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Research paper

Recombinant production of *Mtb* antigens and their purification by affinity chromatography

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ABSTRACT

Effective control of TB, the second largest cause of human deaths, is based on early diagnosis, and proper treatment with the availability of an efficacious vaccine. The antigen-based detection of antibodies and formulation of subunit anti-TB vaccines require highly purified immunogenic *Mtb* antigens. In this study three immunogenic *Mtb* antigens EspC, HspX, and TB10.4 were cloned and expressed in *E. coli* through recombinant DNA techniques. SDS-PAGE was performed to check the molecular sizes of EspC (13kDa), HspX (18kDa), and TB10.4 (11kDa) proteins. After expression, all the polyhistidine-tagged (6x-His) proteins were purified through an automated AKTA purifier FPLC system using the HisTrap^{TM} FF column. This purification system separates the proteins of interest through a continuous rather than step-wise imidazole gradient. The fractions containing the required protein were pooled in each of the EspC, HspX, and TB10.4 preparations thus obtained were more than 90% purified. The percentage recoveries of HspX, EspC, and TB10.4 proteins were 12.0, 5.5, and 7.1, respectively. These preparations should be suitable for validation through immunological studies.

KEYWORDS Anti-TB vaccines, *Mtb* antigens, polyhistidine-tagged proteins.

INTRODUCTION

Tuberculosis (TB) is a highly contagious infection occur owing to the directly inhalation of droplets from active patients to healthy individuals via air current. The emergence and prevalence of MDR-TB and XDR-TB strains, control, early diagnosis and vaccine development is a dire need of the time [1].

Mtb contains a number of immunogenic antigens having good potential as serodiagnostic agents or vaccine candidates [2, 3]. It elicits liberation of IL-2, IFN-γ, and TNF-α therefore it can be exploited as promising candidate of vaccine against tuberculosis [4] with diagnostic potential [5]. TB10.4 i.e. *Rv0288* is essentially required for mycobacterial pathogenesis [6]. It causes a massive induction of IFN-γ in patients [7]. HspX (Rv2031c) is a cytosolic protein of *Mtb* with a chaperone-like activity [8]. It can induces both humoral as well as cell mediated immunity in the active and latent stages of tuberculosis [9]. Moreover, variations in the ELISA sensitivities i.e. 34-62% for the screening of specific antibody in the serum of patients [10,11]. In our previous study synergistic effect of heat shock protein (HspX) with other antigens of TB Bacilli has been explored as serodiagnostic approach [12]. Truncation of the PstS1 antigen of *Mtb* was found to improve the diagnostic efficiency of the antigen [13]. The other antigens studied for their immunological evaluation in serodiagnosis of TB include HSP, FbpC1, PstS1, and PE35 [14, 15].

Fast Protein Liquid Chromatography (FPLC) is an automated system for the separation of different types of biopolymers including proteins. It has an advantage for

the higher resolution of separation of biomolecules and more loading capacity. The automated AKTA Fast Protein Liquid Chromatography is being widely for the purification of proteins. Different types of chromatographic modes can be performed by FPLC like ion exchange, size exclusion, affinity, and reverse phase [16].

In the present study, cloning of three immunogenic *Mtb* antigens namely, EspC, HspX, and TB10.4 was carried out and then it was proceeded with recombinant expression in *E. coli* BL21 CodonPlus (DE3)-RIPL cells. Then, proteins that were 6xHis-tagged were subjected to purification through affinity HisTrap^{TM}-based AKTA FPLC system further for their application as sero-diagnostic and vaccine interventions.

MATERIALS AND METHODS

Molecular cloning of the antigens

Genomic DNA of *Mtb H37Rv* was isolated from bacterial cultures by CTAB/NaCl method [17]. Full-length ORFs encoding the antigens HspX (435bp), EspC (312bp) and TB10.4 (294bp) were PCR amplified from the genomic DNA using gene specific primers. PCR amplicons were purified and ligated into pTZ57 R/T cloning vector and sub-cloned into pET-28a(+) expression vector according to the protocols described previously [18]. Competent cells of *E. coli* DH5α were prepared by $CaCl_2$ method. The competent cells were transformed with recombinant plasmids harboring genes of interest. The positive transformants were further screened by colony PCR and restriction analysis of all the recombinant plasmids was done to confirm the genes of interest.

Recombinant expression and cell lysis

The culture of *E. coli* BL21-CodonPlus (DE3)-RIPL (Stratagene, USA) was used as model organism for expression and pET28a (+) was selected as expression vector (Novagen EMB Biosciences, Germany). *E. coli* BL21-CodonPlus (DE3)-RIPL competent cells were subjected to transformation with the help of recombinant plasmids harboring *Mtb* antigens such as, pET-EspC, pET-TB10.4, and pET-HspX. For protein expression, BL21 cells were grown in Luria broth and incubated at 37^0C under shaking condition. IPTG (0.5 mM) was added as inducer after 4 h of incubation when OD_{600} reached 0.6–0.8. Lysate of BL21 cells was prepared using a lysis buffer (20mM Tris-Cl buffer (pH 8.0), containing 0.3 N NaCl, 10mM imidazole and 1 mM PMSF) and then disruption of cells was carried out using Sonics Vibra-Cell VCX-500 Ultrasonic Processor. The soluble or insoluble fractions of proteins were collected [18]. Washing of proteins with 0.5% Triton X-100 and solubilization in 8M urea containing 20 mM Tris–Cl (pH 8.0), 0.3 N NaCl and 1 mM PMSF was done, refolding of insoluble proteins was attained by sequential removal of urea through dialysis against 20 mM Tris–Cl (pH 8.0). The estimation of molecular sizes (kDa) was made via 12% SDS-PAGE. The densitometric analysis was carried out to evaluate the percentage of expression in total cell proteins via Syngene gel documentation system (United Kingdom).

Purification of the Recombinant Proteins

All the 6x-His tagged recombinant proteins were purified through FPLC system with AKTA purifier (GE Healthcare). For this purpose, 1ml of pre-packed HisTrap^{TM} FF column was equilibrated at flow rate of 1 ml/min. Elution of proteins tagged with 6x-histidine was done using linear continuous imidazole gradient (5-500 mM). Fractions were taken at OD_{280} and dialyzed against 20 mM Tris–Cl (pH 8.0). Analysis of purified protein was done via SDS-PAGE. Protein

eastimation was made using BSA as a standard [19].

RESULTS

Expression of *Mtb* proteins

HspX, EspC and TB10.4 proteins were expressed in LB media upon induction of IPTG (0.5mM) in BL21 transformants carrying pET-HspX, pET-EspC and pET-TB10.4 plasmids showing expression level of 29%, 16% and 14%, respectively of the total bacterial cell proteins. SDS PAGE was done to check sizes and expression level of all the transformed proteins (Figure 1). The recombinant proteins HspX, EspC and TB10.4 showed the correct apparent sizes 18kDa, 13kDa and 11kDa respectively.

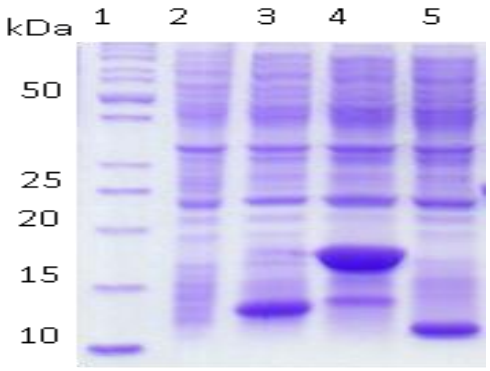

Figure 1. 12% SDS-PAGE showing *E. coli* cells expressing *Mtb* proteins. 1: protein marker; 2: Uninduced cells; 3: EspC (13kDa); 4: HspX (18kDa); 5: TB10.4 (11kDa).

Purification of recombinant proteins

Elution of Poly-histidine tagged EspC, HspX and TB10.4 proteins was accomplished via linear imidazole gradient of 5mM-500mM (Figures 2a,3a and 4a). SDS-PAGE was performed to check the size and purity of the eluted fractions of protein as shown in Figure 2b, 3b and 4b, respectively. The purity level of each of the proteins EspC (13kDa), HspX (18kDa) and TB10.4(11kDa) as determined by SDS-PAGE gel, were found to be more than 90%. Recoveries obtained in the cases of EspC, HspX and TB10.4 were 5.5%, 12% and 7.1 %, respectively (Table 1).

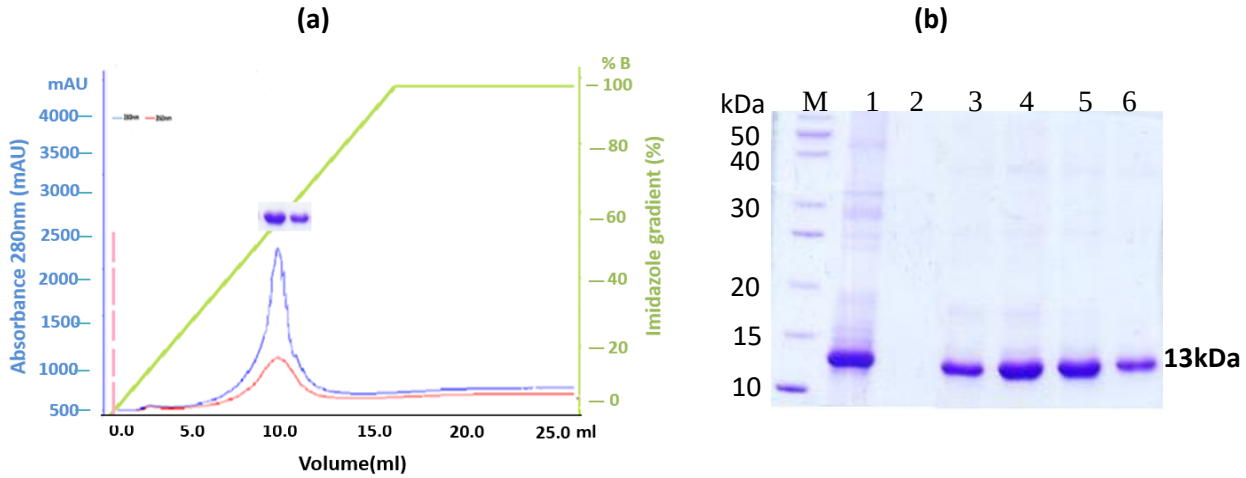

Figure 2: Purification of EspC through Ni^{2+} affinity AKTA FPLC system.

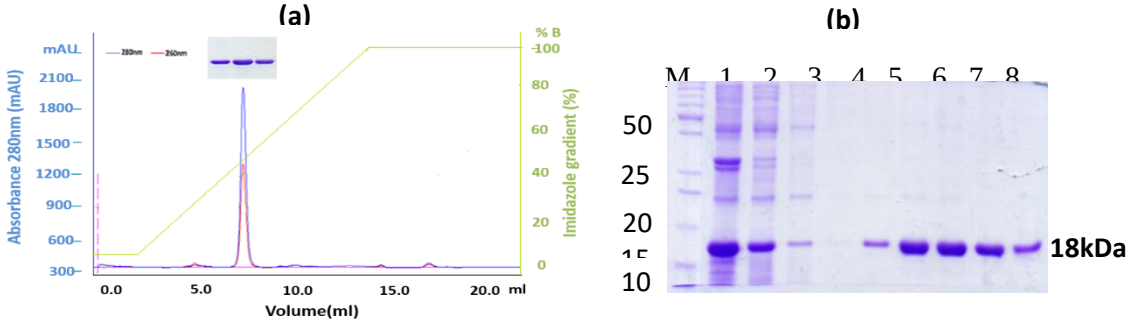

Figure 3: Purification of HspX through Ni^{2+} affinity AKTA FPLC system.

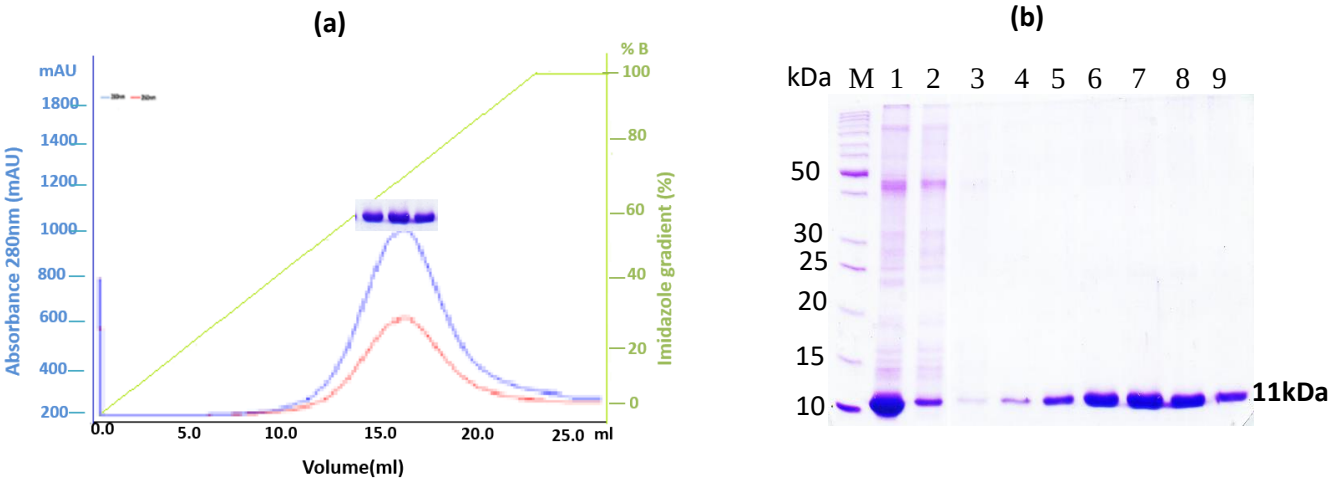

Figure 0: Purification of TB10.4 through Ni^{2+} affinity AKTA FPLC system.

Table 1 Purification summary of recombinant antigens of Mycobacterium tuberculosis.

No.	Antigen	Expression level (%)*	Yield ($mg/l/OD_{600}$)*	Purity (%)	Recovery (%)
1	HspX	29	52	>90	12.0
2	EspC	16	29	>90	5.5
3	TB10.4	14	26	>90	7.1

*See ref. 14 for details.

DISCUSSION

Better purification and recovery is advantageous for the economical production of the *Mtb* antigens on commercial scale for their serological testing and vaccine development. Fast Protein Liquid Chromatography (FPLC) based on an affinity column provides rapid and reproducible protein purification. The most engrossing outcome of FPLC system is the accuracy and reliability. The FPLC system includes samplers, gradient program control and peak collection. It helps to monitor several parameters at a time such as UV level, pH and conductance and it can also run multiple purification columns in tandem [20].

In the present study, we develop three recombinant constructs pET-EspC, pET-HspX and pET-TB10.4 which were

expressed in *E. coli* at different levels. All the antigens were selected on the basis of their immunogenic potentials. The expression levels of all the proteins were determined using Syngene gel documentation system. All the 6x-His tagged recombinant HspX (18kDa), EspC (13kDa) and TB10.4 (11kDa) proteins showed expression levels of 29%, 16 and 14%, respectively. All the proteins were purified through automated AKTA purifier FPLC system using HisTrapTM FF column. Following the continuous imidazole gradient of 5mM-500mM the eluted fractions were collected when sharp peaks of OD_{280nm} appeared as shown in Figures 2a, 3a and 4a. The sizes and purity of the eluted fractions of proteins were checked on SDS-gels. Position of the three antigens appeared on gel according to their sizes. Purity of each of the antigen thus obtained was found to be >90%. The purified TB10.4, HspX and EspC proteins showed percentage recoveries of 7.1%, 12.0% and 5.5%, respectively. This study results in an efficient and rapid purification of *Mtb* antigens, which would be evaluated for their serodiagnostic and prophylactic potentials.

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