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# 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. centuries, co-evolution 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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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 trifused protein was designed. Enzyme restriction sites were placed between the three genes. The enzyme cutting sites for Sall, 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-1thiogalactopyranoside (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-polyhistidine peroxidase monoclonal antibody (Sigmaaccording the manufacturer's Aldrich) to 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 immobilized metal purified by 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.

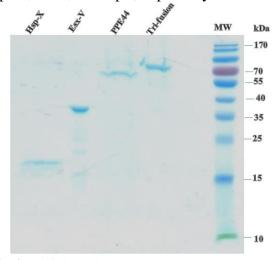
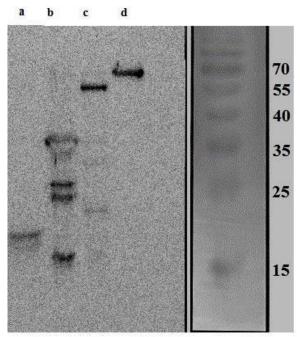


Fig. 2. Purified proteins were electrophoresed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R250. Trifusion, Ppe44, EsxV, and HspX were detected as 70, 65, 40, and 16 kDa bands.



**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, hydrophobicity, charge average, and physicochemical properties, contribute to the formation inclusion bodies (38). Several strategies, such as coexpressing 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 °C, 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 Mth infection.

## References

- 1. Kochi A. Tuberculosis: distribution, risk factors, mortality. Immunobiology. 1994;191(4):325-336.
- 2. Ravn P, Demissie A, Eguale T, Wondwosson H, Lein D, Amoudy HA, et al. I. Human T cell responses to the ESAT-6 antigen from Mycobacterium tuberculosis. Journal of Infectious Diseases. 1999; 179(3):637-645.
- 3. Skeiky YAW, Sadoff JC. Advances in tuberculosis vaccine strategies. Nature Reviews Microbiology. 2006; 4(6):469-476.
- 4. Wang L, Shi C, Fan X, Xue Y, Bai Y, Xu Z. Expression and immunogenicity of recombinant Mycobacterium bovis Bacillus Calmette-Guérin antigen ESAT-6 strains secreting the Mycobacterium tuberculosis in mice. CHINESE JOURNAL-BEIJING-ENGLISH **MEDICAL** EDITION. 2007; 120(14):1220.
- 5. Young DB, Stewart GR. Tuberculosis vaccines. British medical bulletin. 2002; 62(1):73-86.
- 6. Andersen P. TB vaccines: progress and problems. Trends in immunology. 2001; 22(3):160-168.
- 7. Babiuk LA. Broadening the approaches to developing more effective vaccines. Vaccine. 1999; 17(13):1587-1595.
- 8. Brown F. Peptide vaccines: fantasy reality?World Journal of Microbiology and Biotechnology. 1992; 8:52-53.
- 9. Levine MM, Sztein MB. Vaccine development strategies for improving immunization: the role of modern immunology. Nature immunology. 2004; 5(5):460-464.
- 10. Siddiqui KF, Amir M, Agrewala JN. Understanding the biology of 16 kDa antigen of Mycobacterium tuberculosis: scope in diagnosis, vaccine design and therapy. Critical reviews in microbiology. 2011; 37(4):349-357.

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- 11. He X-Y, Zhuang Y-H, Zhang X-G, Li G-L. Comparative proteome analysis of culture supernatant proteins of Mycobacterium tuberculosis H37Rv and H37Ra. Microbes and infection. 2003; 5(10):851-856.
- 12. Mattow J, Schaible UE, Schmidt F, Hagens K, Siejak F, Brestrich G, et al. Comparative proteome analysis of culture supernatant proteins from virulent Mycobacterium tuberculosis H37Rv and attenuated M. bovis BCG Copenhagen. Electrophoresis. 2003; 24 (19-20):3405-3420.
- 13. Målen H, Berven FS, Fladmark KE, Wiker HG. Comprehensive analysis of exported proteins from Mycobacterium tuberculosis H37Rv. Proteomics. 2007; 7(10):1702-1718.
- 14. Hanif S, Al-Attiyah R, Mustafa A. Molecular Cloning, Expression, Purification and Immunological Characterization of Three Low-Molecular Weight Proteins Encoded by Genes in Genomic Regions of of Mycobacterium Tuberculosis. Difference Scandinavian journal of immunolog. 2010; 71(5):353-361.
- 15. Ansari MA, Zubair S, Mahmood A, Gupta P, Khan AA, Gupta UD, et al. RD antigen based nanovaccine imparts long term protection by inducing memory response against experimental murine tuberculosis. PLoS One. 2011; 6(8):e22889.
- 16. Marongiu L, Donini M, Toffali L, Zenaro E, Dusi S. ESAT-6 and HspX improve the effectiveness of BCG to induce human dendritic cells-dependent Th1 and NK cells activation. PLoS One. 2013; 8(10):e75684.
- 17. Xin Q, Niu H, Li Z, Zhang G, Hu L, Wang B, Li J, et al. Subunit vaccine consisting of multi-stage antigens has high protective efficacy against Mycobacterium tuberculosis infection in mice. PLoS One. 2013; 8(8):e72745.

- 18. Behr M, Wilson M, Gill W, Salamon H, Schoolnik G, Rane S, et al. Comparative genomics of BCG vaccines by whole-genome DNA microarray. *Science*. 1999; 284(5419):1520-1523.
- 19. Gordon SV, Brosch R, Billault A, Garnier T, Eiglmeier K, Cole ST. Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. *Molecular microbiology*. 1999; 32(3):643-655.
- 20. Bonanni D, Rindi L, Lari N, Garzelli C. Immunogenicity of mycobacterial PPE44 (Rv2770c) in Mycobacterium bovis BCG-infected mice. *Journal of medical microbiology*. 2005; 54(5):443-448.
- 21. Romano M, Rindi L, Korf H, Bonanni D, Adnet P-Y, Jurion F, Garzelli C, et al. Immunogenicity and protective efficacy of tuberculosis subunit vaccines expressing PPE44 (Rv2770c). *Vaccine*. 2008; 26(48):6053-6063.
- 22. Rindi L, Peroni I, Lari N, Bonanni D, Tortoli E, Garzelli C. Variation of the expression of Mycobacterium tuberculosis ppe44 gene among clinical isolates. *FEMS Immunology & Medical Microbiology*. 2007; 51(2):381-387.
- 23. Uk L. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227(259):680-685.
- 24. Niu H, Peng J, Bai C, Liu X, Hu L, Luo Y, et al. Multi-stage tuberculosis subunit vaccine candidate LT69 provides high protection against Mycobacterium tuberculosis infection in mice. *PloS one.* 2015; 10(6):e0130641.
- 25. Wang X, Zhang J, Liang J, Zhang Y, Teng X, Yuan X, et al. Protection against Mycobacterium tuberculosis Infection Offered by a New Multistage Subunit Vaccine Correlates with Increased Number of IFN-γ+ IL-2+ CD4+ and IFN-γ+ CD8+ T Cells. *PLoS One.* 2015; 10(3).
- 26. Geluk A, Lin MY, van Meijgaarden KE, Leyten EM, Franken KL, Ottenhoff TH, Klein MR. T-cell recognition of the HspX protein of Mycobacterium tuberculosis correlates with latent M. tuberculosis infection but not with M. bovis BCG vaccination. *Infection and immunity*. 2007; 75(6):2914-2921.
- 27. Taylor JL, Wieczorek A, Keyser AR, Grover A, Flinkstrom R, Karls RK, et al. HspX-mediated protection against tuberculosis depends on its chaperoning of a mycobacterial molecule. *Immunology and cell biology*. 2012; 90(10):945-954.

- 28. Yuan X, Teng X, Jing Y, Ma J, Tian M, Yu Q, et al. A live attenuated BCG vaccine overexpressing multistage antigens Ag85B and HspX provides superior protection against Mycobacterium tuberculosis infection. *Applied Microbiology and Biotechnology*. 2015;1-9.
- 29. Cuccu B, Freer G, Genovesi A, Garzelli C, Rindi L. Identification of a human immunodominant T-cell epitope of mycobacterium tuberculosis antigen PPE44. *BMC microbiology*. 2011; 11(1):167.
- 30. Knudsen NPH, Nørskov-Lauritsen S, Dolganov GM, Schoolnik GK, Lindenstrøm T, Andersen P, et al. Tuberculosis vaccine with high predicted population coverage and compatibility with modern diagnostics. *Proceedings of the National Academy of Sciences*. 2014; 111(3):1096-1101.
- 31. Zhao HL, Yao XQ, Xue C, Wang Y, Xiong XH, Liu ZM. Increasing the homogeneity, stability and activity of human serum albumin and interferon- $\alpha$ 2b fusion protein by linker engineering. *Protein expression and purification*. 2008; 61(1):73-77.
- 32. Arai R, Ueda H, Kitayama A, Kamiya N, Nagamune T. Design of the linkers which effectively separate domains of a bifunctional fusion protein. *Protein engineering*. 2001; 14(8):529-532.
- 33. Afshar S, Olafsen T, Wu AM, Morrison SL. Journal of Experimental & Clinical Cancer Research. *Journal of Experimental & Clinical Cancer Research*. 2009; 28:147.
- 34. Bai Y, Shen W-C. Improving the oral efficacy of recombinant granulocyte colony-stimulating factor and transferrin fusion protein by spacer optimization. *Pharmaceutical research*. 2006; 23(9):2116-2121.
- 35. E. LaVallie, E. DiBlasio, S. Kovacic, K. L. Grant, P. F. Schendel, and J. M. McCoy, "A thioredoxin gene fusion expression system that circumvents inclusion body formation in the E," *coli cytoplasm. Biotechnology.* 1993; vol. 11, pp. 187-193.
- 36. Francis DM, Page R. Strategies to optimize protein expression in E. coli. *Current protocols in protein science*. 2010;5.24. 21-25.24. 29.
- 37. Sørensen HP, Mortensen KK. Advanced genetic strategies for recombinant protein expression in Escherichia coli. *Journal of biotechnology*. 2005; 115(2):113-128.
- 38. Hannig G, Makrides SC. Strategies for optimizing heterologous protein expression in Escherichia coli. *Trends in biotechnology*. 1998; 16(2):54-60.