

Original Article

Purification and characterization of *Toxoplasma gondii* immune mapped protein-1

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Received December 10, 2015; Accepted February 18, 2016; Epub June 1, 2016; Published June 15, 2016

Abstract: Objective: To express and purify the *Toxoplasma gondii* RH strain immune mapped protein 1 (IMP1) protein through the prokaryotic expression system, and to identify the purified protein. Method: Gene of TgIMP1 was attained by RT-PCR and the amplified product was identified by TA-clone and sequencing. The identified fragment of IMP1 was connected into the expression vector pET28b and the recombinant pET28b-TgIMP1 was identified by double digestion and sequencing. The fusion protein TgIMP1 with 6 × His tag was expressed in *E. coli* BL21 (DE3) host and the optimal inducing conditions were investigated by adjusting the harvesting time interval, inducing temperature and concentration of isopropyl β-D-thiogalactoside (IPTG). The solubility detection of TgIMP1 protein and the massive purification of it were conducted with Ni²⁺-affinity purification. The purified product was identified by Coomassie-stained SDS-PAGE and immune-blotting. Results: The prokaryotic expression recombinant pET28b-TgIMP1 was successfully established on the basis of the identified TgIMP1 gene by TA-clone. Recombinant proteins of TgIMP1 could be efficaciously and largely expressed in *E. coli* BL21 strain with 0.3 mM IPTG for 9 h at 20 °C, and with the characteristics of good stability, high solubility and purity, and fine immunity. Conclusion: TgIMP1 protein could be expressed in the prokaryotic host strain with satisfied stability and solubility. Our findings may lay the foundation for the future protein-based investigations such as development of subunit vaccine and structural determination.

Keywords: *Toxoplasma gondii*, TgIMP1, expression, purification

Introduction

Toxoplasma gondii (*T. gondii*) is an obligate intracellular protozoan which can infect a broad range of warm-blooded animals worldwide, in humans, the infection rate ranged from 15% to 85% [1]. As one of the most widespread zoonoses in the world, Toxoplasmosis is lethal for immunodeficient patients with AIDS, or organ transplant recipients [2]. Pregnant women who infected with *T. gondii* can cause severely damages to fetus and newborns, such as hydrocephalus, miscarriage, mental retardation and blindness [3]. Moreover, *T. gondii* infection can cause enormous economic losses due to neonatal deaths in livestock, and the infected livestock will become a potential source of infection to humans [4, 5]. Therefore, it is urgently required to develop new vaccines with strong protective efficacy for the prevention and treatment of toxoplasmosis. At present, the primary method using to control toxoplasmosis is mainly relied on the chemotherapy, which is associ-

ated with various of adverse reactions such as toxicity, dose dependent and sequelae [6], and vaccines fully protective against toxoplasmosis are still scarce, thus prepare and identify new vaccine candidates with good immunogenicity is necessary and urgent [7].

Immune mapped protein 1 (IMP1) is a newly discovered protective protein and was initially identified in *Eimeria maxima* (*E. maxima*), and it was proved to be immunogenic and conferred protection against *E. maxima* infection [8, 9]. IMP1 is conserved across most apicomplexan parasites [8]. On the basis of bioinformatics analysis, the TgIMP1 was theoretically predicted to be a membrane protein with multiple epitopes and immunogenicity, which suggested that it may be a potential vaccine candidate against toxoplasmosis [10]. Currently, the homologues of IMP1 protein was identified as immunoprotective antigens in both *T. gondii* [11] and *Neospora caninum* [12], in particular, Xia Cui et al. demonstrated the strong protective

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activity of TgIMP1 on the BALA/c mouse infected with tachyzoites of *T. gondii* RH strain [11]. Although the EtIMP1 has been identified and purified in *Escherichia coli* cells [9, 13], there have been no reports demonstrating the characterization and purification of TgIMP1. Additionally, the mechanism of its protection role remains unclear, the structure, the interaction factors and physiology pathways involved in its function also need further investigations.

Thus, the objectives of this study were to purify the protein of TgIMP1 concisely and efficiently through prokaryotic expression system. We successfully cloned the TgIMP1 genes from the virulent *T. gondii* RH strain, and the optimal inducing expression conditions of TgIMP1 were extensively explored. TgIMP1 protein expressed in *Escherichia coli* presented to be soluble and stable. Our findings may lay a basis for further studies such as development of sununit vaccine based on TgIMP1, crystal structure determination and interaction factors screening.

Material and methods

T. gondii tachyzoites culture and purification

Tachyzoites of *T. gondii* RH strain (Type I) were stored well in liquid nitrogen in our laboratory, maintained and passaged by female BALB/c mice (purchased from Animal Center of Shandong Academy of Medical Sciences). The frozen vial was thawed in 37-42°C water bath rapidly. The BALB/c mice were infected with the thawed tachyzoites intraperitoneally. After 4-7 days, tachyzoites were harvested from the peritoneal fluid of mice, washed 3 times with phosphate-buffered saline (PBS, pH 7.2), and pelleted at 3000 rpm for 10 min.

Cloning of TgIMP1

Total RNA was extracted from *T. gondii* tachyzoites using a RNA Isolation kit (TIANGEN, China), according to the manufacturer's instructions. The cDNA was synthesized by reverse transcription using a First Strand cDNA Synthesis Kit (Thermo scientific, USA). Primers (forward primer: 5'-ATACATATGGAACCGTTTGCACGAAG-3'; reverse primer: 5'-ATACTCGAGCGCATTTGCTCTGTCCACC-3') correspond to the TgIMP1 gene sequence (GeneBank ID: JN657189.1), and the open reading frame of TgIMP1 was amplified by temperature gradient PCR with the introduced *Nde I* and *Xho I* sites (underlined).

PCR programs were 6 min at 96°C, 35 cycles of 30 s at 95°C, 30 s at 52-60°C, 70 s at 72°C and 10 min at 72°C. After recovered by 1.0% agarose gel electrophoresis, the PCR products were cloned into the pMDTM18-T Vector (TaKaRa, Japan). Positive clones were identified by PCR amplification and double digestion with *Nde I* and *Xho I* enzymes (TaKaRa, Japan), verified by a commercial sequencing service (Sangon Biotech, China). The sequencing results were analyzed by MegAlign software (LaserGene, USA).

Construction of recombinant pET-28b-TgIMP1

The verified fragments of TgIMP1 was isolated by digesting the pMD-TgIMP1 plasmid with *Nde I* and *Xho I*, and then inserted into the corresponding sites of prokaryotic expression vector pET-28b by T4 DNA ligase (TaKaRa, Japan) to construct recombinant pET28b-TgIMP1. The recombinant was transformed into competent cell *E. coli* BL21 (DE3) and the positive clones were verified by double digestion and sequencing as described above. The C-terminal of TgIMP1 was introduced with a 6 × His tag for affinity-purification.

Expression of TgIMP1 protein

Recombinant pET28b-TgIMP1 plasmid containing TgIMP1 was expressed in *E. coli* BL21 (DE3). The transformed cells were first cultured in 10 mL LB broth medium with kanamycin (50 µg/mL) overnight at 37°C and 220 rpm min⁻¹, then transferred into 100 mL LB medium and incubated until the OD₆₀₀ reach to 0.6. The gradients of harvesting time interval (3 h, 6 h, 9 h), inducing temperature (20°C, 28°C, 37°C) and concentration of isopropyl β-D-thiogalactoside (IPTG) (0.3 mM, 0.6 mM, 1.0 mM) were set up to optimize the expression conditions. Proteins expressed in transformed cells were separated using a 12% SDS-PAGE gel.

Massive purification of TgIMP1 protein

After the optimal expression conditions were determined, the culture volume was increased to 1 L for massive purification. TgIMP1 protein was induced with 0.3 mM IPTG for 9 h at 20°C, bacterial were pelleted by centrifugation at 4000 rpm for 15 min at 4°C, resuspended in the lysis buffer (25 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 0.4 mM PMSF), and lysed by sonication on ice. After centrifugation at

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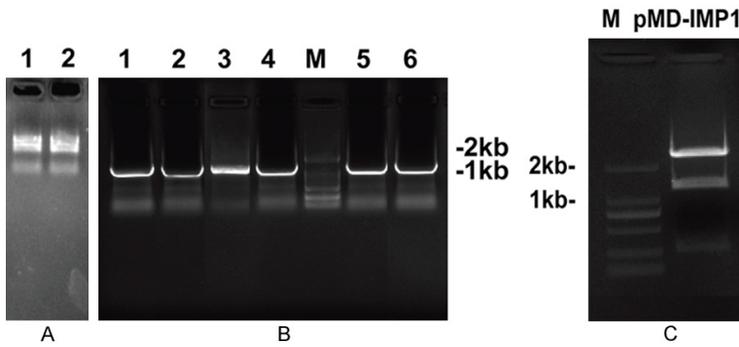


Figure 1. TA-clone of IMP1. A. Gel electrophoresis of total RNA extraction products from *T. gondii* tachyzoites (Lane 1-2). B. RT-PCR amplification products of TgIMP1 gene (Lane M: DNA marker; Lane 1-6: the RT-PCR products amplified at different annealing temperatures 60, 58, 56, 54, 52 and 50°C). C. Plasmids of the pMD-TgIMP1 TA-cloning digested by *Nde I* and *Xho I*.

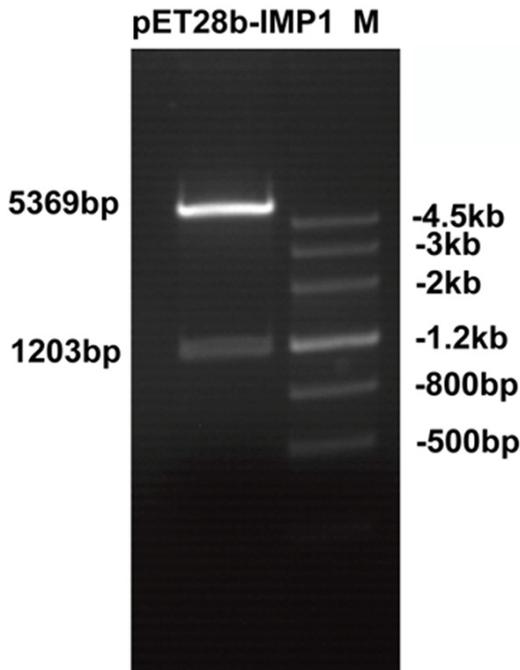


Figure 2. Identification of the recombinant prokaryotic expression plasmid pET28b-TgIMP1 by restriction enzyme digestion with *Nde I* and *Xho I*.

21,170 × g for 45 min at 4°C, both precipitates and supernatants were examined by Coomassie blue-stained SDS-PAGE to examine the solubility of TgIMP1. Supernatants containing expressed protein were loaded on the Ni²⁺-NTA Chelating Sepharose Fast Flow column (GE Healthcare, USA), the column was washed with wash buffer (25 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 15 mmol/L imidazole pH 8.0) of five column volumes to remove nonspecific protein banding. The target protein was eluted

with 5 mL of elution buffer (25 mmol/L Tris-HCl pH 8.0, 100 mmol/L NaCl, 250 mmol/L imidazole pH 8.0). Products in the sonication, centrifugation, flow, wash and elution steps were analyzed by 12% SDS-PAGE respectively.

Identification of TgIMP1 protein by immune-blotting

Proteins detected in SDS-PAGE gel were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA) at 150 mA for 2 h. The PVDF membrane was blocked in 5% (w/v) skim

milk diluted in PBS for 1.5 h, and then incubated with an anti-His-tag monoclonal antibody (1:5000, Sigma, USA) for 2 h at room temperature. After washing three times with 0.01 mM PBST, the membrane was incubated with goat anti-mouse IgG antibodies conjugated with HRP (1:8,000; Sigma, USA) for 1 h. The 6 × His tagged TgIMP1 proteins were detected with the HRP-ECL chemiluminescence kit (CoWin Biotech Co, LTD, China).

Results

TA-clone of pMD-TgIMP1

The total RNA isolated from RH tachyzoites of *T. gondii* was identified by 1% agarose gel electrophoresis. The clearly visible 28 S and 18 S RNA bands (**Figure 1A**) indicated that the quality of extracted RNA met the requirement for being the template of the following RT-PCR. Products of the temperature gradient RT-PCR separated in 1% agarose gel (**Figure 1B**) showed that positive bands with the length of 1200 bp were obtained at each annealing temperatures, the optimal temperature was at 56°C. The positive IMP1 gene product preliminarily amplified by RT-PCR was connected into the TA cloning vector pMDTM18-T to construct the recombinant pMD-TgIMP1. The plasmid of pMD-TgIMP1 were verified using double digestion, and the result (**Figure 1C**) showed that the corresponding bands in the length of 2692 bp and 1203 bp were clearly appeared, indicating that IMP1 gene has been successfully connected into the pMD-T vector. The result of sequencing of the identified plasmid has a 99% identity to published IMP sequence in GenBank (data not shown).

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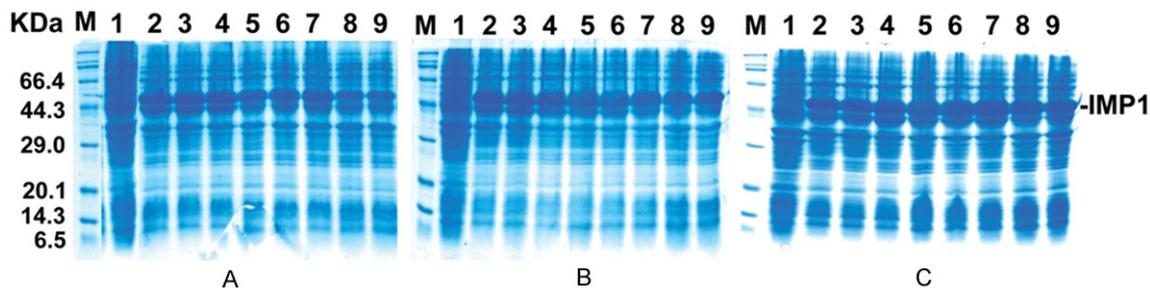


Figure 3. Optimum expression condition analyses of TgIMP1 protein in *E. coli* by Coomassie-stained SDS-PAGE. A. Proteins expressed for 3 hours after induction. B. Proteins expressed for 6 hours after induction. C. Proteins expressed for 9 hours after induction. Lane M: DNA marker; Lane 1: Bacteria incubated without IPTG; Lanes 2-4: Bacteria incubated with a final concentration of 0.3, 0.6, 1.0 mM IPTG at 37 °C; Lanes 5-7: Bacteria incubated with a final concentrations of 0.3, 0.6, 1.0 mM IPTG at 28 °C; Lanes 8, 9: Bacteria incubated with a final concentrations of 0.3, 0.6 mM IPTG at 20 °C.

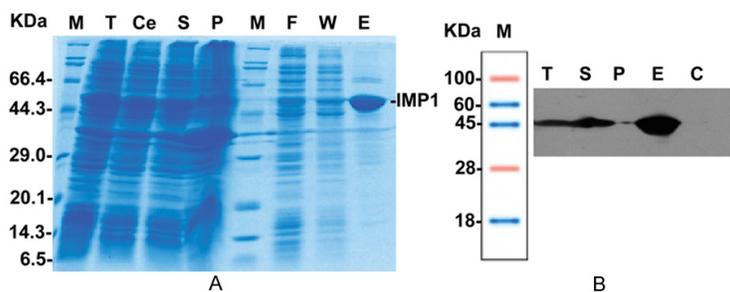


Figure 4. A. SDS-PAGE analyses of the purified 6 × His tagged TgIMP1 protein by Ni²⁺-affinity purification. B. Recombinant TgIMP1 protein confirmed by immune-blotting. Lane M: DNA marker; Lane T: *E. coli* cells induced under optimal expression conditions; Lane Ce: the broken bacterial products by sonification; Lane S: supernatants of Ce; Lane P: precipitations of Ce; Lane F: outflow products of supernatants loaded onto the Ni²⁺-NTA column; Lane W: washing products of Ni²⁺-NTA column; Lane E: elution products of Ni²⁺-NTA column; Lane C: Negative control.

Construction of the prokaryotic expression recombinant pET28b-TgIMP1

The identified fragment of IMP1 was removed from the positive plasmid of pMD-TgIMP1 by enzymatic digestion with *Nde I* and *Xho I*, and then connected into the corresponding sites of prokaryotic expression vector pET28b to construct pET28b-TgIMP1. After transforming, the plasmid was extracted from positive colony and identified by double digestion as mentioned above. Double digestion with *Nde I* and *Xho I* displayed a 5369 bp and a 1203 bp restriction products respectively, coincide to the expected sizes of empty plasmid pET-28b and TgIMP1 gene (Figure 2). The following result of sequencing was identical to pMD-TgIMP1, indicating that the prokaryotic expression recombinant pET28b-TgIMP1 has been successfully established.

Optimal expression conditions of IMP1 protein

The IMP1 protein with a molecular mass of 46 kDa was obviously over expressed in all of the inducing conditions by Coomassie-stained SDS-PAGE, except for the control group without IPTG in the process of induction (Figure 3). The maximum amount of IMP1 protein was attained with the extension of induction time, as shown in Figure 3C. Gradients of inducing temperature and IPTG concentration showed no apparent effect on the production amount of target-

ed protein, and hence the lower temperature of 20 °C and 0.3 mM concentration of IPTG was selected to be the optimal conditions for the following enlarged purification, in consideration of achieving active IMP1 protein with more stably folding characteristic, and the inducing time was up to 9 hours.

Purification and identification of IMP1 protein

The 6 × His tagged IMP1 protein was produced in 1 L LB medium and bacteria transfected with pET28b-TgIMP1 plasmid were induced according to the optimal conditions. The fusion proteins of IMP1 were analyzed by SDS-PAGE followed by Coomassie Blue staining or immune-blotting. SDS-PAGE results (Figure 4A) showed that a large proportion of fusion IMP1 protein was presented in the supernatants of broken bacteria, whilst only small amount of corre-

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sponding band appeared in precipitation, suggesting that IMP1 had a relatively higher solubility when purified in *E. coli* BL21. The eluted product from Ni²⁺-NTA column was approximately 46 kDa with high purity, corresponding to the expected size of IMP1, as well as the results of immune-blotting (**Figure 4B**), confirmed that the purified product was 6 × His tagged IMP1 fusion protein with high solubility and stability in the elution buffer. The result suggested that the TgIMP1 protein could be expressed in prokaryotic expression system and the product was soluble and stable.

Discussion

IMP1 was initially identified in *E. tenella* as a potential vaccine candidate, and was also demonstrated to be a membrane protein in both *N. caninum* and *T. gondii* [12, 13]. Efforts have been devoted to the expression of fusion EtIMP1 and EtIMP1-C proteins in *E. coli* with the introduced 6 × His tag and the proteins were purified by Ni²⁺-affinity chromatography [14, 15], moreover, it has been verified that both a bacterial flagellin-fused EtIMP1 and a C-terminal derivative of EtIMP1 expressed in the bacterial host system can elicit strong protective immunity in broiler chickens with the *E. tenella* challenge [14, 15]. Recombinant NcIMP1 (rNcIMP1) was also expressed in *E. coli* with 6 × His tag [16] but only the DNA-based vaccine of NcIMP1 was evaluated to have the protective ability against neosporosis [12]. Inconsistent with the present study, the fusion protein EtIMP1 expressed in *E. coli* was unstable in non-denaturing buffer, and the degradation was irrelevant to the expression vector and absence of protease inhibitors, but it was consistent with the stability analysis of the EtIMP1 amino acid sequence [13].

In this study, IMP1 of *T. gondii* was identified and purified by cloning, expression and Ni²⁺-affinity purification. Sequence analysis showed that our fragment of TgIMP1 has a 99% identity to the registered sequence of *T. gondii* RH strain in GenBank with the accession number of JN657189. Protein of IMP1 purified in this study was in a quite stable status in the pH 8.0 buffer containing low concentration of Tris-HCl and high concentration of NaCl and imidazole, and after the proteins were stored at room temperature for six days, the degradation bands of

TgIMP1 began to appear by the detection with Coomassie-stained SDS-PAGE (data not shown). Therefore, the proteins of TgIMP1 in the present study could be directly used for preparation of monovalent or polyvalent subunit vaccines against toxoplasmosis, and was also stable enough to carry out following crystallization, subcellular localization or function investigations.

Previous studies have predicted the characteristics of IMP1 among the apicomplexan parasites *N. caninum*, *E. maxima* and *T. gondii* [8, 10, 12, 16]. Analyzed by bioinformatics, the secondary structure of TgIMP1 contains 6 transmembrane domains and of a certain hydrophobicity [10]. Whereas in this study, the full length of TgIMP1 protein could be efficiently expressed using prokaryotic expression system and the purified protein has a high solubility, suggesting that the predicted multiple transmembrane domains may not exist in the three dimensional structure of TgIMP1, though its structure needs further investigations. Bai et al. [10] also predicted that the protein has 25 post translational modifications including one tyrosine kinase phosphorylation sites, two N-glycosylation sites, five Protein kinase C phosphorylation sites, eight Casein kinase II phosphorylation sites and nine N-myristoylation sites, and has a total of 16 predicted epitopes. On the basis of the previous study [17], the complete NMR assignments of IMP1-like protein in *Plasmodium falciparum* have been published, but the structure of pflIMP1 is still unsolved.

In conclusion, our study established a convenient method to prepare a large amount of TgIMP1 protein via prokaryotic expression system. The purified fusion TgIMP1 proteins were stable and soluble. Our study laid the foundation for the future protein-based investigations.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (31300617, 31502057, 81501770), the Natural Science Foundation of Shandong Province (BS2013SW015, ZR2014YL039), and the Medicine and Health Science Technology Development Plan of Shandong Province (WS-0370).

Disclosure of conflict of interest

None.

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