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Study of the chemical stability of the capsular polysaccharide produced by *Haemophilus influenzae* type b

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ABSTRACT

Haemophilus influenzae type b (Hib) is a human pathogen that causes severe infections such as pneumonia, sepsis and meningitis. Vaccines for Hib infections are based on its capsular polysaccharide conjugated to a protein. This conjugated Hib antigen is included as one of the components of polyvalent vaccines and accounts for more than 50% of the total cost of the formulations. The instability of the polysaccharide is responsible for the high cost of the vaccine. In this study, the factors affecting the spontaneous degradation of the polysaccharide from Hib were evaluated based on the decrease in its molecular mass, as measured by size-exclusion chromatography. Temperature and pH were found to be the most significant variables, and the results showed that the conditions of bacterial cell growth (37 °C and pH 7.5) are favourable for depolymerization. An increase in the concentration of sodium ions up to 200 mM intensified the effect of pH, allowing higher rates of depolymerization at lower pH values, whereas the presence of magnesium ions showed no effects.

1. Introduction

Haemophilus influenzae type b (Hib) is a human pathogen associated with various health complications such as pneumonia, sepsis and meningitis, mainly in children under two years of age and immunocompromised individuals (Wilfert, 1990). Vaccination against Hib was introduced in 1987 in North America and, in the rest of the developed world in the 1990s and is currently being extended to developing countries by international organizations, such as the Global Alliance for Vaccine Innovation (GAVI) and the United Nations Children's Fund (UNICEF) (Bisgard et al., 1998; GAVI, 2013; WHO, 2013). Today, the World Health Organization (WHO) recommends the use of polyvalent formulations, which combine the Hib antigen with the Diphteria–Tetanus–Pertussis (DTP) vaccine (tetravalent) or both DTP and Hepatitis B (HepB) vaccines (pentavalent). However, the inclusion of the Hib antigen increases the cost of the polyvalent vaccines, in fact, it; represent more than half of the overall cost per dose of the formulation (UNICEF, 2013).

One of the major factors contributing to the high cost of the Hib vaccine is the inherent instability of the polysaccharide molecule. It has been demonstrated that the polysaccharide molecule of Hib undergoes spontaneous degradation under certain ambient conditions, and a molecular mass decrease has been observed during the cell growth steps (Egan, Schneerson, Werner, & Zon, 1982; Sturgess et al., 1999). The instability and consequent molecular mass decrease of the polymer has great significance on the final global productivity of the vaccine because many steps of the most recent purification and conjugation processes are based on ultrafiltration (Albani, da Silva, Takagi, & Cabrera-Crespo, 2012; Takagi et al., 2008).

The Hib vaccine is composed of a polysaccharide-protein conjugate, and the polysaccharide is the molecule present in the extracellular capsule of the bacterium. The capsular polysaccharide of Hib is formed by units of ribosyl-ribitol, that are linked together by a phosphodiester linkage, denominated poly-ribosyl-ribitol-phosphate, PRP (Egan et al., 1982).

The chemical structure of PRP is responsible for its natural instability. As shown in Fig. 1, the PRP molecule is structurally similar to the molecule of RNA: the hydroxyl at the carbon 2 of ribose is located in the proximity of the phosphate group, and in the presence of free hydroxyls in the milieu this carbon undergoes transesterification and consequent depolymerization (Egan et al., 1982). In the case of RNA, this reaction is not only affected by the concentration of hydroxyls, but also by the presence of mono and divalent metal ions, such as potassium and magnesium, which are capable of stabilizing the reactive form of the hydroxyl from ribose by reducing its pKa of ionization, by facilitating proton transfer or by acting as a Lewis acid catalyst (Li & Breaker, 1999).

Through their analysis of the PRP molecule, Egan et al. (1982) demonstrated that ions of sodium and calcium do not become strongly associated with the polysaccharide chain, even though the

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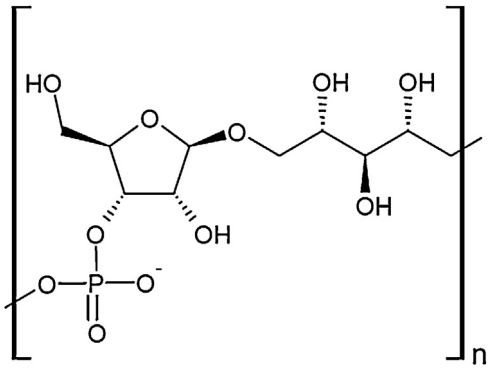

Fig. 1. Chemical structure of the poly-ribosyl-ribitol-phosphate repetitive unit.

divalent cation is indeed capable of catalysing the transesterification reaction and the monovalent cation has a significant effect on the rate of depolymerization.

The study of the depolymerization rate of PRP as functions of ambient conditions, such as pH, temperature, and ions concentrations, is of great importance to the search for vaccine production processes that are less expensive and economically viable. During all stages of production, i.e., cell growth, purification and chemical conjugation, the molecule is exposed to specific conditions that may promote chain cleavage. In the PRP production process, cell growth is conducted at a mildly alkaline pH of 7.5 (Takagi, Cabrera-Crespo, Zangirolami, Raw, & Tanizaki, 2006), demanding the addition of sodium hydroxide for pH control and thus increasing the concentration of monovalent cations. In the purification step, proteolytic enzymes and endonucleases are used for the removal of proteins and nucleic acids, and this reaction is conducted in the presence of magnesium ions at higher pH values (Albani et al., 2012).

Under these premises, this work proposes the study and quantification of the depolymerization rate of PRP by the measurement of molecular mass decrease, regarding the effects of pH, temperature and, sodium and magnesium cations concentration.

2. Materials and methods

2.1. Preparation of PRP

Strain GB3291 of *H. influenzae* type b was purchased from Instituto Adolpho Lutz (São Paulo, Brazil). The cells were cultivated in 15 L of the Modified Medium Peptone (MMP) described by Takagi et al. (2006) in a BioFlo 5000 bioreactor (New Brunswick Scientific Co., USA), until all of the glucose was consumed, as measured using at Bioliquid glicose colorimetric enzymatic kit (Laborclin LTDA, Brazil). At this point, the feeding of a concentrated solution of glucose and yeast extract (20% each) at a constant flow rate of 1.2 L h^{-1} was initiation, and this feeding was ended when the total volume in the reactor reached 60 L. Throughout the cultivation, the pH was controlled at 7.50 with 5 M NaOH, the temperature was maintained at 37 °C, the air flow rate was controlled at 15 L min^{-1}, and the dissolved oxygen was maintained at 30% of the saturation by controlling the agitation speed, which ranged from 100 to 500 rpm. The cells were separated from the culture broth in a continuous tubular centrifuge with automatic piston discharge (APD75 Celeros Inc, USA) at 20,000 g and 4 °C with a feed flow rate of approximately 200 mL min^{-1}. The isolation of PRP from the supernatant was performed by a series of diafiltrations on 100 kDa cut-off membranes, ethanol precipitations and enzymatic hydrolysis, as described by Albani et al. (2012). The final purified PRP was lyophilized and stored at −20 °C until use.

Table 1
Values of the variables at different levels in the 2^4 RCCD for the analysis of PRP stability.

Variables	Levels				
	−2	−1	0	1	2
NaCl (mM)	0	50	100	150	200
$MgCl_2$ (mM)	0	0.75	1.50	2.25	3.00
pH	5.00	5.75	6.50	7.25	8.00
T (°C)	25.00	28.75	32.50	36.25	40.00

2.2. Experimental design

A rotational central composite design (RCCD) was used for the study of the effects of the four variables (2^4), namely pH, temperature (T) and, the molarity of NaCl and $MgCl_2$ according to Table 1. The central point was performed in duplicate. The experiments were analysed using the STATISTICA® 11 software.

2.3. Measurement of PRP depolymerization rate

The solutions of PRP were prepared to obtain a concentration of 1000 mg L^{-1} and incubated in a water bath at the conditions specified by the experimental design. Samples were collected over time for the measurement of the number average molecular mass (M_n). The M_n was measured by high-performance size exclusion chromatography (HPSEC) using two serially connected 30 cm TKS Gel GMPWxl columns (TOSOH Bioscience, Japan). The chromatography was performed on a Shimadzu HPLC system, composed of an isocratic pump (10ADVp), a column oven (CTO-10ASvp), an autosampler (SIL-10ADvp), a refraction index (RI) detector (RID-10A), a system control unit (SCL-10AVp) and the Class VP version 6.2 software for data acquisition (Shimadzu Corp., Japan). A frequency of 2 Hz was used for the collection of the elution data. The mobile phase consisted of a solution of 150 mM NaCl, 10 mM Na_2HPO_4, and 0,02% NaN_3, pH of which was adjusted to 7.50 using 6 M HCl; this solution was pumped at a rate of 0.6 mL min^{-1}, and the temperature of the column and the detector cell were maintained at 40 °C. The injection volume of all of the samples was 50 μL. Under these parameters, the exclusion volume/time of the system was determined to be 21.85 min or 13.11 mL with Blue Dextran (Fluka Analytical, Sweden), whereas the total permeation volume/time was determined to be 35.00 min or 21 mL with dextrose; both reagents were prepared at a concentration of 1 g L^{-1}. The elution time of the polysaccharide was converted into values of molecular mass through a linear correlation between the elution times of low-dispersity dextrans and the logarithm of their known molecular mass; six dextran standards with nominal molecular mass (M_p) values of 1.5, 6, 10, 40, 70 and 229 kDa were used (Fluka Analytical, Sweden). The data were then used to calculate the number average molecular mass (M_n) using Eq. (1) and the mass average molecular mass (M_w) using Eq. (2), where h_i is the height of the chromatographic signal and M_i is the molecular mass value relative to the elution time of fraction i (Meunier, 1997). The ratio between the two averages, M_w/M_n, was defined as the dispersity $Đ$ of the distribution (Stepto, 2009).

$$M_n = \frac{\sum h_i}{\sum h_i/M_i} \quad (1)$$

$$M_w = \frac{\sum h_i M_i}{\sum h_i} \quad (2)$$

For the estimation of the rate of depolymerization, a linear curve was fitted to the plot of the inverse of the M_n values as a function of time. Based on the theory of random scission of polymers, the angular coefficient of this curve is proportional to the rate of