# PURIFICATION OF UNTAGGED PNEUMOCOCCAL SURFACE PROTEIN A OBTAINED ON A DETOXIFIED EXPRESSION PLATAFORM

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## ABSTRACT

The production of biopharmaceuticals, including vaccine components, involves the cultivation of cells, in which the product is synthesized, followed by a sequence of recovery and purification steps with the objective of eliminating impurities and obtaining the product suitable for therapeutic use. The costs of purification operations range from 20 to 80% of the overall expenses of the production process of a biotechnological product, being the determining factor of its price. In the present work, the purification of an antigenic and untagged fragment of the Pneumococcal Surface Protein A (PspA4Pro) obtained from ClearColi® cultures, a strain of recombinant *Escherichia coli,* free of endotoxic activity, was investigated. The mentioned protein is a vaccine candidate which can be used to prevent diseases caused by *Streptococcus pneumoniae*. Two PspA4Pro purification processes with starting materials obtained from biomasses harvested from different culture media were carried out to investigate an efficient protocol, suitable for the purification of the untagged target protein. The experimental procedure adopted in both purifications included cell disruption, precipitation with cetyltrimethylammonium bromide (CTAB), anion exchange chromatography and cryoprecipitation. The results showed that by combining the fractionation of the central elution with cryoprecipitation, a product with high purity can be obtained.

**Keywords:** Anion exchange chromatography. Purification. Pneumococcal vaccine.

## 1 INTRODUCTION

*Streptococcus pneumoniae*, the causative agent of pathologies such as pneumonia, sinusitis and meningitis[1]. The pneumococcal cell membrane is covered by a polysaccharide capsule, which is the main virulence factor of the microorganism[2]. Immunization using pneumococcal vaccines is the most effective way to prevent diseases caused by *S. pneumoniae*. Most vaccines on the market are formulated with a combination of polysaccharides from different serotypes, whether or not conjugated to a protein, and have limited coverage, as they include only few polysaccharides from more than 100 serotypes. In order to improve immunity coverage, a new vaccine is being developed to offer serotype-independent protection using protein antigens such as PspA, a protein of the pneumococcal cell wall exposed beyond the polysaccharide capsule[3]. In the present work, PspA4Pro is the protein of interest.

The large-scale production of recombinant proteins involves the cultivation of the host microorganism that produces the protein of interest at high titers, followed by a sequence of steps to obtain the product within the specifications defined for pharmaceutical regulations. These purification processes account for up to 80% of total production costs[4]. The choice of the host and cloning strategy can contribute to increase process complexity and its cost as well. When expression platforms based on conventional *E. coli* strains are used, the purification process has to handle with the endotoxin content. Similarly, when tags are used in the cloning procedure, extra steps have to be included to remove them during the purification. Therefore, the development of simple, reliable and scalable purification processes for recombinant proteins represents an important contribution for affordable vaccine production.

In the present work, special attention is given to the anion exchange chromatography (AEC) step, because it plays the main role on separating host cell proteins (HCP) from PspA4Pro, increasing its purity. Besides, it impacts process cost too due to the high market value of the chromatographic resin. The main objective of this work was to evaluate the purification of untagged PspA4Pro obtained in ClearColi®, a strain of *Escherichia coli* free of endotoxic activity, under different cultivation conditions. Fractionation of AEC elution with 250 mmol.L$^{-1}$ was also studied[5].

## 2 MATERIAL & METHODS

The purification of PspA4Pro produced in two different cultures, using complex medium (purification 1) or defined medium (purification 2), were evaluated. Both cultures were carried out in a 5 L bioreactor with 3.5 L working volume at a temperature of 32°C, and isopropyl β-d-thiogalactopyranoside (IPTG, 0.7 mmol.L$^{-1}$) was used as inducer. The biomass was centrifuged, the pellet was frozen at -80°C and sent to the Laboratory of Vaccine Development of Instituto Butantan for storage. The biomass from purifications 1 and 2 had 175 and 152 mg PspA4Pro/g cells, respectively, as PspA4Pro yield[6].

The next step was to carry out the cell lysis using a high-pressure homogenizer. Thirty grams of frozen pellets (wet mass) of each stored biomass were resuspended in 300 mL of lysis buffer at pH 6.5, composed of sodium phosphate 10 mmol.L$^{-1}$, Triton X-100 0.1%, 1 mmol.L$^{-1}$ phenylmethylsulfonyl fluoride (PMSF) and EDTA 5 mmol.L$^{-1}$. The contents were shaken for 15 min, stored in containers and sent to the homogenizer. The pressure of operation selected was 500 bar and the materials were recirculated nine times through the equipment to obtain the cell lysate[5].

The subsequent procedure was to perform precipitation with CTAB, adding 0.1% by volume of CTAB to the cell lysates obtained from previous step. The mixtures were stirred for 1 h and, then, centrifuged at 10000 rpm for the same time. The resulting supernatants were placed in beakers and homogenized, being called the clarified cell extracts of each purification[7].

Next, the clarified cell extracts were processed by anion exchange chromatography. A Q-Sepharose resin packed into a column of 74 mL connected to an Äkta Avant 150 chromatograph was used. The operating flow rate was 13.5 mL/min. The elution protocol adopted in both chromatographies used a discontinuous gradient of NaCl with the concentrations of 100, 250 and 1000 mmol.L$^{-1}$. Each elution used 5 column volumes. The elution with 250 mmol.L$^{-1}$ NaCl was fractionated into ten identical volumes, with the aim of achieving higher purity values of the target protein. The output samples included the non-adsorbed fraction ($Q_{NADS}$) and the fractions of each elution (Q100, the ten subfractions of Q250, identified as 250_1 to 250_10, and Q1000).

The last step in the purification process was the cryoprecipitation at pH 4.0. The ten subfractions of the elution with 250 mmol.L$^{-1}$ NaCl from each purification were pooled in two different beakers, each corresponding to one purification. The contents were homogenized. The pH of each pool was set to 4.0 using acetic acid. The two suspensions were stored in bottles and placed in a freezer for a minimum of 24 h. Lastly, they were thawed, centrifugated at 10000 rpm for 1 h, and the supernatant transferred to other containers[7].

Using the collected samples of all materials, the concentration of total soluble proteins was determined by the BCA method and absorbances of each sample were read at 595 nm wavelength, using a spectrophotometer[8].

The purity of PspA4Pro was assessed by SDS-PAGE combined with densitometry. The stained gels were inserted into a Loccus DS-5000 densitometer and scanned. Their images were analyzed by the software LabImage 1D L-340[5]. Using band densitometry, the purity of PspA4Pro (%) in all fractions from the two purifications were obtained[9].

The overall purification factor ($PF_{overall}$) and the overall recovery ($R_{overall}$) of each sample from the purifications were calculated by using the Equations 1 and 2. The masses of PspA4Pro were estimated multiplying the mass of total soluble proteins by the respective purity of PspA4Pro. The cryoprecipitation step purification factor ($PF_{cryo.}$) and step recovery ($R_{cryo.}$) of the pool composed of all ten subfractions of the elution with 250 mmol.L$^{-1}$ NaCl from each purification was determined by using the Equations 3 and 4[7].

$$PF_{overall}\,(i) = \frac{PspA4Pro\ (\%)\ of\ fraction\ i}{PspA4Pro\ (\%)\ of\ clarified\ cell\ extract} \quad (1)$$

$$R_{overall}\,(i) = \frac{PspA4Pro\ (g)\ of\ fraction\ i}{PspA4Pro\ (g)\ of\ clarified\ cell\ extract}.100\% \quad (2)$$

$$PF_{cryo.} = \frac{PspA4Pro\ (\%)\ of\ pool\ after\ cryoprecipitation}{PspA4Pro\ (\%)\ of\ initial\ pool} \quad (3)$$

$$R_{cryo.} = \frac{PspA4Pro\ (g)\ of\ pool\ after\ cryoprecipitation}{PspA4Pro\ (g)\ of\ initial\ pool}.100\% \quad (4)$$

## 3 RESULTS & DISCUSSION

Table 1 presents the main results of purification 1. The highest purity values were observed in the initial subfractions of the elution with 250 mmol.L$^{-1}$ NaCl, as expected[5]. They were represented by Q250_2, Q250_3 and Q250_4, as shown in Table 1. Subfractions Q250_1 and Q250_5 to Q250_10 were not showed due to the low values of purity, purification factor and recovery, but they were included in the preparation of the pool for cryoprecipitation, along with the subfractions Q250_2, Q250_3 and Q250_4.

**Table 1** AEC results of P1. Clarified from biomass cultivated in complex medium. PspAPro yield = 175 mg PspA4Pro /g cells.

| Fraction | Purity (%) | $PF_{overall}$ | $R_{overall}$ (%) |
|---|---|---|---|
| Clarified cell extract | 58.8 | 1.00 | 100.00 |
| Q100 | 12.6 | 0.84 | 5.64 |
| Q250_2 | 91.2 | 1.55 | 10.40 |
| Q250_3 | 87.0 | 1.48 | 9.37 |
| Q250_4 | 74.7 | 1.27 | 4.00 |
| Q1000 | 55.7 | 0.95 | 3.81 |

As expected, the purity of PspA4Pro in the Q100 fraction was low (12.6%). The Q1000 fraction provided an unexpected high purity result, 55.7%, albeit the low recovery. This high purity may be related to the excess of negative charges on the protein of interest, which could cause it to adhere more firmly to the resin sites and, consequently, a higher concentration of NaCl was

necessary to elute the target molecule. The highest values of purity, overall purification factor and overall recovery were recorded in subfraction Q250_2.

Table 2 presents the main results of purification 2. As noted in the previous purification, the highest purity values were observed in the initial subfractions of the elution with 250 mmol.$L^{-1}$ NaCl. They were represented by Q250_2 and Q250_3[5], as shown in Table 2. Subfractions Q250_1 and Q250_4 to Q250_10 were not showed in the table due to the low values of purity, purification factor and recovery, but they were included in the preparation of the pool for cryoprecipitation, along with the subfractions Q250_2 and Q250_3.

**Table 2** AEC results of P2. Clarified from biomass cultivated in defined medium. PspAPro yield = 152 mg PspA4Pro/g cells

| Fraction | Purity (%) | $PF_{overall}$ | $R_{overall}$ (%) |
|---|---|---|---|
| Clarified cell extract | 25.8 | 1.00 | 100.0 |
| Q100 | 28.4 | 1.10 | 24.11 |
| Q250_2 | 67.1 | 2.60 | 13.15 |
| Q250_3 | 80.0 | 3.10 | 32.92 |
| Q1000 | 40.2 | 1.56 | 10.76 |

Comparing the results of P1 and P2 (Table 1 and 2), the purity of the clarified cell extract from purification 2 was expected to be lower than the purity of the clarified from purification 1, because of the lower PspA4Pro yield of its biomass. Subfractions Q250_2 and Q250_3 had higher purities in purification 1 than in purification 2, which can be justified by the higher purity of the starting material of the former. The overall purification factor and recovery of these subfractions were high in purification 2 only because of the low values of purity and mass of PspA4Pro of the clarified cell extract. The proposed AEC strategy applied to both clarified cell extracts presented good performance, regardless the cultivation conditions used for protein production.

Table 3 illustrates the comparison between the results of the initial pool composed by the ten Q250 subfractions of each purification, and the same pool after cryoprecipitation. P1 represents purification 1 and P2, purification 2.

**Table 3** Purity, purification factor and recovery for cryoprecipitation of P1 and P2.

| Sample | Purity (%) | $PF_{cryo.}$ | $R_{cryo.}$ (%) |
|---|---|---|---|
| Initial pool (P1) | 54.9 | - | - |
| Pool after cryoprecipitation (P1) | 88.1 | 1.60 | 100.0 |
| Initial pool (P2) | 57.8 | - | - |
| Pool after cryoprecipitation (P2) | 77.0 | 1.33 | 100.0 |

According to Table 3, the purities of the initial pool were close in both purifications. The values of purity of the pool after cryoprecipitation were in the same range. The values of step purification factor of cryoprecipitation ($PF_{cryo.}$) were similar in both purifications, making it possible to increase the purity of the pool by up to 1.6 times. Furthermore, the entire mass of PspA4Pro contained in the pool was recovered after cryoprecipitation in both purifications.

## 4 CONCLUSION

The fractionation of the elution with 250 mmol.$L^{-1}$ NaCl is a good strategy to increase the purity of the protein of interest. The combination of fractionation with cryoprecipitation of the each subfraction can lead to purity values in compliance with the minimum required by regulatory agencies, eliminating the need for additional chromatography steps.

## REFERENCES


1 SENKOVICH, O., COOK, W. J., MIRZA, S., HOLLINGHEAD, S. K., PROTASEVICH, I. I., BRILES, D. E. 2007. J. Mol. Bio. 370 (4), 701-713.

2 MADHI, S. A., PELTON, S. I. 2008. Epidemiology, diagnosis, and treatment of serious pneumococcal infections in children. *In*: Pneumococcal Vaccines. SIBER, G. R., KLUGMAN, K. P., MAKELA, P. H. (eds), 93-116.

3 BARAZZONE, G. C., CARVALHO, R., KRASCHOWETZ, S., HORTA, A. L., SARGO, C. R., SILVA, A. J., ZANGIROLAMI, T. C., GOULART, C., LEITE, L. C. C., TANIZAKI, M. M., GONÇALVES, V. M. 2011. Proc. Vaci. 4, 27-35.

4 BANKI M. R., GERNGROSS T. U., WOOD T. U. 2005. Prot. Sci. 14(6). 1387-1395.

5 BENEDINI, L. J. 2019. Modeling and optimization of anion exchange chromatography in the pneumococcal protein production process. Thesis (Doctorate in Chemical Engineering) - Center for Exact Sciences and Technology, Federal University of São Carlos, São Carlos.

6 CARDOSO, V. M. 2019. Production of recombinant pneumococcal surface protein A in Escherichia coli: economic analysis and influence of the expression platform. Thesis (Doctorate in Chemical Engineering), Federal University of São Carlos, São Carlos.

7 FIGUEIREDO, D. B. 2014. Development of the purification process for clade 4 pneumococcal surface protein A (PspA4Pro). Dissertation (Master's in Biotechnology) - Institute of Biomedical Sciences, University of São Paulo, São Paulo.

8 SMITH, P. K., KROHN, R. I., HERMANSON, G. T., MALLIA, A. K., GARTNER, F. H., PROVENZANO, M. D., FUJIMOTO, E. K., GOEKE, N. M., OLSON, B. J., KLENK, D. C. 1985. Anal. Biochem. 150(1), 76-85.

9 VILLELA, S. M. A., KRAIEM, H., BOUHAOUALA-ZAHAR, B., BIDEAUX, C., LARA, C. A. A., FILLAUDEAU, L. 2020. Microbiologyop. 9(6), 1175-1182.


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