High-level expression and evaluation of the antigenicity of a recombinant *Toxoplasma gondii* GRA2 protein

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Abstract
Toxoplasmosis is a worldwide infection which is commonly asymptomatic but can cause serious health problems in immunocompromised individuals and fetus. GRA2 is a dense granule protein of Toxoplasma gondii, which induces strong antibody and T-cell response in human and mice. The purpose of this study was to prepare recombinant GRA2 and evaluate its antigenic properties using infected mice sera. To reach this point, GRA2 gene was highly expressed as a fusion protein with Thioredoxin (TRX) in Escherichia coli BL21pLysS strain. The protein was purified in a single step on Ni-NTA affinity column. TRX-GRA2 was confirmed by Western blot technique using a monoclonal antibody specific for GRA2. Sera from mice infected with Toxoplasma gondii showed high reactivity toward GRA2 and the level of IgG2a isotype was predominant and significantly higher than IgG1.Taken together, TRX-GRA2 might be considered as an ideal antigen to be used as a vaccine target as well as diagnostic tool for detection of toxoplasmosis.
Keywords: *Toxoplasma gondii, GRA2, Thioredoxin, E. coli, Expression.*

INTRODUCTION

Toxoplasma gondii is an obligate intracellular pathogen that can probably infect all warm-blooded animals (Desolme *et al*., 2000). In humans with a competent immune system, the acute infection commonly resolves without treatment, but the host remains chronically infected, perhaps for the rest of his or her life. In contrast, humans with compromised immune system, such as those infected with human immunodeficiency virus (HIV) or those receiving immunosuppressive therapy, are at risk for life-threatening disease following recently acquired *T. gondii* infection or following reactivation of previously dormant infection (Bout *et al*., 2002). In pregnant women primary infection can place the child at risk for serious medical problems so it is of great importance to identify newly acquired infection in pregnant women. In livestock, particularly sheep and pigs, infection can lead to abortion, causing significant losses to the industries (Desolme *et al*., 2000; Angus *et al*., 2000 and Bout *et al*., 2002).

In view of developing a subunit vaccine for prevention or attenuation of the disease, we focused our efforts on GRA2, a 28 kDa protein located in the parasite dense granules. GRA2 gene codes for a polypeptide of 185 amino-acid residues, comprising a highly hydrophobic 23 amino-acid signal sequence, two amphipatic alpha helices and a highly charged C-terminal domain (Mercier *et al*., 1993).

It has been shown that GRA2 induce humoral and cellular immune response in several models including human and mice (Prigione *et al*., 2000; Pistoia *et al*., 1996; Murray *et al*., 1993; Brinkmann *et al*., 1993 and Sharma *et al*., 1984). Immunological responses to GRA2 may be important in controlling infection, as immunization with the native protein partially protects mice against acute toxoplasmosis (Brinkmann *et al*., 1993 and Sharma *et al*., 1984). Moreover, GRA2 is expressed in the two stages of the parasite life cycle (tachyzoite and bradyzoite) and anti- GRA2 antibodies are present in more than 80% of sera from acutely infected patients and in about 95% of sera from chronically infected patients (Murray *et al*., 1993). It was also shown that targeted disruption of GRA2 gene

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decreases acute virulance in mice (Mercier *et al*., 1998). Taken together, these data suggested that GRA2 antigen constitutes a potential vaccine candidate and also is of interest to study humoral immune response during *T. gondii* infection.

The evaluation of GRA2 immunogenicity requires sufficient quantities of highly purified protein. Its purification from the parasite needs huge amount of parasites and results in very few amount of the protein (Mercier *et al*., 1993). Initial efforts on expression of recombinant full-length GRA2 as fusion with glutathione S-transferase in *E. coli*, resulted in very low level expression. This may be due to hydrophobic identity of the protein or its sensitivity against host proteases (Murray *et al*., 1993 and Parmley *et al*., 1993).

The problems encountered with overexpression of proteins in bacteria, have been most successfully overcome by the use of fusion proteins (Terpe, 2003 and Ford *et al*., 1991). Among the fusion systems, Thioredoxin (TRX) is a commonly used fusion partner that has been shown to increase expression of hydrophobic proteins. Moreover, it has been shown that heterologus proteins particularly sensitive to proteolysis became stabilized when fused with TRX (Martinez *et al*., 1995; Terpe, 2003; Murby *et al*., 1991; La Vallie *et al*., 1993 and Yang *et al*., 2004).

In this study, the production and purification of antigenic TRX-GRA2 protein was accomplished as a basis for evaluating vaccine potential and diagnostic value of GRA2 protein. Hence, Recombinant GRA2 was highly expressed in *E. coli* and purified near to homogeneity. Expression of TRX-GRA2 was then confirmed using a monoclonal antibody (mAb) against GRA2. In addition, sera from CBA/J mice infected with *T. gondii* were obtained to measure the level of total IgG and specific IgG isotypes against GRA2.

MATERIALS AND METHODS

Reagents: The *E. coli* DH5α (Invitrogen, USA) and BL21 (DE3) pLysS (Promega, USA) strains were used for cloning purpose and expression of the recombinant protein, respectively. The expression plasmid pET-32a was obtained from (Novagen, USA). Ni-NTA agarose resin was purchased from (Qiagen, Germany).

PCR amplification of GRA2 cDNA: The coding region for the mature GRA2 protein, residues 23 through 185, (excluding signal sequence) was amplified from a pBluescript-GRA2 plasmid which contains complete cDNA of GRA2 (Mercier *et al*., 1993) (kindly provided by Marie-France Cesbron-Delow). A pair of specific primers was designed according to the published sequence of GRA2 gene (Accession No: L01753). *Nco*I and *Bam*HI restriction sites were introduced at the 5′ end of the forward and reverse primers, respectively.

Forward primer: 5′ACTCCATGGACATGAGGGCTGCCGAGTTTTC3′
*Nco*I

Reverse primer: 5′TACGGATCCTGCGAAAAGTCTGGGAC3′
*Bam*HI

The GRA2 cDNA was amplified from 50 ng of pBluescript-GRA2 plasmid as template in a total volume of 50 ml containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.2, 2.5 U AmpliTaq (Promega, USA) and 100 pmol of each oligonuleotide primer. Amplification conditions were one cycle of 94°C for 5 min then 25 cycles of 94°C for 30s, 55°C for 1 min as an annealing temperature, and 72°C for 30 s and the final primer extension at 72°C for 5 min. The PCR product was electrophoresed on 1.0 % agarose gel and the corresponding band of 500 bp cut and purified. Restriction analysis was performed on the purified PCR product using *Rsa*I enzyme.

Cloning of GRA2 in the bacterial expression vector pET-32a: GRA2 PCR product was cloned into *Nco*I and *Bam*HI restriction sites of the pET-32a expression plasmid downstream of T7 promoter. The ligated product was used to transform DH5α competent cells. Recombinant clones were screened and one recombinant clone was selected for sequencing. There was no amplification error in the sequenced recombinant clone, designated as pET-GRA2.

Expression of TRX-GRA2: The recombinant expression plasmid pET-GRA2 was transformed into *E.coli* BL21 (DE3) pLysS strain and cultivated in LB agar (10 g/l NaCl; 10 g/l tryptone; 5 g/l yeast extract) cotaining ampicilin (100 µg/ml). The expression of TRX-GRA2 fusion protein of several colonies under the control of isopropyl-B-D-Thiogalactopyranoside (IPTG)-inducible promoter was tested on an analytical scale (1 ml of induced culture) by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE). A single fresh colony was selected for inoculation of 20 ml LB and incubated over night at 37°C, 200 rpm. Cells were diluted into 1:50 and allowed to grow at 37°C until the OD_{600} reached 0.4-0.5. For induction, 1 mM IPTG was added and cells were harvested 3h later by centrifugation at 6000×g for 10 min.

Fractionation of soluble and insoluble proteins: Pellet of 3 ml induced culture of the recombinant pETGRA2 clone was resuspended in 300 µl of lysis buffer (20 mM Tris-HCl, pH 9.0, 0.5 M NaCl, 0.1% Triton X- 100, 1 mg/ml lysozyme, protease inhibitor cocktail without EDTA) and incubated at 4 for 30 min. The cells were lysed with gentle vortexing, and then insoluble proteins and cell debri were pelleted by centrifugation at 16000×g for 12 min. The pellet and supernatant and also crude lysate before fractionation were analyzed by SDS-PAGE (Laemmli, 1970)

Purification of recombinant TRX-GRA2: Cells from 500 ml induced culture were resuspended in 8 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris- HCl, 0.1% Triton X-100, pH 9.0 containing protease inhibitor cocktail without EDTA) and broken by sonication.

After centrifugation (16000×g for 20 min at 4°C), the supernatant was incubated with 2 ml Ni-NTA agarose resin for 1h (shaking at 4°C and 100 rpm) and loaded onto a column. The column was washed three times with 5 ml washing buffer 1 (binding buffer, containing 40 mM imidazole and 0.01% Triton X-100) and three times with washing buffer 2 (binding buffer, containing 80 mM imidazole and 0.01% Triton X-100) and eluted with 4 ml elution buffer (binding buffer, containing 0.5 M imidazole and 0.01% Triton X-100). The eluted fractions were dialyzed against phosphate-buffered saline (PBS pH 7.4) buffer (1% w/v NaCl, 0.075% w/v KCl, 0.014% w/v $NaHPO_4$, and 0.0125 w/v $KH2PO_4$).

GEL electrophoresis and western blotting: Protein samples were analyzed by SDS-PAGE on 13% acrylamide gel and Blotting was carried out at 90 V for 1h using a transfer system (Bio Rad, Hercules, CA) according to the conditions of the supplier. Nitrocellulose membranes were saturated for 1h with 5% fat-free milk in PBS followed by sequential incubation with the anti-GRA2 monoclonal antibody Tg 17-179 (Charif *et al.,* 1990) and peroxidase-conjugated goat anti-mouse secondary antibody (Jackson immunoresearch laboratories, West Grove, PA) (both 1/20000 dilution). Signals were detected using super signal ECL system (Pierce chemical, Rockford).

ELISA: TRX-GRA2 and *T. gondii* lysate (TLA) were diluted to the optimized concentration of 5 µg/ml and 10 µg/ml, respectively, in bicarbonate buffer (PH 9.6) and microtiter plate was coated using 100 ml of each diluted antigen. Control wells were coated with TRX protein (5 µg/ml) purified from *E. coli* BL21 (DE3) pLysS transformed with pET-32a plasmid using the same protocol as for TRX-GRA2. Coated plate was stored overnight at 4°C then washed three times with PBS-Tween 20 and blocked for 2 h at 37°C with blocking buffer (1% BSA and 0.05% Tween 20 in PBS, pH 7.4). Sera from 5 CBA/J mice infected with PRU strain of *T. gondii*, 5 naive CBA/J mice and 3 *Leishmania major* (*L. major*) infected BALB/c mice were pooled and diluted 1 to 10000 with blocking buffer.

After adding 100 µl of diluted sera to each well, the plate was incubated for 1h at 37°C and washed three times with PBS-Tween 20. Bound mouse IgG was detected using horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma, Germany) diluted 1:3000 with blocking buffer and incubated for 2h at 37°C. The plate then washed three times with PBS-Tween 20. The binding of conjugate was visualized with 0-phenylendiamine dihydrochloride (OPD, Sigma), prepared according to the manufacturer's instructions. After 20 min at room temperature in dark, the color development reaction was stopped by adding 100 µl of 1 M sulfuric acid and the color intensity was read in a microtiter plate reader (organon) at 490 nm.

RESULTS

Construction of the recombinant expression plasmid: GRA2 cDNA without the signal sequence was amplified from a pBluescript-GRA2 plasmid which contains full length GRA2 cDNA. To allow directional cloning of GRA2 into pET-32a plasmid, *Nco*I and *Bam*HI restriction sites were introduced at the ends of amplified gene by means of PCR amplification. Specific PCR product of 500 bp was viewed on 1% agarose gel (Fig. 1). The band was cut and PCR product was purified. Restriction analysis using *Rsa* I enzyme was performed on the PCR product and confirmed it (data not shown). GRA2 PCR product and pET-32a plasmid were cut with *Nco*I/ *Bam*HI restriction enzymes and GRA2 was cloned at 3´end of TRX coding region. PET-GRA2 construct was sequenced and the result confirmed the correct sequence of recombinant GRA2 gene.

Expression and purification of recombinant GRA2 in *E. coli:* The pET-GRA2 construct was transformed into *E. coli* BL21(DE3) pLysS strain. After induction of the expression by IPTG, bacteria were lysed and cell extract was analyzed on SDS-PAGE (Fig. 2). The lysate was also subjected to centrifugation to analyze

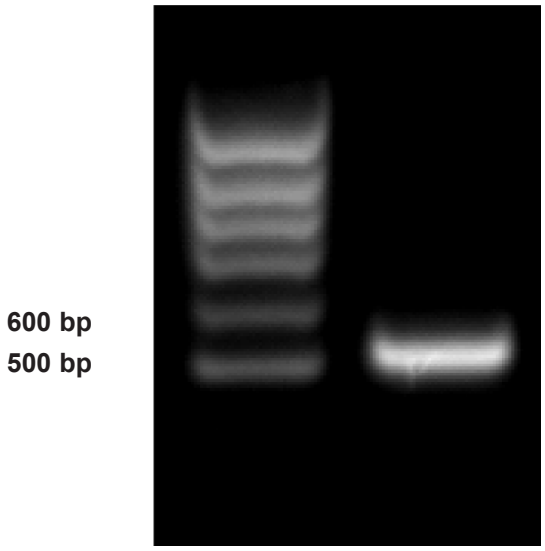

Figure 1. PCR amplification of GRA2 cDNA from a pBluescript-GRA2 plasmid.

the solubility of recombinant TRX-GRA2. It was shown that the main part of TRX-GRA2 is in soluble form as well as there is considerable amount in insoluble form (Fig. 3). Soluble TRX-GRA2 was purified by Ni–NTA chromatography. The recombinant fusion protein required detergent to remain soluble (0.01% Triton X-100), otherwise decreasing the concentration of the detergent caused the precipitation of purified TRX-GRA2.

Western blot analysis: Purified TRX-GRA2 (about 50 kDa) was visualized by Coomassie blue staining as a major band and some smaller weak bands, which probably resulted from protease degradation (Fig. 4A). The single band and smaller bands were also detected by Western blot analysis using monoclonal antibody anti-GRA2 Tg 17-179 (Fig. 4B).

Sera reactivity toward GRA2 using *T. gondii* infected mice sera: To evaluate the seroreactivity due to TRX-GRA2, pooled sera from CBA/J mice infected with *T. gondii* and naive CBA/J mice were obtained. As a control, sera from 3 *Leishmania major* (*L. major*) infected BALB/c mice (an irrelevant disease) were used in parallel. Since the expressed GRA2 is fused with TRX, we tested sera reactivity to TRX as well as to TLA. As it was shown in figure 5, Panel A, the infected mice had high level of total IgG against TLA in compare to naïve mice. In addition, there was high and significant level of total IgG against TRX-GRA2 in compare to naïve mice. This reactivity was antigen specific since there was no specific absorbance when TRX, the fused part, was used as coating antigen or when seroreactivity of TRX-GRA2 against sera of *L. major* infected mice was examined.

To see if the immune response against TRX-GRA2 in the natural course of infection is toward T-helper1 (Th1) or T-helper2 (Th2) immune response, the level

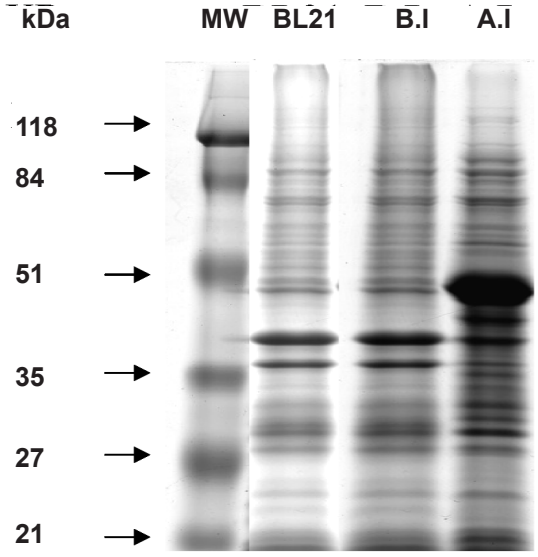

Figure 2. Expression of TRX-GRA2 in *Escherchia coli.* pET-GRA2 construct was transformed into *E. coli* BL21 (DE3) pLysS and expressed as described under Materials and Methods. Aliquots of BL21 cells, non-induced cells (B.I) and induced cells (A.I) were analysed by SDS-PAGE on 13% polyacrilamide gels followed by Commassie blue staining. MW; Molecular Weight maker.

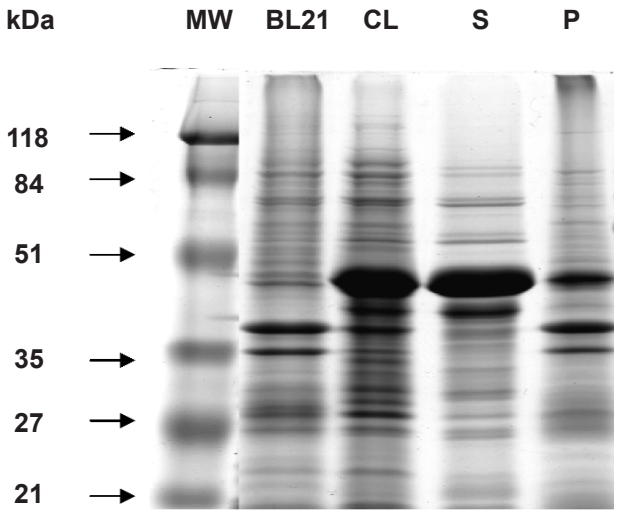

Figure 3. Evaluation of solubility of recombinant TRX-GRA2 expressed in *E. coli*. Induced cells were lysed and aliquots of crude lysate, solubale and insoluble (pellet) fractions were analysed by SDS-PAGE. Proteins were detected by Commassie blue staining. CL: crude lysate., S:solubale., P: pellet., TRX: thioredoxin. MW; Molecular Weight maker.

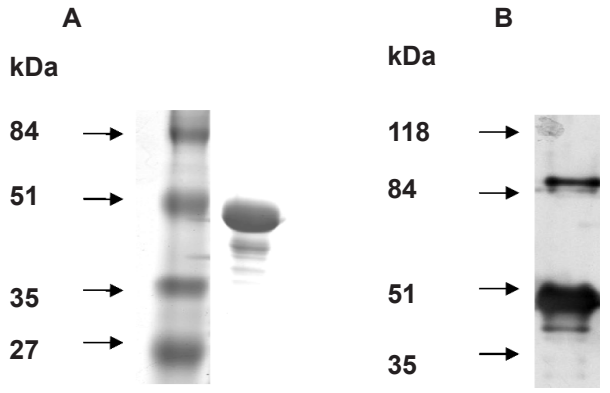

Figure 4. Analysis of purified recombinant TRX-GRA2. A: TRX-GRA2 was purified by Ni-NTA chromatogeraphy was analyzed by SDS-PAGE and detected using Commassie blue staining. B: Western blot analysis of purified TRX-GRA2 protein. Membrane was probed with GRA2-specific monoclonal antibody Tg 17-179 and developed with horseradish peroxidase-conjugated goat anti-mouse antibody. Signals were detected using super signal ECL (Enhanced Chemiluminescence) system.

of specific IgG2a and IgG1 antibodies against TRX-GRA2 in sera of infected mice were measured. As controls, the level of IgG1 and IgG2a antibodies against TLA and TRX were also tested. As it was indicated in figure 5, Panel B and Panel C the type of IgG antibody response against GRA2 and TLA in the infected mice was of IgG2a isotype and there was no specific IgG1 production.

DISCUSSION

GRA2, a *T. gondii* dense granule protein displays immunological properties making it a suitable vaccine candidate. This antigen is recognized by specific human T-cell clones (Prigione *et al*., 2000 and Pistoia *et al*., 1996) and anti-GRA2 IgG antibodies have been detected in most of the infected people (Murray *et al*., 1993).

Expression of GRA2 in *E. coli* is associated with some difficulties since the protein has hydrophobic nature and is prone to protease degradation (Murray *et al*., 1993; Parmley *et al*., 1993). Efforts on the expression of soluble form of full-length GRA2 without fusion or as a fusion with GST resulted in a very low expression level, which was probably due to hydrophobic nature of the protein, the presence of highly hydrophobic signal peptide and sensitivity to bacterial protease function (Murray *et al*., 1993 and Parmley *et al*., 1993). To increase the expression level

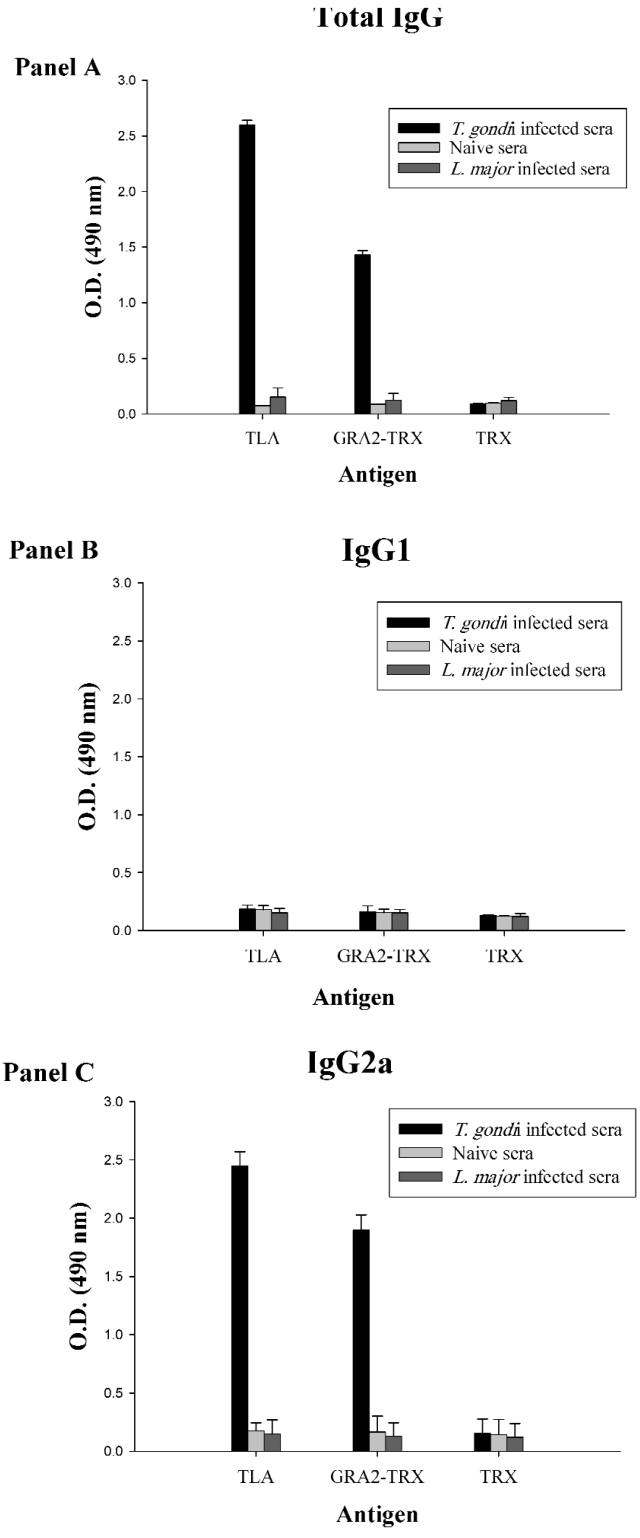

Figure 5. Specific total IgG (Panel A), IgG1 (Panel B) and IgG2a (Panel C) against TLA, TRX-GRA2 and TRX antigens were detected in sera from *T. gondii* infected CBA/J mice (n = 5), naïve CBA/J mice (n = 5) and *L. major* infected BALB/c mice (n = 3) through ELISA. Data in each group of mice represented as mean of OD_{490} ± S.D.

in *E. coli*, GRA2 cDNA was expressed in fusion with TRX, an *E. coli* protein that is well expressed and increase the solubility of protein of interest (Terpe, 2003 and Ford *et al*., 1991), in absence of signal peptide.

Fusion of TRX to the N-terminus of GRA2 resulted to a high-level expression of GRA2 fragment which was soluble as most of the fusion proteins with TRX described in literature (Martinez *et al*., 1995; Terpe, 2003; Murby *et al*., 1991; La Vallie *et al*., 1993 and Yang *et al*., 2004).

The presence of Histidine tag at the ends of TRX-GRA2 enabled single step purification on Ni-NTA chromatography and purification resulted in a single band of about 50 kDa. There was also a protein band with approximate size of 100 kDa in the blot of purified TRX-GRA2 (Fig. 4B) which is probably related to dimerization of GRA2 protein as it has been suggested by other studies (Golkar *et al*., 2005; Mercier *et al*., 1993 and Parmley *et al*., 1993).

As a first step for evaluating the antigenicity and seroreactivity of TRX-GRA2 recombinant protein, pooled sera from inbred CBA/J mice infected with *T. gondii* was used.

The results of ELISA experiments showed recombinant TRX-GRA2 was recognized by the infected sera (Fig. 5 Panel A). The possibility that the observed seroreactivity was because of TRX moiety was rejected in a control ELISA experiment. TRX antigen was used as coating antigen and recognition of TRX by sera of infected and naive mice were tested and the results showed no specific reaction. To further confirm the specific reaction of TRX-GRA2 and its potential as a diagnostic tool in infected individuals, seroreactivity assay was performed against sera of *L. major* infected mice (as an unrelated infection) and the results showed no specific reaction.

A proper candidate vaccine against intracellular parasites such as *T. gondii* should be able to induce immune response of Th1 type (Bout *et al*., 2002 and Angus *et al*., 2000). Knowing the type of induced immune response against a specific antigen in the course of infection gives some information about the immunogenic nature of the protein and could help to design a proper vaccine. To see if immune response against GRA2 in *T. gondii* infected mice was of Th2 or Th1 type, IgG1 and IgG2a isotypes were measured. The results (Fig. 5, Panel B and Panel C) showed that in the course of infection, GRA2 merely induces the production of IgG2a, a marker of Th1 response that is necessary to control *T. gondii* infection. The next step would be to examine diagnostic potential of TRX-GRA2 in infected humans and also to evaluate its immunogenicity and protectivity in animal models.

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