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Original Article

Molecular Cloning and Expression of Iranian *Leishmania major* Pteridine Reductase 1

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Abstract

Background: *Leishmaniasis* is an endemic disease in 88 countries. Reports on *Leishmania* drug resistance are growing in number. The mechanism of unresponsiveness against glucantime in Iranian cutaneous leishmaniasis has not yet been characterized. To begin the first step in finding an anti-*Leishmania* chemotherapy, we prepared recombinant *L. major* PTR1 enzyme and characterized its activity by enzymatic assay.

Methods*: Leishmania* promastigote DNA was extracted and the *ptr1* gene amplified using specific primers. The PCR product was cloned in pQE30 expression vector, transformed into *E.coli* and expressed. The recombinant protein was purified, its enzymatic activity was assayed and anti-PTR1 antibody prepared in rabbit.

Results: The PCR product of *ptr1* gene was sequenced and deposited in GenBank. The amino acid sequence of Iranian *L.major* PTR1 was compared with other *Leishmania* PTR1 and showed some identities and diversities. Purified protein was reacted by anti PTR1 antibody in gel diffusion and western blot assy. Enzyme activity of purified recombinant PTR1 was 38 nmol/min per 0.4 mg of protein and it showed pteridine reduction by PTR1

Conclusion: We cloned and expressed Iranian *L. major ptr1* gene and assayed its enzymatic activity. This enzyme will be used for further investigation about *Leishmania* antifolate therapy that is effective against PTR1

Keywords: *Pteridine reductase*, Leishmania major, *Enzyme activity*, *Methotrexate resistance*

Introduction

Leishmaniases are endemic diseases in 88 countries, with over 12 million people infected, 1.5-2 million new cases per year and 350 million at risk (1). As safe vaccines are not available, there is considerable interest in the identification and characterization of biochemical pathways with the aim of developing new chemotherapies.

Pteridine auxotrophs, *Leishmania* species have an absolute requirement for external sources of these compounds (2-6). The main role of dihydrofolate reductase (DHFR) is the reduction of dihydrofolate to tetrahydrofolate, necessary for the synthesis of thymidylate, in addition to its major role in purine nucleotide synthesis in bacteria and higher eukaryotes (7). Hence, DHFR has been the major target of chemotherapy against *Leishmania.* Although antifolate drugs, such as methotrexate (MTX), are used in the treatment of other parasitic diseases like malaria (8), they have no effect on *Leishmania*, primarily due to the enzyme pteridine reductase 1 (PTR1), which sequentially reduces oxidized biopterin to dihydrobiopterin and then to tetra-

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hydrobiopterin (9,10-12). DHFR-TS shows no activity with biopterin or dihydrobiopterin (9), however, pteridine reductase 1 (PTR1) shows significant activity with folate and dihydrofolate (10). Furthermore, PTR1 is 2000-fold less sensitive than DHFR-TS to the inhibitory activity of MTX (13). The mainstay of *Leishmania* chemotherapy has been antimonial drugs, yet reports of drug resistance against, such as the recent unresponsiveness to glucantime in Iranian cutaneous leishmaniasis due to *L. tropica* (14), are on the rise.

In this study, we cloned and expressed Iranian *L. major* PTR1 for further studies of potential chemotherapy.

Materials and Methods

DNA extraction

Clinically isolated *L.major* was grown in NNN medium and mass cultured in RPMI-1640 cell culture medium enriched with 10% fetal bovine serum. *Leishmania* promastigotes were harvested by centrifugation at 12,000 rpm for 15 min, washed three times in phosphate buffered saline, and lysed in a lysis buffer(composed of 320 mM glucose, 10 mM Tris base pH 8, 1.5 mM $MgCl_2$, 2% Triton-x100), for 3 h at 37 ^oC. The solution was then boiled for 10 min, centrifuged at 12,000 rpm for 10 min and the supernatant was transferred to a new microfuge tube. The DNA was extracted using phenol-chloroform and precipitated with ethanol (15).

PCR reaction

A set of primers were designed based on the published *L.major ptr1* sequence (GenBank Accession No. L01699) with BamHI and KpnI restriction sites at the 5′end of the forward and reverse primers, respectively. Primers included: PTR F 5′-GGA TCC ATG ACT GCT CCG ACC-3′ and PTR R 5′-GGT ACC TCA GGC CCG GGT AAG-3′. The PTR1 coding region was amplified from genomic DNA.

The PCR mix contained 0.5 µg of DNA, 0.1 mM dNTP, 1.5mM $MgCl_2$, 20 pico moles each of PTR1 specific primers, 1.25 units of *Taq* DNA polymerase in a final volume of 50 µl.

The PCR reaction was carried out using 30 cycles of 30 sec at 94 ^oC, 30 sec at 61 ^oC, and 40 sec at 72 ^oC. The reaction solution was incubated for 10 min each at 94 ^oC and 72 ^oC before and after PCR cycling, respectively (16). The PCR product was separated by electrophoresis using a 1.5% agarose gel and then stained with ethidium bromide. The DNA band was visualized under ultraviolet light (UV Tran illuminator). The PCR product was purified and subjected to sequencing by the dideoxy chain termination method.

Cloning

The PCR product was electrophoresed on a low melting point (LMP), 1% agarose gel and the DNA fragment containing the PTR1 gene was sliced under long-wave UV and recovered using a DNA extraction kit (Fermentas Cat. No. K0 513). The recovered PCR product was ligated to a 3′ T tailed EcoRV digested pBluescript (17), transformed in *E. coli* XL1-blue strain competent cells (18) and dispensed on an LB agar plate containing 50 µg/ml ampicillin, 20 µg/ml X-gal and 0.1 mM IPTG.

White colonies containing the recombinant plasmid (19) were mass cultured on LB medium and subjected to plasmid extraction using the alkaline method (20). The recombinant plasmid (pBSC-ptr) was digested with BamHI and KpnI restriction enzymes, electrophoresed through LMP agarose gel, and the DNA fragment containing the *ptr1* gene was sliced by scalpel under long-wave UV and purified using a DNA extraction kit (Fermentas Cat. No. K0 513). The purified DNA fragment (PTR1 gene) was sub cloned and digested using BamHI and KpnI, in a pQE-30 expression vector and named pQE-ptr.

Gene expression

pQE-ptr was transformed in *E.coli* M15 strain, in medium containing 50 µg/ml ampicillin. A bacterial colony was inoculated into 5 ml X medium [1.2% Bacto tryptone, 2.4% yeast extract, 0.04% glycerol, 1% M9 salts (6.4% Na_2HPO_4-$7H_20$, 1.5% KH_2PO_4, 0.025% NaCl,

0.05% NH_4Cl)] and incubated overnight at 37°C in a shaker incubator at 200 rpm. The next day, the cultured bacteria was inoculated into a 50-ml flask and placed in a 37 °C shaker incubator at 200 rpm.

Culture logarithmic phase (at OD_{600} of ~0.5-0.6) was induced for 5 h with 1mM IPTG (21). Samples were collected before and after induction and lysed in 2x sample buffer (100 mM Tris-HCl pH8, 20% glycerol, 4% SDS, 2% 2-ME 0.2% bromo phenol blue) and separated by 10% SDS-PAGE (22). The gel was stained with Coomassie brilliant blue R-250. Uninduced bacteria containing pQE-ptr were analyzed in parallel.

Protein purification

A colony containing the recombinant plasmid was pre-inoculated in LB medium containing 50 µg/ml ampicillin and subcultured the next day in 50 ml culture media until an OD_{600} of 0.6-0.8 was observed, followed by induction by 1mM IPTG for 5 h at 37 °C. The 50-ml induced culture was used for protein purification. The cells were harvested by centrifugation at 8000 rpm for 10 min at 5 °C. The cell pellet was resuspended in 5 ml equilibration buffer (0.4 M urea, 50 mM Tris, 0.5M NaCl) and sonicated (5 x 10 sec) on ice. The resulting cell lysate was centrifuged at 10,000 rpm for 5 min at 5 °C and suspended in 2 ml equilibration buffer and then incubated overnight at 5 °C. It was centrifuged the next day at 10,000 rpm for 5 min at 5 °C. The clear supernatant was colleted and used for protein purification (21).

The recombinant protein was purified by affinity chromatography, based on its N-terminal His_6 tag. The cell lysate was applied to the column at a flow rate of 15 drops/min, and allowed to bind. The bound protein was eluted with elution buffer (4 mM urea, 50 mM Tris, 0.5 M NaCl, 1 mM imidazol).

Production of antibody against PTR1 and western blot analysis

The purified recombinant PTR1 was subjected to antibody production. Briefly, a white rabbit was first immunized using 50 µg of the recombinant PTR1 in Freund's complete adjuvant. After three weeks, the rabbit was boosted with 50 µg recombinant PTR1 in incomplete Freund's adjuvant. After 10 days, the rabbit was boosted with 50 µg recombinant PTR1, and the serum was obtained after the last bleeding.

For immunoblotting, purified recombinant PTR1 and *Leishmania* cell lysate were separated on 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membrane (22). After UV crosslinking for protein fixation, the membrane was blocked with 3% BSA at room temperature. After 1 h, the membrane was washed twice with 1x TBS (10mM Tris, 150 mM NaCl), and was then incubated with the rabbit anti-PTR1 antiserum at a 1:1000 dilution for 1 h at 37 °C. The membrane was washed three times with 1x TBS, containing 0.1% Tween 20 (TBST) and incubated in goat anti-rabbit IgG HRP conjugate solution at a 1:5000 dilution for 1 h at 37 °C (22, 23). Binding was detected using DAB and H2O2.

Western blot analysis using His-tag* monoclonal *Ab

Purified protein was electrophoresed on 10% SDS-PAGE and transferred to a nitrocellulose membrane. Western blot was carried out as described previously (23). Briefly, the membrane was incubated using a His-tag monoclonal antibody as the primary antibody (1:1000) and sheep anti-mouse IgG horse radish peroxidase (HRP) conjugate as secondary antibody (1:1000) and detected by colorimetry with diamino benzoic acid (DAB) and H_2O_2.

Pteridine reductase activity assay

Purified PTR1 activity was assayed using 0.8 mM pteridine (6-biopterine) as the substrate and 0.04 mM NADPH as the co-factor in 20 mM Tris HCl (pH 4.7) at 30 °C (9). NADPH oxidation was monitored at 340 nm at 30 min, 1.5 h, 2 h, 3 h and 20 h. As pteridine exhibits absorbance changes while being reduced, the enzyme activity was measured by $A=\varepsilon CL$, where A is the absorbance of reduced pteridine, ε is extinction coefficient (for NADPH: 6.22×10^{-3}), C is nmol/min per mg of protein and L is

the diameter (cm) of the spectrophotometer cuvette.

Results

Leishmania major promastigote DNA was extracted and subjected to PCR amplification using PTR1 specific primers. The 866-bp PCR product of PTR1 gene (Fig. 1) was purified, sequenced and deposited to GenBank at accession number EF113119. The PCR product was cloned in pBluescript. Recombinant plasmid was digested with BamHI and KpnI and purified by LMP agarose gel (Fig. 2).

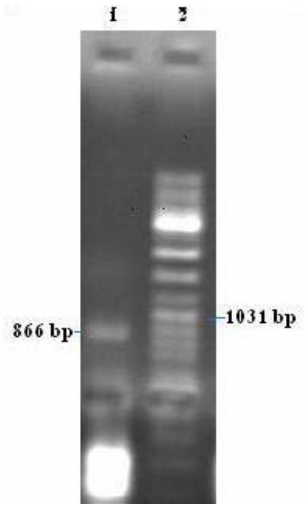

Fig. 1: Electrophoresis of PCR product on 1% agarose gel.
Lane 1: The 866-bp as PCR product of *leishmania major ptr1* gene
Lane 2**:** 100-bp DNA ladder marker

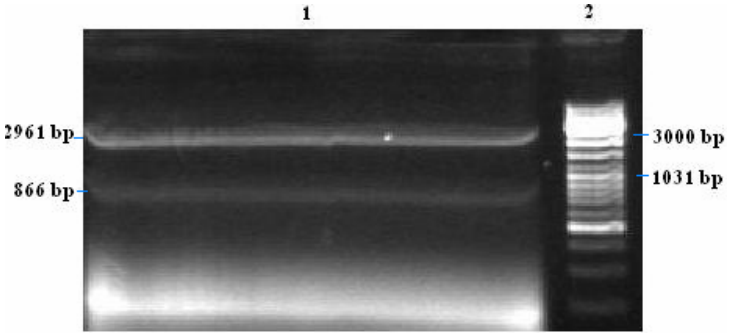

Fig. 2: Electrophoresis on 1% LMP agarose gel
Lane 1: Digested pBSC-ptr by BamH1 & Kpn1
Lane 2: 100bp DNA ladder marker

Purified fragment (PTR1 gene) subcloned in pQE-30 expression vector (pQE-ptr) and confirmed by restriction digestion using BamHI and KpnI enzymes (Fig. 3).

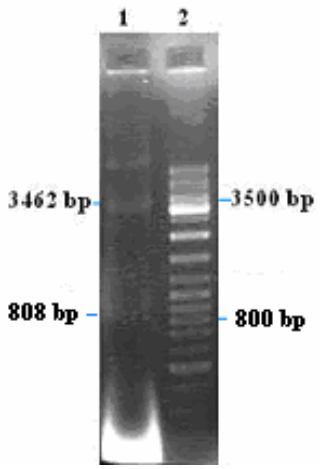

Fig. 3: 1.5% Agarose gel electrophoresis.
Lane 1: Digested pQE-ptr by BamH1, Kpn1
Lane 2: 100bp DNA ladder marker

Recombinant pQE-ptr was mass cultured and induced with IPTG. Samples were collected before and after induction, lysed and separated by 10% SDS-PAGE. Un induced bacteria containing pQE-ptr were analyzed in parallel. Figure 4 shows the 10% SDS-PAGE of the 33-kDa expressed protein (PTR1 enzyme).

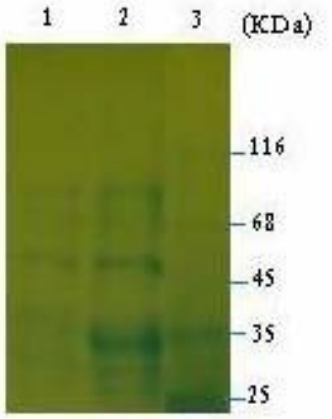

Fig. 4: Analyses of expressed PTR1 protein (33 kDa) by 10% SDS-PAGE.
Lane 1: Cell lysate before induction.
Lane 2: Cell lysate after induction by IPTG.
Lane 3: Protein marker

The recombinant protein was purified by affinity chromatography. Purified protein was electrophoresed on 10% SDS-PAGE and transferred to a nitrocellulose membrane. Western blot was carried out with two different antibody groups. Figures 5a and 5b show western blot analysis of lysate cell transferred on nitrocellulose membrane. The induced lysate cells and purified recombinant PTR1 protein were reacted by antibodies (His tag monoclonal antibody and anti PTR1 antibody) but were not reacted induced non recombinant pQE30 or M15 bacterial cells as showing in Fig. 5a and 5b.

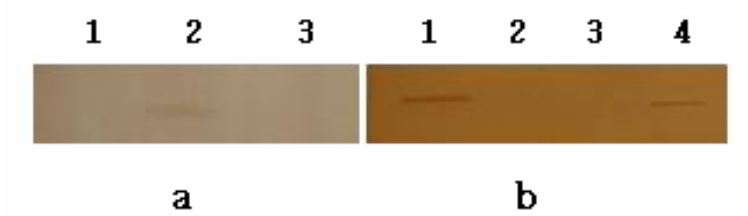

Fig. 5a: Recombinant PTR1 analyzed by western blot. Induced pQE-30 and M15 as antigen and anti-His-tag monoclonal Ab as primary antibody and detected by goat anti-mouse IgG HRP conjugate. Lane 1: Induced pEQ-30. Lane 2: Affinity purified recombinant *L. major* PTR1. Lane 3: Induced M15 bacterial cell

Figure 5b: Identification of purified PTR1, pQE-ptr, pQE-30 and M15 by western blot. Induced pQE-ptr, pQE-30, M15 bacteria and purified PTR1 as antigen (lanes 1-4, respectively), with rabbit serum as the primary antibody, detected by sheep anti-rabbit IgG HRP conjugate. Lane 1: Induced pQE-ptr. Lane 2: Induced pQE-30. Lane 3: Induced M15 bacterial cell. Lane 4: Purified recombinant PTR1.

We compared PTR1 amino acid sequences of *L. major* (Iran) (Accession number EF113119), *L. major* (Accession number L01699), *L.tropica* (Iran) (Accession number DQ313225) and L. *donovani* (Accession number AY547305) by CLC free workbench3 software and showed some differences (Fig. 6).

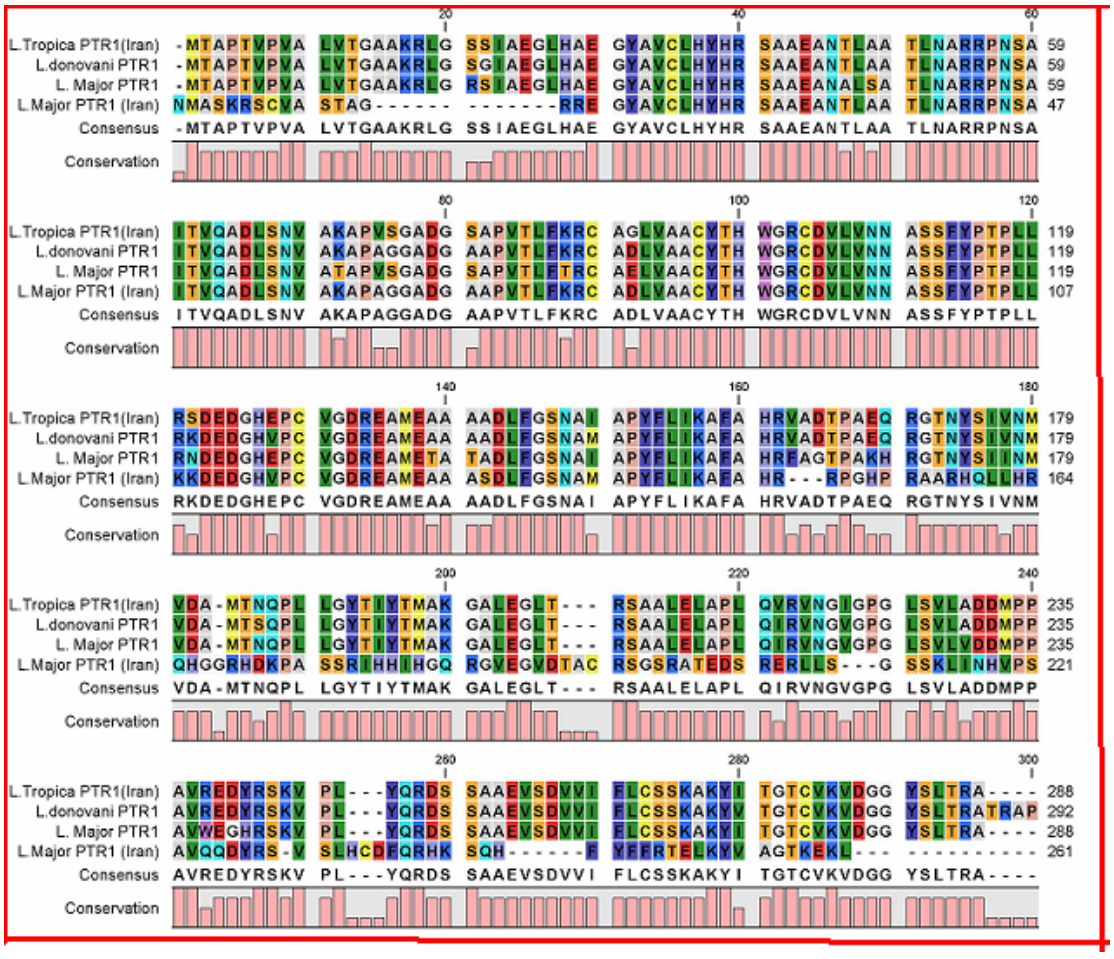

Fig. 6: Amino acid sequence of Iranian L.*major* PTR1 (Accession number EF113119) was compared with amino acid sequence of L.*major* PTR1 (Accession number L01699), Iranian L.*tropica* (Accession number DQ313225), L.*donovani* PTR1 (Accession number AY547305).

The enzyme activity of purified recombinant PTR1 was 38 nmol/min per 0.4 mg of protein and it shows pteridine reduction by PTR1. Optimum pH for the reduction of 6-biopterin was 4.7 (Table 1).

Table 1: Measurement of enzyme activity of *L. major* PTR1

Protein quantity	30 min[a]	1.5 h[a]	2 h[a]	3 h[a]	20 h[a]	C/60min[b]	C/min[b]	
Pr PTR[c]	0.4 mg	6.3	8.76	9.04	11.74	27.42	2276.5	38

[a] Absorbance of reduced pteridine at 340 nm
[b] Concentration of purified *L. major* PTR1 enzyme
[c] Pr PTR: purified recombinant *L. major* PTR1 enzyme

Discussion

Nutritional, biochemical and genetic studies show that conjugated folates and unconjugated biopterin are essential for leishmanial growth (2-7, 24, 25), due to the fact that *Leishmania* parasites cannot synthesize pteridine from GTP, but actually prepare it through a salvage pathway (2). This characteristic shows the importance of PTR1 in the oxidized pterin salvage. The PTR1-mediated modulation of MTX potency is considered important in anti *Leishmania* chemotherapy in vivo, since antifolates are effective against *Leishmania* in cultured macrophages only in 'folate-free' media (26). Schuttelkopf et al demontrated that PTR1 enzyme and represents a target for the development of improved therapies for infections caused by these parasites as it participates in the salvage of pterins. They proposed that three valuable enzyme activities, DHFR-TS and PTR1 are drugs target for trypanosomatid infection including *Leishmania* (27).

One of the major targets of chemotherapy against *Leishmania* is DHFR and antifolate drugs such as methotrexate (MTX), are used in the treatment of other parasitic diseases like malaria (8, 28). Because *Leishmania* PTR1 reduces oxidized biopterin to dihydrobiopterin and tetrahydrobiopterin (9-12), antifolate drugs have no effect on this parasite. Leishmaniasis is an important health problem in Iran and antimonial drugs are used as anti *Leishmania*, but there are some reports on *leishmania* drug resistance in Iranian cutaneous leishmaniasis (8, 14). As a first step toward overcoming this problem, for the first time, we have cloned and expressed Iranian *L. major* PTR1.

The amino acid sequence of Iranian L.*major* PTR1 was compared with other *Leishmania* PTR1 and showed some identities and diversities. As the first report on the expression and enzyme activity assay of Iranian *L. major* PTR1, we found the specific activity of *L. major* PTR1 was less than *lizard leishmania*, as measured by Kazemi *et al.* (29).

In conclusion we cloned and expressed Iranian *L. major ptr1* gene and successfully assayed enzymatic activity. This enzyme will be used to continue the research for *Leishmania* antifolate therapy that is effective against PTR1.

Abbreviations

PTR1: pteridine reductase 1; MTX: methotrexate; DHFR-TS: dihydrofolate reductase-thymidylate synthase; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophesis; NADPH: nicotinamide adenine dinucleotide phosphate; IPTG: isopropyl-β-D-1-thiogalactopyranoside; LMP: low melting point; X-gal: 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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