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Profiling pneumococcal type 3-derived oligosaccharides by high resolution liquid chromatography–tandem mass spectrometry

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ABSTRACT

Pneumococcal type-3 polysaccharide (Pn3P) is considered a major target for the development of a human vaccine to protect against *Streptococcus pneumoniae* infection. Thus, it is critical to develop methods for the preparation and analysis of Pn3P-derived oligosaccharides to better understand its immunological properties. In this paper, we profile oligosaccharides, generated by the free radical depolymerization of Pn3P, using liquid chromatography (LC)–tandem mass spectrometry (MS/MS). Hydrophilic liquid interaction chromatography (HILIC)–mass spectrometry (MS) revealed a series of oligosaccharides with an even- and odd-number of saccharide residues, ranging from monosaccharide, degree of polymerization (dp1) to large oligosaccharides up to dp 20, generated by free radical depolymerization. Isomers of oligosaccharides with an even number of sugar residues were easily separated on a HILIC column, and their sequences could be distinguished by comparing MS/MS of these oligosaccharides and their reduced alditols. Fluorescent labeling with 2-aminoacridone (AMAC) followed by reversed phase (RP)-LC–MS/MS was applied to analyze and sequence poorly separated product mixtures, as RP-LC affords higher resolution of AMAC-labeled oligosaccharides than does HILIC-based separation. The present methodology can be potentially applied to profiling other capsular polysaccharides.

1. Introduction

Despite a long history of in-depth investigation of Streptococcus pneumoniae, these bacteria remain major human pathogens. Pneumococcal diseases such as pneumonia, otitis media, bacteremia, and meningitis are particularly worrisome, and oftentimes fatal to highly susceptible immunocompromised individuals, infants, and the elderly [1–3].

S. pneumoniae can be divided into over 90 serotypes based on the structural differences of the capsular polysaccharide. The capsules of these Gram-positive bacteria are considered a major virulence factor, based on the decreased virulence of non-encapsulated strains, their inability to activate the alternative complement pathway, and their resistance to phagocytosis [1]. Surface exposure of the capsular polysaccharide, and its role in the virulence capacity of *S. pneumoniae* make it an ideal candidate for vaccine development. Multiple studies have shown the ability of anti-capsular polysaccharide antibodies to provide protection from bacterial challenge [3]. A capsular polysaccharide-based vaccine (PPV23) first became available in 1983, encompassing 23 serotypes of the pneumococcus. This purely polysaccharide-based vaccine was proven effective in healthy adults, but was largely inadequate in its immunogenicity in young children. Polysaccharide-protein conjugate vaccines

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have been designed to enhance anti-capsular polysaccharide antibody production [2,3]. By coupling capsular polysaccharides to a carrier protein, a T-cell dependent response is achieved along with immunoglobulin class switching, immunological memory, and rapid antibody production [4,5]. Since the introduction of the first conjugate vaccine, PCV7, the incidence rate of pneumococcal disease has been reduced dramatically [3]. However, a global serotype distribution shift after this introduction has highlighted the importance of generating improved conjugate vaccines to include a wider range of serotypes. The current pneumococcal conjugate vaccine available is a 13-valent PCV13 (Prevnar13®), effective against the 13 most prevalent serotypes of *S. pneumoniae* [3].

By investigating the mechanism of adaptive immune activation by glycoconjugate vaccines, more effective vaccines can be thoughtfully designed. We recently characterized this mechanism, and indicated the critical role that reactive oxygen species (ROS) in the endolysosomal compartments of antigen presenting cells (APCs) has in polysaccharide processing [6]. Processed glycoconjugates are presented to T-cells in the context of major histocompatibility complex type II (MHCII) molecules to initiate the adaptive immune response against the carbohydrate epitopes. A major discovery in this study was evidence of the existence of a carbohydrate-specific T-cell repertoire (Tcarbs) that plays a critical role in the production of protective antibodies against the capsular polysaccharide portion of the glycoconjugate [6,7]. We have also shown that optimizing a glycoconjugate vaccine for these carbohydrate-specific T-cell epitopes yields a robust and strong immune response and protection in a disease model [7].

Pn3P, a repeating linear co-polymer of glucuronic acid (GlcA) and glucose (Glc) (Fig. 1a), was isolated from Pneumococcal type-3 (Pn3), which is considered a major target for the development of a human vaccine to protect against *S. pneumoniae* infection. Pn3 is a highly virulent serotype of the pneumococcus [8]. The low efficacy of the current glycoconjugate vaccine against this capsular serotype highlights the importance of generating highly immunogenic, Pn3P-based glycoconjugate vaccines [8]. Thus, it is critical to develop methods for the preparation and analysis of Pn3P-derived oligosaccharides to better understand the immunological properties of Pn3P. Previously, small oligosaccharides containing Pn3P repeating units have been prepared either through acid hydrolysis of Pn3P [9] or synthesis [10] as there are no well-characterized enzymes available for its controlled, preparative depolymerization. In a cellular environment, reactive oxygen species are likely to degrade Pn3P in the endosomes of antigen presenting cells. Therefore, the *in vitro* free radical depolymerization used in the current study potentially mimics cellular depolymerization of Pn3P and the knowledge gained in this study should be useful for future biochemical and immunological investigations. While HILIC–FTMS and RP-FTMS followed 2-AMAC labeling have each been previously reported in glycosaminoglycan analysis [11–17], their integrated application as a systematic strategy for profiling Pn3P, a major target for the development of a human vaccine to protect against *S. pneumoniae* infection, is novel. The retention characteristics of acidic Pn3P-derived oligosaccharides on diol HILIC column and their AMAC-derivatives on C18 column are investigated. The analysis of endosomally processed Pn3P has not previously been systematically studied, and the methods developed in this study will be generally useful in the preparation of oligosaccharides to study the antigen processing mechanisms of this and other capsular polysaccharides in the endosomal compartments of APCs. In addition to describing methods for characterizing their products, this study can also inform biochemists of possible endosomal depolymerization mechanisms. Structurally defined Pn3 oligosaccharides may be useful in constructing future vaccines that are optimized for eliciting strong adaptive immune responses against the capsular polysaccharides.

2. Experimental

2.1. Materials

Pneumococcal type-3 polysaccharide (Pn3P) (Fig. 1a) was either obtained from ATCC (Manassas, VA) or purified, as described in Section 2.2. Methanol, dimethylsulfoxide, sodium borodeuteride ($NaBD_4$), hydrogen peroxide, copper (II) acetate, acetonitrile (HPLC grade), ammonium acetate (HPLC grade), and 2-aminoacridone (AMAC) (Fig. 1c) were purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Purification of pneumococcal type-3 polysaccharide

Crude bacterial extract from a capsular polysaccharide type 3 strain of *S. pneumoniae* was acquired from the laboratory of Dr. Moon Nahm at the University of Alabama at Birmingham (Birmingham, AL, USA). Purified capsular polysaccharide was obtained through a series of enzymatic treatments and purification steps. Approximately 10 g of the crude extract was treated with DNase (Sigma–Aldrich, St. Louis, MO, USA), RNase (Sigma), and Proteinase K (Sigma) as previously described [18], followed by base treatment [19]. After extensive dialysis, the soluble polysaccharide was separated by size exclusion chromatography (Superdex 200, Sephacryl S-300, GE Healthcare) on an FPLC system (NGC Discover, Biorad). Fractions were tested for type 3 polysaccharide content in an immunoblot using type 3 specific-IgM antibody (Hyp3M6, Nahm Lab Birmingham, AL). Pooled reactive fractions were dialyzed against deionized water, and lyophilized. The purified capsular polysaccharide was obtained with a 5% yield. Purity was assayed through 1D and 2D Nuclear Magnetic Resonance Spectroscopy (Varian Inova 600 FT-NMR). The purified capsular polysaccharide of average molecular weight of >400 kDa was identical in structure and size to that obtained from the ATCC.

2.3. Preparation of Pn3P-derived oligosaccharides by free radical depolymerization

Pn3P was partially degraded through the controlled oxidative depolymerization by ROS generated using hydrogen peroxide and cupric acetate (Fig. 1a). The polysaccharide samples (200 μg) were dissolved in 100 μL of 0.1 M sodium acetate-acetic acid solution containing 0.2 mM copper (II) acetate and adjusted to pH 7.0. Hydrogen peroxide (4 μL of 3% solution) was added with mixing and reacted at 45 °C for 3 h. Sodium bisulfite was added to terminate the reaction by removing excess unreacted hydrogen peroxide and the reaction mixture was divided into three portions for lyophilization. One portion of lyophilized product was dissolved in 50% acetonitrile solution (v/v) for direct HILIC–MS^n analysis. The second portion was reduced prior to analysis and the third portion was labeled with AMAC prior to analysis.

2.4. Pn3P-derived oligosaccharides reduction

Freshly prepared $NaBD_4$ reagent (20 μL of 0.05 M $NaBD_4$ in 0.01 M NaOH) was added to the freeze-dried Pn3P-derived oligosaccharides (typically 20 μg), and reduction was carried out overnight at 4 °C as previously described (Fig. 1b) [20]. The reaction solution was then neutralized to pH 7 with a solution of AcOH/H_2O (1:1) to destroy borohydride before passing through a 100-μL mini-column packed with cation exchange resin (AG50W-X8, H^+ form). After loading the sample on the column, the column was washed with 500 μL of water and the eluent solution was collected and