DOI 10.1007/s10517-023-05785-3


# IMMUNOLOGY AND MICROBIOLOGY

# Preparation a Recombinant Form of Pneumolysin Protein from *Streptococcus pneumoniae*

**D. S. Vorobyev, A. V. Sidorov, A. A. Kaloshin, N. A. Mikhailova, A. V. Poddubikov, and I. M. Gruber**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 174, No. 12, pp. 723-727, December, 2022
Original article submitted October 19, 2022

A recombinant form of pneumolysin from *Streptococcus pneumoniae* was obtained. By using Vector NTI Advance 11.0 bioinformatic analysis software, specific primers were designed in order to amplify the genome fragment of strain No. 3358 *S. pneumoniae* serotype 19F containing the nucleotide sequence encoding the full-length pneumolysin protein. A PCR product with a molecular weight corresponding to the nucleotide sequence of the *S. pneumoniae* genome fragment encoding the full-length pneumolysin was obtained. An expression system for recombinant pneumolysin in *E. coli* was constructed. Sequencing confirmed the identity of the inserted nucleotide sequence encoding the full-length recombinant pneumolysin synthesized in *E. coli* M15 strain. Purification of the recombinant protein was performed by affinity chromatography using Ni-Sepharose in 8 M urea buffer solution. Confirmation of the recombinant protein was performed by immunoblotting with monoclonal antibodies to pneumolysin.

**Key Words:** *Streptococcus pneumoniae; nucleotide sequence; sequencing; affinity chromatography; recombinant pneumolysin*

Pneumococcal infections remain relevant for young children and the elderly for several decades [1,2]. On the one hand, the infections caused by *Streptococcus pneumoniae* are vaccine-controlled, on the other hand, the use of preventive drugs has led to a significant increase in pneumococcal diseases caused by non-vaccine *S. pneumoniae* serotypes [3,4]. Vaccines based on pneumococcal proteins are now actively developed, some studies are at the stage of clinical trials [5]. Pneumolysin, one of the main protein virulence factors of *S. pneumoniae*, is responsible for the transmission of the pathogen and destruction of cell–cell contacts [6]. Previously, we proved the protective activity of native protein-containing pneumococcal antigens obtained from different strains of *S. pneumoniae*, among which pneumolysin was identified by using commercial monoclonal antibodies [7]. However, the role of individual proteins in these preparations cannot be determined because purified native proteins should be obtained for this purpose, which is a very laborious process. Instead, recombinant *S. pneumoniae* proteins can be obtained, in particular, recombinant pneumolysin, a conserved protein of pneumococcus [6] (despite the large number of pathogen serotypes) that demonstrates cross-reactivity. This feature of pneumolysin attracts the attention of researchers for the development of a pneumococcal protein vaccine with serotype-independent protective activity.

The aim of this work was to obtain a recombinant form of the *S. pneumoniae* pneumolysin protein.

I. I. Mechnikov Research Institute of Vaccines and Sera, Moscow, Russia. ***Address for correspondence:*** vorobievdenis@yandex.ru. D. S. Vorobyev

## MATERIALS AND METHODS

The study was carried out using strains from the collection of the Common Use Center of I. I. Mechnikov Research Institute for Vaccines and Sera with the support of the project by the Ministry of Science and Higher Education of the Russian Federation (Agreement No. 075-15-2021-676, July 28, 2021). A genomic DNA sample was obtained from *S. pneumoniae* 19F strain No. 3358. The precipitated bacterial biomass of *S. pneumoniae* was dissolved in 200 μl of TE buffer (pH 8.0). After that, 10 μl of proteinase K (10 mg/ml) and 200 μl of lysing solution were added to the tube. The mixture was incubated at 65°C for 15 min and periodic agitation. Then, 200 μl of the precipitating solution was added and the mixture was transferred to a K-sorb-100 column (Synthol) and genomic DNA was isolated according to the manufacturer's protocol. The resulting DNA solutions were stored at -20°C.

To obtain the pneumolysin gene, the sequence of the reference strain Spain6B *S. pneumoniae* was selected from the available database of nucleotide sequences [8]. Using Vector NTI Advance 11.0 software for bioinformatic analysis, primers for PCR were designed to further clone the genome fragment of the selected *S. pneumoniae* strain containing the nucleotide sequence encoding the full-length pneumolysin protein.

The following primers were used to amplify the pneumolysin gene: F: 5'-GGATCACTTAGTCCAACCAC-3'; R: 5'-GCAAACATTCTTCTCTCCTA-3' flanking the nucleotide sequence encoding the full-length pneumolysin protein. PCR was carried out in a Biometra amplifier using the amplification program: 98°C, 1 min; 30 cycles: 15 sec at 98°C, 20 sec at 55-65°C, and 50 sec at 72°C; then, 5 min at 72°C. The concentration of nucleic acids was measured on a Nanodrop 2000 spectrophotometer using the manufacturer's software. PCR results were assessed visually by agarose gel electrophoresis in the presence of EtBr. Reagents from Fermentas were used for PCR.

The following vectors were used: pAL2-T (Eurogen) for cloning the PCR product and pQE-30 (Qiagen) for protein expression in a bacterial system. For re-cloning from the pAL2-T vector into the pQE-30 vector, a forward primer containing the BamHI restriction site at the 5'-end was used, while the reverse primer contained the XmaI restriction site. Restriction was carried out with enzymes and buffers from Fermentas.

*E. coli* CC001 cells (genotype XL-Blue, Eurogen) were used for transformation of the resulting construct into the pAL2-T vector, and *E. coli* M15 cells were used for the pQE-30 vector.

The resulting recombinant expression vector was transformed into competent *E. coli* cells (M15/pRep4, Qiagen) that were stored at -70°C. The tube with competent cells was thawed on ice. The cell suspension (100 μl) was incubated with 3 μl of the ligation mixture on ice for 30 min. After that, the tube with cells was heated in a water bath for 45 sec at 42°C and placed back on ice for 5 min for cooling. Then, 200 μl of a commercial synthetic medium (C-medium, Fermentas) was added to the cell solution, and the biomass was incubated in an Ecotron shaker-incubator (Infors) at 200 rpm and 37°C for 1 h. The culture was plated on dished with LB medium containing two antibiotics (75 μg/ml ampicillin and 25 μg/ml kanamycin) and grown in a thermostat at 37°C for 16-18 h.

Plasmid DNA from the colonies grown on LB medium after transformation was isolated using a commercial kit (Eurogen) in accordance with the manufacturer's recommendations. The identity of the inserted sequences was verified by sequencing.

Colonies obtained as a result of transformation *E. coli* strains with previously selected and confirmed by sequencing plasmids were seeded with a bacteriological loop in 5 ml of liquid commercial LB medium with antibiotics, grown overnight in a shaker-incubator at 200 rpm and 37°C. Then, 100 μl culture was added to 5 ml fresh liquid medium with antibiotics and incubated under the same conditions until optical density of the medium reached 0.6. To induce expression of the recombinant pneumolysin protein, 1 M isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and culturing was continued for 1-4 h. After culturing for 1, 2, 3, and 4 h, the resulting biomass was precipitated, and protein products were analyzed by electrophoresis in PAGE according to Laemmli to detect recombinant protein with a certain molecular weight. The protein concentration was measured on a Nanodrop 2000 spectrophotometer.

Immunoblotting was carried out according to the generally accepted method. Mouse monoclonal antibodies IgG1 to pneumolysin (100 μg/ml; 1:400; Santa Cruz Biotechnology) were used as primary antibodies, anti-mouse anti-IgG1 antibodies (1:10,000) were used as secondary antibodies. For detection of the antigen–antibody complex, we used diaminobenzidine with $H_2O_2$ in PBS. Protein specificity was assessed visually by membrane staining.

To obtain the recombinant pneumolysin protein in a preparative amount, purification was performed by affinity chromatography using Ni-Sepharose (GE Healthcare) in 8 M urea buffer solution [9]. The biomass containing the recombinant protein was denatured in a buffered urea solution (8 M urea, 0.1 M $NaH_2PO_4$, and 0.01 M Tris; pH 8.0) overnight at room temperature on an incubator shaker at 120 rpm. Then, the obtained lysate was centrifuged twice at 10,000 rpm/min for 30 min to remove undissolved cell components. The supernatant was transferred to

a flask, a suspension of Ni-sepharose (1 ml of suspension per 25 ml of lysate) was added, and the mixture was incubated for 2 h at room temperature on a shaker at 60 rpm. The resulting suspension was passed through the column using a Biologic LP chromatography system (Bio-Rad). The protein-bound sorbent was washed with 8 M urea buffer solution at pH 6.3, then at pH 5.9. The elution was carried out using 8 M urea buffer at pH 4.5. A preparation of purified recombinant pneumolysin protein was dissolved in 50 mM Tris-HCl buffer (pH 8.0) using stepwise dialysis against several buffers containing 6, 4, 2, 1, and 0.5 M urea, and then against 50 mM Tris-HCl buffer (pH 8.0). Dialysis was performed overnight at 4°C. The

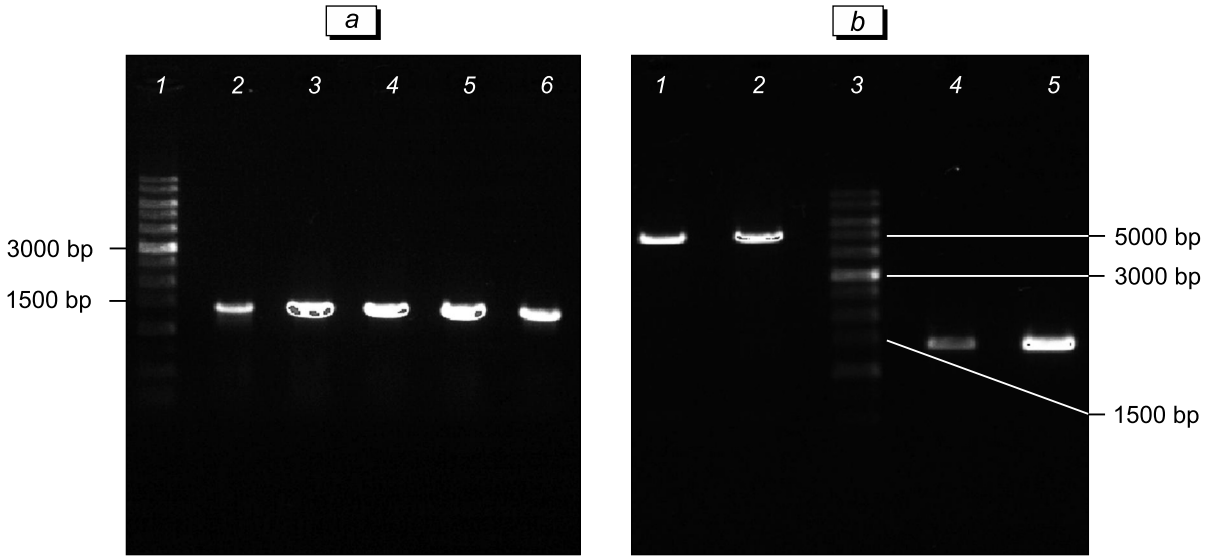


**Fig. 1.** Electrophoresis in 0.8% agarose gel with visualization of EtBr. *a*: *1*) Molecular weight marker; *2-6*) a 1500-bp PCR product. *b*: *1*, *2*) Linear form of pQE-30 plasmid with a 5000 bp insert after treatment with restriction endonuclease BamHI; *3*) molecular weight marker; *4*, *5*) a 1500-bp insert after treatment with two restriction endonucleases BamHI and XmaI.

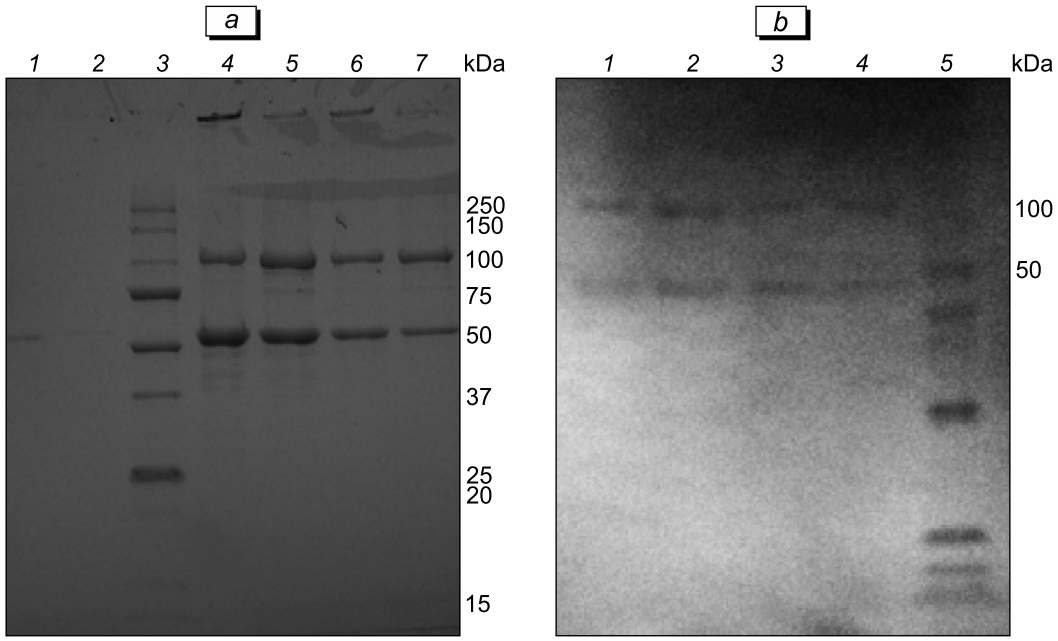


**Fig. 2.** Electropherogram and immunoblotting of protein products resulting from the expression of the recombinant pneumolysin gene inserted into the pQE-30 plasmid in *E. coli* M15 cells. *a*: *1*, *2*) Protein products of recombinant pneumolysin gene expression during *E. coli* biomass culturing without IPTG induction; *3*) molecular weight marker; *4-7*) protein products of recombinant pneumolysin gene expression during culturing of *E. coli* biomass with IPTG after 1, 2, 3, and 4 h, respectively. *b*: *1-4*) Stained protein bands formed during interaction of monoclonal antibodies to pneumolysin with recombinant protein during cultivation of the producer strain after 1, 2, 3 and 4 h, respectively; *5*) molecular weight marker.

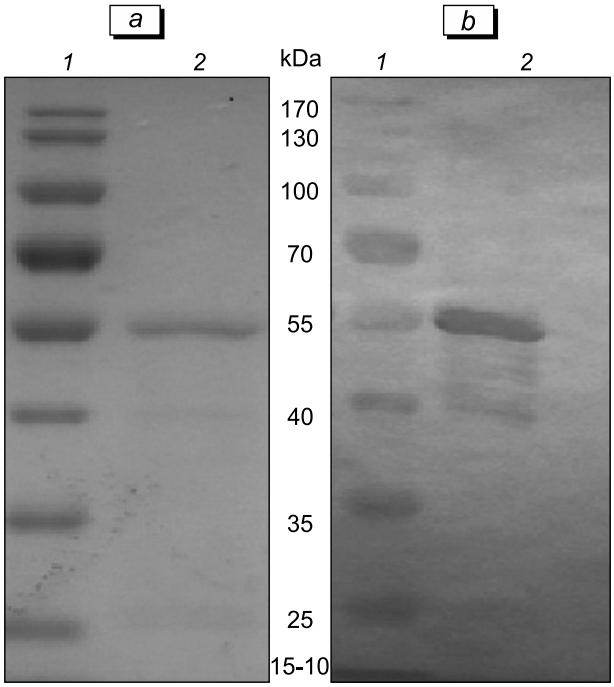


**Fig. 3.** Electropherogram and immunoblotting of the purified protein resulting from the expression of the recombinant pneumolysin gene inserted into the pQE-30 plasmid in *E. coli* M15 cells. *1*) Marker of molecular weights of proteins; *2*) protein band corresponding to the molecular weight of recombinant pneumolysin (*a*), stained protein band formed by the interaction of monoclonal antibodies to pneumolysin with recombinant protein (*b*).

protein content was determined spectrophotometrically (Genesys 6, Thermo Scientific) at 280 nm. When calculating the concentration of the recombinant protein, we used extinction coefficient of 0.7 determined using the Vector NTI Advance 11.0 software.

Bioinformatic calculations were performed using Vector NTI Advance 11.0 software.

## RESULTS

The fragment of the *S. pneumoniae* genome encoding the full-length pneumolysin protein was amplified by PCR. As a result, specific 1500-bp products were obtained (Fig. 1, *a*) corresponding to the nucleotide sequence of the *S. pneumoniae* genome fragment encoding full-length pneumolysin protein. The DNA concentration varied from 50 to 100 ng/μl. The PCR product was first cloned into the non-expressing vector pAL2-T, and after confirmation by sequencing, it was cloned into the expression vector pQE-30. For this purpose, PCR was additionally performed to add restriction sites (the forward primer containing the 5'-terminal BamHI restriction site, the reverse primer containing the XmaI restriction site), at which the resulting PCR fragment was inserted into the pQE-30 plasmid carrying regulatory regions for expression in *E. coli* cells (strain M15). Correct insertion was confirmed by sequencing. After transformation, isolated colonies grown on solid LB medium were selected for plasmid DNA isolation. The concentration of plasmid DNA was approximately 100 ng/μl. Analysis of clones after treatment of plasmid DNA with the appropriate restriction enzymes (BamHI and XmaI) revealed the presence of DNA bands corresponding to the pQE-30 vector (3500 bp) and a ~1500-bp insert (Fig. 1, *b*).

After sequencing, we selected a clone of strain No. 3358 of *S. pneumoniae* serotype 19F containing a nucleotide sequence encoding the synthesis of the full-length pneumolysin protein. Analysis of the biomass extract obtained after the induction of recombinant pneumolysin gene expression by culturing in the presence of IPTG for 1-4 h revealed the presence of specific protein bands that were absent in the cells of the same producers grown without IPTG. The molecular weight of the synthesized recombinant protein pneumolysin determined by electrophoresis in PAGE revealed two protein bands corresponding to 53 kDa (calculated molecular weight of pneumolysin [3]) and 106 kDa (Fig. 2, *a*). The presence of a protein band with a molecular weight of 106 kDa indicates the synthesis of a pneumolysin dimer, which can affect the immunobiological properties of the protein during its further study (Fig. 2, *a*). The recombinant protein and its dimer showed high specificity during interaction with monoclonal antibodies of the IgG1 subisotype to pneumolysin (Fig. 2, *b*). This verifies the resulting recombinant protein as pneumolysin.

The recombinant pneumolysin has an additional N-terminal amino acid sequence Arg-Gly-Ser-His-His-His-His-His-His-Gly-Ser encoded in the polylinker sequence. This allowed purifying the synthesized recombinant protein by affinity chromatography on columns with Ni-Sepharose in a preparative amount (Fig. 3, *a*). The protein concentration was 500 μg/ml. The resulting recombinant pneumolysin was also confirmed by immunoblotting with monoclonal antibodies of the IgG1 subisotype to pneumolysin (Fig. 3, *b*).

Thus, a recombinant pneumolysin of *S. pneumoniae* was obtained. We have previously shown that triple immunization with recombinant pneumolysin protected mice from intraperitoneal infection with *S. pneumoniae* serotype 3 and induces the synthesis of specific antibodies [10]. In further experiments, the immunobiological properties of the isolated recombinant protein against other pneumococcal serotypes should be studied and the absence of toxicity should be proven, because pneumolysin is considered as a promising antigen for the development of a vaccine with serotype-independent protective activity.

This work was supported by the Russian Science Foundation (project No. 22-25-20221).

## REFERENCES


1. Brooks LRK, Mias GI. Streptococcus pneumoniae's virulence and host immunity: aging, diagnostics, and prevention. Front. Immunol. 2018;9:1366. doi: 10.3389/fimmu.2018.01366
2. Weiser JN, Ferreira DM, Paton JC. Streptococcus pneumoniae: transmission, colonization and invasion. Nat. Rev. Microbiol. 2018;16(6):355-367. doi: 10.1038/s41579-018-0001-8
3. Briles DE, Paton JC, Mukerji R, Swiatlo E, Crain MJ. Pneumococcal vaccines. Microbiol. Spectr. 2019;7(6). doi: 10.1128/microbiolspec.GPP3-0028-2018
4. Wagner A, Weinberger B. Vaccines to prevent infectious diseases in the older population: immunological challenges and future perspectives. Front. Immunol. 2020;11:717. doi: 10.3389/fimmu.2020.00717
5. Masomian M, Ahmad Z, Gew LT, Poh CL. Development of next generation streptococcus pneumoniae vaccines conferring broad protection. Vaccines (Basel). 2020;8(1):132. doi: 10.3390/vaccines8010132
6. Nishimoto AT, Rosch JW, Tuomanen EI. Pneumolysin: pathogenesis and therapeutic target. Front. Microbiol. 2020;11:1543. doi: 10.3389/fmicb.2020.01543
7. Vorobyev DS, Semenova IB, Volokh YuV, Romanenko EE, Baturo AP, Mikhailova NA. Properties of native protein-containing antigens of Streptococcus pneumoniae. Zh. Mikrobiol. Epidemiol. Immunol. 2019;(1):22-28. Russian. doi: 10.36233/0372-9311-2019-1-22-28
8. Eutsey RA, Powell E, Dordel J, Salter SJ, Clark TA, Korlach J, Ehrlich GD, Hiller NL. Genetic stabilization of the drug-resistant PMEN1 Pneumococcus lineage by its distinctive DpnIII restriction-modification system. mBio. 2015;6(3):e00173. doi: 10.1128/mBio.00173-15
9. Kaloshin AA, Gatypova EV, Mikhailova NA. Obtaining recombinant forms of the outer membrane protein F of pseudomonas aeruginosa and assessment of their immunogenic properties. Appl. Biochem. Microbiol. 2011;47(8):780-788. doi: 10.1134/S0003683811080060
10. Petukhova ES, Vorobyev DS, Sidorov AV, Semenova IB, Volokh YV, Leonova AY, Sidorova AV, Mikhailova NA. Immunization with recombinant pneumolysin induces the production of antibodies and protects mice in a model of systemic infection caused by Streptococcus pneumoniae. Bull. Exp. Biol. Med. 2020;168(4):485-487. 10.1007/s10517-020-04736-6