CHAPTER

5

CLONING, OVEREXPRESSION, AND PURIFICATION OF *LEISHMANIA DONOVANI* ADENOSYLHOMOCYSTEINASE AND TRYPANOTHIONE REDUCTASE

5. Cloning, Overexpression, and Purification of *Leishmania donovani* Adenosylhomocysteinase and Trypanothione reductase

5.1 Introduction

As reviewed in the preceding chapters (1&2), chemotherapy remains the mainstay for the treatment of leishmaniasis and till date no vaccine is available to combat this disease. The recent trend in vaccine development has been the identification and characterization of defined leishmanial antigens as well as the elucidation of the range and specificity of antileishmanial immune responses. Advances made in clinical proteomic technologies have further enhanced the mechanistic understanding of leishmanial pathobiology thereby defining novel vaccine targets (Garg et al., 2006; Gupta et al., 2007; Kumari et al., 2008a). The evaluation of such vaccine targets for their prophylactic potential will provide further lead towards the development of a candidate vaccine(s). Taking a rationale and systematic approach discovery of a potent vaccine candidate is feasible. Earlier studies in our laboratory, by using classical activity based fractionation and sub-fractionation of the soluble proteins from promastigote stage of *L. donovani,* led to the identification of a potent sub-fraction ranging from 89.9 to 97.1 kDa which induced Th1 type cellular responses in cured *Leishmania* patients and hamsters and alongwith yielded significant prophylactic efficacy in hamsters (Kumari et al., 2008c). Further characterization of this sub-fraction by proteomic approaches led to the detection of 18 potential Th1 stimulatory proteins.

This chapter deals with the cloning, expression and purification of two of the identified proteins viz adenosylhomocysteinase and trypanothione reductase that have been reported to be potential drug targets, immunogenic or T-cell stimulating or vaccine candidates in other parasitic species but were not explored in case of VL. The proteins thus developed as recombinant proteins were further assessed for their immunogenicity and the most potential was further evaluated for its prophylactic efficacy. For cloning of these two genes, primers were designed on the basis of the *L. major* sequences of these proteins available at National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) (Table 5.1). The nucleotides underlined in forward and reverse primers correspond to restriction sites respectively to facilitate cloning in expression vector.

Table :5.1 Size of genes and their primers required for cloning.

Gene	Size (bp)	Forward (FR) and Reverse (RR) Primers
Adenosylhomocysteinase	1314	FR 5'-CATATGATGGCGGACTACAAGGTAAAGGACATC-3' RR 5'-AAGCTTGTAGCGGTAGTGGTCCGGCTTGAACG -3'
Trypanothione reductase	1476	FR 5'GATCCATGTCCCGCGCGTACGACCTCGTGGTGCTTGG 3' RR5'GAATTCGAGGTTGCTGCTCAGCTTTTCGACGCGCTTGC3'

5.1.1 *L. donovani* Adenosylhomocysteinase (LdADHT)

Adenosylhomocysteinase or S-Adenosylhomocysteine hydrolase (AdoHcy)2 (EC 3.3.1.1) catalyzes the reversible conversion of AdoHcy to adenosine (Ado) and homocysteine (Hcy) (Yin *et al.*, 2000), (Chiang *et al.*, 1996). Because AdoHcy is a product inhibitor of all S-adenosylmethionine (AdoMet)-dependent methyltransferases, the catalytic activity of AdoHcy hydrolase is critical in eukaryotic cells to maintain the normal cellular level of AdoHcy and to permit the numerous transmethylation reactions required in normal cell functions to proceed (Yin *et al.*, 2000), (Chiang *et al.*, 1996). Inhibition of AdoHcy hydrolase in eukaryotic cells causes an increase in the cellular level of AdoHcy and inhibition of AdoMet-dependent methyltransferases that result in various pharmacological effects (e.g., antiviral, antiarthritic, immunosuppressive, antitumor, etc.) (Yin *et al.*, 2000), (Chiang, 1998). Because parasites such as *L. donovani* (Henderson *et al.*, 1992), (Avila *et al.*, 1997), *Trypanasoma cruzi* (Avila *et al.*, 1997), (Seley *et al.*, 1997), and *Plasmodium falciparum* (Creedon *et al.*, 1994), (Whaun *et al.*, 1986) express their own AdoHcy hydrolase, these parasitic enzymes are potential targets for developing antiparasitic agents.

5.1.2 *L. donovani* Trypanothione reductase (LdTPR)

Trypanothione, an unusual form of glutathione found in parasitic protozoa of the Kinetoplastidae family, plays a crucial role in regulating the intracellular thiol redox balance and in the defence against chemical and oxidative stress (reviewed in (Muller, 2004). It contains two molecules of glutathione joined by a polyamine linker. Trypanothione reductase (TPR; EC 1.8.1.12) is a NADPH-dependent flavoprotein oxidoreductase essential in maintaining the intracellular concentration of reduced trypanothione ($T[SH]_2$), and plays a

central role in the thiol metabolism of all trypanosomatids (Cunningham & Fairlamb, 1995),(Fairlamb & Cerami, 1985), (Fairlamb & Cerami, 1992), (Shames *et al.*, 1986), (Taylor *et al.*, 1994). It is also an essential enzyme in the detoxification process. Genes encoding TPR have been described from several trypanosomatid species, such as the human pathogens *Trypanosoma cruzi* (Sullivan & Walsh, 1991) and *T. brucei* (Aboagye-Kwarteng *et al.*, 1992), the cattle pathogen *T. congolense* (Shames *et al.*, 1988), the insect pathogen *Crithidia fasciculate* (Aboagye-Kwarteng *et al.*, 1992), and the Old World Leishmania species *L. donovani* (two diverent strains, one from Ethiopia and one from India—(Taylor *et al.*, 1994), (Mittal *et al.*, 2005). TPR sequences can also be derived from the *L. major*, *L. braziliensis* and *L. infantum* genome projects (http://www.sanger.ac.uk).

There have been no reports available regarding the immunogenicity and prophylactic potential of these gene ADHT and TPR in case of VL, hence, it was pertinent to assess their immunogenic and prophylactic potentials.

5.2 Materials and methods

The methodologies for cloning, expression and purification of each protein are described as follows:

5.2.1 Cloning, expression and purification of ADHT

5.2.1.1 Genomic DNA isolation

The isolation of genomic DNA was carried out following the protocol of (Kelly, 1993). Briefly, 10^8 promastigotes from 10–15ml of culture was harvested and centrifuged at 3500 rpm for 10 min at 4 °C. The supernatant was decanted, cell pellet was resuspended in 3-6 ml NET buffer (0.1M NaCl, 1.0mM EDTA (pH 8.0), and 10mM Tris (pH 8.0), and centrifuged at 3500 rpm for 10 min at 4°C. The supernatant was discarded and pellet was redissolved in 750 µl NET, 7.5 µl proteinase K (10mg/ml stock), and 50 µl of 15% sarcosyl. Sample was incubated overnight at 37°C in a waterbath for proteinase K activity and 1 µl of RNase was added to it and further incubated for 1 hour. The cell lysate was centrifuged at 18,000 rpm for 1 h at 4°C. The supernatant containing nuclear DNA was extracted first with one volume each of phenol (750 µl), then with phenol/ choloroform/ isoamyl alcohol (25:24:1) and finally with chloroform. DNA was precipitated from the aqueous phase obtained after centrifugation with two volumes of pre-chilled absolute ethanol and kept at -20°C. Following day, the DNA

pellet was obtained by briefly spinning the tube at 13000 rpm for 1 min and stored at 4°C for future use.

5.2.1.2 PCR amplification

The PCR reaction mixture contained 2X PCR Master mix (Fermentas), 1µg of genomic DNA, 10 pmol of forward and reverse primers each, and final volume made upto 20 µl with nuclease free water. The PCR was performed in MJ mini thermal cycler (BioRad) with initial denaturation at 96°C for 4 min, followed by 30 cycles at 94°C for1 min, 58°C for1 min and 72°C for 1 min 30 sec with a final extension at 72°C for 10 min.

5.2.1.3 Elution of amplified gene from the Gel

The amplified PCR product was visualized on a 1% agarose gel stained with ethidium bromide (EtBr) and the gel slice containing the amplified gene was excised out and purified using a QIAquick GEL Extraction Kit (Qiagen). Briefly, the excised gel slice was weighed and 3 volumes of buffer QG was added to it. Further it was heated to 50°C for 10 minutes or more until the gel slice was completely dissolved and then precipitated with isopropanol and applied to the QIAquick column for binding of DNA. After washing, the DNA was eluted in 20 µl of Nuclease free water (Sigma).

5.2.1.4 Cloning of gene in pTZ57R/T (T/A cloning) vector

The eluted product was ligated onto linearised pTZ57R/T vector (Fermentas) (**Fig**. 5.1) using T4 DNA ligase. The vector takes advantage of the 3'A overhang at each end of the PCR product, added by Taq DNA polymerase. The linearized pTZ57R/T vector contains the 5'ddT overhang at both ends and is ligated to the PCR product with high specificity. In a 15µl ligation reaction, following components were added in a 1.5 ml microcentrifuge tube.

Component	**Concentration**	**Volume used**
pTZ57R/T vector	50ng/ µl	3 µl
Amplified PCR product	75ng/ µl	4 µl
5 X ligation buffer	3µl	3 µl
T4 DNA ligase	5U/ µl	1 µl
Nuclease free Water	-	Up to 15 µl

The ligation mixture was incubated overnight at 16^0C.

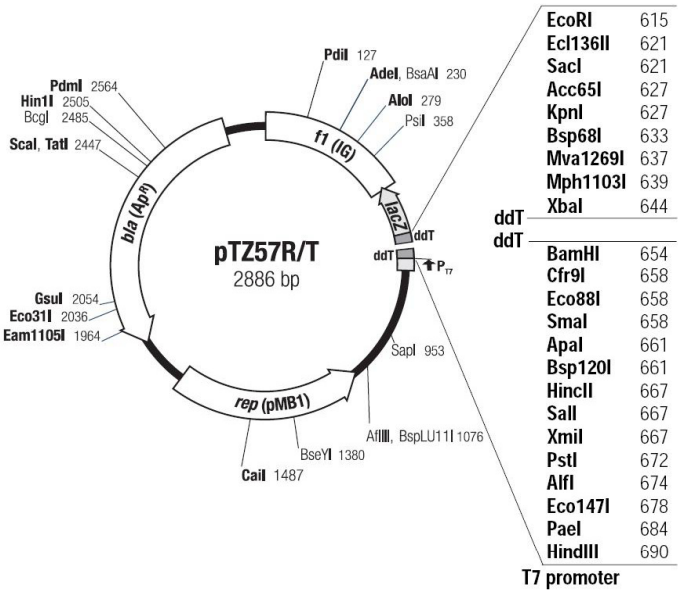

Figure. 5.1: Restriction map of pTZ57R/T vector.

5.2.1.5 Preparation of media for bacterial cell culture

Composition of Luria Bertani (LB) broth

Tryptone	10g/l
Yeast extracts	5g/l
NaCl	10g/l

The pH of LB was adjusted between 7.2-7.4.

Composition of LB agar

Tryptone	10g/l
Yeast extract	5g/l
NaCl	10g/l
Agar	20g/l

5.2.1.6 Preparation of chemically competent Escherichia coli using calcium chloride method

The chemically competent cells were prepared by the method described by (Cohen, 1972). A single colony of E. coli DH5α cells was picked from LB agar plate and inoculated aseptically into a 5ml LB tube and incubated overnight at 37^{o}C in a shaker incubator at 200 rpm. 1% of the overnight grown primary culture was inoculated into 25ml LB and incubated for 2 hrs or more at 37^{o}C, 200 rpm till the OD_{600} reached approximately 0.5-0.6. The culture was then transferred into sterile prechilled 50ml poly propylene tube. Bacterial cells were harvested by centrifugation at 5000Xg for 15min at 4^{o}C and the supernatant was discarded by inverting the tubes on a paper towel for 2 min to completely drain off the media. The cell pellet was resuspended in 10 ml of ice cold 100mM $CaCl_2$ solution and incubated on ice for 1 hr. The cells were then collected by centrifugation at 4^{o}C for 15min at 5000Xg and the pellet obtained was resuspended in 2 ml of chilled 100mM $CaCl_2$+15% glycerol solution. Finally aliquots of 200µl of cells were prepared and stored at -70ºC until use.

5.2.1.7 Transformation

A test transformation was set with 1ng of pTZ57R control plasmid to check the transformation efficiency by heat shock treatment as described by (Hanahan, 1983). For this process, the control plasmid was added to the chemically competent E. coli DH5α cells and the mixture was incubated on ice for 20 minutes, after which it was transferred rapidly to a preheated 42°C circulating waterbath for 90 sec without shaking. Reactions were then transferred rapidly onto ice for 2 min, followed by immediate addition of 800µl of C-medium (Fermentas) at room temperature and incubated at 37°C for 1 hr with agitation (~200 rpm) to allow the cells to recover. About 100µl of recovered cells were plated onto appropriate antibiotic selection agar plates and incubated overnight in inverted position at 37^{o}C. The transformation efficiency was calculated on the following day and when it was found approximately 10^4 CFU µg-1 DNA, remaining competent cells were used to transform the ligation mix. 15µl of ligation mix was transformed according to the above mentioned procedure. After recovery, the transformants were incubated overnight at 37^{o}C and selected by plating onto selective LB Ampicillin (100µg/ml) with X-gal (40µg/ml) plates. Positive white colonies were picked up for further screening.

5.2.1.8 Confirmation of positive clone(s)

5.2.1.8.1 Preparation of master plate and isolation of plasmid DNA from transformed *E. coli* (Mini Prep)

Master plate was prepared by streaking the positive white colonies picked from Amp/X-gal plate with pipette tip and further inoculating the same tip aseptically into a 5ml LB-Amp tube. Both the master plate and the inoculated tubes were incubated for overnight at 37^{o}C in a shaker incubator at 200 rpm. Plasmid was isolated from the overnight culture by using QIAprep spin miniprep kit (Qiagen) as per manufacturer's protocol. Briefly, the cells harbouring the recombinant plasmid were harvested by centrifugation at 6000 rpm for 5 min and resuspended in 250µl of buffer P1 (containing RNAse) followed by addition of 250µl P2 and mixed by inverting the tubes 4-6 times. The mixture was then precipitated by adding 350µl of buffer N3 and the contents of the tube were mixed by inverting the tubes 10 times. The supernatant was obtained by centrifugation at 13000 rpm for 10 min and applied to the QIAprep spin column. After washing the columns with 750µl of buffer PE the plasmid was eluted in 30µl of nuclease free water (Sigma). The isolated plasmids were visualized on 1% agarose gel by electrophoresis.

5.2.1.8.2 Colony *PCR*

The positive transformants were confirmed by colony PCR wherein isolated plasmids were used as template for PCR of the respective gene using the gene specific primers and the same PCR conditions as used previously for the same gene.

5.2.1.8.3 Restriction Digestion of Plasmid DNA

Clone confirmation was further assessed by restriction digestion of the isolated plasmid with *NdeI* and *HindIII* (Fermentas) restriction enzymes in 2X Tango buffer supplied with the enzymes. Nearly 1µg of Plasmid DNA was digested in a 20µl reaction volume for 3-5 hrs at 37ºC temperature. The reaction was terminated by heating the reaction mixture at 70°C for 10min. Restriction digestion was analyzed by agarose gel electrophoresis using DNA molecular weight markers.

5.2.1.8.4 Sequencing of ADHT gene

One positive clone was selected and sequenced by automatic sequencer ABI Prism (Version 3.0.Model 3100) at Department of Biochemistry, University of Delhi, South campus, New

Delhi. The sequence homology of *L. donovani* ADHT was compared with *L. infantum* and other species using ClustalW software and finally the sequence was submitted to NCBI

5.2.1.9 Subcloning of ADHT in pET-28a expression vector

ADHT gene cloned in pTZ57R/T vector was digested with *Nde*I and *Hind*III restriction enzymes. The digested product was run on 1% agarose gel and the insert was excised and purified from agarose gel by using gel extraction and purification kit (Qiagen) as described earlier and ligated into pET28a vector (Novagen) predigested with the same enzymes (**Fig**. 5.2). The reaction mixture is as follows.

Component	Concentration	Volume used
NdeI / *HindIII* digested pET 28a vector	25ng/µl	4 µl
NdeI / *HindIII* digested pTZ57RT+ADHT	50 ng/ µl	4 µl
Ligation master mix	5 X	4 µl
T4 DNA ligase	5U/ µl	1 µl
Nuclease Free Water	-	Up to 20 µl

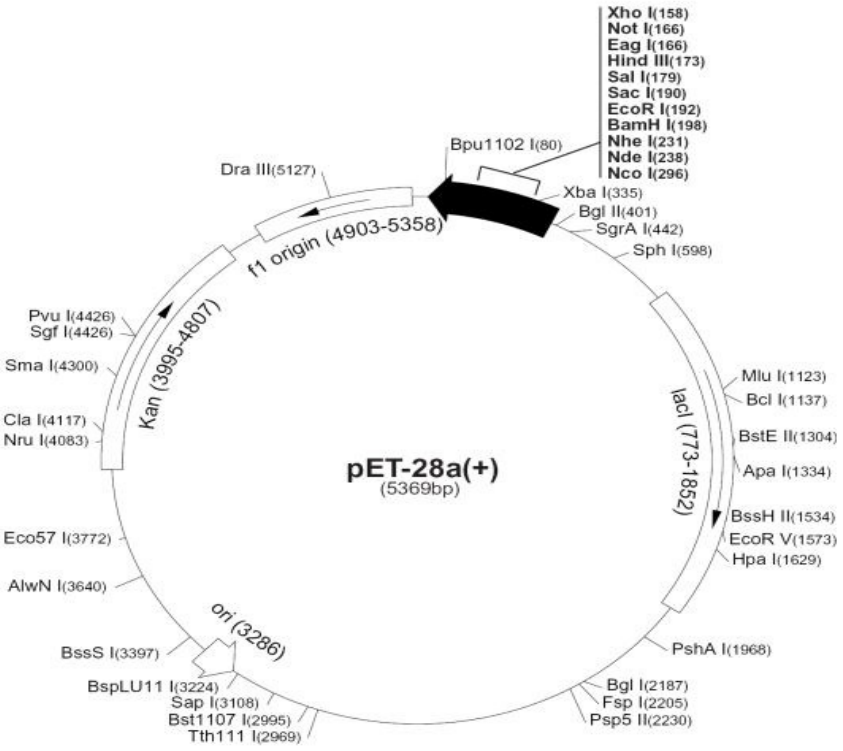

Figure.5.2 : Restriction map of pET-28a vector

5.2.1.10 Transformation in *E. coli* Rosetta (DE3) cells

5.2.1.10.1 Transformation procedure

Appropriate amount of plasmid construct was added to 200µl of *E. coli* Rosetta (DE3) competent cells in pre-chilled 1.5 ml microfuge tube and the mix was incubated on ice for 30 minutes. Heat shock at 42°C for 90 sec was given to the mixture and it was immediately chilled on ice again for 5 minutes. After 5 minutes, 800µl of C-medium was added immediately to the mix and the cells were mixed by pipetting up and down and incubated for additional 1hr at 37^{o}C with gentle shaking for recovery. Following recovery, the transformants were selected by plating onto selective kanamycin plates and incubated overnight at 37^{o}C. Positive clones were used for protein expression.

5.2.1.11 Clone confirmation

The positive clones were confirmed by colony PCR and restriction digestion using *Nde*I / *Hind*III restriction enzymes as described earlier.

5.2.1.12 Recombinant protein Expression

A single positive colony was inoculated to 5 ml LB tubes supplemented with kanamycin and after the OD_{600} of the culture reached 0.6, the culture was induced with 1mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Sigma) for 12 hours. After induction, cells were lysed in 5X sample buffer (0.313M Tris–HCl, pH 6.8, 50% glycerol, 10% SDS, and 0.05% bromophenol blue, with 100mM DTT) and analyzed by 12% SDS–PAGE. Uninduced control culture was analyzed in parallel. The expression of ADHT was visualized by staining the gel with commassie brilliant blue R-250 stain (Sigma). To increase the solubility of the protein, different IPTG concentrations (0.1, 0.25, 0.5. and 1.0 mM) and different time intervals (12, 16 and 18 hrs) were tried.

5.2.1.13 Purification of recombinant ADHT (rLdADHT)

For recombinant protein purification a 1.5% inoculum of overnight grown culture was seeded to a 100 ml LB flask supplemented with kanamycin and after shaking at 37°C for nearly two hours when the $O.D._{600}$ reached to 0.6, the culture was induced with 1 mM IPTG and incubated again for 12 h with constant shaking at 180 rpm. After 12 h, the culture was centrifuged at 6000 rpm for 10 minutes and the pellet dissolved in 10 ml of lysis buffer (20 mM Tris, 100 mM NaCl, pH 8.0) containing 3 mM phenylmethylsulphonyl fluoride (PMSF, Sigma). The suspension was sonicated 10 X with 30 sec ON and 30 sec OFF pulse on Soniprep at 30 amplitude. The sonicated cells were subjected to centrifugation at 15,000 g for 30 min, and the supernatant was incubated at 4°C for 1 h with 2 ml of Ni–NTA Superflow resin (Qiagen) previously equilibrated with lysis buffer. The suspension was loaded in chromatography column (2: 1 cm). After washing with 10 column volumes of buffer (20 mM Tris, 100 mM NaCl, pH 8.0), the purified recombinant ADHT (rLdADHT) was eluted with elution buffer (20 mM Tris, 100 mM NaCl, 10% glycerol and 250 mM imidazole, pH 7.5). The eluted fractions were analyzed for purity by 12% SDS–PAGE and the protein content of the fractions was estimated by the Bradford method using bovine serum albumin (BSA) as standard.

5.2.1.14 Production of polyclonal antibodies against rLdADHT and Western Blotting

The purified recombinant protein was used for raising antibodies in rabbit. Rabbit was first immunized using 100µg of recombinant protein emulsified in Freund's complete adjuvant. After 15 day, the rabbit was boosted three times with 50µg recombinant protein each in

incomplete Freund's adjuvant at 2-weeks interval. The serum was obtained 7 days after the last bleeding.

For immunoblots, whole cell lysate and SLD were electrophoretically separated using 10% polyacrylamide gels and transferred on to nitrocellulose membrane using a semi-dry blot apparatus Hoefer Semiphor (Pharmacia Biotech) (Towbin et al., 1979). After overnight blocking in 5% BSA, the membrane was incubated with antiserum to the recombinant protein at a dilution of 1:10,000 for 2h at room temperature (RT). The membrane was washed three times with phosphate buffer saline (PBS) containing 0.5% Tween 20 (PBS–T) and then incubated in peroxidase conjugated goat anti-rabbit IgG (Banglore Genei) at a dilution of 1:10,000 for 1h at RT. Finally, the blot was developed by using diaminobenzidine (DAB), imidazole and hydrogen peroxide (H_2O_2).

5.2.2 Cloning, Overexpression and purification of TPR

5.2.2.1 Amplification, Cloning and Sequencing

Gene specific primers were designed on the basis of *L. major* gene sequence available from the gene bank and described in **table** 5.1. TPR gene of 1476 bp was amplified by PCR as described in previous section with the annealing temperature of 55ºC for 1.30 min and extension time 72ºC for 2 min in the PCR cycle. Following amplification the gene was cloned and subcloned in pET28a vector as described previously. The sequencing was done as described above and submitted to NCBI. The pET28a+TPR construct was transformed in *E. coli* C41 (DE3) cycles and checked for overexpression.

5.2.2.2 Overexpression and Purification of recombinant protein (rLdTPR)

Two hundred milliliters of LB medium containing 35µg/ml kanamycin was inoculated with *E. coli.* C41 (DE3) strain transformed with pET28a+TPR, and grown at 37°C till OD_{600} reached 0.6. Recombinant protein expression was induced by addition of 1 mM IPTG (Sigma) and the culture was incubated for an additional 5 hrs. The recombinant TPR was purified by affinity chromatography using Ni^2+chelating resin to bind the His6-tag fusion peptide derived from the pET28a vector. The cell pellet was resuspended in 4 ml of lysis buffer (20 mM Tris, 100 mM NaCl, 6M Urea and 10% glycerol, pH 8.0) containing 3 mM PMSF (Sigma) . The suspension was sonicated for 10X 20 sec (with 20 s intervals between each pulse) on ice. The sonicated cells were centrifuged at 15,000 g for 30 min, and the supernatant was incubated at 4°C for 1 hr with the 2 ml of Ni–NTA Superflow resin (Qiagen)

previously equilibrated with lysis buffer. The suspension was loaded in chromatography column (2: 1 cm). After washing with 10 column volumes of buffer (20 mM Tris, 100 mM NaCl, 6M Urea and 10% glycerol, pH 8.0) with increasing concentrations of imidazole (20, 30 and 50 mM), the purified recombinant TPR was eluted with elution buffer (20 mM Tris, 100 mM NaCl, 6M Urea, 10% glycerol, and 250 mM imidazole pH 7.5). The eluted fractions were analyzed by 12% SDS–PAGE and the gels were stained with Coomassie brilliant blue R-250 (Sigma, USA). The protein content of the fractions was estimated by the Bradford method using BSA as standard.

5.2.2.3 Polyclonal antibody generation and **western blot** were described as above in section5.2.1.14.

5.3 Results

5.3.1 rLdADHT was cloned, overexpressed, purified and antibody raised

The ADHT gene was successfully amplified and cloned (**Fig.** 5.3 A and B) in pTZ57R/T (T/A) cloning vector and transformed into competent *E.coli* DH5α cells. The positive transformants were sequenced from Delhi University (New Delhi) and submitted to the National Centre for Biotechnology Information (http: //www. ncbi.nlm.nih.gov/; Accession no. **GU353334**). The gene was further subcloned into pET28a vector and the recombinant plasmid was characterized with double enzymatic digestion (**Fig.** 5.3 C) and checked for over-expression. 1314 bp amplicon of ADHT gene encoded 438 amino acids with a predicted molecular mass of ~52 kDa. Comparisons of both non-induced and induced culture in SDS-PAGE revealed that the protein was overexpressed successfully and the induced protein bands corresponded to its predicted size (**Fig**. 5.3 D). Purification of His-tagged rLdADHT by metal affinity chromatography yielded ~4 mg of pure protein from a 1-L bacterial culture (**Fig.**5.3 E). Further western blotting using the antiserum to rLdADHT, detected single band at ~52 kDa in the soluble promastigote lysate, thus exhibiting its specificity (**Fig.** 5.3F).

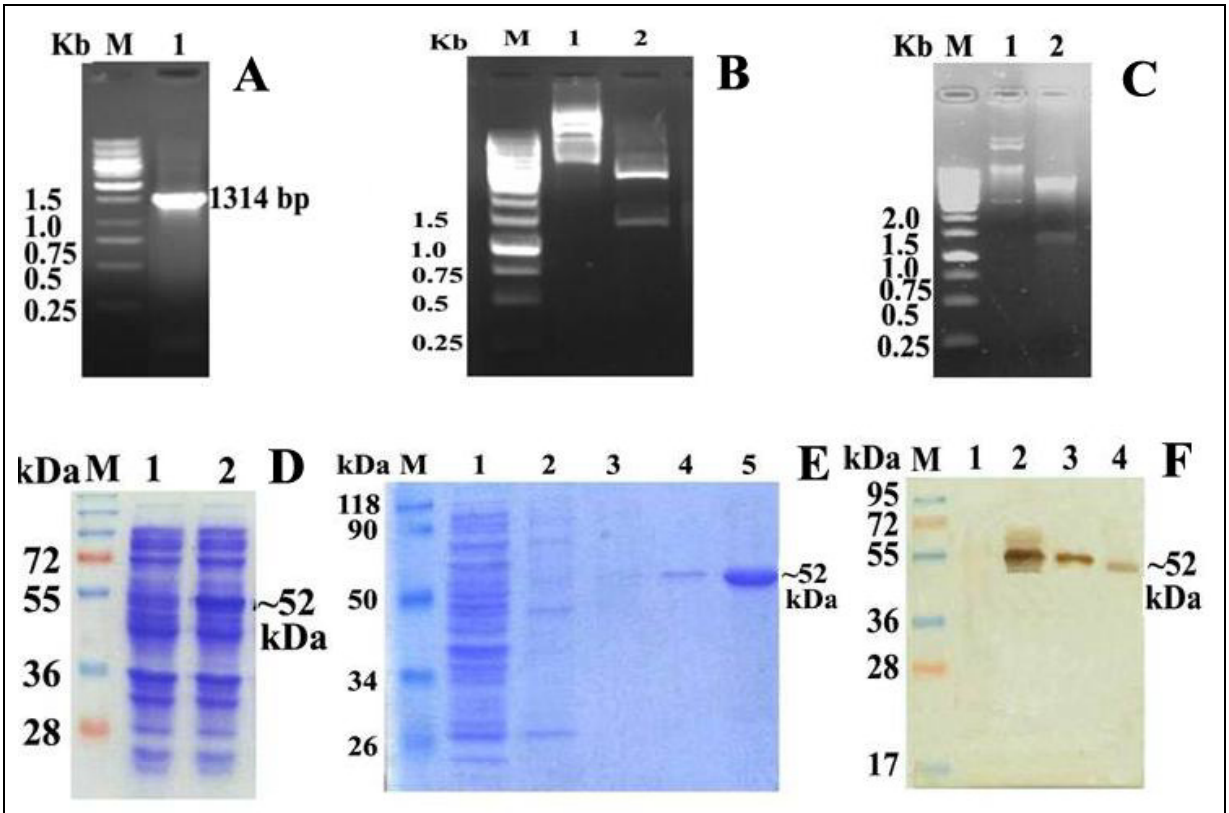

Fig 5.3: Cloning, expression, purification and western blot analysis of Adenosylhomocysteinase (ADHT). **(A)** Specific PCR of ADHT, Lane M: 1 kb ladder, Lane 1: amplified PCR product at 1314 bp; **(B)** Clone confirmation in pTZ57R/T, Lane M: 1Kb ladder, Lane 1: Undigested plasmid, Lane 2: *NdeI-HindIII* digested pTZ57R/T+ADHT; **(C)** Clone confirmation in pET28a, Lane M: 1 kb ladder, Lane 1: Undigested plasmid, Lane 2: *NdeI-HindIII* digested pET28a+ADHT; **(D)** Overexpression of rLdADHT: Lane M: Molecular weight marker, Lane 1: WCL of pET28a+ADHT before IPTG induction, Lane 2: WCL of pET28a+ADHT after IPTG induction (1mM IPTG), **(E)** Purification of rLdADHT : Lane M: Molecular weight marker, Lane 1: Supernatant of induced and sonicated lysate of pET28a+ADHT, Lane 2: Flowthrough of induced and sonicated lysate after equilibration in Ni-NTA column, Lane 3 & 4 : Wash fractions, Lane 5 : Eluted purified rLdADHT, **(F)** Western blot using anti-ADHT antibody. Lane M: Molecular weight marker; Lane 1: WCL of pET28a+TPR before IPTG induction, Lane 2 : WCL of pET28a+TPR after IPTG induction, Lane 3: Purified rLdTPR, lane 4: Soluble *L. donovani* antigen.

5.3.2 rLdTPR was cloned, overexpressed, purified and antibody raised

The TPR gene was successfully amplified and cloned (**Fig.** 5.4 A and B) in pTZ57R/T (T/A) cloning vector and transformed into competent *E.coli* DH5α cells. The positive transformants were sequenced from Delhi University (New Delhi) and submitted to the National Centre for Biotechnology Information (http: //www. ncbi.nlm.nih.gov/; Accession no. **JN007485**). The gene was further subcloned into pET28a vector and the recombinant plasmid was characterized with double enzymatic digestion (**Fig.** 5.4 C) and checked for over-expression.

1476 bp amplicon of TPR gene encoded 492 amino acids with a predicted molecular mass of ~55 kDa. Comparisons of both non-induced and induced culture in SDS-PAGE revealed that the protein was over expressed successfully and the induced protein bands corresponded to its predicted size (**Fig.** 5.4D). Purification of His-tagged rLdTPR by metal affinity chromatography yielded ~3.2 mg of pure protein from a 1-L bacterial culture (**Fig.** 5.4 E). Further western blotting using the antiserum to rLdTPR, detected single band at ~55 kDa in the soluble promastigote lysate, thus exhibiting its specificity (**Fig.** 5.4 F).

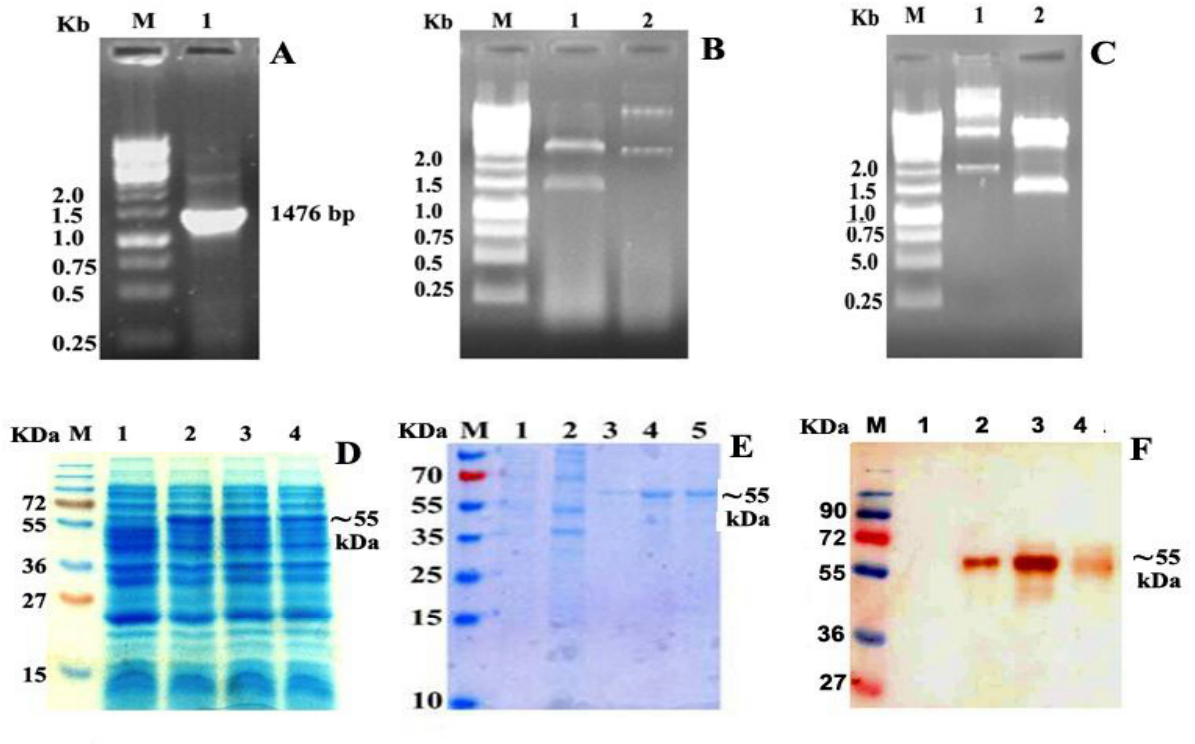

Fig 5.4: Cloning, expression, purification and western blot analysis of Trypanothione reductase (TPR). **(A)** Specific PCR of TPR, Lane M: 1 kb ladder, Lane 1: amplified PCR product at 1476 bp; **(B)** Clone confirmation in pTZ57R/T, Lane M: 1Kb ladder, Lane 1: *Bam HI-EcoRI* digested pTZ57R/T+TPR, Lane 2: Undigested plasmid; **(C)** Clone confirmation in pET28a, Lane M: 1 kb ladder, Lane 1: Undigested plasmid, Lane 2: *Bam HI-EcoRI* digested pET28a+TPR; **(D)** Overexpression of rLdTPR: Lane M: Molecular weight marker, Lane 1: WCL of pET28a+TPR before IPTG induction, Lane 2,3 & 4: WCL of pET28a+TPR after IPTG induction (1mM,1.5mM & 2mM IPTG respectively), **(E)** Purification of rLdTPR : Lane M: Molecular weight marker, Lane 1&2: Wash fractions, Lane 3, 4 & 5:: Eluted purified rLdTPR, **(F)** Western blot using anti-TPR antibody. Lane M: Molecular weight marker; Lane 1: WCL of pET28a+TPR before IPTG induction, Lane 2 : WCL of pET28a+TPR after IPTG induction, Lane 3: Purified rLdTPR, lane 4: Soluble *L. donovani* antigen.

5.4 Discussion

For assessing the immunogenicity and prophylactic potential of Th1 stimulatory proteins large quantity of the protein is required and its purification from the crude soluble extract is a tedious process alongwith the chance of losing good amount of protein. Also from practical point of view a defined recombinant rather than crude or purified vaccinal products would be desirable in terms of standardization, availability, and cost. Several Leishmania genes have been cloned for different research purposes and related proteins expressed in and purified from *E. coli* cultures. Therefore recombinant DNA technology offers the best possible way to obtain large quantities of purified proteins and hence, this method was used in this thesis for cloning and purification of the Th1 stimulatory proteins. In the present study, ADHT and TPR were successfully cloned, overexpressed and purified in large quantities using *L. donovani* genomic DNA as template. They were confirmed by sequencing and exhibited very close homology with the similar proteins of *L. infantum* and other Leishmania species. To evolve their functions, both of the proteins were expressed in the *E. coli* strains (C41 (DE3) and Rossetta) with pET28a vectors. Immunoblot studies of *L. donovani* promastigote lysates with the polyclonal antibodies raised against –rLdADHT and rLdTPR, revealed one dominant protein each of ~52 kDa and ~55 kDa, respectively. It is therefore to be noted that these proteins have been identified in the higher molecular weight fraction through proteomic studies, which is different from its observed molecular mass. This could be attributed to the fact that post-translational modifications are common in Leishmania and hence this could be due to their multiple 'charge' and/or 'mass' forms.