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Production and affinity purification of diverse recombinant *Mtb* antigens

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ABSTRACT
Tuberculosis (TB) remains a global health threat, and effective disease control depends on timely diagnosis alongside appropriate treatment, while a robust vaccine could further reduce disease incidence. For both serological diagnosis and vaccine development, highly purified *Mtb* (*Mycobacterium tuberculosis*) antigens with strong immunogenicity are essential. In this study, we aimed to clone, express, and purify three *Mtb* recombinant proteins — Hrp1 (15.5kDa), HspX (16kDa), and Cfp17 (22kDa) — in soluble form using *Escherichia coli*. The target genes were amplified by PCR and inserted into the pET28a(+) vector. Recombinant proteins, carrying an N-terminal 6×His tag, were expressed in *E. coli* BL21-CodonPlus (DE3)-RIPL cells under IPTG induction. Purification was carried out by Ni-NTA affinity chromatography. Densitometric analysis indicated expression levels of approximately 32% for Hrp1, 25% for HspX, and 16% for Cfp17. Final purified products exceeded 90% purity, with recoveries of 38.3% (Hrp1), 73% (HspX), and 45% (Cfp17). The recombinant Hrp1, HspX and Cfp17 proteins, obtained with high purity are therefore well suited for further evaluation in TB serodiagnostic assays and subunit-vaccine development.

KEYWORDS: Cfp17, Hrp1, HspX, *Mtb* antigens, Ni-NTA affinity chromatography

INTRODUCTION

Tuberculosis (TB) is a severe infectious disease and remains the second leading cause of mortality worldwide. Transmission occurs through airborne droplets expelled by individuals with active TB, which contain the intracellular pathogen *Mycobacterium tuberculosis* (*Mtb*). The emergence of drug-resistant TB, which is increasingly difficult to treat with conventional antibiotics, underscores the urgent need to curb the spread of this disease [1,2]. Effective TB control requires early and accurate diagnosis, along with the development and deployment of potent vaccines.

The dormancy survival regulator (DosR) regulon in the *M. tuberculosis* genome consists of 48 co-regulated genes, many of which encode antigens with strong immunogenic potential in both humans and mice [3]. Among these, Hrp1 and HspX have been reported to elicit robust immune responses [4]. Hrp1 (Hypoxic Response Protein 1) is highly expressed in *Mtb* and induces both humoral and cellular Th1-type immune responses, making it an attractive candidate for TB diagnostics and vaccine design [5]. Similarly, HspX, also known as the 16-kDa antigen and a member of the HSP20 family, is well recognized for triggering immune responses during both latent and active TB [6,7]. The gene encoding HspX, *Rv2031c*, contributes to humoral and cell-mediated immunity, and its chaperone-like function, combined with its strong serological reactivity, makes it suitable for constructing fusion proteins used in TB serodiagnosis [8,9].

In addition to these DosR-regulated proteins, several secreted antigens—such as ESAT-6, PstS1, Cfp10, Ag85B, and HSP16.3—have been identified as promising targets for serological assays [10]. Another antigen of interest, Cfp17, is abundantly present in the plasma of TB patients and shows high

diagnostic sensitivity and specificity [11]. Also known as the Mtb GarA protein, Cfp17 contains a forkhead-associated (FHA) domain at its C-terminus [12].

In this study, three immunogenic native Mtb antigens—Hrp1, HspX, and Cfp17—were cloned and expressed in soluble form in *E. coli* BL21 CodonPlus (DE3)-RIPL cells using recombinant DNA technology. Following expression, all three proteins, engineered with an N-terminal 6×His tag, were purified using Ni-NTA affinity chromatography. This approach employs nitrilotriacetic acid (NTA) as a chelating ligand in immobilized metal affinity chromatography (IMAC), coordinating nickel ions at four binding sites. With a binding capacity of up to 60 mg of protein per milliliter of resin, this system provides an efficient and reliable purification strategy [13]. HisPur™ Ni-NTA resin (catalog number 88222) was used for the purification procedures in this study. The resulting purified proteins are suitable for downstream immunological applications, contributing to research efforts in TB diagnosis and vaccine development.

MATERIALS AND METHODS

Primers designing and gene amplification

Genomic DNA from *Mycobacterium tuberculosis* strain H37Rv was isolated using a modified CTAB/NaCl protocol [14]. The complete open reading frames (ORFs) of genes *Rv2626c, Rv2031c*, and *Rv1827* — which encode the *Mtb* antigens Hrp1, HspX, and Cfp17, respectively — were chosen for further study. For each gene, forward and reverse primers were designed using an oligonucleotide design tool, and primer specificity was confirmed via a Primer-BLAST search. Restriction enzyme recognition sites compatible with downstream cloning were added at the 5′ ends of the primers using an *in-silico* digestion tool. The primer sequences employed for antigen amplification are provided in Table 1.

Table 1 List of primers used for the PCR amplification

Antigens	Primers	Nucleotide Sequences 5′→ 3′	Restriction sites	T m (°C)
Cfp17	cF1	AACATATGGTGACGGACATGAACCCG	*Nde*l	67.9
	cR1	ATAAGCTTTCACGGGCCCCC GGTA	*Hin*dIII	68.5
Hrp1	hF2	GAACATATGACCACCGCACGCGACATC	*Nde*l	71.5
	hR2	AGAGAATTCCTAGCTGGCGAGGGCCAT	*Eco*R1	71.5
HspX	xF3	GCAGCAGGATCCATGGCCACCACCCTT	*Bam*HI	74.4
	xR3	AGCGCAAAGCTTTCAGTTGGTGGACCG	*Hin*dIII	71.3

The PCR mixture contained 125 ng of *Mtb* H37Rv genomic DNA as template in a final volume of 50 µl, along with 5 units of *Taq* DNA polymerase. Amplification was carried out using a thermal cycler (Kyratec Super Cycler): the program began with an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 45 seconds, annealing for 30 seconds (set at 66.3 °C for Hrp1, 67.9 °C for HspX, and 63.3 °C for Cfp17), and extension at 72 °C for 1 minute, with a final extension at 72 °C for 10 minutes. The resulting PCR products were analyzed on a 1% agarose gel to confirm amplification.

Molecular cloning of selected antigens

Each PCR amplicon was purified and ligated into the cloning vector pTZ57R/T. Following sequence verification, the genes encoding Hrp1, HspX and Cfp17 were sub-cloned into the pET-28a(+) expression vector using the established protocol [15]. The resulting recombinant plasmids were chemically transformed into competent *E. coli* DH5α cells (prepared by the $CaCl_2$ method) [16]. Randomly chosen single colonies were picked on LB agar plates supplemented with 100 µg/mL ampicillin, and screened by colony PCR to identify positive transformants. Finally, the presence of the correct inserts in Hrp1, HspX and Cfp17 plasmids was confirmed by restriction enzyme digestion analysis.

Protein expression

Recombinant plasmids carrying the *Mtb* antigen genes — pET-Hrp1, pET-HspX, and pET-Cfp17 — were transformed into competent BL21-CodonPlus (DE3)-RIPL (Stratagene, USA) cells. For protein expression, transformed cells were grown in Luria–Bertani (LB) medium at 37 °C with shaking. When the culture reached an optical density at 600 nm (OD_{600}) of 0.6–0.8, expression was induced with 0.5 mM IPTG, and incubation continued under the same conditions for an additional four hours. After induction, the bacterial pellet was resuspended in lysis buffer (20 mM Tris–Cl, pH 8.0) supplemented with 0.3 M NaCl, 10 mM imidazole, and 1 mM PMSF. Cells were disrupted using a Sonics Vibra-Cell VCX-500 ultrasonic processor. The lysate was then separated into soluble and insoluble fractions by centrifugation. Expression levels and solubility were assessed by running the samples on a 12% SDS-PAGE gel, and protein expression yield was quantified via densitometric analysis using a gel-documentation system.

Purification of recombinant Hrp1, Hspx and Cfp17

Recombinant Hrp1, HspX, and Cfp17—all bearing an N-terminal 6×His tag—were purified using nickel-based affinity chromatography (Ni–NTA). We employed commercially available HisPur™ Ni-NTA resin (cat. no. 88222). After binding and washing to remove non-specific proteins, the target proteins were eluted using a gradient of imidazole (ranging from 100 mM to 300 mM), with final elution achieved at 300 mM imidazole. Elution fractions were collected carefully in separate tubes and then dialyzed against 20 mM Tris–Cl (pH 8.0) to remove imidazole and equilibrate the buffer. Purity and integrity of the purified proteins were verified by running the samples on 12% SDS–PAGE followed by Coomassie Brilliant Blue staining. Protein concentration was estimated using bovine serum albumin (BSA) as a standard for comparison.

RESULTS

Expression of *Mtb* proteins

Recombinant Hrp1, HspX, and Cfp17 were expressed in LB medium after induction with 0.5 mM IPTG in *E. coli* BL21 transformants harboring the respective pET-Hrp1, pET-HspX, and pET-Cfp17 plasmids. The expression levels reached approximately 32%, 25%, and 16% of the total cellular protein content for Hrp1, HspX, and Cfp17, respectively. Following induction, bacterial cells were lysed and centrifuged, and the lysates were fractionated into soluble and insoluble (pellet) components. SDS–PAGE analysis (Figure 1a. 2a and 3a) confirmed protein expression, with the recombinant bands migrating at the expected apparent molecular masses of ~15.5 kDa for Hrp1, ~16 kDa for Hrp1, and ~22 kDa for Cfp17. A significant portion of the expressed protein partitioned to the soluble fraction — indicating correct folding under the given

expression conditions and suitability for downstream purification and functional assays.

Purification of recombinant proteins by affinity chromatography

Elution of Poly-histidine tagged hrp1, HspX and cfp17 proteins was accomplished via linear imidazole gradient of 100mM-300mM 12 % SDS-PAGE was performed to check the size and purity of the eluted fractions of protein followed by dialysis as shown in Figure 1b, 2b and 3b respectively. The purity level of each of the proteins hrp1, Hspx and Cfp17 as determined by SDS-PAGE gel, were found to be more than 90%. Recoveries obtained in the cases of purified Hrp1, Hspx and Cfp17 were 38.3% ,73% and 45%. respectively (Table 2)**.**

Table 2 Purification summary of *Mtb* recombinant proteins

Purification levels	**Hrp1**	**HspX**	**Cfp17**
Expression level (%)	32	25	16
Yield (mg/L/0D_{600})	57.6	45	28
Purity (%)	>90	>90	>90
Recovery (%)	38.3	73	45
Amount of purified protein (mg/L/OD_{600})	22.08	32.88	12.8

DISCUSSION

The successful cloning, expression, and purification of the *Mtb* antigens Hrp1, HspX, and Cfp17 highlight the efficiency of the recombinant expression system and Ni–NTA affinity purification strategy utilized in this study. Affinity tagging—especially the use of a 6×His tag—remains one of the most reliable approaches for distinguishing recombinant proteins from host cellular proteins, while enabling their rapid purification and easy detection. Commercially available His-tag expression systems and nickel-based affinity resins greatly simplify this process and ensure reproducibility for both research and industrial-scale applications [17].

All three constructs—pET-Hrp1, pET-HspX, and pET-Cfp17, were successfully expressed in *E. coli*, yielding expression levels of 32%, 25%, and 16%, respectively. These relative differences likely reflect inherent variations in protein size, folding kinetics, and metabolic load placed on the expression host. The BL21-CodonPlus (DE3)-RIPL strain, with its enhanced supply of rare tRNAs, likely contributed to improved translation efficiency of *Mtb* genes, which often contain rare codons for *E. coli [18].*

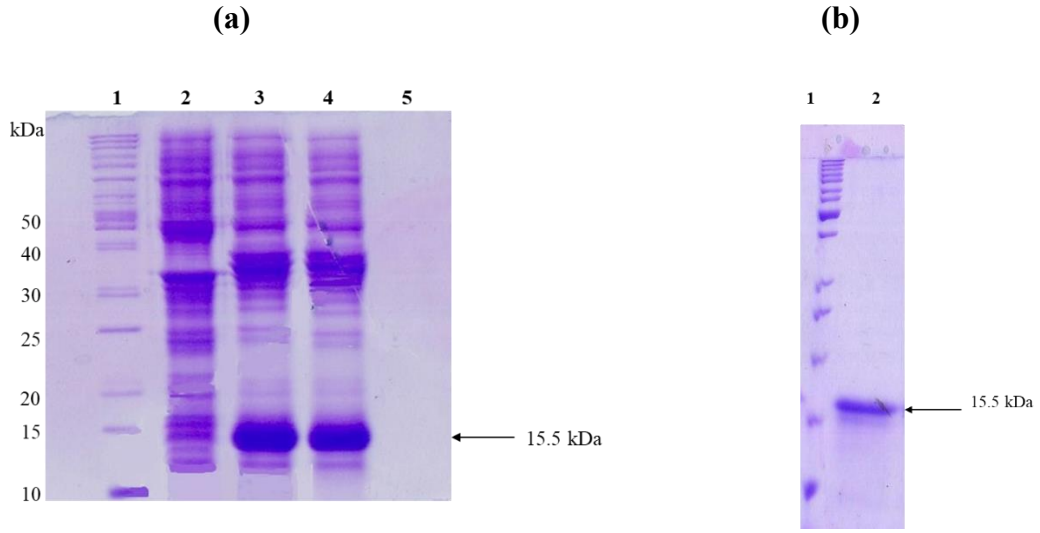

Figure 1. (a) 12% SDS PAGE analysis exhibiting protein expression of pET-Hrp1. Lane 1: Protein ladder, Lane 2: Uninduced cellular proteins, Lane 3: Total cell proteins, Lane 4: Soluble fraction (supernatant), Lane 5: Insoluble fraction (pellet) **(b)** Lane 1: Protein Ladder, Lane 2: purified Hrp1 after dialysis.

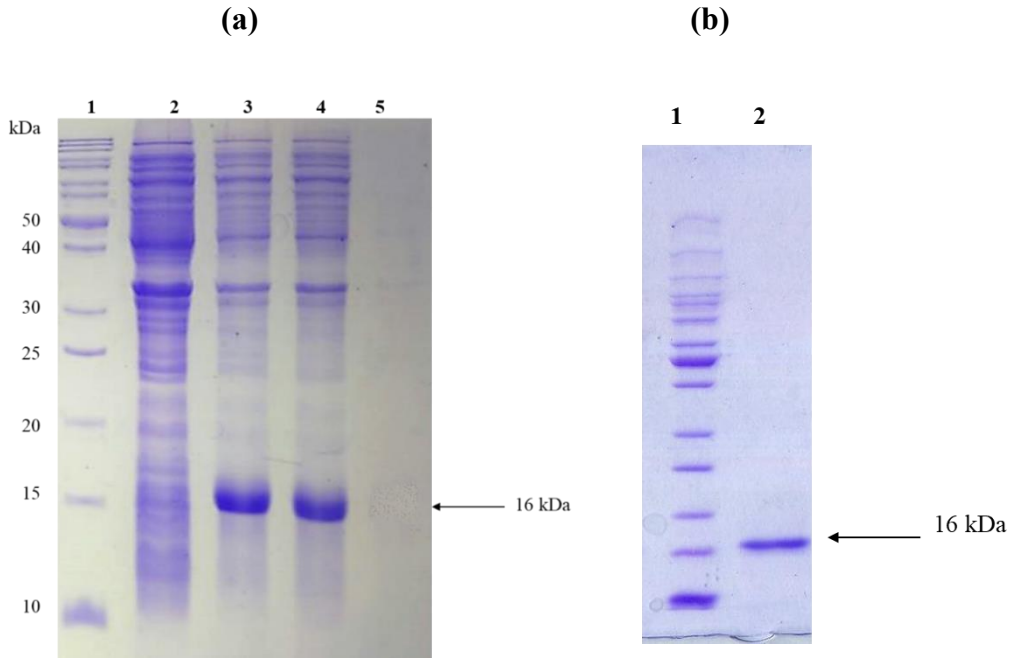

Figure 2. (a) 12% SDS PAGE analysis exhibiting protein expression of pET-HspX. Lane 1: Protein ladder, Lane 2: Uninduced cellular proteins, Lane 3: Total cell proteins, Lane 4: Soluble fraction (supernatant), Lane 5: Insoluble fraction (pellet). **(b)** Lane1: protein marker, Lane 2: Purified Hspx after dialysis.

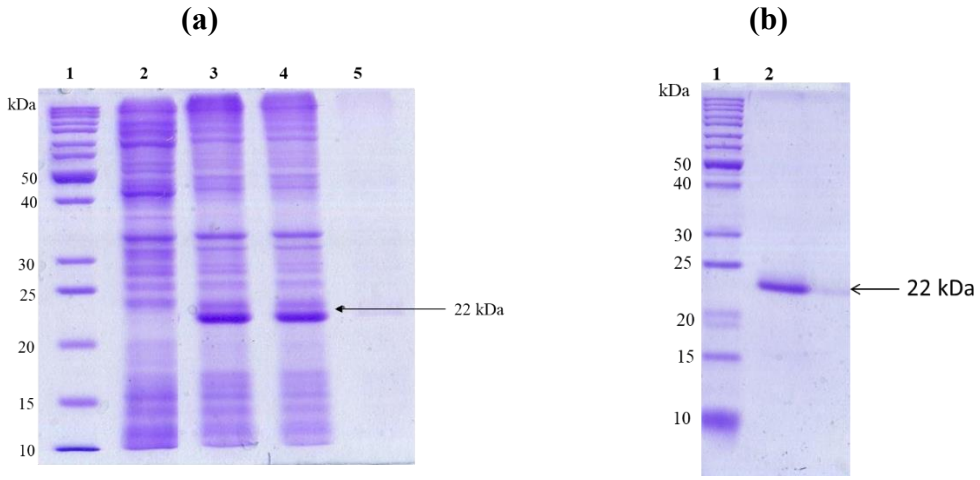

Figure 3. (a) 12% SDS PAGE analysis exhibiting protein expression of pET-Cfp17. Lane 1: Protein ladder, Lane 2: Uninduced cellular proteins, Lane 3: Total cell proteins, Lane 4: Soluble fraction (supernatant), Lane 5: Insoluble fraction (pellet). **(b)** Lane 1: Protein ladder Lane 2: Purified Cfp17 after dialysis.

Purification was carried out solely using Ni–NTA affinity chromatography, which selectively binds the polyhistidine-tagged proteins. A linear imidazole gradient was applied, and elution fractions were manually collected as the gradient increased. This approach ensured good separation between weakly bound contaminants and the specific recombinant proteins. The manual collection of fractions provided flexibility in isolating only those elution tubes containing visibly enriched protein fractions after SDS–PAGE analysis.

Analysis by SDS–PAGE confirmed that the purified antigens migrated at their expected molecular weights—approximately 15.5 kDa for Hrp1, 16 kDa for HspX, and 22 kDa for Cfp17. Each protein exhibited a purity level exceeding 90%, demonstrating that Ni–NTA chromatography was highly effective [19] for obtaining clean preparations suitable for immunological studies. The recovery yields were 38.3% for Hrp1, 73% for HspX, and 45% for Cfp17. The relatively high recovery of HspX is consistent with its known structural stability, while the lower recovery of Cfp17 may reflect the greater complexity of its folding or tendency toward partial loss during purification.

The high purity and solubility of these recombinant antigens make them ideal candidates for downstream applications. Hrp1 and HspX, both DosR-regulon antigens, are documented to elicit strong immune responses during latent and active TB infection. Cfp17 (GarA), which is frequently detected in clinical specimens from TB patients, possesses considerable serodiagnostic potential [20]. The availability of purified recombinant forms of these antigens supports their evaluation in ELISA-based serodiagnostic assays, antigenic screening, and potential inclusion in multi-antigen diagnostic platforms. Furthermore, these antigens may serve as components of future subunit vaccine formulations.

Overall, the present work demonstrates a rapid and reliable workflow for producing highly pure recombinant Mtb antigens using Ni–NTA affinity chromatography. This method yielded proteins of adequate quality

and quantity for subsequent immunological and diagnostic studies and contributes valuable reagents for TB research, serodiagnosis, and prophylactic development.

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