Original Article

Cloning and Expression of *TRYP6* Gene from *Leishmania major* (MRHO/IR/75/ER)

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Abstract

Background: *Leishmania,* needs to detoxify the macrophage derived potent peroxides (H2O2). Tryparedoxin pathway contains tryparedoxin peroxidase (TSA or TRYP). The aim of the study was to detect the full-length gene sequence and its encoded protein of the *LmTRYP6* gene (EU251502), and comparison the gene sequence with *LmTRYP6* (LmjF15.1140), another previously reported member of this gene family.
Methods: *L.major* (MRHO/IR/75/ER) promastigotes were cultured, DNA and RNA were extracted and the interested gene was amplified using PCR and RT-PCR methods. PCR/ RT-PCR fragments were purified and cloned first in pTZ57R/T and then in pET15b expression vector. The expressed protein was verified using western blot method. Characterization of the expressed protein was performed bioinformatically.
Results: Molecular evaluation revealed that the cloned *LmTRYP6* gene (EU251502) encoded a predicted 184 amino acid long protein with a theoretical isoelectric point of 6.1101. Alignment showed a number of changes in amino acid composition including the replacement of highly conserved Trp177 by Cys in *Lm*TRYP6 (ABX26130).
Conclusion: So far no study has been done on this group, i.e. *TRYP6* gene, from tryparedoxin peroxidase family. The low homology with *LmTRYP6* (LmjF15.1140) and vast array of differences observed in the gene under study (*LmTRYP6;* EU251502) could open new windows in the field of anti-*Leishmania* combat. Based on its important role in the viability and successful establishment of the parasite in the host organism it looks to be very good candidate for vaccine development and any other sort of novel drug development.

Keywords: Tryparedoxin peroxidase, *L. major,* Peroxiredoxin, TRYP6, Cloning

Introduction

Leishmania, similar to other aerobic organisms, is exposed to Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (NOS) producing by macrophage as a part of defense mechanisms against invading parasites. ROS damage various cellular components, including membrane lipids and nucleic acids, leading to parasite death. *Leishmania* possesses a unique dithiol trypanothione (N^1N^8- bisglutathionylspermidine) pathway which eliminates H_2O_2 (Nogoceke et al. 1997, McGonigle et al. 1998, Muller et al. 2003). This pathway consists of a cascade of low molecular weight thiol specific oxidoreductases acting in order

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of trypanothione reductase (TR), trypan-othione (T [SH] 2), tryparedoxin (TXN), and tryparedoxin peroxidase (TXNPx) in a NAD (P)H dependent manner to detoxify peroxides.

As shown in genedb (www.genedb.org), the genes encoding tryparedoxin peroxidase in *L. major* comprise TRYP1 (tryparedoxin per-oxidase), TRYP2 (tryparedoxin peroxidase, TXNPx, PXN1, TSA), TRYP3 (tryparedoxin peroxidase, TXNPx, PXN3, TSA), TRYP4 (tryparedoxin peroxidase, TSA, TXNPx, PXN), TRYP5 (tryparedoxin peroxidase, TXNPx, PXN, TSA), TRYP6 (tryparedoxin peroxidase, TXNPx, PXN, TSA), and TRYP7 (tryparedoxin peroxidase) that present on chromosome 15 in a tandem array. TRYPs 1, 3, 5 and 7 en-code a predicted protein with 199 amino acids whereas TRYPs 2, 4 and 6 code a predicted protein with 191 amino acids (Levick et al.1998).

Tryparedoxin peroxidase is a potential target area of specific trypanocidal agent (Nogoceke et al. 1997) due to its unique anti-oxidant system in *Trypanosomatidae* which is highly crucial for survival of parasite in an unfavorable oxidative environment (Zarley et al. 1991, Wilson et al. 1994). Also, other func-tions have been attributed to tryparedoxin peroxidase including protection of the mi-tochondrial genome from direct or indirect peroxide-mediated damage (Harder et al. 2006). Recently, its role in arsenite resistant (Lin et al. 2005) and metastasis (Walker et al. 2006) are defined. The association of tryparedoxin peroxidase with metastasis in *Leishmania* strengthens the link between parasite virulence and antioxidant defence (Walker et al. 2006). Peroxiredoxins are shown to possess peroxini-trite reductase activity and participate in de-toxification of ROS (Chen et al. 1998, Bryk et al. 2000). Therefore, due its involvement in vast array of biological phenomenon, trypare-doxin peroxidase aimed as a target for in-vestigation. To our knowledge there is no re-port on *TRYP6* gene sequence from *L. major* (MRHO/IR/75/ER), as an approved Iranian isolate which is used for leishmanization and preparation of Old World experimental *Leishma-nia* vaccine and leishmanin (Khalil et al. 1988, Nadim and Javadian 1998, Sharifi et al. 1998, Momeni et al. 1999, Bahar et al. 2000, Khamesipour et al. 2005).

In this study the full-length gene se-quence and its encoded protein of the *LmTRYP6* gene (EU251502) is reported, the gene se-quence was also compared with *LmTRYP6* (LmjF15.1140), another previously reported member of this gene family.

Materials and Methods

Parasites

L. major promastigotes (MRHO/IR/75/ ER) were grown at 26±1° C in RPMI 1640 medium (Sigma, USA) supplemented with 10% Fetal Calf Serum (FCS, Sigma), 100 U/ml penicillin G and 100 µg/ml streptomycin (Hendricks et al.1978, Ozbilgin et al. 1995, Castro et al. 2004).

DNA extraction

DNA extraction was done based on method described by Eisenberger and Jaffe (1999) with a minor modification. SDS (10%) was used instead of Triton-X 100 (1%). The proteinase K and RNase A were added at the same time and incubated for 24 h. The extracted DNA sample was quantified and analyzed by agarose gel electrophoresis.

RNA extraction

RNA extraction was performed using RNX^{TM} solution (CinnaGen) according to the manufacturer instruction. The sample was quan-tified and analyzed by agarose gel electro-phoresis under RNAse free condition.

cDNA synthesis

Using RevertAid^{TM} First Strand cDNA Synthesis Kit (#K1621, Fermentas), cDNA was synthesized according to the manufac-turer instruction.

Primers

Sense and antisense oligonucleotide prim-ers were designed based on the nucleotide se-

quence data of *LmTRYP6* gene (LmjF15. 1140) obtained from GeneBank. The sequences of sense and antisense primers used in this study are: 5′-ATGTCCTGCGGTAACGCCAAG-3′ and 5′-TTACTTGTTGTGGTCGACCTTC-ATGC-3′.

PCR, RT-PCR and sequence analysis

PCR amplification was performed using *L. major* genomic DNA or cDNA as template. PCR master mix contained 10mM Tris-HCl pH 8.3, 50mM KCl, 1.5mM MgCl2, 0.2mM each dNTPs, 20 pmol of each primer and 0.5 unit of Taq polymerase (Fermentas). Thermal cycling was applied as follow: 94º C for 5 min as initial denaturation, 30 cycles with 94º C for 45 seconds, 63º C for 45 seconds and 72º C for 45 seconds. The final 1 cycle of 72º C applied for 20 min. The PCR product was analyzed by agarose gel and the bands contained amplified products were purified using High Pure PCR Product Purification Kit (#11732668001, Roche).

Cloning of *LmTRYP6* in pTZ57R/T

This step was performed using InsT/ Aclo-ne™ PCR Product Cloning Kit according to the manufacturer instruction. The recombinant plasmid was transformed into *E. coli* XL1-Blue. To confirm the ligation reaction success, restriction digest was performed on isolated plasmids and the size of the linearized recombinant vector was assessed by agarose gel electrophoresis.

Cloning of *LmTRYP6* in pET15b

The insert of pTZ57R/T was isolated using restriction enzyme digestion and ligated into pET15b expression vector using T4 DNA ligase. The recombinant DNA was transformed into *E. coli* BL21. The in-frame cloning was verified by sequence analysis of the isolated insert from purified vector. After this verification step, *in vitro* protein expression of *Lm*TRYP6 was carried out.

Expression of *Lm*TRYP6 protein *in vitro*

The cultured bacteria harboring recombinant plasmids were induced to express by add-ing IPTG (final concentration 1mM) into the culture medium. The protein was purified after 2, 4, 6 and 24 h post induction and analyzed by SDS-PAGE and western blot.

Characterization and molecular analysis

The interest cloned gene and its encoded protein was analyzed separately using software tools accessible at NCBI, SRS, SWISS_PROT and Predict Protein server.

Results

Amplification

The PCR and RT-PCR products showed exactly equal size of *LmTRYP6* gene on agarose gel (Fig. 1).

Cloning of *LmTRYP6* gene into pTZ57R/T

Cloning of *LmTRYP6* gene was confirmed by restriction enzyme digestion using BamHI and NdeI (Fig. 2).

Cloning of *LmTRYP6* gene into pET15b

The 555 bp fragments were cut from pTZ57R/T vectors and cloned in the dephosphorylated pET15b. The recombinant pET15b plasmids were transferred into *E. coli* BL21 in order to propagating through bacteria. Isolated recombinant DNA plasmids were confirmed by restriction enzyme digestion (Fig. 3) and sequencing.

In vitro expression of *Lm*TRYP6 protein

IPTG induced recombinant *Lm*TRYP6 protein (ABX26130) was expressed in *E. coli* BL21, analyzed and characterized using SDS-PAGE (Fig. 4) and western blot methods (Fig. 5).

**Characterization and molecular analysis**

Molecular characterization of the amplified *LmTRYP6* gene at DNA level showed that the coding region of *LmTRYP6* (EU251502) contained 555 bp. A deletion in non-conserved sequence of the gene towards its C-terminal domain was seen. The homology of *LmTRYP6* gene (EU251502) with *LmTRYP6* (LmjF15.1140) was 79.6%. The coding region of DNA sequence was G_C rich (62.7%) and

codon usage was biased for G or C occupancy in the third base position (97.3 %). 44.56% of the amino acids were encoded by ten codons

UUC, CUG, AUC, CCG, AAC, AAG, GAC, GAG, CGC, and GGC.
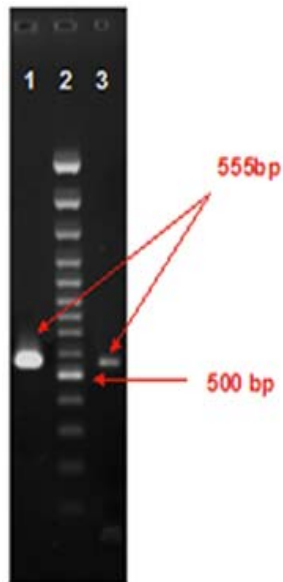
Fig. 1. Agarose gel electrophoresis of PCR products (555bp) resulting from DNA (line 1) and cDNA (line 3) amplification of the *LmTRYP6* gene (Gene Bank Accession No EU251502). As shown in this Fig., both amplicons have the same size. Line 3 shows 100bp ladder.
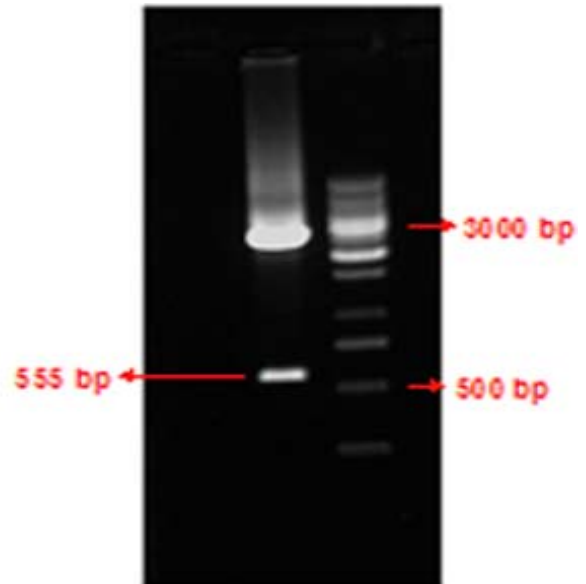

Fig. 2. Double digestion of recombinant T-vector by BamHI and NdeI enzymes. Line 1 shows 555 bp insertion cutting from T-vector (2886bp). Line 2 shows a 1Kb ladder.
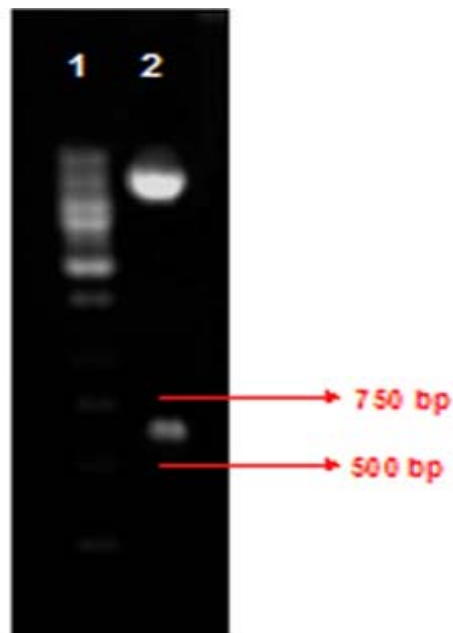
Fig. 3. Double digestion of pET15b by BamHI and NdeI restriction enzymes. Line 2 shows the linearized 5708 bp pET15b plasmid and the cut off 555 bp fragment insert from plasmid. Line 1 shows a 1kb ladder
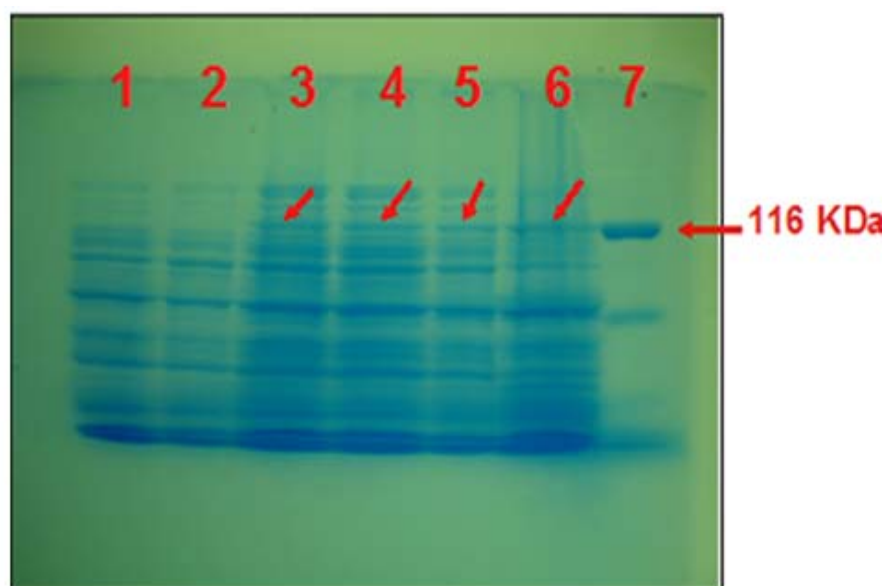
Fig. 4. SDA-PAGE analysis of extracted *Lm*TRYP6 protein (Gene Bank Accession No. ABX26130) from induced BL21 bacteria harboring recombinant plasmids. Line 1, extracted protein from culture containing BL21 bacteria with no plasmid, as control; line 2, extracted protein form culture containing BL21 bacteria harboring recombinant pET15b before inducing; line 3, extracted protein from culture containing BL21 bacteria, two hour after inducing; line 4, extracted protein from culture containing BL21 bacteria, four hour after inducing; line 5, extracted protein from culture containing BL21 bacteria, six hour after inducing; line 6, extracted protein from culture containing BL21 bacteria, an overnight after inducing. Lines 3-6 have additional band that was not in lines 1 and 2. It was considered as recombinant protein but for verifying, the two hour induced culture containing BL21 bacteria harboring recombinant plasmids were analyzed using western blot. Line 7 shows a protein size marker.
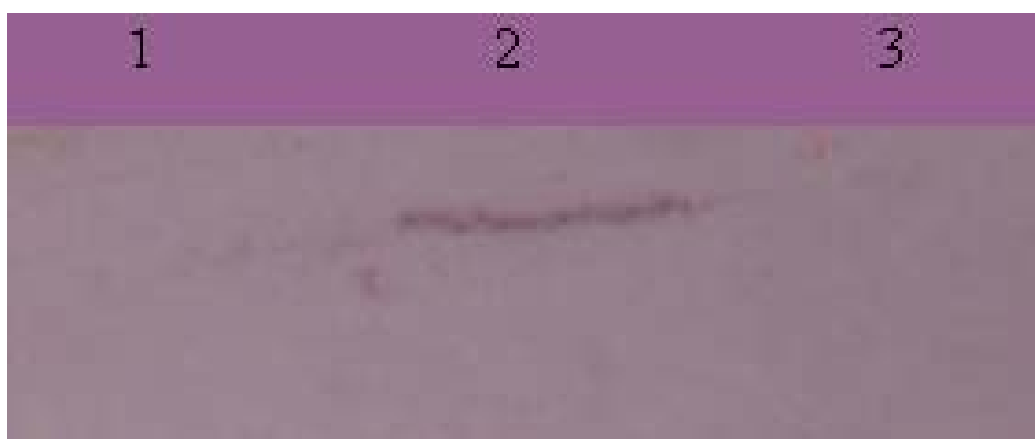
Fig. 5. Western blot analysis of extracted *Lm*TRYP6 protein (ABX26130) from two hour induced culture containing BL21 bacteria harboring recombinant plasmids. Line1, the extracted protein from culture containing BL21 bacteria with no plasmids, as control; line2, the extracted protein from two hour induced culture containing BL21 bacteria harboring recombinant plasmids; line 3, the extracted protein from culture containing BL21 bacteria harboring recombinant pET15b plasmid without any inducing. Line 2 show expressed *Lm*TRYP6 (ABX26130).

Discussion

Due to the nature of *Leishmania* genome which is comprised of intron free genes, the products of PCR and RT-PCR, as was expected, showed the identical size of 555bp (Fig. 1), on the other hand, its size was smaller than *LmTRYP6* (LmjF15.1140; 573bp). As shown in alignment with *LmTRYP6* (Lmj15.1140), a homology 79.6% between *LmTRYP6* (EU251502) and *LmTRYP6* (Lmj15.1140) was seen. There is a deletion in nonconserved sequence towards C-terminal domain of *LmTRYP6* (EU251502) (531-548; 3.1% of the gene). The CAI (Codon Adaptation Index) for *LmTRYP6* (EU251502) was 0.897, and for *LmTRYP6* (Lmj15.1140) was 0.814. This index is also a useful criterion to predict the level of expression of a gene. Therefore, *Lm*TRYP6 protein (ABX26130) encoded by *LmTRYP6* gene (EU251502) would categorized as an abundantly expressed protein. On the basis of its abundance and high level of expression, it is concluded that the expressed protein is of vital importance in the life cycle of parasite. Based on differences between the cloned gene and its encoded hypothetical protein compared to those reported previously, they were proposed and accepted in Gene Bank as new gene and its predicted protein with Accession No EU251502 and ABX26130, respectively.

The predicted protein encoded by this gene contained 184 amino acids with molecular weight of 20547.56 Daltons and an isoelecterical point (PI) of 6.1101. Its homology with *Lm*TRYP6 protein (LmjF15. 1140) was 74%. Based on the protein sequence, it is clearly a member of the 2-Cys peroxiredoxins. The characteristic feature of this category is the presence of 2 highly conserved redox active-cysteine residues: the peroxidatic cysteine (Cys52) and the resolving cysteine (Cys173) present in the valine-cysteine-proline motif.

As shown in alignment, the conserved tryptophan (Trp) 177 in some other 2Cys tryparedoxin peroxidases is replaced by Cys in *Lm*TRYP6. As far as our knowledge concern, there are two conserved tryptophans, Trp87 and Trp177, in all remaining tryparedoxin peroxidases (Montemartini et al. 1999). These two tryptophan residues contribute to the activation of the active-site cysteines (Cys52, Cys173) in other 2-Cys peroxiredoxins. Trp87 most probably interacts with Cys52, while Trp177 might similarly interact with Cys173 in the C-terminal domain (Montemartini et al. 1999, Chauhan and Mande 2002, Koshkin et al. 2004). The same consideration seems true for Trp177 with respect to Cys173 (Montemartini et al. 1999). Based on another explanation, His169 and His 182, located in the vicinity of the Cys173 may be involved in stabilizing the thiolate form (Choi et al. 1998), as one or both of these histidines might take part in Trp177 activities.

On the other hand, in similar nonparasite models, the third Cys residue is highly reactive and readily participates in non-specific intermolecular disulfide bond which is involved in catalytic activity of enzyme (Guimaraes et al. 2005). Chauhan and Mande (2002) suggested that in 3 Cys models, the N-terminal Cys, Cys52, is capable of forming a disulphide linkage with both C-terminal Cysteines, i.e. conserved Cys173 and the additional Cys located at the position of 177 in the protein under study. It is also shown that the N-terminal Cys, Cys52 in our case, assumes a central role in catalysis, the two C-terminal Cys, Cys173 and Cys177 in our case, are also important for peroxidase activity. After peroxide reduction, the N-terminal Cys, Cys52, changes to sulfenic acid (SOH), which is in turn attacked by the sulfhydryl group of the resolving cysteine, one of the C-terminal Cys, either Cys173 or Cys177 (Chauhan and Mande 2002, Koshkin et al.

2004). A possible proposed scenario might be the attack of two conserved Cys, Cys52 and Cys173 in *Lm*TRYP6 (ABX26130), by the sulfhydryl group of Cys177 to form a Cys173-Cys177 disulfide. As a consequence, the disulfide is in the flexible C-terminal arm of a single monomer, free to move and highly exposed for subsequent reduction by an external thiol (Guimaraes et al. 2005). The afore-said accounts could be summarized as presented in table1.

In conclusion, an attractive molecular target was studied. Further studies are needed for using this molecule in analyzing of vaccination experiments, designing of drugs to fight an important human pathogen and investigating the proposed theories.

Table 1. Difference between *Lm*TRYP6 (ABX26130) and *Lm*TRYP6 (LmjF15.1140)

conserved	**substitution of conserved Trp177 with Cys177**
	deletion of 6 amino acids from C-terminal (178-183)
Non-conserved	Adaptive changes with no phenotypic expression: Ile8→Leu8, Glu16→Asp16, Val18→Met18, Ser25→Thr25, Ile29→Val29, Trp38→Tyr38, Leu46→Met46, Ser49→Thr49, Val56→Ile45, Leu69→Ile69, Leu74→Ile74, Ala75→Ser75, Ala83→Ser83, Ser111→Ala111, Ser115→Ala115, Glu120→Asp120, Leu130→Val130, His136→ Asn136, Val144→Ile144, Val149→Ile149, Leu157→Ile157, Leu160→Val160, Phe163→Leu163, Lys168→Glu168 Changes with the same polarity persistence: Ser14→Glu14, Ser62→Asp62, Val63→Ala63, Ser64→Lys64, Thr99→Pro99, Ser122→Asp122, Gln123→Ser123, Ser152→Asn152 Changes with phenotypic expression: Ser10→His10, Glu17→Val17, Ala58→Gln58, Asn67→Ala67, Cys71→Thr71, Ile78→Cys78, Leu89→Ser89, Gln90→Val90, Met138→Lys138, Thr143→Ile143

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