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Short communication

# Purification of preparative quantities of group B *Streptococcus* type III oligosaccharides

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**Abstract**

Many bacterial capsular polysaccharides are regularly repeating units of oligosaccharides. Bacterial oligosaccharides have been used in neoglycoconjugate vaccines and as reagents in the study of specific antibody binding. Unfortunately, separation methods have not been adequate for the purification of preparative quantities of bacterial oligosaccharides. Here we describe a size-exclusion procedure that resulted in the resolution of group B *Streptococcus* type III oligosaccharides composed of 4–25 sugars.

## 1. Introduction

Oligosaccharides are composed of more than 2 but less than 10 monosaccharides [1]; however, in the vaccine field more common usage refers to an oligosaccharide as a polymer smaller in molecular size than that of the native polysaccharide [2–5]. Many bacterial capsular polysaccharides, composed of several repeating units of oligosaccharide, are well characterized virulence factors. Some bacterial polysaccharides serve to evade the host immune mechanisms by impeding antibody binding and/or complement activation. Oligosaccharides, including those of *Haemophilis influenzae* [2,6,7] and group B *Streptococcus* (GBS) type III [8,9], have been used in the design of neoglycoconjugate vaccines.

GBS type III capsular polysaccharide (CPS) is composed of pentasaccharide repeating units of galactose, glucose, N-acetylglucosamine and N-acetylneuraminic (sialic) acid (Fig. 1). The sialic acid moiety is critical in maintaining the conformation of the antigenic epitope of GBS type III cps [10]. High-affinity binding of specific antibody to the type III CPS is dependent on the presence of the negatively charged sialic acid moiety positioned as the terminal saccharide of the disaccharide side chain of GBS type III CPS [5]. Protective antibody to GBS recognizes the conformationally determined epitope on the type III CPS with negligible binding to a single type III pentasaccharide. However, increasingly higher affinity binding is found as the chain length of the oligosaccharide increases beyond two pentasaccharide repeating units [4]. Desialylation of GBS type III CPS results in a structure identical to that of the CPS of *S. pneumoniae* type 14 (Fig. 1) and a diminution of binding by type III-specific antibody [11]. Therefore, desialylated GBS type III CPS has been a useful reagent in

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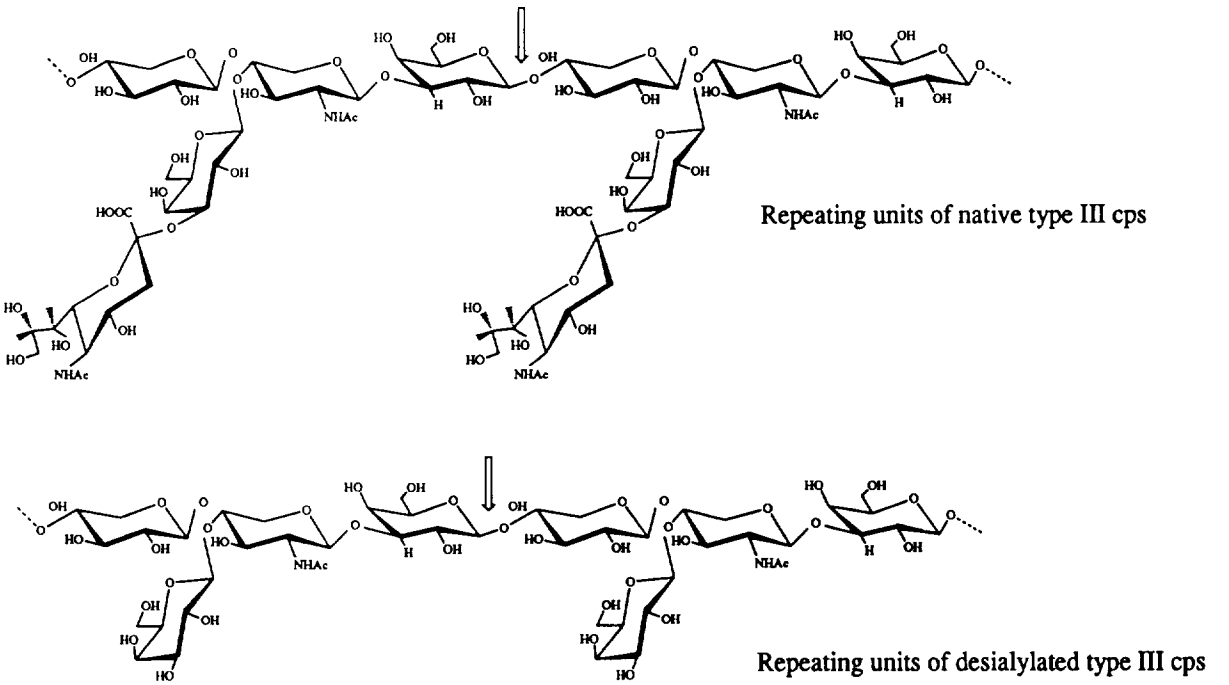


Fig. 1. Two repeating unit oligosaccharides structure of native, sialylated GBS type III [5] and desialylated GBS type III [11]. The arrow indicates site of depolymerization by endo-β-galactosidase.

experiments designed to study the role of sialic acid in binding of type III-specific antibody.

The need to study further the effect of GBS type III oligosaccharide chain length on the binding of specific antibody prompted an investigation into ways of generating preparative amounts of pure GBS type III oligosaccharides. Depolymerization of GBS type III CPS can be accomplished by digestion of the native CPS with crude preparations of endo-β-galactosidase prepared from spent culture fluids of *Citrobacter freundii* [8]. Endo-β-galactosidase cleaves internal β-D-galactose(1→4) linkages [12] and digestion of type III CPS with this enzyme results in the generation of oligosaccharides (Fig. 1) of one or more pentasaccharide repeating units [8]. Moreover, this method of depolymerization does not affect the acid-labile, α(2→3) ketosidic linkage between the side chain galactose and sialic acid of GBS type III CPS. Endo-β-galactosidase also cleaves desialylated GBS type III CPS, thus allowing for generation of type III oligosaccharides that lack sialic acid.

In general, chromatographic techniques have been used to analyse analytically component saccharides of oligosaccharides found on mammalian glycoproteins. Guile et al. [13] have recently reported the use of ammonium formate buffers in preparative anion-exchange high-performance liquid chromatographic (HPLC) separation of anionic sugars. Although excellent separation of anionic sugars was achieved by these investigators, the amount of oligosaccharides purified by this method was not reported. Previous attempts for the purification of GBS type III oligosaccharides using anion-exchange HPLC was labor-intensive and resulted in low ($\leq$5 mg) yields [5]. Here we describe a procedure for the separation and purification of GBS oligosaccharides by size-exclusion chromatography with use of a Superdex 30 preparation grade column and an fast protein liquid chromatography (FPLC) system. Superdex 30 is a new size-exclusion medium composed of dextran covalently coupled to highly crosslinked porous agarose beads (Pharmacia LKB Biotechnology

product sheet). This matrix has a molecular mass separation range of $\leq 10\,000$ useful for the separation of proteins, peptides and nucleic acids. This is the first report of the application of this chromatographic matrix for the separation of bacterial oligosaccharides.

## 2. Experimental

### 2.1. Chemicals

Native, sialylated GBS type III CPS was isolated and purified from GBS strain M781 as described previously [14]. Desialylated type III CPS was generated with an acid extraction procedure as described by Lancefield [15]. Endo-$\beta$-galactosidase was partially purified from spent culture fluids of *C. freundii* and was used to depolymerize GBS type III as described previously [8]. All other chemicals were purchased from Sigma (St. Louis, MO, USA).

### 2.2. Equipment

An FPLC system consisting of an LCC-501 plus controller, P-500 pumps, MV-7 mixing valve, a Sephadex G-75 column (31 cm × 25 mm I.D.), a HiLoad 16/60 Superdex 30 prep grade column (60 cm × 16 mm I.D.) and a Superfrac fraction collector were purchased from Pharmacia (Uppsala, Sweden). A differential refractometer Model 401 (Waters, Division of Millipore, Marlborough, MA, USA) was used as the detector.

### 2.3. Chromatographic conditions

*Sialylated type III oligosaccharides*

Sialylated type III oligosaccharides were purified in a two-step procedure. Depolymerized type III CPS was placed on a G-75 column equilibrated with 10 m*M* Tris, pH 7.0 and fractions that corresponded to large (eluting close to the void volume of the column), medium, and small (eluting close to the bed volume of the column) were pooled and lyophilized to dryness. This procedure was repeated with several depolymerized batches of type III CPS. Each of three size pools was suspended in 0.7 ml of water and 0.5 ml was placed onto a Superdex 30 column equilibrated with 330 $\mu M$ phosphate, 5 m*M* NaCl buffer, pH 7.3. The flow-rate was 1.0 ml/min. Oligosaccharide peaks were detected with a refractometer and fractions corresponding to each peak were individually collected and dried by lyophilization.

*Desialylated type III oligosaccharides*

Desialylated type III oligosaccharides were purified by loading enzyme-depolymerized, desialylated type III material directly onto the Superdex column. Fractions containing different-size oligosaccharides were collected as described above.

### 2.4. Thin layer chromatography and NMR analysis

Type III oligosaccharides composed of 10 sugars or less were visualized by TLC by methods described previously [5]. The number of repeating units in the oligosaccharides obtained from each of the peaks in the fractionation was determined by NMR spectroscopic analysis of coded samples in the laboratory of Dr. Harold J. Jennings, National Research Council of Canada, Ottawa. The intensity of the reducing-end galactose anomeric ($\alpha$ plus $\beta$) signals (doublets at 5.234 and 4.571 ppm, respectively) were compared with the coincident methyl signals (singlet at 2.03 ppm) of the N-acetylglucosamine and sialic acid residues. NMR spectra were run in $^2H_2O$ on a Bruker 600-Mz spectrometer at 290 K. For this analysis, acetone was used as an internal standard.

## 3. Results and discussion

### 3.1. Separation of sialylated GBS type III oligosaccharides

The elution profiles of the three sialylated type III oligosaccharide pools are shown in Fig. 2. The smallest-size pool recovered from the

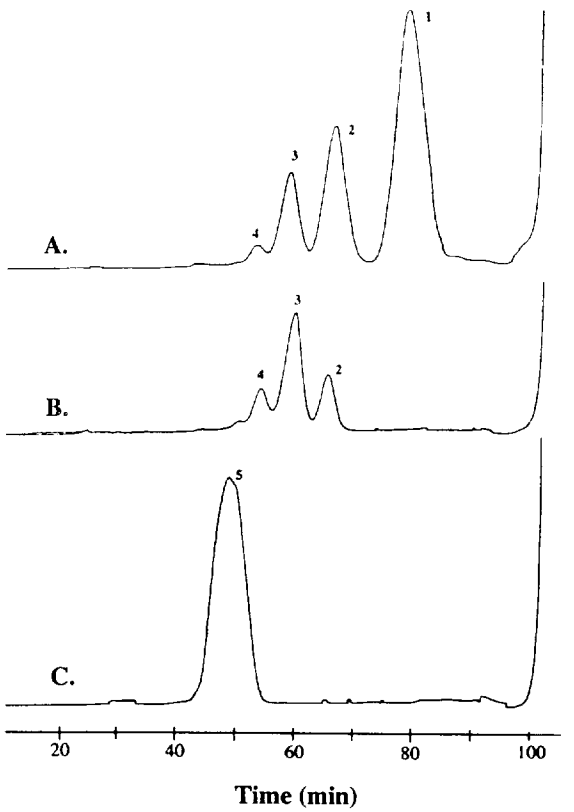


Fig. 2. Elution profiles of sialylated type III oligosaccharides. The numbers of type III repeating units are indicated above each peak. The attenuation setting on the refractometer was 16×, 16× and 8× for profiles A, B and C, respectively.

Sephadex G-75 column resolved into four fractions on the Superdex 30 column with peak volumes of 81 ml (peak 1), 68 ml (peak 2), 61 ml (peak 3), and 55 ml (peak 4) as shown in Fig. 2A. The medium-size pool recovered from the Sephadex G-75 column resolved into three fractions on the Superdex 30 column with peak volumes of 68 ml (peak 2), 61 ml (peak 3), and 55 ml (peak 4) as shown in Fig. 2B. The largest-size pool from the G-75 column resolved into a single fraction with a peak volume of 48 ml (peak 5) as shown in Fig. 2C. Elution volumes and $K_{av}$ of these oligosaccharides are shown in Table 1. These data indicate baseline separation of sialylated type III oligosaccharides in the range of 5 to 25 sugars.

### 3.2. Separation of desialylated GBS type III oligosaccharides

Desialylated type III polysaccharide possessed 1% (w/w) sialic acid as measured by the thiobarbituric acid assay [16] with commercially available sialic acid as the standard. Desialylated type III (dIII) oligosaccharides were separated into distinct fractions of 6 repeating units (24 sugars) to 1 repeating unit (4 sugars). The elution volumes and $K_{av}$ of these dIII oligosaccharides are shown in Table 1. During these experiments,

Table 1
Separation of GBS type III sialylated or desialylated oligosaccharides with use of a Superdex 30 size-exclusion column

| Type III oligosaccharides | Repeating units (No. sugars) | FM ($\log_{10}$) | Elution volume ($V_e$) (ml) | $K^a_{av}$ |
|---|---|---|---|---|
| Sialylated | 5 (25) | 3.702 | 48 | 0.067 |
| | 4 (20) | 3.606 | 55 | 0.186 |
| | 3 (15) | 3.481 | 61 | 0.288 |
| | 2 (10) | 3.307 | 68 | 0.407 |
| | 1 (5) | 3.009 | 81 | 0.627 |
| Desialylated | 6 (24) | 3.633 | 56 | 0.203 |
| | 5 (20) | 3.554 | 61 | 0.288 |
| | 4 (16) | 3.458 | 66 | 0.373 |
| | 3 (12) | 3.333 | 71 | 0.458 |
| | 2 (8) | 3.159 | 79 | 0.593 |
| | 1 (4) | 2.864 | 92 | 0.814 |

[a] $V_o$ (44 ml) and $V_t$ (103 ml) were determined using native type III polysaccharide and acetone, respectively. FM = formula mass.

we learned that digested CPS can be placed directly onto the Superdex 30 column eliminating the need for the Sephacel G-75 size-exclusion step and without loss of resolution. Furthermore, void volume material that contained both enzyme and native polysaccharide was collected and again incubated at 37°C for further digestion and processing of native polysaccharide.

Recovery of pure type III oligosaccharides typically ranged from 5 to 17 mg with greater amounts obtained with the smaller-size oligosaccharides.

### 3.3. TLC analysis of type III oligosaccharides

Type III oligosaccharides consisting of 4, 5, 8, and 10 saccharides were resolved by TLC (Fig. 3). The resolution limit of type III repeating units by TLC is 2 pentasaccharide repeating units or 10 sugars. These results are in complete agreement with published reports on the purification of these oligosaccharides obtained using more labor-intensive methods of purification [5,17]. In addition to TLC analysis, purity of all type III oligosaccharides was independently confirmed by NMR (not shown) as described above.

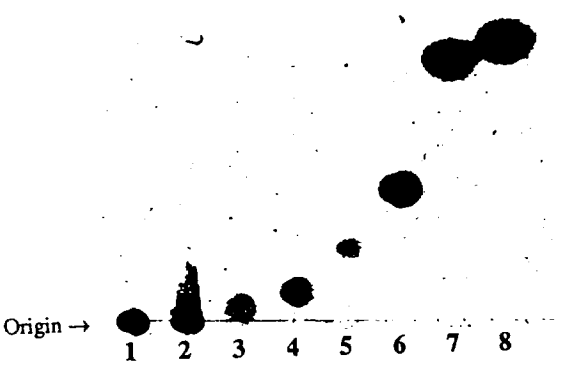


Fig. 3. TLC analysis of GBS type III oligosaccharides. Lane 1 = native type III polysaccharide; lane 2 = 2 pentasaccharide repeating units; lane 3 = 2 tetrasaccharide repeating units; lane 4 = 1 pentasaccharide repeating unit; lane 5 = 1 tetrasaccharide repeating unit; lane 6 = sialic acid; lane 7 = galactose; lane 8 = glucose.

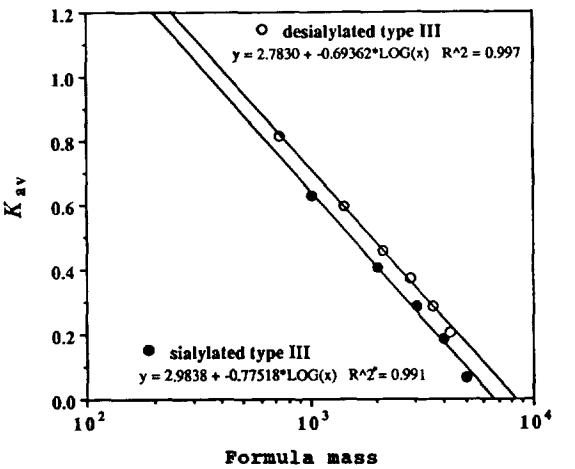


Fig. 4. Relationship between $K_{av}$ and the formula mass of purified sialylated (●) and desialylated (○) type III oligosaccharides.

### 3.4. Relationship between type III oligosaccharide molecular mass and $K_{av}$

The formula mass and $K_{av}$ of purified, native and desialylated type III oligosaccharide is shown in Table 1. There was a direct, positive relationship between the log-transformed formula mass and determined $K_{av}$ of sialylated ($r^2 = +0.991$) and desialylated ($r^2 = +0.997$) GBS type III oligosaccharides as shown in Fig. 4. The Superdex 30 column matrix also resolved a four (desialylated single repeating unit) and a five (sialylated single repeating unit) sugar structure as indicated by the different $K_{av}$ values for these oligosaccharides (Table 1 and Fig. 4).

## 4. Conclusions

Preparation of pure sialylated and desialylated GBS type III oligosaccharides consisting of 25 sugars or less was accomplished by size-exclusion chromatography using a Superdex 30 column. The procedure for generating oligosaccharides described here is efficient and rapid and results in baseline separation of preparative quantities of oligosaccharides of different chain length. A

direct linear correlation existed between the $K_{av}$ measured and the log formula mass of type III oligosaccharides. These oligosaccharides will be valuable reagents for the study of the influence of polysaccharide conformation to the binding of GBS type III-specific antibody.

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