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Original article

Molecular Cloning, Expression and Purification of Protein TB10.4 Secreted by *Mycobacterium Tuberculosis*

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Abstract

Objective(s)
Tuberculosis (TB) is the leading cause of mortality among the infectious diseases, especially in developing countries. One of the main goals in tuberculosis research is to identify antigens which have the ability of inducing cellular and/or humoral immunity in order to use them in diagnostic reagents or vaccine design. The aim of this study was to clone and express the TB10.4 protein in *Escherichia coli* expression system.

Materials and Methods
DNA was extracted from *Mycobacterium tuberculosis* H37Rv. Gene specific primers were designed using Gene Runner software according to sanger sequence database. Gene tb10.4 fragment was amplified by PCR method and purified tb10.4 gene was cloned into pET 102/D vector. Plasmid containing pET102/D-10.4 was transformed into competence *E. coli* TOP10. A positive transformant was chosen and plasmids DNA was isolated and subsequently transformed into competence *E. coli* BL21(DE3). The bacterium was induced by IPTG and its lysates were loaded directly onto SDS-PAGE. Purified recombinant protein was achieved using metal affinity chromatography (Ni-nitrilotriacetic acid).

Results
TB10.4 molecule was successfully cloned, expressed, and purified. An approximately 26.4 kDa exogenous protein was observed on the SDS-PAGE. The recombinant protein was confirmed by DNA sequencing of correct insert.

Conclusion
The success of expressing the TB10.4 protein could serve as a basis for further studies on the usefulness of the gene and its expression product in the development of subunit vaccine and diagnostic method.

Keywords: Gene tb10.4, Molecular Cloning, *Mycobacterium tuberculosis*, Protein TB10.4

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Introduction

Tuberculosis (TB), caused by the intracellular pathogen *Mycobacterium tuberculosis*, is a major health problem in the developing world. It is a disease which is re-emerging as a major health threat in the developed countries (1). It has been estimated that one-third of the world's population is currently infected. More than 80% of the world's population is now vaccinated with the available live attenuated bacilli Calmette Guerin (BCG) vaccine and even with this effort; TB remains the second leading cause of death worldwide among all infectious diseases and is responsible for approximately 2 million deaths annually (2).

The high mortality induced by TB has been attributed to various reasons: BCG inefficacy, HIV co-infection and appearance of multiple drug-resistant strains of *M. tuberculosis*. Development of new and improved vaccines against TB is therefore an urgent need. One of the main goals in most of the studies in tuberculosis field is to identify antigens which have the ability of inducing cellular and/or humoral immunity in order to use them in diagnostic reagents or vaccine design.

Biochemical, immunological, and biomolecular characterization of *M. tuberculosis* has opened the way to discovery of many new MT antigens such as ESAT-6, Ag85A, Ag85B, TB16.3, and TB9.7 (3-5) which have been shown to be useful in the development of improved diagnostic methods and/or vaccines (6).

The immunogenicity of proteins that are actively secreted by *M. tuberculosis* during growth have been shown to generate protective immune responses in animal models (7-12). Furthermore, secreted proteins of *M. tuberculosis* induce delayed type hypersensitivity responses in guinea pigs and antibody production in TB patients (13-15). For this reason, secreted proteins of *M. tuberculosis* have attracted significant attention both in vaccine research and as potential tools for diagnostic skin test and serological diagnosis of TB.

TB10.4 is a secreted protein and belongs to the ESAT-6 family which is encoded by the Rv0288 gene located in the esx cluster 3 and appears to be essential for the virulence of *M. tuberculosis* (16). The data obtained from previous studies suggest that TB10.4 protein is a target for antimicrobial immune responses in humans (17, 18). In fact TB10.4 is strongly recognized by BCG-vaccinated individuals as well as by approximately 70% of TB patients and induces high levels of gamma interferon (INF-γ) in these individuals (17).

Considering the above information we sought to find and express the TB10.4 and thus the aim of this study was to clone and express the TB10.4 immunoreactive protein in *E. coli* expression system.

Materials and Methods

Mycobacteria

M. tuberculosis H37Rv, kindly provided by Dr Khajekaramaddini from Pasture Institute of Tehran, Iran, was cultured on Lowenstein-Jensen (LJ) medium at 37 ˚C. Extraction of genomic DNA was performed by Cinnagen DNA extraction kit (Tehran, Iran) as described by manufacture.

PCR cloning

Primers were designed, based on the DNA sequences from the Sanger sequence database. Cloning was carried out directionally. The primers were as follows: tb10.4-sense CAC CAT GTC GCA AAT CAT GTA CAA CTA CC; tb10.4-antisense, GCC TCC CCA TTT GGC GGC TT. In this system, the primers used include the 4 base pair sequences (CACC) necessary for directional cloning on the 5′ end of the forward primer (underlined). PCR was carried by using 35 cycles of amplification (95 ˚C for 30 sec, 60 ˚C for 45 sec, and 72 ˚C for 60 sec) in a Techne thermocycler by using the pfu DNA polymerase. The DNA purification column was from Fermentas. The tb10.4 blunt-end PCR product was ligated to the pET102/D-TOPO (Invitrogen) cloning vector to generate the recombinant plasmid pET102/D-tb10.4. The recombinant plasmid was transformed into One Shot® TOP10 chemically competent *E. coli* (Invitrogen) by a heat shock method as described by manufacturer. The clones were selected on LB (Luria-Bertani, MERCK, Germany) agarose plates supplemented with ampicillin (100 µg/ml). TOP10 *E. coli* cells were used for cloning and

maintenance of plasmid DNA, whereas BL21 *E. coli* cells (Invitrogen) were used for expression studies. Plasmid DNA extraction was performed using the QIAprep® Miniprep DNA purification system (Qiagen). Nucleotide sequencing was carried out by Seq lab (Germany).

Expression and purification studies

The purified plasmids were further introduced into *E. coli* BL21. The transformed *E. coli* cells were grown on LB solid medium containing ampicillin (100 µg/ml). An overnight culture was used to inoculate 200 ml LB broth medium supplemented with ampicillin. When the OD_{600} had reached 0.5 expression was induced by the addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a final concentration of 2 µl/ml for 24 hr at 37 ˚C. The induced cells were then harvested by centrifugation at 4000 g for 15 min at 4 ˚C. Pellets were suspended in lysis buffer (300 mM NaCl, 50 mM Tris-HCl, pH 7.6, 100 mM KCl, 10 % glycerol, 0.5 %Triton X) and frozen in liquid nitrogen and thawed in 37 ºC for 3 times and then centrifuged at 4000 g for 10 min. Both the supernatant and the pellet were analyzed by SDS–PAGE using a 12.5% polyacrylamide gel. The gel was stained for 4 hr by standard coomassie brilliant blue G-250 and destained with 45% methanol/10% acetic acid solution. The supernatant of the cell lysate was passed through the nickel–NTA column (Qiagen). The column was washed with 15 ml lysis buffer containing 10 mM imidazol. Then the protein passed through the column. Next the column was eluted with increasing imidazole concentration (10, 20 and 200 mM).

Purity of the recombinant protein was assessed by SDS–PAGE using a 12% polyacrylamide gel, and then dialyzed overnight against PBS at 4 °C. Quantitation of recombinant proteins was carried out using Bradford protein assay.

Results

Cloning was based on the polymerase chain reaction (PCR). We amplified tb10.4 from H37Rv genomic DNA. To reduce the possibility of errors caused by amplification, we used pfu, a high-fidelity DNA polymerase with proofreading activity. The PCR product was confirmed 291 bp DNA segment by using agarose gel electrophoresis, as expected (Figure1).

The PCR-amplified coding region of the tb10.4 gene was gel purified and inserted into the pET102/D-TOPO vector. Recombinant colonies were selected on LB agar plate which was supplemented with ampicillin. Only one white colony was picked and the tb10.4 recombinant plasmids were extracted from this clone. Plasmid sequencing was performed in order to confirm the identity of cloned gene and to verify that the DNA encoding tb10.4 is inserted in correct frame. Alignment of sequence revealed that recombinant DNA was 99% homologous with different strains of *M. tuberculosis*. The recombinant plasmids were then transformed into expression host *E. coli* BL21 (DE3). Expression of the recombinant protein was assessed in the presence of inducer IPTG. The recombinant TB10.4 was abundant in the total lysate. As expected, TB10.4 was expressed with an apparent molecular mass of 26.4 kDa on SDS–PAGE and was completely found in the soluble phase (Fiure 2).

The recombinant protein was purified by Ni-affinity chromatography from lysates of *E. coli* BL21. The flow through and wash fractions were collected before the gradient former was switched on and fraction was collected after increasing imidazole concentration (10, 20 and 200 mM). The aliquot from the fraction was run on SDS-PAGE. Pure protein containing fraction was dialysed to get rid of imidazole. The collected fractions were then run on SDS-PAGE (Figure 3).

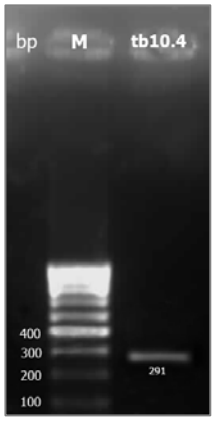

Figure 1. Agarose gel electrophoresis of PCR product showing the amplification of gene of interest (tb10.4). Lane tb10.4: band of 291 bp; Lane M:100 bp DNA marker (Fermentas).

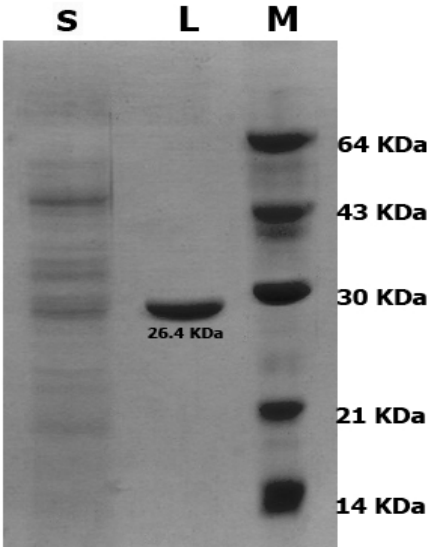

Figure 2. SDS-PAGE showing induction of the expressed TB10.4 protein in liquid phase before purification. Lane M, molecular weight markers (kDa); lane L, soluble protein fraction obtained from the *Escherichia coli* transformant pellet; lane S, insoluble protein fraction

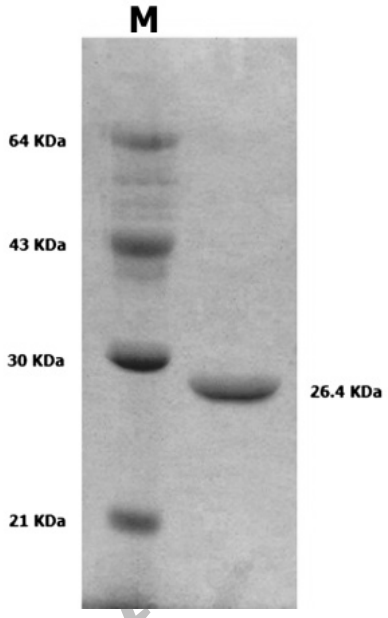

Figure 3. Coomasie blue staining of purified TB10.4 recombinant protein. Lane M, molecular weight markers (kDa); lane P, purified and dialysed TB10.4 protein (26.4 KDa)

Discussion

For many years, great efforts have been made to develop a new vaccine against tuberculosis and designing better methods for the diagnosis of the disease.

Considering these facts, the TB10.4 protein from the highly active low-mass fraction of culture filtrate has been formerly shown to induce strong Th1 responses following TB infection both in humans and animal models (17-20).TB10.4 was strongly recognized by around 70% of the TB patients as well as by BCG-vaccinated individuals (17, 18).

For the first time in 2000, TB10.4 was identified in culture filtrate from *M. tuberculosis* by Skjot *et al*. The gene was cloned in pMST24 vector but the recombinant protein was expressed in inclusion body (18). In a previous study, cloning of tb10.4 was achieved in pET28b vector. The recombinant protein was produced as an insoluble one with no protein detectable in soluble fraction (21). In mentioned studies, the plasmid and the inserted gene were cut by restriction enzymes. In the present study, pET TOPO directional technology was used for cloning of TB10.4 from H37Rv. Accordingly, the blunt end PCR product was directly inserted to the expression vector. There was no need to digest the inserted gene or plasmid with the restriction enzymes. Protein TB10.4 was expressed in *E. coli* expression system having a poly histidine purification tag and purified to near-homogeneity by nickel-affinity chromatography. We applied a simple method of releasing cytoplasmic proteins by repeated cycles of freezing and thawing which was proved to be efficient for a number of recombinant proteins (22). Another interesting point of our work in comparison with the others was that the entire TB10.4 protein was expressed in liquid phase (Figure 2).

Conclusion

The success of expressing the TB10.4 protein could serve as a basis for further studies on the usefulness of this gene and its expression product in the development of subunit vaccine and diagnostic methods. As a matter of fact, it is important to determine whether TB10.4 evokes immune responses in TB patients and BCG vaccinated healthy subjects in an endemic region. We are currently working on the process of optimizing enzyme-linked immunosorbent assay (ELISA) with TB10.4 recombinant protein for serological diagnosis of tuberculosis.

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