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Research Article

PURIFICATION OF *STREPTOCOCCUS PNEUMONIAE* CAPSULAR POLYSACCHARIDES USING ALUMINIUM PHOSPHATE AND ETHANOL

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ABSTRACT

Introduction: *Streptococcuspneumoniae* is one of the major causes of invasive pneumococcal infections in humans, including both infants and adults. Predominantly capsular polysaccharide (CPS) is the virulence factor associated with these bacteria and is extensively used in vaccine preparation.

Methods: The method involves collection of detergent lysed culture followed by concentration, ethanol precipitation, aluminium phosphate adsorption and diafiltration using a 300kDa (kilodalton) tangential flow filtration system.

Results: Cumulative percentage of process impurities were reduced to < 1.5% w/w of purified CPS with 65-80% CPS recovery. The final pure capsular polysaccharide meets the Technical Report Series (TRS) specification of World Health Organization.

Conclusion: Methods describing the purification of CPS are limited in the public domain and are confined to procedures involving the usage of nucleases, proteolytic enzymes and corrosive chemicals like phenol. In the current study, we have developed a simple and efficient method for purification of capsular polysaccharides using ethanol and $AlPO_4$ substituting the usage of nucleases, proteases and phenol.

Keywords: *Streptococcus pneumonia*, capsular polysaccharide, purification, ethanol, aluminum phosphate.

INTRODUCTION

Streptococcus pneumoniae is one of the major pathogens of humans causing pneumonia, meningitis, septicemia and otitis media.[1]Globally pneumococcal infections accounted for ~14.5 million cases of serious illness and 735,000 deaths every year in children less than five years of age.[2]Incidents of invasive pneumococcal diseases showed geographic variation from 21 to 33 cases per 100,000 populations as per the report generated by Centre for Disease Control.[3,4]In developing countries like India pneumococcal attributable deaths among children less than five years of age is up to 19-20%. *S. pneumoniae* is also a major cause of illness and death in the developed nations like United States, with an estimated 43,500 cases and 5,000 deaths among persons of all ages in 2009, as reported by Advisory Committee on Immunization Practices (ACIP), Pneumococcal Vaccines Working Group.

Capsular polysaccharide (CPS) is associated with the cell wall of this bacterium and protects it against the host cell immune system.[5]CPS based vaccines are effective only in elderly patients.[6]Conjugating CPS with carrier proteins like cross-reactive material 197 builds the immunogenicity in adults and infants.[7,8]Pneumococcal infections were reduced to a large extent by the introduction of conjugate vaccines but even with advanced vaccine technology, the cost per dose remains as major hurdle for a truly affordable vaccine. The current cost of the conjugate vaccine exceeds $50 per dose.[9]Three dose regime per infant in the developing world countries is infeasible with current price.

More than 90 different serotypes have been identified based on the difference in the chemical composition and size of capsular polysaccharide out of which 23 serotypes are known to cause infection in humans.[10]Serotypes isolated from child and adult infections are different but cumulative prevalence of the five serotypes 3, 6B, 14, 19F and 23F accounted for 56 % of pneumococcal infections in humans.[11] Considering the above mentioned statistics, we focused to produce the native polysaccharides of these five serotypes. Purified CPS can be used as drug substance in polysaccharide vaccine preparation or as conjugate vaccine by conjugating with carrier protein.

MATERIALS AND METHODS

Bacterial strain: Bacterial strains used in the current study were procured from ATCC (American Type Culture Collection, Manassas, VA, U.S.A.). Following are the details of the strains with ATTC No's: Serotype3 - 6303, Serotype6B - 6326, Serotype14 - 6314, Serotype19F - 6319, Serotype23F – 6323

Seed culture preparation

Streptococcus pneumoniae is categorized under biological safety level-2 containment. All precautionary measures were taken accordingly while handling the cultures. Lyophilized cultures were revived on tryptic soy agar (TSA) containing 5% (w/v) defibrinated sheep blood (Difco, Detroit, MI, U.S.A.) at 37^{0}C for 24h. An isolated colony was inoculated into 5ml of Holt's medium.[12]Incubated at 37^{0}C, 220 rpm for 24 hrs. Seed cultures were developed by sub culturing into 100 ml of Holt's medium and incubated in a rotary shaker for 12h to 16h at 37^{0}C/220rpm.

Fermentation medium and process parameters

Modified Holts medium was used for fermentation. Composition included 30g of Hisoya (Kerry), 20 g of Glucose (Merck), 0.7g of KH_2PO_4 (Merck), 0.02g of $CaCl_2.2H_2O$ (Merck), 0.2g of Cystine. HCl (Merck), 2g of NaCl (Merck) and 0.5g of $MgSO_4.7H_2O$ (Merck) in a final volume of one liter distilled water. The pH was adjusted to 7.0 using 1M NaOH. The medium was sterilized at 121^{0}C for 30 minutes except Glucose, $MgSO_4$ and Cystine stocks which were filter sterilized with 0.2 micron filter and added aseptically. Fermentation was performed at 37°C temperature, pH 7.0, 200 - 600 rpm of agitation and 0.1-0.2VVM (volume of air per volume of medium per minute) of air. Deoxycholate detergent at 0.5% - 1.0% w/v was added to culture broth after attaining the plateau of optical density (OD_{590}) to extract CPS.

Clarification and concentration

Biomass was separated using micro filtration skid (Cassette type Millipore, Bedford, MA, USA) and the filtrate was concentrated to 10~20 fold using a 0.1m^2/300kDa tangential flow ultra-filtration (TFUF) membrane followed by diafiltration with normal saline.

Ethanol precipitation with aluminum phosphate ($AlPO_4$)

Sodium deoxycholate (0.3% w/v) and absolute ethanol were added to the crude concentrate (30%v/v) and incubated for 4 hrs at 4^{0}C. The concentrate was then centrifuged at 10,000g/4^{0}C for 30mins. To the supernatant, 8% w/v of $AlPO_4$ was added and incubated for 8hrs at 25^{0}C followed by centrifugation. Absolute ethanol was added to

the collected the supernatant to a final concentration of 70%v/v and incubated for 4hrs at 4^{0}C. The pellet collected after centrifugation was dissolved in ~300ml of distilled water, concentrated and diafiltered using a 100kDa tangential flow ultra filtration cutoff membrane (Millipore), followed by carbon filtration and 0.2micron filtration. Purified CPS was stored at -20^{0}C.

Analytical procedures

Total CPS content was determined by colorimetric method using rhamnose as standard.[13]Nucleic acids were estimated spectrophotometrically at A260 and the amount was calculated assuming an absorbance of 1.0A=50µg.[14]Protein was determined by Lowry's method.[15]Endotoxins were determined using Limulus Ameobocyte Lysate test.[16] The relative purity (RP) is the ratio of amount of CPS and contaminant in each purification step (mg product/mg contaminant). Recovery percentage is the ratio of CPS input at crude concentrate to the CPS obtained at the end of purification.

SDS-PAGE

All the samples were diluted to 10 fold using physiological saline and 20µL was loaded into each well. Samples were boiled in sample buffer (5%v/v 2-mercaptoethanol and 0.1%v/v SDS in TRIS buffer pH8.8) for 5mins. The reduced samples were loaded on 10% polyacrylamide gel and electrophoresis was carried out at 150 V for 1 hour at room temperature. The electrophoresed gel was stained with 1% commassie blue dye for an hour and de-stained (40% v/v methanol along with 10% v/v acetic acid solution). High molecular weight protein marker (Bangalore Genei) was loaded to determine the approximate molecular size of protein impurities.

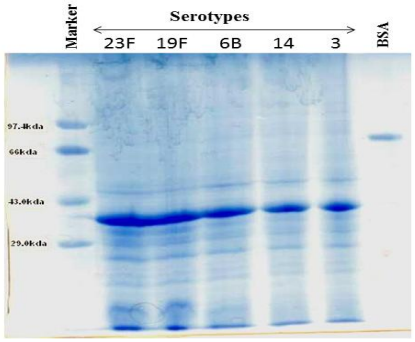

Fig. 1: SDS- PAGE of crude CPS

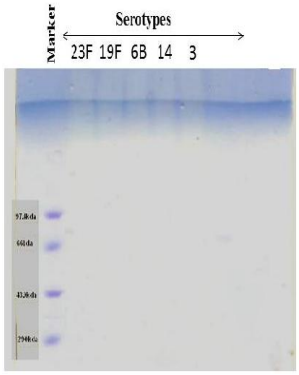

Fig. 2: SDS- PAGE of purified CPS

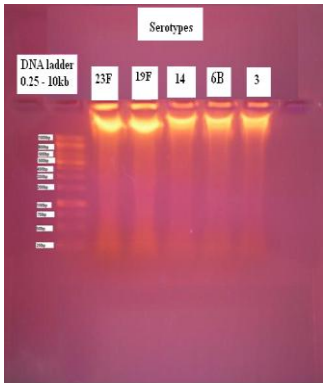

Fig. 3: Agarose gel of crude CPS

Agarose Gel electrophoresis

All the samples were diluted to 10fold using physiological saline and 20µL was loaded into each well. Electrophoresis was performed on 1% agarose gel at 100V for 90mins. Ethidium bromide (10µg/100ml) was used to stain the gel. DNA ladder (1kb, Fermentas) was loaded to determine the approximate molecular size of nucleic acid impurities.

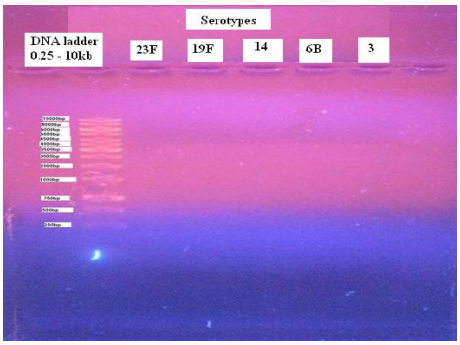

Fig. 4: Agarose gel of purified CPS

RESULTS AND DISCUSSION

Nucleicacids, proteins, endotoxins and other host cell impurities[17] associated with CPS were successfully eliminated. Minimal CPS loss 16.2% was observed with serotype-3 while a maximum of CPS loss of 35% was observed with serotype-6B. In serotypes 3&6B cumulative percentage of nucleic acid and protein impurities reduced from 31.1% to 1.0% and from 37.3% to 1.3% respectively. Similar observations were reported by Gonclaves et.al[18]using ethanol and nucleases for purification of serotypes 6B and 23F but comparatively the product recovery was on the lower side and removal of endotoxins was not demonstrated. CPS of serotype 14 was purified with maximum relative purity (111.1) and with a recovery percentage of 81.2%.

Impurities reduced from 27.6% to 0.9% in serotype 14. Crude CPS of serotype 19F with maximum initial impurities (53%) was reduced to 1.2% with a recovery percentage of 75 and a relative purity of 83.3. Nucleic acid and protein impurities were reduced from 43.6% to 1.4% for serotype 23F with minimum relative purity of 71.4% and with a CPS recovery of 73.7% [Table I]

Table 1: Purification Summary

Serotype	Stage	CPS in (grams)	Endotoxin (EU/µg of CPS)	Protein (% w/w)	Nucleic acid (% w/w)	Relative purity
3	Before purification	8.0	750-800	5.6	25.5	3.2
	After purification	6.7	2.5-5.0	0.6	0.4	100.0
6B	Before purification	8.0	1000-1100	6.2	31.3	2.6
	After purification	5.2	5-10	0.8	0.5	76.9
14	Before purification	8.0	500-600	7.8	19.8	3.6
	After purification	6.5	5-10	0.3	0.6	111.1
19F	Before purification	8.0	700-800	12	41	1.9
	After purification	6.0	5-10	0.4	0.8	83.3
23F	Before purification	8.0	1000-1100	15.6	38.0	1.9
	After purification	5.9	5-10	0.5	0.9	71.4

Hostcell protein impurities present in crude CPS [Fig 1] were successfully eliminated at the end of purification [Fig 2]. Nucleic acid impurities in crude CPS [Fig 3] were not detectable in the agarose gel of purified CPS [Fig 4]. Protein and nucleic acid impurities were effectively removed by ultra filtration.[19]The current method recovered maximum CPS from all the serotypes (varying from 65% to 83%) making the method flexible for all the serotypes with minimal residual process impurities i.e < 1.5% w/w of CPS

Aluminium phosphate is the most common adjuvant used in preparation of human vaccines[20] but we used $AlPO_4$ for purification of capsular proteins. Phenol precipitation and usage of nucleases, proteases and hydrolytic enzymes [21-23] were replaced by $AlPO_4$ adsorption and ultra filtration. We successfully demonstrated the negatively charged particle ($AlPO_4$) discriminates between the two negatively charged compounds (CPS & endotoxin) and selectively binds to the endotoxin. The developed method has the advantage over the other serotype specific purification methods[24]as all the five serotypes can be purified with minimal impurities. In conclusion, we have developed a simple, efficient, eco-friendly and cost effective method that could be easily scaled-up to produce purified CPS at commercial scale.

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