SL Vaccines And Vaccination Journal

Special Issue Article "Hib Vaccine"

Research Article

Purification of a Phosphated Biopolymer by Selective Ethanol Precipitation in Presence of Surfactant and Sodium Acetate

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ARTICLE INFO

Received Date: February 20, 2022
Accepted Date: March 24, 2022
Published Date: March 25, 2022

KEYWORDS

Surfactant
SDS
PRP purification
Ethanol
Sodium acetate

Citation for this article: Simas R, Dias Vieira L, De Marco R, Cabrera-Crespo J and Takagi M. Purification of a Phosphated Biopolymer by Selective Ethanol Precipitation in Presence of Surfactant and Sodium Acetate. SL Vaccines And Vaccination Journal. 2022; 3(1):116

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ABSTRACT

Bacteria have the ability to produce biopolymers with different chemical properties, for different purposes and vary according to the bacterial strains and their physiological status, and these can be used as vaccine antigens. Haemophilus influenzae type b is a microorganism pathogenic to humans, which causes several types of infections. It is classified into six serotypes, the biopolymer of serotype b (Hib) being the most virulent, known as Poly Ribosylribitolphosphate (PRP). The aim of this work was to evaluate different candidate surfactants to be used in the PRP purification step, as well as the effects of ethanol in combination with sodium acetate. From all the surfactants used, 0.5% SDS proved to be potent in eliminating protein impurities and nucleic acids and in accordance with criteria of regulatory agencies. Regarding the combination of ethanol and sodium acetate to precipitate impurities, in the first fractionation step and polysaccharide, in the second fractionation step; the best conditions were: 40% ethanol without sodium acetate in the first stage and 60% ethanol containing 7% sodium acetate in the second stage. This improved condition resulted in nearly 100% polysaccharide recovery with relative purities higher than 100 for both protein and nucleic acid. In the traditional PRP purification process the final polysaccharide recovery was around 20% at the end of the process, while the new condition will result in at least 80% and within the purity criteria established by WHO for this polysaccharide.

INTRODUCTION

Haemophilus Influenzae Type b (Hib) is a Gram-negative coccobacillus and responsible for more than 90% of systemic infections, prior the introduction of conjugated vaccines, causing pneumonia and meningitis mainly in infants. It remains to be significant public health concern in many parts of the world nowadays. During invasive infections, the capsule serotype confers resistance to phagocytosis and complement mediated host defenses.

Exopolysaccharides produced by *Haemophilus influenzae* are antigens of great importance for clinical studies. Of these, serotype b capsular polysaccharide (PRP) is the most important because it serves as an antigen for tetravalent (Hib + DTP) and pentavalent (Hib+DTP+HepB) vaccines. The first approaches to isolate this exopolysaccharide from the culture began in the mid-1940s. Dingle and Forthergill [1] introduced multiple selective ethanol precipitation; Anderson and Smith [2] proposed a combination of detergents and selective extractions to isolate polysaccharide; Joseph Kuo [3], presents a new methodology for the purification of

PRP, where the fermented broth, after cell inactivation and centrifugation to remove them, undergoes the addition of ethanol to precipitate the PRP. Then, the Cetavlon, a nitrogen quaternation detergent, is added to form a complex with negative charged molecule including PRP, and precipitate it. Repeated precipitations using ethanol in different concentrations are performed to remove detergent and contaminants such nucleic acid, proteins and endotoxins.

An improved methodology to purify the polysaccharides for vaccines against *Neisseria meningitidis* serotype C and *Streptococcus pneumoniae* serotype 23 and 6B was developed, in which phenol treatment was replaced by enzymatic treatment in presence of detergents [4,5]. Takagi et al, [6] reported that this protocol showed to be reproducible for PRP purification, however the relatives purities concerning to proteins and nucleic acid were not achieved. Years later, Albani et al innovated the downstream process based on membrane technology besides incorporated sodium deoxycholate (DOC)/cocoamidopropyl betaine in the washing buffer to improve the quality of the product. Combining detergents such as cocamidopropyl betaine, a zwitterionic, and sodium deoxycholate, an anionic, helps for breaking up the hydrophobic interactions of the LPS. On the other hand, EDTA chelates the divalent ions present in the polysaccharide fraction of the LPS causing it to be unstable and generate monomers with low molecular mass which are removed easily [7,8].

Detergents or surfactants are agents that reduce the surface tension of liquids and act on the solution interfaces, due to the amphiphilic characteristic, given by their structures. These compounds are divided into classes and can be anionic (negative), cationic (positive), amphoteric (negative and positive) and neutral (no charge) serving different applications. Surfactant-biopolymer interactions can occur through different mechanisms, which have been extensively studied and documented [9-11]. Such interactions depend primarily on the nature of the components present, with regard to their classifications and also on the concentrations of each species in the medium to be studied, causing the solubility of species in the solution to be altered [12]. In this work the effects of different detergents, ethanol, acetate concentration and its combination were evaluated considering relative purities and recovery for application in the PRP purification process.

MATERIAL AND METHODS

Polysaccharide production, harvesting and concentration

Haemophilusinfluenzae type b strain GB3291 was purchased from Nucleo de Coleção de Microrganismos - Instituto Adolf Lutz (São Paulo, Brazil). The working seed was stored at −80 °C according to Takagi et al. [13]. Experiments were carried out inbioreactors Bioflo 2000 (New Brunswick Scientific Co., USA) with 6.5 liters. The pH value was controlled at 7.5 with addition of NaOH5 M, temperature controlled at 37 °C, air supply at 1.0 vvm (volume of air per minute per volume ofmedium), agitation varying between 200 to 800 rpm in order to control pO2 at 30 % of air saturation. After the glucose consumption in the batch phase, the fed-batch started with a specific feeding rate of 21 mL/L.h. Glucose and yeast extract were increased to 200 g/L in the MMP medium [13] to elaborate the feed solution.

The production process was finished after 20 hours of cultivation and the culture broth was inactivated and centrifuged at 17,725g; 4 °C for 30 min (Beckman Avanti® J-25I) to remove cell, and the supernatant was concentrated to 1/10 of initial volume by tangential ultra filtration with a 100 kDa cut-off spiral membrane (Prep/Scale-TFF-6, Merck Millipore, MA, USA) to eliminate culture medium and low molecular mass molecules. The concentrated fraction, containing PRP, was diafiltered with 6 volumes of phosphate buffer 10 mM, pH 6.3 containing EDTA 2 mM and NaCl 150 mM. This fraction was named concentrate 100 kDa or [100k] and used in the following experiments.

Experiments

Two strategies were drawn as shows the (Figure 1). In the first experiment the effectiveness of different detergents was evaluated, and in the second one the effects combining ethanol and sodium acetate to remove the main impurities, protein and nucleic acids.

Experiment 1: Effect of different detergents in the first ethanol precipitation steps

In the fraction of [100k], sodium acetate (NaAC) was added to achieve a final concentration of 5% and the pH was adjusted to 5.8 with glacial acetic acid. An aliquot of 30 mL was distributed in centrifuge tubes of 50 mL and submitted to different detergents. Five detergents were tested: Deoxycholate sodium (DOC), Sodium Dodecil Sulphate (SDS),

Triton X-100 (TT), Tween-20 (TW), Cocoamidopropyl betaine (CAPB). Five different concentrations (0.1-1%) were studied for each detergent and one control - without detergent was carried out together. Samples were incubated for 30 minutes, at room temperature under agitation of 100 rpm. All samples were precipitated with Ethanol at 30%, the insoluble were removed by centrifugation, obtaining the EtOH30 soluble fraction containing PRP (Figure 1 – Exp1).

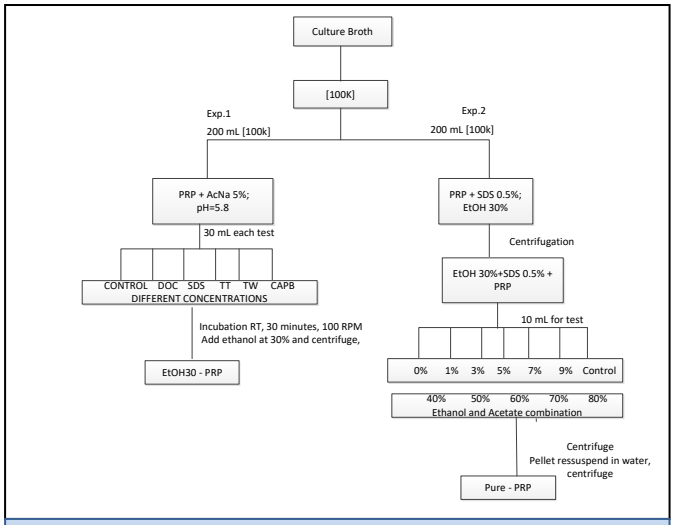

Figure 1: Scheme of the experiments carried out: Exp 1. Effects of different detergents and their concentrations, Exp 2. Effect of ethanol and sodium acetate, and its concentrations on the removal of impurities.

Experiment 2: Effect of the combination between ethanol and acetate, in different concentrations, in the second precipitation step

A volume of 200 mL of [100k] fraction was added concentrated SDS solution to a final concentration of 0.5%. Following, ethanol was added to 30% v/v, mixed and centrifuged at 9.000 g for 1 hour to obtain the supernatant fraction named EtOH30SDS. A volume of 10 mL of this supernatant was used in each condition tested. A solution of NaAc 30%, pH 5.8 was poured to each tube containing EtOH30SDS to achieve final concentrations ranging from 0 – 9% in relation to the aqueous phase. Ethanol was finally added to final concentration varying from 40 - 80% for each sodium acetate concentration tested (Figure 1 – Exp2). The samples were stirred for 1h at room temperature and left undisturbed for 3h. The Precipitates (PRP) were recovered by centrifugation (9000 g, 1h, 20°C) and solubilized in ultrapure water. These soluble PRP fraction was centrifuged again (9000g, 1h, 20°C) to remove insoluble materials. Control condition refers to first precipitation step with Ethanol at 30% and NaAc 5%, followed by the second precipitation with Ethanol at 80% and NaAc 7% as mentioned by Albani et al [7].

Analytical methods

The amount of PRP was determined by a modified Bial's method, using ribose as standard [14]. One gram of ribose corresponds to 2.55 grams of PRP [15]. Nucleic Acids (NA) concentration was estimated by measuring the optical density at 260 nm, where one absorbance unit corresponds to 50µg/mL [16]. Protein (Prt) amount was determined by the Lowry method, using BSA as standard [17].

Data analysis

Samples collected from the culture broth and concentrated in 100 kDa ([100k]) considered as 100%, supernatant from the first ethanol precipitation (30%) and from the second precipitation step/solubilized in water. Recovery of PRP is represented as (wt PRP_{step}/wt $PRP_{[100k]}$)*100. Relative Purity (RP) of PRP in relation to protein is expressed as RP_{Prot} (wt PRP_{Step}/wt Prt_{Step}) and in relation to nucleic acid as RP_{NA} (wt PRP_{step}/wt NA_{step}). Purification factor (PF) expresses how many times the RP improved in relation to the [100k] fraction, $RP_{step}/RP_{[100k]}$. In order to better interpret the results obtained and correlate the different variables involved, experimental design of the centered faces was used. The results were statistically evaluated with data and contour graphics produced in the Protimiza Experimental Design® software.

RESULTS AND DISCUSSION

Effect of Different Detergents in the First Ethanol Precipitation Steps

Detergents are amphipathic molecules that contain a polar head at the end of a long tail composed by hydrophobic carbon. In the aqueous milieu, the polar groups form tension of water and they are used in several biological process as proteins precipitation, LPS isolation and polysaccharides purification [7,8,18,19].

In the polysaccharides downstream process, detergents are used in different ways, sometimes before ethanol precipitation, others use in combination with ethanol in order to be more effective [8,20-22]. More specifically for the polysaccharide produced by *Haemophilus influenzae* b, PRP, Takagi et al, [6]

used sodium deoxycholate (DOC 0.3%) after enzymes treatment step to remove residual impurities from protein, nucleic acid or even the residual LPS. Albani et al, [7] introduced in the first and last step of purification, buffers containing Cocoamidopropryl Betaine (CAPB) and sodium deoxycholate (DOC), respectively to eliminate impurities, during the concentration in100kDa by ultrafiltration system.

In this study were considered others detergents as SDS (anionic) a strong protein denaturant, Triton X-100 and Tween-20(both non-ionic), besides CAPB and DOC already applied for PRP purification. However, it was considered different concentrations intervals of each surfactant used in order to evaluate the efficiency of each one. Cationic detergent as CTAB was not considered in the present study because it forms a complex with PRP, nucleic acid and others negatively charged polymers. Nevertheless, CTAB was used to treat samples for analytical determination of molecular weight and PRP concentration measurement protocol as established by Cintra & Takagi [23]. In this case CTAB (hexadecyltrimethyl ammonium bromide) works in the precipitation of polysaccharide [22] a negative charged polymer as showed in (Figure 2).

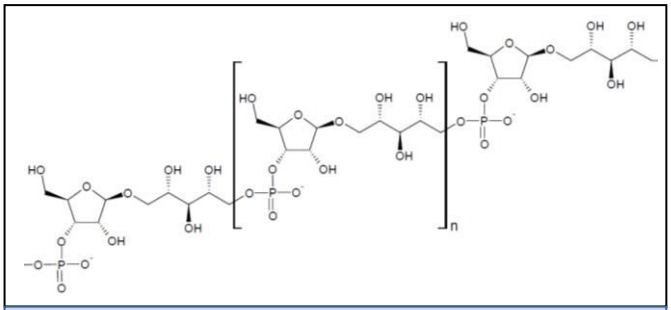

Figure 2: Chemical structure of the poly-ribosyl-ribitol-phosphate repetitive unit.

The Figure 3 illustrates the profiles of recovery of PRP, relative purity for protein and nucleic acids evaluated in presence of different detergents and its concentrations considering PRP in the [100kDa] fraction (Figure 1 – Exp1.). The polysaccharide recovery in the presence of DOC draws attention because it is less than that the control, with an increase in recovery as the concentration increases. In the presence of 0.3% of DOC, the recovery of PRP was 67%, which is in accordance with the data obtained by Albani et al [8]; increasing DOC concentration to 0.5 – 1.0% the recovery increases to 71%.All other detergents presented recovery of PRP around 84~97%, better than the control of 80%. Concerning to relative purities it is possible to observe in the figure 2 that Triton X-100 did not show improvements, in any concentrations, with respect both contaminants protein and nucleic acids, whose values are quite similar to the control. In presence of DOC the relative purity at concentrations of 0.5% to 1% for both, protein and nucleic acids, increases in the range from 3 to 15, and it was higher than the control, without detergents. Albani et al used DOC at 0.3% thus below the optimal level for the elimination of impurities. However, the use of DOC was ruled out in our study by the fact that it was from animal origin and therefore it is nonconformity with regulatory agencies criterium [24].

The relative purities for SDS varied between 23 – 30 and 4 – 15 for protein and nucleic acids respectively showing a good result in the concentration of 0.3% -1.0%. Tween 80 worked well to eliminate proteins with a relative purity of 25, but not so well for nucleic acids with RP_{AN} of 19. From all detergents evaluated CAPB in the concentration of 1% showed the best results for relative impurities of 30 for protein and more than 20 for nucleic acids. However, this surfactant cannot be used in the PRP purification process because it interferes strongly in the further process of conjugation, even in residual amounts.

The second-good results in term of elimination of impurities are the Tween 80 with ~25 for nucleic acid and 10-15 for protein respectively, and it showed PRP recovery superior to 88%. Tween 80 is known as Polysorbate 80, is a synthetic nonionic surfactant available as chemically diverse mixture of different fatty acid esters and the main component is a sorbitan. Tween 80 has both hydrophobic and hydrophilic moieties where hydrophobic moieties drive an interaction with the air-water interface resulting in formation of micelles at concentrations above the critical micelle concentration of 0.01% in aqueous solutions [25-28]. The formation of those micelles can play a critical role in the elimination of impurities, mainly in concentrations of 0.1 and 0.3% as observed in the Figure 2 and where the relative purities presented superior value than other concentrations.

Recent data indicated that polysorbate 80 is a biologically and pharmacologically active compound and as the causative agent for the anaphylactoid reaction of nonimmunologic origin in the patient [27,29]. Based on this information, SDS at

concentration of 0.5% was selected as a surfactant to be used in the next steps of this study.

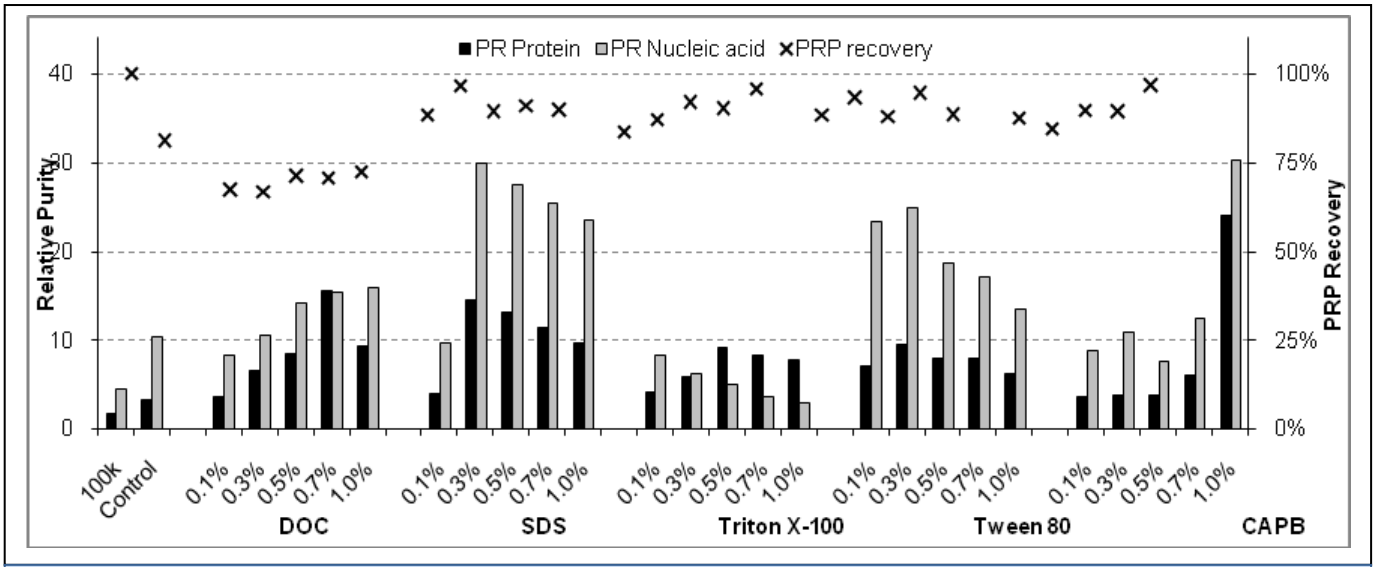

Figure 3: Influence of selected detergents used in [100 kDa] fraction on the PRP recovery and its relative purity in relation to protein ($RP_{Protein}$) or nucleic acids ($RP_{Nucleic\ Acid}$).

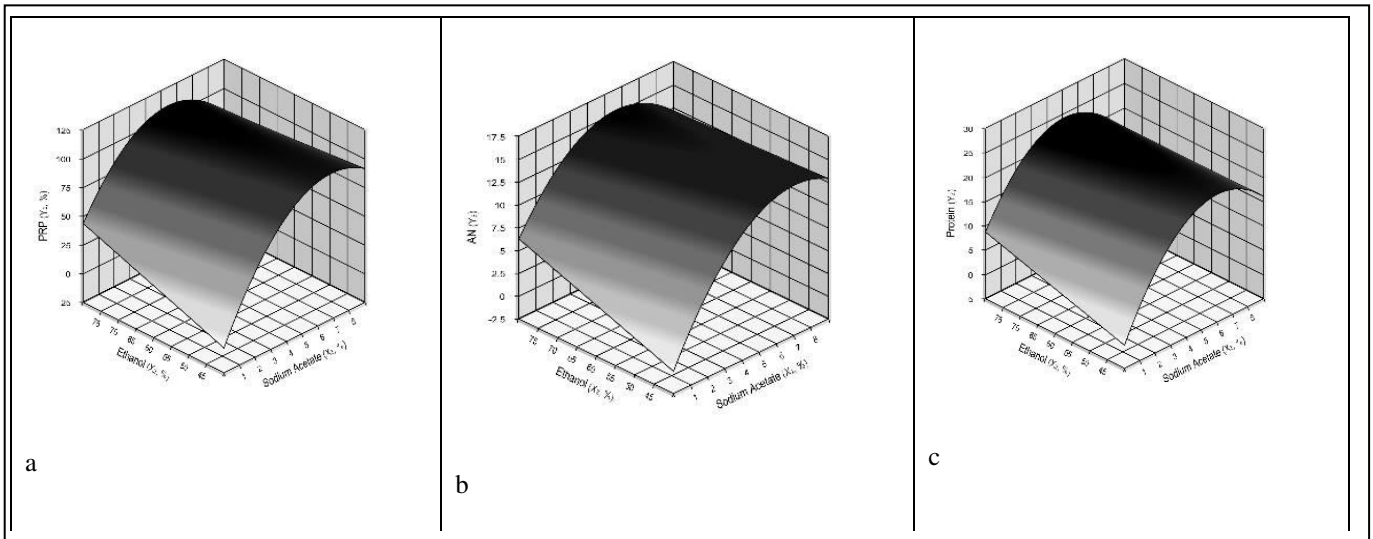

Figure 4: Three-dimensional surface response effects of the sodium acetate (%) and ethanol (v/v) on (a) PRP Recovery, (b) RP Nucleic acid and (c) RP Protein after the second precipitation step.

After first precipitation with ethanol 30% (Exp.2)

Effect of the combination between ethanol and Sodium Acetate in the second ethanol precipitation steps

Study carried out by Simas et al, [30] showed PRP precipitation is dependent on the presence of salts. Under these conditions, precipitation occurs abruptly with increasing solvent concentration. A practical application of this behavior is that the concentrations of ethanol used in the purification of PRP can be changed. This change is not commonly followed, for instance according to the state of the art, usually polysaccharide precipitation is performed with ethanol at 70% or above [6-8].

To evaluate the changing of the ethanol concentration is a fundamental issue in the downstream process. For this, a study was carried out to verify the best condition for precipitation of PRP considering both the combination of the ethanol and sodium acetate.

Figure 4 below shows the response surface to: PRP recovery, RP_{NA} and RP_{Prot} in the supernatant phase after the second ethanol precipitation, considering different concentration of ethanol and sodium acetate. According to the Figure 3 at 40% of ethanol was not enough to precipitate all polysaccharide

even adding 9% of sodium acetate. Increasing ethanol to 50%, still is necessary to add sodium acetate at 3% to precipitate 100% of polysaccharide; and from 60% of ethanol all polysaccharide is recovered without add acetate as is shown in the Figure 4a. It is also possible to verify that increasing the concentration of NaAc, reduced the concentration of ethanol needed for precipitation. To achieve the relative purity of 15 for nuclei acid only was necessary increase ethanol to 60% and NaAc to 5% (Figure 4b) and the best values for relative purity concerning to protein of 25, were with EtOH 60% containing NaAc 5% or 7% (Figure 4c).

The results from this study were proved that, PRP precipitation occurs in a narrow range of concentration of ethanol in the presence of sodium acetate. In the first step of ethanol precipitation, the most desirable condition is where PRP is soluble and the impurities precipitate. In tests performed varying the concentration of ethanol in the first step, it was found that 40% ethanol without any addition of sodium acetate was the best condition resulting in the recovery of polysaccharide (data not shown).

Considering the product`s purity data, a new condition was established for the precipitation of the PRP: 40% ethanol in the first stage and 60% ethanol plus 7% acetate sodium in the second precipitation step. It was defined 0% NaAc and 40% EtOH to be used in the first ethanol precipitation step, because the absence of NaAc generated less foam than any other condition, besides almost 100 % PRP recovery. The control condition was ethanol 30% and 5% sodium acetate in the first precipitation; ethanol 80% and sodium acetate 7% in the second precipitation [6-8].

Figure 5 shows PRP recovery in supernatant phase after the second precipitation. Efficiency of the process was compared with traditional methodology through sequential precipitation of the fermented broth under the conditions described earlier [6-8]. Four tests (A-D) were performed using different batches of culture broth. It was found that in all assays, the relative purities achieved in the new condition was superior to the traditional condition (control). The relative purity for protein was represented in dark gray color and nucleic acid in light gray; the black lines crossing the bars indicate the respective values found in the control. In all assays, PRP recovery was around 100%; RP_{prot} and RP_{NA} varied due to different culture broth, however the relative purities were higher than 100 (much higher than the control), which is in accordance with WHO [31] recommendation purity criterium for pure polysaccharide from *Haemophilus influenzae*.

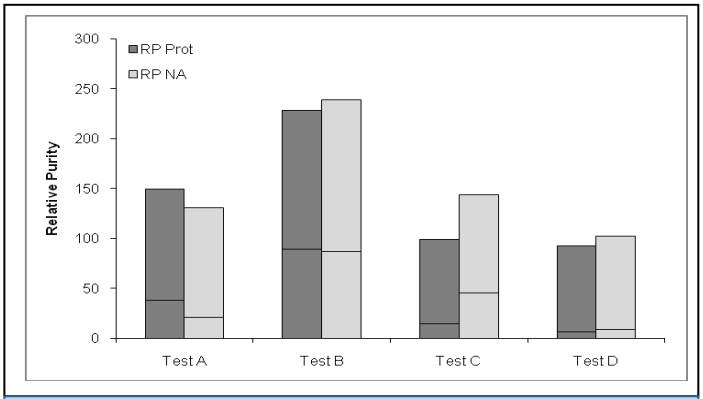

Figure 5: Efficiency of improved PRP purification condition. RRP_{Prot} (dark gray) and RP_{NA} (light gray) for different purification batches of polysaccharide. Black lines crossing the bars indicate the result achieved in control.

First and second precipitation steps with 40 and 60% ethanol, respectively. SDS 0.5% and NaAc 7% were added to the first and second precipitation steps, respectively.

CONCLUSION

Surfactants proved to have a good effect on PRP purification by helping to remove impurities without affecting polysaccharide recovery. From all the surfactants tested, only Triton X-100 did not present a favorable result in lower polysaccharide yield and relative purities values than the control. However, for pharmaceutical grade products such as PRP, safety issues for users must be taken into account. In this sense, DOC that has been used in cell lysis and in the purification of vaccine polysaccharide production, however it does not meet the requirements of regulatory agencies because it is from animal origin. As well as Tween 80, which showed promising results in the elimination of protein and nucleic acids, and do not negatively affect the polysaccharide recovery. On the other hands there are publications on polysorbate 80 being biologically and pharmacologically active, and can serve as the causative agent of the anaphylactoid reaction of non-immunological origin in the patient. CAPB at a concentration of 1% would be the perfect detergent due to excellent result in the removal of impurities, without affecting the recovery of PRP. However, previous results with the use of this surfactant proved that its use

significantly affects the process of conjugating this polysaccharide to the tetanus toxoid to generate the Hib vaccine. In this way 0.5% SDS, known as a potent protein denaturant, was chosen as the detergent to be used in this study, showing to be very effective in eliminating impurities of 13 and 27 for nucleic acids and proteins respectively.

Studies on the combination of ethanol and sodium acetate have shown that the polysaccharide precipitation occurs in a very narrow range between the concentrations of these both components. It was found that in the presence of 40% ethanol, it was not enough to precipitate the entire polysaccharide present in the supernatant, even when sodium acetate was added in its maximum concentration. Increasing the concentration of ethanol to 50%, there was still the need to add 3% acetate. However, 60% ethanol or higher, the entire polysaccharide precipitates. The improved condition performing the precipitation of the [100kDa] fraction with 40% ethanol without adding sodium acetate and in the second fractionation with 60% ethanol and 7% sodium acetate resulted in nearly 100% polysaccharide recovery with relative purities higher than 100 for both protein and nucleic acid. This improved condition will contribute intensively to the polysaccharide purification process, which until then the final polysaccharide recovery was around 20% at the end of the process, while the new condition will result in at least 80% final yield and within the purity criteria established by WHO for this polysaccharide.

ACKNOWLEDGMENTS

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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