Comparison of the carbohydrate biological response modifiers Krestin, schizophyllan and glucan phosphate by aqueous size exclusion chromatography with in-line argon-ion multi-angle laser light scattering photometry and differential viscometry detectors

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Abstract

A major barrier to the development, preclinical and clinical application of natural carbohydrate biological response modifiers has been the difficulty involved in accurately characterizing carbohydrate polymers with molecular masses ranging from 10^4 to 10^7 g/mol. Herein, we employed size exclusion chromatography with multi-angle laser light scattering and differential viscometry to compare and contrast structural properties of the biological response modifiers Krestin, schizophyllan and glucan phosphate. Krestin, schizophyllan and glucan phosphate exhibit significant differences in molecular mass moments, molecular mass distribution, polymer sizes, intrinsic viscosity and perhaps their solution behaviour. This knowledge of precise physicochemical data is required for a better understanding of the properties and higher structure of complex carbohydrate biological response modifiers.

1. Introduction

Water soluble, complex carbohydrate polymers, derived from natural sources (i.e. bacterial and fungal), have been shown to augment vari-

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ous facets of immune responsiveness in humans [1] and animals [2–5]. These natural product carbohydrate polymers belong to the class of drugs known as biological response modifiers (BRMs). The ability of complex carbohydrate BRMs to exert beneficial effects in a wide variety of experimental [2–5] and clinical disease states [1] has stimulated research into their potential biomedical applications. Among the more widely-known drugs of this class are the BRMs

Krestin [6,7], schizophyllan [8–10], and glucan phosphate [2,4,5], all of which exist as water soluble, pharmaceutical grade preparations. Schizophyllan and glucan phosphate are composed of $(1\rightarrow3)$-β-D-linked glucopyranose backbones with varying degrees of $(1\rightarrow6)$-β side-chain branches. Krestin, a proteoglycan, contains $(1\rightarrow3)$-β, $(1\rightarrow4)$-β and $(1\rightarrow6)$-β intrachain linkages [6].

A major barrier to the development, understanding and ultimate clinical utilization of complex, natural product, carbohydrate BRMs has been the difficulty involved in accurately characterizing carbohydrate polymers with molecular masses ranging from 10^4 to 10^7 g/mol. Recent advances in aqueous size exclusion chromatography (SEC), in concert with development of new detectors, microcomputer-based data acquisition systems and software make it possible to rapidly, simply and accurately determine a number of important physicochemical characteristics of complex polymer systems including molecular mass moments [number- (M_n), mass- (M_w), and z- (M_z) average molecular mass], root mean square radius of gyration moments (rms$_n$, rms$_w$, and rms$_z$), polydispersity (I), intrinsic viscosity $([\eta])$, branching characteristics [11,12] as well as the radius of gyration exponent, "ν", and the Mark–Houwink exponent, "α". Therefore the purpose of this study is to compare and contrast the molecular masses, sizes and conformations in aqueous solution of Krestin, schizophyllan and glucan phosphate by means of aqueous SEC with multi-angle laser light scattering (MALLS) and differential viscometry (DV).

2. Experimental

2.1. Carbohydrate BRMs

Krestin (PSK), a protein-containing ($\sim$25–38%) polysaccharide derived from *C. versicolor*, was obtained as a powder from Kureha Chemical Industries (Tokyo, Japan). Schizophyllan (SPG, derived from *S. commune*) was obtained in sterile water (10 mg/ml) from Kaken Chemical Co. (Tokyo, Japan). Glucan phosphate from *S.*

cerevisiae was prepared in our laboratory [13]. Dextran (Pharmacia, Piscataway, NJ, USA), and pullulan standards (Showdex P-82 series, J.M. Science, Grand Island, NY, USA) were used for calibration of the system.

2.2. High-performance aqueous SEC

To evaluate the polymer distribution of the various BRMs, Krestin, schizophyllan and glucan phosphate were analyzed by aqueous high-performance SEC. The basic SEC system consisted of an SSI 222D (Scientific Systems, State College, PA, USA) single-piston isocratic, pulse-dampened HPLC pump, a Rheodyne sample injector (Rheodyne, Cotati, CA, USA) and a 505 LC column oven (SSI, State College, PA, USA). The mobile phase, 0.05 M sodium nitrite, was filtered (0.45 μm) and stored in a sterile reservoir. Mobile phase was delivered at a flow-rate of 0.5 ml/min. Three Ultrahydrogel (Waters Chromatography Division, Milford, MA, USA) aqueous SEC columns having exclusion limits of $2\cdot10^6$, $5\cdot10^5$ and $1.2\cdot10^5$ g/mol were connected in series along with an Ultrahydrogel guard column. The columns were maintained at 30°C. The system was calibrated using narrow-band pullulan and dextran standards. For analysis, Krestin and schizophyllan were dissolved in mobile phase at a concentration of 2–3 mg/ml by gentle rocking until completely hydrated ($\sim$2–3 h). The lyophilized glucan phosphate preparation was solved under the same conditions, but in 50 mM NaOH to separate the aggregate peak and then injected with 50 mM sodium nitrite mobile phase. A 200-μl injection volume was used for all analyses.

2.3. Determination of the specific refractive index increment [dn/dc]

The dn/dc values for Krestin, schizophyllan and glucan phosphate were determined with an Optilab 903 interferometric refractometer (Wyatt Technology, Santa Barbara, CA, USA) at 25°C in 50 mM sodium nitrite mobile phase.

2.4. Determination of molecular mass and rms$_z$ by argon-ion and helium–neon MALLS photometry

Absolute molecular masses of the BRMs were determined by on-line MALLS (15 angles ranging from 21.5° to 158.5°) photometry employing a Dawn-DSP argon-ion (488 nm) MALLS photometer fitted with a K5 flow-cell (Wyatt Technology) at ambient temperature. Absolute molecular mass distribution, molecular mass moments (number-average, z-average, mass-average), polydispersity and rms$_z$ moments were established with ASTRA software (v. 3.04). Values for ν were established with EASI software (v. 7.02, Wyatt Technology). Significant fluorescence was observed when Krestin was analyzed by argon-ion MALLS at 488 nm. To eliminate the fluorescence interference, Krestin was analyzed by helium–neon MALLS photometry at 632.8 nm as described by Williams et al. [14]. Reported molecular masses of pullulan and dextran standards used to check column calibration showed good agreement with MALLS data.

2.5. Determination of intrinsic viscosity by DV

Intrinsic viscosity of the BRM polymers was determined by in-line DV. For determination of [η] the column eluent was passed through a Viscotek Model 200 differential refractometer/viscometer and data were analyzed with Unical software (v. 4.03, Viscotek, Porter, TX, USA). Molecular mass determinations of standards using this technique showed good agreement with MALLS data. Intrinsic viscosities of pullulan standards were determined to be in close agreement with previous data [15].

3. Results

3.1. Specific refractive index increments (dn/dc) for Krestin, schizophyllan and glucan phosphate

We established the dn/dc values for the BRMs in 50 mM sodium nitrite mobile phase. The values are given in Table 1. Krestin has a dn/dc

Table 1
Specific refractive index increments (dn/dc) for Krestin, schizophyllan and glucan phosphate at 488 nm and 25°C[a]

BRM	dn/dc (ml/g)[b]
Krestin	0.147 ± 0.001
Schizophyllan	0.253 ± 0.006
Glucan phosphate	0.158 ± 0.001

[a] Data were acquired with an OptiLab 903 interferometric refractometer employing 50 mM sodium nitrite as the mobile phase.
[b] Data presented are averages ± standard error of two separate runs ($n = 2$) with nine different concentrations for each BRM.

of 0.147 ml/g. Schizophyllan has a dn/dc of 0.253 ml/g. Glucan phosphate shows a dn/dc of 0.158 ml/g.

3.2. Average molecular mass moments, rms$_z$ moments, intrinsic viscosities and polydispersity of Krestin, schizophyllan and glucan phosphate

Fig. 1 shows a plot of molecular mass vs. elution volume and concentration vs. elution volume for Krestin, schizophyllan and glucan phosphate. The average molecular mass moments and rms$_z$ moments for the carbohydrate BRMs, as determined by SEC–MALLS–DV are presented in Table 2. Polydispersity and the intrinsic viscosity of each polymer are also shown in Table 2.

Krestin, a proteoglycan [6,7] showed an unusual light scattering chromatogram at 488 nm, suggesting that a portion of Krestin (i.e. peak 3) exhibits fluorescence at this wavelength. Therefore, establishing correct molecular mass moments, rms$_z$ moments and polydispersity for Krestin with argon-ion MALLS was not possible. Attempts to remove the fluorophore from Krestin by SEC were unsuccessful. We speculate that the fluorophore is covalently bonded. To circumvent this problem, Krestin was analyzed by helium–neon laser photometry ($\lambda = 632.8$ nm) as previously described [14]. We confirmed and extended our previous observations for Krestin [14] by detecting three polymer peaks with M_ws

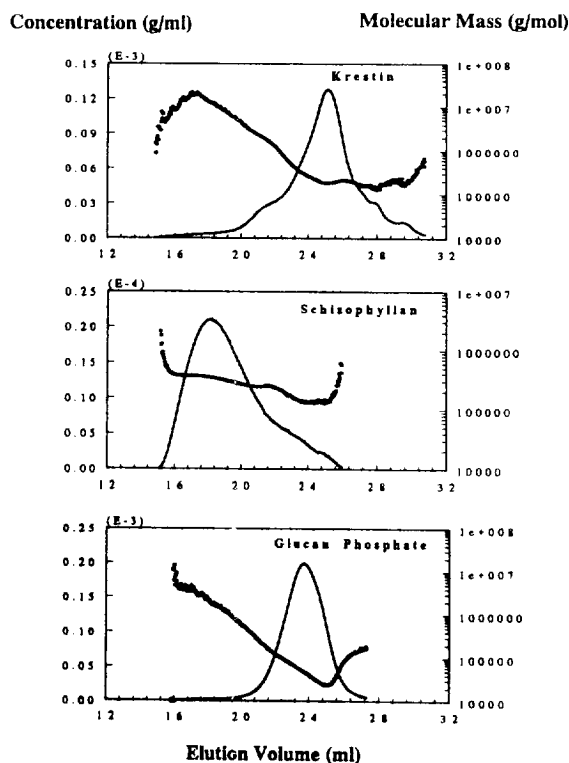Concentration (g/ml)   Molecular Mass (g/mol)

Elution Volume (ml)

Fig. 1. Molecular mass vs. elution volume and concentration vs. elution volume chromatograms for Krestin, schizophyllan and glucan phosphate as established by SEC–MALLS–DV. The molecular mass vs. elution volume and concentration vs. elution volume chromatograms for Krestin, schizophyllan and glucan phosphate are presented in the top, middle and bottom panel, respectively. Schizophyllan and glucan phosphate were analyzed by argon-ion (488 nm) MALLS. Krestin, due to significant fluorescence at 488 nm, was analyzed by helium–neon (633 nm) MALLS. The gaussian curve represents the concentration chromatograms, whereas the linear relationship represents the molecular mass vs. elution volume chromatograms.

of $1.1 \cdot 10^7$, $1.3 \cdot 10^6$, and $2.0 \cdot 10^5$ g/mol, respectively, and rms$_z$ radii of gyration of 40.2 nm for the first peak and 35.0 nm for the second peak, respectively. The limited instrument sensitivity of the laser photometer at 632.8 nm made it impossible to detect an rms$_z$ moment for the third peak. The [η] of Krestin was 17 ml/g. Molecular mass distribution across all three peaks was $8.2 \cdot 10^5$ g/mol with an rms$_z$ moment of 38 nm. The first M_w peak accounted for ~3.5% of the total polymer mass. The second

M_w peak accounted for 21.7% of the total polymers. The third M_w peak ($2.0 \cdot 10^5$ g/mol) contained the majority (74.8%) of Krestin polymers and also the fluorophore observed at 488 nm.

Schizophyllan, a branched $(1 \rightarrow 3)$-β-D-glucan polymer [8], was observed to have a narrow polymer distribution ($I = 1.08$, M_w/M_n) with one polymer peak ($M_w = 3.06 \cdot 10^5$ g/mol). The z-average rms moment for schizophyllan was 37.3 nm and [η] was 732 ml/g.

Glucan phosphate, a $(1 \rightarrow 3)$-β-linked polymer [13], was observed to have two peaks (with M_ws of $2.24 \cdot 10^6$ and $7.16 \cdot 10^4$ g/mol, respectively) with z-average rms moments of 36.1 and 17.7 nm, respectively. The [η] of glucan phosphate was 49 ml/g. The high-molecular-mass peak was only observed by the light scattering detector, suggesting the presence of a very-high-molecular-mass complex occurring at very low concentration. The first glucan phosphate peak may represent aggregate formation. The lower M_w peak ($7.16 \cdot 10^4$ g/mol) accounted for >99.99% of total polymers.

We also attempted to analyze the molecular mass of lentinan, another fungal BRM derived from *L. edodes*. Because of lentinan's lack of aqueous solubility, we were unable to perform the analysis.

3.3. Relationship between molecular mass and polymer size

The slope of the linear relationship between the logarithm of the radius of gyration and the logarithm of the molecular mass moment ($R_G = K_\nu \cdot M^\nu$) has been termed "ν" [16–18]. Establishing ν may provide additional insights into polymer solution conformation [16–18]. The ν-values for Krestin, schizophyllan and glucan phosphate are presented in Table 3. Krestin has a ν-value of 0.24. Schizophyllan showed an average ν-value of 0.599. Glucan phosphate was observed to have an average ν of 0.354.

The slope of the linear relationship between log intrinsic viscosity and log molecular mass ([η] = $K_\alpha \cdot M^\alpha$) is known as the Mark–Houwink or α-value for a polymer system [16,19]. The

Table 2
Molecular mass moments, rms radii of gyration moments, polydispersity and intrinsic viscosity of Krestin, schizophyllan and glucan phosphate as determined by SEC–MALLS–DV

	Number-average MW (g/mol)[a]	Mass-average MW (g/mol)[a]	z-Average MW (g/mol)[a]	z-Average RMS radii of gyration (nm)[a,b]	I (polydispersity, M_w/M_n)[a]	Intrinsic viscosity [η] (ml/g)[c]
Krestin[d]						
Peak 1	$9.1 \cdot 10^4$	$1.1 \cdot 10^7$	$1.3 \cdot 10^7$	40.2	1.22	
Peak 2	$7.9 \cdot 10^5$	$1.3 \cdot 10^6$	$2.1 \cdot 10^6$	35.0	1.68	
Peak 3	$2.0 \cdot 10^5$	$2.0 \cdot 10^5$	$2.1 \cdot 10^5$	n.d.[e]	1.03	
All peaks	$2.5 \cdot 10^5$	$8.2 \cdot 10^5$	$6.9 \cdot 10^6$	38.3	3.3	17.0 ± 2.0
Schizophyllan						
Peak 1	$2.8 \cdot 10^5$	$3.06 \cdot 10^7$	$3.25 \cdot 10^7$	37.3	1.08	732.0 ± 27.0
Glucan phosphate						
Peak 1	$1.7 \cdot 10^6$	$2.24 \cdot 10^6$	$2.94 \cdot 10^6$	36.1	1.28	
Peak 2	$4.8 \cdot 10^4$	$7.16 \cdot 10^4$	$1.4 \cdot 10^5$	17.7	1.50	49.0 ± 14.0

[a] The values were chosen from a pool of at least two single runs and are representative for each sample.
[b] The root mean square radius of gyration was estimated by multi-angle laser light scattering.
[c] The standard errors for the intrinsic viscosity were calculated from at least three single experiments.
[d] The MW moments, z-average rms radius of gyration and polydispersity for Krestin were acquired with the helium–neon laser photometer.
[e] not detectable

Mark–Houwink relationship provides another means of examining the solution conformation of polymers. The α-values for Krestin, schizophyllan and glucan phosphate are presented in Table 4. We observed two α-values (i.e. two slopes) for Krestin. The first slope over the molecular mass range from $1 \cdot 10^5$ to $4 \cdot 10^6$ g/mol yields an α-value of 0.643, while the second slope for molecular masses ranging from $4 \cdot 10^6$ to $4 \cdot 10^7$ g/mol yields $\alpha = 1.161$ (Table 4).

Table 3
Scaling relationship between the rms radius of gyration and molecular mass for Krestin, schizophyllan and glucan phosphate by SEC–MALLS

BRM	ν[a]	Molecular mass range employed for calculation of ν[b]
Krestin[c]	0.24 ± 0.001	$\sim 2 \cdot 10^6 - \sim 5 \cdot 10^7$
Schizophyllan	0.599 ± 0.043[d]	$\sim 2 \cdot 10^5 - \sim 4 \cdot 10^5$
Glucan phosphate	0.354 ± 0.028[e]	$\sim 5 \cdot 10^4 - \sim 4 \cdot 10^5$

[a] The ν-value was calculated as an average ± standard error.
[b] The molecular mass ranges represent the linear dependence of the scaling relationship between rms radius of gyration and molecular mass [27].
[c] The ν-value for Krestin was derived from the high-molecular-mass fraction (peaks 1 and 2). The ν-value for Krestin was averaged from two single runs ($n = 2$).
[d] The ν-value for schizophyllan was calculated as an average from four single experiments ($n = 4$) and found to be highly reproducible.
[e] The ν-value for glucan phosphate was averaged from eleven single experiments ($n = 11$).

Table 4
Scaling relationship between the average intrinsic viscosity and molecular mass (Mark–Houwink plot) of Krestin, schizophyllan and glucan phosphate[a]

BRM	α	Molecular mass range employed for the calculation of α[b,c]
Krestin[d]	0.643 ± 0.082	$\sim 1 \cdot 10^5 - \sim 4 \cdot 10^6$
	1.161 ± 0.249	$\sim 4 \cdot 10^6 - \sim 4 \cdot 10^7$
Glucan phosphate[e]	0.649 ± 0.024	$\sim 1 \cdot 10^4 - \sim 5 \cdot 10^5$

[a] The values are presented as averages ± standard error.
[b] The molecular mass ranges represent the linear dependence of the scaling relationship between intrinsic viscosity and molecular mass [27].
[c] The α-value for schizophyllan could not be established due to the narrow molecular mass distribution.
[d] The α-values for Krestin were averaged from three single runs.
[e] The α-value for glucan phosphate was averaged from three single runs.

Glucan phosphate shows an α-value of 0.649 (Table 4). It was not possible to ascertain the α-value for schizophyllan due to the narrowness of the distribution.

4. Discussion

In this study we have compared and contrasted the molecular mass moments, molecular mass distribution, polymer size and solution characteristics of the natural product carbohydrate BRMs Krestin, schizophyllan and glucan phosphate by aqueous SEC with in-line MALLS photometry and DV. Previously, we have demonstrated the utility of SEC–MALLS–DV in the isolation, development and characterization of various natural product BRMs [13,14,20]. Our previous studies employed a helium–neon MALLS photometer with a wavelength of 632.8 nm [13,14,20]. In the present study, we have employed an argon-ion MALLS system ($\lambda = 488$ nm) which provides increased analytical capability over a broader polymer range. In addition, we have employed interferometric refractometry to accurately establish the dn/dc values for each polymer system.

The M_w of Krestin has been reported to be $\sim 1.0 \cdot 10^5$ g/mol with a single peak observed by classical concentration-dependent SEC [7,21]. Recently, we have analyzed Krestin by SEC–MALLS–DV using the helium–neon MALLS system ($\lambda = 632.8$ nm) and an assumed dn/dc of 0.146 ml/g [14]. We observed three polymer peaks [14]. In the present study, employing the argon-ion SEC–MALLS system, we observed that Krestin showed a fluorescence effect at 488 nm, making an accurate determination of molecular mass moments and rms radii of gyration moments at this wavelength impossible. To alleviate this interference we analyzed Krestin by helium–neon laser photometry, using the determined dn/dc of 0.147 ml/g. We established the overall dn/dc for Krestin, since it would have been virtually impossible to collect enough material from each individual peak for separate dn/dc determinations. By comparing the data acquired with the argon-ion and the helium–neon laser systems we found that the molecular mass moments for peaks 1 and 2 are in good accordance. Moreover, both the light scattering chromatogram at 488 nm and the molecular mass moments at 488 nm and 633 nm for peak 3 indicate that the Krestin fluorophore is located within the third polymer peak, which contained the majority of the polymers. Krestin exhibits the lowest intrinsic viscosity of all three BRMs. We speculate that this is due, in part, to the protein component. The relationship between

molecular mass and rms radius of gyration yielded a ν-value of 0.24 for Krestin, which is suggestive of a spherical or branched polymer [17]. This value was primarily derived from peaks 1 and 2. We were unable to derive a ν-value for peak 3 because of the limited resolving power of the helium–neon MALLS photometer for this polymer system. The Mark–Houwink plot for Krestin revealed two slopes. The α-values for Krestin were 1.161 ± 0.249 (peaks 1 and 2) and 0.643 ± 0.082 (peak 3, major peak). These values suggest that Krestin exhibits a coiled structure, which is partially solvated with increasing portions of more rigid conformations as molecular mass increases in aqueous phase.

Schizophyllan is one of the better known $(1 \rightarrow 3)$-β-D-glucan BRMs [8,10,19,22,23]. The schizophyllan examined in this study is a pharmaceutical grade preparation. Previous reports concerning the physicochemical characterization of schizophyllan must be considered equivocal, because many of the preparations examined were not highly purified and/or the analytical techniques employed were not comparable to argon-ion SEC/MALLS [19]. The observed $\mathrm{d}n/\mathrm{d}c$ for schizophyllan (0.253 ml/g) is considerably higher than previously reported [19,23]. The precise reasons for this discrepancy are not known. However, we speculate that differences in the purity of the compound and/or differences in the method of $\mathrm{d}n/\mathrm{d}c$ estimation may account for the variation. The polydispersity of schizophyllan was observed to be very narrow ($I = 1.08$). The rms_z moment of schizophyllan was observed to be 37.3 nm by light scattering and the z-average R_g was 41.29 nm by viscometry (data not shown). This is in good agreement with previous reports concerning the radius of gyration of schizophyllan [24]. Schizophyllan is considered to behave as a semi-flexible, worm-like coil in solution, which corresponds to a ν-value of ~ 0.6 [19]. In our study, schizophyllan showed a reproducible ν-value of 0.599, which is consistent with the results of Kashiwagi et al. [19]. We conclude that schizophyllan behaves predominantly as a semi-flexible, rod-like structure in aqueous solution. However, our results cannot rule out the possibility that schizophyllan may be a mixture of randomly coiled polymers and more rigid rod-like conformations.

Glucan phosphate is a non-branched $(1 \rightarrow 3)$-β-D-linked glucopyranose polyelectrolyte with randomly distributed phosphate groups on the main chain [13,25]. Glucan phosphate shows a very-high-molecular-mass peak with no detectable concentration which may be due to aggregate formation during the lyophilization process. The aggregate, which comprises $<0.01\%$ of total polymers, can be separated by suspending the sample in dilute sodium hydroxide solution (unpublished results). This BRM has a lower average molecular mass than either Krestin or schizophyllan. The solution structure of glucan phosphate is characterized by a ν-value of 0.354 indicating a solution behaviour between a spherical and a randomly coiled conformation. However, the α-value of 0.649 for glucan phosphate suggests a different solution structure of a partially solvated or perturbed coil. Although this α-value is valid over a broader molecular mass range than the ν-value of 0.354 it is currently very difficult to say which conformational behaviour predominates for glucan phosphate. The ν-value for glucan phosphate differs from schizophyllan, suggesting that glucan phosphate behaves differently in aqueous solution, although they possess a similar primary structure. We assume that glucan phosphate is not behaving as a true random coil in dilute aqueous solution, but that there may be different types of coiled structures.

Since Krestin, schizophyllan and glucan phosphate show considerable differences in their molecular mass, polymer size, polydispersity and intrinsic viscosity, they also behave differently in aqueous solution. Krestin is a very complex proteoglycan, containing three different molecular mass moments as well as a fluorophore group, which causes fluorescence at 488 nm. Krestin is probably behaving as a coil with an excluded volume effect in aqueous solution, although there are portions which seem to be more rigid. While schizophyllan and glucan phosphate share a similar primary structure, they seem to exhibit different conformations in aque-

ous solutions. Only schizophyllan, which is reported to form a triple helix in water [8,9], is observed to behave as a rod-like structure. The behaviour of glucan phosphate in aqueous solution may be caused by the presence of phosphate groups on the polymer and/or differences in the complex structure of the triple helix itself [26]. Other factors such as molecular mass, polymer size, ionic strength of the solvent and/or the type of salt in the solution, especially for polyelectrolytes may also play a role in the expression of solution conformations.

In conclusion, Krestin, schizophyllan and glucan phosphate exhibit substantial differences in molecular mass moments, molecular mass distribution, polymer sizes, intrinsic viscosities and perhaps their solution behaviour. The relationship between molecular mass and polymer size needs to be studied more extensively.

The observation of significant structural differences between these BRMs might be of special importance with regard to the known biological activities of these compounds, which are assumed to be partially based on structural properties. We speculate, that there might be different ways of expressing structure–biological activity relationships through which these BRMs act. The application of other techniques to increase our knowledge about the solution conformation and behaviour of these BRMs will provide additional insights into these structure–activity relationships.

Acknowledgements

References

[1] W. Browder, D. Williams, H. Pretus, G. Olivero, F. Enrichens, P. Mao and A. Franchello, Ann. Surg., 211 (1990) 605.

[2] D.L. Williams, E.R. Sherwood, R.B. McNamee, E.L. Jones, I.W. Browder and N.R. Di Luzio, Hepatology, 7 (1987) 1296.

[3] E.R. Sherwood, D.L. Williams, R.B. McNamee, E.L. Jones, I.W. Browder and N.R. Di Luzio, J. Biol. Resp. Modif., 7 (1988) 185.

[4] W. Browder, D. Williams, P. Lucore, H. Pretus, E. Jones and R. McNamee, Surgery, 104 (1988) 224.

[5] W. Browder, D. Williams, E. Sherwood, R. McNamee, E. Jones and N. DiLuzio, Surgery, 102 (1987) 206.

[6] M. Ikuzawa, K. Matsunaga, S. Nishiyama, S. Nakajima, Y. Kobayashi, T. Andoh, A. Kobayashi, M. Ohhara, Y. Ohmura, T. Wada and C. Yoshikumi, Int. J. Immunopharmac., 10 (1988) 415.

[7] R. Hirai, Y. Oguchi, N. Sugita, K. Matsunaga and K. Nomoto, Int. J. Immunopharmacol., 15 (1993) 745.

[8] T. Yanaki, W. Ito, K. Tabata, T. Kojima, T. Norisuye, N. Takano and H. Fujita, Biophys. Chem., 17 (1983) 337.

[9] T. Norisuye, T. Yanaki and H. Fujita, J. Polym. Sci., 18 (1980) 547.

[10] T. Kojima, K. Tabata, W. Itoh and T. Yanaki, Agric. Biol. Chem., 50 (1986) 231.

[11] P.J. Wyatt, Anal. Chim. Acta, 272 (1993) 1.

[12] L.P. Yu and J.E. Rollings, J. Appl. Polym. Sci., 35 (1988) 1085.

[13] D.L. Williams, R.B. McNamee, E.L. Jones, H.A. Pretus, H.E. Ensley, I.W. Browder and N.R. Di Luzio, Carbohyd. Res., 219 (1991) 203.

[14] D.L. Williams, H.A. Pretus and I.W. Browder, J. Liq. Chromatogr., 15 (1992) 2297.

[15] L. Weaver, L.P. Yu and J.E. Rollings, J. Appl. Polym. Sci., 35 (1988) 1631.

[16] H.G. Elias, Macromolecules, Vol. 1, Huethig and Wepf, Basel, 1990.

[17] R.G. Beri, J. Walker, E.T. Reese and J.E. Rollings, Carbohyd. Res., 238 (1993) 11.

[18] T.E. Perkins, S.R. Quake, D.E. Smith and S. Chu, Science, 264 (1994) 822.

[19] Y. Kashiwagi, T. Norisuye and H. Fujita, Macromolecules, 14 (1981) 1220.

[20] D.L. Williams, H.A. Pretus, R.B. McNamee, E.L. Jones, H.E. Ensley, I.W. Browder and N.R. Di Luzio, Immunopharmacology, 22 (1991) 139.

[21] T. Taguchi, Personal communication, 1978.

[22] S. Tanaka, J. Aketagawa, S. Takahashi and Y. Shibata, Carbohyd. Res., 218 (1991) 167.

[23] B.T. Stokke, A. Elgsaeter, C. Hara, S. Kitamura and K. Takeo, Biopolymers, 33 (1993) 561.

[24] K. Van and A. Teramoto, Polym. J., 17 (1985) 409.

[25] H.E. Ensley, B. Tobias, H.A. Pretus, R.B. McNamee, E.L. Jones, I.W. Browder and D.L. Williams, Carbohyd. Res., 258 (1994) 307.

[26] M. Kopecka and M. Gabriel, Arch. Microbiol., 158 (1992) 115.

[27] M. Kurata and Y. Tsunashima, in J. Brandup and E.J. Immergut (Editors), Polymer Handbook, Wiley Interscience, New York, NY, 1989, p. 7/1.