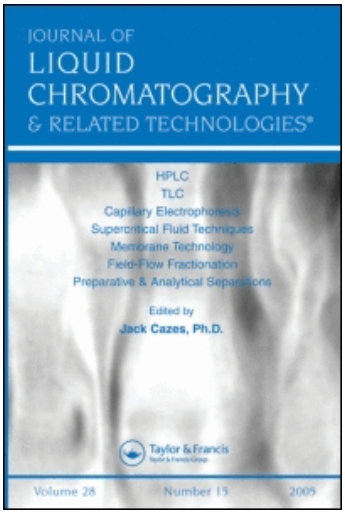

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Hennessey Jr., John P. , Bednar, Bohumil and Manam, Veda(1993) 'Molecular Size Analysis of*Haemophilus Influenzae* Type B Capsular Polysaccharide', Journal of Liquid Chromatography & Related Technologies, 16: 8, 1715 — 1729
To link to this Article: DOI: 10.1080/10826079308021683
URL: http://dx.doi.org/10.1080/10826079308021683

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(8), 1715-1729 (1993)

MOLECULAR SIZE ANALYSIS OF *HAEMOPHILUS INFLUENZAE* TYPE B CAPSULAR POLYSACCHARIDE

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ABSTRACT

The molar mass and molar mass distribution of polyribosyl ribitol phosphate (PRP) was determined using high performance size-exclusion chromatography with light scattering, viscometric, and refractive index detection. Light scattering data showed the PRP preparations to have an M_w of *ca.* 75,000, and a polydispersity (M_w/M_n) of about 1.46. Based on integration of the molar mass and intrinsic viscosity data, the Mark-Houwink-Sakurada coefficients for PRP were determined to be a = 1.45, and K = 3.57×10^{-6} cm^3/g for 0.2 M ammonium acetate ,pH 7.0, and a = 1.54 and K = 0.6×10^{-6} cm^3/g for 0.1 M sodium phosphate, pH 7.2. The values of a indicate that PRP is a rigid, rod-like molecule. The rigidity of the PRP molecules is characterized by unperturbed dimensions ($\langle R_o \rangle / M^{1/2}$) of 0.24 nm in ammonium acetate buffer and 0.22 nm in sodium phosphate buffer. These results suggest that PRP likely has a highly ordered secondary structure.

INTRODUCTION

Polyribosyl ribitol phosphate (PRP) is an anionic polysaccharide that encapsulates *Haemophilus influenzae* type B (HIB). PRP is one of the primary immunogenic determinants on this organism, such that levels of anti-PRP antibodies are the primary immunologic indicator of current or past HIB infection, and are a predictive indicator of protection against HIB infection for those immunized with vaccines containing PRP. Though initial HIB vaccines consisted primarily of purified PRP, more recent semi-synthetic vaccines consist of PRP linked to carrier protein complexes (1-5). The design and understanding of these and subsequent new HIB vaccines should benefit from detailed understanding of the molecular characteristics of the PRP molecule.

The chemical structure of PRP was defined in 1975 by Crisel *et al.* (6) to contain ribitol, ribose, and phosphate, in equimolar ratios. Subsequent work by Branefors-Helander *et al.* (7) using NMR analysis, showed the chemical linkage of the PRP polymer to be β-D-ribose linked through the 1-hydroxyl to the 1-hydroxyl of D-ribitol, which in turn was linked though the 5-hydroxyl to a phosphate moiety. The phosphate is linked to the 3-hydroxyl of the next ribose. There are no indications of branching in this polymer.

The relative molar mass of PRP preparations is commonly determined using size-exclusion chromatographic (SEC) methods, typically using a secondary standard such as dextran as a calibration standard. Absolute molar mass information cannot be derived directly from such analyses given the dramatic differences in the intrinsic viscosity, and therefore hydrodynamic volume per unit mass, for polymer molecules. Less commonly, the absolute molar mass of PRP has been estimated by using chemical means to determine the ribose content, and then by either chemical or spectroscopic means to determine either reducing end groups or terminal phosphate groups (8, 9).

However, this approach relies on assumptions about the uniformity of the end-groups of PRP and generally must be limited to polymers of molar mass less than *ca.* 10,000 Da to maintain the error in the values within reasonable bounds.

A more conventional, though less convenient, approach to determining the absolute molar mass and molar mass distribution of polymeric preparations incorporates high performance SEC (HPSEC) analysis with on-line determination of molecular size and mass by light scattering, concentration by differential refractometry, and specific viscosity by differential viscometry (10,11). This approach not only yields direct information on the molar mass and molar mass distribution of polymer samples, but can provide information about the shape, polymer chain stiffness, and hydrodynamic interactions of the polymer molecules, as well as provide indications as to whether the properties of the polymer molecules change as a function of the molar mass (12-15).

In this study, the above chromatographic analyses have been applied to PRP preparations to determine the absolute molar mass, molar mass distribution, and some physical parameters characterizing the behavior of PRP molecules in solution. Such analyses provide considerable information about the hydrodynamic interactions and partly also about the physical structure of PRP in solution.

METHODS AND MATERIALS

PRP samples were obtained from Merck Manufacturing Division. PRP samples were purified from HIB cultures by a combination of enzymatic digestion, liquid-phase extraction, and alcohol precipitation steps (16). PRP samples were dissolved in deionized water at a concentration of 5 mg/mL,

and were subsequently diluted 5-fold with mobile phase for the HPSEC analysis. PRP concentrations were determined by RI detector response relative to a moisture-corrected PRP standard solution.

HPSEC analysis.

HPSEC analyses were performed using Toyo-soda (TSK) high performance size-exclusion columns. The column oven and detectors were set at 30°C. The HPSEC analyses utilized a TSK PWXL guard column (0.7 x 4 cm), two TSK G4000 PWXL analytical columns (0.7 x 30 cm each), and a mobile phase containing 0.2 M ammonium acetate, 0.05% (w/v) sodium azide, pH 7, at a flow rate of 0.5 mL/min., hereafter referred to as the TSK/NH_4Ac system, or a Polymer Laboratories guard column (0.75 x 5 cm; 8 um) and PL Aquagel-40, -50, and -60 analytical columns (0.75 x 30 cm each; 8 um) in a mobile phase containing 0.1 M sodium acetate, 0.05% (w/v) sodium azide, pH 7.2, at a flow rate of 1.0 mL/min, hereafter referred to as the PL/$NaPO_4$ system.

HPSEC-MALLS/RI analysis.

HPSEC analysis with multi-angle laser light scattering (MALLS) and refractive index (RI) detection was performed using a Waters model 715 UltraWISP autosampler, a Waters 510 HPLC pump, a Waters column oven, a Dawn-F multi-angle laser light scattering detector (Wyatt Technology Corp.), and a Waters model 410 refractive index detector. Data collection and analysis was done using Wyatt's ASTRA® and EASI® software (17).

HPSEC-SV/RI analysis.

HPSEC analysis with specific viscosity (SV) and RI detection was performed using an ERMA model ERC-3322 on-line degasser, a Waters model 715 UltraWISP autosampler, a Hewlett-Packard 1090 LC pump, a

Shimadzu model CTO-6A column oven, a Viscotek model 100 differential viscosity detector, and a Waters model 410 differential refractive index detector. Data collection and analysis was done using Viscotek Unical 3.12 software.

Universal calibration analysis.

Pullulan standards were obtained from Polymer Laboratories, Inc. Standards were prepared at 2.0 mg/mL in deionized water, and further diluted to concentrations of 1.0 mg/mL with mobile phase.

dn/dc determination.

A PRP solution was subjected to analysis of the change in refractive index as a function of change in PRP concentration using a Brice-Phoenix interferometry refractometer set at 30°C and 546 nM. Values were interpolated to λ = 633 nm via a Cauchy dispersion.

RESULTS AND DISCUSSION

HPSEC analysis

The molecular parameters defined in this study were based on the average results from duplicate analysis of two independently purified PRP preparations. Analysis of the two PRP preparations showed no significant differences between the preparations, with intra- and inter-sample variations being identical for both samples, and typically less than 5% for any given parameter.

HPSEC analysis of PRP samples resulted in essentially complete recovery of the samples from the two HPSEC systems used. In contrast, initial attempts to use Waters Ultrahydrogel linear columns with either a sodium phosphate or ammonium acetate mobile phase showed very poor

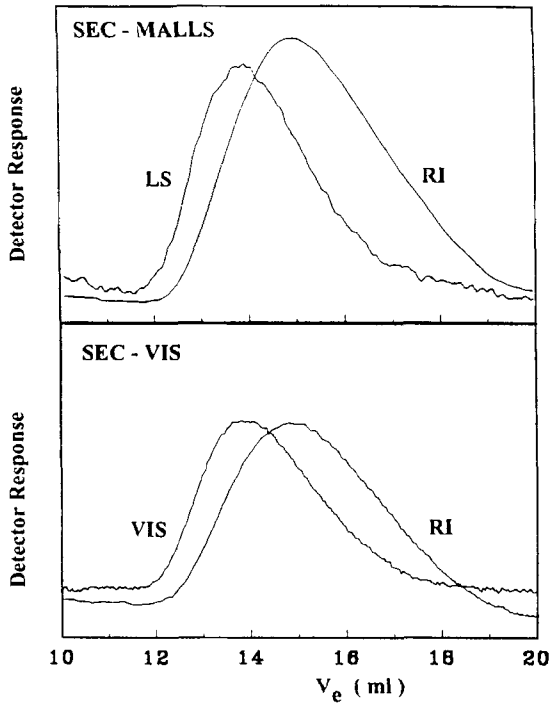

Figure 1. MALLS (at 90°), RI, and SV detector profiles from HPSEC analysis of PRP.

recovery of the PRP from the column. Overlaid RI, MALLS, and SV profiles for a typical TSK/NH_4Ac PRP chromatogram are shown in Figure 1. The PL/$NaPO_4$ system yielded similar profiles (not shown), though the relative elution volume (K_d) was greater in this system than in the TSK/NH_4Ac system.

Figure 2 shows a universal calibration plot (log([η]M) versus retention volume) for pullulan standards ranging from a molar mass of 12,000 to 835,000. The calibration curve was fit with a third-order polynomial and was used for universal calibration analyses of the PRP samples.

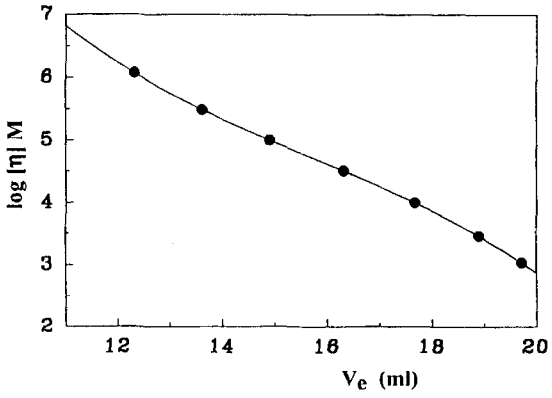

Figure 2. Universal calibration curve for pullulan standards on the TSK G4000 PWXL columns with ammonium acetate, pH 7, mobile phase. Flow rate was 0.5 mL/min.

Molar mass determinations

As shown in Table 1, the weight-average molar mass (M_w) of the PRP samples as determined by HPSEC-MALLS/RI is *ca.* 105,000, with a index of polydispersity (M_w/M_n) of 1.45. Determination of M_w for PRP using the universal calibration method based on pullulan standards resulted in a value that was *ca.* 30% higher than that obtained by MALLS/RI, whereas calculation of the M_w using the Mark-Houwink-Sakurada (MHS) coefficients (see below) defined by the joint HPSEC-MALLS/RI and SV/RI analyses resulted in an M_w in very good agreement with the value determined by MALLS/RI alone. Though use of the MHS coefficients for calculation of M_n, and therefore polydispersity (M_w/M_n), results in a value in better agreement with the MALLS/RI determined value than the result by universal calibration analysis, the inherent instability in calculating an absolute value of M_n still results in significant deviation in results between the different methods. However, the consistency of the M_n values determined within a given method was typically $\pm$ *ca.* 10% (compared to $\pm$ <5% for M_w within and between

TABLE 1

Molar mass parameters for PRP derived from HPSEC-MALLS/RI and HPSEC-SV/RI analyses using the TSK/NH_4Ac system.

Method	M_w	M_n	M_w/M_n
MALLS/RI	105,000	72,500	1.45
SV/RI			
Universal calibration	132,500	105,000	1.27
MHS coefficients	99,000	87,500	1.14

methods), suggesting that the relative values of M_n determined by a given method still allow for reasonably reliable determination of a relative measure of polydispersity, the absolute value of which will vary depending on the method used to define M_w and M_n.

The above results indicate that universal calibration does not apply to PRP in the HPSEC system utilized here. Similar conclusions resulted from analyses of *Streptococcus pneumoniae* polysaccharide preparations (18) and for the neutral polysaccharide schizophyllan (19), both analyzed in different aqueous chromatographic systems, reinforcing the precaution that universal calibration cannot be assumed to apply to all polymers, particularly with regard to polysaccharides.

Figure 3 shows a graphical representation of the molar mass distribution for PRP. The asymmetry in the profile is due to the presence of smaller molecular weight fragments of the PRP in the preparation, which are likely attributable to the inherent lability of the PRP polymer. Given that the repeat unit structure of PRP has a molecular mass of 345 Da, these PRP preparations contain, on average, approximately 300 repeat units per

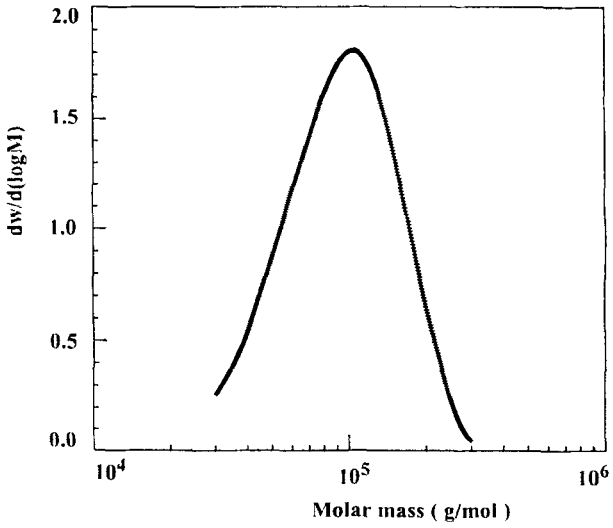

Figure 3. Molar mass distribution of PRP on the TSK/NH_4Ac HPSEC system.

molecule, with *ca.* 80% of the molecules having greater than *ca.* 60 repeat units (M_w = 21,000) but less than *ca.* 550 repeat units (M_w = 190,000).

Calculation of MHS coefficients and unperturbed dimensions

Figure 4 shows plots of [η] versus M_w for PRP in the TSK/NH_4Ac and PL/$NaPO_4$ systems used, utilizing the combined data from all sample analyses in a given system (typically a total of three to four measurements on each HPSEC system for each column/mobile phase combination). Least squares analysis of this data produces a regression lines described by the equation $[\eta] = 3.57 \times 10^{-6} M^{1.45}$ for the TSK/NH_4Ac system and $[\eta] = 0.6 \times 10^{-6} M^{1.54}$ for the PL/$NaPO_4$ system, thus defining the MHS coefficients *a* and K (see Table 2). These coefficients are appropriate for the molar mass range from approximately 20,000 to 600,000 g/mol. The relatively high values of *a* indicates that the PRP molecules are a very stiff polymer chain, with a shape

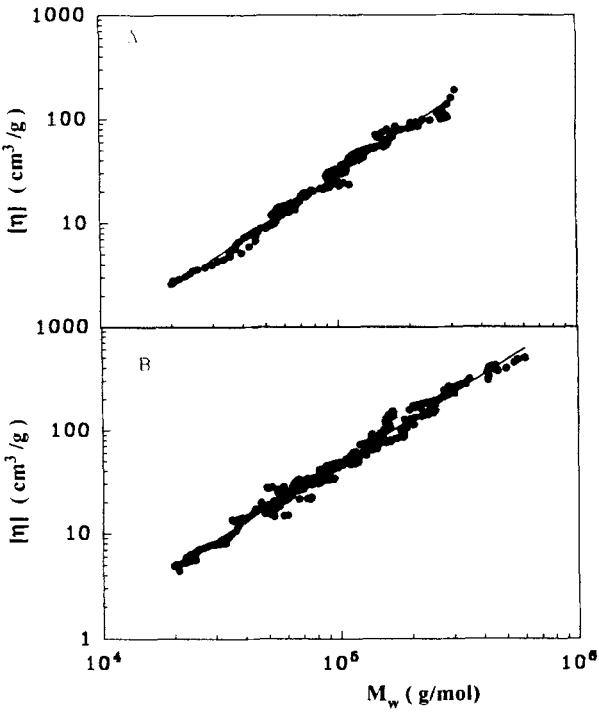

Figure 4. Mark-Houwink-Sakurada plots for PRP. The regression lines are defined by the equation $[\eta] = K M^a$. **A.** 0.1 M Sodium phosphate, pH 7.2; $K = 0.6 \times 10^{-6}$ and $a = 1.53$. **B.** 0.2 M amonium acetate, pH 7.0; $K = 3.57 \times 10^{-6}$ and $a = 1.45$.

approaching that of a rigid rod. This is in contrast to the random coil shape of anionic polysaccharides previously examined, where the *a* values ranged from 0.65 to 0.84 (18) and for the uncharged type 14 pneumococcal polysaccharide (Pn14) and pullulan where *a* is equal to 0.72 and 0.59, respectively ((18); see Table 2).

Figure 5 shows Bohdanecky plots ($(M_w^2/[\eta])^{1/3}$ versus $M_w^{1/2}$) for PRP in the TSK/NH_4Ac and PL/$NaPO_4$ systems. From these plots estimates of

TABLE 2

Summary table of physical parameters for PRP, Pn14, and pullulan.

Parameter	PRP		Pn14	pullulan
Buffer	NH_4Ac	$NaPO_4$	$NaPO_4$	$NaPO_4$
$[\eta]$ (cm^3/g)	77	57	181	114
$Kx10^6$ (cm^3/g)	3.57	0.65	7690	39700
a	1.45	1.53	0.72	0.59
A	349	463	261	29
B	0.67	0.73	1.52	1.93
$(<R_o>/M^{1/2})_\infty$ (nm)	0.24	0.22	0.105	0.082

A_η and B_η can be calculated using the Yamakawa-Fujii (20) equation as proposed by Bohdanecky (15)

$$[M^2/[\eta]_o]^{1/3} = A_\eta + B_\eta M^{1/2}$$

where $A_\eta = A_o M_L \Phi_{o,\infty}^{-1/3}$, and $B_\eta = B_o \Phi_{o,\infty}^{-1/3}(<R_o^2>/M)_\infty^{-1/2}$. Regression analysis of this data shows $A_\eta = 373$ and $B_\eta = 0.67$ in ammonium acetate, and $A_\eta = 463$ and $B_\eta = 0.73$ in sodium phosphate. The results of similar calculations for pullulan and Pn14 (18) are provided in Table 2 for comparison. This in turn allows calculation of the unperturbed dimensions, a fundamental conformational characteristic of polymer molecules, defined as $(<R_o>/M^{1/2})_\infty$, by the following equation

$$(<R_o>/M^{1/2})_\infty = \frac{B_o}{B_\eta \Phi_o^{1/3}}$$

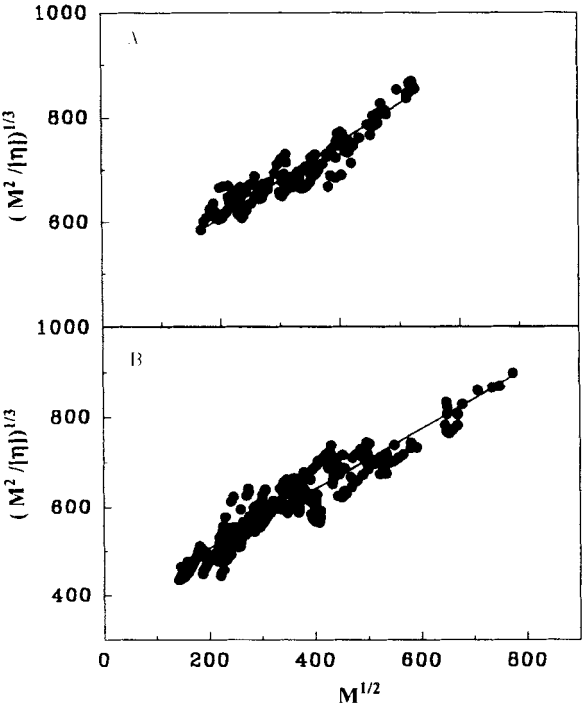

Figure 5. Bohdanecky plots of $(M^2/[\eta])^{1/3}$ vs. $M^{1/2}$ for PRP. **A.** 0.1 M Sodium phosphate, pH 7.2; A_η = 463 ; B_η = 0.73. **B.** 0.2 M amonium acetate, pH 7.0 A_η = 373 ; B_η = 0..67

where B_o is approximated as 1.05 (15), and Φ_o = 2.86×10^{23}. The value of $(<R_o>/M^{1/2})_\infty$ for PRP, 0.22 nm or 0.24 nm in ammonium acetate and sodium phosphate, respectively, is over twice the value calculated for Pn14 and pullulan (see Table 2). The value of $(<R_o>/M^{1/2})_\infty$ for the polysaccharide schizophyllan in water, 0.12, (calculated from (21)) shows that PRP seems to be in fact more rigid than this polysaccharide, which has a comparably high value of a (1.7 at M < 0.5×10^6 and 1.2 at M > 5×10^6) (21).

The rigid rod-like nature of the PRP suggests that this molecule should have a highly ordered secondary structure, possibly of a helical nature, and the relatively large unperturbed dimensions suggests that even very large PRP molecules are unlikely to have significant tertiary structural interactions. However, a more detailed study of PRP structure in solution will be necessary to confirm this.

Understanding of the physical structure of PRP may have significant implications for understanding the critical immunogenic features of PRP. Given the rigid, rod-like character of the PRP, and the apparent independence of both antigenicity (22) and immunogenicity (8, 23-26) on PRP size, except at very low molecular masses (*i.e.* less than *ca.* 10,000 Da), it would appear that the antigenic and immunogenic epitope(s) are defined by linear regions along the PRP chain, or by a conformational epitope dictated by the secondary structural features of the polymer. This suggests that the vast majority of the rod-like PRP molecules should exist as extended rods, with little potential for tertiary structural interactions that might result in more complex conformational epitopes such as have been found in pneumococcal polysaccharide types 3 (27) and 14 (28).

In conclusion, these studies have provided an absolute measure of the molar mass and molar mass distribution of these PRP preparations, as well as values for several physical parameters which provide information about conformational properties of PRP. The MHS coefficients defined in these studies have a practical application in that they can now be used to measure the molar mass and molar mass distribution of other PRP samples from a broad range of PRP molecular sizes from HPSEC-SV/RI analyses alone. Additionally, these studies have provided experimental evidence of the rigid, rod-like shape of PRP molecules, an insight that may be useful in obtaining a more detailed understanding of the immunologic response to PRP and other polysaccharides.

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