Original Article

Cloning, Expression and Characterization of *Toxoplasma gondii* P35 protein in *E. coli*

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Abstract

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Introduction: *Toxoplasma gondii* is a protozoan parasite which is globally prevalent in human and animals. Toxoplasma infection is commonly asymptomatic, but can cause serious medical problems in immunocompromised individuals and in fetus. Recombinant antigens of the parasite may be helpful in diagnosing the infection more precisely. The goal of this study was to construct and evaluate the functionality of a prokaryotic expression plasmid pGEX-P35, harboring P35 surface antigen gene of *T. gondii* and to perform preliminary studies on its ability to detect *T. gondii* specific antibodies.

Material and Methods: A 450 bp fragment of the P35 gene was amplified and inserted into pGEM-T plasmid, sequenced, cut, and then inserted into pGEX-4T-1 plasmid to produce the recombinant plasmid pGEX-P35. In order to confirm that the plasmid construct was capable of expressing P35 in bacterial cells, it was transformed into BL21 strain of *E. coli* and expressed. The resultant recombinant protein was purified and subjected to SDS-PAGE and Western-blot analysis.

Results: A 450 band of PCR product was visualized on 1% agarose gel. Comparison of resultant DNA sequence with GenBank databases showed 100% identity with AF01275. Restriction enzyme analysis confirmed subcloning and correction of orientation. SDS-PAGE analysis showed a 42 kDa band of purified expressed protein. Western-blot analysis using mouse antibody against RH strain of *T. gondii* showed that the recombinant P35 antigen could be recognized by specific antibodies.

Conclusion: Purified and specific recombinant antigens obtained by molecular biology techniques are attractive alternatives for detection of serum antibodies. We amplified and cloned a fragment from P35 gene of *Toxoplasma gondii* encoding P35 tachyzoite-specific surface antigen. Sequenced fragment was accepted by GenBank with an accession number of DQ092625. This confirms previous results from other countries estimating just 1% divergence at the level of DNA sequence between lineages isolated from different geographical areas. As the recombinant P35 (rP35) antigen is recognized by specific antibodies, it is suggested to evaluate the (rP35) for diagnosis of clinical *Toxoplasma gondii* infections.

Keywords: Toxoplasmosis, Gene Expression, Recombinant Protein, Toxoplasma P35 Protein

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Introduction

Toxoplasma gondii is a ubiquitous obligate intracellular protozoan parasite with a broad host range (1). Toxoplasmosis is generally asymptomatic in immunocompetent adults, whereas intrauterine transmission of the parasite during gestation can result in severe fetal and neonatal complications (2). Toxoplasmosis is also a serious complication following organ transplantation (3) and AIDS (4, 5).

In Iran, about 51.8% of the people are estimated to have antibodies against the infection (6). Accurate diagnosis of recent infection by *Toxoplasma gondii* is important for proper clinical management. Laboratory diagnosis of *Toxoplasma* infection by serologic methods is usually based on the detection of specific antibodies using enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) tests. Most commercial kits use prepared tachyzoites grown in mice and/or tissue culture and probably contain varying amounts of non-parasitic materials.

Due to inherent limitations of the tachyzoite antigen in serologic tests, the use of purified and tachyzoite-specific recombinant antigens obtained by molecular biology techniques is an attractive alternative for the detection of serum antibodies. The use of recombinant antigens would allow better standardization of the tests and reduce the production costs.

The surface of *T. gondii* tachyzoites is covered by glycosyl phosphatidylinositol (GPI)-anchored antigens (7), most of them are members of the surface antigen 1 (SAG1). P35 surface antigen is a member of this group (8).

These molecules appear to play a role in immune modulation, host cell invasion, and virulence attenuation, although they may also protect the parasite to survive in the environment (9). In this study, we amplified, cloned, and expressed a fragment of 450 bp from *toxoplasma gondii* P35 gene encoding a truncated protein of P35 tachyzoite- specific surface antigen.

Several basic methods for cloning polymerase chain reaction (PCR) products and five major expression systems have been described (10, 11). We will discuss our methods of choice in this article. Using the recombinant antigens would allow better standardization of the tests and reduce the production costs.

Materials and Methods

Toxoplasma gondii P35 gene sequence was accessed via the World Wide Web (GenBank accession no. AF01275) and found to contain no introns (12).

Parasite

Tachyzoites of *T. gondii* RH strain were harvested from the peritoneal cavity of infected mice as described previously (13). The study was performed according to the guidelines for laboratory animal use and care set forth by the Research Council at Shahid Beheshti University of Medical Sciences.

DNA extraction and PCR amplification

Genomic DNA for polymerase chain reaction (PCR) was obtained by proteinase K treatment, phenol/chloroform extraction, and ethanol precipitation (11). We designed and ordered a set of primers (Gene Works, Australia) to amplify only the sequence coding for the mature part of P35 protein (12) with *BamHI* and *SalI* restriction sites at 5` end of forward and reverse primers, respectively. In order to minimize non-specific annealing, we used longer oligonucleotide primers.

(F:5'AATTCACGGATCCAACGGTCCTTT GAGTTATCATCCAAGCAGT3')
(R:5'AATCGAAGCTTAGTCGACTTAGAT GGTGAACTGCCGGTATCTCC-3')

PCR reaction contained 0.5 μg DNA, 40 pmol of forward and reverse primers, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1X PCR buffer, 1.5 IU of Taq DNA polymerase (CinnaGen, Iran), and dH_2O up to 50 μl. PCR amplification was carried out with 30 cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 1 min. PCR reaction was incubated at 94 °C and 72 °C for 5 min before and after the PCR cycling, respectively.

Gene cloning

PCR product was electrophoresed on low melting point (LMP) agarose gel and the DNA band was sliced under long-wave ultraviolet (UV) light, recovered by purification kit (QIA quick PCR Purification Kit or QIA II Agarose Gel Extraction Kit), ligated into pGEM-T vector (Promega) via T/A cloning method, and submitted for sequencing (Griffith University, Queensland, Australia). Sequence homology search was done using BLAST (Basic Local Alignment Search Tool)

program to compare the results with *T. gondii* P35 gene (GenBank accession no. AF01275). T-vector/P35 was transformed into DH5α competent bacterial cells. Bacterial colonies containing recombinant plasmids were screened and confirmed by PCR and restriction analysis. One positive clone was sequenced, digested by *BamHI* and *SalI*, and the cut out DNA was subcloned into *BamHI* and *SalI*-digested pGEX-T4-1 prokaryotic expression vector (kindly provided by Dr. Michael De Veer, Centre for Animal Biotechnology, University of Melbourne, Australia).

Gene expression and protein purification

Escherichia coli strain BL21 (Pharmacia Biotech, Piscataway, N.J.) was transformed using the recombinant plasmids and grown in Luria Bertani (LB) medium supplemented with 50 µg/ml ampicillin at 37 °C overnight. Two-liter culture flasks containing 400 ml of LB containing 50 µg/ml ampicillin were inoculated by 5-ml samples of the overnight cultures. The cultures were grown at 30 °C with vigorous shaking until the OD_{600} = 0.8 - 1.0. The culture was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 hr at 30 °C. Induced cells were pelleted by 7,700 ×*g* at 4 °C for 10 min. The pellet was resuspended in 20 ml of 1× Tris-buffered saline (0.05 mol/L Tris, 0.1 mol/L NaCl, pH 7.4) (TBS)-1% Triton X-100 and frozen at 20 °C overnight. The cell pellet was sonicated eight times on ice in pulses of 15 sec at preset amplitude #3 and medium speed (Ultrasonic Heat System Model W-220), and centrifuged at 12,000 ×*g* for 10 min at 4°C. Recombinant proteins were purified from the supernatants (crude extracts) using a batch purification protocol (Pharmacia Biotech). Briefly, 50% glutathione-Sepharose 4B slurry was prepared according to the protocol. Two milliliters of slurry was added to crude extracts and incubated with gentle agitation at room temperature for 30 min. The resin was pelleted by 500 ×*g* at room temperature for 5 min and washed twice with 50 ml of 1× TBS-1% Triton X-100 followed by two washes with 50 ml of 1× TBS. Recombinant proteins were eluted with glutathione elution buffer (10 mM glutathione, 50 mM Tris-HCl [pH 8.0]). Right molecular weight of the recombinant protein was confirmed by SDS-PAGE in comparison with protein marker.

Western-blot analysis

The samples were prepared for sodium dedocyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by adding 5.0 µL sample buffer to 20 µL of the samples. They were then boiled for 5 min and 20 µL was loaded onto a 10% polyacrylamide gel. The proteins were transferred to nitrocellulose membrane at 90 V for 1 hr, using a transfer system (Bio-Rad, Hercules, CA) according to manufacturer's recommended conditions. The membrane was saturated for 1 hr by 5% fat-free dried milk in phosphate buffered saline (PBS) and probed using the mouse antibody against RH strain of *T. gondii* (kindly provided by Dr. David Piedrafita, Centre for Animal Biotechnology, University of Melbourne, Australia) diluted 1:5000 in 5% fat-free dried milk in PBS. The bound antibodies were detected using peroxidase-conjugated goat anti-mouse secondary antibody (Jackson Immuno-research Laboratories, West Grove, PA) diluted 1:20,000 in fat-free dried milk, and signals were detected using Super Signal ECL (enhanced chemiluminescence) system (Pierce chemical, Rockford, IL).

Results

Extracted DNA was subjected to a 30-cycle PCR.

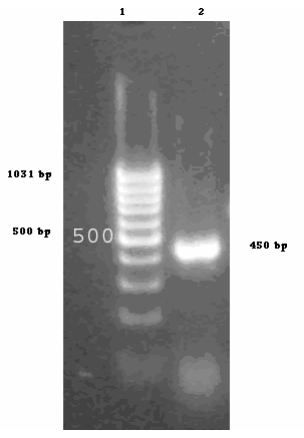

Fig. 1. PCR product on 1% agarose gel electrophoresis. Lane 1: 100 bp DNA ladder marker.

Lane 2: a 450bp band as the PCR product of P35.

Figure 1 shows 1% agarose gel electrophoresis containing a 450 bp band as the PCR product. It was cloned into pGEM-T plasmid, sequenced, and submitted to GenBank with an accession number of DQ092625. Sequencing results were compared with the GenBank sequence of P35 gene, showing 100% identity with AF01275. Inserted DNA was then sub-cloned into pGEX-T4-1 expression vector. The integrity of the DNA plasmid was checked by agarose gel electrophoresis after digestion with *NotI* restriction enzyme (Fig. 2).

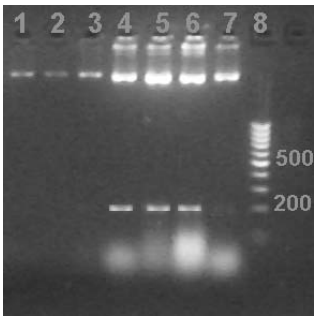

Fig. 2. Restriction analysis of pGEX-4T1-P35. There is a NotI restriction site at position 959 on the reading frame of pGEX vector. The insert has also a NotI restriction site at position 238. Finding a 200 bp band on 2% agarose gel indicates a correct direction of the insert into expression vector. Lanes 1-3: purified plasmids from pGEX-4T1P35-negative colonies. Lanes 4-6: purified plasmids from pGEX-4T1-P35-positive colonies. Lane 7: empty pGEX-4T-1 vector. Lane8: 100 bp DNA ladder.

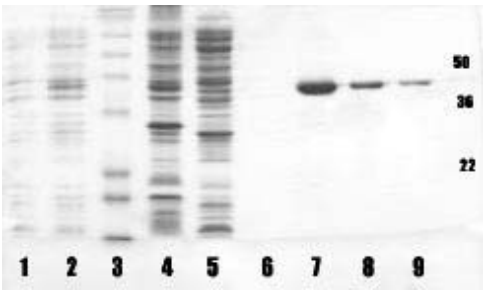

Fig. 3. Coomassie blue-stained SDS-PAGE analysis of recombinant P35 preparation. Lanes 1–2: cell extract from pGEX-4T1-P35-transformed E. coli before (1) and after (2) induction by IPTG; lane 3: molecular weight marker (kDa); supernatant (lane 4) and pellet (lane 5) resulted from sonication of induced pGEX-4T1-P35-transformed E. coli; lane 6: collected solution after passing the PBS through the chromatography column before purification; lanes 7-9: purified GST-P35 fusion protein. Collected solution after passing the elution buffer three times through the chromatography column.

Expression was done and SDS-PAGE of recombinant protein showed a 42 kDa band including GST molecular weight (26kDa) plus P35 molecular weight (16kDa) (Fig.3). To confirm the detection of specific anti toxoplasma antibody by recombinant P35 antigen the western blotting analysis was carried out. The result showed that the recombinant antigen was recognized by anti toxoplasma antibody (Fig.4).

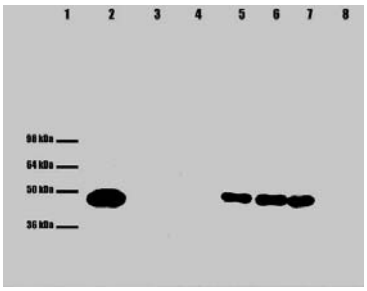

Fig. 4. Results of reacting recombinant protein on Toxoplasma gondii-positive serum.
Lane 1: protein marker (kDa).
Lane 2: P35 recombinant protein.
Lane 3: Non-recombinant GST for detection of possible anti-GST antibody in serum.
Lane 4: Collected solution after washing with PBS.
Lane 5: Post-sonication supernatant.
Lane 6: Post-sonication pellet.
Lane 7: Lysate of bacterial cells after induction by IPTG.
Lane 8: Lysate of bacterial cells from non-induced culture.

Discussion

Although 21 *Toxoplasm*a surface antigen (SAG) genes have already been cloned, much remains to be learned in regard to their pattern of expression (9). Current evidence suggests that gene expression in *T. gondii* is transcriptionally regulated (14, 15, 16). Unlike other protozoan parasites such as *Trypanosoma* spp. and *Leishmania* spp., polycistronic transcription and RNA editing have not been detected. Although conventional *cis*-acting eukaryotic promoters such as the TATA box or SP1 motif have not been observed, upstream sequence analysis of several genes has identified a common highly conserved T/AGAGACG heptanucleotide core element (17).

Five major antigens on the tachyzoite surface (P43, P35, P30, P23, and P22) were initially defined using monoclonal antibodies (18). While P43 (SAG3) and P23 are also expressed by bradyzoites (19), others are developmentally regulated. P35 is a 35-KDa surface antigen of *T.*

gondii. It was detected in immunoblots of tachyzoite extracts probed with serum obtained from individuals shortly after they became infected (20, 21). Several studies have found that the recombinant P35 protein can distinguish acute from chronic *T. gondii* infection (22, 23, 24, 25). They have used different methods and vectors for cloning and expression of this gene in *E. coli*. Aubert, Li, Suzuki, and their colleagues cloned the PCR product directly in frame with glutathione S-transferase (GST) gene in the pGEX-5X-1 expression vector (23, 24, 25). Lu and coworkers cloned the PCR product into T-vector and sub-cloned the P35 gene from the pGEM-T easy vector to the GST fusion vector pGEX-2TK (22). In present study, we cloned the PCR product into T-vector because many restriction enzymes including *SalI* fail to cleave recognition sequences located close to the ends of DNA fragments, particularly those generated by PCR (26). However the single 3′ adenosyl extension generated by *Taq* DNA polymerase provides a highly efficient method to clone PCR product into a T-vector containing a complementary unpaired 3′ thymidyl residue (27). We also subcloned the P35 gene from pGEM-T to the GST fusion vector (pGEX-4T-1) as an expression vector. We chose the GST gene fusion system because it is a versatile system for expression, purification, and detection of fusion proteins produced in *Escherichia coli*. The system is based on inducible, high-level expression of genes or gene fragments as fusions with *Schistosoma japonicum* GST (28). Expression in *E. coli* yields fusion proteins with the GST moiety at the amino terminus and the protein of interest at the carboxyl terminus. The protein accumulates within the cell cytoplasm. GST fusion proteins are purified from bacterial lysates by affinity chromatography using immobilized glutathione. GST fusion proteins are captured by the affinity medium, and impurities are removed by washing. The purification process preserves protein antigenicity and function. The GST Gene Fusion System has been used successfully in many applications (22, 24).

Compared with other protozoans including related apicomplexans such as *Plasmodium falciparum*, *T. gondii* maintains a remarkably conserved nuclear genome despite a wide host range and nonobligatory sexual cycle (29). Comparing our sequencing data with the GenBank sequence of P35 gene showed 100% identity. Previous comparative sequence analyses of individual genes estimated only 1% divergence at the DNA sequence level (29). So, our data confirms results obtained from previous studies.

Conclusion

We amplified, cloned, sequenced, and expressed a 450 bp fragment of P35 gene of *Toxoplasma gondii* encoding P35 tachyzoite-specific surface antigen. We observed that produced recombinant P35 could be recognized by anti-Toxoplasma antibodies in serum. Our next step would be evaluation of the P35 antigen as a potential tool in diagnosis of Toxoplasmosis, particularly for differentiating acute from chronic infection.

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