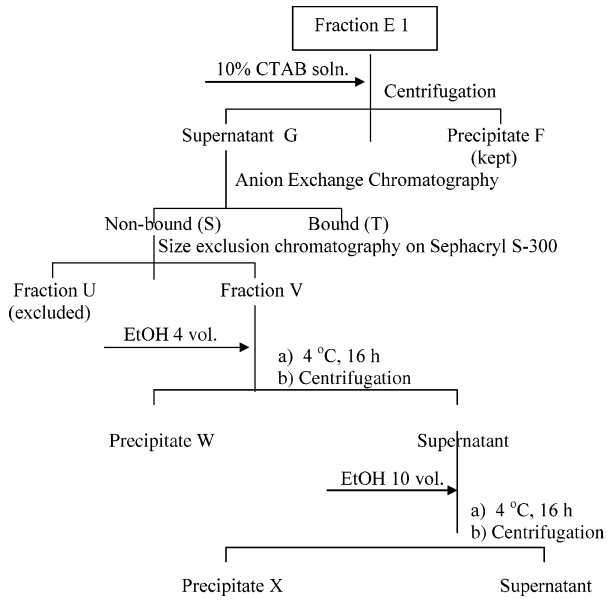
where $P(D)$ represents the SDC distribution function. Assuming a normalized Gaussian distribution as the functional form of $P(D)$, the moments of $P(D)$, D_o, and σ, the self-diffusion coefficient at the center of the DSDC (or weight-averaged self-diffusion coefficient) and the half-width of the DSDC (standard deviation of the distribution), respectively, were obtained by fitting the experimental signal decay to eq 4 using a nonlinear least-squares fitting procedure.

Figure 6, set A, shows that fitting the experimental signal decay for the anomeric signal at 5.32 ppm gave the same result for the weight average molecular weight whether the mathematical expression used considered the galactofuranan to be monodisperse or allowed a DSDC. This observation confirmed the low polydispersity present in the galactofuranan sample. In both cases, excellent agreement with the experimental data was achieved. The similarities in the D_o values, 7.26×10^{-11} ($m^2\ s^{-1}$) for the T1/T2 fitting routine and 7.05×10^{-11} ($m^2\ s^{-1}$) for the DSDC distribution also confirm the agreement between fits. The σ value of 1.3×10^{-11} ($m^2\ s^{-1}$) can be used to determine of the self-diffusion coefficients range as $D_o \pm {}^1/_2\sigma$, which leads to the weight average molecular weight range of (15.3 ± 2.9) kDa using eq 1 and the Viel group values of the scaling parameters, K and α.[28]

The weight average molecular weight of the arabinogalactans of fractions L and P were also evaluated. In both cases, DOSY experiments were recorded at a very dilute regime (1.43 mg/mL)[28] (Figure 5 left and center), and the experimental signal decay for the anomeric signal at 5.46 ppm was fitted to eq 2 (T1/T2 *Xwinnmr* fitting routine) and eq 4, the latter considering a normalized Gaussian function to describe the shape of the hypothetical DSDC. For the arabinogalactan of fraction P, the experimental plots are linear and both fitting procedures gave similar results, in excellent agreement with the experimental data (Figure 6, set B). This suggests a low polydispersity for this version of the arabinogalactan. The D_o and σ values of 3.99×10^{-11} and 0.51×10^{-11} ($m^2\ s^{-1}$), respectively, obtained from the DSDC fitting procedure were employed as described above to estimate the weight average molecular weight range of 50.2 ± 6.6 kDa. For the arabinogalactan of fraction L (Figure 6, set D), the experimental plots were not linear, implying a polydisperse sample. The DSDC fitting procedure gave D_o and σ values of 1.6×10^{-11} and 1.04×10^{-11} ($m^2\ s^{-1}$), respectively. These values were employed as described above to estimate the weight average molecular weight range 1020 ± 370 kDa. The large molecular weight range is consistent with a polydisperse sample.

The weight average molecular weight of 47 kDa reported by us[2] from SEC-MALS measurements for the arabinogalactan isolated previously in fraction I falls into the molecular weight range estimated for the arabinogalactan of fraction P. However, as the 47 kDa value was calculated on the basis of the amount of light scattered over the whole width of the peak that was centered at ~12 mL,[2] it inherently includes contributions from the edges of the peak, one of which was partially overlapped with that of a small amount of a higher molecular weight component that was present. Reevaluation of the SEC-MALS data for the original arabinogalactan (fraction I) using the CMF versus molar mass representation (not shown) showed that 60% of the SEC peak had a weight average molecular weight centered at 29 kDa, representing the center of the peak at 11.9−12.1 mL.

Chart 2. Flowchart Describing the Process to Obtain Fraction X Starting from Fraction E1, the Sephadex G-100 Excluded Fraction[2]



The results shown above indicate that, by using the fractionation protocol described, three different versions (29, 50.1, and 1020 kDa) of an arabinogalactan with a repeating unit consisting of →2)-α-l-Ara*f*-(1→3)-[α-l-Ara*f*-(1→4)]-*β*-D-Gal*p*-(1→ [2] can be isolated from the *Chlorella pyrenoidosa* extract. In fact, the observation of an arabinogalactan with molecular weight value of 1020 kDa is consistent with our hypothesis[2] that the original structure was larger than 47 kDa.

Another fractionation of a different batch of freeze-dried *C. pyrenoidosa* cells was performed exactly as before except that ethanol precipitation of fraction G was not done (see Chart 2). Instead, fraction G (535.0 mg) was fractionated further by anion exchange chromatography on Q-Sepharose Fast Flow under the conditions previously described to yield a neutral (S, 127.1 mg) and an acidic fraction (T, 295.3 mg) after dialysis and freeze-drying. Size exclusion of fraction S on Sephacryl S-300 (conditions as described; chromatogram not shown) yielded two fractions, U (22.3 mg), that was excluded, and V (66.4, mg), that was included.

The ^{13}C NMR spectrum of fraction U (not shown) contained the set of signals associated to the arabinogalactan[2] plus other polysaccharidic components present in minor quantities (anomeric signals at 108.1, 105.1, and 94.6 ppm). For this arabinogalactan, the experimental signal decay for the anomeric signal at 5.46 ppm obtained from a DOSY experiment recorded at 1.43 mg/mL shows a noticeable deviation from the linear behavior associated with a monodisperse system. This observation suggests a large polydispersity for this version of the arabinogalactan. A normalized Gaussian distribution could be used to describe the DSDC of this arabinogalactan, as judged by the agreement between the experimental data and the signal intensities predicted using the DSDC fitting procedure (Figure

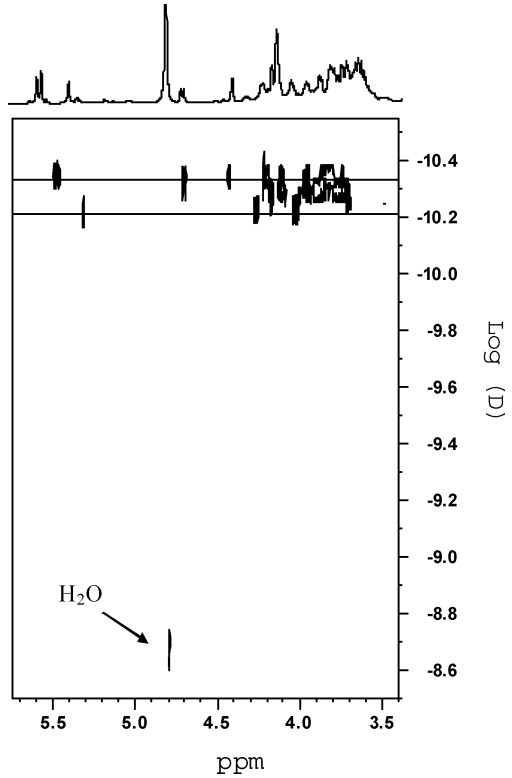


Figure 8. 500.1 MHz ^{1}H DOSY spectra of fraction X recorded at 14.57 mg/mL.

6, set C). The weight average molecular weight of 188 ± 109 kDa was obtained from the D_o and σ values of 2.09 and 1.04 × 10^{-11}, respectively, of the DSDC fitting procedure.

On the other hand, the ^{13}C NMR spectrum of fraction V displayed the sets of signals for either the arabinogalactan or the galactofuranan plus a number of minor signals (12) in the anomeric region that arise from other polysaccharidic components. Fraction V was purified by dissolving it in 2 mL of water and treating it with 95% ethanol. At an ethanol/water ratio of 4:1, fraction W (25.2 mg after dialysis and freeze-drying) precipitated. At an ethanol/water ratio of 10:1, fraction X (21.1 mg after dialysis and freeze-drying) precipitated. Fraction W was kept, while fraction X was characterized further.

From its ^{13}C NMR spectrum (not shown), fraction X contained only the major polysaccharides of fraction V (arabinogalactan and galactofuranan). The intensities of the signals of the three anomeric carbons of the three sugar residues of the arabinogalactan (109.1, 108.7, and 102.8 ppm) were double the intensity of the anomeric signal of the galactofuranan (107.0 ppm). A similar ratio of intensities is observed in the ^{1}H NMR spectrum (not shown) for the anomeric protons of the sugar residues of both polysaccharides, indicating a 2:1 molar ratio of the arabinogalactan and the galactofuranan.

The molecular weight estimation of the individual polysaccharides of fraction X was accomplished as described above for the molecular weight estimation of the galactofuranan of fraction R. For the polysaccharides mixture of fraction X, the anomeric signals from both spin systems were well-isolated in the diffusion dimension of the DOSY spectrum (Figure 8), which allowed the utilization of the T1/T2 *Xwinnmr* fitting routine to estimate the *D* values accurately, assuming a narrow SDC distribution for both polymers. However, only two out of the remaining five signals for the galactose residue of the galactofuranan at 4.18 (H-2) and 4.27 (H-3) were separated at a diffusion coefficient value of 5.78 × 10^{-11} m^2 s^{-1} due to signal overlapping in the ring region (Figure 8). The inaccurate separation of signals differing by diffusion coefficient values closer than 30% (28% in this case) is due to difficulties in fitting a biexponential curve with a nonlinear least-squares regression algorithm.[21]

The dependence of *D* versus concentration (Figure 7, bottom) was linear and with a negative slope as expected.[42,43] The D_o values obtained by extrapolation of the individual curves to zero concentration 5.40 and 7.01 × 10^{-11} m^2 s^{-1} were substituted in eq 1, and the resulting weight average molecular weight values were 27.1 and 15.9 kDa for the arabinogalactan and the galactofuranan, respectively.

The weight average molecular weights of fraction V were also evaluated by SEC-MALS using the cumulative weight fraction (CWF) versus molar mass representation, since the normal RI plot showed one overlapping peak. The curve in the former graph contained two regions with distinct slopes, consistent with the presence of two components as established above (Figure 9). The first component, representing 28% of the mixture (0.07−0.35 on the CWF axis) had a weight average molecular weight centered at 18.5 kDa and corresponds to the galactofuranan. The second component (arabinogalactan) represents 48% of the mixture (0.52−0.9 on the CWF axis), and the average molecular weight is centered at 29.0 kDa. These results are in excellent agreement with those from the DOSY experiments and further validates the equation advocated by Viel et al.[28]

As outlined above, two fractionation protocols on different batches of *C. pyrenoidosa*, that either included ethanol precipitation (G) or did not, both yielded at least two arabinogalactans of the type described previously[2] differing in their molecular weights (27.1 and 188 kDa without ethanol precipitation and 29, 50.1, and 1020 kDa with ethanol precipitation). For both protocols, only a single galactofuranan with a molecular weight of ∼15.6 kDa was obtained. The fact that the protocol that included the extra step, ethanol precipitation of fraction G, yielded an arabinogalactan with a larger molecular weight value (1020 kDa) suggests that this step is not the cause of any possible degradation of a larger arabinogalactan.

It appears that the arabinogalactan is present in *C. pyrenoidosa* in a number of different molecular weight forms. The low polydispersity of the ∼28 and 50 kDa versions suggests that more disperse larger molecular weight polymers are biosynthesized by assembly of variable numbers of these preassembled units.

Biological Activity. The results of the bioactivity testing are shown in Figure 10. As we described previously,[2] fraction H contains the bulk of the immunostimulatory activity of the supernatant G, measured from its stimulation of NO synthesis. None of the bioactivity of fraction H was present in either the galactofuranan or in the 50 or 28 kDa arabinogalactans. However, for the latter type of polymer, an increase in NO production is observed for the larger versions (188 and 1020 kDa; Figure 10), which indicates that the bioactivity of this polysaccharide is correlated with its size.

Publications linking biological activity and molecular size of the branched 1,3-glucans, scleroglucan, lentinan, and schizophyllan, have emphasized the importance of rigid conformations such as triple helices[14,15] for activity. The arabinogalactans described here, with both arabinosides present as furanosyl residues, seem unlikely candidates to adopt rigid conformations.

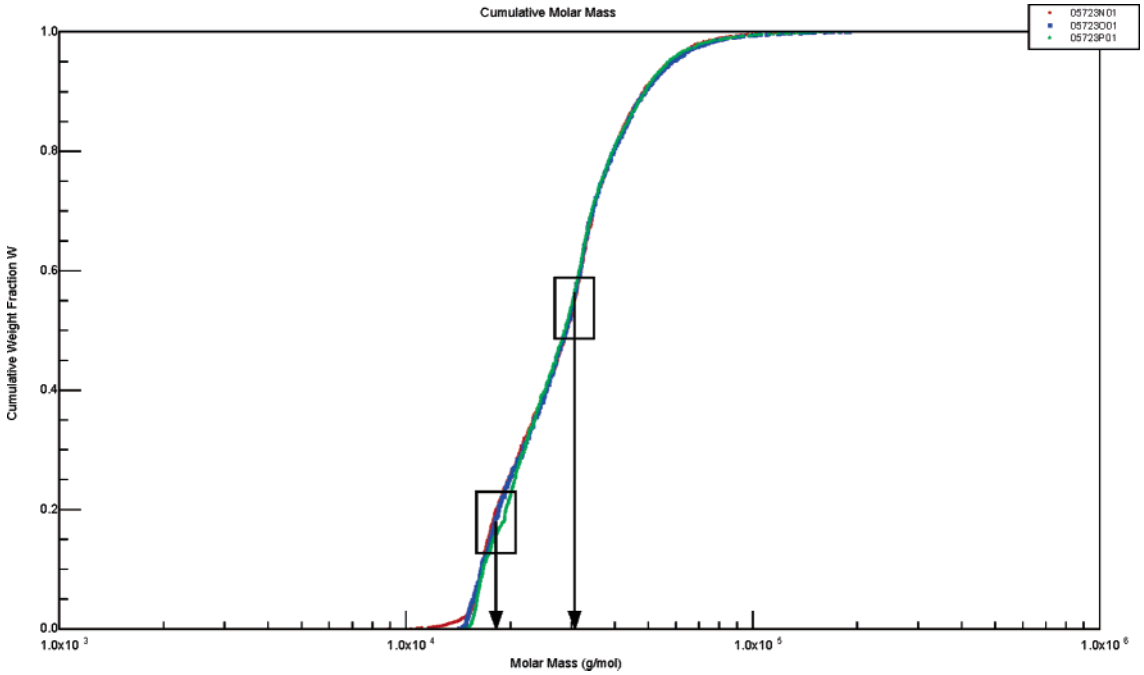


Figure 9. Cumulative molecular weight curve from SEC-MALS of fraction X. The center of the two distinct regions representing the weight average molecular weights is highlighted.

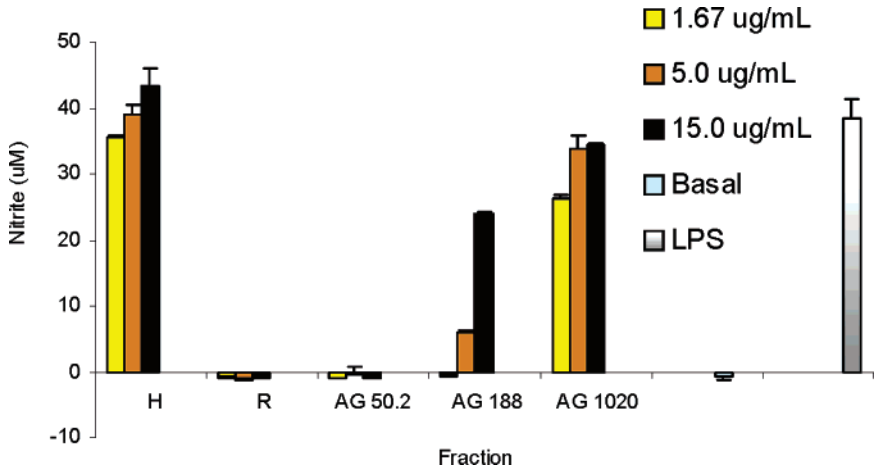


Figure 10. Stimulation of NO synthesis in RAW cells culture supernatants.

Conclusion

The unique arabinogalactan previously isolated from *Chlorella pyrenoidosa* cells was shown to be present as a variety of different molecular weight variations. The versions isolated had molecular weights of 27, 50, 188, and 1020 kDa as determined by DOSY NMR measurements using the relationship between self-diffusion coefficient and molecular weight established by Viel et al.[28] A new polysaccharide, a 1→2- linked α-D-galactofuranan, was also isolated, and its structure was determined from its 1D and 2D ^{1}H and ^{13}C NMR spectra. Its molecular weight was determined to be 15 kDa from DOSY NMR measurements. For those polysaccharides where molecular weights were also determined by SEC-MALS, very similar molecular weights were obtained by both techniques, extending the types of neutral polysaccharides for which the relationship established by Viel et al.[28] between molecular weight and self-diffusion coefficients is valid. Very little information has been available about molecular weight dispersion in natural polysaccharides. The DOSY data was analyzed by two methods; one assumed a monodisperse polymer, the other assumed a Gaussian distribution of self-diffusion coefficients about the average. Both methods gave the same results for the three lower molecular weight polysaccharides, implying close to monodisperse polymers. The two larger arabinogalactans had considerable variation in their molecular weights (188 ± 109 kDa and 1020 ± 370 kDa). Since very mild conditions were used in the isolation of these different arabinogalactans, it seems likely that little degradation took place during isolation, and different molecular weight arabinogalactans are present in *Chlorella pyrenoidosa* cells. Only the two larger arabinogalactans showed immunostimulatory activity.

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CDV

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