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Expression and purification of pneumococcal surface protein a of clade 4 in *Escherichia coli* using hydroxylapatite and ion-exchange column chromatography

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ABSTRACT

Streptococcus pneumoniae is a major pathogen that causes life-threatening diseases, such as pneumonia, otitis media, bacteremia, and meningitis, worldwide and especially in young children and the elderly. Pneumococcal surface protein A (PspA) is a widely studied candidate protein vaccine that represents a promising replacement for current polysaccharide and polysaccharide-conjugate vaccines. In this study, we describe a simple method to produce PspA of clade 4 from an *Escherichia coli* expression system using hydroxylapatite and ion-exchange chromatography. Using this method, we successfully expressed soluble PspA4 in 10 L of autoinducing culture medium, with a wet-cell yield of 19 g/L and a final PspA4 concentration of 22.8 mg/L. Additionally, we improved PspA4 purity from 17% to 70% in a single step through the use of hydroxylapatite, resulting in acquisition of recombinant PspA4 (> 95% purity) at a final yield of 43% from the starting cell-lysis solution. We subsequently verified the secondary structure molecular weight of recombinant PspA4 by circular dichroism and mass spectrometry, respectively. These results demonstrated a highly efficient method for mass producing PspA4 protein and that can also be applied for purification of PspA proteins from other clades.

1. Introduction

The Gram-positive bacteria *Streptococcus pneumoniae* represent a major cause of pneumonia, otitis media, meningitis, and bacteremia in young children, the elderly, and those with weakened immune systems. Additionally, pneumonia is the leading cause of death in children < 5-years old [1,2]. As antibiotic resistance becomes more prevalent through the overuse of antibiotics, pneumococcal polysaccharide vaccines have been used to prevent and reduce rates of pneumococcal disease over the previous 30 years [3,4]. Although the efficacy and safety of polysaccharide vaccines are very good, they still harbor certain limitations, including weak immunogenicity and an inability to elicit immunologic memory. Moreover, there are > 90 different pneumococcal capsular serotypes for pneumoniae, and current polysaccharide vaccines are unable to contain all of them [5,6].

Several pneumococcal proteins have been identified as potential vaccine candidates to replace the polysaccharide vaccine [7]. One well-known candidate protein antigen is pneumococcal surface protein A (PspA), which exists in all clinical isolates and is necessary for pneumococcal virulence [8]. PspA proteins are grouped into three families and six different clades, and although there are sequence differences between families, the antibodies induced by PspA-based vaccines reveal different degrees of cross-reactivity and cross-protection [9,10]. A previous study reported that serum from humans immunized with PspA passively protected mice against challenge with various pneumococcal strains during Phase I clinical trials [11], suggesting that using one or several members of various PspA families might be sufficient for designing a broad-coverage vaccine.

Although the PspA protein elicits efficient immunogenicity and represents a promising vaccine candidate, the availability of simple and reliable methods for PspA preparation and purification is essential. Currently, *Escherichia coli* is used as a protein-expression system for many immunogens, as well as PspA, because of its simplistic operation and low cost. Separation and purification is a key phase of the downstream process of vaccine preparation [12]; therefore, a cost-effective and straightforward purification method for scaled production requires

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consideration. When designing a downstream processing strategy for a protein, the inclusion of an affinity tag might be attractive to facilitate purification [13]. According to substantial PspA protein studies, affinity approaches represent the most selective methods used for purification; however, adding an affinity tag also reportedly causes toxicity, altered protein-structure conformation, and lower yields, making it a potential disadvantage for projected application of the protein for clinical use [14,15]. Therefore, to obtain high-quality PspA products, the development of novel and effective purification methods is required. Specifically for the purpose of use in vaccines, recombinant proteins are required to be highly pure and accompanied by minimal concentrations of impurities, such as host-cell proteins, DNA, and endotoxins, at the end of the downstream purification process [16,17]. Additionally, there currently exist few reports concerning PspA protein production using non-affinity chromatography methods. In this study, we describe a process for PspA (family 2, clade 4) production and purification from *E. coli* using non-affinity chromatography. These methods allow the production of high concentrations of PspA proteins and can be applied to obtain PspA proteins of other families and clades based on their amino acid sequence homology and similar isoelectric points.

2. Materials and methods

2.1. Preparation of bacteria expressing soluble PspA4

The gene encoding PspA from clade 4 of family 2 (GenBank: U89711.1) was obtained from *S. pneumoniae* strain EF5668. The amino acid sequence included residues 32 to 450 and included the N-terminal domain and the proline-rich region. The *PspA4* gene was digested with the restriction enzymes *Nde*I and *Xho*I, followed by cloning the fragment into the pET-20 b (+) vector. The recombinant vector was expressed in *E. coli* BL21(DE3) cells following transformation. For bacterial production, 30 mL overnight culture was inoculated into a flask of 500 mL Luria-Bertani/ampicillin medium. Both cultures were grown with shaking at 200 rpm and at 37 °C. When the optical density at 600 nm (OD_{600}) was ∼2.0, the 500 mL culture was added to 10 L of complex autoinducing medium and cultured in a 15-L bioreactor (New Brunswick; Eppendorf, Hauppauge, NY, USA). Two carbon sources, 5 m $props_value{literPattern}/L glycerol and 2 g/L glucose, were used for protein production. The original lactose concentration in the medium was 2 g/L. The medium also contained 8.95 g/L Na_2HPO_4, 3.4 g/L KH_2PO_4, 0.71 g/L Na_2SO_4, 0.49 g/L $MgSO_4{\cdot}7H_2O$, 2.67 g/L NH_4Cl, 10 g/L tryptone, and 10 g/L yeast extract. Anti-foam agent (Sigma-Aldrich, St. Louis, MO, USA) was used as necessary. The temperature of the culture was set at 37 °C, and the pH was controlled at 7.0 b y addition of phosphoric acid and ammonium hydroxide. The dissolved oxygen was maintained at ≥20% by automatic agitation and addition of gas. The culture was sampled at 1-h intervals, and OD_{600} measurements were taken. After fermentation, cells were harvested by centrifugation at 8000 × *g* for 10 min and stored at −20 °C. Cells were resuspended in phosphate buffer (PB; 20 mM; pH 6.5) with 5 mM EDTA to a wet weight of 20 mL/g and disrupted using an ultrasonic cell disruptor. The disruption progress was monitored by cell density (OD_{600}) and released protein concentration (A_{280}) during the process. The mixture was then centrifuged at 16,000 × *g* for 30 min at 4 °C.

2.2. Clarification and initial purification of PspA4 using hydroxylapatite

The cell mixture (30 g; 500 mL) was used for initial rounds of purification. Hydroxylapatite (Aladdin Biological Technology Co., Ltd, Xi'an, China) (10 g) was resuspended in 500 mL of the cell mixture and allowed to incubate with stirring for 20 min. After centrifugation at 9000 × *g* for 10 min, the medium was washed with 500 mL PB (20 mM; pH 6.8) containing 1 M NaCl and 1% Triton X-100. PB (200 mL; 100 mM; pH 6.8) containing 100 mM NaCl was used to elute PspA4 protein from the hydroxylapatite-containing medium, followed by filtration of the supernatant through a 0.45-μm membrane.

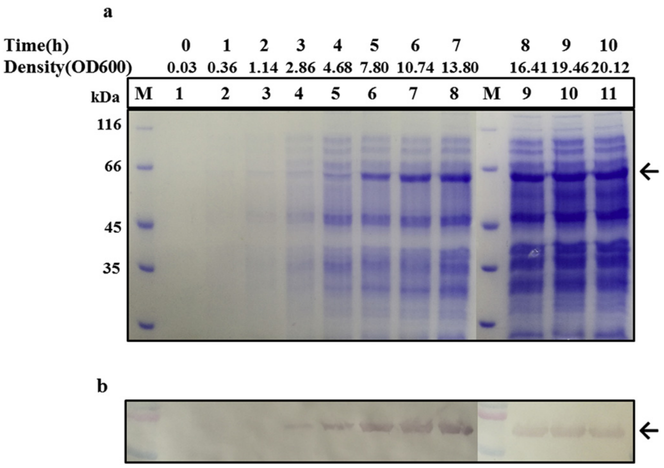

Fig. 1. Expression and identification of PspA4 culture from *E. coli* in a 15 L fermentor. (a) Analysis of PspA4 protein expression of *E. coli* BL21. Lane M, protein molecular weight marker; Lanes 1–11, protein expression at different time points from 0 h to 10 h. The target protein PspA4 was labeled by arrows. (b) Western Blot of PspA4 cell culture at different time points among the fermentation. Detection was performed by using mouse anti-PspA4 monoclonal antibody 3G8H (Prepared by our lab) and alkaline phosphatase conjugated anti-mouse IgG.

2.3. Purification and chromatographic processing

Anion-exchange chromatography (AEC) and cation-exchange chromatography (CEC) were performed for further purification using an AKTA Explorer 10 system (GE Healthcare, Little Chalfont, UK). For AEC and CEC, 25 mL of anion-exchange HiTrap Capto DEAE beads or cation exchange SP HP beads (GE Healthcare), respectively, were packed into XK16 columns (GE Healthcare). For all chromatographic processes, sample flow rate was 60 cm/h, and the elution flow rate was 144 cm/h.

DEAE column was equilibrated with five column volumes (CVs) of 20 mM PB (pH 6.5), which was also used for the washing and elution steps. After protein adsorption, the column was washed with PB containing 300 mM NaCl to eliminate contaminants. The target protein was eluted with PB containing 500 mM NaCl, and fractions were collected (Fig. 2a). For CEC, the SP column was equilibrated with 5 CVs of 20 mM sodium acetate buffer (pH 5.0), which was also used for the washing and elution steps. The washing step was performed with buffer containing 200 mM NaCl, and the elution step was performed with buffer containing 300 mM NaCl (Fig. 2b). The columns were regenerated by cleaning in place with 2 M NaCl and 500 mM NaOH.

In order to get better purification results, a study of the sequence of chromatography steps was performed by changing AEC and CEC sequence. Buffer changing was performed by a desalting column (GE Healthcare) between ion-exchange chromatography steps.

2.4. Protein purity and contamination analyses

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine relative purity by 12% electrophoresis, followed by analysis on a near-infrared laser-imaging system (Odyssey; LI-COR Biosciences, Lincoln, NE, USA). Purity was calculated as the percentage of the target band relative to the whole band, and protein concentration was determined by bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA, USA). The final purified PspA4 protein (100 μg) was applied to the pre-equilibrated TSKgel G2000SWxl column (TOSOH, Japan) to determine the purity. The mobile phase was 20 mM PB buffer containing 100 mM NaCl (pH 7.0) at a speed of 0.4 mL/min.