

Bacterial Production of Dense Granule Antigen GRA8 of *Toxoplasma gondii*

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ABSTRACT

Background: Dense granule antigens (GRA antigens) of *Toxoplasma gondii* induce strong antibody response in humans and are considered as useful diagnostic antigens. Previous studies reported expression of amino terminal GRA8 protein in fusion with large tags such as glutathione-S-transferase. The present study aimed to produce soluble full length immunogenic GRA8 in bacteria. **Methods:** GRA8 complementary DNA (cDNA), encoding amino acids 24 to 258, was amplified from tachyzoites of RH strain and cloned in prokaryotic expression vector pET-28b(+). Expression of recombinant GRA8 (rGRA8) was analyzed by SDS-PAGE. Antigenicity and immunogenicity of the protein were evaluated by Western-blotting. **Results:** The cloned gene fragment exhibited complete similarity with the published sequence of *gra8* gene by sequence analysis. rGRA8 was expressed in *Escherichia coli* in fusion with a very small tag and the soluble protein was purified by immobilized metal ion affinity chromatography. In immunoblot, serum sample from a rabbit immunized with rGRA8 recognized a single antigen of *T. gondii* tachyzoite at the expected molecular weight of native GRA8. Sera from acutely-infected pregnant women strongly reacted with rGRA8 in Western-blotting, while sera from chronically-infected or *T. gondii*-negative women failed to recognize the protein. **Conclusion:** The full length soluble rGRA8 was successfully produced in *E. coli* and shown to be a highly immunogenic protein. As a result it could be used in immunological as well as molecular biology experiments. *Iran. Biomed. J.* 13 (3): 145-151, 2009

Keywords: *Toxoplasma gondii*, Dense granule antigen, GRA8, Expression, *Escherichia coli*

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite which can infect warm-blooded animals including human beings. It is estimated that up to one third of the world's human population has been exposed to the parasite [1]. Although Toxoplasmosis is generally benign in immunocompetent individuals, it is a significant cause of morbidity and mortality in congenitally-infected and immunocompromised individuals. Primary infection with *T. gondii* acquired during pregnancy can spread to the fetus and may result in premature birth, permanent neurological damage and visual impairment [2, 3]. Toxoplasmic encephalitis, the most common clinical presentation of Toxoplasmosis in immunocompromised individuals, occurs in 25 to 50% of HIV-positive individuals and

can be fatal if not recognized and treated soon [4]. In livestock, abortion of ewes also causes considerable economic losses [5].

Laboratory diagnosis of *Toxoplasma* infection relies on detection of specific *Toxoplasma* immunoglobulin G (IgG) and IgM antibodies, mainly by ELISA and indirect immunofluorescence assay [6, 7]. On the other hand, definitive diagnosis in immunocompromised individuals is mostly undertaken by direct detection of the parasite by means of PCR, histology, cell culture and mouse inoculation [7]. Serological tests are primarily based on crude *Toxoplasma* antigens. The complex nature of *Toxoplasma* antigens makes standardization of the assays difficult, and frequently gives rise to insufficient specificities for differentiation between acute and chronic infection [8, 9]. The use of recombinant antigens presents several advantages

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compared to whole parasite extract such as ease of production, constant quality and cheaper price [10, 11]. Moreover, application of *T. gondii* antigens which are representative of acute and/or chronic infection provides a valuable tool for development of new tests capable of discriminating acute from chronic infection [12, 13]. Dense granule antigens (GRA antigens) of *T. gondii* are secreted in abundance and constitute an important fraction of antigens which circulate in the bloodstream during the first days following infection [14]. Previous studies introduced several GRA antigens including GRA2 [15], GRA6 [13, 16, 17], GRA7 [18], and GRA8 [18, 19, 20] as markers of acute infection and revealed their diagnostic potential. The sequence of GRA8 is composed of 267 amino acids and 64 of them are hydrophobic proline (24% overall). GRA8 contains an amino terminal signal peptide, three degenerate proline-rich repeats in the central region and a potential transmembrane domain near the carboxyl terminus [21]. Previous studies demonstrated that GRA8 is a marker of acute infection and IgG and IgM ELISA with the protein are useful in detection of *T. gondii* infection and discriminating acute from chronic infection [19, 20].

We previously produced recombinant GRA2 and GRA6 antigens and reported efficacy of ELISA tests using these antigens for detection of acute Toxoplasma infection [13, 15]. In this study, bacterial production and purification of recombinant GRA8 protein (rGRA8) was accomplished as a basis for developing an ELISA test using combination of different GRA antigens for serodiagnosis of Toxoplasma infection.

MATERIALS AND METHODS

Parasite. Tachyzoites of *T. gondii* (RH strain) were injected into peritoneal cavity of Swiss mice. Three days later, tachyzoites were harvested, washed with PBS and stored at -80°C.

Sera. Sera were obtained from pregnant women referred to Tehran medical diagnostic labs for *T. gondii* infection. Sera were analyzed with Euroimmun IgG and IgM diagnostic kits (Euroimmun, Lübeck, Germany). Pooled sera, used in this study, were prepared from 5 women with positive IgG and IgM responses (acute infection), 5 women with positive IgG and negative IgM response (chronic infection), and 5 women with negative IgG and IgM responses (no toxoplasma infection).

RNA isolation. Total RNA from tachyzoites was extracted using RNX-Plus (CinnaGen, Tehran, Iran) according to the manufacturer's instruction and stored at -80°C.

Reagents. *E. coli* DH5α strain (Invitrogen, Carlsbad, CA) was used for cloning purpose and *E. coli* Rosetta (DE3) strain (Promega, Madison, WI) was used for expression of rGRA8. The bacterial expression plasmid pET-28b(+) was obtained from Novagen, Madison, WI. Ni2+-nickel-nitrilotriacetic acid (Ni2+-NTA) agarose resin was purchased from Qiagen, Hilden, Germany.

PCR amplification of GRA8 complementary DNA (cDNA). Single strand cDNA was synthesized using First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) and random hexamer primers. To amplify GRA8 cDNA, a pair of specific primers was designed according to the published sequence of *gra8* (AF150729). For cloning purpose, *Bam*HI and *Xho*I restriction sites were introduced at 5' end of the forward and reverse primers, respectively.

Forward primer: 5'-GCG GAT CCC GCC ATG
AAC GGT CCT TTG AGT TAT- 3'

Reverse primer: 5'- GC CTC GAG GTG GAG ATT
TGG AAA CAT CCG TAC CG -3'

The coding region for GRA8 protein excluding the first 23 amino acids, representing the signal peptide, and the 9 C-terminal amino acids were amplified from the *T. gondii* cDNA. PCR amplification was performed according to the following conditions: 1 cycle of 94°C for 5 min then 35 cycles of 94°C for 1 min, 57°C (+ 0.1°C increment per cycle) for 1 min, and 72°C for 50 s. Final primer extension was 7 min at 72°C. PCR product was run on 1.0% agarose gel and presence of specific band of GRA8 was investigated.

Cloning GRA8 cDNA. The purified PCR product was digested with *Bam*HI/*Xho*I restriction enzymes, purified, and ligated with the *Bam*HI/*Xho*I digested pET-28b(+) plasmid. Ligation product was transformed into *E. coli* DH5α bacteria. Screening of correct clone harboring recombinant plasmid pET-28b-GRA8 (pGRA8) was performed by restriction digestion using *Eco*RV enzyme followed by sequence analysis of the cloned gene.

Expression of rGRA8. The pGRA8 plasmid was transformed into *E. coli* Rosetta (DE3) strain and bacteria were cultivated in Luria-Bertani (LB) broth containing kanamycin (25 µg/ml). Expression of rGRA8 from several colonies under the control of isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter was tested on an analytical scale by SDS-PAGE. A single fresh colony was selected for inoculation of 10 ml LB broth and incubated at 200 rpm at 30°C overnight. The day after, cells were diluted 1:50 and allowed to grow at 37°C until the OD₆₀₀ reached 0.4-0.5. Induction of protein expression was performed using 1 mM of IPTG and bacteria were harvested 5 h later by centrifugation at 6,000×g for 15 min.

Fractionation of soluble and insoluble proteins. Pellet of 1 ml induced bacteria was resuspended in 100 µl of lysis buffer (20 mM Tris, pH 9.0, 0.5 M NaCl, 0.1% Triton X-100 and 1 mg/ml lysozyme) and incubated at 4°C for 30 min. The cells were lysed by gentle vortexing, and insoluble proteins and cell debris were pelleted by centrifugation at 16000×g for 15 min. The pellet, supernatant and crude lysate before fractionation were analyzed by SDS-PAGE.

Purification of rGRA8. Bacteria from 500 ml induced culture were resuspended in 8 ml buffer A (5 mM imidazol, 0.5 M NaCl, 0.1% Triton X-100, 20 mM Tris, pH 9.0, containing protease inhibitor cocktail without EDTA (Roche, Mannheim, Germany). The cells were sonicated for 6 min using an MSE ultrasonic disintegrator (Fisons, Loughborough, UK) at 60% of power, centrifuged at 12000 × g at 4°C for 20 min and the supernatant was passed through a 0.2-µm (pore size) filter. Two milliliters of Ni²⁺-NTA resin, previously washed with buffer A, was added to the supernatant and stirred gently at 4°C for 1 h. The resin was then applied to an empty column and washed sequentially with 20 mL of buffer B, C, and D (the same composition as buffer A but containing 20, 40, and 80 mM imidazol, respectively). The rGRA8 protein was eluted with buffer E (the same composition as buffer A but containing 400 mM imidazol and 0.01% Triton X-100) and dialyzed against PBS. Protein concentration was determined using DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Rabbit immunization with rGRA8. One rabbit was immunized intramuscularly (i.m.) with 100 µg

of purified rGRA8, emulsified in Freund's complete adjuvant. Injection was repeated two times with two weeks intervals using Freund's incomplete adjuvant and serum sample was collected two weeks after the last immunization. Another rabbit was injected with the adjuvant alone and serum was taken accordingly.

Western-blotting. SDS-PAGE was performed on 13% polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes, saturated for 1 h with 2% BSA in PBS-0.05% Tween 20 and probed with pooled sera from pregnant women diluted 1:100 or rabbit sera diluted 1:100. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody diluted 1:2000 (Sigma, Hilden, Germany) or HRP-conjugated goat anti-rabbit IgG antibody diluted 1:500 (Ray Biotech, Kermanshah, Iran). Human sera, rabbit sera and secondary antibodies were diluted in blocking buffer. Signals were detected using 3, 3'-diaminobenzidine tetrahydrochloride substrate (Sigma, Hilden, Germany).

RESULTS

Cloning GRA8. GRA8 cDNA corresponding to the amino acids 24 to 258, excluding the putative signal sequence and the 9 C-terminal amino acid was amplified from total cDNA of *T. gondii* RH strain by means of PCR. Specific PCR product of 725 bp was observed in gel agarose electrophoresis (Fig. 1) and cloned in prokaryotic expression plasmid pET-28b(+). Screening recombinant clones harboring GRA8 cDNA was performed by digestion with EcoRV restriction enzyme (Fig. 2). While digestion of recombinant plasmids containing GRA8 cDNA produced two fragments of 4136 and 1904 bp, non-recombinant plasmid gave rise to only one DNA band. Sequence analysis of the correct clone revealed 100% homology with the published sequence of *gra8* (AF150729) (data not shown). In pGRA8 construct, 34 amino acids including T7 tag and a cluster of 6 His residues were fused to the N-terminal and a second 6 His tag was fused to C-terminal of the target ORF.

Expression and purification of rGRA8. The pGRA8 construct was transformed into *E. coli* Rosetta (DE3) and expression of rGRA8 was induced by IPTG. Optimized expression and

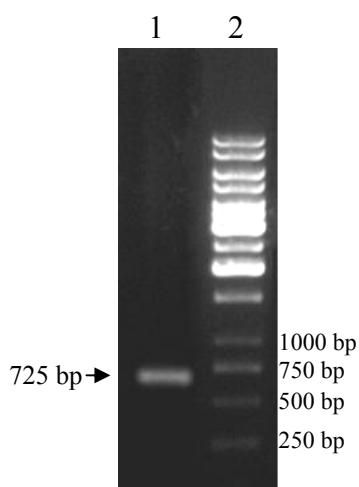


Fig. 1. PCR amplification of GRA8 cDNA. A pair of specific primer was designed and used to amplify GRA8 cDNA (encoding amino acids 24-258) from tachyzoites of *T. gondii* RH strain. Lane 1, PCR product was analyzed by 1% gel agarose electrophoresis and a specific band of 725 bp was observed; lane 2, DNA molecular weight.

solubility of rGRA8 were obtained with the following experimental conditions: 1.0 mM of IPTG was added to a culture of $OD_{600} = 0.4-0.5$ and expression was induced at 37°C for 5 h. SDS-PAGE analysis of induced bacteria showed weak expression of a doublet with the apparent molecular mass of 36 and 41 kDa (Fig. 3). The smaller band, which was almost completely in insoluble form, is either related to the onset of expression from an internal ATG codon located in

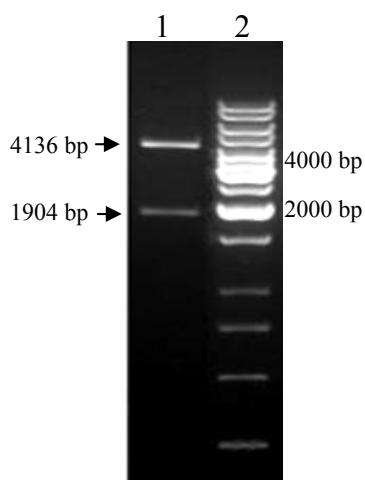


Fig. 2. Screening of recombinant clones containing GRA8 cDNA. GRA8 PCR product was ligated with pET-28b(+) plasmid and ligation product was transformed into DH5 α bacteria. Screening of recombinant clones was performed by EcoR V restriction digestion. Lane 1, digestion of recombinant plasmid harboring GRA8 cDNA resulted in two DNA fragments of 4136 and 1904 bp; lane 2, DNA molecular weight.

forward primer or is a degradation product of rGRA8. The calculated molecular mass of rGRA8 was about 30.6 kDa (278 aa). The discrepancy between the calculated molecular mass and the apparent molecular weight on SDS-PAGE was expected as it is a common feature of GRA proteins. It has been attributed partly to the presence of 64 proline residues in the sequence of GRA8 [22, 23]. To gain an estimate of solubility of rGRA8, induced bacteria were lysed. Crude bacteria lysate was fractionated into both soluble and insoluble fractions and separated on SDS-PAGE; while part of rGRA8 was soluble, the main fraction of the protein was in insoluble form (Fig. 3A). Soluble fraction of induced bacteria was applied to Ni^{2+} -NTA chromatography and rGRA8 protein was purified in a single step (Fig. 3B). The yield of purified rGRA8 was about 1.5 mg per liter of bacterial culture.

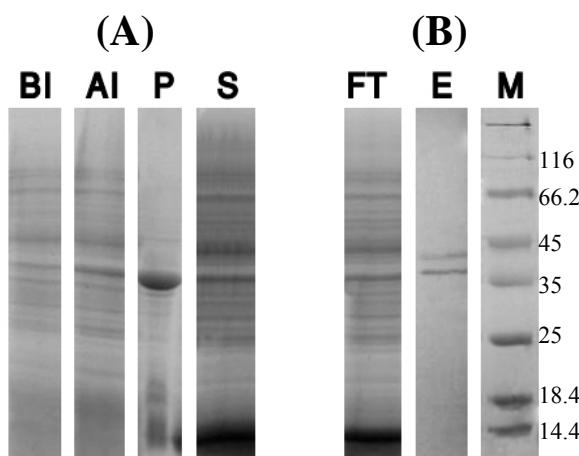


Fig. 3. Expression and purification of rGRA8. Expression of rGRA8 was induced by IPTG. (A) Expression level and relative solubility of rGRA8. The pGRA8 plasmid was introduced into *E. coli* Rosetta (DE3) bacteria and expression of rGRA8 was induced by IPTG. Aliquots of the soluble (S) and pellet (insoluble) (P) fractions from induced bacteria were analyzed by SDS-PAGE on 13% polyacrylamide gels followed by Coomassie blue staining. M: protein molecular weight (kDa). The equivalent of 0.25 ml of induced bacteria culture was loaded onto each lane. BI: before induction; AI: after induction. Note that the 14 kDa band observed in both the P and S fractions correspond to the lysozyme added to lyse the bacteria. (B) Purification of rGRA8. The soluble recombinant protein was purified by Ni^{2+} -NTA affinity chromatography and analyzed by SDS-PAGE. FT: Flow through; E: Elution.

Antigenicity of rGRA8. Antigenicity of rGRA8 was investigated in Western-blot analysis. One microgram of purified protein was transferred onto nitrocellulose membrane and probed with pooled human sera representing acute, chronic or no toxoplasma infection. While acute sera strongly reacted with rGRA8, chronic sera and negative sera

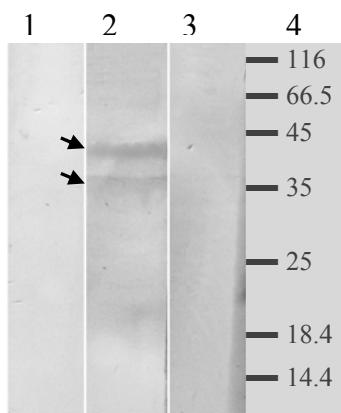


Fig. 4. Reactivity of rGRA8 with human sera. The purified rGRA8 was analyzed by SDS-PAGE, transferred onto nitrocellulose paper and probed with human pooled sera from acute (lane 2), chronic (lane 3) or no Toxoplasma infection (lane 1). Only acute sera reacted with rGRA8, and chronic and negative sera failed to recognize rGRA8. Lane 4, protein molecular weight (kDa).

failed to recognize the protein (Fig. 4). The results demonstrate antigenicity of rGRA8 and superior reactivity of rGRA8 toward acute sera, as compared to chronic sera.

Immunogenicity evaluation of rGRA8. In order to confirm immunogenicity of rGRA8, one rabbit was i.m. immunized with the purified protein three times with 2 weeks intervals. Two week after last immunization, sera was collected and used to probe RH tachyzoites in Western-blotting. Immunized rabbit serum recognized a single protein band of 35 kDa, the expected size of native GRA8 antigen. In contrast, there was no specific band in the blot of RH tachyzoites probed with serum from the rabbit injected with the adjuvant alone or serum from an unimmunized rabbit (Fig. 5).

DISCUSSION

Serodiagnosis of *T. gondii* infection is performed primarily using live or chemically treated tachyzoites or whole extracts of RH tachyzoites. Large-scale amplification of the parasite, grown either in host cell monolayers or in mice peritoneal cavities, is an expensive and laborious process. Moreover, inconstant quality of crude *T. gondii* antigen, obtained in different batches, could cause difficulties in developing well-standardized immunodiagnostic tests. Several studies have proposed a combination of individual antigens can successfully replace crude *T. gondii* antigen in

ELISA tests [18, 24]. Recombinant technology has enabled fast, efficient and less-expensive preparation of highly pure proteins. Application of recombinant proteins could minimize possible variations in the quality of antigens and help standardizing the assays [10, 11]. The use of recombinant antigens representative of acute or chronic infection might further enhance their application for discriminating acute from chronic infection [13, 20, 24].

In the present study, cDNA encoding GRA8 protein of *T. gondii*, residue 24 to 258, was amplified from RH strain and cloned. The rGRA8 protein was expressed in bacteria and purified by an immobilized metal ion affinity chromatography. The purified protein was reactive toward sera from acutely-infected women and induced specific antibody response upon immunization of a rabbit. Despite its high content of proline, several studies reported a high-level expression of N-terminal part of GRA8 fused to long fusion tags such as CMP-2-keto-3-deoxy octulosonic acid synthetase and glutathione-S-transferase (GST) [18, 24]. In fact, numerous studies have shown that application of soluble large fusion tags could improve expressivity and solubility of hydrophobic proteins [25]. The low expression level of rGRA8 observed in this study could be attributed to the presence of the C-terminal hydrophobic transmembrane domain and/or use of a small fusion tag. We chose to add a small fusion tag

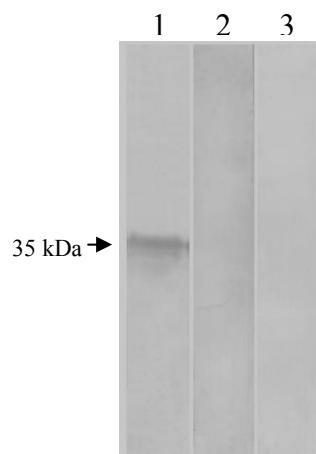


Fig. 5. Evaluation of immunogenicity of rGRA8. One Rabbit was injected i.m. with 100 µg of rGRA8 three times with two weeks intervals. Two weeks after the last injection, rabbit serum sample was obtained. Tachyzoites of *T. gondii* RH strain (5×10^6 tachyzoites) were analyzed by SDS-PAGE, transferred onto nitrocellulose membrane and probed with sera from the rGRA8-immunized, adjuvant-injected or unimmunized rabbits. Immunized rabbit serum recognized a single protein band of 35 kDa, the expected size of native GRA8 antigen (lane 1), while serum from an adjuvant-injected (lane 2) or unimmunized (lane 3) rabbit failed to react with any specific protein of RH tachyzoites.

to GRA8, as long fusion partners are not desirable in ELISA tests and might cause non-specific reactions. To improve expression level of rGRA8, we aim at using a large fusion partner such as GST and Thioredoxin. The stronger reactivity of rGRA8 with acute *T. gondii* sera is consistent with previous studies introducing GRA8 as a marker of acute infection [19, 20]. The rGRA8 was capable of induction of specific immune response in a rabbit, confirming its proper immunogenicity. GRA8, an antigen of dense granules of *T. gondii*, elicits strong IgG and IgM antibodies responses which usually decrease during chronic infection [19, 26]. Several studies have shown that ELISA tests using GRA8 alone or in combination with other antigens are useful for detecting *T. gondii* IgG and IgM antibodies. Suzuki *et al.* [20] applied GRA8 in an IgM ELISA test and showed sensitivity and specificity of 90% and 100%, respectively for distinguishing between acute and chronic Toxoplasma infection. A combination of GRA7, GRA8, and Rhopty 1 in IgM ELISA showed superior performance compared to each individual antigen [18]. Moreover, a combination of GRA7, GRA8, and SAG1 [18] or of GRA7, GRA8, SAG2, and H4 [24] was proposed for detection of *T. gondii* IgG antibodies.

We have shown that GRA8 can be produced in bacteria, in milligrams levels, without using a large fusion tag. The use of full length GRA8 protein, instead of truncated one, in ELISA tests could maximize diagnostic performance of the protein. We are going to apply rGRA8 alone or in combination with other Toxoplasma antigens for developing ELISA tests for detection of *T. gondii* infection and discriminating acute from chronic infection.

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