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Cloning and expression of *Toxoplasma gondii* dense granular protein 4 (GRA4) in *Pichia pastoris*

Lau, Y.L.[1]*, Hasan, M.T.[2], Thiruvengadam, G.[2], Idris, M.M.[1] and Init, I.[1]
[1]Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2]School of Science, Monash University Sunway Campus, Bandar Sunway, 46150 Selangor, Malaysia
*Corresponding author email: *lauyeeling@um.edu.my*
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Abstract. GRA4 of *Toxoplasma gondii* has been shown to prompt IgG, IgM and IgA responses in previous studies and is thus considered one of the major immunogenic proteins from *T. gondii* that can be used for both diagnostics purposes and vaccine development. This study seeks to clone and express the *GRA4* in *Pichia pastoris*, which has numerous advantages over other systems for expression of eukaryotic proteins. In order to achieve this, the gene was cloned into the pPICZα A expression vector, which was then incorporated into the *P. pastoris* genome via insertional integration for expression of the recombinant protein, under the *AOX1* promoter. The antigen was expressed along with the prepro sequence of the α-factor of yeast so that it could be excreted out of the *P. pastoris* cells and obtained from the medium. Upon SDS-PAGE analysis it was found that the recombinant protein was expressed optimally as a 40 kDa protein after 96 hours of induction with 0.75% of methanol. The expressed GRA4 protein showed discrepancy in size with the calculated molecular mass. This may be attributed to the various posttranslational modifications including glycosylation and phosphorylation. Despite the difference in molecular weight, the recombinant protein was able to detect toxoplasmosis in Western blot format. The recombinant GRA4 was expressed with an intact polyhistidine-tag, which could be used for future purification of the antigen.

INTRODUCTION

Toxoplasmosis is a parasitic disease caused by the apicomplexan parasite *Toxoplasma gondii*, which can infect a very wide range of intermediate hosts, including all warm-blooded animals and birds, but the primary host is the Felidae family (domestic or wild cats). It is estimated that up to 50% of the world's human population is infected by the disease (Cesbron-Delauw, 1994). An immunocompetent individual may present no symptoms for the disease or mild flu-like symptom during the first few weeks. However, in immunocompromised patients, such as those with AIDS, cancer, neoplastic disease, or transplant recipients, the disease can cause chorioretinitis, encephalitis and neurological diseases, often proving to be fatal (Mévélec *et al.*, 1992; Suzuki & Remington, 1993). Toxoplasmosis contracted during pregnancy can lead to abortion, stillbirth or neonatal malformations. The infection is not limited to humans and affects the agricultural industry worldwide causing abortion in sheep and goats (Mévélec *et al.*, 1992).

Current diagnostic tests against infection are difficult, expensive and sometimes non-specific because the kits employ total cellular lysate antigen (Hofgartner *et al.*, 1997; Wilson *et al.*, 1997). Moreover the methods and source of antigens involve the usage of parasite culture which can prove to be hazardous to laboratory staff handling the samples. Usage of recombinant protein cloned and expressed in suitable expression systems is a potential alternative source of antigens.

For this purpose, several antigens have been identified in the parasite that can bring about induction of the host's immune system. Most of these proteins are specific to a certain stage of the parasite's life cycle, while some are common to various stages of the life cycle.

Surface antigens (SAGs) are the most comprehensively studied proteins, including SAG1 and SAG2 protein families and their relatives. SAG1 has been the most researched of all vaccine candidates for toxoplasmosis (Shang *et al.*, 2009). Beside SAGs, there are three secretory organelles which produce three distinct proteins of immunogenic importance. These are microneme proteins (MICs), rhoptry proteins (ROPs) and dense granular proteins (GRAs) (Smith, 1995). Amongst this is the GRA4 protein, which is involved in nutrition acquirement and trafficking of proteins between the host and the parasite. Escherichia coli derived recombinant GRA4 has been shown to prompt IgG, IgM and IgA responses in previous studies and is thus considered one of the major immunogenic proteins from *T. gondii* that can be used for both diagnostics purposes and vaccine development (Mévélec *et al.*, 1992; Martin *et al.*, 2004; Zhang *et al.*, 2007; Ferraro *et al.*, 2008). Immunization with GRA4 expressing plasmids were shown to give protection to mice against lethal challenges with *T. gondii* by inducing a Th1-type response and releasing IL-2 and large amounts of IFN-γ (Desolme *et al.*, 2000). Moreover, mice vaccinated with GRA4 expressing plasmids induced protective immunity against acute infection by lethal challenge with tissue cysts (Desolme *et al.*, 2000).

However, it is known that recombinant protein produced in *E. coli* often looses its antigenic value due to an incorrect folding (Meek *et al.*, 2003). Thus, this study seeks to clone and express the *GRA4* gene in *Pichia pastoris*, which has numerous advantages over other systems for expression of eukaryotic proteins. The *Pichia* expression system possesses all the post-translational mechanisms which produce recombinant proteins having conformation that is almost similar to the native proteins (Cregg *et al.*, 1993). This yeast has a very high growth rate and expression rate of recombinant proteins, yielding up to 12 g/L of fermentation culture in the case of recombinant tetanus toxin fragment C production (Clare *et al.*, 1991). On the other hand, unlike other eukaryote expression systems, this yeast does not require expensive growth media such as fetal bovine serum. In fact it is capable of growing using methanol as its sole carbon source (Cregg *et al.*, 1993; Kalidas, 2004). This yeast does not produce large amounts of native proteins, so purifying the recombinant protein from the total protein is easy and inexpensive (Kalidas, 2004). The *Pichia* expression system has successfully been used to produce numerous of *T. gondii* recombinant antigens (Biemans *et al.*, 1998; Lau & Fong, 2008).

MATERIALS AND METHODS

Parasite

Tachyzoites of the *T. gondii* RH strain were obtained from the Institute for Medical Research Kuala Lumpur, Malaysia. The tachyzoites were grown in MBDK cell monolayers in RPMI supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 environment.

PCR amplification of *GRA4* gene

The *T. gondii* tachyzoite genome was used as the template for the initial PCR, to amplify the *GRA4* gene using specific primers: 5' GCTGAATTCACCATGCAGGG CACTTGGTT 3' and 5' GCTGAATTCCGCT CTTTGCGCATTCTTTCC 3'. These primers were designed based on published sequence (GenBank accession number: M76432.1). *Eco*RI (Fermentas Life Sciences, Canada) cutting site (GAATTC) was incorporated into the primers to facilitate splicing of the PCR fragment into the corresponding *Eco*RI site of the expression plasmid vector, pPICZα A (Invitrogen Corp., USA). PCR was carried out in a 25 µl reaction mixture containing

1 U *Taq* DNA polymerase (Fermentas Life Sciences, Canada). The PCR mixture was initially pre-heated at 95ºC for 10 min for initial denaturation before 30 cycles of amplification, which consisted of denaturation at 94ºC for 1 min, annealing at 54ºC for 1 min, and elongation at 72ºC for 2 min. Final extension of the reaction was carried out at 72ºC for 10 min.

Recombinant plasmid construction

The amplified DNA fragment was digested with *Eco*RI and spliced into the corresponding cloning site in pPICZα A. The recombinant vectors were transformed into *E. coli* TOP10F'. This bacterial strain was grown in either Luria Bertani broth or on Luria Bertani agar, supplemented with zeocin (50 µg/ml, Invitrogen Corp., USA). Positive recombinant clones were selected and their plasmids were extracted and sent to a commercial laboratory for sequencing to confirm the orientation and integrity of the *GRA4* gene.

Transformation and expression of recombinant GRA4 in *P. pastoris*

Transformation of *P. pastoris* with the recombinant pPICZα A was done according to the manufacturer's protocol of the EasySelect™ *Pichia* Expression kit (Invitrogen Corp., USA). Positive recombinant *P. pastoris* clones were selected for expression study. A single recombinant *P. pastoris* colony was inoculated into 25 ml of buffered complex medium containing glycerol. The culture was grown at 28ºC for 24 h. The cells were harvested and resuspended in 100 ml of buffered complex medium containing methanol. The culture was allowed to grow for 72 h. Methanol was added every 24 h to a final concentration of 0.5% to induce expression of the recombinant *GRA4* gene. *Pichia pastoris* cells culture was collected every 12 h after methanol induction for protein extraction and analysis. Non-recombinant *P. pastoris* host cells (X-33 strain) and X-33 transformed with parent vector (without insert) were similarly treated and analyzed as negative controls. The growth conditions in shake flasks such as methanol concentration and inducing time were optimized.

Protein precipitation

The recombinant *P. pastoris* cells were removed from the growth medium by pelleting at low speed centrifugation. An equal volume of 20% (v/v) trichloroacetic acid was added to the growth medium. The mixture was incubated on ice for 1 h or overnight at -20ºC. The mixture was then centrifuged at 12,000g for 30 min to harvest the protein precipitate. The pellet was then washed with acetone, and centrifuged at 12,000g for 5 min, followed by air drying at room temperature for 10 min. The protein was resuspended in phosphate-buffered saline (PBS).

SDS-PAGE and Western blot

The recombinant proteins were evaluated in Western blot assays using sera of patients diagnosed with toxoplasmosis (n = 20) and healthy donors (n = 20). Specificity of the recombinant protein was also tested with sera of patients diagnosed as having amebiasis (1 samples), cysticercosis (3 samples), filariasis (2 samples), malaria (3 samples), and toxocariasis (1 samples). These samples were obtained from the Diagnostic Laboratory at the Department of Parasitology, University of Malaya. The harvested proteins were separated by SDS-PAGE and transferred by electroblotting to polyvinylidene difluoride membranes (Bio-Rad, USA). The proteins were probed with human serum (at 1:200 dilution) or anti-His antibody (Invitrogen, USA) (at dilution 1:1000). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgM/IgG (Bio-Rad, USA) or goat anti-mouse IgG and IgM (KPL, USA), respectively.

RESULTS

Cloning and expression of *T. gondii* GRA4 in *P. pastoris*

GRA4 protein was extracellularly expressed in yeast and obtained from the culture medium by TCA precipitation. Two

negative controls were included in the expression study, one is *P. pastoris* cells alone, and another one is cells transformed with pPICZα A vector without the *GRA4* insert. These two samples provided the background native proteins that the yeast generally expressed and the proteins that were encoded by the vector in absence of the insert of interest. A sample was also collected from the recombinant clone culture at 0 hours to observe the bands representing proteins right before induction. SDS-PAGE analysis through a 12% resolving gel showed the appearance of a novel band at approximately 40 kDa upon coomassie staining, which was absent in the negative controls. This size corresponds to the predicted molecular weight of recombinant GRA4, together with the polyhistidine-tag (Figure 1).

Optimization of the expression condition for production of recombinant antigen

Qualitative analysis of the intensity of the bands at different hours showed that it had the highest intensity for the 96 hour sample. This was thus deemed as the optimum time of expression and thereafter samples were only analyzed for this time interval. The expression of the GRA4 protein was induced under 5 different methanol concentrations: 0.25%, 0.5%, 0.75%, 1% and 1.5%. A band was present for each of the 5 lanes at approximately 40 kDa (Figure 2). However, as the concentration of methanol was increased, the band appeared to become more intense, until a peak was reached at 0.75% of mathanol concentration. After this concentration, the intensity of the band was observed to decrease.

Western blot analysis on the expression of recombinant GRA4 in *P. pastoris*

The antigenicity of the recombinant GRA4 was tested by Western blot using a positive human serum. When total recombinant proteins were analyzed with serum of a toxoplasmosis patient, non-specific bands were seen in all lanes including the negative controls (Figure 3). A novel 40 kDa protein was observed in the lanes containing the proteins from recombinant GRA4 clone (Figure 3). The recombinant protein was further evaluated by Western blot using 20 *Toxoplasma*-infected patients, 20 seronegative samples from healthy donors and 10 samples from patients suffering helminthic and other protozoal infections. The results of Western blot revealed that all sera from toxoplasmosis patients reacted with the recombinant GRA4 (Figure 4). None of the serum from the toxoplasmosis negative control including serum from patients suffering helminthic and other protozoal infections reacted with the recombinant GRA4 (Figure 4). The recombinant GRA4 identity was further confirmed in a Western blot probed with anti-His antibody. A single band was present at the same position (Figure 5).

DISCUSSION

The predicted peptide size of GRA4 is 36 kDa. The protein at the downstream region including *myc* epitope and the polyhistidine-tag contribute 5 kDa to the size of the expressed protein. Thus, the total size of the recombinant GRA4 was expected to be around 41 kDa. According to previous studies, the GRA4 protein migrates at approximately 40 to 41 kDa on SDS-PAGE gels (Mévélec *et al.*, 1992). A pilot study was carried out to determine the presence of this 40 kDa protein and its optimal time for expression. The induction was initially carried out with 0.5% methanol concentration as recommended by the Invitrogen EasySelect™ *Pichia* Expression Kit Manual. SDS-PAGE gel analysis showed a novel band at 40 kDa and the intensity of the band was highest after 96 hours of induction.

Another factor that affected the expression of the recombinant GRA4 in *Pichia* expression system was methanol concentration. For the expression of protein using *AOX1* promoter, it is important to keep the methanol level within a relatively narrow range. High expression of foreign protein expression depends on meticulous control of methanol level and maintenance

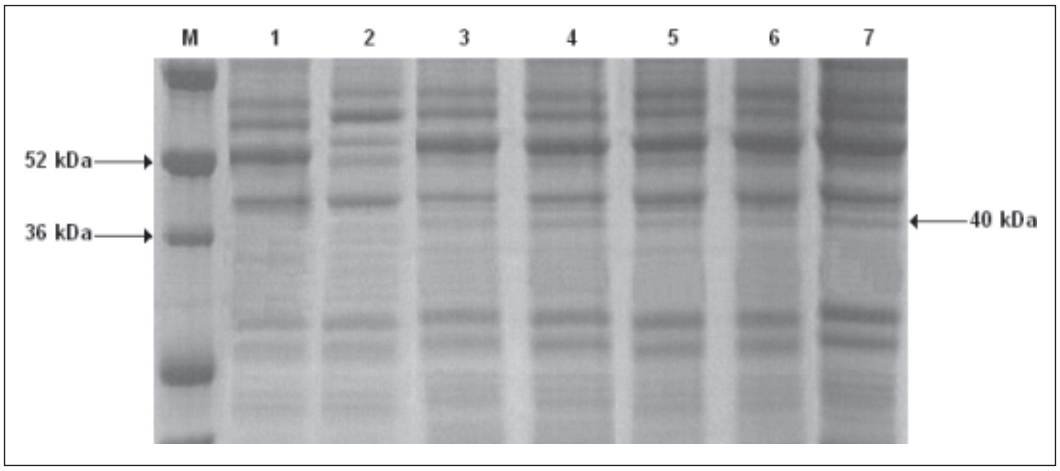

Figure 1. SDS-PAGE analysis on the expression of recombinant GRA4 in *P. pastoris*. Lane M contained the Bio-Rad Prestained Broad Range Protein Marker; Lane 1 contained supernatant of untransformed *P. pastoris* X33 cell; Lane 2 contained supernatant of *P. pastoris* X33 cells transformed with the pPICZα A vector alone; Lane 3-7 contained supernatant of *P. pastoris* X33 cells transformed with recombinant pPICZα A-GRA4 plasmid at 0 hours, 24 hours, 48 hours, 72 hours and 96 hours respectively. All samples shown were induced with 0.5% methanol. Supernatant of protein from recombinant GRA4 *P. pastoris* cell showed the appearance of a novel 40 kDa protein post induction, and its intensity peaked after 96 hours

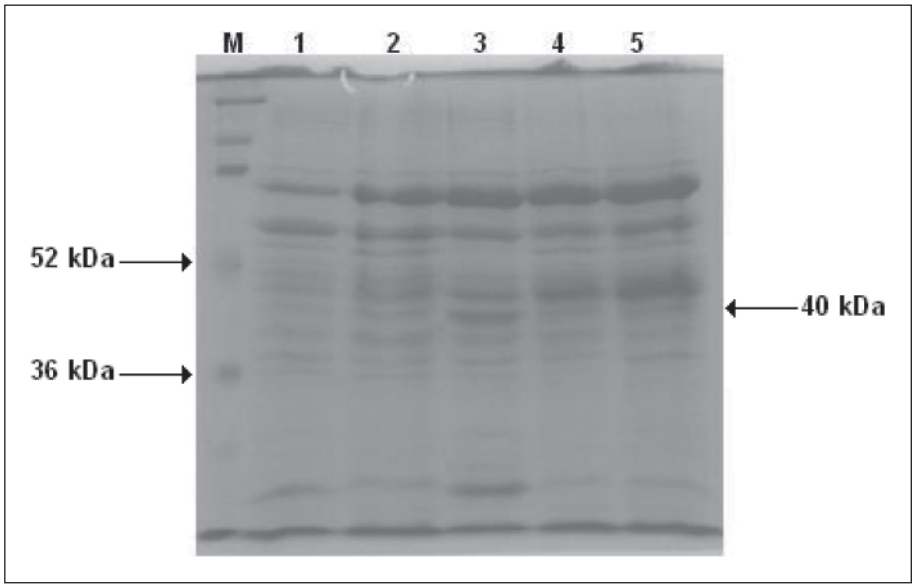

Figure 2. SDS-PAGE analysis of the effect of various methanol concentrations on the induction of protein expression of the transformed *P. pastoris* X33 cells containing the recombinant pPICZα A-GRA4 plasmid. All samples shown were collected after 96 hours of induction. Lane M contained the Bio-Rad Prestained Broad Range Protein Marker; Lanes 1-5 contained the recombinant GRA induced by a methanol concentration of 0.25%, 0.5%, 0.75%, 1% and 1.5% respectively. While all bands were seen to become more intense with increasing concentration of methanol, the 40 kDa band appeared as maximum intensity with 0.75% methanol concentration (lane 3)

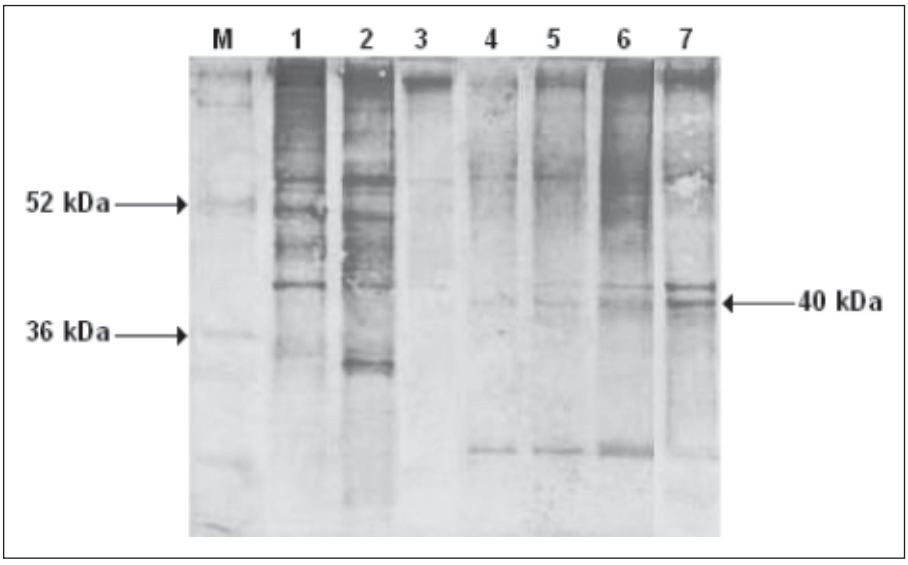

Figure 3. Western blot analysis of the recombinant GRA probed with human toxoplasmosis positive serum (IgM and IgG positive). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgM/IgG. Lane M contained the Bio-Rad Prestained Broad Range Protein Marker; Lane 1 contained supernatant of *P. pastoris* X33 cell; Lane 2 contained supernatant of *P. pastoris* X33 cells transformed with the pPICZα A vector alone; Lane 3-7 contained supernatant of *P. pastoris* X33 cells transformed with recombinant pPICZα A-GRA4 plasmid at 0 hours, 24 hours, 48 hours, 72 hours and 96 hours respectively. It was observed that the novel 40 kDa band was only present on the lanes containing the recombinant GRA4

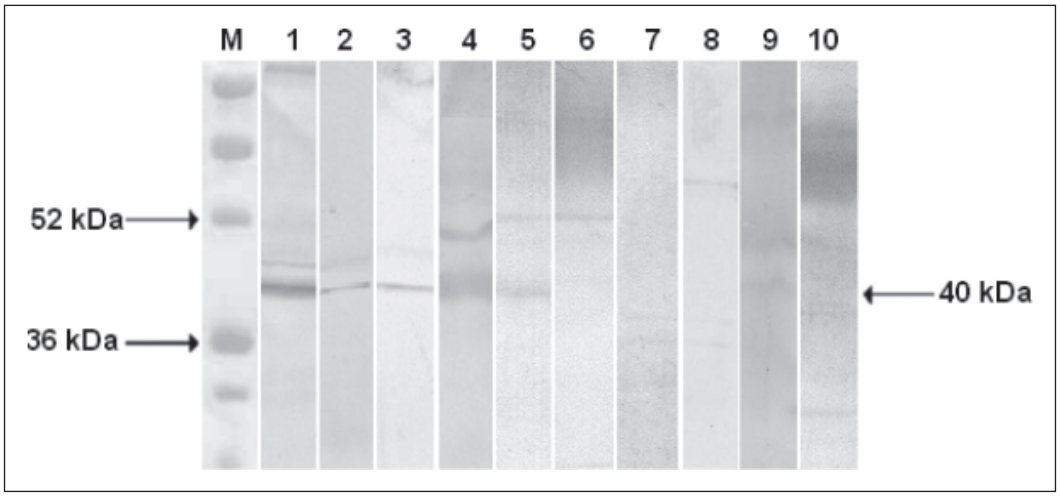

Figure 4. Western blot analysis of recombinant GRA4 probed with human serum of toxoplasmosis positive and toxoplasmosis negative control. Lane M contained the Bio-Rad Prestained Broad Range Protein Marker. All lanes contained supernatant of *P. pastoris* X33 cells transformed with recombinant pPICZα A-GRA4 plasmid. Lane 1 to 5 represents results of 5 sera of toxoplasmosis positive patients. Lanes 6-10 represents results of sera of healthy donors, amebiasis patient, cysticercosis patient, filariasis patient and malaria patient respectively. It was observed that the novel 40 kDa band was only present on the lanes probed with sera of toxoplasmosis positive patients

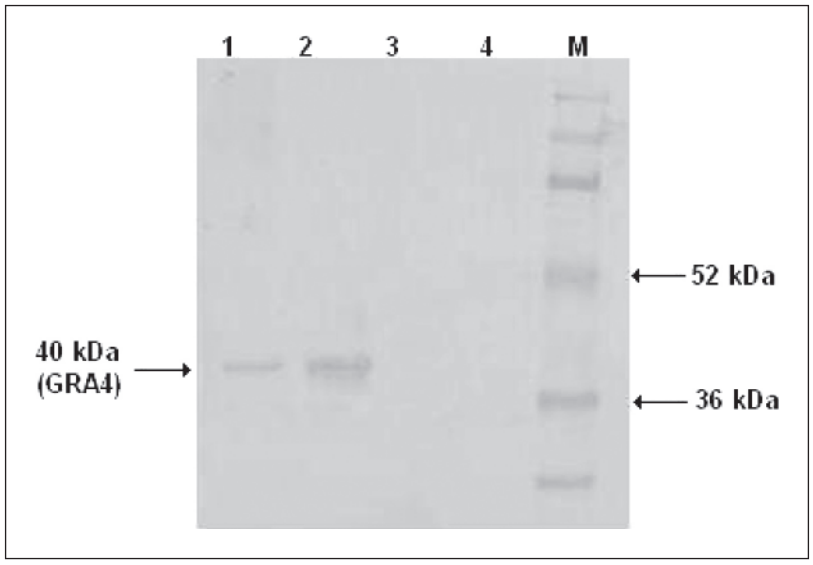

Figure 5. Western blot analysis of the recombinant GRA probed with anti-His antibody. Lanes 1 and 2 showed distinct bands at approximately 40 kDa, after 96 hours of induction with 0.5% methanol, representing the recombinant GRA which was expressed with a His tag tail. Lanes 3 and 4 represented supernatant of untransformed X33 cells and X33 cells transformed with the pPICZα A vector alone respectively. Neither lane showed any positive band for anti-His. Lane M contained the Prestained Broad Range Protein Marker (Bio-Rad)

of dissolved oxygen in the growth medium. Excess methanol can be toxic to the cell and drastically reduce promoter AOX activity, which may lead to cell death (Brierley *et al.*, 1990; Chiruvolu *et al.*, 1997; Minning *et al.*, 2001). In addition, methanol metabolism requires a high concentration of oxygen and recombinant gene expression is negatively affected by limiting oxygen (Chen *et al.*, 1997). In this study, different volumes of pure methanol were added to the media daily in order to maintain induction of the *AOX1* promoter. A methanol feeding of 0.75% was optimal for yeast growth and expression of GRA4. Slightly reduced growth and expression were observed when methanol feeding of 0.75% was used. This might probably be due to the limited carbon or the toxic effect of accumulated methanol.

Detection of specific antibodies is the method of choice to diagnose toxoplasmosis. The recombinant proteins were preliminary analyzed in Western blot with the serum of a confirmed toxoplasmosis patient. The 40 kDa recombinant protein was found to display antigenic reactivity in the Western blot. To further evaluate the usage of recombinant GRA4 as immunoreagents in Western blot for toxoplasmosis serology, positive and negative human sera were reacted against the recombinant GRA4 in Western blot. The results obtained show that the recombinant GRA4 could differentiate very clearly positive from negative sera. All Western blots using *Toxoplasma*-infected patient serum detected a specific band at approximately 40 kDa. The specificity of the recombinant protein as immunodiagnostic antigen was also evaluated by testing against serum from patients suffering helminthic and other protozoal infections. The recombinant GRA4 did not react with any of these serum samples, demonstrating that the recombinant antigen is specific for *T. gondii*. These results suggest that the recombinant GRA4 not only could detect

antibodies against *T. gondii*, but also could distinguish between toxoplasmosis and other infections.

Recombinant expression permits the protein to be tagged, *e.g.* by a polyhistidine-tag, to facilitate purification, which means that the purification can be done in fewer steps. Polyhistidine-tag affinity purification is often used for affinity purification of polyhistidine-tagged recombinant proteins. In this study, polyhistidine-tag was incorporated at the C-terminal of the recombinant protein. Thus, the recombinant protein could easily be detected using anti-His antibody. In the current study, the Western blot performed using the anti-His antibody indicated a clear band at 40 kDa. This showed that the His-tag was intact and in frame with the recombinant GRA4 protein, and can thus be used to purify the protein in the future by affinity media contain bound metal ions, either nickel or cobalt to which the polyhistidine-tag binds with micromolar affinity.

In conclusion, the recombinant GRA4 was successfully expressed in *P. pastoris* under controlled conditions. The putative GRA4 protein was able to detect toxoplasmosis that could prove to be valuable in diagnostic applications or vaccine development. Optimizing the other conditions such as pH, culture speed and culture medium is expected to give a better yield of the recombinant antigen. Our next step will be to produce the recombinant protein under a control environment such as fermentor and purify the recombinant protein by polyhistidine-tag purification method.

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