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Characterisation of a truncated *Toxoplasma gondii* surface antigen 2 (SAG2) secreted by the methylotrophic yeast *Pichia pastoris*

Lau, Y.L.[1], Shamilah Hisam[2] and Fong, M.Y.[1]
[1] Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
[2] Parasitology Unit, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia
Corresponding author email: fongmy@um.edu.my
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Abstract. A truncated form of surface antigen 2 (SAG2) of the protozoan parasite *Toxoplasma gondii* was cloned and expressed in the methylotrophic yeast *Pichia pastoris*. This recombinant antigen, designated as recSAG2-N, contained only the N-terminal half of the native SAG2. The recSAG2-N was secreted by the *Pichia pastoris* into the culture supernatant, and it was harvested by using the trichloroacetic acid precipitation method. Specificity of recSAG2-N was evaluated in western blot assays. Fifty human serum samples, including 32 from confirmed cases of toxoplasmosis, were tested. Results from the assays showed that recSAG2-N reacted with sera from the toxoplasmosis cases only. *In vivo* experiments showed that serum from mice which received recSAG2-N reacted with the native SAG2 of *T. gondii*.

INTRODUCTION

Toxoplasmosis is a widespread disease which is caused by the protozoan parasite *Toxoplasma gondii*. In human, severity of toxoplasmosis ranges from mild fever and lymphadenopathy, to chronic insidious infection of the eyes, encephalitis in immunodeficient individuals and severe CNS manifestations in congenital infections (Hill & Dubey, 2002).

Routine diagnosis of toxoplasmosis is based on serological detection of antibody in the patient. Antigens used in these serological assays are usually proteins derived from *T. gondii* cells which are propagated in mouse or *in vitro* culture. Growing and maintaining this parasite is laborious, time-consuming, and expensive. Most worryingly, it poses a biological hazard to laboratory personnel handling the culture. Thus, there have been attempts to produce antigens through safer means such as recombinant DNA technology. Majority of these endeavours have focused on the major surface antigens of the parasite such as SAG1 and SAG2 (Prince *et al.*, 1990; Parmley *et al.*, 1992; Kim *et al.*, 1994; Harning *et al.*, 1996; Chen *et al.*, 2001).

Pichia pastoris is a non-pathogenic methylotrophic yeast that can be manipulated to generate recombinant proteins (Cregg *et al.*, 1993). This non-fastidious yeast can be cultured in simple inexpensive medium. Recombinant genes in *P. pastoris* can be induced to high level of expression. In addition, this eukaryotic yeast has post-translational mechanisms to modify recombinant proteins into structures similar to the native proteins.

It has been reported previously that recombinant truncated forms of the *T. gondii* SAG1 produced by *P. pastoris* were antigenically adequate for serorecognition (Biemans *et al.*, 1998; Letourneur *et al.*, 2001). Furthermore, the truncated SAG1 could induce protection of mice against lethal challenge with *T. gondii* tachyzoites. Hence, in our present study, we postulated that a truncated form of SAG2 produced in *P.*

pastoris would also possess sufficient antigenicity for serorecognition. Our strategy was to construct a truncated SAG2 gene, which upon expression in *P. pastoris*, would produce and secrete only the N-terminal half of the SAG2 antigen. We subsequently characterised this truncated antigen in western blot assays and *in vivo* experiments.

MATERIALS AND METHODS

Parasite

Tachyzoites of the *T. gondii* RH strain were grown in MBDK cell monolayers in RPMI supplemented with 10% fetal calf serum at 37°C in a 5% CO_2 environment.

Bacterial strains and growth conditions

Escherichia coli TOP10F' was used as host for plasmid DNA manipulation experiments. In all experiments, this bacterial strain was grown in either Luria Bertani broth or on Luria Bertani agar, supplemented with zeocin (50 µg/ml) when appropriate.

Mice

Six- to eight-week-old female ICR mice used for *in vivo* experiments were obtained from the Animal Experimental Centre, University of Malaya, Kuala Lumpur.

PCR amplification of partial SAG2 gene

Toxoplasma gondii genomic DNA was directly used as template for PCR amplification as there are no introns within the SAG2 gene. The genomic DNA was extracted from the cell culture using a commercial kit (QIAGEN, Germany). The amplification was carried out using a primer pair consisting of the forward primer 5' ATAGAATTCATCCACCACCGAGACGCCA 3'; and reverse primer 5' TATGAATTC GCGGGCTGCTGGACCTTA 3'. The primers were designed based on published sequence of Prince *et al.* (1990). EcoRI cutting site (GAATTC) was incorporated into the primers to facilitate splicing of the PCR fragment into the corresponding EcoRI site of the expression plasmid vector, pPICZαC (Invitrogen Corporation, USA). PCR was carried out in a typical 25 µl reaction mixture containing 1 U *Taq* DNA polymerase (Fermentas Life Sciences, Canada). The PCR mixture was initially pre-heated at 95°C for 10 min before 30 cycles of amplification, which consisted of incubations at 94°C for 1 min, 54°C for 1 min and 72°C for 2 min.

Recombinant plasmid construction

The amplified DNA fragment was digested with EcoRI and spliced into the corresponding cloning site in pPICZαC. The recombinant plasmid was transformed into *E. coli* TOP10F'. Several positive clones were selected and sequenced in a commercial laboratory to confirm the orientation and integrity of the partial SAG2 gene.

Transformation and expression of recombinant truncated SAG2 in *P. pichia*

Transformation of *P. pastoris* with the recombinant pPICZαC was carried out using the EasySelect™ *Pichia* Expression kit (Invitrogen Corporation, USA). Positive recombinant *P. pastoris* clones were selected for expression. A single recombinant *P. pastoris* colony was picked and inoculated into 10 ml of buffered complex medium containing glycerol (BMGY). The culture was grown at 28°C for 24 hours. The cells were harvested and resuspended in 50 ml of buffered complex medium containing methanol (BMMY). The culture was allowed to continue growing for 72 hours. Methanol was added every 24 hours to a final concentration of 0.5% to induce expression of the recombinant SAG2 gene. Culture supernatant was collected at 12 hour-intervals for protein extraction and analysis. Nonrecombinant *P. pastoris* host cells (X-33 strain) and X-33 transformed with parent vector pPICZαC (without insert) were similarly treated and analysed as negative controls.

Protein extraction

Total protein was extracted from the culture supernatant by precipitation with 10% trichloroacetic acid. The protein precipitate was washed several times with acetone and finally resuspended in water.

SDS-PAGE and western blotting

The harvested proteins were separated by SDS-PAGE and transferred by electroblotting to polyvinylidene difluoride (PVDF, Bio-Rad Laboratories, USA) membranes. The proteins were probed with a positive anti-*Toxoplasma* human serum (at 1:200 dilution). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgM or IgG (Bio-Rad Laboratories, USA).

Evaluation of recombinant truncated SAG2 with human serum samples

The recombinant truncated SAG2 was then further tested in western blot assays with 50 human serum samples (at 1:200 dilution) of the following category: [A] IgG +ve, IgM –ve (20 samples); [B] IgG –ve, IgM +ve (12 samples); and [C] IgG –ve, IgM –ve (negative control, 18 samples). The serological status of these samples was initially determined by the Diagnostic Laboratory at the Department of Parasitology, University of Malaya. We reconfirmed the serological status (Table 1) using Captia™ *Toxoplasma gondii* IgG and *Toxoplasma gondii* IgM kits (Trinity Biotech, Ireland).

Immunization of mice

Two mice were injected subcutaneously with extracted protein (10 ng per mouse) derived from a recombinant *P. pastoris* culture. The mice were boosted with two further injections with five-day intervals. Control groups included mice receiving total extracted protein of nonrecombinant *P. pastoris* and water only. The sera of mice from each group were pooled and then examined for reaction with total cellular protein of *T. gondii* tachyzoites in a western blot.

Table 1. Serological status of human serum samples. The samples were tested using commercial kits (Trinity Biotech, Ireland). Immune Status Ratio (ISR) values of >1.1 were recorded as positive for *Toxoplasma* antibody. Hence, samples 1 – 20 were positive for IgG (category A), samples 21 – 32 positive for IgM (category B), and samples 33 – 50 negative for both IgG and IgM (category C)

Category A			Category B			Category C		
Sample number	IgG ISR	IgM ISR	Sample number	IgG ISR	IgM ISR	Sample number	IgG ISR	IgM ISR
1	2.01	0.03	21	0.25	1.23	33	0.40	0.11
2	1.14	0.16	22	0.31	1.47	34	0.38	0.28
3	1.93	0.42	23	0.58	1.25	35	0.40	0.16
4	1.66	0.15	24	0.21	2.29	36	0.42	0.12
5	1.55	0.15	25	0.66	1.19	37	0.46	0.35
6	1.78	0.58	26	0.45	1.13	38	0.45	0.34
7	1.45	0.49	27	0.29	1.18	39	0.45	0.11
8	1.34	0.46	28	0.32	1.15	40	0.56	0.02
9	1.79	0.25	29	0.87	2.19	41	0.61	0.01
10	3.30	0.47	30	0.71	1.28	42	0.57	0.25
11	3.65	0.21	31	0.36	1.56	43	0.68	0.06
12	2.37	0.71	32	0.21	1.15	44	0.40	0.11
13	3.92	0.45				45	0.38	0.28
14	3.45	0.46				46	0.70	0.14
15	2.19	0.34				47	0.56	0.25
16	2.14	0.29				48	0.40	0.16
17	2.22	0.90				49	0.77	0.35
18	2.50	0.32				50	0.16	0.81
19	2.04	0.38						
20	2.58	0.21						

RESULTS

Specific PCR primers were designed to generate a DNA fragment containing the N-terminal half of the SAG2 gene. This partial SAG2 gene was sequenced and the result showed complete (100%) identity with the published sequence of Prince *et al.* (1990) (Figure 1). The recombinant pPIC±C clone bearing the partial SAG2 gene was transformed into *P. pastoris* host cells.

A time course expression study was carried out to determine the optimal time and conditions for maximum expression of the recombinant clone. The culture was methanol-induced for 72 hours. The recombinant clone showed high expression of a novel protein of »33 kDa (Figure 2) at the 60th hour when induced with 0.5% methanol. This protein was not detected in the control samples. Thus, this »33 kDa protein was most likely the putative recombinant truncated SAG2. The identity of this truncated antigen was confirmed in a western blot which was probed with a positive anti-*Toxoplasma* human serum (Figure 3). We designated this recombinant truncated SAG2 as 'recSAG2-N'.

RecSAG2-N was further tested in western blot assays with human serum samples of: (A) IgG +ve, IgM –ve; (B) IgG –ve, IgM +ve; and (C) IgG –ve, IgM –ve (negative control). The serological status of these samples was pre-determined using a commercial immunoassay kits (Table 1). The western blot assays showed that all 20 samples from category A and all 12 from category B reacted with recSAG2-N (ten positive reactions from each category are shown in Figures 4a and 4b, respectively). None of the 18 serum samples from category C, which consisted of samples from non-toxoplasmosis individuals, reacted with recSAG2-N (Figure 4c). These results thus show that the recSAG2-N was specific for anti-*Toxoplasma* IgG and IgM antibodies. The results also suggest that the N-terminal half of SAG2 possesses antigenic sites or epitopes which can evoke the production of IgM and IgG.

Results from the western blot assays indicate that recSAG2-N shared common epitopes with the native SAG2. To further evaluate the antigenicity of recSAG2-N, immunization of ICR mice was carried out with total protein of the recombinant clone. Two weeks after the final immunization, sera from the mice were used to probe cellular proteins of *T. gondii* tachyzoites. It was observed that only the pooled serum from mice which received proteins containing recSAG2-N reacted with a protein of »22

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tccaccaccgagacgccagcgcccattgagtgcactgccggcgcaacgaagactgttgat
 S  T  T  E  T  P  A  P  I  E  C  T  A  G  A  T  K  T  V  D
 *  *  *     *                    *           *     *

gcaccctccagtggttccgttgtcttccaatgtggggataaactaaccatcagtcccagt
 A  P  S  S  G  S  V  V  F  Q  C  G  D  K  L  T  I  S  P  S


ggcgaaggtgatgtcttttatggcaaggaatgcacagactcgaggaagttgacgactgtc
 G  E  G  D  V  F  Y  G  K  E  C  T  D  S  R  K  L  T  T  V


cttccaggtgcggtcttgacagctaaggtccagcagccc
 L  P  G  A  V  L  T  A  K  V  Q  Q  P
                  *
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Figure 1. Nucleotide sequence of the PCR-amplified partial SAG2 gene (N-terminal half) and the encoded amino acids. The sequence shows 100% identity to that published by Prince *et al.* (1990). Asterisks (*) indicate potential O-glycosylation sites, as predicted by the Expert Protein Analysis System (Swiss Institute of Bioinformatics).
(*Note: the single alphabet code is used to represent the amino acids*)

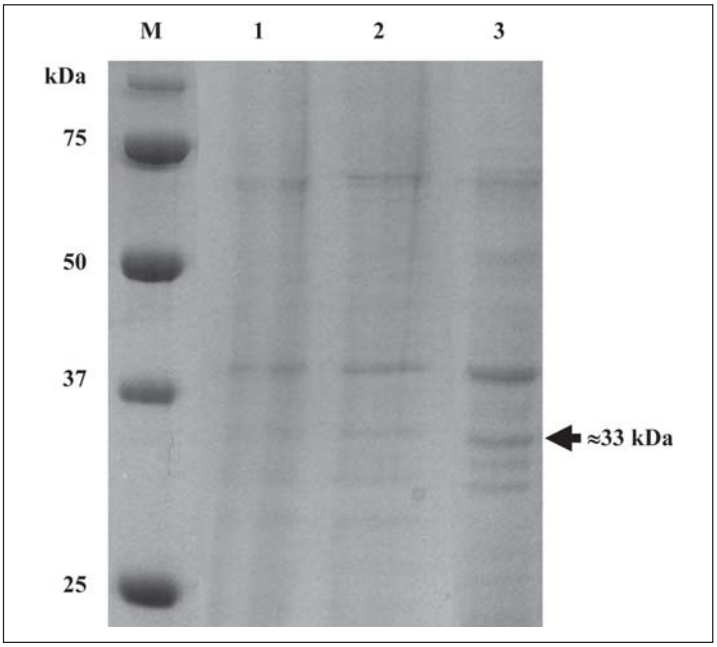

Figure 2. SDS PAGE analysis of total protein extracted from the culture supernatant of *P. pastoris*. Lanes 1 and 2 were loaded with total protein extracted from *P. pastoris* X-33 host cell and a nonrecombinant *P. pastoris* clone, respectively. Lane 3 was loaded with total protein extracted from a recombinant *P. pastoris* clone after 60 hours of induction with 0.5% methanol, showing a novel »33 kDa protein (arrow). Lane M contained the protein molecular weight standard.

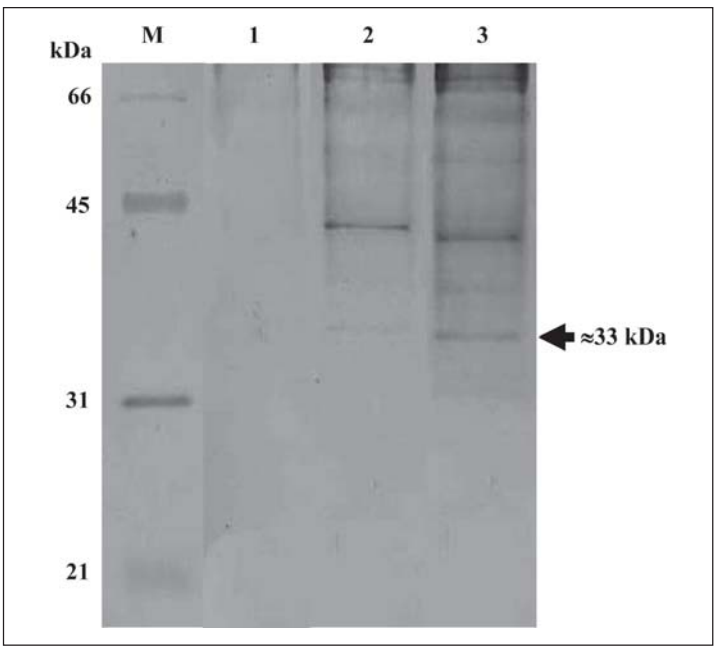

Figure 3. Preliminary western blot analysis of total protein extracted from the culture supernatant of *P. pastoris*. Lanes 1 and 2 were loaded with total protein extracted from *P. pastoris* X-33 host cell and a nonrecombinant *P. pastoris* clone, respectively. Lane 3 was loaded with total protein extracted from a recombinant *P. pastoris* clone after 60 hours of induction with 0.5% methanol. The human serum detected the novel »33 kDa protein in lane 3 (arrow), thus confirming the identity of the recombinant truncated SAG2 (recSAG2-N). Lane M contained the protein molecular weight standard.

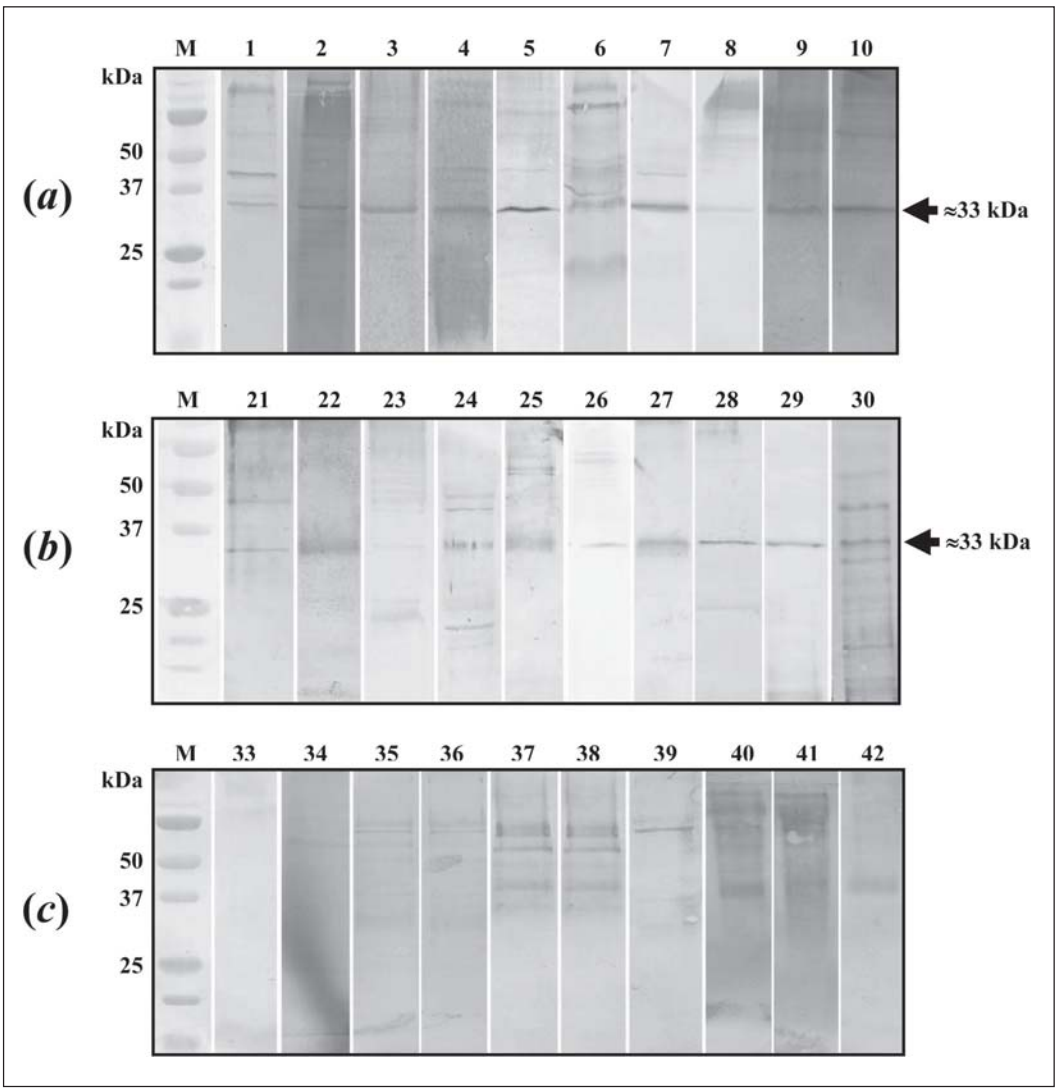

Figure 4. Detection of recSAG2-N with human serum samples. Each immunoblot strip, containing extracted proteins from a recombinant *P. pastoris* clone, was tested with a serum sample from either category A (IgG +ve, IgM –ve), B (IgG –ve, IgM +ve) or C (IgG –ve, IgM –ve). Only ten samples from each category are shown here. Strip numbers correspond to the sample numbers in Table 1. Strip M in each panel is the protein molecular weight standard. All samples from categories A and B (panels a and b, respectively) reacted with the »33 kDa recSAG2-N (arrow). None of the samples in category C (panel c) showed positive reaction.

kDa, the estimated size of the native SAG2 (Figure 5).

DISCUSSION

SAG2 is an attachment ligand which plays an important role in *T. gondii* invasion of host cells (Grimwood & Smith, 1996). Many attempts have been made to produce recombinant SAG2 using various expression systems. For example, recombinant SAG2 expressed in *E. coli* has been shown to be effective in detecting IgG antibody to *T. gondii* in human patients with toxoplasmosis (Prince *et al.*, 1990; Parmley *et al.*, 1992), but challenge studies using animal models revealed that the recombinant antigen

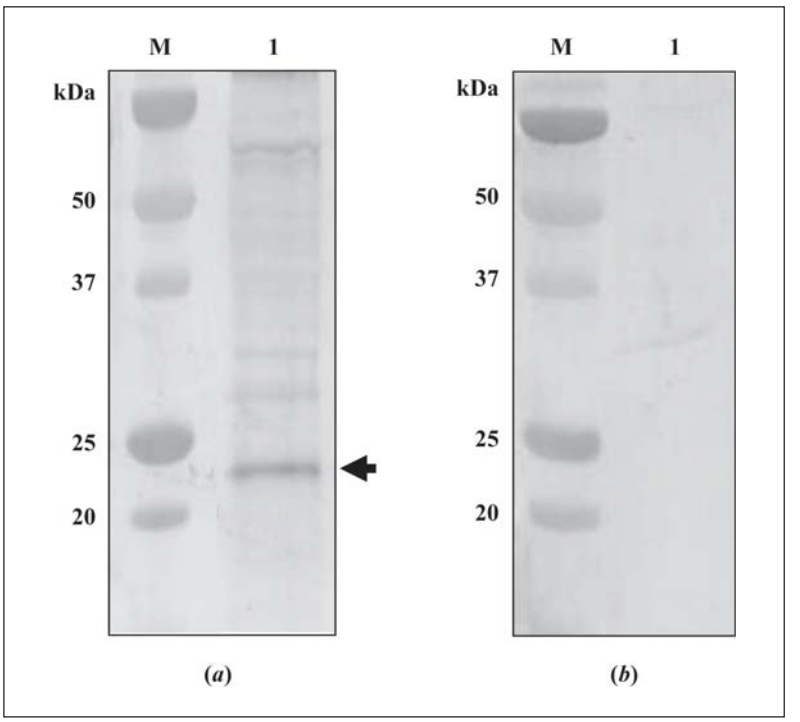

Figure 5. Reaction of mouse serum with total protein of *T. gondii* tachyzoites in a western blot. In panel a, the blot was probed with pooled serum from mice which were injected with total cellular protein of a recombinant *P. pastoris* clone expressing the recSAG2-N. The serum reacted with a protein of »22 kDa, (arrow, lane 1), the native *T. gondii* SAG2. Serum of mice which received total cellular protein of a nonrecombinant *P. pastoris* did not react with any protein of *T. gondii* (panel b, lane 1).

provided only partial protection against lethal infection of *T. gondii* (Mishima *et al.*, 2001). This lack of immunogenicity was likely due to incorrect folding of the recombinant SAG2.

In our study, we used the recombinant secretory system of the yeast *P. pastoris* to express the N-terminal half of SAG2 of *T. gondii*. We chose this expression system for its high efficiency to produce recombinant proteins, and also its ability to modify and fold the recombinant proteins into conformations that are similar to those of the native proteins.

The results of our studies showed that the N-terminal half of SAG2 was sufficiently specific to detect human anti-*Toxoplasma* IgG and IgM antibodies. In addition, *in vivo* experiments revealed that recSAG2-N was immunogenic enough to evoke antibody production against the native SAG2. Therefore, it would be worthwhile in future studies to examine the protective potential of recSAG2-N in immunised mice by challenging with live tachyzoites of *T. gondii*.

It was noted, however, that the molecular mass of recSAG2-N (»33 kDa) was much larger than the calculated truncated SAG2 (»11 kDa, i.e. half the size of native SAG2). This discrepancy in molecular mass might be attributed to the difference in posttranslational modification, such as glycosylphosphatidylinositol linkage or glycosylation. Recombinant proteins produced in the *Pichia* expression system are known to be hyper-glycosylated, causing significant increase in the molecular mass (Letourneur *et al.*, 2001). Using the Expert Protein Analysis System of the Swiss Institute of Bioinformatics (URL: http://www.expasy.org), we identified eight potential O-glycosylation sites in recSAG2-

N (Figure 1). Increase in the molecular mass was also seen in our previous work on *P. pastoris* expression of a *Toxocara canis* worm antigen (Fong & Lau, 2004). Nevertheless, the difference in the size and degree of glycosylation does not markedly affect the antigencity of the recombinant antigens.

In conclusion, the findings in this study have laid the foundation for our further endeavours in producing a highly specific recombinant antigen for use in immunodiagnosis assays, and possibly as vaccine for toxoplasmosis.

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