SL Vaccines And Vaccination Journal

Special Issue Article "Hib Vaccine"

Research Article

Instability and Depolymerization of the Exopolysaccharide Produced by *Haemophilus Influenzae Type B* during Fermentation

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ARTICLE INFO

Received Date: March 20, 2022
Accepted Date: April 21, 2022
Published Date: April 22, 2022

KEYWORDS

Polysaccharide
Haemophilus influenzae b
Instability
Depolymerization
Molecular weight

Citation for this article: Felipe de Oliveira Cintra and Mickie Takagi. Instability and Depolymerization of the Exopolysaccharide Produced by *Haemophilus Influenzae Type B* during Fermentation. SL Vaccines And Vaccination Journal. 2022; 3(1):117

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ABSTRACT

Haemophilus influenzae type b (Hib) is a pathogenic bacterium and the major cause of sequalae and deaths among infants due to meningitis. Hib vaccine is constituted of the exopolysaccharide from the cell capsule and conjugated to a carrier protein. The production of the polysaccharide is complicated due to low yields of production and product recovery in the downstream. In this work, the profiles of molecular mass throughout fermentation were investigated. The molecular mass decreased along fermentation time, despite the increase in concentration. The mechanisms of hydrolysis were investigated, with three possibilities considered: alkaline hydrolysis caused by the solution used to maintain pH of the fermentation; presence of hydrolytic activity from the fermentation metabolites and spontaneous hydrolysisat the temperature and pH was evaluated in the conditions of the fermentation and with purified polysaccharide. The results have suggested that there is not significant influence of the strength of the alkali solution used for pH control neither the presence of hydrolases in the supernatant; however spontaneous hydrolysis was verified in a temperature dependent manner and confirmed with purified polysaccharide.

INTRODUCTION

Haemophilus influenzae is a Gram-negative bacterium, with pleomorphic morphology, usually assuming a coccobacillar shape that colonizes the upper respiratory tract of human being and may become pathogenic in infants, elderly and immunodepressed patients [1]. Clinical manifestations include pneumonia, otitis, cellulites, infectious arthritis, septicemia and meningitis [2,3]. Some strains have no capsule and termed non encapsulated *Haemophilus influenzae* or non-typeable (NTHi). Other strains present an extracellular polysaccharide (encapsulated or typeable strains). These strains are classified into 6 different serotypes (a-f) based on the specific chemical composition of their capsules. *H. Influenzae type b* (Hib) is the most virulent and invasive, being responsible for deaths and sequelae due to meningitis in young children [4,5].

Vaccination against Hib infections was first introduced in the 1980's, using as antigen the extracellular polysaccharide capsule conjugated to a carrier protein [6]. The polysaccharide produced by Hib is a linear heteropolymer built up of repetitive units of D-ribitol-(1→1)-β-D-ribose-3-phosphate (Figure 1), also represented as Poly-Ribosyl-Ribitol-Phosphate (PRP) [7,8].

In vitro, the PRP is naturally released from the outer membrane into the supernatant [9], whence it may be recovered after cell removal. Recent trends on purification

techniques advocate the use of tangential micro and ultrafiltration techniques in replacement of serial precipitation/extraction and centrifugation steps [10,11], diminishing demands on organic solvents, energy and being an environmental friendly alternative. On the other hand, application of tangential filtration technique to the PRP process has shown not be capable to satisfactorily recover polysaccharide in the first step of purification (a 100kDa cut-off diafiltration), achieving recovery yields lower than 50% [12]. This fact highlights that the PRP produced in the fermentation has a small molecular mass and cannot be retained by the ultra-filtration membrane.

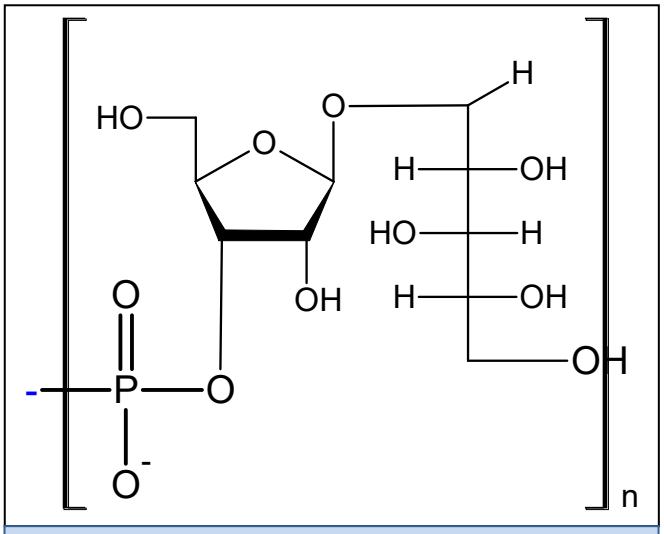

Figure 1: Chemical structure of the capsular polysaccharide PRP produced by *Haemophilus influenzae* b.

The molecular mass of the polymer has a significant role in the immunological effectiveness of the conjugated vaccine. World Health Organization (WHO) and the European Pharmacopeia require defined molecular mass ranges and stability of the material for using in vaccination campaigns [13,14].

Other studies have demonstrated the instability of polymers alike PRP, i.e. molecules that hold a phosphodiester bond in its composition, as the extracellular capsule of the *Streptococcus pneumoniae* serotypes 6A, 6B, 19A and 19F [15], ribonucleic acid [16] and the capsule of *H. influenzae* types a, b, c and f [8]. In these cases, the instability of the polysaccharides may happen due to spontaneous hydrolysis of the phosphodiester bonds in alkaline medium and in the presence of bivalent metallic ions. Enzymatic hydrolysis may also be accounted for the polysaccharide instability during fermentation. Pham et al. [17] demonstrated that long period of fermentations time result in polysaccharides with lower molecular mass due to the action of specific hydrolases expressed by the microorganism [17].

This work reports the profile of the polysaccharide molecular mass produced by *H. influenzae* type b during fermentation and its molecular behavior when subjected to different conditions in order to evaluate and characterize the depolymerization process.

MATERIALS AND METHODS

Strain

Haemophilus influenzae type b GB 3291 was purchased from Nucleo de Coleção de Microrganismos - Instituto Adolf Lutz (São Paulo, Brazil).

Experiments

Fermentations were carried out on a BioFlo 2000 bioreactor (New Brunswick Scientific)with a working volumes of 5L (batch) and 10L (fed-batch), at 37°C; pH controlled at 7.5 with three different alkali solutions: sodium hydroxide 5M, ammonium hydroxide 14% and sodium carbonate 20%; aeration was maintained at 0.5VVM and pO2 controlled at 30% of air saturation.

Culture media

Modified MP medium (MMP) as described by Takagi et al. [18] was used in the batch phase, and for feeding phase MMP medium was incremented with glucose (Merck) and yeast extract (BD) up to 20% [18]. Feeding was started upon exhaustion of the glucose (evaluated by a pO2 spike) at 1.8 mLmin-1. Samples were taken from the reactor and centrifuged at 4°C, 16,000g for cell removal and the supernatant was used for the analysis.

Polysaccharide determination

Supernatants from the culture were treated according to procedure described by Cintra & Takagi (2012) [19]. The PRP concentration was determined by the modified Bial's method [20].

Molecular mass determination: An amount of 2 mL of the supernatant were treated with CTAB(Cationic hexadecyl trimethyl ammonium Bromide) as described by Cintra & Takagi (2012) and precipitated by 80% ethanol in the presence of 5% sodium acetate pH 5.8 and following small modification. The pellets were resuspended in a solution of Na_2HPO_4 10mM, pH adjusted to 7.5 containing NaCl 150mM and sodium azide

0.02%. These samples were applied in a gel filtration column (2 serial TSK gel GMPWXL columns) through a Shimadzu HPLC system composed of an isocratic pump (LC-10ADvp) at a flow rate of 0.6mL/min, an oven set at 40°C (CTO-10ASvp), a UV-VIS detector (SPD-10Avp) and a refractive index detector (RID-10A). The relative molecular mass values (Mw) were obtained from the refractive index elution times and a calibration curve constructed with dextran of different defined molecular mass.

Stability test of polysaccharide in the culture broth: Cell free sample from the different fermentation times was collected and submitted to the following treatment: a) frozen right away (control); b) incubated at 37°C overnight; c) incubated at 37°C overnight with addition of formaldehyde (2%); d) PRP was partially purifiedby CTAB and ethanol precipitation as described by Cintra & Takagi (2012) [19].

RESULTS AND DISCUSSION

The polysaccharide produced by *H. influenzae* type b is a linear chain that comprises a phosphodiester bond in its backbone which greatly reduces its stability, mainly with respect to extreme pH values. In the fermentation process, strong alkali such as sodium hydroxide (5M)is added into the culture broth in order to adjust pH, but this procedure could hydrolyze the PRP being produced during cultivation and released to the culture broth, and thus affect the final yields of production and recovery.

Firstly, it has been investigated if the addition of strong concentrated alkali in the broth during the fermentation might be causing hydrolysis due to local pH increase. For this purpose, screening experiments were carried out with three identical batches cultures, where only the alkali solution used was changed. The traditional base, sodium hydroxide5M solution, was replaced by ammonium hydroxide 14%a weak base and usually used in microorganism cultures, and sodium carbonate 20% based on the study carried out by Crienan et al, 2010 who used this base due to its efficiency in preventing alkaline hydrolysis

The Figure 2 shows the PRP concentration and relative MW profiles from the experiments carried out in three different alkali solutions. The concentration of PRP increases with a parallel decreasing in molecular mass values during the fermentation for the tested bases. Although absolute values of MW in the experiments with NH3OH is higher than that one with NaOH and Na_2CO_3, this difference may be considered insignificant once the slope, ie, the hydrolysis rate are similar among them and in this way it can be concluded that none of the alkali solutions were effective in preventing molecular mass decrease during fermentation.

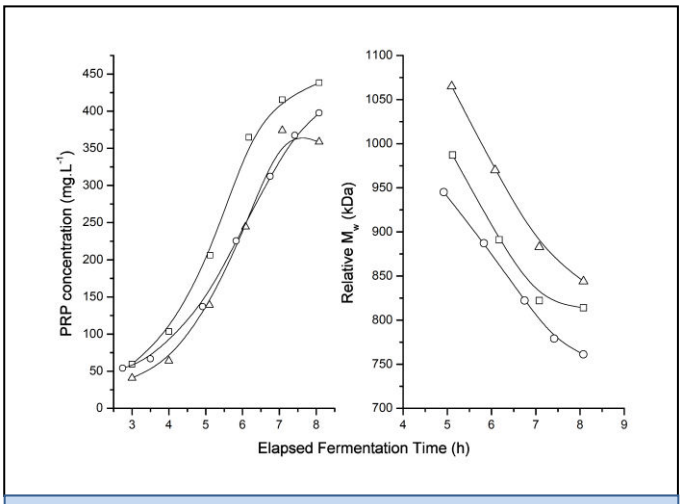

Figure 2: Evolution of PRP concentration and molecular mass during fermentation carried out with different alkali solutions used for pH control: sodium hydroxide (○), sodium carbonate (□) and ammonium hydroxide (Δ).

Some encapsulated bacteria are able of hydrolyzing their own polysaccharide through the expression of specific hydrolases. Although genomic databases do not acknowledge the presence of such sequence in the *H. influenzae* genome, the presence of enzymatic activity was tested. For that purpose, screening experiment was conducted with fed-batch fermentation (with $NaCO_3$ as the alkali solution and feeding initiated 7 hours after batch start), where samples were taken on five different points during the fermentation and the cell free supernatant was fractionated in different ways. Figure 3A illustrates the kinetics profile of polysaccharide formation and the respective decreasing molecular mass along of fermentation time, as confirmed in the previous assay. Figure 3B shows the Mw of the samples incubated for the stability test.

Sample collected at 5 hours of cultivation (sample 1) was frozen immediately (control) and its molecular mass was calculated as 923 kDa, while the same sample incubated at 37°C showed a different value of 622kDa, a reduction of almost 33% in molecular size. This pattern of reduction was observed for all the other four samples, although the

magnitude of reduction decreased along fermentation time, which may be explained by the fact that the molecules of the latter samples have already gone through degradation in the reactor and show a molecular mass value close to the minimum. The fact that the molecular mass decreases during incubation at 37°C contributes to conclusion that the addition of alkali to the broth has no effect in the hydrolysis of the polysaccharide.

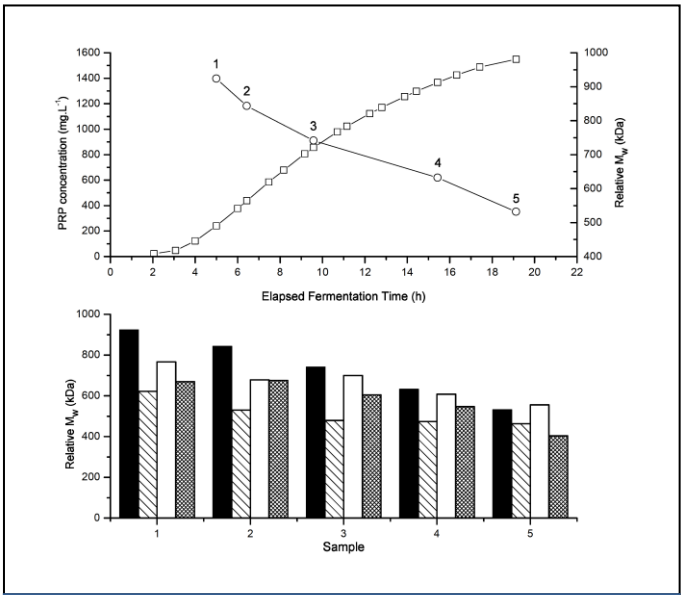

Figure 3: Evaluation of PRP stability in the presence and absence of fermentation metabolites. A) Evolution of PRP concentration (□) and molecular mass (○). B) Samples indicated in A were tested for stability under different conditions: frozen control (■), native sample incubated at 37°C (▨), sample incubated in the presence of formaldehyde (□), sample purified by CTAB and ethanol precipitations and incubated at 37°C (▩).

As the native sample incubated at 37°C was in the presence of cell metabolites, there is a possibility that the supernatant contains some enzymatic activity. If enzymatic hydrolysis is the factor responsible for the reduction of the MW, then the addition of formaldehyde would prevent any enzyme's activity present and the MW would not be changed after the incubation period. By observing samples 1 and 2, it is clear that the MW decreases even with the addition of formaldehyde, although the values are still greater than the native sample. In samples 3 and 4, the difference in the MW is not very large, and in sample 5 it is actually a little greater. Nevertheless, as formaldehyde promotes cross-linking of the organic molecules, it may be masking the higher values observed. Knowing that, in the samples 1 and 2 formaldehyde was unable to maintain the MW values constant, we may conclude that a non-enzymatic process is contributing to the degradation of the polysaccharide and that the presence of an enzyme should be considered doubtful.

The last assay confirmed the evidence that MW reduction is not caused by enzymatic activity, where the polysaccharide was isolated from the cell metabolites by CTAB and ethanol treatment. After incubation at 37°C, this isolated polysaccharide also had its MW reduced. The values of MW do not match the incubated sample probably because the incubation time was not exactly the same. The effect of time on the level of polysaccharide depolymerization will be determined in future studies.

CONCLUSION

Despite the fact that alkaline hydrolysis of the PRP is well described in the literature, this seems not to be the responsible agent for depolymerization during fermentation. This conclusion is asserted by the fact that incubation of the cell-free supernatant also resulted in hydrolysis. The purified PRP incubated in the same conditions as the fermentation demonstrate that instability of the molecule is not dependent of enzymes. In conclusion, the PRP instability observed during fermentation is caused by spontaneous hydrolysis depending on fermentation conditions such as pH, temperature and duration of the culture.

ACKNOWLEDGEMENT

This work was supported by BNDES (The Brazilian Development Bank)Grant number 11.2.0322.1/2012 and Butantan Foundation. We thank Mrs. Ana Maria Rodrigues Soares and Mr. Lourivaldo Ignacio de Souza for technical assistance.

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