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# Production and Purification of Mycolyl Transferase B of *Mycobacterium tuberculosis*

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**Background:** Antigen 85 complex of *Mycobacterium tuberculosis* includes three immunogenic proteins which are TB vaccine candidates of great importance. As they are very hard to be achieved in natural form, recombinant production of them fuels immunological experiments. Production of such apolar mycobacterial proteins located in the cell wall faces substantial challenges mainly regarding their solubility. This study reports the production of soluble recombinant Ag85B with an efficient yield.

**Materials and Methods:** Ag85B gene was cloned in pJET1.2 and subsequently in pET32a (+). Both recombinant plasmids were sequenced. Expression of the recombinant protein was induced with 1mM IPTG. Recombinant Ag85B was purified through dissolving inclusions in 8M urea buffer, absorbing to Ni-NTA resins, washing by buffers with decreasing urea concentrations and finally eluted in imidazole. Western blot analysis was performed using anti-6His tag antibody, rabbit anti- *M. tuberculosis* polyclonal antibody and serum of hospitalized TB patients.

**Results:** Ag85B gene was successfully cloned in both plasmid vectors. The recombinant Ag85B was expressed in *E. coli* host and purified with significant yield.

**Conclusion**: Western blot results along with those of sequencing ensured accurate production of recombinant Ag85B and retaining of its antigenic structure.

**Key words:** Ag85 complex, Ag85B, TB vaccine, *Mycobacterium tuberculosis*

## INTRODUCTION

Despite the widespread use of BCG vaccine to confront TB and its causative agent *Mycobacterium tuberculosis*, this disease is still one of the most important lethal infections in the world and especially in developing countries claiming lives of about 2 million patients annually (1). Considering the increased prevalence of immunodeficiency diseases like AIDS, emergence of antibiotic resistant *M. tuberculosis* strains and inefficiency of BCG vaccine in adults during the recent years, attempts are being made to develop preventive and therapeutic strategies in order to control TB globally. In this regard many of researchers are studying immunogenic antigens of *M. tuberculosis* to develop efficient vaccines against this disease (2).

The Ag85 complex proteins are among the most important immunogenic antigens of *M. tuberculosis*. Studies have shown that these proteins induce proliferation of Th1 lymphocytes, stimulate production of IFN-γ and activate cytotoxic lymphocytes in TB-infected cases and mice vaccinated by BCG. The ability of these antigens in eliciting a protective immune response has made them promising

candidates for designing TB vaccines (3, 4). This complex comprises three similar secreted proteins namely antigens 85A, 85B and 85C which have mycolyl transferase activity and play a major role in bacterial cell wall synthesis. This enzyme catalyzes the transfer of mycolic acids from a trehalose 6-monomycolate to another resulting in formation of trehalose 6-6′ dimycolate also known as cord factor (5, 6). This confirms that these enzymes play a critical role in physiology and pathogenesis of *M. tuberculosis* (5, 7, 8).

Many vaccine candidates, anti-mycobacterial medication and diagnostic methods have been designed based on members of Ag85 complex (9-11). Recent studies have focused on the use of recombinant DNA vaccines and recombinant BCG strains as the primary vaccine and administration of mycobacterial antigens as a booster (12). On the other hand, use of recombinant antigens per se is suggested as an immunogenic factor and that is why production of recombinant protein antigens has been considered for use in immunization process (13, 14).

Ag85B is one of the most abundant secreted proteins of *M. tuberculosis* which is secreted in the primary phase of illness and is frequently found in infected macrophages (15). Considering the mentioned characteristics and since the immunogenicity of this protein has been confirmed, Ag85B is a promising candidate for immunization against TB. Similar to some other proteins present in the mycobacterial cell-wall, Ag85B shows apolar characteristics consistent with hydrophobic conditions (6, 16). Production of recombinant apolar proteins and their subsequent extraction and purification is challenging and extensively different from purification of other types of soluble proteins and require innovative strategies.

Matsuo et al. in 1988 reported cloning and expression of the *Mycobacterium bovis* BCG gene for extracellular alpha antigen in vector pUC18. The characteristics of this antigen are compatible with those of Ag85B (17). Matsuo et al. defined the G+C ratio of this gene as 86% and a peptide signal was detected in its first 40 amino acids. 0.5 mg/ml of the recombinant Ag85B was produced in pKK233-2 plasmid under the control of the *Trc* promoter (17).

Lakey et al. in 2000 reported cloning and expression of Ag85A and Ag85B (18). For the expression of recombinant protein, they used *pTrcHisB* vector and *E. coli* TOP10 expression host. Their study claims that *pTrcHisB* vector overcomes the problem of low G+C percentage in *E. coli* genome. They purified recombinant proteins under denaturing conditions and urea removal was not performed.

Kremer and colleagues in 2002 cloned Ag85A, Ag85B and Ag85C and evaluated their mycolyl transferase activity (19). For gene expression they used pET23b (+) vector that contains T7 promoter and *E. coli* C41 (DE3) host but they failed to produce recombinant Ag85B to a considerable amount so that preferred to use native Ag85B.

This study aimed at producing a new recombinant plasmid with the ability to significantly express Ag85B protein and purification of this recombinant protein as a solute in non-denaturing conditions

## MATERIALS AND METHODS

**Bacterial strains and plasmids**

*Mycobacterium tuberculosis* H37Rv was obtained from Pasteur Institute of Iran. PJET1.2 plasmid (Fermentas) and pET32a(+) (Novagen) were used as cloning and expression vectors, respectively. *E. coli* GM2163 (Fermentas) and *E. coli* Rosetta gami (Novagen) respectively were used as cloning and expression hosts.

**Cloning of Ag85B**

Registered sequences of Ag85B gene of *M. tuberculosis* were obtained from NCBI database and evaluated using VectorNTI™ 11.0 software. A pair of primers was designed for cloning of the whole coding region of this gene including the sense primer (H85B-F5′-AAA GGA TCC ATG ACA GAC GTG AGC CGA AAG ATT CG-3′) and the antisense primer (H85B-R-5′AAA GAG CTC GCC GGC GCC TAA CGA ACT CTG CAG G-3′). Cutting site for *Bam*HI embedded at 5′ end of the sense primer and at 5′ end of the antisense for *Sac*I restriction enzyme (underlined).

Ag85B gene fragment of *M. tuberculosis* H37Rv strain was amplified by PCR using PrimSTAR®HS DNA

polymerase (TaKaRa). The thermal cycle included 98˚C for 5 min followed by 35 cycles of 98˚C for 10s, 62˚C for 15s, 72˚C for 1 min and ending with 72˚C for 10 minutes.

The amplified Ag85B PCR product was purified and cloned into the plasmid pJET1.2 according to the instructions of the CloneJET™ PCR cloning kit (Fermentas) and then transferred into *E. coli* GM2163. Screening for recombinant plasmids was performed by colony PCR and use of pJET1.2 universal primers followed by Ag85B specific primers. Cloning of Ag85B gene in pJET1.2 plasmid was confirmed by enzymatic digestion of recombinant plasmids. Recombinant plasmids were sequenced and results were analyzed by VectorNTI Advanced ™ 11.0 (Invitrogen).

Ag85B gene fragment was excised from recombinant pJET1.2 by double enzyme digestion (*Bam*HI - *Sac*I) and was subcloned in pET32a(+) lineared by similar restriction enzymes. Recombinant plasmids (pET32a-85B) were transformed into *E. coli* GM 2163 and recombinant clones were screened with specific primers for antigen 85B using colony PCR. Enzyme digestion and sequencing (as explained earlier) was performed to ensure the accurate production of recombinant plasmids (pET32a-85B).

**Expression and purification of recombinant protein Ag85B (rAg85B)**

Recombinant plasmid pET32a-85B was transferred into expression host *E. coli* Rosetta gami (DE3) pLysS. Recombinant strains (RG-85B) were inoculated in 1 ml LB medium containing 50µg/ml ampicillin, 5µg/ml kanamycin, 34µg/ml chloramphenicol and 12.5µg/ml tetracycline and placed in the 37˚C incubator overnight. This overnight culture was used for inoculation of 10 ml LB medium containing 50µg/ml ampicillin. Medium was placed in a rotary incubator shaker (250rpm) to an absorbance at 600 nm (A600) of 0.6. Recombinant plasmids were induced by 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG). Three samples were obtained from the induced bacteria at times "$t_0$" (before induction) to "$t_3$" and were evaluated for protein expression at different times by SDS-PAGE in 12.5% gel followed by Coomassie Blue G-250 staining. Expression of a recombinant protein was induced in 1 liter volume and RG-85B cells were collected 1 to 3 hours after induction. The bacterial cells were washed with phosphate buffered saline, suspended in lysis buffer (50 mM NaH2PO4, 300mM NaCl, 10mM imidazole and PMSF; pH:8.0) and sonicated three times with 0.6 second frequency of 80% amplitude for 5 minutes until the cells were completely destroyed. The lysate was centrifuged for 20 minutes at 18,000×g and the solid precipitate and the supernatant were separated and evaluated for detection of recombinant protein via SDS-PAGE.

In order to produce the recombinant protein rAg85B, an overnight culture of RG-85B bacteria was prepared and used for inoculation of 1 liter of LB medium. When the culture grew up to $OD_{600}$ 0.6, protein synthesis was induced by 1mM IPTG and continued for three hours at 37˚C. Bacterial cells were collected by centrifugation at 9000×g and washed by phosphate buffered saline. Bacterial cells were dissolved in B buffer (100mM NaH2PO4, 10mM Tris-Cl, and 8M urea; pH: 8). Insoluble debris was removed by centrifugation and the lysate was mixed with Ni-NTA resins on a rocker for one hour. The mixture was then transferred to a poly styrene column and washed with buffer C (100mM NaH2PO4, 10mM Tris-Cl, and 8M urea; pH 6.3). Urea was removed by washing buffers with decreasing urea concentrations (8, 6, 4, 2, 1, 0 m urea). Eventually, recombinant Ag85B was eluted from the column using 250mM imidazole solution. In order to remove imidazole, protein solution was dialyzed against phosphate buffered saline (pH 7.4) overnight. Recombinant protein production was quantified spectrophotometrically using NanoDrop device and the total amount of purified solubilized protein from 1 lit of bacterial culture was calculated.

**Western Blot**

Recombinant Ag85B was transferred from polyacrylamide gel to polyvinylidene difluoride (PVDF) membrane using Transblot®SD (Bio-Rad) in semi-dry conditions. Ponceau S staining was used to control protein transfer. Membrane was cut to narrow strips each containing a blot of protein. PVDF membrane strips were blocked by bovine serum albumin (BSA) 5% for an hour.

Afterwards separate membranes were incubated with rabbit polyclonal antiserum of *Mycobacterium tuberculosis* H37Rv (1:5000), Anti-His-tag monoclonal antibody (1:10,000) and serum of a hospitalized TB patient (1:2000) for 2 hours. Washing was done with tris-buffered saline with 0.05% Tween 20 (TBS-T). Membranes were incubated with secondary antibodies (mouse 1:10,000, rabbit 1:10,000, human 1:5000) for 2 hours. Membranes were washed and incubated with enhanced chemiluminescent substrate (ECL) under standard conditions and immediately appearance of fluorescent bands was recorded on radiographic films.

## RESULTS

### Cloning and Sequencing of Ag85B

Ag85B gene with 996 base pairs was amplified with PrimSTAR®HS DNA polymerase which is a high fidelity enzyme and successfully cloned in pJET1.2 plasmid. The length of this gene is 978 base pairs but due to the addition of recognition sequences of *Bam*HI and *Sac*I enzymes and three extra nucleotides as anchor on both sides, the final size of product would be 996 base pairs.

Comparison of the sequencing results of recombinant plasmids with reference sequences showed that no change or mutation had occurred on the cloned gene. Subcloning of Ag85B gene in pET32a (+) was done successfully. Enzyme digestion and sequencing ensured the correct insertion of Ag85B gene in this plasmid.

### Expression and purification of recombinant protein Ag85B

Protein expression was observed on the SDS-PAGE gel in the range of 50kDa which is compatible with the molecular weight of our respective protein which was estimated to be 53kDa (Figure 1). Ag85B weighs about 34KD and fusion of thioredoxin to its N-terminus in pET32a(+) adds a further 19kDa to its weight. Expression of proteins was not significantly different between 1 to 3 hours after induction. Evaluation of the SDS-PAGE gel containing the supernatant and the sediment resulted from cell destruction by sonication showed that the highest amount of recombinant protein is in the sediment phase as insoluble solid inclusions. Electrophoresis of Ag85B showed significant purification of this protein (Figure 2). The amount of soluble protein obtained from 1 lit of bacterial culture was determined to be 7.5 mg. Western blot test results show that rAg85B is detectable by rabbit polyclonal antibody, Anti-His monoclonal antibody and patient's serum (Figure 3).

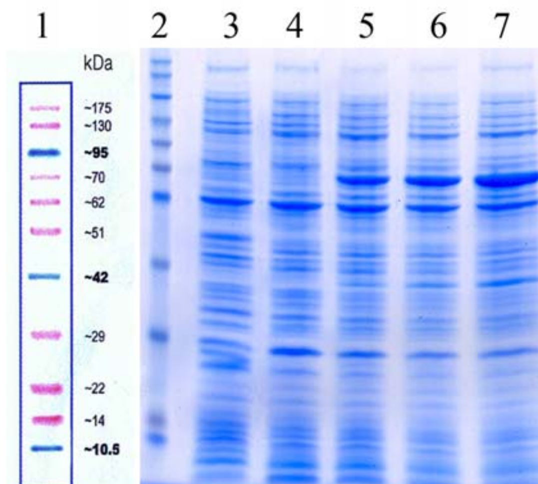


Figure 1. Expression of recombinant Ag85B cloned in pET32a (+).

1- Standard protein molecular weight marker
2- Protein molecular weight marker
3- *E. coli* Rosetta gami strain containing intact pET32a(+)
4- *E. coli* Rosetta gami strain before induction at time 0
5- *E. coli* Rosetta gami strain one hour after induction
6- *E. coli* Rosetta gami strain two hours after induction
7- *E. coli* Rosetta gami strain three hours after induction

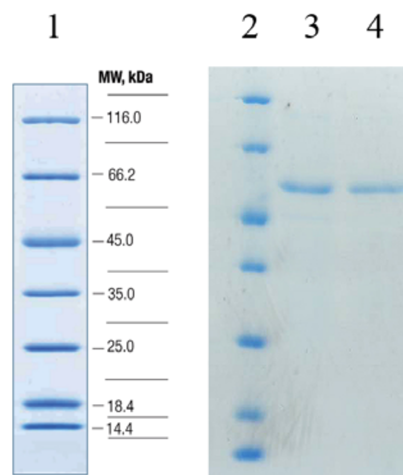


Figure 2. Results of recombinant Ag85B protein purification of 2 separate attempts

1- Standard protein molecular weight marker
2- Protein molecular weight marker
3- Sample of first purified protein
4- Sample of second purified protein

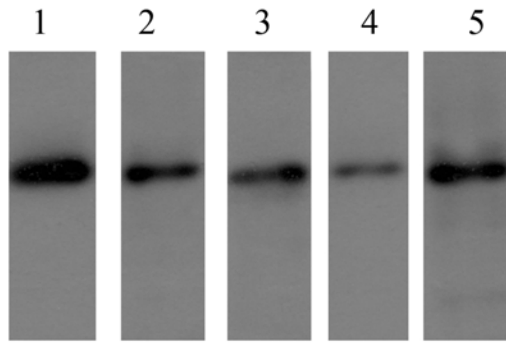


**Figure 3**. Western blots results.

1- Western blotting with Anti-His antibody
2- Western blotting with rabbit polyclonal antibody
3- Western blotting with patient #1 serum antibody
4- Western blotting with patient #2 serum antibody
5- Western blotting with patient #3 serum antibody

## DISCUSSION

High prevalence of TB in the world and disappointing efficacy of its only vaccine BCG has made researchers to look for an alternative vaccine or an efficient treatment for this disease (20, 21). Various immunogenic cell components are among the suggested candidates for TB vaccine. Numerous studies have been conducted on the development of different types of DNA vaccines, recombinant BCG variants and subunit vaccines (14). Recently, it has been confirmed that using protein booster for DNA vaccines or recombinant BCG can considerably stimulate the immune system and induce a more appropriate response. Additionally, some protein compounds alone or in combination with other proteins and various adjuvants have the ability to produce protective immune responses (14, 22-24). In order to use TB bacilli proteins as the immunogenic factor or booster vaccine, recombinant production of them is necessary. Fragments of Ag85 complex have a critical role in mycobacterial cell wall synthesis and are considered among the most abundant secreted proteins of this microorganism. Immunogenicity of Ag85 complex (Ag85A, Ag85B, and Ag85C) has been reported in guinea pig models. Ag85 complex proteins are promising candidates for designing vaccines, diagnosis and treatment of TB (25-29).

Mycobacterial cell wall structure and its components have significant differences with those of other bacteria. Presence of several hydrophobic compounds confers specific characteristics that differentiate this microorganism from other prokaryotes (30). Ag85 complex proteins are located in mycobacterial cell wall and play a major role in its synthesis. Since the mycobacterial cell wall is hydrophobic, most compounds especially proteins should be compatible with that conditions. Components of Ag85B complex are also non-polar (5, 7, 8). Apolar structure of Ag85B and misfolding in the heterogeneous prokaryotic host such as *E. coli*, results in high insolubility during expression. However, for many research purposes, proteins must be water soluble under native conditions. Use of plasmid vectors is an acceptable routine solution for production of recombinant apolar proteins. A soluble protein segment (like beta-galactosidase or thioredoxin) is added to the respective protein resulting in production of a soluble or relatively soluble chimeric protein. Efficacy and function of these protein segments depend on the structure of the target protein and its level of apolarity or hydrophobic characteristic. PET32a(+) is among the best plasmid vectors for this purpose. This plasmid places thioredoxin in the N-terminal of the target protein sequence (Trx-tag). Thioredoxin is a highly soluble protein and is easily produced in *E. coli*. Addition of thioredoxin to an apolar protein causes a relative polarity and increased solubility of the whole protein product. Type of immunogenic response against the protein product is important. When presented by immune system cells, protein antigen is broken down into minute peptide segments in the phagolysosome. These peptide segments contain epitopes of protein antigen and are presented by MHC-II molecules to immune system cells (31, 32). Therefore, primary structure or amino acid sequencing of a protein antigen is more important than its three dimensional structure in creating an immune response. Physical configuration, orientation and folding of a protein

have a less significant role in the process of antigen presentation. Evidence show that this chimeric protein compound can be used for immunogenicity purposes (32). In our study, Ag85B protein was produced in pET32a(+) plasmid and a protein segment containing thioredoxin with 19kDa weight was added to the N-terminus. Expression of recombinant protein in the bacterial host is significant and evaluations with SDS-PAGE confirm presence of a protein with 53kDa weight.

Most of the recombinant Ag85B produced in *E. coli* is in the form of intracellular inclusions. The reason is lack of complete folding of mycobacterial protein in this host. The main reason for this incomplete folding is the lack of specific chaperone system in the heterogeneous host. In order to dissolve the proteins collected as inclusion bodies in the host cell, the conventional method is to use substances with denaturing ability for such proteins among which urea and guanidinium chloride are the most common. Denaturing compounds break the hydrogen bonds and cause the inclusion bodies to dissolve. Denaturation accelerates proteins with polyhistidine terminal to be accessed and absorbed with Ni-NTA resins. Presence of a denaturing agent itself limits the biologic use of dissolved protein because it is highly capable of damaging living cells. Several methods have been suggested for removal of denaturing compounds with various results for different proteins. Insoluble inclusion bodies containing recombinant Ag85B protein were dissolved in water using 8M urea solution. Primary removal of urea by dialysis did not show good results (related data are not presented here) and caused the protein to coagulate. In order to obtain recombinant soluble Ag85B several methods were used. Eventually, it was successfully achieved using a series of washing solutions with decreasing urea concentrations. By doing so, urea is eliminated and protein is refolded when binding to Ni-NTA resins and does not allow the free proteins to cross link, aggregate or coagulate. This is a considerable advantage of this method over others. The amount of soluble recombinant protein which is the final product of this method is significant when compared to other methods used for purification of other proteins in Ag85 complex (17, 33-35).

Western blotting with anti-His-tag monoclonal antibody confirms the synthesis of a protein in pET32a(+) plasmid which is compatible with sequencing results. Western-blot results with rabbit polyclonal antibody against *M. tuberculosis* H37Rv strain confirm production of a protein with antigenic characteristics of a mycobacterial protein. This finding along with sequencing results of the plasmid containing Ag85B has approved the production of this recombinant protein and partial retaining of its antigenic structure. Therefore, we can state that the purification method confers solubility in aqueous solutions under native conditions and relatively preserves the 3D structure of the recombinant Ag85B. Detection of recombinant Ag85B by serum of hospitalized patients shows that recombinant protein produced in this study is similar in structure and configuration to the protein produced by TB during infection. Protein produced and purified by this method can be used in various investigation fields including immunization experiments and serodiagnosis.

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