Asian Journal of Biological and Life Sciences | Original Research

Purification of recombinant fusion proteins carrying foreign antigens

Sivasankar Murugan*, Stephen M.L. Raj

Department of Biotechnology, MepcoSchlenk Engineering College, Sivakasi, India.

E-mail : m.sivasankar75@gmail.com Contact No. : +91-8148517784

Submitted : 14.05.2015 Accepted : 20.07.2015 Published : 30.08.2015

Abstract

Tuberculosis the well-known infectious disease in human beings is caused by *Mycobacterium tuberculosis*. The major antigens produced by *M. tuberculosis* during infection is CFP10, ESAT6, Ag85. Present study focuses on CFP10 which is a protective antigen that could induce immunity against tuberculosis. Being a lower molecular weight protein, CFP10 and IFN-γ is vulnerable for proteolytic degradation and consequently often failed to enhance the immunogenicity for longer time. Hence, it was fused with potyvirus coat protein to form virus like particles (VLPs) that could enhance the immunogenicity of the fusion partner. The potyvirus is a largest group of plant virus consist of coat protein (CP) synthesis from Johnsongrass mosaic virus (JGMV) expressed in bacteria or yeast resulted in the formation of potyvirus like particles (PVLPs). The aim of this study was to purify the recombinant fusion proteins (CFP10, CP:CFP10 and CP:IFN-γ) by chromatographic techniques and ultracentrifugation methods. Further, SDS-PAGE analysis was performed to confirm the purified fusion proteins to estimate the molecular weight. The purified protein was already immunized in mice to raise antibodies against CFP10 was determined by western blot. The purified recombinant fusion proteins CFP10, CP:CFP10 and CP:IFN- γ can be used as a diagnostic tool and development of vaccine against tuberculosis.

Key words : *Mycobacterium tuberculosis*, Fusion proteins, Chromatography, Ultracentrifugation.

INTRODUCTION

Tuberculosis is well known infectious disease in human beings caused by an intracellular pathogen, *M. tuberculosis*. Currently, attenuated *M.bovis* Bacillus Calmette-Gue´rin (BCG) is the vaccine against tuberculosis, but it has proven to be problematic because of instabilityduring diagnosis due to cross-reactivity[1].BCG is not an ideal vaccine and has major limitations, especially its poor efficacy against adult pulmonary tuberculosis.BCG induced protection, wanes with age. The fact that immunological memory induced by BCG at an early age (neonates or infants) at which the immune system is not yet fully matured. However, other factors (helminths and viruses) may also decreases the efficacy of BCG and or enhanced vulnerability of young adults to tuberculosis[2]. Recently the BCG vaccine shows that induces variable protection in infants, after routine BCG vaccination.Mycobacteria specific T cell expression ofIFN-γ, or combinations of other Th1 cytokines does not predict risk of tuberculosis disease in newborn 10 weeks after BCG vaccination[3].The efficacy rates of BCG vaccination have varied from 0-80% in studies of different patient populations. It reduces the risk of tuberculosis in all forms by 50% and severe, non-pulmonary forms such as childhood tuberculosis meningitis, by at least 70%[4]. BCG vaccine had lost the protective effect for last 10 years but there is little evidence to support more prolonged benefit, when administrated to adults. Large studies have shown very limited efficacy of revaccination with BCG in adolescents and adults[5].After vaccination it requires an *in vitro* analysis for identification of relevant vaccine candidate for monitoring the action of a protective cell-mediated immune response[6]. BCG is a live vaccine and the development of protective immunity after vaccination appears to require BCG replication in the host for a certain period of time. However, this can be prevented by a pre-existing immune response that can react to BCG. Currently there are two major categories of potential vaccine candidates against *M. tuberculosis*[7]. The first category follows the approach to improve the current BCG vaccine through recombinant BCG strains constructs with improved vaccine efficacy that are intended to replace BCG[8]. Another approach includes the use of booster vaccines such as fusion protein subunits, inactivated whole cell, whole cell lysates, and naked DNA vaccines. Boosting BCG-induced immunityincludes administration of BCG as the prime with a subunit vaccine (DNA or protein) either to infants and young children before they are exposed to tuberculosis (boosting shortly after BCG vaccination) or as a separate booster to young adults (boosting several years after BCG vaccination)[9].The first subgroup includes viral vectors Modified Vaccinia Ankara (MVA), replication-deficient adenovirus (Ad) that expresses immunodominant*M. tuberculosis* antigens for the strong Th1-dominated immune response to the expressed heterologous antigen and induce CD^{8+} T-cell response. The MVA and Ad5 virus carriers express Ag85A whereas the Ad35 co-expresses the antigens Ag85A and Ag85B[10]. Current research has focused on several antigens with subunit vaccines as the booster that induce strong INF-γ production[9].During *M. tuberculosis* infection, including initiate stage, active tuberculosis and latent tuberculosis, tubercle bacilli population consists of growing and non-growing subpopulations with various metabolic states. Therapeutic ideal subunit vaccines should target the mycobacterial subpopulations. If subunit vaccine consisting of key antigens of *M. tuberculosis* is used, it helps to restore the immune response even in adults which overcomes the limitation of conventional BCG vaccine[7]. In order to develop effective tuberculosis vaccines, antigens mainly expressed by the bacteria in various metabolic stages were selected to construct fusion proteins.Several strategies were devised to construct vaccines that could replace BCG with improved antigenicity and immunogenicity. The recombinant BCGΔUreC:Hly vaccine developed by Kaufmann group has increased immunogenicity compared to parental BCG[11]. Hly (listeriolysin from *Listeria monocytogenes*) is a component of vaccine which is active only in acidic pH because BCG neutralizes of phagosomal pH rendering

Hly biologically inactive.In order to overcome this as a first step, deletion of urease C (UreC) gene is done so that acidic pH is maintained in the phagosome[10].New antigens currently assessed for next generation tuberculosis subunit vaccines include the heparin-binding hemagglutinin (HBHA), a protein shared by *M.tuberculosis* and BCG but differentially methylated. The HBHA is an adhesion molecule for lack of phagocytic cells and is involved in extrapulmonary propagation of *M.tuberculosis*[12]. Currently, the strategies for developing a new tuberculosis vaccine safer and more effective to replace the BCG with stronger vaccine which provides a longer duration of protection. The *M. tuberculosis* contains lot of protective antigens of relevance for the generation of new anti-tuberculosis vaccine and the proteins are CFP10, ESAT6 and Ag85B[13]. Two major antigens produced by *M. tuberculosis* during infection are CFP10 and ESAT6 presently being used for the development of subunit vaccines. CFP10 from *M. tuberculosis* is a well characterised immunodominant 14kDa protein known to evoke a very potent IFN-γ response in mice and human.CFP10 is a prominent target protein found in culture filtratesand forms a complex with other protein, ESAT6.CFP10 contains at least two epitopes, one containing 10 amino acids and another containing 9 amino acids. Deleting a single amino acid from amino or carboxy terminus of either peptide reduces the IFN-γ production[14]. INF-γ is currently used as a biomarker for tuberculosis vaccine, INF-γ alone is insufficient for protection against tuberculosis. As CFP10 and INF-γ are small peptides, they could not able to enhance the immunogenicity for longer period. So CFP10 is fused with coat protein (CP) assembled to form virus like particles (VLPs) and it enhances the immunogenicity for longer time when compared to the individual antigens. Likewise, INF-γ is also fused with coat protein to enhance the immunogenicity[15]. The potyvirus is a largest group of plant virus consist of coat protein (CP) synthesis from Johnsongrass mosaic virus (JGMV) expressed in bacteria or yeast resulted in the formation of potyvirus like particles (PVLPs)[16]. Development of new vaccines have two basic strategies: i) Replacing BCG by either improved BCG by recombinant DNA technology or by genetically attenuated *Mycobacterium tuberculosis*. The genetically improved recombinant BCG could be safer, more immunogenic and induce longer protection. ii) Improving BCG by introducing genetic modifications for targeting essential immune pathways[17].For vaccine development, expression of the antigenic peptides in the context of the viral CP is desired because of the high levels of accumulation of this protein when heterologously expressed in *E. coli*. The coat protein gene of sugarcane streak mosaic virus (SCSMV) was expressed in *E. coli* found virus like particles (VLPs) when purified by sucrose density gradient centrifugation. The CFP10 is fused with ESAT6 shows result in a sensitivity of 90% for detection of *M. tuberculosis* infection with a specificity of 95%[18].

MATERIALS AND METHODS

Purification of recombinant fusion protein using metal affinity chromatography

Sample preparation

The IPTG induced pellet (pCFP10 transformed into *E. coli* BL21/DE3 cells) was taken from -80°C and resuspended in 30 mL of binding buffer (buffer A: 20 mMTrisHCl (pH 8.0),100 mMNaCl, 1 mM PMSF). The resuspended pellet was sonicated using ultrasonicator (Sartorius, Germany) for 30 cycles of 30 sec strike with 1 min pause at frequency of 80 MHz. The sonicated samples were centrifuged at 10,000 rpm for 30 min at 4°C and the supernatant was filtered through a 0.2 micron membrane at 4°C[19].

Column preparation

HiTrap™ Chelating HP 1 mL column (GE Healthcare, CA, USA) was taken and washed with 5 column volumes (CV) of nanopure water in order to wash away the 20% ethanol. Then 5 CV of 0.1 M $NiSO_4$ solution was loaded in the column, followed by 5 CV of nanopure water and was saturated with buffer A.

Sample loading into the column

The crude filtrate was loaded into the column using the peristaltic pump (GE Healthcare, CA, USA) and the resultant flow from the column was collected as flow through. The column was washed with buffer A and the unbound protein was collected as wash. The column was then connected to FPLC(GE Healthcare, CA, USA) and elution was carried out with elution buffer (buffer B; 20 mMTrisHCl (pH 8.0), 100 mMNaCl, 1 mM PMSF, 0.5 M Imidazole)[20]. The eluted samples were collected as 1 mL fractions in test tubes and the chromatogram obtained was also noted. 10 µL of the identified peak fractions were aliquoted and 10 µL of SSB was added, boiled for 5 min, resolved on 10% SDS PAGE (Bio Rad, CA, USA) and the gel was documented after staining with Coomassie Brilliant blue R 250. Similar purification procedure was carried out for two protein samples (CP:CFP10 and CP:IFN-γ) as described above.

Purification of recombinant fusion protein CFP10 using anion exchange chromatography (AIEC)

The anion exchangecolumn was taken and washed with 10 CV of nanopure water proceeding with 5 CV of elution buffer B1 (20 mMTrisHCl (pH 8.0), 500 mM NaCl,1 mM DTT), 10 CV of nanopure water and 10 CV of start buffer A1 (20 mMTrisHCl (pH 8.0), 1 mM DTT). The entire process was performed at a flow rate of 0.5 mL/min. The column was equilibrated by washing with 10 CV of start buffer. The eluted protein samples from metal affinity column were pooled and the protein samples were passed through the anion exchange column and the resultant flow was collected as flow through. The column was washed with 10 CV of buffer A1 and unbound proteins were collected as wash. The column was then connected to FPLC (GE Healthcare, CA, USA) and elution was carried out with buffer B1[21]. The eluted samples were collected as 1 mL fractions in test tubes and the chromatogram obtained was also noted. 10 µL of the identified peak fractions were aliquoted and 10 µL of SSB was added, boiled for 5 min, resolved on 12.5% SDS PAGE (Bio Rad, Hercules, CA, USA) and the gel was documented after staining with Coomassie Brilliant blue R 250.

Purification of recombinant fusion protein by sucrose density gradient centrifugation

The IPTG induced pellet (CP:CFP10 transformed into *E. coli* DH5α cells) was taken from -80°C and resuspended in 30 ml of buffer II (100 mMTrisHCl (pH 8.0), 10 mM EDTA, 1 mM PMSF). The resuspended pellet was sonicated using ultrasonicator (Sartorius, Germany) for 40 cycles of 1 min strike with 2 min pause at frequency of 100 MHz. The sonicated samples were centrifuged at 10,000 rpm for 30 min at 4°C and the supernatant was filtered through a 0.2 micron membrane at 4°C. The ultracentrifuge tubes were soaked in 70% ethanol for 20 min and washed with nanopure water. The samples were loaded into the ultracentrifuge tubes. These tubes were balanced and placed

on the ultracentrifuge (Beckman Coulter, USA) using Ti70 rotor. The samples were centrifuged at 50,000 rpm for4 hr at 4C and the pellet was resuspended in buffer II. The resuspended pellet was centrifuged at 10,000 rpm for 10 min at 4C. Initially 40% sucrose stock was prepared. From the prepared stock, different concentration of sucrose 30%, 20% and 10% were prepared using buffer II. 2 mL of each sucrose solution was added to the centrifuge tube with the descending order of concentration. The tubes were weighed and balanced with the protein sample and ultracentrifuged for 4 hr at 30,000 rpm at 15C in ultracentrifuge (Beckman Coulter, USA) using SW41 rotor. After 3 hr, 500 µL of sample was collected in eppendorfs by making a hole in the bottom of the tube and the samples were resolved on 10% SDS PAGE followed by silver staining.Similar purification procedure was carried out for two protein samples (CP and CP:IFN-γ) as described above.

Silver staining

After electrophoresis, gel was removed from the cassette and incubated with fixative solution for 20 min and the gel was washed thrice with nanopure water. 50 mL of reducing solution was added and incubated for 2 min. The gel was washed twice with nanopure water. Silver nitrate solution was added and incubated for 7-10 min to allow the silver ions to bind to proteins. After incubation, the gel was rinsed twice with nanopure water for 20-60 sec. The gel was rinsed with developing solution for 2-5 min and the reaction was terminated by addition of50 mL of stopping solution. The protein bands were visualized on a white platform and the gel was documented.

Quantification of purified proteins

The purified protein was quantified using Bradford assay with BSA as a standard. An aliquot of the purified protein samples was incubated with Bradford reagent (Sigma, Bangalore, India) and the absorbance was recorded at 595 nm using a spectrophotometer (Hitachi, Japan). The concentration of the purified proteins CP, CP:CFP10 and CP:IFN-γ were estimated by extrapolation.

Western blotting

20 µL of the purified protein fractions were resolved on a 12.5% polyacrylamide gel and electrophoresed at 90 V. The Western blot transfer was performed at 250 mA for 2 hr. After transfer, the membrane was incubated with blocking solution at room temperature for overnight.Then, the membrane was washed thrice for 7 min with phosphate buffer saline with tween-20 (PBST: 0.5 M Phosphate buffer, 15 mMNaCl, 0.05% Tween-20), and incubated with primary antibody (1:200 dilution) for 1 hr.This was followed by washing the membrane thrice for 7 min in PBST and later incubated with secondary antibody (1:5000 dilution) for 1hr.This was followed by washing the membrane thrice for 7 min in PBST.After incubation, the membrane was soaked in alkaline phosphatase buffer (100 mMTris Base (pH 9.5), 100 mMNaCl, 5 mM $MgCl_2$) for 2 min and developed using BCIP/NBT solution (Sigma, Bangalore, India) for 5 min.

Immunization and Raising antisera

Purified coat protein was immunized in mice to raise antibody against the coat protein. 6-8 week old albino mice were procured and was left for about two weeks as a period of adaptation in our laboratory. Dosage is prepared initially by adding coat protein (300 µg) along with PBS (30 µL). This mixture is then added to Freund's complete adjuvant (50 µL) that was administered intraperitoneally, using an insulin syringe. Booster dosages were provided at regular intervals of time (two weeks) and the control mice was supplemented only with that of the PBS buffer. Once the dosage is completed, blood should be collected through tail bleeding.

RESULTS

Purification of recombinant fusion protein (CP:IFN-γ) using metal affinity chromatography

IPTG induced pellet CP:IFN-γ (pSTE47 plasmid transformed into*E. coli* BL21/DE3) subjected into sonication and purification by metal affinity chromatography using nickel chelating column. The column was washed and any bound protein into the column

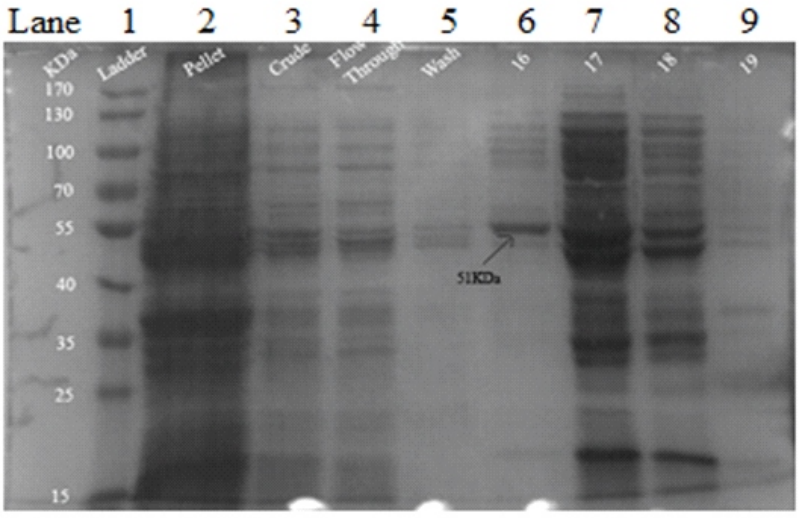

Fig. 1: The chromatogram obtained during the purification of fusion protein (CP:INF-γ) using Ni^{2+} NTA column by fast performance liquid chromatography. Imidazole at a concentration of 250 mM elutes the fusion protein (CP:INF-γ). Fractions obtained during the purification of recombinant fusion protein (CP:INF-γ) using metal affinity chromatography were resolved on 10% SDS PAGE. Lane 1, 2, 3, 4 and 5 corresponds to the protein marker, pellet, crude, flow through and wash respectively. Lane 6, 7, 8 and 9 corresponds to the FPLC peak fractions.

was eluted by buffer B. The target protein were eluted in the elution fractions and contaminant proteins was collected as flow through (FT). The elution fractions and FT obtained during chromatography were collected and the fractions were resolved on 10% SDS PAGE and the molecular weight of the fusion protein (CP:IFN-γ) was determined as 51 kDa as shown in figure 1.

Figure 1.The chromatogram obtained during the purification of fusion protein (CP:IFN-γ) using Ni^{2+} NTA column by fast performance liquid chromatography. Imidazole at a concentration of 250 mM elutes the fusion protein (CP:IFN-γ). Fractions obtained during the purification of recombinant fusion protein (CP:IFN-γ) using metal affinity chromatography were resolved on 10% SDS PAGE. Lane 1, 2, 3, 4 and 5 corresponds to the protein marker, pellet, crude, flow through and wash respectively. Lane 6, 7, 8 and 9 corresponds to the FPLC peak fractions.

Purification of recombinant fusion protein (CP:CFP10) using metal affinity chromatography

IPTG induced cell pellet (CP:CFP10 plasmid transformed into *E. coli* DH5α) cells was resuspended in buffer A were subjected into sonication and purification by metal affinity chromatography using nickel chelating column. The column was washed and any bound protein into the column was eluted by buffer B. The target protein were eluted in the elution fractions and contaminant proteins was collected as flow through (FT). The elution fractions and FT obtained during chromatography were collected and the fractions were resolved on 10% SDS PAGE and the molecular weight of the fusion protein (CP:CFP10) was determinedas 48 kDa as shown in figure 2.

Figure 2. The chromatogram obtained during the purification of fusion protein (CP:CFP10) using Ni^{2+} NTA column by fast performance liquid chromatography. Imidazole at a concentration of 125 mM elutes the fusion protein (CP:CFP10). Fractions obtained during the purification of recombinant fusion protein (CP:CFP10) using metal affinity chromatography were resolved on 10% SDS PAGE. Lane 1, 2, 3 and 4 corresponds to the protein marker, crude sample, flow through and wash respectively. Lanes 5, 6, 7 and 8 corresponds to the FPLC peak fractions.

Purification of recombinant CFP10 protein using metal affinity chromatography

IPTG induced cell pellet (pCFP10 plasmid transformed into *E. coli* BL21/DE3) cells was resuspended in buffer A were subjected into sonication and purification by metal affinity chromatography using nickel chelating column. The column was washed and any bound protein into the column was eluted by buffer B. The target protein were eluted in the elution fractions and contaminant proteins was collected as flow through (FT). The elution fractions and FT obtained during chromatography were collected and the fractions were resolved on 12.5% SDS PAGE and the molecular weight of the fusion protein was determinedas 14 kDa as shown in figure 3.

Figure 3.The chromatogram obtained during the purification of fusion protein (CFP10) using Ni^{2+} NTA column by fast performance liquid chromatography. Imidazole at a concentration of 150 mM elutes the fusion protein (CFP10).Fractions obtained during the purification of recombinant fusion protein CFP10 using metal affinity chromatography were resolved on 12.5% SDS PAGE. Lane 1, 2, 3 and 4 corresponds to the protein marker, crude sample, flow through and wash respectively. Lanes 4, 5, 6, 7, 8 and 9 corresponds to the FPLC peak fractions.

Purification of recombinant CFP10 protein using AIEC

IPTG induced pellet pCFP10 was resuspended in buffer A were subjected into sonication and purification by metal affinity

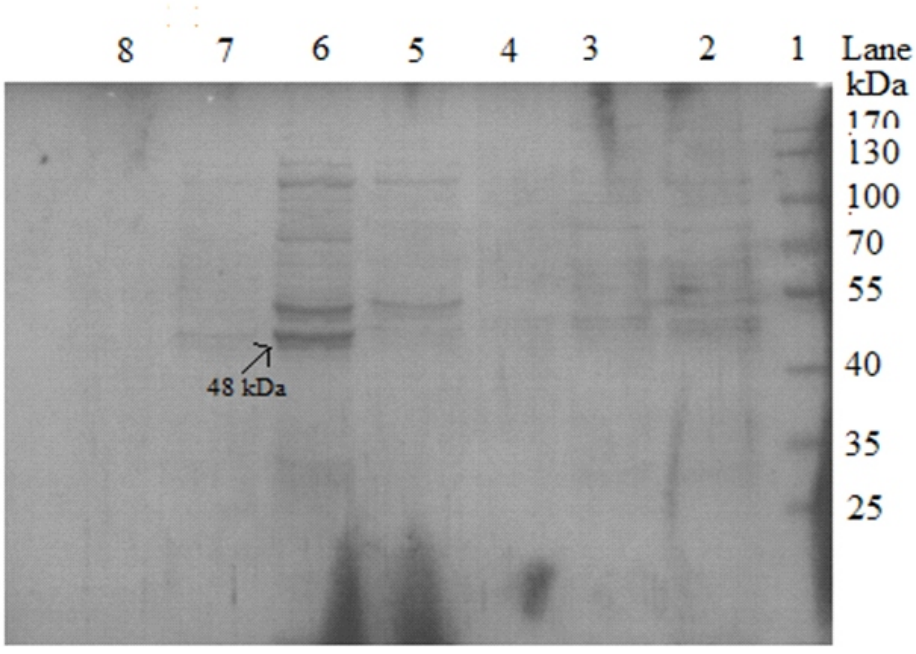

Fig. 2: The chromatogram obtained during the purification of fusion protein (CP:INF-γ) using Ni^{2+} NTA column by fast performance liquid chromatography. Imidazole at a concentration of 250 mM elutes the fusion protein (CP:INF-γ). Fractions obtained during the purification of recombinant fusion protein (CP:INF-γ) using metal affinity chromatography were resolved on 10% SDS PAGE. Lane 1, 2, 3, 4 and 5 corresponds to the protein marker, pellet, crude, flow through and wash respectively. Lane 6, 7, 8 and 9 corresponds to the FPLC peak fractions.

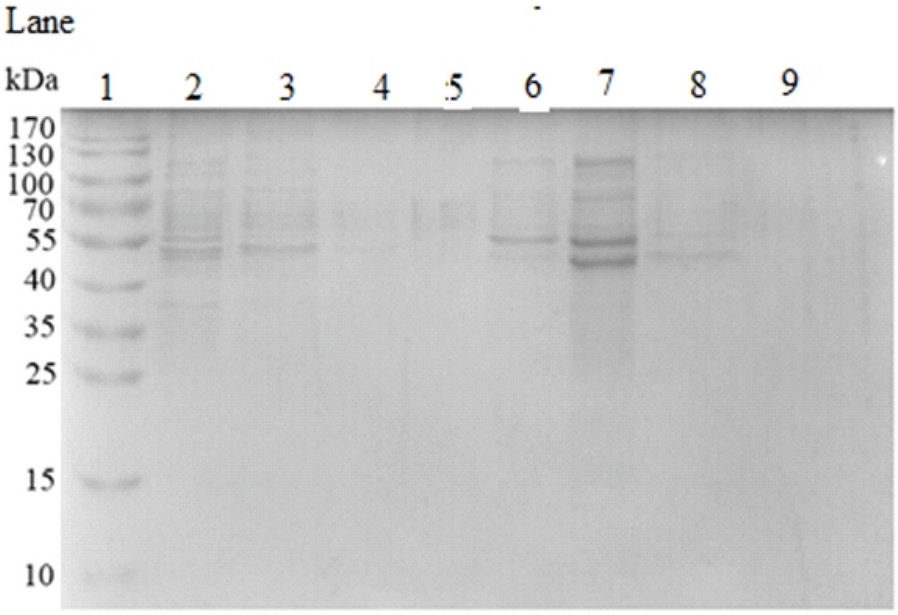

Fig. 3: The chromatogram obtained during the purification of fusion protein (CFP10) using Ni^{2+} NTA column by fast performance liquid chromatography. Imidazole at a concentration of 150 mM elutes the fusion protein (CFP10). Fractions obtained during the purification of recombinant fusion protein CFP10 using metal affinity chromatography were resolved on 12.5% SDS PAGE. Lane 1, 2, 3 and 4 corresponds to the protein marker, crude sample, flow through and wash respectively. Lanes 4, 5, 6, 7, 8 and 9 corresponds to the FPLC peak fractions.

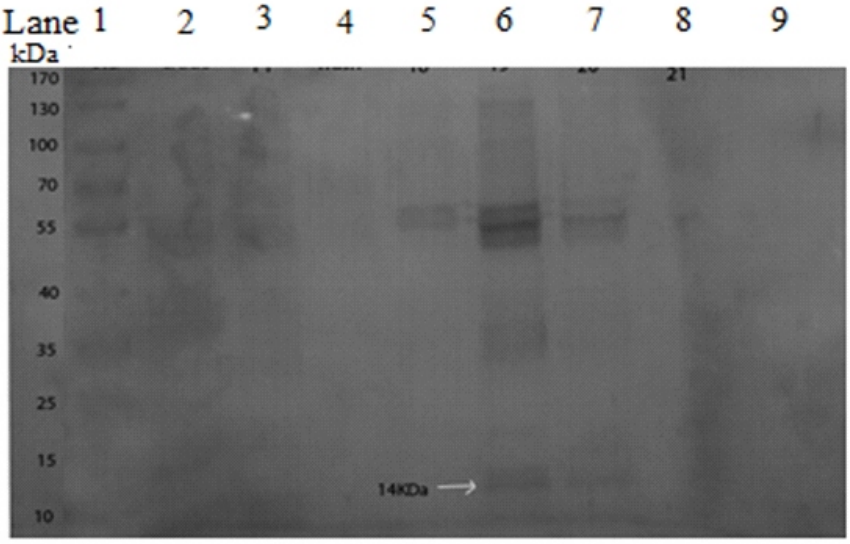

Fig. 4: The chromatogram obtained during the purification of fusion protein (CFP10) using anion exchange column by fast performance liquid chromatography. Imidazole at a concentration of 280 mM elutes the fusion protein CFP10. Fractions obtained during the purification of recombinant fusion protein CFP10 using anion exchange chromatography were resolved on 12.5% SDS PAGE. Lane 1, 2, 3 and 4 corresponds to the protein marker, crude sample, flow through and wash respectively. Lanes 5, 6, 7, 8 and 9 corresponds to the FPLC peak fractions.

chromatography using nickel chelating column. The column was washed and bound proteins were eluted by buffer B. To further purify the partially purified protein, the collected fractions from metal affinity chromatography were pooled, and subjected to anion exchange chromatography using HQ sepharose column. The column was washed and bound proteins were eluted by buffer B1. The target protein were eluted by increasing the salt concentration. The eluted fractions of chromatography were resolved on 12.5% SDS PAGE. The molecular weight of the fusion protein CFP10 was determinedas 14 kDa as shown in figure 4.

Figure 4. The chromatogram obtained during the purification of fusion protein (CFP10) using anion exchange column by fast performance liquid chromatography. Imidazole at a concentration of 280 mM elutes the fusion protein CFP10. Fractions obtained during the purification of recombinant fusion protein CFP10 using anion exchange chromatography were resolved on 12.5% SDS PAGE. Lane 1, 2, 3 and 4 corresponds to the protein marker, crude sample, flow through and wash respectively. Lanes 5, 6, 7 and 8 corresponds to the FPLC peak fractions.

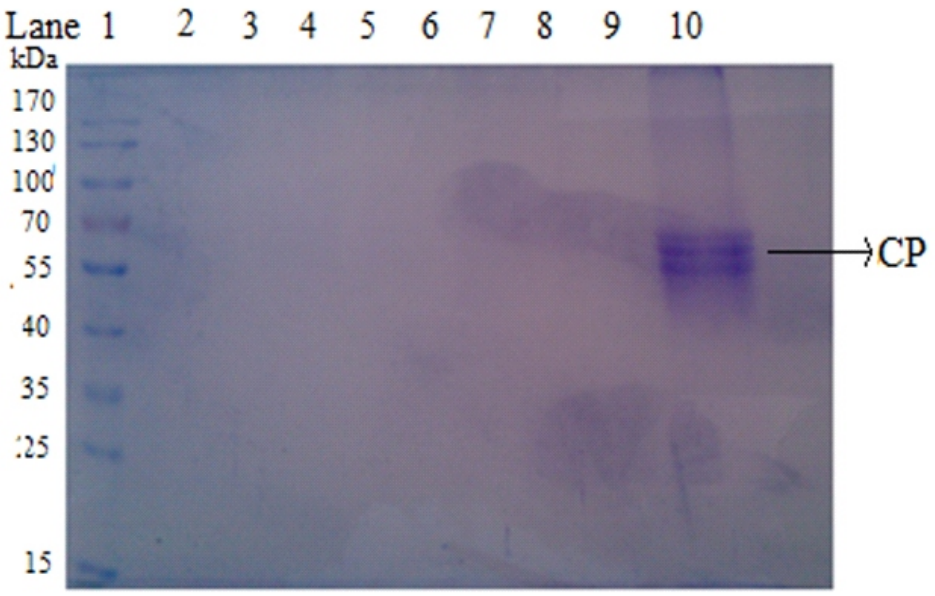

Fig. 5: Tetermination of molecular weight of the coat protein by resolving the fractions on 10% SDS PAGE. Lane 1 corresponds to the protein marker and the consecutive lanes that ranges from 2 to 10 corresponds to the sucrose density gradient fractions.

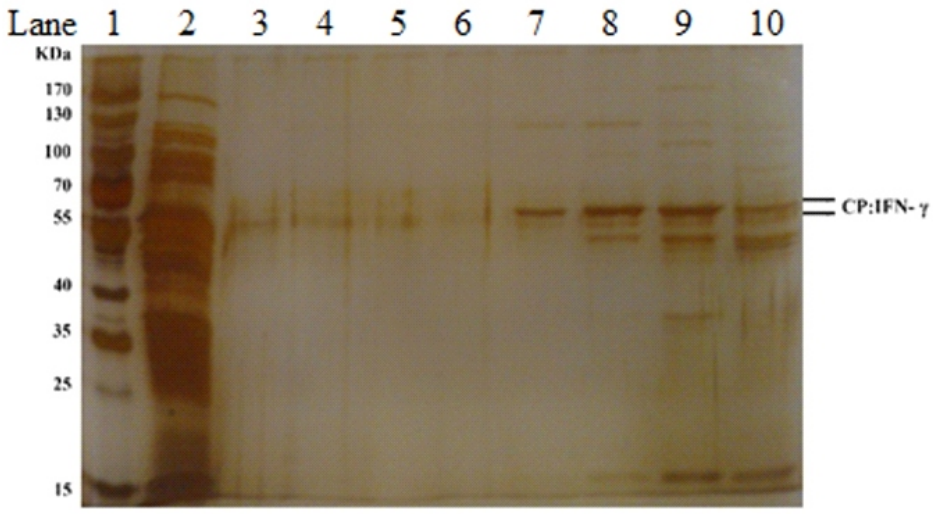

Fig. 6: Fractions obtained from sucrose density gradient centrifugation were resolved on 10% SDS PAGE. Lane 1 and 2 corresponds to the protein marker and ultracentrifuge supernatant respectively. Consecutive lanes from 3 to 10 corresponds the sucrose density gradient fractions.

Purification of recombinant protein CP by sucrose density gradient centrifugation

The recombinant coat protein was purified by sucrose density gradient centrifugation. The fractions that were collected from ultracentrifugation were resolved on 10% SDS PAGE and the molecular weight of the coat protein was found to be 34 kDa as shown in figure 5.

Figure 5. Determination of molecular weight of the coat protein by resolving the fractions on 10% SDS PAGE. Lane 1 corresponds to the protein marker and the consecutive lanes that ranges from 2 to 10 corresponds to the sucrose density gradient fractions.

Purification of recombinant fusion protein CP:IFN-γ by sucrose density gradient centrifugation

The fusion protein CP:IFN-γ was purified by sucrose density gradient centrifugation. The fractions were collected from ultracentrifugation were resolved on 10% SDS PAGE and the molecular weight of the fusion protein (CP:IFN-γ) was determined as 51 kDa as shown in figure 6.

Figure 6. Fractions obtained from sucrose density gradient centrifugation were resolved on 10% SDS PAGE. Lane 1 and 2 corresponds to the protein marker and ultracentrifuge supernatant respectively. Consecutive lanes from 3 to 10 corresponds the sucrose density gradient fractions.

Purification of recombinant fusion protein CP:CFP10 by sucrose density gradient centrifugation

The fusion protein CP:CFP10 was purified by sucrose density gradient centrifugation. The fractions obtained from sucrose

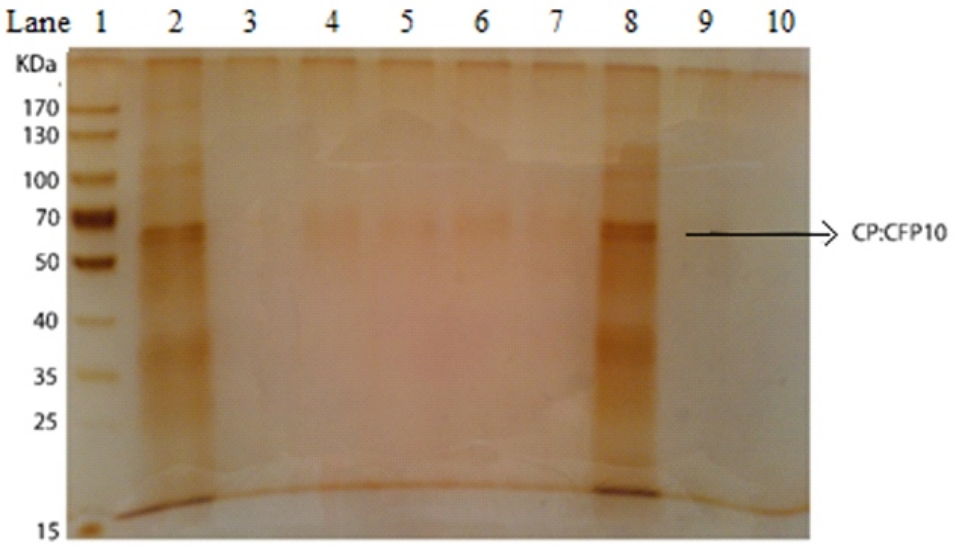

Fig. 7: Fractions obtained from ultracentrifugation were resolved on 10% SDS PAGE. Lane 1 and 2 corresponds to protein marker and ultracentrifuge supernatant. Consecutive lanes from 3 to 10 corresponds the sucrose density gradient fractions.

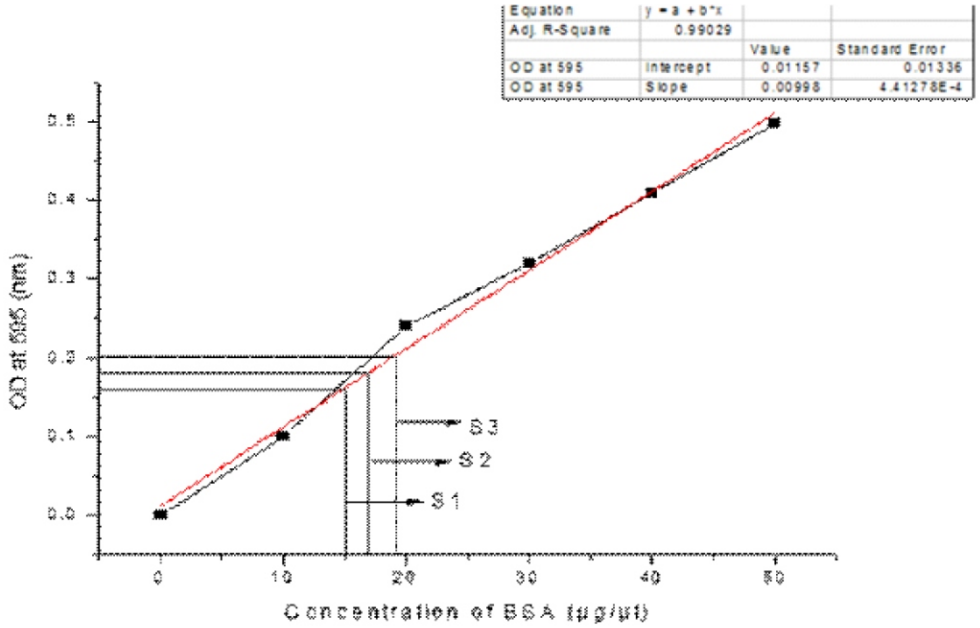

Fig. 8: Quantification of the purified proteins by Bradford assay using BSA as the standard. The concentration of the purified protein S1 (CP), S2 (CP:CFP10), S3 (CP:IFN-γ) were found to be15 µg μL^{-1}, 16 µg μL^{-1} and 19 µg μL^{-1} respectively.

density gradient centrifugation were resolved on 10% SDS PAGE and the molecular weight of the fusion protein (CP:CFP10) was found to be 48 kDa as shown in the figure 7.

Figure 7. Fractions obtained from ultracentrifugation were resolved on 10% SDS PAGE. Lane 1 and 2 corresponds to protein marker and ultracentrifuge supernatant. Consecutive lanes from 3 to 10 corresponds the sucrose density gradient fractions.

Quantification of purified protein by Bradford assay:

The concentration of the purified proteins was quantified by Bradford assay. The standard graph was plotted and the relative optical density was measured at 595 nm [Figure 8].

Figure 8. Quantification of the purified proteins by Bradford assay using BSA as the standard. The concentration of the purified protein S1 (CP), S2 (CP:CFP10), S3 (CP:IFN-γ) were found to be15 µg μL^{-1}, 16 µg μL^{-1} and 19 µg μL^{-1} respectively.

Western Blot

The western blot analysis was performed for all the three purified proteins (CFP10, CP:CFP10 and CP:IFN-γ) using antibody raised against CFP10. CP:IFN-γ is used as control. The presence of CP:CFP10 was confirmed, by the presence of band in lane 3 of figure 9. Lane 1 corresponding to CFP10 does not show any band as it might be due to degradation of protein.

Figure 9. Western blot was carried out using the CFP10 antibody to confirm the presence of CFP10 and CP:CFP10.

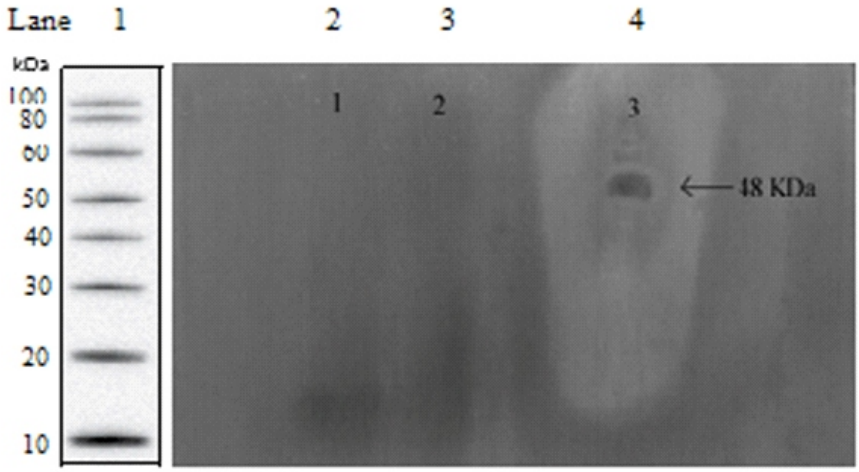

Fig. 9: Western blot was carried out using the CFP10 antibody to confirm the presence of CFP10 and CP:CFP10. Protein bands were detected by incubating with alkaline phosphatase conjugate antibody (Sigma, Bangalore, India) and is visualized using BCIP/NBT reagent. Lane 1, 2, 3 and 4 corresponds to protein marker, CFP10, CP:IFN-γ as control and CP:CFP10 respectively.

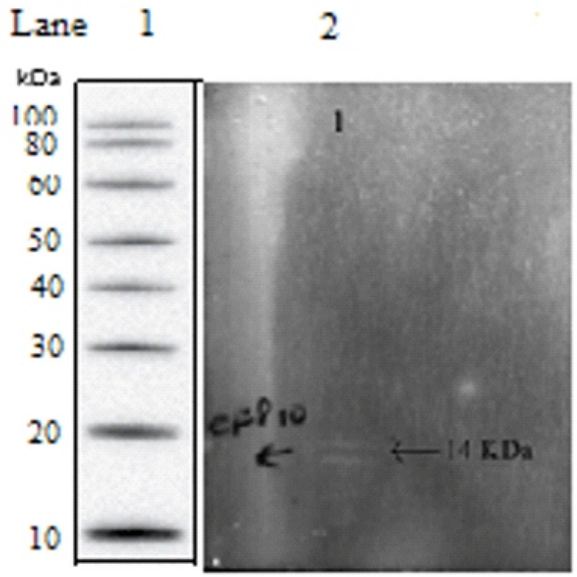

Fig. 10: Western blot was performed using the CFP10 antibody. Protein bands were detected by incubating with alkaline phosphatase conjugate antibody (Sigma, Bangalore, India) and visualized BCIP/NBT reagent. Lane 1 and 2represents protein marker and CFP10.

Protein bands were detected by incubating with alkaline phosphatase conjugate antibody (Sigma, Bangalore, India) and is visualized using BCIP/NBT reagent. Lane 1, 2 and 3 corresponds to CFP10, CP:IFN-γ as control and CP:CFP10 respectively.

The CFP10 protein was again purified and subjected to western blot analysis as shown in the figure 10. Lane 1 which corresponds to CFP10 protein shows band which is unclear which might be due to over exposure of nitrocellulose membrane to substrate.

Figure 10.Western blot was performed using the CFP10 antibody. Protein bands were detected by incubating with alkaline phosphatase conjugate antibody (Sigma, Bangalore, India) and visualized BCIP/NBT reagent. Lane 1represents CFP10.

DISCUSSION

Currently there are two major categories of potential vaccine candidates against *M. tuberculosis*. One is BCG replacement vaccines, including live recombinant BCG (rBCG), attenuated mutants of *M. tuberculosis*, as well as modified non-pathogenic mycobacteria (*M. vaccae* and *M. smegmatis*) and the other is booster vaccines such as fusion protein subunits, inactivated whole cell, whole cell lysates, and naked DNA vaccines. One major reason for the failure of BCG in adults is that the protective immune responses induced by BCG inoculation at infant waned as children grow up. For this reason, it is believed that using subunit vaccine consisting of key antigens of *M. tuberculosis* as a booster vaccine in adolescence or adulthood to restore the immune response[7].Immune response of host could be a good indicatorof exposure to *M. tuberculosis.*Proteins produced by *M. tuberculosis* is culture filtrate protein (CFP-10), early secreted antigenic target protein (ESAT-6), Ag85 etc. These proteins are well characterised immunodominant that induces and increase the production of IFN-γ cells. The recombinant CFP-10 was produced in fusion with astretch of eight histidines at its N-terminus. The present study focuses on the optimization of various purification techniques adopted to purify the recombinant proteins. RecombinantCFP-10 was expressed in the insoluble form and waspurified by metal ion affinity chromatography[22].This form of protein could only be purified andrefolded in low-yield after a time-consuming and laborintensive process[23, 24].Solubilizing and purifying of desired proteins is one ofthe difficulties in other studies, while in our study thisprotein was expressed substantially in the soluble formthat could be easily purified by metal affinity chromatography.In order to confirm the presence of CFP10, it was further purified by anion exchange chromatography. Though, CFP10and IFN-γ is a protective antigen it could not able to enhance the immunogenicity for longer time because of lower molecular weight protein and also vulnerable for proteolytic degradation. Hence, it was fused withpotyvirus coat protein to form virus like particles (VLPs) that could enhance the immunogenicity of the fusion partner. For vaccine development, expression of the antigenic peptides in the context of the viral CP is desired because of the high levels of accumulation of this protein when heterologously expressed in *E. coli*. The fusion of an

epitope to a large carrier molecule such as the coat protein (CP) of plant virus could enhance the immunogenicity and the stability of the epitope. The fulllengthCP of a potyvirus is expressed in *E. coli* or *S. cerevisiae*, the resulting products readily assemble to formPVLPs.The PVLPs were flexuous, similar inwidth to that of JGMV particles but heterogeneous inlength.It is unclear at this stage whether the CPmonomers assembled to form PVLPs inside the cellsand/or during processing of the cell extracts[16].Proteins such as CFP10, CP:CFP10 and CP:IFN-γ were purified using metal affinity chromatography and sucrose density gradient fractionation. Purified recombinant mycobacterial antigenic proteins can act as a biomarker, potential vaccine candidate and it could also help diagnosing tuberculosis.

CONCLUSION

Antigens suchas CFP10, ESAT6, Ag85 etc., play important role in causing tuberculosis and can induce IFN-γ release in the host cell. CFP10 is a small secreted protein found in the culture filtrate of *M. tuberculosis*. As CFP10 and INF-γ are small peptides, they could not able to enhance the immunogenicity for longer period. So CFP10 is fused with coat protein (CP) assembled to form virus like particles (VLPs) and it enhances the immunogenicity for longer time when compared to the individual antigens. The fusion proteins CFP10, CP:CFP10 and CP:IFN-γ was successfully purified by using chromatography techniques and ultracentrifugation method. The purifiedrecombinant fusion proteins can be used as diagnostic tool and development of vaccine against tuberculosis.

ACKNOWLEDGEMENT

Author are thankful to Dr.M.L Stephan Raj, Professor, Department of Biotechnology, MepcoSchlenk Engineering College, for his guidance throughout the period of study.

REFERENCES

1. Antas P R, Castello-Branco L R. New vaccines against tuberculosis: lessons learned from BCG immunisation in Brazil.Trans. R. Soc. Trop. Med. Hyg. 2008: 102(7): 628-30.

2. Ottenhoff T H. New pathways of protective and pathological host defense to mycobacteria. Trends. Microbiol. 2012: 20: 419-28.

3. Kagina B M, Abel B, Scriba T J, Hughes E J, Keyser A, Soares A, Gamieldien H, Sidibana M. Specific T cell frequency and cytokine expression profile do not correlate with protection against tuberculosis after bacillus Calmette-Guérin vaccination of newborns. Am. J.Respir. Crit. Care. Med. 2010: 1073-9.

4. Colditz G A, Brewer T F, Berkey C S, Wilson M E, Burdick E, Fineberg H V,Mosteller F. Efficacy of BCG vaccine in the prevention of tuberculosis. Meta-analysis of the published literature. JAMA.1994: 271: 698-702.

5. Sterne J A, Rodrigues L C, Guedes I N. Does the efficacy of BCG decline with time since vaccination?. Int. J.Tuberc. Lung Dis.1998: 2: 200-7.

6. Brandt L, Oettinger T, Holm A, Andersen A B, Andersen P. Key epitopes on the ESAT-6 antigen recognized in mice during the recall of protective immunity to Mycobacterium tuberculosis. J.Immunol. 1996: 157: 3527-33.

7. Xin Q, Niu H, Li Z, Zhang G, Hu L, Wang B, Li J, Yu H, Liu W, Wang Y, Da Z, Li R, Xian Q, Zhang Y, Jing T, Ma X. and Zhu B.Subunit vaccine consisting of multi-stage antigens has high protective efficacy against Mycobacterium tuberculosis infection in mice.PLoS One.2013: 8: e72745.

8. Michael J Brennan, JelleThole. Tuberculosis Vaccines: A Strategic Blueprint for the Next Decade. Tuberculosis (Edinb). 2012.

9. Claus Aagaard, TrucThi Kim Thanh Hoang, Angelo Izzo, Rolf Billeskov, JoLynnTroudt, Kim Arnett, Andrew Keyser, Tara Elvang, Peter Andersen, Jes Dietrich. Protection and Polyfunctional T Cells Induced by Ag85B-TB10.4/IC31 against Mycobacterium tuberculosis Is Highly Dependent on the Antigen Dose.PLoS ONE. 2009: 4(6): e5930.

10. Stefan HE Kaufmann. Future Vaccination Strategies against Tuberculosis: Thinking outside the Box.J.Immuni.2010: 33(4): 567-77.

11. Grode L, Seiler P, Baumann S, Hess J, Brinkmann V, Nasser Eddine A, Mann P, Goosmann C, Bandermann S, Smith D. Increased vaccine efficacy against tuberculosis of recombinant Mycobacterium bovisbacilleCalmette-Gue´ rin mutants that secrete listeriolysin. J. Clin. Invest. 2005: 115: 2472–2479.

12. HosungSohn, Jong Seok Kim,Sung Jae Shin, Kwangwook Kim, ChoulJae Won, Woo Sik Kim, KiNam Min, Han Gyu Choi, Je Chul Lee, Jeong-Kyu Park, Hwa Jung Kim.Targeting of Mycobacterium tuberculosis Heparin-Binding Hemagglutinin to Mitochondria in Macrophages.PLoS.Pathog. 2011: 7(12): e1002435.

13. Li L, Qiao D, Li Q, Zhang X, Lao S.and Wu C. Distinct polyfunctional CD4+ T cell responses to BCG, ESAT-6 and CFP-10 in tuberculous pleurisy. Tuberculosis (Edinb).2012: 92: 63-71.

14. Shams H, Klucar P, Weis S E, Lalvani A, Moonan P K, Safi H, Wizel B, Ewer K, Nepom G T, Lewinsohn D M, Andersen P. and Barnes P F. Characterization of a Mycobacterium tuberculosis peptide that is recognized by human CD4+ and CD8+ T cells in the context of multiple HLA alleles. J.Immunol. 2004: 173: 1966-77.

15. Jun Liu. New Vaccine Against Tuberculosis: CurrentDevelopments and Future Challenges.Science Foundation in China. 2009: 17(2).

16. Mittur NJagadish, Colin W Ward, Keith H Gough, Peter A Tulloch, Loyce A Whittaker, Dharma D Shukla. Expression of potyvirus coat protein in Escherichia coli and yeast and itsassembly into virus-like particles.Journal of General Virology. 1991: 72: 1543-1550.

17. Ottenhoff T H. and Kaufmann S H. Vaccines against tuberculosis: where are we and where do we need to go?.PLoS.Pathog.2012: 8: e1002607.

18. Hema M, Subba Reddy Ch V, Savithri H S. and Sreenivasulu P. Assembly of recombinant of coat protein ofsugarcane streak mosaic virus into potyvirus like particles. Indian Journal of Experimental Biology. 2008: 46: 793-96.

19. J. X. Feliu, R. Cubarsi, A. Villaverde. Optimized release of recombinant proteins by ultrasonication of E. coli cells. Biotechnology and Bioengineering. 1998: 58: 5.

20. Block H, Kubicek J, Labahn J, Roth U, and Scha¨fer F. Production andcomprehensive quality control of recombinant human Interleukin-1b: A case study fora process development strategy. Protein Expr. Purif. 2008: 27: 244–254.

21. Levison P R, Badger S E, Jones R M H, Toome D W, Streater M,Pathirana N D and Wheeler S. Validation studies in the regeneration of ion-exchange celluloses. J. Chromatogr. 1995: 702: 59–68.

22. Berthet FX, Rasmussen PB, Rosenkrands I, Andersen P, Gicquel B. A Mycobacterium tuberculosis operon encoding ESAT-6 anda novel low-molecularmass culture filtrate protein (CFP-10). Microbiology. 1998: 144: 3195-3203.

23. Meher AK, Bal NC, Chary KV R, Arora A. Mycobacterium tuberculosis H37Rv ESAT-6– CFP-10 complex formation confersthermodynamic and biochemical stability. FEBS J. 2006: 27:1445–1462.

24. Mukherjee P, Dutta M, Datta P, Dasgupta A, Pradhan R, Pradhan M,Kundu M, Basu J, Chakrabarti P. The RD1-encoded antigenRv3872 of Mycobacterium tuberculosis as a potential candidate forserodiagnosis of tuberculosis. Clin. Microbiol. Infect. 2007: 13(2):146-152.