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Full Length Research Paper

Cloning, expression and purification of 10 kd culture filtrared protein (CFP-10) of *Mycobacterium tuberculosis*

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Tuberculosis is a well-known infectious disease in human beings and domestic animals since ancient times. Tuberculin skin test as the only indicator of latent infection with *Mycobacterium tuberculosis* has a low specificity and sensitivity value. This test also cannot distinguish between tuberculosis infection and *Mycobacterium bovis* BCG vaccination, or exposure to environmental mycobacteria. Recent identification of the RD1 region of *M. tuberculosis* provides a new opportunity for the development of novel diagnostic tools. The purpose of this study was to clone and express the 10-kda culture filtrate protein (CFP-10) of *M. tuberculosis* in soluble form to be applicable for diagnostic purposes. To reach this aim, DNA was extracted from *M. tuberculosis* H37Rv and CFP-10 gene was then amplified by using specific primers. Specificity of PCR products were confirmed, and then were cloned into the pET102/D vector. After sequencing and confirming the insertion of desired PCR product into expression vector, recombinant plasmid was initially transformed into *Escherichia coli* TOP10 and was subsequently transformed into *E. coli* BL-21 to an expression recombinant protein. Recombinant CFP-10 was purified from the soluble supernatant by metal affinity chromatography. SDS-PAGE analysis was performed to confirm expression of CFP-10 as 28 kDa fusion protein. In this study, we cloned, expressed and purified sufficient amounts of CFP-10 that could be usable in sero-diagnostic tests in future.

Key word: *Mycobacterium tuberculosis*, CFP-10, cloning.

INTRODUCTION

Tuberculosis is a well-known infectious disease affecting human beings and domestic animals since ancient times. This disease is prevalent throughout the world including the developing and industrialized countries (Mustafa and Al-Attiyah, 2003). Diagnosis of tuberculosis in its early stages can lead to a decrease in the spreading of the disease (Jureen et al., 2006). Tuberculin skin test as the only indicator of latent infection with *Mycobacterium tuberculosis* has low specificity and sensitivity value (Colangeli, 2000).

Therefore, the accurate diagnosis of the infection in its latent phase with sensitive and specific tests based on specific *M. tuberculosis* antigens is desirable for early treatment and prevention of infection spreading (Meher et al., 2006).

In recent years, *M. tuberculosis*-specific 10-kda culture filtrate protein (CFP-10) has been identified that is, encoded by Ihp gene (Renshaw et al., 2005). This gene is located in a specific region of the *M. tuberculosis* genome called "region of difference" (RD1). The RD1 region has 9.5 kb in length and contains nine open reading frames (ORFs) and CFP-10 is more significant among them (Meher et al., 2006; Guinn et al., 2003). The RD1 region is conserved in *M. tuberculosis* and is not present in environmental mycobacteria and BCG strains (Arend et al., 2001; Lyashchenko et al., 1998). Therefore, CFP-10 is not present in BCG substrains and most non-tuberculosis mycobacteria. The identification of CFP-10 as a potent stimulator of T-cells makes for great

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advances in tuberculosis diagnosis (Mustafa, 2005; Harboe et al., 1996).

It is documented that RD1-encoded proteins which are recognized by the immune system, like CFP-10 antigen, can induce delayed-type hypersensitivity reaction in guinea pigs. Therefore, it could be an appropriate alternative for TST (Colangeli, 2000; Jafari et al., 2006; Pinxteren et al., 2000; Okkels et al., 2003; Brock et al., 2004). It also seems that in contrast to TST, responses to CFP-10 antigens can differentiate between active tuberculosis and BCG vaccination (Lalvani et al., 2001; Lalvani and Millington, 2007).

Recombinant protein expression in *E. coli* is a valuable strategy to provide desirable amount of protein for diagnostic purposes. There are some reports describing the cloning and expression of CFP-10 antigen (Berthet et al., 1998; Dilon et al., 2000; Meher et al., 2006). Unfortunately, in most cases, this protein was expressed in the insoluble form that could only be purified and refolded in low-yields after a time-consuming and labor-intensive process.

This study aimed to clone and express CFP-10 antigen in the soluble form to be applicable for establishing a sensitive and specific test as an alternative to traditional diagnostic procedures.

MATERIALS AND METHODS

Bacterial strains and DNA extraction

M. tuberculosis standard strain H37RV was obtained from tthe Pasteur Institute of Iran and subcultured on Leuwenstien-Jensen media at 37°C for 4 weeks. Cauliflower colonies were stained by Ziehl-Neelson to confirm the presence of acid-fast bacilli. DNA was extracted from H37RV by DNA extraction kit (Cinnagen, Iran), according to the manufacturer's instructions.

Primer design and PCR

Target sequence for primer designing was the coding sequence of the CFP-10 gene which is located in the RD1 region, called RV3874 or Ihp (GenBank accession no. FJ014498). According to this sequence, one set of forward (5'-CAC CAT GGC AGA GAT GAA GAC CGA T) and reverse (5'-GAA GCC CAT TTG CGA GGA C) primers was designed by Gene Runner software and to amplify specifically Ihp gene, giving a fragment 304 nucleotides in length.

Extraction of PCR products from agarose gel

For removing additional materials such as dNTP, Pfu enzyme and nonspecific bands from amplified specific products, the PCR product was electrophoresed on a low melting 2% agarose gel (Fermentas) and the desired band removed and purified by DNA gel extraction kit (Fermentas). The purified PCR product was then electrophoresed through a 2% agarose gel in a TAE buffer and analyzed by the Kodak 1D 3.5 imaging software (Eastman Kodak Company, USA).

Ligation and transformation

Cloning was performed using the Champion pET directional TOPO Expression kit (Invitrogen) to insert and ligate the PCR product into pET102/D expression vector according to kit protocol. Subsequently, recombinant plasmid was transformed into competent *E. coli* TOP10 cells. This organism provides a host for stable propagation and maintenance of recombinant plasmids (Invitrogen TM, 2006). Transformed bacteria were then cultured on an LB agar media containing ampicillin (0.1 mg/ml).

Confirmation of CFP-10 gene cloning into pET102/D

Recombinant plasmid was extracted from *E. coli* TOP10 colonies on an LB agar media by QIAGEN Plasmid Mini kit (Qiagen, Germany). PCR analysis of recombinant plasmid was performed by T7 primers, provided from the Invitrogen kit. To confirm the identity of the construct, purified recombinant plasmids were sequenced by the SeqLab Laboratory (Germany).

Expression and purification of recombinant CFP-10

Extracted recombinant plasmids were transformed into *E. coli* BL21 by heat shock and were plated on an LB agar containing ampicillin at 37°C overnight. Overnight incubated colonies were inoculated into an LB broth and was grown to OD600 0.55, then isopropyl β- D thiogalactoside (IPTG) added and growth was continued in a 37°C shaker incubator. Then, the total broth media was centrifuged and the bacteria cell pellet was dissolved in a binding buffer (10 mM imidazole, 0.3 M NaCl, 0.1 M KCl, 10% glycerol, 0.5% Triton X-100, 50 mM Tris-HCl, pH 7.6) (Sankian et al., 2007). Recombinant CFP-10 (rCFP-10) was purified from the cell-free supernatant by chromatography on an Ni2+-NTA agarose column. After washing the column with 10 mM imidazole in a lysis buffer (50 mM Tris-HCl, PH 7.8, containing 300 mM NaCl, 100 mM KCl, 10% glycerol and 0.5% Triton X-100 1% v/v), CFP-10 was eluted with 200 mM imidazole in the lysis buffer. Fraction containing rCFP-10 was dialyzed against a PBS buffer pH 7.5 and was analyzed by SDS-PAGE electrophoresis.

RESULTS

Amplification of CFP-10 gene using specific primers resulted in a single 304 bp fragment (Figure 1) that was subsequently cloned into pET102/D (Figure 2). Recombinant and non-recombinant plasmids showed 6618 and 6318bp bands (respectively) on a 1% agarose gel using a 1 kbp DNA Ladder (Figure 2B). This implied the presence of the inserted fragment (CFP-10 gene) in the mentioned vector.

Further analysis of construct by PCR showed a 538 bp band on a 2% agarose gel. Since the interval fragment between forward and reverse T7 primers was 235 bp in length and the length of the inserted fragment was 304 bp, the appearance of 538 bp could be due to the presence of the desired insert in the mentioned vector (Figure 2A).

Two of these clones were sequenced. Sequencing analysis revealed that the insert corresponds to a 300 bp open reading frame which encodes CFP-10, a 100-amino-acid polypeptide with an average molecular mass of 10.7 kDa. The obtained sequences were searched for homology identity with the NCBI BLAST software. The results showed that the sequences were completely

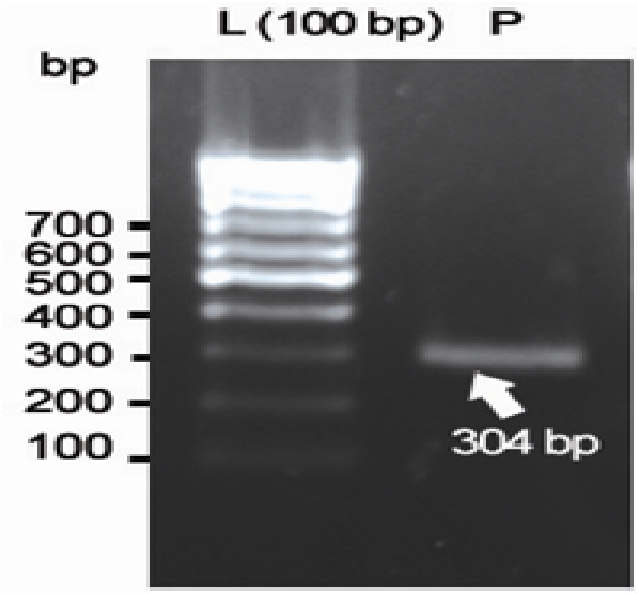

Figure 1. Amplification of the CFP-10 gene using specific primers. Lane P indicates a band of 304 bp corresponding to the CFP-10 gene plus an additional sequence (CACC). Lane L shows 100 bp DNA ladder.

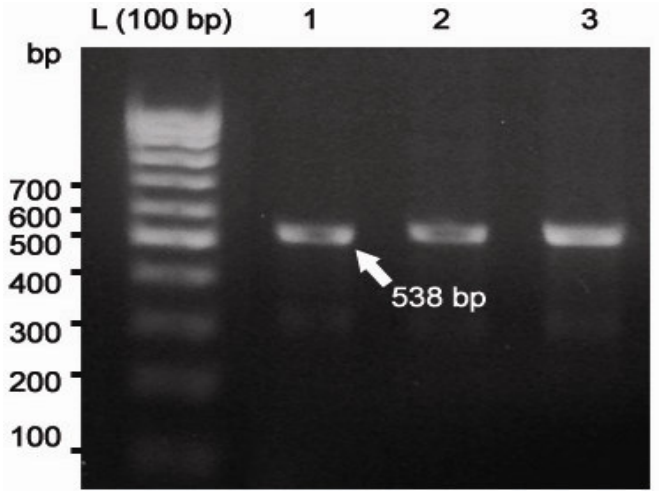

Figure 2A. Confirmation of CFP-10 gene cloning into pET102/D; extracted plasmid from clone 1, 2 and 3 (lanes 1, 2 and 3, respectively) showed 538 bp bands in PCR amplification by T7 primers, corresponding to the CFP-10 gene and a 203 bp fragment from plasmid.

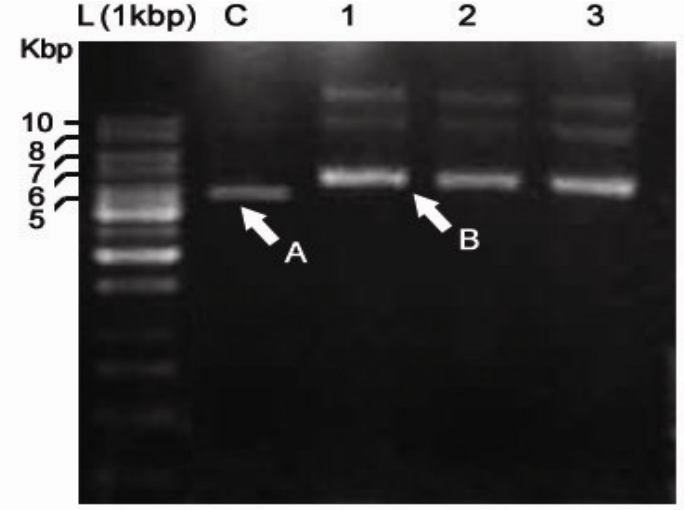

Figure 2B. Confirmation of CFP-10 gene cloning into pET102/D; recombinant plasmids (arrow B) and empty plasmid (arrow A) indicated a clear difference in electrophoretic mobility on a 1% agarose gel.

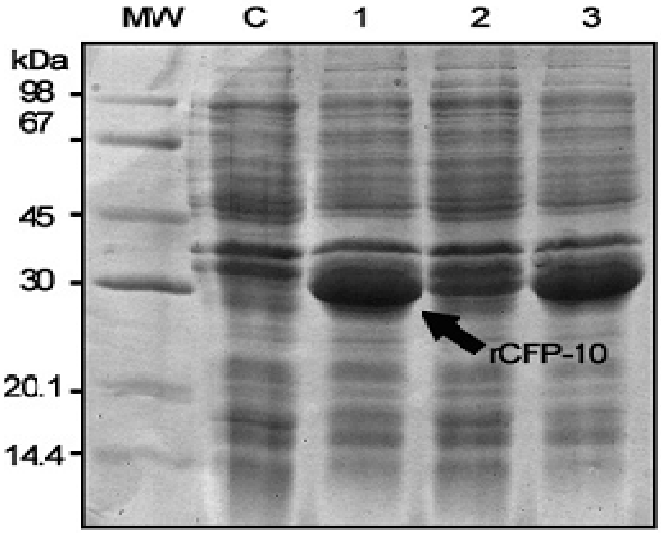

Figure 3. Expression of rCFP-10 fusion protein in *E. coli* BL21. SDS-PAGE analysis of IPTG –induced BL-21 (DE3) containing recombinant plasmids (lanes 1, 2 and 3) showed a 28 kDa band corresponding to the CFP-10 fusion protein. Uninduced bacteria showed no recombinant protein expression (lane C).

identical with the CFP-10 sequence (Gene Bank Accession no. FJ014498). After the expression of the recombinant CFP-10 protein by *E. coli* BL21, a 28 kDa protein band was detected by SDS-PAGE analysis (Figure 3). This protein band implied on a fusion protein that consists of a CFP-10 core protein fused to a C-terminal His-tag (3 kDa) and an N-terminal Hispatch thioredoxin (13 kDa) (Figure 3). SDS-PAGE electrophoresis was performed for the soluble and insoluble fractions of the crude lysate of the bacteria. This analysis indicated that rCFP-10 is present in the soluble fraction (Figure 4A). In Figure 6, SDS-PAGE analysis of the elution fraction of Ni2+-NTA agarose chromatography showed that rCFP-10 was completely purified (Figure 4B).

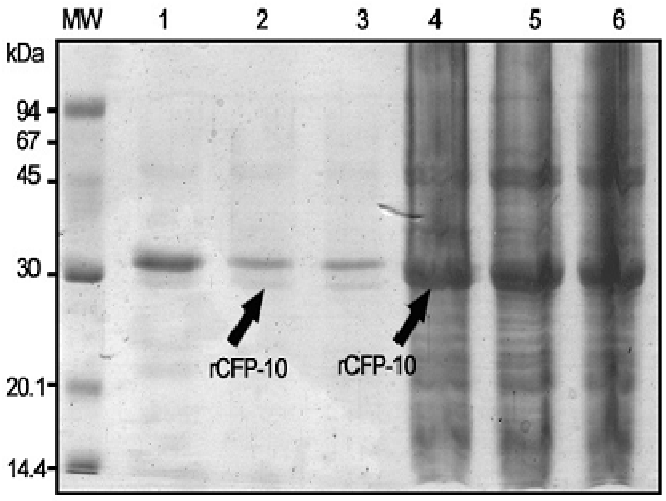

Figure 4A. Purification of CFP-10 fusion protein as a soluble recombinant protein: SDS-PAGE analysis of the soluble phase (lanes 1, 2 and 3) and insoluble phase (lanes 4, 5 and 6) of IPTG –induced BL-21 (DE3) containing recombinant plasmids.

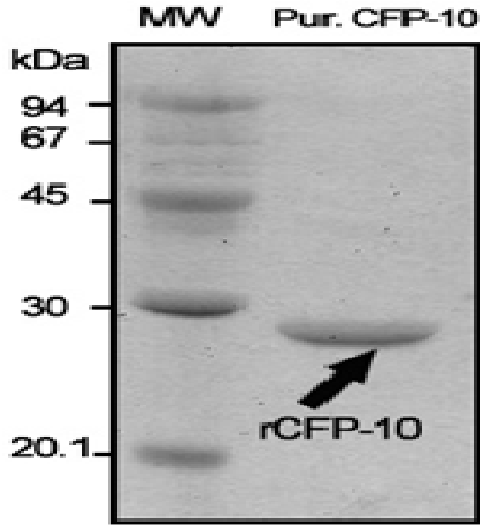

Figure 4B. Purification of CFP-10 fusion protein as a soluble recombinant protein: SDS-PAGE analysis of CFP-10 fusion protein, purifying by Ni-NTA chromatography.

DISCUSSION

The studies from countries with low prevalence of tuberculosis show that RD1 gene-based diagnosis is more accurate than the TST (Arend et al., 2001). Ihp gene (CFP-10) (Okkels and Andersen, 2004), also called RV3874 (Flint et al., 2004), esxB (Brodin et al., 2006) and MTSA-10 (Colangeli, 2000) is one of the major antigens in the RD1 region of the *M. tuberculosis* genome (Meher et al., 2006). This gene is located upstream of ESAT-6 gene and codes the protein CFP-10 which is a member of the ESAT-6 family (Brodin et al., 2004).

There are several reports that described the cloning and expression of the CFP-10 gene in different expression vectors such as pMTC6 (Berthet et al., 1998), pET17b (Dilon et al., 2000), pET28a (Mukherjee et al., 2007), pET28b and pQE60 (Meher et al., 2006). Berthet et al. (1998) amplified Ihp gene by PCR and cloned it into the pMCT6 expression vector digested with XmaI/BamHI. Recombinant CFP-10 was produced in fusion with a stretch of eight histidines at its N-terminus. Recombinant CFP-10 was expressed in the insoluble form and was purified by metal ion affinity chromatography (Berthet et al., 1998). This form of protein could only be purified and refolded in low-yield after a time-consuming and labor-intensive process (Meher et al., 2006; Mukherjee et al., 2007).

Solubilizing and purifying of desired proteins is one of the difficulties in other studies, while in our study this protein was expressed substantially in the soluble form that could be easily purified by metal affinity chromatography. In the present study, Champion pET Directional TOPO kit that facilitates direct cloning by topo-isomerase I reaction was used (Invitrogen TM, 2006). The pET102/D vector has a thioredoxin stretch in the N-terminus that makes the expression of protein in the soluble phase.

As we mentioned earlier, this vector has a 6xHis tag stretch in the C-terminus that facilitates protein purification with the Ni-NTA column affinity chromatography (Invitrogen TM, 2006). Furthermore, this vector also has a cleavage site for enterokinase to eliminate additional peptide stretch from the protein's N-terminus. This strategy has been designed to maintain a conformational structure of the recombinant protein (Invitrogen TM, 2006).

Finally, CFP-10 was cloned and expressed in a soluble form to be applicable for establishing a sensitive and specific test as an alternative to traditional diagnostic procedures.

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