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Molecular Cloning, Expression and Enzymatic Assay of Pteridine Reductase 1 from Iranian Lizard *Leishmania*

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ABSTRACT

Background: Currently, there are no effective vaccines against leishmaniasis, and treatment using pentavalent antimonial drugs is occasionally effective and often toxic for patients. The PTR1 enzyme, which causes antifolate drug resistance in *Leishmania* parasites encoded by gene *pteridine reductase 1* (*ptr1*). Since *Leishmania* lacks pteridine and folate metabolism, it cannot synthesize the pteridine moiety from guanine triphosphate. Therefore, it must produce pteridine using PTR1, an essential part of the salvage pathway that reduces oxidized pteridines. Thus, PTR1 is a good drug-target candidate for anti-*Leishmania* chemotherapy. The aim of this study was the cloning, expression, and enzymatic assay of the *ptr1* gene from Iranian lizard *Leishmania* as a model for further studies on *Leishmania*. **Methods:** Promastigote DNA was extracted from the Iranian lizard *Leishmania*, and the *ptr1* gene was amplified using specific primers. The PCR product was cloned, transformed into *Escherichia coli* strain JM109, and expressed. The recombinant protein (PTR1 enzyme) was then purified and assayed. **Results:** *ptr1* gene was successfully amplified and cloned into expression vector. Recombinant protein (PTR1 enzyme) was purified using affinity chromatography and confirmed by Western-blot and dot blot using anti-*Leishmania major* PTR1 antibody and anti-T7 tag monoclonal antibody, respectively. The enzymatic assay was confirmed as PTR1 witch performed using 6-biopterin as a substrate and nicotinamide adenine dinucleotide phosphate as a coenzyme. **Conclusion:** Iranian lizard *Leishmania ptr1* was expressed and enzymatic assay was performed successfully. *Iran. Biomed. J. 14 (3): 97-102, 2010*

Keywords: Pteridine reductase 1 (PTR1), *Leishmania*, Gene expression

INTRODUCTION

Leishmania parasites infect millions of people worldwide [1]. No effective vaccine is available and treatment by pentavalent antimonial drugs is only occasionally effective and often toxic for patients [2]. Furthermore, Hadighi *et al.* [3] reported unresponsiveness to glucantime treatment in Iranian cutaneous leishmaniasis due to drug-resistant *Leishmania tropica* parasites. Although antifolate drugs are used in the treatment of other parasitic diseases like malaria, they have no effect on leishmaniasis [4] because of the presence of the gene *pteridine reductase 1* (*ptr1*) in the *Leishmania* parasite [5].

Purines and pyrimidines perform many vital functions in cells. *Leishmania* parasites lack the metabolic machinery to prepare purine nucleotides *de novo* and rely on their hosts for preformed purines. This mechanism of purine salvage can be used as a potential target for anti-parasitic drugs. Because the pyrimidine biosynthetic pathways of *Leishmania* and its host, i.e., human, are similar, it is thought that therapeutic manipulation of pyrimidine metabolism in *Leishmania* would be less effective as compared to manipulation of the purine salvage pathway [6-8]. Owing to its purine salvage dependency, *Leishmania* requires an exogenous source of pteridines. In the absence of pteridines, *Leishmania* uses a salvage pathway in which the

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enzyme PTR1 reduces pteridines, such as biopterin and folate [9-11], thereby reducing the effectiveness of methotrexate—a dihydrofolate reductase (DHFR) inhibitor—in *Leishmania antifolate* therapy [reviewed in 2, 8]. The sensitivity of PTR1 to the inhibitory activity of methotrexate is 2000-folds less than that of DHFR-thymidylate synthase [7].

In 1964, Adler [12] reported nine species of lizard *Leishmania*. Each species of lizard *Leishmania* has individual characteristics. In 1966, Hoar and Wallace [13] suggested that lizard *Leishmania* promastigotes are observed in NNN (Novy-MacNeal-Nicolle) medium or insect vectors, whereas amastigotes are observed in mammalian hosts. However, we have isolated a lizard *Leishmania* promastigote [14] which differs from lizard *Leishmania* isolated previously in other countries because that lived in heart blood. This lizard *Leishmania* was isolated using heart blood culture [14]. In 1990, the WHO Experts Committee has classified lizard *Leishmania* as belonging to the *Sauroleishmania* genus, but others believe that lizard *Leishmania* belongs to the trypanosome genus [15]. Gomes-Eichelmann *et al.* [16] reported some differences between lizard *Leishmania* and mammalian *Leishmania* with regard to kinetoplast nucleic acid sequences, chromosomes, and membrane lipids, which are not the same as those reported in mammalian *Leishmania*.

In this study, for the first time, we cloned and expressed *ptr1* from Iranian lizard *Leishmania* and characterized the resultant recombinant PTR1 enzyme by performing an enzymatic assay. This model can be used for further investigations into *Leishmania* drug resistance and chemotherapy.

MATERIALS AND METHODS

DNA extraction. Because there is no intron in *Leishmania* genes [17], PCR was performed using genomic DNA. Iranian lizard *Leishmania* [14]. Promastigote DNA was extracted as previously described [18]. Briefly, *Leishmania* promastigotes were grown in NNN medium and mass cultured in RPMI-1640 medium enriched with 10% fetal bovine serum. *Leishmania* promastigotes were harvested by centrifugation at 12,000 ×g and washed three times with phosphate-buffered saline. Washed promastigote were lysed with lyses buffer (320 mM glucose, 10 mM Tris base pH 8, 5 mM $MgCl_2$, 2% Triton-X 100) at 37°C for 3 h and boiled for 10 min. Samples were centrifuged at 12,000 ×g for 10 min, and the supernatant was transferred to a new microfuge tube, where it was subjected to DNA extraction using phenol-chloroform and precipitated with ethanol.

PCR. The PCR mix contained 0.5 µg of DNA, 0.1 mM dNTP, 1.5 mM $MgCl_2$, 20 pmole of each *ptr1*-specific forward and reverse primers [5] (Ptr F 5′-GAG CTC ATA TGA CGA CTT CTC CGA-3′ and Ptr R 5′- GAA TTC TCA GGC CCG GGT AAG GC-3′), and 1.25 units of *Taq* DNA polymerase (Cinnagen, Iran) in a final volume of 50µl. PCR was carried out within 30 cycles: denaturation at 94°C for 30 s, annealing at 65°C for 30 s, and elongation at 72°C for 40 s [19]. The PCR product was subjected to electrophoresis on 1% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light (UV transilluminator). *Sac*I and *Eco*RI restriction sites were add to the 5′ end sequences of the forward and reverse primers, respectively. PCR product was purified, sequenced and deposited to GenBank. (Accession number EF197114.2)

Cloning. PBlueScript was digested using a blunt-end cutter *EcoR*V restriction enzyme, and a 3′ terminal T nucleotide was added by the terminal transferase enzyme during PCR. The PCR product was ligated to the T vector and transformed in the XL1-blue *Escherichia coli* strain [20]. Recombinant plasmids were screened by using X-gal and isopropyl-β-D-thio galactopyranoside (IPTG) [21]. The recombinant plasmid was extracted [22] and subjected to electrophoresis on 0.8% agarose gel. The plasmid was digested with *Sac*I and *Eco*RI enzymes, and the products were separated by electrophoresis with a low-melting-point agarose gel. The gel contained the DNA fragment with the *ptr1* gene, which was cut with a scalpel under a long-wave UV and purified using a DNA extraction kit No k0513(Fermentas, Lithuania). The purified DNA fragment (*ptr1* gene) was sub cloned in a *Sac*I- and *Eco*RI-digested pGEMEX-1 expression vector and named pKBPTR.

Gene expression. Gene expression was performed as previously described [23], with some modifications. Briefly, *E. coli* strain JM109 (DE3) was transformed with the pKBPTR plasmid and selected using Luria-Bertani (LB) agar containing 50 µg/ml ampicillin. The transformed colony was inoculated into a 3-ml culture tube containing X medium (1.2% Bacto Tryptone, 2.4% yeast extract, 0.04% glycerol and 1% M9 salts). M9 salts

contained 6.4% $Na_2H_2O_4$-$7H_2O$, 1.5% KH_2PO_4, 0.025% NaCl and 0.05% NH_4Cl and allowed to grow overnight at 37°C in a shaker at 160 rpm. The next day, the cultured bacteria were inoculated into a 50-ml flask and allowed to grow at 37°C in a shaker at 200 rpm. Cultures in the logarithmic phase (OD_{600} of 0.6) were induced for 6 h with 1 mM IPTG. After induction, cells were lysed in 2X sample buffer (100 mM Tris HCl pH 8, 20% glycerol, 4% SDS, 2% beta mercaptoethanol and 0.2% bromophenol blue) and analyzed by 12% SDS-PAGE [24]. The gel was stained with Coomassie brilliant blue R-250 and an uninduced control culture was analyzed in parallel.

Protein purification. Colonies from LB agar plates were used to prepare the pre-inoculation in X medium containing 50 µg/ml ampicillin. The pre-inoculums were used to grow 500 ml of cell culture in X medium with 50 µg/ml ampicillin at 37°C to an OD_{600} of 0.6-0.8 followed by IPTG (1 mM) induction after 6 h at 37°C. After centrifugation at 6,500 ×g for 10 min, the cell pellet was suspended in 15 ml equilibration buffer (50 mM Tris and 0.5 M NaCl) containing a protease inhibitor cocktail and the cell suspension was sonicated (2 × 30 s) on ice. The cells were harvested by centrifugation at 4,000 ×g for 15 min, suspended in 5 ml ice-cold buffer containing 6 M urea, and incubated on ice for 1 h. The insoluble materials were removed by centrifugation at 12,000 ×g for 20 min. The supernatant was filtered through a 0.45-micron membrane before allowing it to bind to the resin. The recombinant protein was purified by affinity chromatography by attaching a T7 tag to its N-terminal. The T7 Tag antibody agarose column was equilibrated with 15 ml Bind/Wash buffer (42.9 mM Na_2HPO_4, 14.7 mM KH_2PO_4, 27 mM KCl, 1.37 mM NaCl, 1% Tween 20, and 0.02% Na_3N). The filtered supernatant was dialyzed to remove urea and then applied to the column and allowed to bind at a flow rate of 15 drops/min. The bound protein was eluted using an elution buffer containing 1 M citric acid (pH 2). To neutralize the eluted fractions, 1.5% neutralization buffer (2 M Tris base, pH 10.4) was applied. The sample was dialyzed overnight in 500 ml of Bind/Wash buffer, which was replaced four times over a period of 24 h. Dot blot analysis was carried out by using the purified protein as an antigen and the T7-Tag monoclonal antibody or anti- *Leishmania major* PTR1 [18] as the primary antibodies. Western-blotting was carried out as follows: the purified protein was subjected to SDS-PAGE and was transferred onto a nitrocellulose membrane. Protein expression was detected by using the T7 Tag monoclonal antibody as the primary antibody and a goat anti-mouse IgG-HRP conjugate.

Pteridine reductase assay. *PTR1* activity was measured at 30°C in 20 mM Tris-HCl (pH 4.7) with 0.04 mM NADPH (nicotinamide adenine dinucleotide phosphate, Sigma-Aldrich, 3050 Spruce St., St. Louis, MO) and 0.8 mM pteridine substrate (6-biopterine, Sigma) [5]. Pteridines exhibit absorbance changes when they undergo reduction; therefore, NADPH (oxidation was monitored at 340 nm at 30 min, 1.5 h, and 20 h. Enzyme activity was calculated by using the formula $A = \varepsilon CL$, where A is the absorbance of the reduced pteridine, ε is the extinction coefficient (6.22×10^{-3} for NADPH), C is rate (in nmol/min per mg of protein), and L is the diameter of the spectrophotometer cuvette (cm).

RESULTS

DNA extraction and PCR amplification. Figure 1 shows the results of the separation of the *ptr1* PCR product (870 bp) on a 1% agarose gel. PTR1 sequence was submitted to the GenBank as accession number EF197114.

Gene cloning. PCR product was ligated into pBlueScript and transformed into *E. coli* XL-1 blue. Recombinant plasmid was digested by SacI and

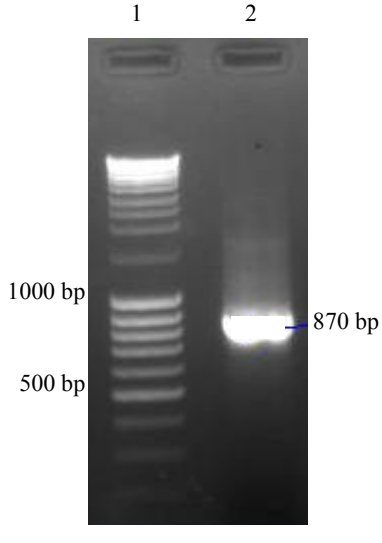

Fig. 1. PCR product of the *ptr1* gene identified on 1% agarose gel electrophoresis. Lane 1, 100-bp DNA ladder marker; Lane 2, *ptr1* PCR product.

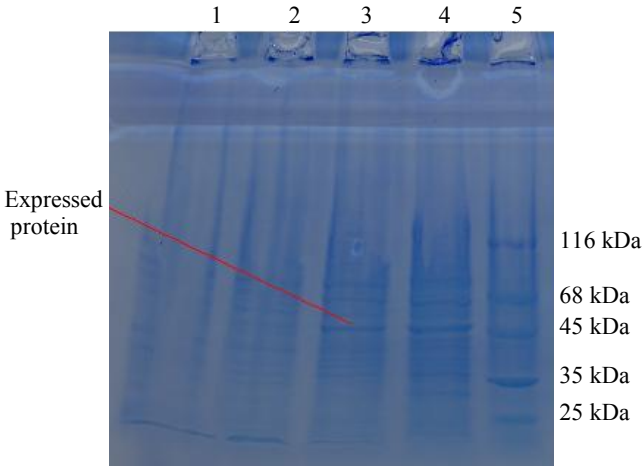

Fig. 2. Cells induced with isopropyl-ß-D-thiogalacto-pyranoside separated on a12% SDS-PAGE gel. Lane 1, cell lysate before induction; lane 2, cell lysate 1 h after induction; PTR was not expressed; lane 3, cell lysate 2 h after induction (PTR1 was expressed); lane 4, cell lysate 4 h after induction (PTR1 was expressed) and lane 5, protein marker.

EcoRI and digested fragment (*ptr1* gene) was subcloned in SacI and EcoRI-digested pGEMEX1 expression vector (pKBPTR) and subjected to protein expression. Figure 2 shows the SDS-PAGE of the expressed protein (The PTR1 and T7 10 gene fusion proteins are approximately 51 kDa in size).

Protein purification and confirmation. Figure 3 shows the results of the dot blot assay performed using the purified enzyme as antigen/anti-*L. major* PTR1 (plate A) and the purified enzyme antigen/T7 Tag monoclonal antibody (plate B). Protein expression was detected by using a goat anti-mouse IgG-HRP conjugate. Figure 4 shows the results of Western-blotting analysis performed using purified recombinant lizard *Leishmania* PTR1 as the antigen and T7 Tag monoclonal antibody as the primary antibody, detected by using a goat anti-mouse IgG-HRP conjugate.

PTR1 enzymatic activity. The enzymatic activity, as measured by the reduction of pteridine with NADPH (Table 1), was 140 nmol/min per 1.6 mg protein (PTR1). The lizard *Leishmania* PTR1 activity measured was 368% greater than that of the Iranian *L. major* [24].

$A = \varepsilon CL$, $65.5 - 13.26 = 6.22 \times 10^{-3} \times C \times L \rightarrow C_{60min} = 6.22 / 8390 \rightarrow C_{min} = 140$

DISCUSSION

PTR1 is an essential enzyme for pterin salvage in the *Leishmania* parasite, and can potentially be used as a target in the development of improved therapies [7, 25]. Bello *et al.* [5] found that the enzyme PTR1 encoded by the ptr1 gene located in the H-region is responsible for methotrexate resistance in *Leishmania* because ptr1-negative mutants in their study required reduced biopterin (dihydropterin or tetrahydropterin), and that PTR1 mediates pterin salvage. Ouellette *et al.* [11] detected acquired resistance to methotrexate in *Leishmania* species that contain an inverted repeat with a 30-kDa product and Papadopoulou *et al.* [10] recognized that the amplification of the H-circle in *Leishmania* species accompanies selection with methotrexate. Kumar *et al.* [26] suggested that PTR1 is degraded during the stationary phase of growth, which is mediated by the proteasome; this leads to decreased levels of H4-biopterin, and subsequently to the highly infective stage of the parasite. They also suggested that PTR1 is important in the identification of a new target molecule for therapeutic intervention. Because leishmaniasis presents a significant health problem in Iran and drug resistance and failure of glucantime treatment have been reported [3, 4], we cloned, expressed, and tested the enzymatic properties of Iranian lizard *Leishmania* PTR1 as a model for further

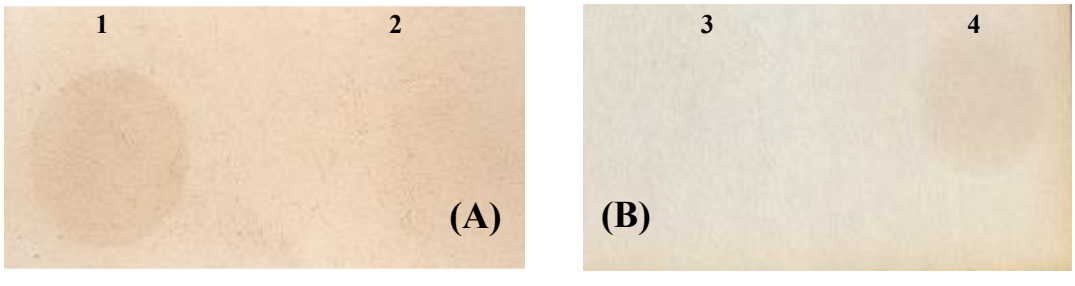

Fig. 3. Dot blot analysis of purified recombinant lizard *Leishmania* PTR1 as the antigen using an antibody against *L. major* PTR1 (plate A) and T7 Tag monoclonal antibody (plate B) as the primary antibodies, as detected by using a goat anti- mouse IgG-HRP conjugate. Lanes 1 and 4, antigen (PTR1) and lanes 2 and 3, control (bacterial cell lysate).

Table 1. Measurement of PTR1 enzymatic activities*.

Parameter Enzyme	Protein quantity	30 min	1.5 h	20 h	$C/_{60min}$	$C/_{min}$
Purified recombinant lizard *Leishmania* PTR1	1.6 mg	14.88	16.8	65.5	8360.0	140
Purified recombinant *L. major* PTR1	0.4 mg	6.30	8.76	27.5	2276.5	38

*340 nm.

investigations into chemotherapy against *Leishmania*. The size of the ptr1 gene has been reported as 867 bp for *L. tropica* [3] and 864 bp for *Leishmania donovani* [27]. Whereas, our study shows that the size of this gene is 870 bp. Leblanc *et al.* [28] isolated a *Leishmania* ptr1 gene and showed that PTR1 is a tetramer that revives pteridines. Iranian lizard leishmania PTR1 specific enzyme activity was 140 nmol/1.6 mg proteins, which is higher than that of *L. major* PTR1, as assessed by its expression and purification [18]. The optimum pH for the reduction of 6-biopterin has been shown to be 4.7 as compared to the optimum pH of 7 for *L. major* [5] and 3.7 for *Trypanosoma brucei* [29].

These tests confirmed that the purified protein was indeed the PTR1 enzyme. As an additional discovery, Iranian lizard *Leishmania* promastigote was found in the host bloodstream [14], Cavazzuti *et al.* [7] suggested that the use of antifolate-targeting enzymes in combination with the new therapeutic may result in an effective anti-parasitic drug.

The model described in this study can potentially be used as a new enzymatic model for *Leishmania* chemotherapy and drug resistance studies, but further investigation is required. This model may be used, with modifications, for similar studies on human *Leishmania* to develop a complete system for identifying an effective antifolate chemotherapy agent for leishmaniasis.

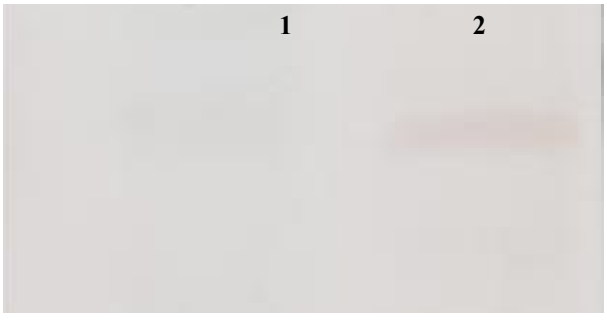

Fig. 4. Western-blotting analysis of purified recombinant lizard *Leishmania* PTR1 as antigen that was detected by T7 tag monoclonal antibody. Lane 1, control (bacterial cell lysate) and lane 2, purified recombinant lizard *Leishmania* PTR1.

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