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Original Article

Designing and Expression of an Immunogenic Poly-Epitope Protein from *Streptococcus pneumoniae*

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HIGHLIGHTS

- The poly epitope PNEU protein is a vaccine candidate for *Streptococcus pneumonia.*
- *PNEU* is a stable and soluble protein.
- PNEU shows a proper folding through *in silico* 3D structure prediction.
- PNEU protein expression in prokaryotic systems was confirmed.

ABSTRACT

A well-designed vaccine against *Streptococcus pneumonia*, a respiratory pathogen, by immunoinformatics approaches can lead to an effective mucosal and local immunity in the upper respiratory tract. In this study, we chose virulence proteins from different strains of *S. pneumonia* (Pneumolysin, Neuraminidase, Zink-Metalloproteinase, and Hydrolase) and designed a new multi-epitope construct by properly linking the individual predicted T and B cell specific epitopes. Then, the polytope, named PNEU, was expressed in *Escherichia coli* as a prokaryotic system. Through computational calculations, PNEU polypeptide with 216 aa has the theoretical pI 8.04 and instability index 33.63, which show that it is a stable and soluble protein. Also, the 3D structure of PNEU was predicted by Phyre2 server with 96.0% confidence. In conclusion, PNEU protein can be considered as a stable and soluble immunogenic protein, which may be efficiently used for immunity stimulation in laboratory animals, investigated in future studies.

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Introduction

Streptococcus pneumoniae with 105 various serotypes as a respiratory pathogen, is the major cause of pneumonia, meningitis, otitis media, and sepsis, especially in young children and elders (Wadhwani et al.,2016; Daniels et al., 2017). In the field of vaccination

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against respiratory pathogens, a desirable approach is the administration of a complex of antigenic epitopes through inhalation, in order to achieve more effective cellular and humoral immunity against invasive microorganisms. *Pneumococci* with the help of its numerous virulence factors can colonize in the host's nasopharynx (Khan and Jan, 2017) and the rapid resistance rate to a variety of antibiotics (Weiser et al., 2018) among serotypes is the major problem. In this study, we chose virulence proteins from *Streptococcus pneumoniae* based on virulence immunogenic proteins (AlonsoDeVelasco et al., 1995). These immunogenic proteins include the following. First, Pneumolysin (Ply), a pro-invasive virulence factor, that is a pore-forming toxin and its cytolysis actions help the bacteria to disseminate and promote its invasion. Second, Zinc metalloprotease B (ZmpB) that is a large protein found in all pneumococcal strains, and about 400 first amino acids of its sequence have been conserved among all strains (Chiavolini et al., 2003). Third, pneumococcal autolysin (LytA) that is an amidase with hydrolytic activity, which digests the bacterial cell wall through its growth and turnover (Mellroth et al., 2012). It has a role in the release of pneumolysin and inflammatory peptidoglycan and teichoic acids from lysed bacterial cells. Moreover, Neuraminidase A (NanA) is another immunogenic protein that assists in bacterial adherence and colonization by exposing cell surface receptors to the pneumococcus (Sharapova et al., 2018).
In present research, we predicted the high score, immunogenic epitopes of mentioned proteins from several strains of *S. pneumoniae* by immunoinformatics, and the proper linkers were applied for connection of them to create a polytope protein, named PNEU. It was synthesized in pET22b expression vector and the recombinant protein was expressed in *Escherichia coli* (*E.coli*) BL21 strain.

Materials and Methods

Proteins sequences retrieval

Complete amino acid sequences of proteins retrieved from the UniProtKB database, in FASTA format with the following accession numbers: Ply, Q04IN8; ZmpB, Q9L7Q2; LytA, P06653; NanA, P62575.

B-cell and T-cell epitopes prediction

By considering haplotype "d" as a dominant allele in the BALB/c mice population, we predicted Major histocompatibility complex (MHC) class I and II restriction epitopes in the IEDB server (Immune Epitope Database and Analysis Resources) (www.iedb.org). Nine-mer long epitopes that are more frequent than 8, 10, or 11-mer peptides were selected in the algorithm.
T-cell epitopes were determined among MHC I and II binding predicted peptides with higher scores for selected BALB/c mouse MHC alleles (H-2Kd, H-2-IAd). The Kolaskar and Tongaonkar scale were used to predict B-cell epitopes in the IEDB server.

Construction, codon optimization and in-silico evaluation of the multi-epitope protein

Selected epitopes linked to each other using GGGS, KFERQ, GPGPG, HHAA, and HHAL linkers, to build the desired multi-epitope construction. S-tag oligopeptide sequence was also added to the C-terminal of the construct by the GGGS linker. To perform the best expression of the PNEU protein with better folding in 3D structure near to natural form, we need to analyze the codon optimization of it in the target host, so we used the Codon Adaptation Tool (JCAT) for reverse translation and codon optimization of the PNEU construct. The *NdeI* and *XhoI* restriction enzyme sites were added to 5' and 3' ends of the sequences. The DNA construct was then ordered to be cloned to the vector pET26b. Physicochemical parameters of the amino acids sequence was analyzed by the ProtParam server (https://web.expasy.org/protparam/). These parameters that are important in laboratory production of recombinant protein, include molecular weight, instability index, aliphatic index, theoretical pI, amino acid composition, and *in vitro* and *in vivo* half-life. In addition, polytope solubility was predicted using the SolPro server (http://scratch.proteomics.ics.uci.edu/explanation.html#SOLpro). Finally, it is important the designed protein has the nearest 3D structure to target first proteins. Phyre2 (Protein Homology/analogY Recognition Engine V2.0) server (http://www.sbg.bio.ic.ac.uk/~phyre2/) can help in this purpose.

Transformation of E. coli BL21 (DE3) strain by vectors and expression of the PNEU polytope

E. coli BL21 (DE3) used as an expression host. It was transformed with the PNEU recombinant plasmid (pET26b) containing the poly-epitope construct, and was grown overnight at 37°C in Luria Bertani agar (LB agar) medium. The transformed colonies were considered to induce the expression of the recombinant protein by 0.5M IPTG (Merck) that was added in the culture and

the culture was incubated for further 4 hours. After that, bacterial pellet was collected by centrifugation (4000 rpm, 10 min). Protein extraction was performed using ultra-sonication of cells in the protein lysis buffer (Tris 50mM, Glycerol 50%, Triton x-100 0.1%, and PMSF 1mM, and 2λ of PMSF).

Confirmation of expressed protein by SDS-PAGE and Western Blotting

Protein expression was analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). In this regard, 20µL of the sample with 10µL of loading buffer (Bromophenol blue 0.25%, Glycerol 40%) was loaded on 12% SDS-PAGE gel. Coomassie blue stain (Merck) (0.25% (w/v) Coomassie blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid) was used for gel staining. Transmission of protein bands from gel to nitrocellulose membrane was carried out by western blot apparatus (APELEX, Belgium) for one hour. Then, protein bands was visualized by alkaline phosphatase conjugated anti-S-tag antibodies (Abcam, UK) in the presence of the enzyme-substrate (NBT-BCIP) (Sigma, USA).

Results and Discussion

Current vaccines against *Streptococcus pneumoniae*

have some limitations and shortcomings; therefore, it is necessary to develop vaccines based on novel approaches though choosing appropriate epitopes to elicit more comprehensive cellular and humoral immune responses against different serotypes of this pathogen. The epitopes could be further assessed as novel immunogenic agent for the development of a peptide vaccine. Based on immunoinformatics predictions, pneumococcal poly-tope constructs have been designed in several studies, and have been evaluated in various aspects, such as secondary and tertiary structure, physicochemical properties, antigenicity and allergenicity. All of their constructs were predicted robust to elicit humoral and cellular immunities (Bahrami et al., 2020).

Protein sequences retrieval and epitopes prediction

We chose virulence proteins from *S. pneumoniae* based on their function and role during colonization and infection, by epitope mapping, considering haplotype "d" as a dominant allele in the BALB/c mice population and predicted MHC I and II restriction epitopes in the IEDB server. Table 1 shows the selected epitopes from each bacterial protein.

Table 1. Chosen high score B cell linear epitopes

Protein	Amino acid	Position	Score
Ply	PLVYISSVAY	233-242	1.164
	KQIYYTVSVDAVKNP	196-210	0.96
NanA	DYRVVVDPVKPAYSD	490-504	1.155
	YPQVKDVYVQ	688-697	1.136
	TSLRSGNFI	244-252	0.7
ZmpB	VGAMGLVVLPSAGAV	109-123	1.173
	AFGLLTVGSLLLI	85-97	1.158
	YSTASYNAL	436-444	0.4
	GNKTRASLLVPKVDY	927-941	1.31
LytA	SDHVDPYPYLAK	146-157	1.105
	LYIELLRNLA	104-113	0.5
	AETYAAVELIESHSTK	80-95	1.64

Construction of the multi-epitope polypeptide and 3D structure prediction

Based on IEDB server predictions, epitopes from the aligned consensus sequences of the proteins with higher scores, which expected to have strong binding affinity to MHC alleles, were chosen. Fig. 1 shows *S. pneumoniae* determinant epitopes fused with proper linkers randomly. Then, the prediction of the conformational structure and modeling was performed using Phyre2 server (Fig. 2).

LYIELLRNLAGGGSSDHVDPYPYLAKKFERQAETYAAVELIESHSTKGPGPGDYRVVVDPVK
PAYSDGGGSYPQVKDVYVQHHAATSLRSGNFIGPGPGVGAMGLVVLPSAGAVGGGSAFGLL
TVGSLLLIGGGSYSTASYNALGPGPGGNKTRASLLVPKVDYGGGSPLVYISSVAYHHAAKQI
YYTVSVDAVKNPGGGSKETAAAKFERQHMDS

Figure 1. The sequence of the PNEU poly-tope. The epitopes were joined to each other using underlined linkers.

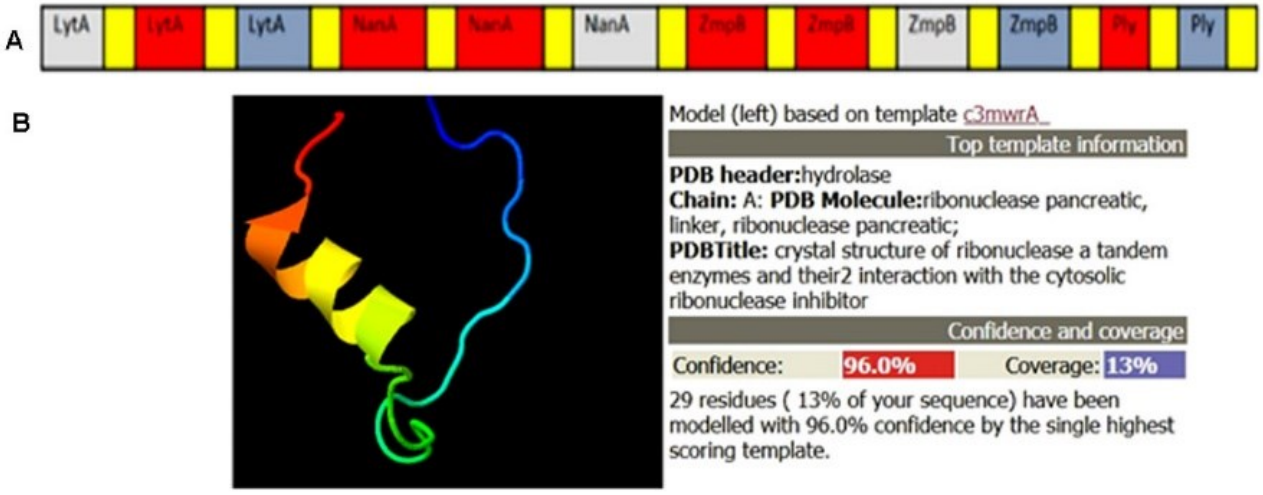

Figure 2. A) Schematic representation of the poly-epitope PNEU. The yellow segments represent the linkers. B) The 3D structure prediction of the polypeptide by Phyre2 server that showed 96.0% confidence. The single highest scoring template has been modeled for the PNEU construct.

In silico evaluation of PNEU polytope

Various pneumococcal virulence proteins were investigated in a number of studies as potential vaccine candidates through the development process of protein or peptide-based vaccines (Chiavolini et al., 2003; Genschmer et al., 2013; Cui et al., 2014; Dorosti et al., 2019). The designed polypeptide evaluation showed PNEU has properties as follows:

The physicochemical properties of the PNEU polypeptide with 216 amino acids consist of molecular weight 22169.89 Da, theoretical pI 8.04 and instability index 33.63 that shows PNEU is a stable protein during experimental production. Based on Solpro server results, the PNEU protein would be soluble upon overexpression with a probability of 0.85. Based on our *in silico* structural and physicochemical evaluations, PNEU is a proper polypeptide for vaccine development.

PNEU protein expression and confirmation by Western blotting

Reverse translation and codon optimization were performed simultaneously by the JCAT server. CAI-Value (Codon Adaptation Index) of the improved sequence for the construct was 1.0 and the GC-content of the improved sequence for the PNEU polypeptide was 53.08. Then, the construct was cloned and recombinantly expressed in *E. coli*. The size and purity of the purified protein was confirmed by SDS-PAGE (~ 20 KDa) and western blot using anti-S-tag monoclonal antibody (Fig.3).

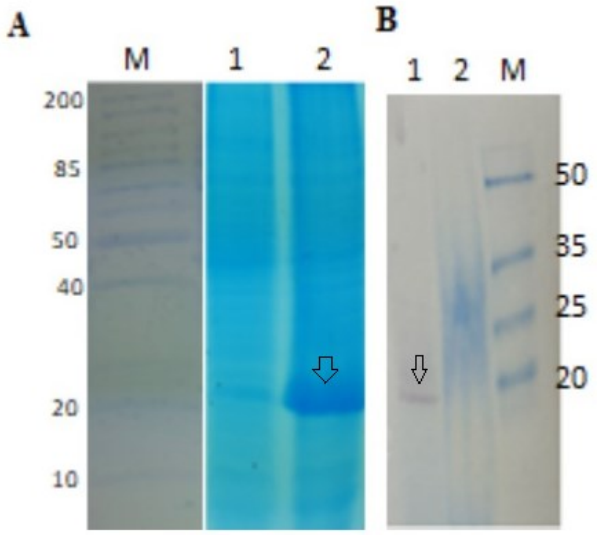

Figure 3. The PNEU recombinant protein expression analysis. (A) SDS-PAGE (12%) followed by staining with Coomassie Blue R250. M: Protein Molecular Weight Marker (KDa), Lane 1: Non-induced control, Lane 2: poly-tope protein. (B) Confirmation of PNEU expression by Western blot. M: Protein Molecular Weight Marker (KDa), Lane 1: poly-tope protein, Lane 2: Non-induced control.

Conclusion

Our study showed that the immunogenic poly epitope PNEU protein is a stable and soluble protein with acceptable 3D structure, which can be proper for immunity stimulation in laboratory animals. We evaluate the immune stimulation properties of this protein in future studies. It was showed that formulations including an antigenic protein alone (Fang

et al., 2015, Da Silva et al., 2009) or a combination of several antigens (Da Silva et al., 2010; Da Silva et al., 2008), as well as, antigen fusions and conjugations (Ritchie et al., 2018) could provoke protective immune response against *S. pneumoniae* and have had promising results in preclinical and clinical trials. Similar studies can be performed for this new designed polytope (PNEU).

Ethical Statement

This article does not contain human subjects and/or animals study.

Competing Interests

The authors declare that they have no conflict of interest.

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Authors Contributions

Mojgan Bandehpour conceived and designed the study conception. Sahar Sadeghi performed the experiments and analyzed the results. Sahar Sadeghi and Mostafa Haji Molla Hoseini prepared the draft manuscript. Mojgan Bandehpour supervised, revised and edited of the article. Bahram Kazemi scientifically consulted the project and finally approved the manuscript.

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