# HETEROLOGOUS PRODUCTION OF DENSE GRANULE GRA7 ANTIGEN OF *TOXOPLASMA GONDII* IN *ESCHERICHIA COLI*

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**Abstract.** Infection with *Toxoplasma gondii* causes serious health problems in congenitally-infected and immunocompromised individuals. Numerous studies have shown usefulness of dense granule antigens of *T. gondii* in serodiagnosis of the infection and induction of protective immunity. This study describes cloning, expression, purification and antigenicity evaluation of recombinant GRA7 protein (rGRA7). DNA encoding GRA7, amino acids 18 to 236, was obtained from *Toxoplasma gondii* RH strain by polymerase chain reaction amplification and cloned in prokaryotic expression plasmid pET-28b(+). Sequence analysis showed 97% similarity between GRA7 gene fragment and published sequence of *gra7*. Recombinant protein was expressed in *Escherichia coli* and purified in a single step by immobilized metal ion affinity chromatography. Antigenicity of the protein was evaluated in Western blot analysis showing human sera from acute *T. gondii* infection strongly reacted with rGRA7 while sera from chronic infection weakly recognized the protein. Negative sera failed to react with rGRA7. The antigenic rGRA7 might be used, in combination with other *T. gondii* antigen, to develop more efficacious diagnostic tests and/or in vaccine formulations.

## INTRODUCTION

Toxoplasmosis is caused by the protozoan parasite *Toxoplasma gondii* in humans and other warm-blooded animals. Primary *T. gondii* infection in pregnant women can result in neurological and ocular complications in the fetus or cause abortion (Remington, 1995). *T. gondii* also can cause severe encephalitis via acute infection or reactivation of chronic infection in immunocompromised individuals such as AIDS patients (Remington, 1995). In farm animals, abortion of ewes causes considerable economic losses (Remington, 1995). So far, no effective and safe vaccine is available to prevent Toxoplasmosis. Fortunately, if the infection is diagnosed promptly and accurately chemotherapy can prevent both transmission of the parasite to offspring and development of fatal toxoplasmic encephalitis (Remington, 1995; Jones *et al*, 2001; Montoya and Liesenfeld, 2004).

Diagnosis of Toxoplasma infection can be established by isolation of the parasite from blood or body fluids, demonstration of the parasite in tissues, detection of specific

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nucleic acid sequences with DNA probes, or detection of *T. gondii*-specific immunoglobulins (Montoya and Liesenfeld, 2004; Remington *et al*, 2004). Diagnosis of Toxoplasmosis in immunocompetent individuals is based mainly on serological tests as primary infection is associated with clinical signs in only 5% of pregnant women (Ferrandiz *et al*, 2004). Available serological tests require preparation of parasite antigens from tachyzoites harvested from mice peritoneal cavities or cell cultures (Aubert *et al*, 2000; Beghetto *et al*, 2006; Golkar *et al*, 2008). These methods are quite expensive, laborious and yield antigens with inconstant quality. Application of recombinant *T. gondii* antigens could result in less expensive and standardized serological tests (Van *et al*, 1993; Liesenfeld *et al*, 1997; Wilson *et al*, 1997; Beghetto *et al*, 2006). Moreover, antigen(s) that induce antibodies preferentially in acute or chronic infection could be applied for differentiating recent *T. gondii* infection from an infection previously acquired (Li *et al*, 2000a, b; Golkar *et al*, 2007a, 2008). As serologic testing may give rise to a negative result if a single antigen is used for the detection of antibodies, a combination of properly selected antigenic proteins would detect all serologically positive individuals, as well as differentiating between acute and chronic infections (Aubert *et al*, 2000; Beghetto *et al*, 2006; Golkar *et al*, 2008).

Dense granule (GRA) antigens of *T. gondii* induce strong antibody response during acute infection and are considered as useful diagnostic markers (Redlich and Muller, 1998; Jacobs *et al*, 1999; Li *et al*, 2000b; Golkar *et al*, 2007a, 2008). Diagnostic performance of GRA antigens, such as GRA2, GRA6, GRA7 and GRA8 have been investigated in ELISA for discriminating acute from chronic Toxoplasma infection (Redlich and Muller, 1998; Jacobs *et al*, 1999; Li *et al*, 2000b; Golkar *et al*, 2008). Several studies have reported vaccine potential of GRA antigens including GRA1 (Vercammen *et al*, 2000; Doskaya, 2007), GRA2 (Golkar *et al*, 2007b), GRA4 and GRA7 (Vercammen *et al*, 2000; Jongert *et al*, 2007) for induction of protective immunity against *T. gondii* infection. GRA7, a 236 amino acids protein, plays an important role in the long term stimulation of the immune response (Jacobs *et al*, 1999; Neudeck *et al*, 2002). GRA7 induces higher levels of specific IgG antibodies during acute infection as compared to chronic infection (Jacobs *et al*, 1999; Pietkiewicz *et al*, 2004, 2006; Pfrepper *et al*, 2005; Gatkowska *et al*, 2006). Several studies applied recombinant GRA7 DNA in vaccination experiments and found that GRA7 could partially protect against *T. gondii* infection (Vercammen *et al*, 2000; Jongert *et al*, 2007, 2008). However, a combination of GRA7 antigen and two other *T. gondii* proteins failed to protect mice against infection (Igarashi *et al*, 2008a,b). To our knowledge, no result has been published about the protective ability of recombinant GRA7 alone.

We have produced GRA2 and GRA6 antigens in bacteria and investigated their usefulness in serodiagnosis of and protection against Toxoplasma infection (Golkar *et al*, 2007a,b, 2008). In this study, we produced recombinant GRA7 (rGRA7) antigen in bacteria and evaluated its antigenicity.

## MATERIALS AND METHODS

### Parasitic and bacterial strains

*T. gondii* RH strain was used for extraction of genomic DNA. Tachyzoites were injected into peritoneal cavity of Swiss mice. Three days later, tachyzoites were harvested from peritoneal fluid, washed with phosphate-buffered saline (PBS) and stored at -80°C until use. *Escherichia coli* Top 10F' (Invitrogen, Carlsbad, CA) and *E. coli* Rosetta (DE3) (Promega, Madison, WI) were used for cloning and expression of recombinant antigen respectively.

**Construction of the recombinant expression plasmid**

DNA sequence of *gra7* was obtained from GenBank database (accession no. Y13863). Genomic DNA was extracted from tachyzoites of *T. gondii*, RH strain, by a genomic DNA extraction kit (Bioneer, Seoul, Korea) and used as template for polymerase chain amplification (PCR) of GRA7 gene fragment, encoding amino acids 18 to 236, using specific primers GCG GGA TCC TTT GCC CCA GTT CGC TAC CG and CGC GAA TTC CCT GGC GGG CAT CCT CCC CAT C (coding sequences are underlined). PCR amplification was performed as follows: 1 cycle of 94ºC for 5 minutes, 35 cycles of 94ºC for 1 minute, 58ºC (+ 0.1ºC increment per cycle) for 50 seconds, and 72ºC for 50 seconds; and a final primer extension for 30 minutes at 72ºC. Amplicon of 670 bp was ligated into T/A cloning vector, pTZ57R/T (Fermentas, Vilnious, Lithuania). Ligation product was transformed into *E.coli* TOP10' cells and recombinant clones were selected by blue/white screening. Recombinant plasmid was extracted from culture of a white bacterial colony and digested with *Bam*HI and *Eco*RI restriction enzymes. The products of restriction digestion were analyzed by agarose gel-electrophoresis and 670 bp fragment was purified from the gel using DNA gel purification kit (Bioneer, Seoul, Korea). The purified DNA fragment was inserted in-frame into expression vector pET-28b(+) previously digested with *Bam*HI/*Eco*RI restriction enzymes. The presence of GRA7 insert in the recombinant plasmid was checked by restriction analysis using *Rsa*I enzyme, and also by sequence analysis. The recombinant plasmid was named pET-28(+)-GRA7 (pGRA7).

**Expression of recombinant GRA7 (rGRA7)**

*E. coli* Rosetta (DE3) were transformed by pGRA7 and grown in Luria Bertani (LB) broth supplemented with kanamycin (25 μg/ml) and chloramphenicol (34 μg/ml). The culture was grown with vigorous shaking at 37ºC to an optical density of 600 nm of 0.6-0.8. Protein production was induced by 1 mM isopropyl-β-D-thiogalactoside (IPTG), and bacteria were incubated with vigorous shaking for an additional period of 5 hours. Induced cells were harvested by centrifugation and expression of recombinant GRA7 was analyzed by SDS-polyacrylamide gel-electrophoresis (SDS-PAGE). In order to assess solubility of rGRA7, 1 ml pellet of induced bacteria was lyzed in 100 μl of the lysis buffer (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, and 1 mg/ml lysozyme) at 4ºC for 30 minutes. The lysate was centrifuged at 16,000*g* for 15 minutes at 4ºC, and supernatant removed for further analysis.

**Purification of rGRA7**

Bacteria from 500 ml of induced culture were resuspended in 10 ml of buffer A (10 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.5, 0.1% Triton X-100, protease inhibitors cocktail without EDTA (Roche, Mannheim, Germany), containing 1 mg/ml lysozyme. Cells were sonicated for 6 minutes using MSE ultrasonic disintegrator (Fisons, Loughborough, UK) at 60% power and centrifuged at 12,000*g* and 4ºC for 20 minutes, and the supernatant was passed through a 0.2 μm (pore size) filter. $Ni^{2+}$-nickel-nitrilotriacetic acid (NTA) resin (3.5 ml) previously equilibrated with buffer A was added to the supernatant and stirred gently for 1 hour at 4ºC. Then the resin was washed sequentially with 20 ml of buffers B, C, and D (buffer A containing 20, 40, and 80 mM imidazole, respectively). rGRA7 was eluted with buffer E (buffer A containing 400 mM imidazole and 0.01% Triton X-100) and dialyzed against PBS. Protein concentration was determined using DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

**Electrophoresis and Western blot analysis**

Bacterial lysate, soluble and insoluble proteins and purified rGRA7 were analyzed by SDS-PAGE performed using 13% polyacrylamide gel. Protein bands were transferred onto PVDF membrane. Membrane was incubated with 2% bovine serum albumin in PBS-0.05% Tween 20 and probed with 1/100 diluted pooled sera from pregnant women. Bound antibodies were detected using horseradish peroxidase conjugated goat anti-human IgG antibodies diluted 1/20,000 (Sigma, Hilden, Germany). Human sera and secondary antibodies were diluted in blocking buffer. Signals were detected using 3, 3'-diaminobenzidine tetrahydrochloride substrate (Sigma, Hilden, Germany).

## RESULTS

**Cloning of GRA7 gene**

A GRA7 gene fragment corresponding to amino acids 18 to 236 was PCR amplified from genome of tachyzoites of *T. gondii* RH strain yielding an amplicon of 670 bp (Fig 1). The amplicon was inserted into pTZ57R/T and sub-cloned into pET-28b(+) expression vector. Screening of recombinant clones harboring the recombinant pET-28b(+)-GRA7 (pGRA7) plasmid was performed by restriction digestion using *Rsa*I (Fig 2). Sequence analysis of a positive clone demonstrated 97% sequence similarity between the cloned gene and published sequence of *gra7* (accession no. Y13863) (data not shown). In pGRA7, the open reading frame of GRA7 gene was inserted with N-terminal fusion of 32 amino acids including a cluster of 6 histidine residues. A second 6 His tag was placed in C-terminal fusion of the target gene to facilitate subsequent purification of the recombinant protein.

**Expression and purification of the recombinant antigen**

The pGRA7 construct was transfected

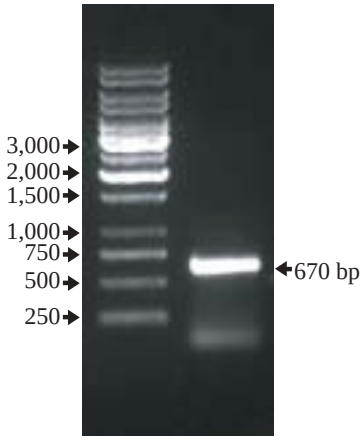


Fig 1–PCR amplification of GRA7 gene. A pair of specific primer was designed and applied to amplify DNA sequence encoding GRA7, amino acids 18-236, from genomic DNA of *T. gondii* RH strain. PCR product was analyzed by 1% agarose gel-electrophoresis.

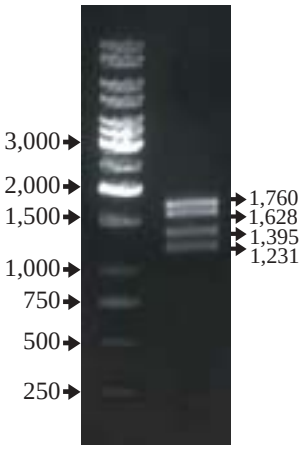


Fig 2–Screening of recombinant clones containing GRA7 gene. GRA7 gene was inserted into pET-28b(+) plasmid and transfected into TOP10F' bacteria. Screening of recombinant clones was performed by restriction digestion using R*sa*I. Digestion of recombinant plasmid harboring GRA7 gene resulted in the expected four DNA fragments of 1,231, 1,395, 1,628 and 1,760 bp.

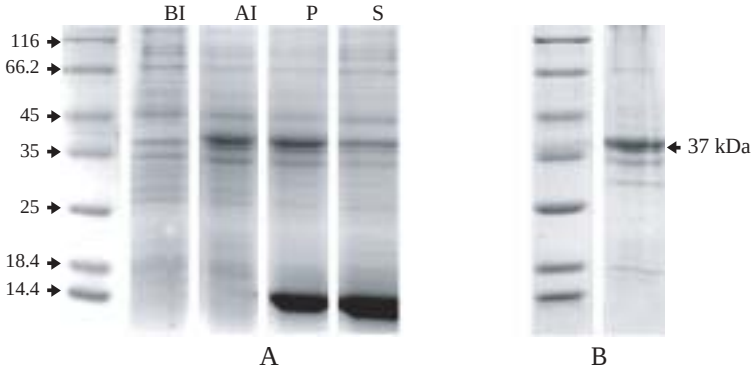


Fig 3–Expression and purification of rGRA7. Expression of rGRA7 was induced by IPTG. (A) Expression level and relative solubility of rGRA7. pGRA7 plasmid was transfected into *E.coli* Rosetta (DE3) and expression of rGRA7 was induced by IPTG. Aliquots of the soluble (S) and pellet (insoluble) (P) fractions from induced bacteria were analyzed in 13% SDS-polyacrylamide gels. The equivalent of 0.25 ml of induced bacteria culture was loaded in each lane. BI: before induction; AI: after induction. Note that the 14 kDa band observed in both P and S fractions correspond to lysozyme added to lyse the bacteria. Left lane shows standard molecular weight markers. (B) Purification of rGRA7. Soluble recombinant protein was purified by $Ni^{+2}$-NTA affinity chromatography and analyzed by SDS-PAGE.

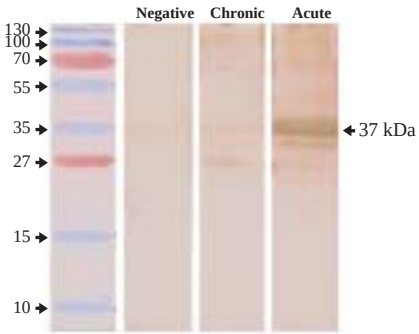


Fig 4–Immunoreactivity of rGRA7. Purified rGRA7 was separated by SDS-PAGE, transferred onto PVDF membrane and probed with pooled sera from pregnant women with acute, chronic or no *T. gondii* infection. Left lane shows standard molecular weight markers.

into *E. coli* Rosetta (DE3) bacteria and expression of rGRA7 was induced by IPTG. SDS-PAGE analysis of induced bacteria showed strong expression of a protein band of 37 kDa, which was absent in uninduced bacteria (Fig 3A). The calculated molecular mass of rGRA7 was 30 kDa (270 amino acids). The discrepancy between the calculated molecular mass and the apparent molecular weight on SDS-PAGE is a common feature of GRA proteins (Mercier *et al*, 2005). Induced bacteria were lyzed and crude bacterial lysate was fractionated into soluble and insoluble fractions. While the majority of rGRA7 protein was in insoluble form, a substantial fraction was soluble (Fig 3A). For purification of native rGRA7, soluble fraction of induced bacteria was applied to $Ni^{2+}$-NTA affinity column and the recombinant protein was purified in a single step. Lower and higher MW bands observed on SDS-PAGE of the purified protein might be bacterial proteins co-purified with rGRA7 or result of degradation of rGRA7 by bacterial proteases (Fig 3B).

**Immunoreactivity of rGRA7**

To investigate immunoreactivity of rGRA7, 1 μg of the purified protein was transferred onto a PVDF membrane and probed with pooled human sera representative of acute, chronic or no Toxoplasma infection. Acute sera

strongly recognized rGRA7 while chronic sera weakly detected the protein (Fig 4). No specific band was observed in immuno blot of rGRA7 probed with *T. gondii*-negative sera.

## DISCUSSION

In this study, we report expression and purification of *Toxoplasma gondii* dense granule GRA7 protein (amino acids 18 to 236) in *E.coli* as a soluble protein using pET-28b(+) expression system. Antigenicity of the purified protein was confirmed in immunoblot using *T. gondii*-positive human sera.

We decided to use an expression plasmid which adds a short His fusion tag to the recombinant protein to prevent possible nonspecific reaction of rGRA7 and proteins of serum in ELISA experiments. The presence of a majority of rGRA7 in insoluble form was expected as there are two C-terminal hydrophobic domains in the protein and we did not use large and solubility-enhancing fusion tags such as thioredoxin or maltose-binding protein. *E. coli* Rosetta (DE3) strain was used for heterologous expression as rGRA7 encodes 26 rare *E. coli* codons for arginine. Preliminary studies showed very low expression of rGRA7 using standard *E. coli* (DE3) bacteria (data not shown). Soluble rGRA7 was subjected to affinity chromatography and purified. Insoluble rGRA7 can be solubilized using urea or guanidine hydrochloride and purified by a denaturing method. In agreement with previous studies, Western blot analysis showed strong immunoreactivity of rGRA7 with sera from acutely-infected women, compared to sera from chronically infected women.

The most important situation encountered in serodiagnosis of Toxoplasma infection is whether a positive serologic profile of a pregnant woman is related to recent infection occurring during pregnancy. Most of the available serological tests apply Toxoplasma native antigens. The quality of Toxoplasma native antigens obtained from cell cultures or peritoneal cavities of mice may vary in different production series and makes standardization of the tests laborious and difficult (Liesenfeld *et al*, 1997). Production of constant-quality recombinant antigens can facilitate standardization of the tests. Besides, insufficient specificities observed in IgM-detecting tests were mainly attributed to the complex nature of parasite extracts and presence of persisting IgM antibodies to *T. gondii* antigens (Gras *et al*, 2004; Golkar *et al*, 2008). Several studies have suggested that utilization of a combination of recombinant *T. gondii* antigens could improve performance of serodiagnosis of infection and successfully replace complex native Toxoplasma antigens (Aubert *et al*, 2000; Li *et al*, 2000b; Beghetto *et al*, 2006).

Application of recombinant GRA7 for detection of Toxoplasma infection has been investigated in IgG and IgM ELISA. Sensitivity of GRA7-ELISA for acute infection sera was shown to be significantly higher than chronic infection sera (Jacobs *et al*, 1999; Aubert *et al*, 2000; Pietkiewicz *et al*, 2004; Pfrepper *et al*, 2007). Only one study investigated efficacy of GRA7-ELISA for distinguishing between acute and chronic Toxoplasma infection (Li *et al*, 2000a), which applied GRA7 in an IgG ELISA test for discriminating 10 acute sera from 10 chronic sera and reported sensitivity and specificity of 80% and 90% respectively. Two studies applied GRA7 in IgM ELISA for detection of acute Toxoplasma infection and reported two different sensitivity values of 20% and 50% (Aubert *et al*, 2000, Pfrepper *et al*, 2005). Production of purified rGRA7 would allow us to investigate the usefulness of GRA7 in IgM and IgG ELISA for detection of *T. gondii* infection and for discriminating acute from

chronic *T. gondii* infection.

We previously produced recombinant GRA6 and GRA2 and reported their diagnostic values in serodiagnosis of Toxoplasma infection (Golkar *et al*, 2007a, 2008). GRA8-IgM-ELISA was also shown to be useful for this purpose. Combination of GRA7 and other diagnostic markers of *T. gondii* might enhance the performance of serodiagnosis of *T. gondii* infection and replace Toxoplasma native antigens with recombinant antigens.

The best way to prevent morbidity and mortality associated with *T. gondii* infection is effective vaccination against the parasite. Despite extensive studies, no safe and effective vaccine is yet available to combat the infection, mainly due to the complex life cycle of *T. gondii* and failure in inducing strong cellular responses (Remington *et al*, 1995). Several antigens of *T. gondii* have been used in different vaccination studies and varying results have been reported (Bhopale, 2003). One promising strategy for improving protective potential of present vaccine antigens is combined usage of highly immunogenic Toxoplasma antigens (Martin *et al*, 2004; Mevelec *et al*, 2005; Golkar *et al*, 2007b; Jongert *et al*, 2008). GRA7 induces strong cellular and humoral immune responses in humans and animals. Vaccination of animals with recombinant GRA7 DNA, alone or in combination with other *T. gondii* antigens, induces IFN-γ production and protects against *T. gondii* infection ( Vercammen *et al*, 2000; Jongert *et al*, 2007, 2008). To our knowledge there is no report of the vaccine potential of GRA7 protein alone. A combination of GRA7, GRA5 and ROP2 antigens failed to protect mice against Toxoplasma infection (Igarashi *et al*, 2008a). We previously reported that recombinant GRA2 protein formulated in monophosphoril lipid (MPL) adjuvant induces strong cellular response and significantly protects mice against brain cyst production (Golkar *et al*, 2007b). Production of purified rGRA7 would allow us to investigate if immunization with rGRA7 could induce protective immunity against *T. gondii* infection or if a cocktail vaccine comprising GRA7 and other immunogenic antigens, such as GRA2 and SAG1, is a step towards an ideal toxoplasma vaccine.

## ACKNOWLEDEGEMENTS

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