Reports of Biochemistry & Molecular Biology
Vol.6, No.2, Apr 2018

Original article

www.RBMB.net

Heterologous Expression, Purification, and Characterization of the HspX, Ppe44, and EsxV Proteins of *Mycobacterium tuberculosis*

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Abstract

Background: Subunit vaccines are appropriate vaccine candidates for the prevention of some infections. In this study, three immunogenic proteins of *Mycobacterium tuberculosis*, including HspX, Ppe44, and EsxV as a new construction, were expressed alone and as a fusion protein to develop a new vaccine candidate against tuberculosis infection.

Methods: To make the fusion protein, the three genes were linked together by AEAAAKEAAAKA linkers and inserted into pET21b and pET32b vectors. *Escherichia coli* (*E. coli)* Top10 cells were transformed with the plasmid, and the purified plasmid was used to transform *E. coli* BL21 cells. Protein expression was induced with IPTG. After optimizing protein expression, the recombinant proteins were purified by Ni-NTA chromatography. Protein purification was confirmed by SDS-PAGE and Western blotting with an anti-poly histidine-peroxidase monoclonal antibody against the 6His–tags at the proteins' C termini.

Results: Directional cloning was confirmed by polymerase chain reaction (PCR), restriction enzyme digestion, and sequencing. The highest expression of the tri-fusion protein and HspX were obtained by the addition of 0.2 mM of IPTG to *E. coli* BL-21 cells at 37 ˚C and 18 h of incubation. For Ppe44 and EsxV, the optimum expression conditions were 18 ˚C and 16 h of incubation. SDS-PAGE and Western blots confirmed that the desired proteins were produced.

Conclusions: The three desired proteins and the fusion protein were successfully expressed and the conditions for optimum expression determined. These recombinant proteins will be evaluated as vaccine candidates against tuberculosis. Further studies are needed to evaluate the abilities of these proteins to induce strong immunological responses.

Keywords: EsxV, Expression, HspX, *Mycobacterium tuberculosis*, Ppe44, Purification.

Introduction

Tuberculosis (TB), a major health problem worldwide, has killed millions of people in recent years. For centuries, co-evolution of *Mycobacterium tuberculosis* (*Mtb*) with the host immune response has allowed remarkable survivalof the bacterium in the human host (1, 2). Recently, in human immunodeficiency virus (HIV) –infected patients, the prevalence of multi-drug resistant TB (MDR-TB) increased up to 9% (3). In addition, after four decades, only two new drugs for treatment of MDR-TB patients have been approved (4, 5). Therefore, vaccination is the best strategy for the control of TB. Bacillus Calmette–Guérin (BCG), which is the only vaccine in use,

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Received: Oct 17, 2016; Accepted: Nov 3, 2016

covered 86% of the world's population in 2000, but has shown variable protective efficacy in different countries (6-8). Therefore, new vaccines are required to reduce the incidence of TB (9).

M. tuberculosis secretes several proteins, including Rv3619c and ESAT-6, which impair the immune response (10). Rv3619c (EsxV), an ESAT-6 family protein, and the 16 kDa heat shock protein (HspX), offer promise as vaccine candidates (11-16).

HspX is secreted during the latency growth phase in the host and is required for persistence of *Mtb* infection in macrophages. Antigenic properties of HspX that induce Th17 and human dendritic cell Th1-dependent responses make it an appropriate and efficient vaccine candidate (16, 17).

Comparative genomic studies based on whole genomic DNA microarrays have detected 16 genomic regions in *Mtb* that are deleted in BCG. *esxV*, one of the deleted regions, has been classified into RD8 and RD9 by Behr et al. (18) and Gordon et al. (19).

Despite the unknown roles of PPE (Pro-Pro-Glu) and PE (Pro-Glu) proteins in *Mtb* infection, they are responsible for 10% of the coding capacity of the *Mtb* genome. Because of the polymorphic nature and stimulation of immune responses in animals and infected humans, Ppe44 may have significant immunological importance (20-22). In the present paper, to develop a new subunit vaccine, three *Mtb* immunogenic proteins were expressed separately and as a fusion protein.

Materials and Methods

Construction of recombinant gene

After obtaining gene sequences from NCBI, the tri-fused protein was designed. Enzyme restriction sites were placed between the three genes. The enzyme cutting sites for SalI, HindIII, BamHI, and XhoI (Fermentas, Lithuania) were placed between hspX and ppe44, ppe44 and esxV, and on the 5' and 3' sides of the hspX/ppe44/esxV fusion, respectively (Fig. 1). After codon optimization using online software (http://www.jcat.de/), the 1.9 kb fragment, after deletion of stop codons, was sent to Generay Company (China) for construction. The DNA construct was cloned into the multiple cloning site (MCS) of pGH.

The construct was used to transform *Escherichia coli* (*E. coli*) Top10 cells. In brief, the Inoue method was used to prepare the *E. coli* competent cells followed by heat shock at 42 ˚C for 90 seconds. Transformed *E. coli* were selected on LB agar plates containing 100 mg/ml of ampicillin. Polymerase chain reaction (PCR), enzyme digestion, and sequencing confirmed transformed colonies. Recombinant plasmids were extracted from *E. coli* with a plasmid extraction kit (Bioneer, Korea) and double digested with enzymes to obtain *hspX*, *ppe44*, *esxV*, and the tri-fused gene according to the manufacturer's recommendations. Double-digested *hspX* and the tri-fused genes were cloned into pET21b+, whereas, *ppe44* and *esxV* were cloned into pET32b+ to form recombinant expression vectors (Novagen, Madison, WI, USA). The competent *E. coli* BL21 cells (Invitrogen, Carlsbad, CA, USA) were transformed with the recombinant vectors. The fidelity of the selected colonies was verified by PCR using T7 primers, enzyme digestions, and sequencing.

Expression of recombinant proteins in E. coli strain BL21 (DE3)

Transformed cells were cultured in 5 ml of Luria Bertani (LB) broth containing 100 mg/ml ampicillin and shaken at 37 ˚C until the optical density (OD) reached 0.4-0.6 at 600 nm. Then, 2 ml of growing culture was used to inoculate 500 ml of LB broth containing 100 mg/ml ampicillin and the culture was shaken at 37 ˚C until the cell density at OD 600 nm reached 0.4-0.6. Protein expression was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at concentrations of 0.2, 0.5, and 1 mM and cultures were shaken as above. To determine the optimum time and temperature, induced cells were incubated at 18, 28, or 37 ˚C for 4, 8, 16 or 24 h. The cells were then pelleted and resuspended in lysis buffer containing 50 mM Tris–HCl pH 6.8, 15 mM imidazole, 100 mM NaCl, 10% glycerol, and 0.5% Triton X-100. Cells were disrupted by three cycles of sonication for 2 min each with 2 min intervals between cycles. The sonicated cells were pelleted by centrifugation at 14,000 x g for 15 min at 4 ˚C. Finally, sample aliquots from both supernatants (periplasmic space) and pellets (inclusion bodies) were evaluated by SDS-PAGE.

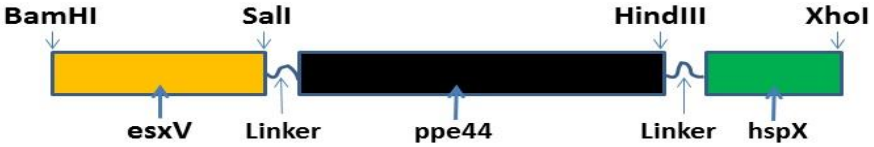

Fig. 1. Schematic illustration of synthetic construct. The AEAAAKEAAAKA linker and different enzyme cutting sites were placed between the selected genes.

SDS-PAGE and Western blotting

Proteins were electrophoresed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (23). Ten ml samples of sonicated cell pellets and supernatants were suspended in sample buffer, heated at 95 ˚C for 7 min, and electrophoresed. Protein bands were stained with Coomassie Brilliant Blue R250 and band sizes were determined using a protein ladder (Thermo Scientific).

For Western blotting, proteins separated by SDS-PAGE were transferred to PVDF membranes (Amersham) and immunoblotted with anti-poly-histidine peroxidase monoclonal antibody (Sigma-Aldrich) according to the manufacturer's recommendations. Protein bands were visualized with a Western Blot Chemiluminescent kit (Parstous Biotechnology, Iran).

Purification of recombinant fusion proteins from E. coli lysates

For the tri-fusion protein, 3 or 6 M guanidine hydrochloride were used to dissolve the inclusion bodies from the insoluble lysate phase. Proteins were purified by immobilized metal affinity chromatography using Ni-NTA (Qiagen, USA) columns. The Ni-NTA columns were equilibrated with binding buffer containing 50 mM potassium phosphate pH 7.8, 150 mM NaCl, 10% glycerol, and 0.5% Triton X-100. Ten ml of denaturing lysisbuffer containing 3 or 6 M guanidine HCl (for the tri-fusion protein), 50 mM potassium phosphate buffer, 150 mM NaCl, and 10% glycerol were used to resuspend the cell pellets and supernatants. After sonication, the filtered supernatants of the soluble lysates and insoluble phases were chromatographed on the Ni-NTA column and washed with 10 column volumes of washing buffer containing 30 and then 50 mM imidazole. The proteins were then eluted with 500 mM imidazole in binding buffer. The eluted proteins were then dialyzed for 24 h in 50 mM phosphate buffer to remove guanidine and imidazole. The purities of the recombinant proteins were assessed by SDS-PAGE and Western blotting. Protein concentrations were determined with a Bicinchoninic Acid Protein Assay Kit (Parstous Biotechnology, Iran).

Results

The tri-fusion, *hspX*, *ppe44*, and *esxV* genes were inserted into the MCS downstream of the pET-21b and pET-32b+ T7 promoters. Cloning was verified by colony PCR using T7 promoter and terminator primers for pET-21b+ and thioredoxin (S) tag and T7 terminator primers for pET32b. Moreover, restriction enzymes analyses and sequencing confirmed correct cloning.

Gene expression

For HspX and the tri-fusion protein, optimum expression was obtained with 0.2 mM IPTG at 37 ˚C for 18 h, but for EsxV and Ppe44, optimum expression was obtained with 0.2 mM IPTG at 18 ˚C for 16 h.

SDS-PAGE and Western blotting

The tri-fusion, Ppe44, EsxV, and HspX proteins were detected as 70, 65, 40, and 16 kDa bands on the SDS-PAGE and Western blots (Figs. 2 and 3). Protein yields were increased after optimization and were 8, 13, 15, and 4.5 mg/mL for tri-fusion, Ppe44, EsxV, and HspX, respectively.

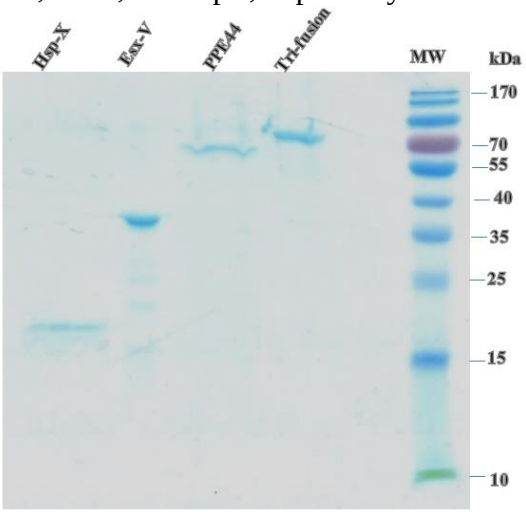

Fig. 2. Purified proteins were electrophoresed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R250. Tri-fusion, Ppe44, EsxV, and HspX were detected as 70, 65, 40, and 16 kDa bands.

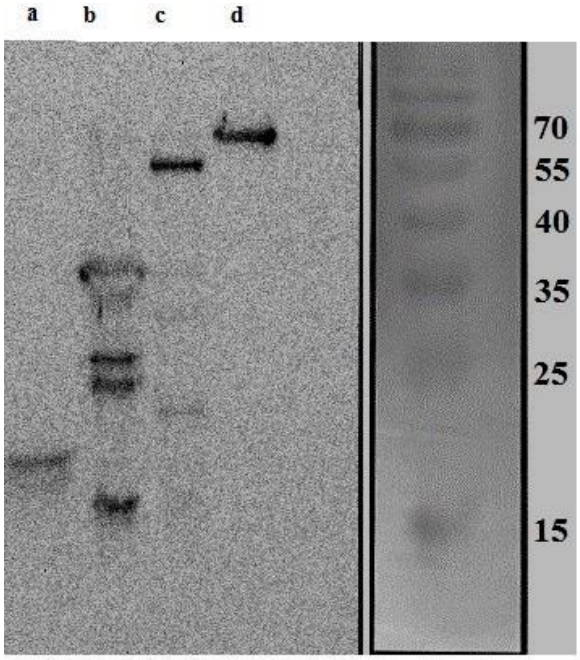

Fig. 3. Western blot of purified proteins using an anti-poly histidine-peroxidase monoclonal antibody. *Lane* a: HspX, *lane* b: EsxV, *lane c*: Ppe44 and *lane* d: tri-fusion

Discussion

This study was conducted to clone, express, and purify three *Mtb* proteins alone and fused, to design a new multi-stage vaccine against *Mtb* infection. Previous studies showed that multi-stage vaccines, which combine antigens from dormant and proliferation stages, induce effective responses to eradicate *Mtb* in all infectious stages (24, 25).

HspX activates peripheral blood mononuclear cells (PBMCs) and induces INF-γ production (26). Gamma interferon immune responses to HspX were significantly greater in TB patients than in BCG-vaccinated individuals (26). Moreover, previous studies reported that HspX alone or fused with other proteins induced strong immunogenic responses against TB in animals (24, 27, 28).

Romano et al. reported that Ppe44 is a promising TB vaccine candidate. TB patients developed no immune responses to Ppe44; however, a strong immune response was observed in PPD-positive and vaccinated individuals. Ppe44 may be associated with immunity against *Mtb* infection (21, 29).

Several studies demonstrated that EsxV, by increasing Th1 immune responses, could be a potential TB vaccine candidate. Knudsen et al. demonstrated that Esx dimer substrates, especially EsxV-EsxW, EsxD-EsxC, and EsxG-EsxH, are protective (14, 30).

Immunogenicity and flexibility are important factors to consider when selecting protein linkers (31). Our multi-stage vaccine proteins were linked via an alpha-helical linker. Previous research has shown that the AEAAAKEAAAKA linker provides flexibility and appropriate spacing for protein separation (32-34).

To improve the poor expression and yield of the EsxV and Ppe44 proteins in *E. coli*, expression vector selection is critical. Hanif et al. used the pGES-TH-1 vector for EsxV expression in *E. coli* (14). First, the pET-21b+ vector was selected, but EsxV and Ppe44 were not expressed. Because the S tag enhances protein solubility and expression, it was used for EsxV and Ppe44 expression (35). The use of pET32b for EsxV and Ppe44 expression provided high protein expression with the S tag and efficient purification on a Ni-NTA column. The tri-fusion protein and HspX were expressed in pET-21b and purified utilizing the 6His affinity tag. In both pET-21b and pET-32b, protein expression was induced with IPTG. *E. coli* was selected as the expression host due to its fast growth, economy, the large number of genetic tools for protein expression, and the ability to increase expression to up to 50% of total cellular protein (36). Moreover, *E. coli* BL21 (DE3) cells are a suitable host for T7 vectors because they have lac permease and B-galactosidase, which is induced by IPTG.

Multiple parameters, including the promoter, protein solubility, and growth temperature can affect protein production (37). Despite the use of different IPTG concentrations, the optimum concentration for all protein expression was 0.2 mM. Incubation time, however, did affect expression; the optimum incubation time was 16 h and increasing the incubation time to 24 h had little effect.

The relatively high rate of protein expression in *E. coli* can result in misfolding, especially for heterologous proteins that require molecular chaperones or additional time to fold correctly (36). Cysteine and proline fractions, turn forming residues, charge average, and hydrophobicity, as physicochemical properties, contribute to the formation inclusion bodies (38). Several strategies, such as co-expressing molecular chaperones, decrease in protein expression rate, and growth in lower temperatures have been applied to improve protein folding. Temperature

may be the most important parameter; the optimum temperature for EsxV and Ppe44 was 18 ℃, while lower temperature had no effect on the tri-fusion protein inclusion body formation.

In this study, three immunogenic proteins of *Mtb* were expressed singly and in fusion form. Further studies are required to evaluate the immunological features of these proteins as vaccine candidates against *Mtb* infection.

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Acknowledgement

This study was financially supported by the Research Council of Mashhad University of Medical Sciences, Mashhad, Iran.

The current study was from a PhD thesis presented to the Mashhad University of Medical Sciences, Mashhad, Iran. This study was supported by Mashhad University of Medical Sciences, Mashhad, Iran (Grant No. 930461).

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