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Isolation and purification of capsular polysaccharide of *Haemophilus influenzae* type b (Hib) by hexadecyltrimethylammonium bromide (CTAB) precipitation and chromatography

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Abstract

Polyribosylribitol phosphate (PRP) is a capsular component from *Haemophilus influenzae* type b (Hib) which is used as an active ingredient in Hib vaccine. PRP was isolated and purified from harvested bulk. This experiment was done to observe the profile of purified PRP. Purification process consists of several steps, which were CTAB (cetyltrimethyl-ammonium bromide) precipitation, chromatography using XK-50 column (Amersham, GE) with CTAB-celite slurry as a stationary phase. CTAB solution and gradient concentration of ethanol in sodium acetate 0.05M was used as mobile phase. Ethanol 60% fraction then precipitated with sodium chloride and ethanol absolute, concentrated, diafiltrated and sterilized with filtration. From three batches of 10 liters purification, about 4.84-7.44 g of PRP could be isolated. PRP was analyzed by orcinol assay. The impurities, nucleic acid and protein, were removed to the minimum level as recommended by WHO guideline for Hib vaccine : less than 1% for both parameter determined by UV scan at 260 nm for DNA and Lowry method respectively. Molecular size of PRP was determined by High Performance Gel Permeation Chromatography (HPGPC) with the result around 165-370 kDa. In conclusion this method could be used to isolate and purify the PRP.

Keywords: Vaccine, Haemophilus influenzae type b, CTAB, PRP purification.

Introduction

Haemophilus influenzae type b (Hib) is an important cause of serious invasive infections in children less than five years old of age. Meningitis is frequent consequence of invasive Hib infection. The capsular polysaccharide, consisting of repeating unit of 5-D-ribitol-(1→1)-*β*-D-ribose-3-phosphate (Fig.1) [1], plays an important role in the virulence of the organism.

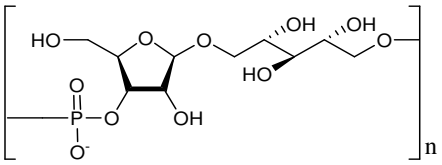

Figure 1 Structure of 5-D-ribitol-(1→1)-*β*-D-ribose-3-phosphate.

Purified polysaccharide (PRP) was one of important material in Hib vaccine production. PRP was isolated from harvested supernatant, precipitated by cetyltrimethylammonium bromide (CTAB) and celite as a matrix. CTAB was also known as hexadecyltrimethylammonium bromide, it was a cationic surfactant that could selectively precipitate the polysaccharide. After precipitation, the polysaccharide (typically in the form of a complex with the cationic surfactant) was re-dissolved. Ethanol has been found to be advantageous to this respect, and it is highly selective for CTAB-polysaccharide complex [2].

Here, we will present a convenient purification method to purify PRP polysaccharide from contaminant such as nucleic acid and protein regarding to the WHO guideline for *Haemophilus influenzae* type b (Hib) conjugate vaccine.

Materials and Methods

Hib sample and other reagents

The PRP crude was obtained from single cultivation batch. It was experimental batch prepared in R/D laboratories in Bio Farma. At the end of the cultivation, the Hib organisms were eliminated by centrifugation. The residual Hib organisms in the culture supernatant were inactivated. Celite for the stationary phase was from Supelco. CTAB for the precipitation was of purity ≥ 98% (Sigma-Aldrich).

Bed preparation

The 10 liters of supernatant/PRP crude were treated by 500 mL of 10% CTAB and 1000 mL of 30% celite, then stirred, to get slurry of CTAB/celite-

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polysaccharide. The mixture was allowed to settle and the supernatant was discarded.

Chromatography

The slurry was loaded into a 5 cm x 50 cm XK-50 column (Amersham, GE) using peristaltic pump and sucked from the bottom outlet to help the packing process using P-1 pump (Amersham, GE). When the slurry was settled, it was eluted with 0,05% CTAB, gradient concentration of 20%, 30% and 60% ethanol using P-50 pump (Amersham, GE) with a flow rate 50 mL/min. All eluent were in 0,05M sodium acetate pH 6. The 60% ethanol fraction was pooled.

Precipitation

5M sodium chloride and ethanol absolute was added to get a final 1M of sodium chloride. The cloudy solution was centrifuged at 3000 rpm, 4^{o}C for 10 min. The supernatant was discarded and the PRP that remained in the bottles was washed with water. This solution was allowed to dissolved completely by stored overnight.

Concentrating, diafiltration and sterilization

PRP solution was concentrated to get 10 mg/mL of final concentration using tangential flow filtration (TFF) pellicon mini 100 kDa membrane (Millipore), and then diafiltrated with water ten times volume of PRP solution. The PRP solution was then passed through a 0,22 μm filter, assayed for ribose by orcinol method [3,4], nucleic acid by UV scan at 260 nm for DNA [5], protein by lowry method [6], and molecular size was determined by High Performance Gel Permeation Chromatography (HPGPC) [7]. Finally it was frozen at ≤ -20^{o}C.

Results and Discussion

PRP was obtained from PRP crude solution by CTAB precipitation. But nucleic acid could also be precipitated by CTAB, as seen in table 1, where nucleic acid content after chromatography before diafiltration was still above the limit (1% w/w nucleic acid per PRP).

Protein content after chromatography were quite low, below 1%. Nucleic acid and PRP content in the pure PRP from three batches were below 1%, as recommended by WHO (Table 2) [8].

From orcinol test result, there were no PRP in the 0.05% CTAB, 20% ethanol and 30% ethanol fractions. PRP were eluted in 60% ethanol.

The molecular size and content of PRP in the three batches were decreasing, because purification process on batch 047-2 was done 4 weeks after cultivation and batch 047-3 was done 7 weeks after. It showed that there was degradation of PRP during storage. Higher degradation rate would increase the quantity of PRP lost in the purification process.

Table 1 PRP, Nucleic acid and protein content in purification samples: before and after concentration and after sterilization.

Sample	PRP (mg/mL)	Nucleic acid (%)[a]	Protein (%)[b]
Batch Hib047-1			
Before conc and UF	3.79	1.88	ND
Retentate	8.48	0.26	0.01
Purified PRP	7.95	0.069	<0.01
Batch Hib047-2			
Before conc and UF	1.62	3.83	ND
Retentate	9.70	0.57	0.05
Purified PRP	10.75	0.28	0.06
Batch Hib047-3			
Before conc and UF	1.99	3.12	ND
Retentate	10.03	0.32	0.026
Purified PRP	9.65	0.29	0.022

[a] % NA/PRP (w/w)
[b] % Protein/PRP (w/w)

Conclusions

Purification process using CTAB precipitation and chromatography with XK-50 column yield pure PRP with acceptable impurities level of nucleic acid and protein as recommended by WHO. Storage of harvested supernatant prior to purification process was not recommended due to PRP degradation.

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Table 2 Purification result from three consecutive batches

No.	Assay	Result			Spec limits (WHO TRS 897)
		Hib047-1	Hib047-2	Hib047-3	
1.	PRP content[a]	7.956 mg/mL (7.44 g)	10.753 mg/mL (4.84 g)	9.6531 mg/mL (5.11 g)	N/A
2.	Protein content[b]	< 0.01 %	0.07 %	0.022 %	< 1%
3.	Nucleic acid [c]	0.069 %	0.278 %	0.292 %	< 1 %
4.	HPGPC[d]	377.5 kDa	171.2 kDa	165.3 kDa	N/A

[a] Orcinol method [3,4]
[b] Lowry method [6]
[c] UV scan at 260 nm [5]
[d] HPGPC method (column : OHpak-SB800P; mobile phase : PBS 0.01M pH 7; flow rate : 1 mL/min) [7]

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