Simple and efficient method of bacterial polysaccharides purification for vaccines production using hydrolytic enzymes and tangential flow ultrafiltration

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Neisseria meningitidis and *Streptococcus pneumoniae* are human pathogens and the main cause of their virulence are the capsular polysaccharides (CPS). Purified CPS are used in the production of vaccines against these bacteria. Although there are several studies about the immunogenic characteristics of CPS vaccines, the know-how of large scale production and purification is not available in the public domain, the publications are scarce and most of them are patents. In Brazil and in other countries, the vaccines are distributed by the Ministry of Public Health and the production cost of these vaccines must be as cheap as possible.

The bacteria are cultivated in industrial bioreactors with appropriated controls. After growth, the CPS are purified up to achieve the purity requirements, while maximizing the recovery and minimizing the production cost. A traditional purification process of bacterial CPS for vaccine production is based on several selective precipitations steps with solvents like ethanol and phenol, and cationic detergents. Separations of solid from liquid are based on continuous centrifugation in explosion proof installations. The lipopolysaccharides, LPS, from Gram-negative bacteria, are separated by ultracentrifugation.

Our laboratory has developed an improved general method for CPS purification from *N. meningitides* CPS-C and *S. pneumoniae* CPS-23F and CPS-6B that greatly reduces the number of ethanol precipitations and eliminates the phenol and the ultracentrifugation steps. The process consists of one ultrafiltration step using membranes with cut-off of 30 or 100 kDa, two steps of ethanol precipitation with 25-30% and 50-80%, and elimination of residual proteins and nucleic acids by enzymatic digestion and diafiltration with 30 kDa or 100 kDa cut-off membranes. The LPS are separated by ultrafiltration in the presence of chelating agent and detergent.

The purified CPS-C from N. meningitidis had a yield of 50% with 2% of protein and 1.4% of nucleic acid related to total CPS. The final CPS from S. pneumoniae 23F had a recovery of 89%. The final protein and nucleic acid impurities were 1.4% and 0.1% (w/w) respectively. The final CPS from S. pneumoniae 6B had a recovery of 72% and the protein and nucleic acid impurities were 1.4% and 0.6% (w/w) respectively. The purified CPS were free of proteolytic enzymatic activity and passed the quality tests of molecular mass and phosphate.

Keywords polysaccharide vaccine; process purification; tangential flow ultrafiltration; enzymatic hydrolysis.

1.Introduction

N. meningitidis [1] and *S. pneumonia* [2] are some of the most common agents of meningitis, pneumonia and bacteremia in infants and immunodeficient adults [3]. The capsular polysaccharides (CPS) act as mechanisms of defense against the immune system of the hosts which makes them the main factor of virulence of these bacteria. Vaccines based on polysaccharides are effective for the adult population.

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Polysaccharides are polymers of high molecular mass of repetitive sugar units joint by glycosidic linkage. *N. meningitidis* serogroup C polysaccharide is a homopolymer of α-(2-9)-N-acetyl neuraminic acid [4]. The pathogenic *S. pneumoniae* includes 90 different types of polysaccharides among which there are 23 that compose the 23-multivalent vaccine. The polysaccharide 23F is a repeating tetrasaccharide unit containing β-D-glucose(1-4)-β-D-galactose+[α-L-rhamnose(1-2)]+[phosphate-glycerol] (1-4)-β-L-rhamnose(1-4) [5]. The polysaccharide 6B is a repeating tetrasaccharide unit of α-D-galactopyranosyl(1-3)α-D-glucopyranosyl(1-3)α-D-rhamnopyranosyl(1-4)D-ribitol-5-phosphate [6].

There are several studies about the immunogenic characteristics of polysaccharide vaccines. However, publications are scarce and the know-how on large scale production and purification is not in the public domain [7-11].

The purification process aims to obtain the product with "the desired specification while maximizing yield and minimizing process cost" [12]. To achieve this goal, differences between physical chemical properties of the product of interest and that of the impurities or contaminants are explored. In general, it is necessary more than one step, each one using different physical chemical properties, to obtain a pure product. The most explored properties are molecular size, electrical charge, hydrophilicity, hydrophobicity, solubility/insolubility in solvents, thermoresistance. The final process is a series of purification steps where there is a trade-off between losses of contaminants, as much as possible, with loss of product, as little as possible. The contaminants in the purification of CPS are proteins, nucleic acids, pigments and other polysaccharides, e.g. cell wall polysaccharides or lipopolysaccharides (LPS).

Purification of CPS follows a general method of concentration/purification by ethanol and/or anionic detergent selective precipitation, protein extraction by phenol and, for Gram-negative bacteria, by ultracentrifugation to eliminate the LPS [7-10].

The traditional purification process of polysaccharides from *N. meningitidis* consists of precipitation with cationic detergent hexadeciltrimetilamonium (cetavlon). The cetavlon precipitate is resuspended in 1 M $CaCl_2$, two precipitations with ethanol, desproteinization by three extraction steps with phenol, dialysis and further ethanol precipitation. The LPS are separated from the CPS by ultracentrifugation [9, 10].

S. pneumoniae CPS traditional purification process consists of total cell lyses with detergent deoxycholate, concentration/diafiltration, four steps of ethanol precipitation, desproteinization by phenol treatment and activated charcoal [7, 8].

Our laboratory has developed an improved method for purification of vaccine polysaccharides from *N. meningitidis* C [13], *S. pneumoniae* 23F [14] and *S. pneumoniae* 6B, which largely reduces the number of ethanol precipitations replacing them by ultrafiltration, the phenol desproteinization was replaced by enzymatic treatment and the ultracentrifugation was substituted by ultrafiltration in the presence of chelating agent and detergent.

2. Material and Methods

2.1 Analytical procedures

CPS concentration from *N. meningitidis* C was measured by the resorcinol method [15]. The CPS from *S. pneumoniae* 23F and 6B were measured by a specific method for methyl pentoses using rhamnose as a standard [16, 17].

Protein was determined by the method of Lowry et al. [18]. Nucleic acids (NA) were estimated at 260 nm and the amount was calculated assuming an absorbance of 1.0 A= 50 μg [10].

2.2 Bacteria strains

N. meningitidis serogroup C and *S. pneumoniae* serotype 23F strain St 9995 and serotype 6B strain ST433/03 are clinically isolated and stored in the Instituto Adolfo Lutz, Sec. de Bacteriologia, SP, Brazil.

2.3 Cultivation

The culture was conducted in a 5~10-L fermentor Bioflo 2000 (New Brunswick Scientific Co.) containing the appropriate medium and conditions described in [19-21].

The whole broth of the bioreactor, *Neisseria* and medium broth, was precipitated with 0.1% cetavlon, decanted and centrifuged. Cetavlon precipitated was selectively solubilized with 1 M $CaCl_2$ and precipitated by two cuts of ethanol: one with 25% and another with 80%. The 80% ethanol precipitated

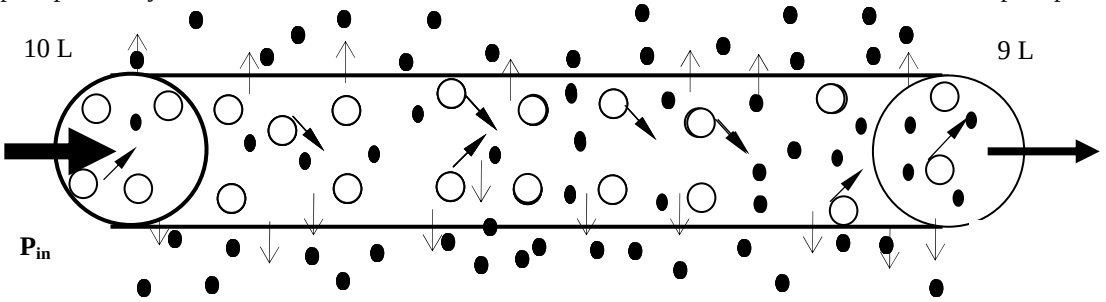

Fig. 1 Scheme of Tangential Flow Microfiltration/Ultrafiltration. *P_{in} =pressure inlet; P_{out} =pressure outlet. Transmembrane pressure - TMP= (Pin+Pout)/2)*

was ressuspended in water, water soluble-CPS-C fraction [9, 10].

The *Streptococcus* cells were separated from the culture broth by tangential microfiltration 0.22 μm, 0.5 m^2, with transmembrane pressure (TMP) of 10 psi (Cassette type Millipore, Bedford, MA, USA) [14, 22]. A peristaltic pump was used to drive the cellular suspension through the membrane (Masterflex, Easy-load I/P model 77601-10, silicone tubing Masterfex 96400-26). The cell-free microfiltrate was used for CPS-23F and CPS-6B purification, Figure 1 and 2. In the Tangential Flow Micro/Ultra filtration, as shown in Figure 1, the feed stream runs tangential to the membrane and the particles and molecules that are bigger than the pore are kept in constants movement, this procedure avoids the formation of a filtration cake and the filtration process can run fast and for long period of time. [23].

2.4 Concentration/Diafiltration

The cell-free CPS-23F or CPS-6B was concentrated to 1/10~1/20 by tangential flow ultrafiltration, TFUF, membranes of 30 or 100 kDa (Prep-Scale spiral type Millipore, peristaltic pump Masterflex, Easy-load I/P model 77601-10, silicone tubing Masterfex 96400-26). The concentrated was washed with 6 volumes of appropriated buffer, Concentrate TFUF 30/100 kDa fraction [14, 22]. The process was performed in an area of 0.1 m^2 with inlet pressure of 20 psi and a TMP of 7.5 psi, Figure 2.

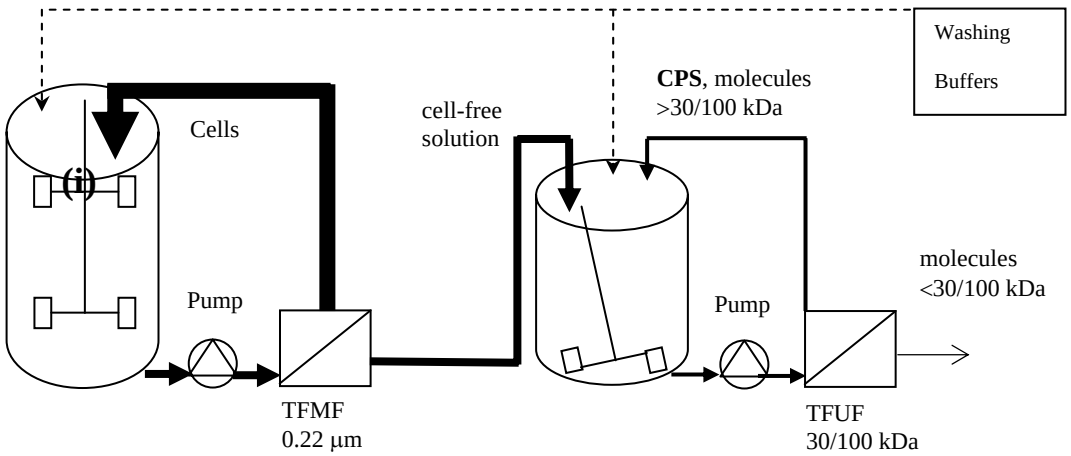

Fig. 2 Scheme of cell separation by Tangential-Flow Microfiltration (TFMF) and concentration/difiltration of cell-free microfiltrate and enzymatic treatment by Tangential-Flow Ultrafiltration (TFUF).

2.5 Precipitations with ethanol

The soluble 1 M $CaCl_2$ fraction of CPS-C was precipitated by 25% (v/v) ethanol and the precipitated discarded. Cold ethanol, up to 80%, was added to the 25% EtOH supernatant and the precipitated recovered with water to obtain the 2nd EtOH Water Soluble-CPS-C fraction [13].

The Concentrated 30 kDa CPS-23F fraction from the first ultrafiltration was processed as above but with 28% and 60% ethanol to obtain the fractions Supernatant 28% EtOH and 2nd EtOH Water Soluble-CPS-23F fractions [14].

The Concentrated 100 kDa CPS-6B fraction was processed with 25% and 50% (v/v) of ethanol as above to obtain the 2nd EtOH Water Soluble-CPS-6B fraction [22].

The ethanol precipitation, 1st and 2nd , was always done at 4°C and 12 hours and the solution centrifuged at 17,696 g for 60 minutes to obtain the precipitated. The 2nd precipitated was ressuspended in water and insoluble materials removed by centrifugation at 17,696 g for 60 minutes.

2.6 Enzymatic treatment and concentration /diafiltration

The pH of Soluble-CPS fractions from *Neisseria* or *Streptococcus* were adjusted to pH=7 with Tris-HCl 50 mM containing 2 mM $MgCl_2$ and 20 mM NaCl. The enzyme Benzonase (Endonuclease EC 3.1.30.2) was added to the Soluble-CPS, 1 Unit per mg NA and incubated for 4 hours at 37°C and 100 rpm. Subsequently the proteases, 1 Unit/mg protein, 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 with a two-hour interval between them and incubated for 12 hours at 37°C and 100 rpm. All the enzymes above were purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, U.S.A.). After the enzymatic treatment, Na-deoxicholate (DOC) and EDTA were added to a final concentration of 0.3 % and 2mM respectively and incubated for 1 hour [13, 14, 22].

Low-molecular-mass contaminants resulting from enzymatic degradation and detergent treatment were eliminated by the second TFUF membrane of 100 kDa cut-off for CPS-C and CPS-6B or 30 kDa for CPS-23F with an area of 0.015 m^2, inlet pressure of 30 psi and TMP of 20 psi (Lab-Scale, cassette type, Millipore), Figure 2. The volume was reduced to ~300 mL and extensively washed with 4 volumes of 50 mM TrisHCl, 2 mM EDTA and 0.3% DOC pH 7.0; 4 volumes of 50 mM TrisHCl, 2 mM EDTA pH 7.0; 4 volumes 150 mM NaCl; 4 volumes distilled water, respectively. The purified CPS was sterile filtered by a 0.2 μm membrane and lyophilized [13, 14, 22].

3. Results and Discussion

The purification processes are summarized in the Figure 3 and Tables 1-3. In the Tables 1 and 2 we can observe that the products, CPS-23F and CPS-6B, are in very low quantities compared to that of the contaminants, presenting a relative purity (RP_{Prt}=mg CPS/mg contaminant) of 0.07 and 0.04 for CPS-23F and CPS-6B, i.e., for every 0.07 mg of CPS-23F we found 1 mg of protein, the same happens for nucleic acids. The purification factor (PF) indicates how many times the relative purity of the product increases in relation to the total process or step by step ($PF=RP_{step}/RP_{initial}$). The final pure polysaccharide can contain ~2 mg of protein and nucleic acids per 100 mg of CPS, giving a RP of ≥50. In the purification process we have to increase the RP initial of 0.07 to 50 or more, this means a PF_{Prt} of at least 715.

In the purification process, this ratio was increased up to RP_{Prt} of 71.2, this means 72.2 mg of polysaccharide per mg of contaminating protein. The purification of CPS-23F related to protein is well distributed among almost all steps: PF of 6.3~7.8, but the last step, enzymatic digestion and TFUF 30 kDa, is not so efficient for protein elimination with a PF of 2.7, nevertheless this step is necessary to reach the required purity. For removal of the NA contamination, there are two steps with high purification factor: the first TFUF 30 (PF_{AN} = 33) and the enzymatic and TFUF treatment (PF_{AN} = 51). In the process, there are several shear forces that damage the nucleic acids and the low molecular mass

oligonucleotides can pass through the pore of 30 kDa from the first concentration. The high molecular mass molecules are further removed by ethanol precipitation and especially by enzymatic treatment. The nuclease, benzonase, hydrolyzes the residual genomic DNA and RNA and the resulting low molecular mass oligonucleotides are filtered through the membrane in the second TFUF.

The elimination of protein and nucleic acids for CPS-6B is well distributed and it seems that the 2nd EtOH water soluble is a little better than the others, PF_{Prt} 18, especially for nucleic acids, PF_{NA} 53. This purification step is actually a series of two precipitations with ethanol and one selective solubilization.

The purification of *Neisseria* CPS-C started with the precipitation of the whole bioreactor, cells and medium, with cetavlon, a cationic detergent. Cetavlon is positively charged and forms a complex with high molecular mass polymers negatively charged like CPS-C, nucleic acids, LPS and some proteins. In Table 1, we can see that the amount of CPS-C is bigger than that of the contaminants, AN, Prt and LPS. The protein and the LPS are eliminated in the last step, after enzymatic treatment and the second concentration/diafiltration with TFUF 100 kDa. The ethanol precipitation steps had a loss of 42% of the CPS-C and a FP_{Prt} 0.8 and a FP_{KDO} 1.3. This means that the trade-off between loss of product and loss of contaminants in this particular series of precipitations was not so good. The last step presented quite good elimination of contaminants and near total recuperation of polysaccharide. The elimination of LPS, for the Gram-negative bacteria, measured as KDO, is done with the TFUF in the presence of detergent and chelating agent, DOC/EDTA, with purification factor of 16. The LPS form aggregates of high molecular mass in aqueous solution, the EDTA complexes the divalent ions from the polysaccharide moisture, and then the electronegative polysaccharides repel each other. 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 mass monomers of LPS which can be freely filtrated in the membrane of 100 kDa.

The final products were free of proteolytic enzymatic activity and passed the quality tests of phosphate and molecular mass (not shown).

The present purification process is based mainly in molecular size. In the first TFUF, molecules with size less than the pore cut off are eliminated, most of them from the culture medium. After the ethanol precipitation the enzymes reduce the size of the contaminants, proteins and nucleic acids, and the second TFUF eliminates them. Low molecular mass monomers from *Neisseria* LPS are ultrafiltrated in the presence of detergent and chelating agent.

The developed processes greatly reduced the number of ethanol precipitations, but it was not feasible to substitute them completely by the enzymatic treatment/ultrafiltration. Experiments were carried out using just enzymatic treatment and the final products did not reach the required purity (not shown). The phenol precipitation and the ultracentrifugation were replaced by enzymatic treatment plus ultrafiltration in the presence of chelating agent and detergent. The new method has the advantage of not using a toxic and corrosive solvent as phenol. Compared to ultracentrifugation, the combination of enzymatic treatment and tangential ultrafiltration is easier to scale-up and much cheaper. The membranes for tangential flow are cleaned in place and stored for repeated use. In conclusion, a simple, efficient and environmentally friendly method that could easily be scaled-up was developed.

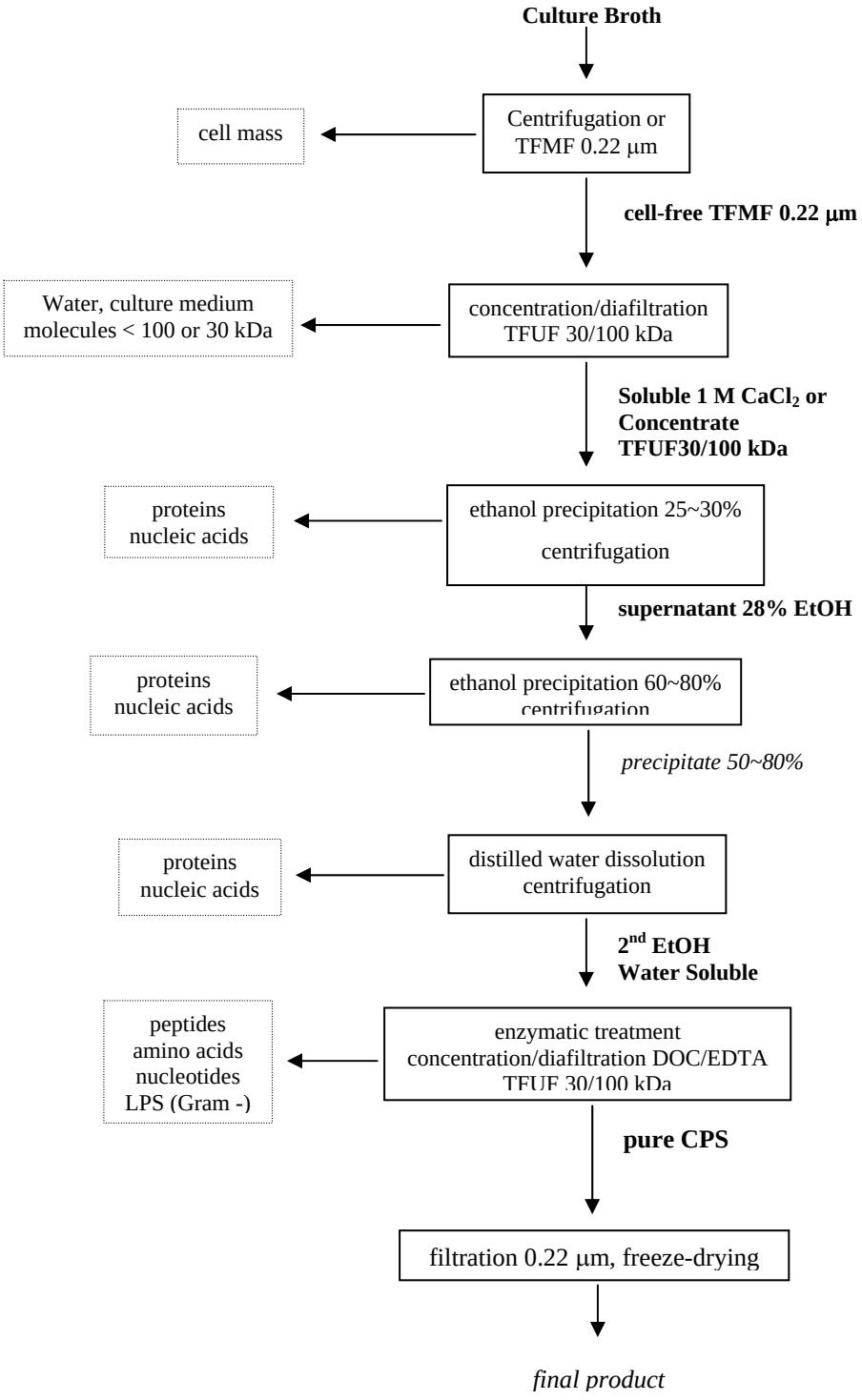

Fig. 3 - Flow diagram of CPS purification process. Unit operations are in solid rectangles, main contaminants are in dotted rectangles, intermediate fractions presented in tables are in bold and intermediate fractions not presented in tables are in italic.

Table 1 Purification of *Streptococcus pneumoniae* polysaccharide CPS-23F

Fractions	CPS-23F	CPS-23F Recovery %	Protein (Prt)	RP_{Prt}	PF_{Prt}	Nucleic Acids (NA)	RP_{NA}	PF_{NA}
Cell free TFMF 0.22μm	*321	#100	4180	0.07	1.0	2288	0.14	1.0
Concentrated TFUF 30	321	100	716	0.44	6.3	67.3	4.7	33
Supernatant 28% EtOH	319	99 (99)	92	3.46	49 (7.8)	41.8	7.6	54 (1.6)
2nd EtOH Water soluble	385	119 (120)	15	25.7	367 (7.4)	20.5	18.8	134 (2.5)
Pure CPS-23F Enzymatic TFUF 30	285	89 (74)	4	71.2	1017 (2.7)	0.3	950	6786 (51)

* and # Interferences in the quantification of CPS-23F, the values of the second step were used.
All values in mg/L of broth culture
Relative Purity: RP_{xx}= mg PS/mg xx; xx is protein (Prot) or Nucleic Acid (NA) or Lipopolysaccharide (LPS) measured as KDO
Purification Factor PF= $RP_{step}/RP_{initial}$
Step Purification Factor: ($FP_{step}= RP_{step}/RP_{previous\ step}$)
( ) previous step

Table 2 Purification of *Streptococcus pneumoniae* polysaccharide CPS-6B

Fraction	CPS 6B	CPS-6B Recovery %	Protein (Prt)	RP_{Prt}	PF_{Prt}	Nucleic Acids (NA)	RP_{NA}	PF_{NA}
Cell free TFMF 0.2μm	286	100	6475	0.04	1	2500	0.11	1
Concentrated TFUF 100 kDa	281	98	651	0.43	10	514	0.5	5
2nd EtOH Water Soluble	292	102 (104)	37	8	179 (18)	10	29	255 (53)
Pure CPS-6B Enzymatic TFUF 100 kDa	277	97 (95)	4	69	1568 (9)	1.7	163	1424 (6)

Table 3 Purification of *Neisseria meningitidis* polysaccharide CPS-C

i) Fraction	CPS-C	CPS-C Recovery %	*2) Protein 3) Prt*	RP_{Prt}	PF_{Prt}	Nucleic Acids NA	RP_{AN}	LPS (KDO)	RP_{KDO}	PF_{KDO}
Soluble 1 M $CaCl_2$	2620	100.0	861	3.0	1.0	ND		134.3	19.5	1
2nd EtOH Water Soluble	1520	58.0	614	2.5	0.8	ND		61.7	61.7	1.3
Pure CPS-C Enzymatic TFUF 100	1380	52.6	30	46.0	15.1 (18.4)	19.7	70	3.5	394.3	20 (16)

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