HETEROLOGOUS EXPRESSION OF *TOXOPLASMA GONDII* DENSE GRANULE PROTEIN 2 AND 5

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Abstract. Toxoplasmosis is a foodborne disease caused by *Toxoplasma gondii*, an obligate intracellular parasite. The parasite remains protected within a parasitophorous vacuole (PV), a specialized compartment formed within the infected host cell during and after invasion. Dense granules (GRA) are *T. gondii* specialized secretory organelles involved in PV development. GRA2 contributes to the formation of intravacuolar network in the PV, allowing nutrients transportation to nourish the parasites. GRA5 helps to inhibit apoptosis of the infected cells thereby protecting the parasites. As such, these two essential antigens have been selected as the target subjects. Heterologous expression in *E. coli* BL21 pLysS (DE3) of *GRA2* and *GRA5* fragment was achieved by transfecting with recombinant expression GRA2- and GRA5-pRSET B plasmid, respectively. His-tagged recombinant proteins were affinity purified using a Nickel-nitrilotriacetic acid column. The identities of recombinant rGRA2 (30 kDa) and 5 (20 kDa) proteins were confirmed by western blotting using immune serum from a patient with toxoplasmosis and by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. The purified *T. gondii* antigens provide candidates for future development of diagnostic kits of human infection as well as vaccines.

Keywords: *Toxoplasma gondii*, GRA2, GRA5, heterologous expression, western blot

INTRODUCTION

Toxoplasmosis is a parasitic disease caused by *Toxoplasma gondii*, which belongs to Phylum Apicomplexa (Igarashi *et al*, 2008). The infection is globally distributed affecting up to one-third of the world's human population (Tenter *et al*, 2000). Infection of *T. gondii* involves the transmission within and between hosts by zoites (Mercier *et al*, 2005). There are three infectious stages of the parasite, namely, tachyzoite, bradyzoite and sporozoite (Dubey *et al*, 1998). Tachyzoite is free living and actively-multiplies in various vertebrate cell types, and readily transform into bradyzoite, a slower-growing stage brought upon by extracellular factors, such as extreme pH changes and presence of cytokines (Lyons *et al*, 2002; Robert-Gangneux and Darde, 2012). Bradyzoites are encysted in the tissues, especially brain and muscle. Sporozoites are protected within the mature oocyts from harsh environment due to the robust multilayer structure of its wall. These three infectious forms are all crescent in shape, varying in length from 4 to 6 μm and from 2 to 3 μm in width with a pointed anterior and a spherical posterior end (Robert-Gangneux and Darde, 2012). They also share comparable ultrastructure

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but with some differences in certain organelles and inclusion bodies (Dubey *et al*, 1998). Humans become infected with this parasite through congenital transmission, consumption of raw or undercooked meat contaminated with *T. gondii* tissue cysts, and consumption of water contaminated with sporulated oocysts from infected cat feces (Dubey, 1996).

T. gondii infection in immuno-competent individuals is usually asymptomatic but becomes an opportunistic disease in immuno-suppressed patient resulting in multisystem organ failure (Ismael *et al*, 2003). Acute or re-activation of chronic toxoplasmosis infection in HIV-AIDS patients can cause toxoplasmic encephalitis, which is lethal if not being treated at the early stages of infection (Mamidi *et al*, 2002). Congenital toxoplasmosis due to transplacental transmission can lead to miscarriage (Gagne, 2001).

T. gondii infection in livestock, especially sheep, imposes a danger from both medical and economic aspects. Infected livestock harboring parasite tissue cysts can transmit the parasite to humans through consumption of undercooked meat. In addition, the meat industry is affected by abortions in infected domestic animals resulting from toxoplasmosis, causing economic loss (Buxton, 1993). Therefore, proper diagnosis (serology tests) and prevention (vaccine) of the disease are crucial. However, for vaccine development the use of antigens originating from tachyzoites is inappropriate due to several factors. Firstly, it is time-consuming to propagate the parasites *in vitro* continuously to obtain large amounts for extraction of total lysate antigens. Secondly, it is hazardous to humans as the preparation involves live parasites, and lastly it requires high cost of production as cell cultures are involved.

In order to overcome these problems, recourse to recombinant DNA technology plays an important role in allowing production of larger quantities of recombinant antigenic proteins for both serodiagnosis and vaccination study of toxoplasmosis in a safer manner with lower production cost (Jana and Deb, 2005). There exists various molecular cloning vectors for heterologous protein production through either prokaryotic or eukaryotic expression systems (Fuerst *et al*, 1986). *Escherichia coli* has been one of the top choices of heterologous protein expression systems among available host cell systems due to its many advantages, such as its fast growth (Yin *et al*, 2007), attractive economical cost of protein production as well as the ease of manipulation of the expression system (Baneyx, 1999; Hewitt and McDonnell, 2004).

Dense granules (GRA) are the major components of vacuoles surrounding both tachyzoites and encysted bradyzoites (Capron and Dessaint, 1988; Cesbron-Delauw and Capron, 1993). They make up most of the circulating antigens in the blood stream of infected hosts, and on account of their high immunogenicity (Mercier *et al*, 2007) can be detected as early as a few hours post-infection (Hughes and van Knapen, 1982). GRA proteins are also found during the chronic stage of *T. gondii* infection (Mercier *et al*, 2007). A total of 12 different GRA proteins (GRA1-10, GRA12 and GRA14) ranging in size from 21 to 41 kDa have been identified (Cesbron-Delauw, 1994; Ahn *et al*, 2005; Mercier *et al*, 2005; Michelin *et al*, 2008; Rome *et al*, 2008) and have been considered as potential vaccine (Scorza *et al*, 2003; Hiszczynska-Sawicka *et al*, 2011; Sun *et al*, 2011) and diagnosis candidates (Redlich and Muller, 1998; Jacobs *et al*, 1999; Golkar *et al*, 2007a).

GRA2 is important to ensure and maintain survival of the parasite after host cell invasion as it is involved in the formation of intravacuolar network (Labruyere *et al*, 1999; Nam, 2009) in the parasitophorous vacuole (PV), which allows transport into the parasite of proteins and nutrients from the invaded host cell (Nam, 2009). GRA5 helps to inhibit apoptosis of the infected host cell by regulating intracellular calcium concentration and thereby protecting the parasite (Feng *et al*, 2002; Ahn *et al*, 2006). In this study we describe the production of recombinant GRA2 and GRA5 (rGRA2 and rGRA5) in *E. coli* for future studies of their immunogenicity.

MATERIALS AND METHODS

Parasite

T. gondii tachyzoites (RH strain) were maintained by serial intraperitoneal passage in BALB/c mice and harvested from peritoneal fluid after 3-4 days of infection. Tachyzoites were washed and resuspended in sterile phosphate-buffered saline (PBS) prior to further use.

Construction of recombinant plasmids

Nucleotide (nt) sequence of *T. gondii GRA2* (corresponding to nt 497-1223) encoding GRA2 was obtained from Genbank (accession no. L01753.1). RNA was extracted from the tachyzoites using TRI reagent (MRC Medical Research Center, Cincinnati, OH) according to the manufacturer's protocol. The extracted RNA was used as template for amplification of *GRA2* using One-step RT-PCR kit (Qiagen, Hilden, Germany) with sense primer GRA2F (5'-GAATTCGCCGAGTTTTCCGGAGTT-3') and antisense primer GRA2R (5'-GAATTCCTGCGAAAAGTCTGGGAC-3'). The primers set contained introduced *Eco*RI restriction site (underlined) to facilitate cloning. RT-PCR amplification was performed in a 50-µl reaction mixture containing 1 µg RNA template, OneStep RT-PCR Enzyme mix, 1X RT-PCR buffer, 400 µM dNTPs and 0.6 µM primers. RT-PCR was initiated with reverse-transcription reaction at 50ºC for 32 minutes, followed by 95ºC for 15 minutes, 37 cycles of 94ºC for 30 seconds, 55-60ºC for 1 minute, and 72ºC for 1 minute and eventually ended with a final heating at 72ºC for 10 minutes. *GRA5* sequence (corresponding to nt 76-360) was obtained from Genbank (accession no. EU918733.1). DNA was isolated from the tachyzoites using DNeasy® Blood and Tissue kit according to the manufacturer's protocol. The extracted DNA was used as template for PCR amplification using forward primer (5'-GCGGAATTCGGTTCAACGCGTGAC-3') and reverse primer (5'-GACGAATTCCTCTTCCTCGGCAACTTC-3'). The primers set contained introduced *Eco*RI restriction site (underlined) to facilitate cloning. PCR amplification was performed in a 20-µl reaction mixture containing 1 µg DNA template, 2.5 U *i*-Taq™ DNA polymerase, 1X PCR buffer, 250 µM dNTPs and 0.5 µM primers. PCR thermocycling conditions were as follows: 95ºC for 10 minutes; 35 cycles of 94ºC for 45 seconds, 55-60ºC for 30 seconds, and 72ºC for 1 minute; with a final step of 72ºC for 10 minutes.

PCR products were purified using QIAquick® Gel Extraction kit according to the manufacturer's protocol before being inserted into the expression vector, pRSET B (Invitrogen, Carlsbad, CA) at the *Eco*RI restriction site. The resulting recombinant pRSET B constructs permitted expression of N-terminally polyhistidine (His)-tagged rGRA2 (amino acid residues 24-185) and rGRA5 (amino acid residues 26-120), each lacking its putative N-terminal signal sequence. GRA2- and

GRA5-pRSET B constructs as well as control pRSET B plasmid were transfected into *E. coli* BL21 (DE3) pLysS by heat shock method (Nishimura *et al*, 1990). The positive recombinant clones were cultured in 100 μg/ml ampicillin-supplemented Luria Bertani broth for plasmids extraction by QIAprep® Spin Miniprep Kit. The extracted plasmids were sequenced (Bioneer Corporation, Daejeon, South Korea) to verify the orientation and integrity of both genes based on the deposited sequences in GenBank database (data not shown).

Heterologous protein expression in *E. coli*

Optimal conditions for recombinant expression of rGRA2 and rGRA5 in *E. coli* were determined prior to scaling up the production protocol. Two main parameters needed to be optimized were i) period of induction and ii) concentration of the inducer, isopropyl β-D-thiogalactopyranoside (IPTG; Invitrogen, Carlsbad, CA). A single GRA2-pRSET B or GRA5-pRSET B transformed colony was inoculated into 5 ml of Luria Bertani (LB) broth supplemented with ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml), grown overnight at 37°C with shaking, then diluted with LB broth to yield an $OD_{600\ nm}$ of 0.1. Aliquots of 10 ml were grown as described above until OD_{600} of 0.4 - 0.5, then 1mM IPTG was added to each culture and incubated further. At 0, 2, 3, 4 and 5 hours, cells were harvested by sedimenting at 5,000*g* for 10 minutes before assessing protein expression using 12% SDS-PAGE. The above procedure was repeated but the cultures were induced with 0.1, 0.5, and 1.0 mM IPTG and incubated for the optimum time.

Purifications of rGRA2 and rGRA5

Cells were harvested from 200 ml of culture optimized for heterologous expression of rGRA2 or rGRA5, lyzed and lysate purified using Probond™ Purification System (Invitrogen, Carlsbad, CA) equipped with a Nickel-nitrilotriacetic acid (Ni-NTA; Qiagen, Hilden, Germany) column as follows.

In brief, for purification of rGRA2 cell pellet was suspended in 8 ml of native binding buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl and 10 mM imidazole), then 8 mg of lysozyme were added and the solution was incubated on ice for 30 minutes prior to sonication on ice using Branson Sonifier® ultrasonic cell disruptor (Branson Ultrasonics, Danbury, CT) at 80% amplitude for 1 minute, 6 times at 10 seconds intervals. Cell lysate was centrifuged at 3,000*g* for 15 minutes and supernatant loaded onto the Ni-NTA column, which then was washed four times with 8 ml of native wash buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl and 20 mM imidazole) and rGRA2 was eluted with 5 ml of native elution buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl and 250 mM imidazole).

For purification of rGRA5, the cell pellet was suspended in 8 ml of guanidine lysis buffer (20 mM sodium phosphate pH 7.8, 6 M guanidine hydrochloride and 500 mM NaCl), incubated for 5 to 10 minutes at room temperature, followed by sonication on ice using Branson Sonifier® ultrasonic cell disruptor at 80% amplitude for 15 seconds, 3 times at 5 seconds intervals. Cell lysate was centrifuged as described above and supernatant loaded onto Ni-NTA column, which was washed sequentially with 4 ml of denaturing binding buffer (20 mM sodium phosphate pH 7.8, 500 mM NaCl and 8 M urea) (two times), followed by 4 ml of denaturing wash buffer (20 mM sodium phosphate pH 6.0, 500 mM NaCl and 8 M urea) (two times), then with 4 ml of denaturing wash buffer (pH 5.3) (two times). rGRA5 was eluted with 5 ml of denaturing elution

buffer (20 mM sodium phosphate pH 4.0, 500 mM NaCl and 8 M urea) and then dialyzed against phosphate-buffered saline (PBS) overnight at 4ºC.

BL21 pLysS (DE3) carrying the empty pRSET B vector was used as a negative control for both protein expression and purification. Protein concentration was measured using Bradford Assay kit (Bio-Rad, Hercules, CA).

Western blot analysis

Purified recombinant proteins were separated by 12% SDS-PAGE and transferred onto a methanol-activated polyvinylidene difluoride (PVDF; Bio-Rad, Hercules, CA) membrane. The membrane was incubated with blocking solution [5% non-fat skim milk in Tris-buffered saline (TBS) for 2 hours at room temperature and subsequently with human toxoplasmosis immune serum sample (1:200 dilution)] [kindly provided by Diagnostic Laboratory (Para: SEAD), Department of Parasitology, University of Malaya and University Malaya Medical Centre] for 2 hours, followed with secondary biotinylated goat anti-human IgG antibodies (KPL, Gaithersburg, MD; 1:2,500) for 1 hour, and lastly with streptavidin conjugated-alkaline phosphatase (KPL, Gaithersburg, MD) (1:2,500) at room temperature for 1 hour. Immunoreactive band was detected using 5-bromo-4-chloro-3-indolyphosphate/nitro blue tetrazolium (BCIP/NBT; Sigma, St Louis, MO).

Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) analysis

Affinity purified recombinant proteins separated by 12% SDS-PAGE were stained with 0.25% Coomassie brilliant blue R-250 dye (Bio-Rad, Hercules, CA) for 2 hours and then incubated de-stained (7% acetic acid and 5% methanol solution) overnight at room temperature. The recombinant protein band of interest (based on size) was excised from the gel and further de-stained several times with 50 µl of 50% acetonitrile (ACN) in 50 mM NH_4HC0_3 until the excised gel was completely de-stained. The gel was incubated with 150 µl of 10 mM dithiothreitol (DTT) in 100 mM NH_4HC0_3 for 30 minutes at 60ºC, cooled to room temperature, and incubated with 150 µl of 55 mM iodoacetamide (IAA) in 100 mM NH_4HC0_3 in the dark for 20 minutes. Gel then was washed four times with 500 µl of 50% ACN in 50 mM NH_4HC0_3, dehydrated by incubating with 50 µl of 100% ACN for 15 minutes, and subjected to speed vacuum using Maxi-Dry Lyo Centrifugal Vacuum Concentrator/Freeze Dryer (Heto, Rockford, IL) for 15 minutes at room temperature. Gel was incubated with 25 µl of trypsin (6 ng/µl) in 50 mM NH_4HC0_3 at 37ºC overnight, then incubated with 50 µl of 50% ACN for 15 minutes to terminate trypsin digestion. The solution was transferred into a new tube (Tube A), and the gel was further incubated with 50 µl of 100% ACN for 15 minutes and this solution was added to Tube A. Tube A was dried using speed vacuum procedure. The digested protein sample was reconstituted in 10 µl of 0.1% formic acid and desalted using a Zip-Tip (Millipore, Billerica, MA) equilibrated with 50% ACN and 0.1% formic acid. The protein sample bound onto the Zip-Tip membrane was washed with 0.1% formic acid, eluted with 10 µl of 0.1% formic acid in 50% ACN and analyzed by MALDI-TOF MS (University of Malaya Center for Proteomics Research).

RESULTS

Optimization of recombinant proteins expressions in *E. coli*

RT-PCR amplification of *T. gondii*

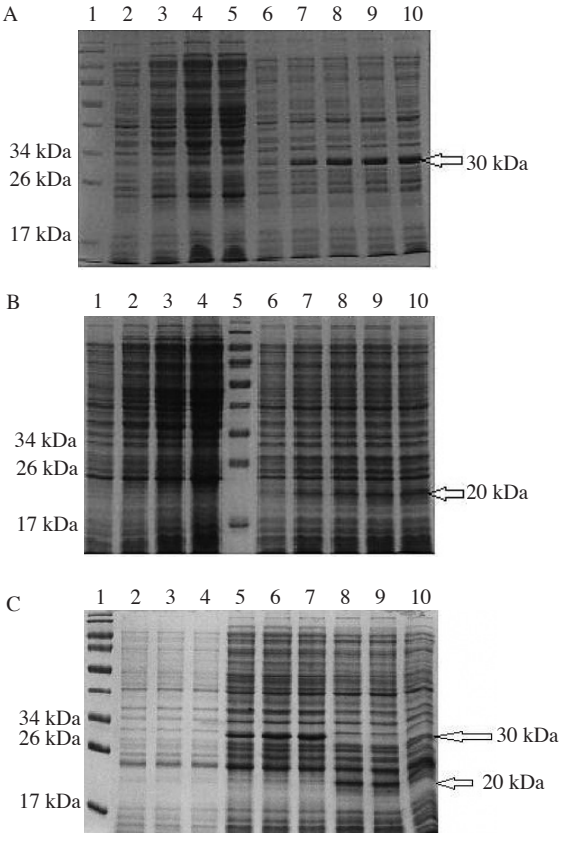


Fig 1–SDS-PAGE analysis of *T. gondii* rGRA2 and rGRA5 heterologous expressions in *E. coli* BL21 pLysS (DE3) optimization conditions. Cells transformed with recombinant expression GRA2-pRSET B and GRA5-pRSET B plasmids were incubated with 1.0 mM IPTG for various times or for optimum period with various IPTG concentrations, then lyzed and lysate subjected to SDS-12% SDS-PAGE followed by Coomassie blue staining. (A) Lane 1, pre-stained broad range protein markers (Fermentas, USA); lanes 2-5, lysate from pRSET B-transfected cells (negative control) at 0, 3, 4, 5 hours; lanes 6-10, lysate from GRA2-pRSET B-transfected cells at 0, 2, 3, 4, 5 hours. (B) Lanes 1-4, lysate from pRSET B-transfected cells (negative control) at 0, 3, 4, 5 hours; lane 5, pre-stained broad range protein markers (Fermentas, USA); lanes 6-10, lysate from GRA5-pRSET B-transfected cells at 0, 2, 3, 4, 5 hours. (C) Lane 1, pre-stained broad range protein markers (Fermentas, USA); lanes 2-4, lysate from pRSET B-transfected cells (negative control) incubated for 2 hours with 0.1, 0.5, 1.0 mM IPTG; lanes 5-7, lysate from GRA2-pRSET B-transfected cells incubated for 5 hours with 0.1, 0.5, 1.0 mM IPTG; lanes 8-10, lysate from GRA5-pRSET B-transfected cells incubated for 2 hours with 1.0, 0.5, 0.1 mM IPTG.

GRA2 and PCR amplification of *GRA5* fragment yielded a 486 and 285 bp product, respectively (data not shown). The PCR products then were cloned into expression vector pRSET B to generate recombinant GRA2-pRSET B and GRA5-pRSET B, whose insert was 99%-100% and 100% identical with published *GRA2* and *GRA5* sequence, respectively (data not shown).

Heterologous expression of recombinant proteins was optimized for duration of induction and concentration of the inducer IPTG as assessed by SDS-PAGE. Optimal rGRA2 (30 kDa) and rGRA5 (20 kDa) expression was achieved using 1.0 mM IPTG for 5 hours and 1.0 mM IPTG for 2 hours, respectively (Fig 1).

Purification and identification of rGRA2 and rGRA5

Following scaled-up (200 ml) heterologous expression rGRA2 and rGRA5 at optimal conditions, the recombinant proteins were purified using Ni-NTA affinity chromatography, producing a yield of 0.2-0.4 mg/ml, and were analyzed by SDS-PAGE, western blotting and MALDI-TOF MS. SDS-PAGE showed that affinity chromatography yielded 95% pure preparations of rGRA2

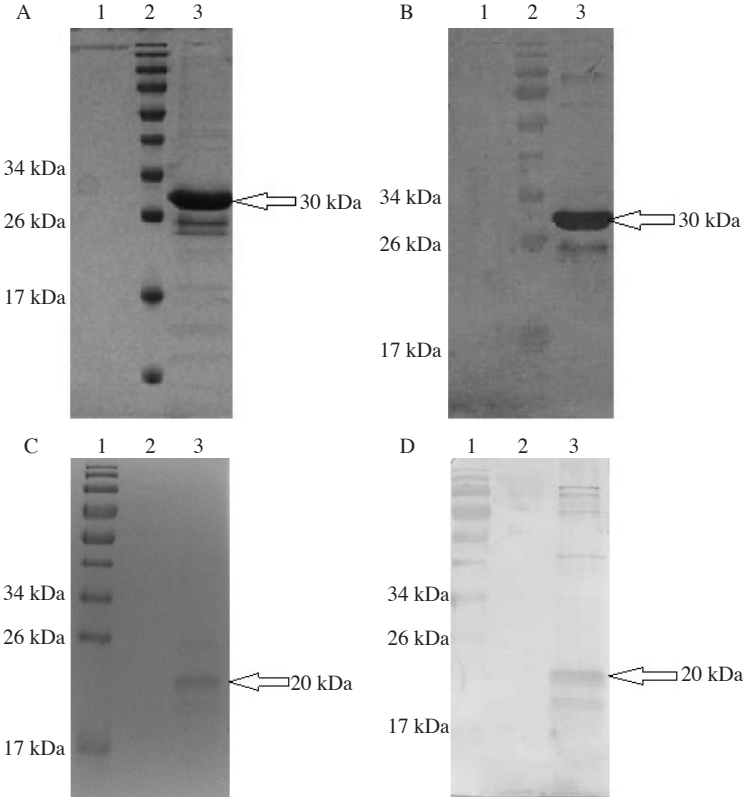


Fig 2–SDS-PAGE and western blot analysis of purified *T. gondii* rGRA2 and rGRA5. Under optimal heterologous expression conditions rGRA2 and rGRA5 were purified using Ni-NTA affinity chromatography. A) 12% SDS-PAGE of rGRA2. Lane 1, negative control; lane 2, pre-stained broad range protein markers (Fermentas, USA); lane 3, affinity purified rGRA2. B) Western blot using immune serum from patient with toxoplasmosis. Lane 1, negative control; lane 2, pre-stained broad range protein markers (Fermentas, USA); lane 3, affinity purified rGRA5. C) 12% SDS-PAGE of rGRA5. Lane 1, negative control; lane 2, pre-stained broad range protein markers (Fermentas, USA); lane 3, affinity purified rGRA5. D) Western blot using immune serum from patient with toxoplasmosis.

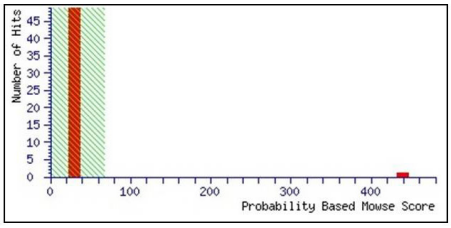
and rGRA5 (Fig 2A and 2C), which could be detected by western blot analysis using immune serum from a *Toxoplasma*-infected patient (Fig 2B and D), although there were minor cross-reactive bands. Furthermore, analysis by MALDI-TOF MS of the tryptic peptides from 20 and 30 kDa bands revealed by the Mascot search results for highest protein scores that the digested peptide masses of these two targets possessed highest match with the amino acid sequences within protein GRA5 and GRA2, respectively ($p < 0.05$) (Fig 3A and 3B).

DISCUSSION

As *T. gondii GRA2* contains a single intron (Mercier *et al*, 1993), it was necessary to amplify the gene from total RNA employing RT-PCR, whereas this was not required in the case of *GRA5*. Both amplified gene fragments were successfully inserted into expression vector, pRSET B harboring T7 promoter. It was important to ensure that *GRA2* and *GRA5* had been inserted into pRSET B in the correct orientation as only a single restriction site (*Eco*RI) was used. This was confirmed by sequencing the resultant recombinant plasmids to be used for subsequent heterologous expression in *E. coli* BL21 (DE3) pLysS.

Recombinant GRA2 was expressed as a soluble protein in the host cytosol allowing standard affinity purification procedures to be employed. However, rGRA5 was produced as inclusion bodies, which required inclusion of a denaturing agent (guanidine hydrochloride) in the buffer to facilitate the solubilization pro-

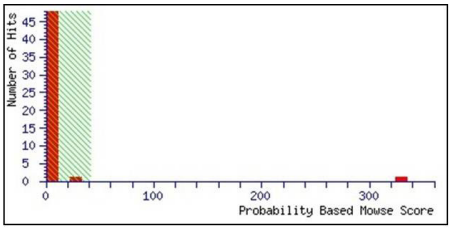
A)



Protein scores >67 are significant (p<0.05)

	Accession	Mass	Score	Description
1.	GRA2_TOXGO	19830	439	Dense granule protein 2 precursor (Protein GRA 2) (28 kDa antigen) (GP28.5) - Toxoplasma gondii
2.	TR25_HUMAN	20264	43	Thyroid hormone receptor-associated protein 6 (Thyroid hormone receptor-associated protein complex
3.	LUXD1_PHOLU	34679	42	Acyl transferase (EC 2.3.1.-) (ACT) (Myristoyl-ACP-specific thioesterase) (C14ACP-TE) - Photorhabdu
4.	LUXD_PHOLL	34710	42	Acyl transferase (EC 2.3.1.-) (ACT) (Myristoyl-ACP-specific thioesterase) (C14ACP-TE) - Photorhabdu
5.	Y367_HAEIN	33991	42	Hypothetical protein HI0367 - Haemophilus influenzae
6.	Y974_HAEIN	41861	41	Hypothetical protein HI0974 - Haemophilus influenzae
7.	DPOD_SCHPO	123490	41	DNA polymerase delta catalytic subunit (EC 2.7.7.7) (DNA polymerase III) - Schizosaccharomyces pomb
8.	KPYM_RAT	57781	40	Pyruvate kinase isozymes M1/M2 (EC 2.7.1.40) (Pyruvate kinase muscle isozyme) - Rattus norvegicus (
9.	YFX2_RHILE	8860	39	Hypothetical 8.8 kDa protein in fixW 5'region - Rhizobium leguminosarum
10.	DNAJ_BACFN	42161	39	Chaperone protein dnaJ - Bacteroides fragilis (strain ATCC 25285 / NCTC 9343)

B)



Protein scores >67 are significant (p<0.05)

	Accession	Mass	Score	Description
1.	GRA5_TOXGO	12969	329	Dense granule protein 5 precursor (Protein GRA 5) (p21) - Toxoplasma gondii
2.	1433_NEOCA	30631	17	14-3-3 protein homolog - Neospora caninum
3.	YM11_PARTE	43730	16	Hypothetical 43.7 kDa protein (ORF11) - Paramecium tetraurelia
4.	TERT_EUPAE	122484	15	Telomerase reverse transcriptase (EC 2.7.7.49) (Telomerase catalytic subunit) (Telomerase subunit P
5.	MTCD_TETTH	16744	14	Cadmium metallothionein precursor (MT-Cd) (Cd-MT) - Tetrahymena thermophila
6.	KNOB_PLAFD	30405	12	Knob-associated histidine-rich protein (KAHRP) (Fragment) - Plasmodium falciparum (isolate CDC / Ho
7.	SSSP_PLAFO	16616	11	Sexual stage-specific protein precursor - Plasmodium falciparum (isolate NF54)
8.	OS25_PLARE	24068	10	25 kDa ookinete surface antigen precursor (Prs25) - Plasmodium reichenowi
9.	KNOB_PLAFG	69107	10	Knob-associated histidine-rich protein precursor (KAHRP) (KP) - Plasmodium falciparum (isolate FCR-
10.	KNOB_PLAFN	71897	10	Knob-associated histidine-rich protein precursor (KAHRP) - Plasmodium falciparum (isolate NF7 / Gha

Fig 3–Histogram of Mascot search of MALDI-TOF MS results. A) Analysis of MALDI-TOF MS of tryptic peptides from 30 kDa band showing highest protein score of 439 corresponding to *T. gondii* protein GRA2 ($p < 0.05$). B) Analysis of MALDI-TOF MS of tryptic peptides from 20 kDa band showing highest protein score of 329 corresponding to *T. gondii* protein GRA5 ($p < 0.05$).

cess. Elution buffer at low pH (pH 4.0) was used to elute rGRA5 as protonation of the histidine residues (pKa = 6.0) in the 6xHis tag would further help rGRA5 to dissociate from the positively-charged nickel ions attached to the column. Washing and elution buffers contained 8M urea to maintain rGRA5 in a soluble form. The eluted rGRA5 was subjected to dialysis against PBS to renature and refold by removing

denaturant/solubilizing agents.

Heterologous expression and purification of rGRA2 and rGRA5 produced protein of about 30 kDa and 20 kDa, respectively, but the predicted molecular weight is 23 kDa and 16 kDa, respectively. These discrepancies can be attributed to the presence of the 6 His residues in both recombinant proteins and the intrinsic error (±10%) in molecular mass determination by SDS-PAGE. Besides, it is also possible that these differences stem from unique features of GRA, such as the proline composition (Mercier *et al*, 2005). In this study, the deduced proline (P) content in rGRA2 and rGRA5 are found to be 8.6% and 1.1%, respectively. The presence of peptidyl-prolyl cis-trans-isomerase in *E. coli* (Liu and Walsh, 1990) may contribute to the catalysis of proline isomerization during protein-folding activity (Lin *et al*, 1988), which eventually may affected the protein migration leading to the observed differences in protein sizes. The same phenomena were noticed in several other *Toxoplasma* proteins as well, such as GRA3, GRA6 and GRA7 with proline content of 5.9%, 4.8% and 5.5%, respectively (Bermudes *et al*, 1994; Lecordier *et al*, 1995; Jacobs *et al*, 1998).

Production of various *T. gondii* recombinant antigens serve as a major stepping stone towards the evaluation of their efficacies for their potential application in serodiagnosis and as vaccine candidates to substitute the use of *T. gondii* total lysate antigens. Several studies have been carried out on the efficacies of *T. gondii* recombinant antigens for the detection of *T. gondii*-specific antibodies in human sera with toxoplasmosis using ELISA (Golkar *et al*, 2007b; Holec-Gasior *et al*, 2009; Holec-Gasior and Kur, 2010). ELISA technique applied in these approaches is actually a sensitive quantitative assay, but it is less specific due to the higher probability of acquiring false-positive results (Gamble *et al*, 2004; Nockler *et al*, 2009). ELISA is also less informative as we are unable to directly visualize the binding between antibodies in the test samples and their respective specific diagnostic antigens (Anderson *et al*, 2007). Thus, further confirmatory test such as immunoblot assay needs to be included.

Immunogenicity and protective efficacies of several *T. gondii* recombinant antigens produced in bacteria have also been performed against *Toxoplasma* infection in experimental mice models (Martin *et al*, 2004; Golkar *et al*, 2007b; Dziadek *et al*, 2009; 2012). A study reported that alum adjuvant-formulated rGRA4 is a potential vaccine candidate either alone or in combination with rROP2 against chronic *T. gondii* infection (Martin *et al*, 2004). It protects vaccinated C57BL/6 and C3H mice against challenge with *T. gondii* ME49 strain through reduction in brain cyst compared to the control mice. MPL adjuvant-formulated rGRA2 either alone or mixed with rGRA6 also demonstrated protective efficacy against chronic *T. gondii* infection through significant reduction in brain cysts formation in immunized CBA/J mice (Golkar *et al*, 2007b). Vaccination of C3H/HeJ mice with rROP2 and rROP4 were shown to elicit mixed Th1/Th2-type immune response with specific IL-2 production (Dziadek *et al*, 2009). These two antigens confer partial protection against challenge with *T. gondii* DX strain (low virulence) with 46% brain cysts reduction. It was reported that combination of the same rROP2 and rROP4 antigens with either rGRA4 or rSAG1 triggered both humoral (generation of high levels of IgG1 and IgG2a) and cellular- (secretion of IFN-γ and IL-2) immunity, with brain cyst loads in the vaccinated BALB/c mice

decreased by 84% and 77%, respectively compared to PBS-injected mice (Dziadek *et al*, 2012).

A preliminary study has been conducted showing that female rats vaccinated using two purified excretory-secretory antigens (ESA), namely GRA2 and GRA5, are significantly protected against congenital challenge with *T. gondii* regardless of the strain used (Zenner *et al*, 1999). This finding encourages the production of these recombinant proteins in order to overcome the limited quantity of purified ESA. Recombinant GRA2 antigen also is a good marker for acute infection as it has higher sensitivity (90%-100%) towards serum samples from *Toxoplasma*-infected subjects compared to other GRAs recombinant antigens (80%-90%) using ELISA assay (Murray *et al*, 1993; Redlich and Muller, 1998; Jacobs *et al*, 1999; Lecordier *et al*, 2000; Beghetto *et al*, 2003; Ferrandiz *et al*, 2004; Golkar *et al*, 2007a; Holec-Gasior *et al*, 2009). Besides contributing to the virulence of *T. gondii* acute infection in mice (Mercier *et al*, 1998), GRA2 is also highly immunogenic and able to induce specific $CD4^+$ T cells with long term immune response against *T. gondii* in chronically infected humans (Prigione *et al*, 2000). This is supported by another study which demonstrated a significant reduction of brain cysts formation observed in CBA/J mice vaccinated with MPL adjuvant-formulated rGRA2 compared to rGRA6 (Golkar *et al*, 2007b). Although GRA5 does not contribute to the virulence of *T. gondii* acute infection (Mercier *et al*, 2001), it enhances the sensitivity and specificity of antigen cocktails in serodiagnosis (Holec-Gasior and Kur, 2010). As limited studies have been performed on GRA5, therefore, it is advantageous to have the ability to produce and evaluate rGRA2 and rGRA5 to compare with other recombinant GRAs.

In summary, *T. gondii GRA2* and *GRA5* were optimally expressed in *E. coli*, their respective recombinant proteins affinity purified and identities verified by western blotting and MALDI-TOF MS. Immunoreactivity of these recombinant proteins and their efficacy as potential vaccine candidates will be evaluated further.

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