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ORIGINAL PAPER

Purification of capsular polysaccharide produced by *Haemophilus influenzae* type b through a simple, efficient and suitable method for scale-up

Mickie Takagi · Rodrigo Barbosa Lima · Silvia Maria Ferreira Albani · Teresa Cristina Zangirolami · Martha Massako Tanizaki · Joaquin Cabrera-Crespo

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Abstract *Haemophilus influenzae* type b, an encapsulated bacterium, causes meningitis in infants worldwide. The capsular polysaccharide conjugated to a carrier protein is effective in the prevention of such infections. The traditional purification process of polysaccharide from bacterial cultures for vaccine production is based on several selective precipitations with solvents such as: ethanol, phenol, and cationic detergents. The separations of solid and liquid phases are based on continuous centrifugation in explosion proof installations. The lipopolysaccharides are separated by ultracentrifugation. A simple and efficient method that can easily be scaled-up was developed for purification of polysaccharides. The ethanol precipitation was reduced to only two steps. The phenol treatment was substituted by ultrafiltration and enzymatic digestion. Lipopolysaccharide was removed by ultrafiltration together with addition of detergent and chelating agent.

Keywords Polysaccharide purification · Tangential ultrafiltration · Enzymatic digestion

Introduction

Haemophilus influenzae type b (Hib), an encapsulated, Gram negative, cocco-bacillus bacterium, is one of the most common agents of meningitis in infants and immunodeficient adults worldwide [1, 2]. In the USA, after the introduction of the vaccines in 1988, the incidence of meningitis by Hib in children up to 5-years-old decreased to less than 2.5 cases per 100,000, in 1993 [3, 4]. In Brazil, the incidence of Hib meningitis under 1 year, between 1987 and 1991, was 17.7 cases per 100,000 ha [5].

The capsular polysaccharide of *H. influenzae* type b, a repeating polymer of ribosyl ribitol phosphate (PRP), is the most important cause of its virulence [6]. The PRP vaccine produces T-cell independent protection and children less than 2 years are not protected. The conjugation of PRP with an immunogenic carrier protein produces T-cell dependent protection and promotes long-term immunological memory in the infant population. Actually there are six conjugated PRP-carrier protein approved [3, 4].

Although there are several studies about the immunogenic characteristics of polysaccharides vaccines, the know-how for large scale production and purification are not in the public domain. Furthermore, the publications are scarce and most of them are patents [7–10]. The vaccination against *H. influenzae* type b will be included in the World Health Organization Expanded Program on Immunization as a tetravalent vaccine (tetanus–diphtheria–pertussis–Hib). The production cost of conjugated PRP vaccines is high because of the several production process stages involved, such as: bacterial cultivation for PRP and protein (in general diphtheria or tetanus toxin) production, PRP and protein purification, chemical conjugation between PRP and protein, and further separation of the PRP-protein from the free reagents. Therefore, any improvement in one of these steps would contribute to enhance the cost–benefit ratio for vaccine production.

According to the patents, the purification of PRP starts with the inactivation of the cells by phenol, formol or

M. Takagi · R. B. Lima · S. M. F. Albani · M. M. Tanizaki · J. Cabrera-Crespo (✉)
Centro de Biotecnologia, Instituto Butantan,
Av. Vital Brasil 1500, São Paulo, SP CEP 05503-900, Brazil
e-mail: jcabrera-crespo@butantan.gov.br

T. C. Zangirolami
Departamento de Engenharia Química,
Universidade Federal de São Carlos-UFSCar,
Rodovia Washington Luiz, km 235, São Carlos,
SP CEP 13565-905, Brazil

thimerosal and the separation of the culture broth by centrifugation. The clarified supernatant is then concentrated by tangential flow ultrafiltration with a 50-kDa cut-off membrane [9, 10], precipitated with cationic detergent cetyltrimethylammonium bromide (cetavlon) and the complex PRP-cetavlon solubilized with calcium chloride [11] followed by several ethanol precipitation and desproteinization by extraction with phenol and further ethanol precipitation. The lipopolysaccharides are separated from the PRP by ultracentrifugation.

Our laboratory has developed an improved method for purification of vaccines polysaccharides against *Neisseria meningitidis* serotype C [12] and *Streptococcus pneumoniae* serotype 23 and 6B [13, 14]. This process reduces considerably the number of ethanol precipitation steps, phenol extraction was replaced by enzymatic treatment and the ultracentrifugation by ultrafiltration in the presence of chelating agent and detergent.

In the present paper, the improved method used for *Neisseria meningitidis* serotype C and *Streptococcus pneumoniae* serotype 23F and 6B was applied to purify the polysaccharide from *H. influenzae* type b in order to check the general applicability of the method, with minor modification, for purification of polysaccharides vaccines.

Materials and methods

Strain

H. influenzae type b GB 3291 was obtained from the Center for Disease Control and Prevention (Atlanta, GA), and the manufacturing master seed was prepared in the Department of Bacteriology at the Instituto Adolfo Lutz (São Paulo, SP, Brazil) [15, 16].

Bioreactor cultivation

The culture was carried out in a 13-L Bioflo 2000 (New Brunswick Scientific Co. USA) bioreactor containing 7.6 L of medium under agitation (100–700 rpm) at 37 °C. The air supply was provided as air sparged aeration and maintained at 0.25 vvm (volume of air per volume of medium per minute). The pH was controlled at 7.4 and dissolved oxygen tension at 30% of air saturation, as described in Takagi et al. [15, 16].

Analytical procedures

PRP concentration was measured by the modified Bial method, using ribose as standard [17] after dialysis of the samples [15, 16]. The PRP concentration was determined by multiplying the ribose concentration by 2.55, according to the PRP structural formula reported by Crisel [18].

Protein (Prt) was determined by Lowry method [19]. Nucleic acids (NA) were estimated at 260 nm and the amount was calculated assuming an absorbance of 1.0 A = 50 μg/mL [20]. Rocket immunoelectrophoresis was carried out according to Laurel [21]. The burro serum against PRP from *Haemophilus influenzae* type b and the standard purified PRP from *H.influenzae* type b were kindly provided by Dr. Carl Frasch [Food and Drug Administration (FDA)]. Lipopolysaccharide (LPS) was determined as KDO (2-keto-3-deoxyoctonate) by Osbom method [22]. Phosphorus concentration was determined by the method of Chen et al. [23]. PRP molecular size was determined in Sepharose CL-4B packed into a XK16/100 column (both from GE Healthcare, Uppsala, Sweden). The distribution coefficient, $K_d = (V_e - V_o/V_f)$, where V_e = elution volume, V_o = void volume and V_f = final volume, was determined using Blue dextran and riboflavin as the respective void-volume and final-volume markers [24]. Residual proteolytic activity of trypsin, pronase and nagarse was determinated in the final product [13].

The purification efficiency was followed by the total amount per liter of culture for: PRP, NA, Prt and KDO (LPS). PRP (%) recovery, relative purity (RP) and purification factor (PF) are defined as follows. Recovery = (step-amount/initial-amount) × 100. Relative Purity of polysaccharide in relation to nucleic acid, protein or KDO (LPS) as RP_{xx} = mg PRP/mg contaminant xx in each purification step (mg of product per mg of contaminant). The purification factor ($PF = RP_{step}/RP_{initial}$) and purification factor in each step ($PF_{step} = RP_{step}/RP_{previous\ step}$) indicate how many times the purity of the product was improved in relation to the initial or previous step of the purification process.

Description of the purification protocol

Cells separation

The culture broth was harvested and the cells were separated by centrifugation at 17,725*g*; 4 °C for 30 min (Beckman Avanti® J-25I) and the supernatant was used for PRP purification—clarified broth fraction.

First concentration by tangential ultrafiltration 100 kDa (1CTUF100)

The clarified broth was concentrated using tangential ultrafiltration membranes with a pore of 100 kDa (TUF 100 kDa.); 0.1-m^2 filtration area with a inlet pressure of 20 psi and a transmembrane pressure (TMP) of 7.5 psi (Prep-Scale spiral type Millipore, Bedford, MA, USA), [25]. The initial volume was reduced to ~1 L and washed

with 4 volumes of saline—concentrated TUF 100-kDa fraction.

Ethanol precipitations

The pH of the concentrated TUF 100 kDa from the first ultrafiltration was adjusted to pH 5.8 and Na-acetate and cold ethanol were added to a final concentration of 5% (w/v) and 30% (v/v), respectively, and kept at 4 °C for 12 h. The 30% ethanol solution was centrifuged at 11.725*g* for 60 min and the precipitated was discarded—supernatant EtOH 30% fraction. The pH of the supernatant EtOH 30% fraction was adjusted pH = 5.8, Na-acetate increased to 7% (w/v) and cold ethanol was added up to 80%, and processed as described above. The precipitated was resuspended in water and insoluble materials removed by centrifugation [15, 16], water soluble EtOH 80% fraction.

Enzymatic treatment and second concentration by tangential ultrafiltration 100 kDa (2CTUF100)

The pH of water soluble EtOH 80% fraction was adjusted to 7.0 with concentrated Tris-HCl buffer containing MgCl and NaCl in order to achieve 50, 2 and 20 mM of final concentrations, respectively. The enzyme endonuclease, (Benzonase® EC 3.1.30.2) was added, 1 unit per mg of NA, to the water soluble EtOH 80% fraction and incubated for 4 h at 37 °C and 100 rpm. Subsequently the proteases pronase E (type XIV; EC 3.4.24.31), trypsin (type I; EC 3.4.21.4) and nargase (type XXVII; EC 3.4.21.62) were added, 1 unit each per mg of Prt, with 2 h interval between them and incubated at 37 °C and 100 rpm. All the above enzymes were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA.). After the enzymatic treatment, Na-deoxicholate (DOC) and ethylenediaminetetraacetic acid (EDTA) were added to a final concentration of 0.3% and 2 mM, respectively, and incubated for 1 h.

Low-molecular-mass contaminants resulting from enzyme hydrolysis and detergent degradation were eliminated by the second 100-kDa cut-off membrane with area of 0.015 m^2; inlet pressure of 30 psi and TMP of 20 psi (Lab-Scale, cassette type, Millipore). The volume was reduced to ~300 mL, washed extensively and sequentially with 4 volumes (vol.) of 50 mM TrisHCl, 2 mM EDTA and 0.3% DOC pH 7.0; 4 vol of 50 mM TrisHCl, 2 mM EDTA pH 7.0; 4 vol 150 mM NaCl; 4 vol. distilled water. The Purified PRP was filtered sterile by a 0.2-μm membrane, lyophilized and submitted to the purity test.

Results and discussion

The results of the purification process are summarized in Table 1. After the separation of cells from the fermented broth, it is possible to verify that the concentration of PRP is quite low if compared to proteins ($RP_{Prt} = 0.24$) and nucleic acids ($RP_{NA} = 0.06$) concentrations. There are around six times and sixteen times more proteins and nucleic acids, respectively, than polysaccharide. The concentration of PRP related to LPS measured as KDO was 28 times more PRP than KDO. This number represents only the amount of KDO (2-keto-3-deoxyoctanate) a sugar component of all LPS [22].

The purification of PRP in relation to the proteins, in the first concentration by ultrafiltration 100 kDa cut-off membrane was $PF_{Prt} = 3.7$ and $RP_{Prt} = 0.87$ (one mg of PRP per 1 mg of protein). Small molecules from the culture medium, salts and others molecules produced by the microorganism lower than 100 kDa can cross easily through the pore of 100 kDa cut-off membrane eliminating large amount of contaminant [13]. The apparent PRP recovery in

Table 1 Purification of capsular polysaccharide from *Haemophilus influenza*e b

	PRP[a] (mg/L)	% Recovery	Protein[a] (mg/L)	PRP/Prot	PF_{prot}	NA[a] (mg/L)	PRP/NA	PF_{NA}	KDO[a] (mg/L)	PRP/KDO
Clarified broth	1,969 ± 6.4	100	8,352 ± 8.3	0.2	1.0	32105 ± 7.3	0.1	1.0	68.5 ± 10.7	29
1CTUF100	1,471 ± 3.6	75 (75)	1,694 ± 15.5	0.9	3.7 (3.7)	3659 ± 20.1	0.4	6.6 (6.6)	57.8 ± 3.8	25
Supernatant EtOH 30%	1,379 ± 3.8	70 (94)	1,706 ± 15.1	0.8	3.4 (0.9)	402 ± 30.9	3.4	56.0 (8.5)	24.3 ± 8.1	57
Water soluble EtOH 80%	1,448 ± 2.0	74 (105)	562 ± 7.8	2.6	10.9 (3.2)	173 ± 25.9	8.4	136.2 (2.4)	7.7 ± 10.7	188
2CTUF100	1,334 ± 2.5	68 (92)	26 ± 0.7	51.8	219.8 (20.1)	2 ± 7.1	579.9	9,457.2 (69.4)	0.4 ± 8.7	3,334

Relative purity: RP_{xx} = mg PRP/mg xx; xx is protein (Prt) or Nucleic acid (NA) or Lipopolysaccharide measured as KDO

Purification factor: $PF = RP_{step}/RP_{initial}$

Step purification factor: ($FP_{step} = RP_{step}/RP_{previous\ step}$). The values between parentheses mean previous step

[a] PRP, protein, nucleic acids and KDO in mg/L of culture broth ± coefficient of variation (%). All values are the average of three cultivations

Table 2 Residual proteolytic activity in purified PRP

Treatment	Sample	Arbitrary activity units (A_{280} at 48 h)
Enzymatic treatment + second concentration 100 kDa	Ultrafiltrate 1.5 volumes saline/Tris/azide	2.5
	Ultrafiltrate 3.0 volumes saline/Tris/azide	1.5
	Ultrafiltrate 2.0 volumes DOC/EDTA/tris	0.02
	Ultrafiltrate 3 volumes Tris	0.0
	2CTUF	0.0
	Pool ultrafiltrates 100 kDa/concentrated 5 kDa	2.7

this step was 75%, the lowest in this process if compared with polysaccharide 6B and 23F of *S. pneumoniae*, [14]. Usually polysaccharides are polydispersed molecules and the lower range of molecular mass can be eliminated through 100-kDa cut-off membrane. Besides, PRP quantification is made by ribose determination and some contaminant molecules that can interfere in the method, but with lower molecular mass are lost in the ultrafiltrate, resulting in an apparent low recovery of PRP. The 30% ethanol precipitation did not show any PRP purification with respect to protein with a $PF_{Prt} = 0.9$. In fact, we observed a bigger loss of polysaccharide than contaminant proteins. The second precipitation step with 80% ethanol, after the resuspension in water, showed a slight purification with a $PF_{Prt} = 3.2$ [13]. The best result for protein elimination was achieved after enzymatic digestion (proteases), where the residual proteins with molecular mass bigger than 100 kDa were hydrolysed and the resulting small peptides ultrafiltrated through 100-kDa cut-off membrane (2CTUF 100), with $RP_{prt} = 51.8$ and $PF_{prt} = 20.1$.

Nucleic acids elimination by the first step 1CTUF 100-kDa cut-off membrane showed a $PF_{NA} = 6.6$. Many components with absorbance at 260 nm and molecular mass less than 100 kDa were easily eliminated in the ultrafiltrated fraction. The precipitation with ethanol 30% had a $PF_{NA} = 8.5$, it was much better than the elimination of proteins, with a good trade off for this step. The same treatment worked very well for one contaminant, nucleic acid, but unsatisfactorily for proteins elimination. The best result for elimination of residual nucleic acids was obtained after the enzymatic treatment with nuclease (Benzoase®) (2CTUF 100) with $RP_{NA} = 579.91$ and $PF_{NA} = 69.4$. The enzyme hydrolyses the high molecular weight residual genomic DNA and RNA and the oligonucleotides produced are filtered freely throughout the membrane.

The lipopolysaccharide, LPS, forms aggregates of high molecular weight in aqueous solution and for this reason, the elimination in the firsts steps of concentration by diafiltration in 100-kDa cut-off membrane was only 16% against 80–90% with nucleic acids and proteins [26, 27]. In the precipitation with ethanol, LPS behaves like a polysaccharide, partially co-precipitated with nucleic acids. The elimination of LPS, measured as KDO, is done with the second TUF in the presence of detergent and chelating agent, DOC/EDTA. The EDTA quenches the divalent ions from the polysaccharide moiety of the LPS and the electronegative polysaccharide repels each other [27]. The detergent deoxycholate, DOC, breaks the hydrophobic interaction of the fatty acids of the lipid part. These combinations of electronegative charge of polysaccharide and diminution of hydrophobic interactions disestablish the aggregate and produce the low molecular weight monomers of LPS; which can be freely filtrated through out membrane of 100-kDa cut-off [26, 27].

Every step in the purification process is necessary to obtain the final purity of Hib polysaccharide, but the highest purification factor was obtained in the enzymatic fraction for the three main contaminants with $PF_{Prt} = 16.6$, $PF_{NA} = 73$ and KDO with elimination of 95%. The purification of polysaccharides from *S. pneumoniae* 23F and 6B showed different results: the elimination of proteins for the 23F was done in the ethanol precipitation steps and the nucleic acid removal in the enzymatic DOC/EDTA TUF 30 kDa [13]. In contrast, the 6B best purification step for proteins and nucleic acid were the ethanol precipitation [14]. On the other hand, the Gram negative *N. meningitidis* behaves similar to the Gram negative *H. influenzae* and the last step of enzymatic treatment is the best step [12].

The residual proteolytic activity in the concentrated fraction and in every washing step was measured using casein as substrate. The proteolytic activity was eliminated by increasing the washing step as showed in the Table 3. In order to make sure whether under the washing conditions the enzymes were eliminated through out the100-kDa cut-off membrane, a pool of the ultrafiltrated fractions was concentrated in the membrane of 5-kDa cut-off. We have observed that all activity was present in the ultrafiltrated fractions.

Figure 1 shows the evaluation of the PRP as immunological antigen in all purification fractions. The Burro serum against PRP from *H.influenzae* type b recognizes PRP in all fractions. The signal in the ultrafiltrated samples (Fig. 1, lane g) was low showing that the majority of PRP was in the concentrated fraction. All PRP concentrations obtained from the Rocket immunoelectrophoresis method were according to that obtained by Bial's method.

Besides the identity, which was verified by immunological test previously discussed, the PRP obtained by the pro-

Table 3 Purified PRP specifications according to WHO reference tests

Test	WHO specification	Purified PRP
Ribose content	Not less than 32.0% of weight	34 ± 2
Nucleic acid content	Less than 1.0% of dry weight	0.58 ± 0.04
Protein content	Less than 1.0% of dry weight	1.9 ± 0.1
Phosphorus content	Between 6.8 and 9.0% of dry weight	6.2 ± 0.2
Molecular size Distribution	Kd	0.49 ± 0.01
Identity	Immunological	Positive (3 samples)

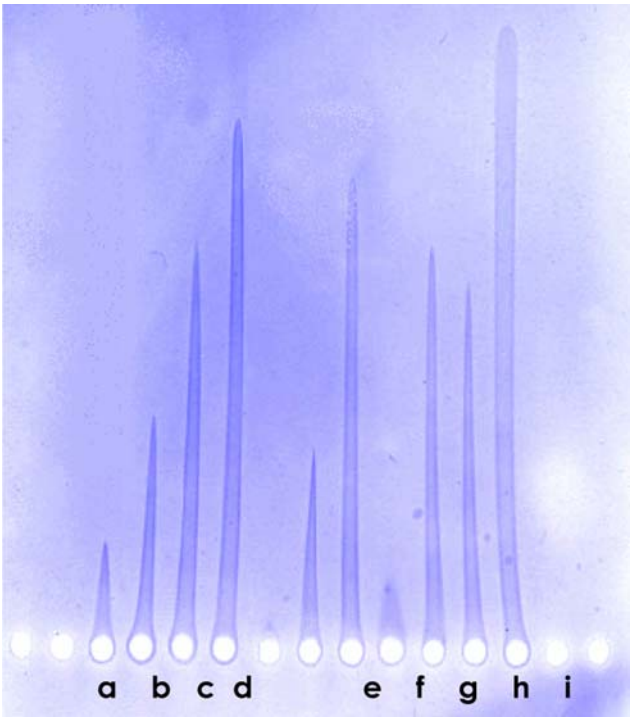

Fig. 1 Rocket immunoeletrophoresis from the all PRP purification fractions. PRP standard solutions (μg/ml) *a* 2.5; *b* 5; *c* 10; *d* 20; *e* clarified broth; *f* concentrated TUF 100 kDa; *g* ultrafiltrated; *h* supernatant EtOH 30%; *i* water soluble EtOH 80%; *j* enzymatic DOC/EDTA TUF 100 kDa

posed purification method was also submitted to further tests to evaluate its compliance to World Health Organization (WHO) specifications [28, 29]. Table 2 shows that the purified PRP fulfills four out of six WHO requirements for this product: ribose and nucleic acid content, molecular size distribution and identity. Phosphorus content is also very close to the specified value. Extensive washing employed after the extraction with 80% ethanol (Table 1) resulted in a 20 times decrease in proteases activity, which were lost in the concentrated of 100 kDa and recovered in the ultrafiltrate [30]. Nevertheless, the protein content is still twice the maximum allowed (Table 2), which implies that more effective washing might be needed. The WHO recommended tests also include endotoxins analysis, which is already being carried out.

The developed process greatly reduces the number of precipitations as well as the volume of ethanol used for this purpose [31, 32], but it was not feasible to substitute it completely by the enzymatic treatment and ultrafiltration. In the experiments that were done using enzymatic treatment without ethanol precipitation, the final product did not reach the required purity (not shown).

The phenol precipitation, for desproteinization, and the ultracentrifugation, for removal of LPS, were replaced by enzymatic treatment and by ultrafiltration in the presence of chelating agent and detergent [26, 27, 33]. The new methods have the advantages of not using a toxic and corrosive solvent as phenol and reducing the volume and number of ethanol precipitation, an explosion prone reagent. The ultracentrifuges are expensive and the running of the process is limited by the volume that each centrifuge can handle. Although enzymatic treatment and tangential ultrafiltration can contribute to increase the downstream processing costs, the new purification method described here is simple, efficient, non-toxic, easy to scale-up and environmentally friendly.

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