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THE PURIFICATION RECOMBINANT TSA PROTEIN AND CONFIRMATION WITH SDS-PAGE AND WESTERN BLOT

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ABSTRACT

The TSA protein may be helpful as an ingredient of a subunit vaccine against leishmaniasis. When tested under similar experimental conditions, it was able to induce similar partial protective effects. The aim of this study was the production, purification new construction of TSA protein. In this regard, the recombinant plasmid pET28-a+used for TSA expression constructed with the TSA gene of cutaneous leishmaniasis fused with his-tag. This recombinant clone over expressed in *Escherichia coli* BL-21 (DE-3).The expressed fusion protein found almost completely in the insoluble form in cell lysate. The purification was performed under denaturing conditions in the presence of 8M urea by Ni–NTA column and dialysis. The purified recombinant proteins confirmed western blot analysis utilized polyclonal antiserum. Therefore, such an association of antigens increased the number of targeted epitopes by immune system with the prospects the responses are at least additive if not synergistic.

KEY WORDS: Vaccine, TSA, Leishmaniasis ,Cloning

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INTRODUCTION

Leishmaniasis is a group of vector-borne diseases precipitated by obligate intracellular protozoan parasites belonging to the genus *Leishmania*. Clinical displays range from self-healing cutaneous lesions to fatal visceral disease. Leishmaniasis is commonplace in six continents and considered endemic in 88 countries[1].Leishmaniasis has recently stated one of the world's most serious parasitic diseases by the World Health Organization (http://www.who.org). About 350 million people are at the moment at risk of getting the various forms of the disease, and the annual incidence of new cases is nearly 2million (1.5 million cases of cutaneous leishmaniasis and 0.5million cases of visceral leishmaniasis). A prophylactic vaccination would prove to be the most effective strategy to control the infection and spreading of diseases [2].Nevertheless, despite substantial effort made in developing a vaccine, there is no licensed vaccine against human leishmaniasis [3]. Different vaccination strategies have tested including the use of heat-killed, genetically adapted or live-attenuated parasites as well as several subunit- and DNA- based vaccines with adjuvant [4].In recent years, important progress has made in identifying vaccine candidates which can induce a protective response. Most of the works have focused on antigens GP63, CP (A, B), Lack, GP64, M2, PSA2, LmSTI1, P20, A2, Leif, P8, Histon H1, Ribosomal like protein [5-9]. Among the vaccine candidates, TSA (thiol-specific antioxidant protein) has been introduced as one of the main vaccine candidates[5]. TSA is *L.major* recombinant protein homologue to eukaryotic thiol-specific-antioxidant protein with molecular weight of 22.1 kDa. It composed of 200 amino acids and placed in the chromosome of 15. TSA gene is abundant and homogeneously spreads on the surface of both extracellular and intracellular promastigote and amastigote. There are multiple copies of the TSA gene in all species of *Leishmania* analyzed. The results recommend that there are at least three copies of TSA in the *L. major* genome. TSA is chosen as vaccine candidates because it elicits a Th1 response in *L. major* infected BALB/c mice comparing to the other selected antigens. TSA DNA–vaccinated mice expose excellent and stronger protection than the mice vaccinated with the other antigens of DNA vaccine. TSA DNA–vaccinated stimulates high titers of specific IgG2 antibody, a phenotypic marker of Th1 response[10-11]. The aim of the present work was to clone *L.major* thiol-specific antioxidant antigen (TSA) gene into appropriate vector for production of recombinant plasmid containing TSA gene. One of the clones thus obtained encoded a novel protein of *L. major* with significant sequence homology to eukaryotic thiol-specific-antioxidant (TSA) proteins. In this study, we presented evidence that the antigens TSA delivered in a plasmid DNA format either as single genes characterization and expression, purification recombinant TSA protein and confirmation it with SDS-Page and Western blot analysis.

MATERIALS AND METHODS

Mice

BALB/c mice obtained from Iran's Razi Serum. The mice maintained under pathogen-free conditions and used at six-week-old.

Parasite culture and DNA isolation

Leishmania major (MHRO/IR/75/ER) parasite provided by Pasteur Institute of Iran.*L. major* promastigotes grown at 23-25°Cin RPMI1640 medium (Sigma®) supplemented with 10% heatinactivated FCS(Gibco®, BRL), and100g/ml streptomycin and 100 IU/ml penicillin G(Sigma®). Stationary-phase of the promastigotes harvested at a density of $2x10^6$/ml.*L. major* cultures were pelleted and washed with PBS followed by DNA isolation using DNA assay Blood and Tissue Kit (Qiagen, USA) according to themanufacturer's instructions. DNA quantitated using a NanoDrop 2000c (Thermo Scientific Inc., Wilmington, DE).

PCR amplification and gel electrophoresis

Genomic DNA isolated from promastigotes used as a template to amplify the TSA gene by PCR. The reaction was performed in 25µl ofthe solution containing: 3µl of template DNA,0.5µl of dNTP (with concentration 10mM andfinal concentration of 200 µM), 0.5µl of TaqDNA polymerase (with concentration 5 Unit/ml),2.5µl of 10X PCR buffer, 0.75µl of MgCl2 (50mM), 15.75µl of distilled water and 1µl of eachof primers (10Pmol/µl).

This study designed a pair of primer based on TSAgene sequence (Accession number LmjF15.1080). Forward primer, 56nt: introduced Hind III recognitionsite, underlined

5' - CAA TTA AA GCT TAT ATG CATCAC CAT CAC CAT CAC ATG TCCTGC GGT AAC GCC AAG- 3' (1-23 nt)For confirmation of gene expression, six-histidine (6-His tag) sequence designed in the forward primer for diagnostic by His-tag monoclonal antibody in western- blotting. Reverse primer, 31nt: introduced *Eco*RI recognitionsite, underlined:5'- CAT

GGA ATT CTT ACT GCT TGCTGA AGT ATCC-3' (579-600 nt), under the following conditions

After an initial five minutes denaturation at 95°C, each cycle consisted of 60s at 95°C, 30s at 60°C and 45s at 72°C. At the end of the 30 cycles of amplification, a final extension continued for five minute at 72°C. These primers contain restriction enzymes that designed for gene expression in the eukaryotic expression vector pcDNA3 downstream T 7 promotor to the CMV promoter The upstream primer for the TSA gene containsa *Hind*III site and the ATG start codon, while the downstream primer contains an *Eco*RI site and the TAA stop codon. The PCR products analyzed by electrophoresis on a 1% agarose gel and photographed. Gel was stained by ethidium bromide and DNA band visualized under UV transilluminator. The size marker used to estimate PCR products was the 1 kbp DNA ladder (Fermentas®) The PCR products were purified using a DNA Extraction Kit from agarose gel (Ferments®) following the manufacturer's recommendations [12-14]. The gel slice containing 600bp fragment band (TSA gene) excised.

Ligation Transformation and Screening of TSA gene

The purified PCR products were ligated to*pTZ57R/T*, following themanufacturer's protocol[15].

The (600bp) sticky ended PCR product cloned in pTZ57R/T plasmid vector (InsT/Aclone ™ PCR product cloning kit, Fermentas®). pTZ57R/T-TSAsequenced with an ABI 3730xl DNA Analyzer machine (Applied BiosystemsTM) using pTZ57R/T sequencing primers.The sequencing results aligned with a reference sequence of TSA(accssion no. EU194915) by vector NTI advanced™ 11.0 software (Invitrogen). TSA coding DNA was sub-cloned by insertion between *Hind*III and *Eco*RI sites in pET28a(+) (designated as pET28-TSA). Constructed recombinant plasmids subsequently verified by PCR, restriction analysis and DNA sequencing.

Expression and purification of the recombinant proteins

Recombinant vector, pET28a-TSA was transformed into *E. coli* BL21 (DE3) and was grown in Luria-Bertani (LB) broth medium. All cultures incubated at 180 rpm and 37°C. Cultures grown until (and if) an optical density (OD) of 0.6 at 600 nm was reached. Then, expression of rTSA induced by adding 1 mM IPTG (isopropyl-l-thio-fl-D-galactopyranoside) to medium. Induction time was 4 hours which the expression levels assessed at one-hour intervals of induction. Induced samples screened and analyzed by SDS-PAGE (Mini-PROTEAN® 3 system; Bio-Rad) with 12.5% resolving gel, followed by Coomassie Brilliant Blue G-250 staining. Cultures (200 ml of 4-hours induced) pelleted, washed with PBS and suspended in lysis buffer (50mM NaH2PO4, 300mM NaCl, 10mM imidazole and 1mM PMSF; pH 8.0). Suspensions were sonicated for 3-5 minutes in 0.6 second pulses of 80% amplitude while they were kept on ice-water. The lysate centrifuged at 18,000×g for 20 minutes at 4ºC to separate soluble (clear lysate) and solid materials (inclusions) and analyzed by SDS-PAGE to detect the recombinant protein. Purification of rTSA performed under denaturing conditions according to the manufacturer's instructions (The QIA expressionistTM, QIAGEN; 2003). The eluted proteins analyzed by SDS-PAGE and quantified using a NanoDrop spectrophotometer (NanoDropTM 2000; Thermo Scientific). Imidazole in elusion buffer removed by dialysis against PBS.

RESULTS

Cloning, sequencing of rTSA and expression plasmid construction

Genomic DNA extracted from promastigote and TSA gene (600bp) successfully expanded by proof reading DNA polymerase. PCR products successfully cloned in pTZ57Rvector and the target DNA fragment was inserted in a multiple cloning site. Sequence analysis of recombinant pTZ-TSA plasmids revealed complete identity with a reference sequence (accession no. EU194915). TSA gene sub-cloned in pET28a (+) expression vector followed by conventional PCR screening and digestion. Sequencing results of recombinant expression plasmid vectors showed entire likeness with reference sequences and confirmed the accuracy of in-frame insertion of target ORF in multiple cloning sites of the vector (Fig1).

Expression Analysis of recombinant TSA (rTSA)

SDS-PAGE analysis of *E. coli* BL21 (DE3), which was transformed with pET28a-TSA and induced with IPTG, showed resulted in expressing a non-glycosylated ~23kDa protein as expected. Express rTSA induced by IPTG 1mM was done. There was a difference in quantitative expression of protein among 1 to 4 hours induced cells. The quantity of expressing protein increased after the 2 hours induction and maximized in 3 hours induced cells (Fig 2).

Purification of rTSA and Western blot analysis

The use of an expression vector adding a six-histidine tag at the protein's N-terminus allowed detecting the recombinant protein by using mouse polyclonal antibody and subsequently purifying it by affinity chromatography. This led to detecting rTSA as insoluble aggregate forming inclusion bodies, thus requiring high concentrations of denaturing agents (8M urea) to solubilize it. The recombinant protein then was purified by affinity chromatography using a Ni(II)-nitrilotriacetic acid or Ni+2-NTA resin and the so collected fractions individually analyzed by SDS-PAGE and Western blot. The recombinant protein was then thoroughly dialyzed against PBS 1X, pH 7.2 to ensure the removal of all denaturing agents and to allow the protein to earn a similar conformation to that of the native one. Finally, purified rTSA detected using mouse polyclonal leishmaniasis antiserum (Fig 3).

Electrophoresis

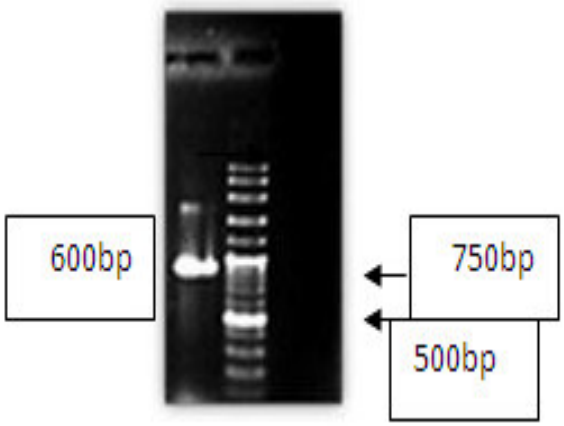

Figure 1
Electrophoresis of purified pcTSA plasmid (Constructed recombinant plasmid verified by PCR) , TSA gene (600bp)

Brad ford

SOLUBLE	OD	Concentration(µg/µl)
E1	0.609	0.5125
E2	0.654	0.5503
MES (Buffer)	0.564	0.4746
E1	0.548	0.46067
E2	1.022	0.8600
MES	0.932	0.78432
E1	0.785	0.6606
E2	0.764	0.6429
MES	0.698	0.5874

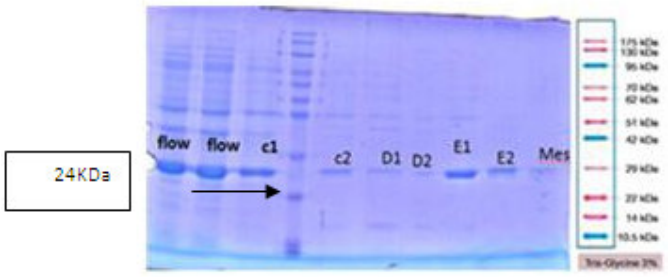

Figure 2
The Purified by affinity chromatography using a Ni+2-NTA resin and collected fractions individually analyzed by SDS-PAGE

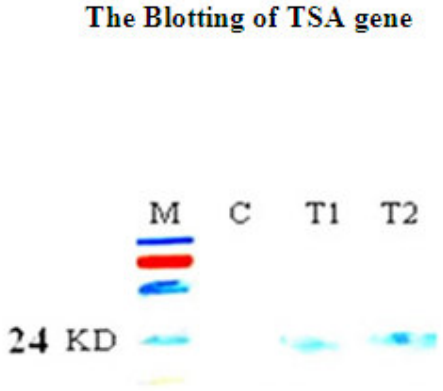

Figure 3
,Protein size marker .C,Non-induced transformed BL21DE3as control .T1,Supernatant of induces transformed BL21BE3. T2,Purified chimeric recombinant protein

DISCUSSION

Leishmaniasis has not shown any tendency towards relief in recent years. Although measures may be taken against vectors and reservoirs, and identifying new drugs is adesirable goal, therefore the emerging drug resistances, developing safe and efficient vaccines remains the best hope of achieving definitive control of the disease. *Leishmania* TSA protein known in both murine and human systems and constitutively expressed in both promastigote and amastigote life stages [5, 10-11]. TSA family from humans to *Saccharomyces cerevisiae* has conserved domains and distinctive similarity in amino acid sequence has been seen in this group. *S. cerevisiae* thiol specific antioxidants confer safety against oxidative stress and damage. Mechanism of TSA action embedded in a thiol oxidation-based enzyme inhibition mode. Function of TSA protein depends on thioredoxin, thioredoxin reductase, and NADPH as reducing equivalents, then been defined as thioredoxin peroxidase. thioredoxin as a reducing equivalent In *S. cerevisiae* help reduction of H2O2 molecules and participate in protection of mechanism against peroxide-mediated oxidative damage Similar situation occurs inside macrophages. Respiratory burst in macrophages mediated by production of H2O2 and TSA molecules as *leishmania* product, guarantee *Leishmania* survival inside the macrophages [16-20]. The recombinant leishmanial antigens LmSTI1 and TSA has been shown that they can induce excellent protection in both murine and non human primate models of human cutaneous leishmaniasis. The recombinant TSA protein with IL-12 induces excellent protection in the BALB/c mice. Recombinant proteins, LACK and TSA have produced at least partial protection against *L. major* in BALB/c mice [21-25]. Successful immunization that induces protection against leishmaniasis is highly dependent on adjuvant that preferentially stimulates the Th1 phenotype of immune response.Currently, the most successful vaccine takes on humans and animals have been achieved with live-attenuated,whole-killed *Leishmania* promastigotes (with or without IL-12) and autoclaved *Leishmania* with BCG also Radio attenuated promastigotes and biochemically altered leishmanial parasites. A single synthetic T-cell epitope from gp63 administered (synthetic peptide) with Th1 stimulating poloxamer 407 conferred protection against *L. major* in BALB/c mice. The conserved antigen among the *Leishmania* genus, TSA, has shown to induce excellent protection in both the murine and non-human primate models of the human disease [16-17] . Therefore production of recombinant TSA protein and evaluating protective effect of this vaccine candidate with various adjuvants could be a good strategy for immunization against *Leishmania* in future studies also this Protein was used for protein booster injection in immunological analysis[26].

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