

RECOMBINANT EXPRESSION OF A TRUNCATED *TOXOPLASMA GONDII* SAG2 SURFACE ANTIGEN BY THE YEAST *PICHIA PASTORIS*

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Abstract. SAG2 is one of the major surface antigens of the intracellular protozoan parasite *Toxoplasma gondii*. In this study, we used the *Pichia pastoris* yeast expression system to produce a truncated form of the SAG2 and determined the serological characteristics of this recombinant antigen. We chose the *Pichia* system because of its high efficiency in expressing recombinant genes, and its ability to modify and secrete the recombinant proteins. Our strategy was to clone and express the part of the SAG2 gene that encodes the carboxyl half of the antigen. The recombinant antigen (recSAG2-C) secreted by the *Pichia* cells was harvested, and then evaluated in Western blot and enzyme-linked immunoassays (ELISA). Sixty human serum samples, including 45 from confirmed cases of toxoplasmosis, were tested against recSAG2-C. Results from the Western blot assay showed that recSAG2-C reacted with all 45 sera from the toxoplasmosis cases but none with the *Toxoplasma*-negative serum samples. Similar results were obtained for ELISA. These results indicated that recSAG2-C was specific for *Toxoplasma* antibody. To investigate the immunological characteristic of recSAG2-C, this recombinant antigen was injected subcutaneously into mice, and their serum was tested against total protein of *T. gondii*. It was observed that the serum specifically detected the native SAG2 (22 kDa) of *T. gondii*. This demonstrates that the recSAG2-C could evoke the production of antibody in mice, which readily recognized the native SAG2.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite that can infect human and a host of warm-blooded animals (Hill and Dubey, 2002). In humans, *T. gondii* infection is mainly asymptomatic in immunocompetent adults, but immunodeficient individuals may suffer encephalitis, pneumonia, and disseminated infection. Congenital infection via transplacental transmission from mother to the fetus during pregnancy may lead to fetal and neonatal complications (Montoya and Liesenfeld, 2004).

The life cycle of *T. gondii* consists of an asexual phase that occurs in all hosts, and a sexual phase occurring only in the intestines of the definitive host, the cat (Frenkel *et al*, 1970). Four functionally distinct forms occur in the life cycle of *T. gondii*: tachyzoite, bradyzoite,

merozoite, and sporozoite. In terms of infection, the tachyzoite is probably the most important, as it proliferates and disseminates rapidly within the host.

Routine diagnosis of *T. gondii* infection is based on serological detection of the antibody in a patient. Antigens used in these serological assays are usually prepared from *T. gondii* cells that are propagated in mouse or *in vitro* culture. Growing and maintaining this parasite is laborious, time-consuming, and expensive. Furthermore, such preparation usually contains extraparasitic materials and may result in interassay variability. Therefore, there have been efforts to produce pure antigens through safer means, such as recombinant DNA technology. The majority of these endeavors have focused on the major surface antigens of *T. gondii*, such as the SAG1 (p30) and SAG2 (p22) (Prince *et al*, 1990; Parmley *et al*, 1992; Harning *et al*, 1996; Chen *et al*, 2001). Most of these recombinant *T. gondii* antigens were produced using the *Escherichia coli* bacterial expression systems. However, such systems lack post-translational mechanisms to modify and fold the recombinant

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antigens into conformations similar to that of the native ones.

Pichia pastoris is harmless methylotrophic yeast that has been manipulated to generate recombinant proteins (Cereghino and Cregg, 2000; Daly and Hearn, 2005). This yeast is non-fastidious and can be cultured in a 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 the recombinant proteins into structures similar to their respective native counterparts.

Previous research work has shown that truncated forms of the recombinant SAG1 produced in *P. pastoris* were sufficiently immunogenic for serorecognition and protection in mice against lethal challenge with *T. gondii* tachyzoites (Biemans *et al*, 1998; Letourneur *et al*, 2001). In our present study, we constructed a truncated SAG2 gene, which upon expression in *P. pastoris*, produced and secreted only the carboxyl-terminal (C-terminal) half of the SAG2 antigen. We postulated that this truncated form of SAG2 would possess antigenic properties. Therefore, this recombinant antigen was evaluated by Western blot assay, ELISA, and *in vivo* experimentation.

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% CO₂ environment.

Bacterial strains and growth conditions

Escherichia coli TOP10F' was used as host for plasmid DNA manipulation experiments. In these 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 Center, University of

Malaya, Kuala Lumpur, Malaysia.

Human serum samples

The recombinant truncated SAG2 was tested in Western blot assays and ELISA with 60 human serum samples of the following categories: (A) IgG+ve, IgM-ve (15 samples); (B) IgG-ve, IgM+ve (15 samples); (C) IgG+ve, IgM+ve (15 samples); and (D) IgG-ve, IgM-ve (negative control, 15 samples). The serological statuses of these samples were 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).

Polymerase chain reaction (PCR) of partial SAG2 gene

T. gondii genomic DNA was directly used as a 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' ACTGAATTCAGCGAAAGGTCTCTGCTACC 3'; and the reverse primer 5' CATGAATTCACCTTGCCCGTGAGAGAC 3'. The primers were designed based on the published sequence of Prince *et al* (1990). The 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 that contained 1 U *Taq* DNA polymerase (Fermentas Life Sciences, Canada). The PCR mixture was initially pre-heated at 95°C for 10 minutes before 30 cycles of amplification, which consisted of incubations at 94°C for one minute, 54°C for 1 minute, and 72°C for 2 minutes.

Recombinant plasmid construction

The amplified DNA fragment was digested with EcoRI and spliced into the corresponding cloning site in the 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 pPICZ α C 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 selected 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, up 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 pPICZ α C (without insert) were similarly treated and analyzed 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) membranes, (Bio-Rad Laboratories, USA). The proteins were probed with human serum samples (at 1:200 dilution). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgM or IgG (Bio-Rad Laboratories, USA).

ELISA for the detection of IgG and IgM

ELISA was carried out using the components of commercial kits (ELISA Ensemble for IgM detection, Alpha Diagnostic, USA; Protein

Detector ELISA kit for IgG detection, KPL Inc, USA). Each well of a microtiter plate (Nunc, Roskilde, Denmark) was coated with 100 μ l of 10 μ g/ml of recSAG2-C in 0.05 M carbonate buffer (pH 9.6). After overnight incubation at 4°C, the antigen solution was removed and replaced with 100 μ l of blocking buffer (1% BSA). The plate was incubated for one hour at room temperature. A serum sample, diluted 1:50 in 1% BSA, was added into each well (100 μ l/well) and incubated for one hour at room temperature. The wells were then washed three times with PBS-T, followed by the addition of 100 μ l antibody-enzyme conjugate (for the detection of IgG, alkaline phosphatase conjugate was used; horseradish peroxidase conjugate was used for IgM) and incubated for one hour at room temperature. The wells were then washed three times with PBS-T and incubated with the appropriate substrate solution for 30 minutes. Optical density (OD) was read at 655 nm and 450 nm for IgG and IgM detection, respectively.

Each sample was run in duplicate wells. The ELISA results were determined for each sample by taking the mean value of the two OD readings, minus the value of the blank. A sample was considered positive if the calculated OD was greater than the mean +2 σ for serum samples of 20 normal individuals.

Immunization of mice

Ten mice were injected subcutaneously with extracted protein (100 μ g/mouse) derived from a recombinant *P. pastoris* culture. The mice were boosted with two further injections at five-day intervals. The negative control group consisted of mice that received total extracted protein of nonrecombinant *P. pastoris*. The sera of the mice from each group were examined for reaction with total protein of *T. gondii* tachyzoites in a Western blot.

RESULTS

The primers for PCR were designed to produce a DNA fragment that contained the C-terminal half of the SAG2 gene. This partial SAG2 gene was sequenced, and the data showed complete (100%) identity with the published

sequence of Prince *et al* (1990) (data not shown). The gene was spliced into the expression vector pPICZ α C and transformed into *P. pastoris* host cells.

A time course experiment was carried out to determine the optimal time and conditions for maximum expression of the recombinant SAG2 gene. The recombinant clone showed high expression of a novel protein of ≈ 33 kDa (Fig 1) after 60 hours of induction with 0.5% methanol, which was absent in the control samples. Thus, this ≈ 33 kDa protein was likely the putative recombinant truncated SAG2. The identity of this truncated antigen was confirmed by a Western blot that was probed with a positive anti-*Toxoplasma* human serum (Fig 2). This recombinant antigen was designated as 'recSAG2-C.'

The recSAG2-C was further evaluated in Western blot assays using panels of human serum samples of categories: (A) IgG+ve, IgM-

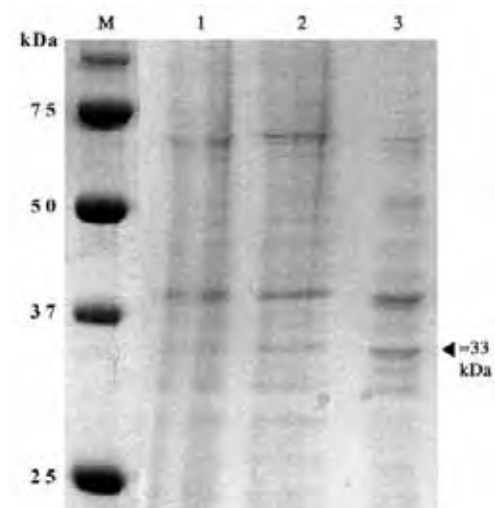


Fig 1- SDS PAGE analysis of total protein extracted from the culture supernatant of *P. pastoris*. Lanes 1 and 2 were loaded with total protein extracted from *P. pastoris* X-33 host cell and a nonrecombinant (*ie*, without SAG2 gene insert) *P. pastoris* clone, respectively. Lane 3 was loaded with total 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.

ve; (B) IgG-ve, IgM+ve; (C) IgG+ve, IgM+ve; and (D) IgG-ve, IgM-ve (negative control). The serological statuses of these samples were pre-determined using commercial immunoassay kits (Table 1). The Western blot assays indicated that all 15 samples from categories A, B, and C reacted with recSAG2-C (five positive reactions from each category are presented in Fig 3a, 3b, and 3c, respectively). None of the serum samples from category D, which consisted of samples from non-toxoplasmosis individuals, reacted with recSAG2-C (Fig 3d, only five blots are shown).

In the ELISA, serum samples from 20 known, normal, healthy individuals were used to determine the cut-off optical density measurement for a positive value. All 15 samples from categories A, B, and C showed OD measurements higher than their respective cut-off values (Table 2). The specificity of the recSAG2-C was evident, as none of the sera in

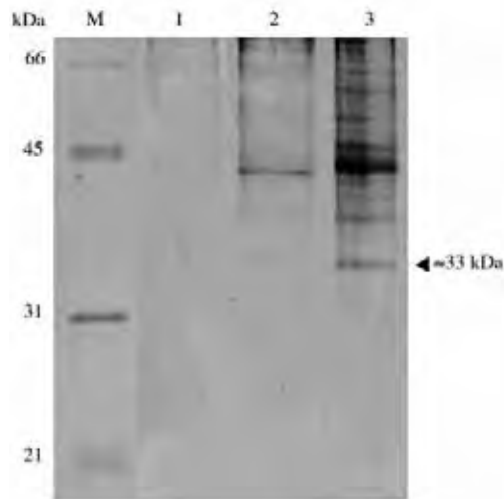


Fig 2- Preliminary Western blot analysis of total protein extracted from the culture supernatant of *P. pastoris*. Lanes 1 and 2 were loaded with total protein extracted from *P. pastoris* X-33 host cell and a nonrecombinant (*ie*, without SAG2 gene insert) *P. pastoris* clone, respectively. Lane 3 was loaded with total protein extracted from a recombinant *P. pastoris* clone after 60 hours of induction with 0.5% methanol. A positive anti-*Toxoplasma* human serum detected the novel ≈ 33 kDa protein in lane 3 (arrow), thus confirming the identity of the recombinant truncated SAG2 (recSAG2-C). Lane M contained the protein molecular weight standard.

Table 1
Serological status of human serum samples.

Sample number	Category A (IgG +ve, IgM -ve)		Category B (IgG -ve, IgM +ve)		Category C (IgG +ve, IgM +ve)		Category D (IgG -ve, IgM -ve)		
	IgG ISR	IgM ISR	Sample number	IgG ISR	Sample number	IgG ISR	Sample number	IgG ISR	IgM ISR
1	2.01	0.03	16	0.25	31	3.67	46	0.40	0.11
2	1.14	0.16	17	0.31	32	3.86	47	0.38	0.28
3	1.93	0.42	18	0.58	33	4.20	48	0.40	0.16
4	1.66	0.15	19	0.21	34	3.26	49	0.42	0.12
5	1.55	0.15	20	0.66	35	3.03	50	0.46	0.35
6	1.78	0.58	21	0.45	36	2.78	51	0.45	0.34
7	1.45	0.49	22	0.29	37	3.53	52	0.45	0.11
8	1.34	0.46	23	0.32	38	3.16	53	0.56	0.02
9	1.79	0.25	24	0.87	39	3.30	54	0.61	0.01
10	3.30	0.47	25	0.71	40	3.83	55	0.57	0.25
11	3.65	0.21	26	0.36	41	2.63	56	0.68	0.06
12	2.37	0.71	27	0.21	42	2.89	57	0.40	0.11
13	3.92	0.45	28	0.34	43	3.77	58	0.38	0.28
14	3.45	0.46	29	0.33	44	2.90	59	0.70	0.14
15	2.19	0.34	30	0.16	45	3.45	60	0.56	0.25

^aThe 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 1-15 were positive for IgG (category A), samples 16-30 positive for IgM (category B), samples 30-45 positive for IgG and IgM (category C), and samples 45-60 were negative for both IgG and IgM (category D).

category D tested positive in the assay.

These results indicate that the recSAG2-C was specific for anti-*Toxoplasma* IgG and IgM antibodies in the human serum samples. The results also suggest that the C-terminal half of SAG2 possesses antigenic sites, or epitopes, which can evoke the production of IgM and IgG.

Results from the Western blots and ELISA indicate that the recSAG2-C has common epitopes with the native SAG2 of *T. gondii*. Immunization of ICR mice was carried out to further evaluate the antigenicity of recSAG2-C. Two weeks after the final immunization, sera from the mice were used to probe the total protein of *T. gondii* tachyzoites. It was observed that sera from mice that received recSAG2-C reacted with the 22 kDa native SAG2 (Fig 4).

DISCUSSION

SAG2 is an attachment ligand that plays an important role in *T. gondii* invasion of host cells (Grimwood and Smith, 1996). Numerous 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 the IgG antibody to *T. gondii* in human patients with toxoplasmosis (Prince *et al*, 1990; Parmley *et al*, 1992). This challenges studies using animal models that suggest that the recombinant antigen provided only partial protection against lethal infection of *T. gondii* (Mishima *et al*, 2001). This lack of immunogenicity might be due to incorrect folding of the recombinant SAG2.

In this study, we exploited the recombinant

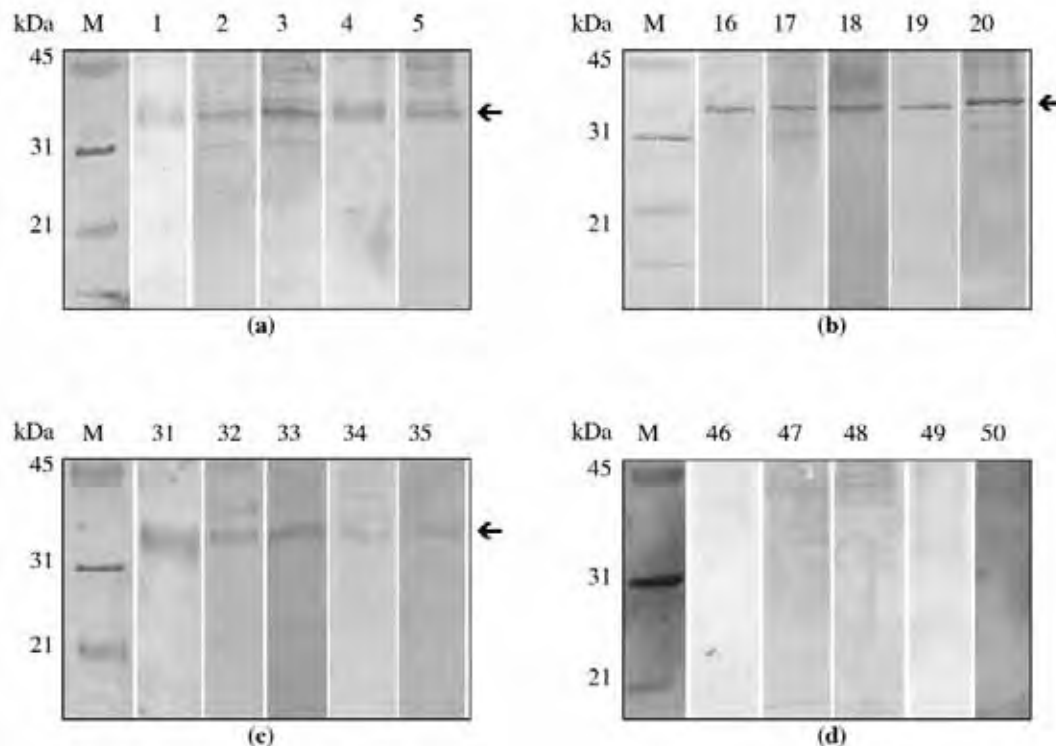


Fig 3- Detection of recSAG2-C with human serum samples. Each Western blot strip, containing the recSAG2-C, was tested with a serum sample from either category A (IgG +ve, IgM -ve), B (IgG -ve, IgM +ve), C (IgG +ve, IgM +ve) or D (IgG -ve, IgM -ve). Only five samples from each category are shown here. 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, B and C (panels a, b, and c, respectively) reacted with the ≈ 33 kDa recSAG2-C (arrows). None of the samples in category D (panel d) showed positive reaction.

Table 2
Results of ELISA using recSAG2-C as antigen.

Sample number	Category A (IgG +ve, IgM -ve)		Category B (IgG -ve, IgM +ve)		Category C (IgG +ve, IgM +ve)		Category D (IgG -ve, IgM -ve)	
	IgG ISR	IgM ISR	IgG ISR	IgM ISR	IgG ISR	IgM ISR	IgG ISR	IgM ISR
1	1.66	0.45	0.22	0.91	1.63	1.22	0.26	0.11
2	1.29	0.32	0.43	1.34	1.40	1.34	0.32	0.18
3	1.00	0.12	0.12	1.25	0.86	0.98	0.38	0.03
4	0.80	0.40	0.33	2.00	0.82	0.99	0.44	0.23
5	0.87	0.13	0.45	1.87	1.96	1.12	0.25	0.23
6	0.82	0.11	0.44	1.05	1.20	0.89	0.39	0.13
7	1.94	0.18	0.29	1.05	1.19	0.99	0.23	0.32
8	1.21	0.22	0.35	0.70	1.44	1.23	0.17	0.24
9	0.99	0.32	0.22	1.93	1.49	1.12	0.42	0.36
10	2.42	0.17	0.23	1.31	1.17	0.92	0.24	0.12
11	1.32	0.45	0.43	0.83	1.43	0.89	0.42	0.22
12	1.31	0.46	0.38	1.69	1.41	0.97	0.22	0.35
13	1.96	0.23	0.29	0.98	1.47	1.23	0.23	0.12
14	1.07	0.11	0.19	1.54	0.91	0.87	0.18	0.15
15	1.85	0.17	0.17	0.61	1.91	0.90	0.31	0.19

Cut-off OD for positive IgG is 0.64; for IgM is 0.46

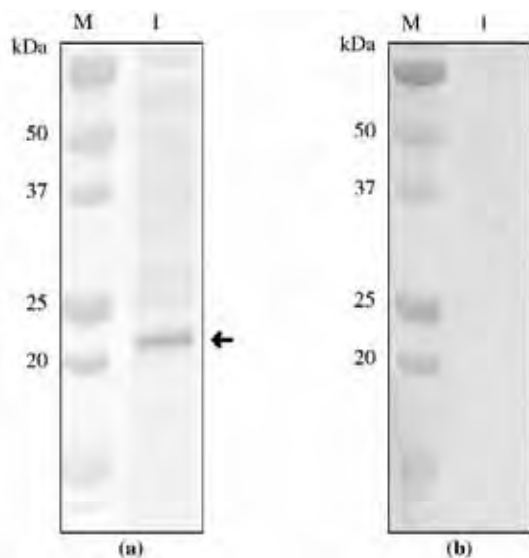


Fig 4- Reaction of mouse serum with total protein of *T. gondii* tachyzoites in a Western blot. In panel a, the blot was probed with serum from a mouse that was injected with recSAG2-C. The serum reacted with the native *T. gondii* SAG2, a protein of 22 kDa (arrow, lane 1). Serum of a mouse that received total cellular protein of a nonrecombinant *P. pastoris* did not react with any protein of *T. gondii* (panel b, lane 1). Lane M in each panel is the protein molecular weight standard.

system of the yeast *P. pastoris* to express and secrete the C-terminal half of SAG2 of *T. gondii*. Our reason for using a truncated SAG2 was primarily to increase its specificity by reducing any possible cross-reaction with surface antigens of other closely related protozoan parasites. The results of our studies indicated that the C-terminal half of SAG2 was sufficiently specific to detect human anti-*Toxoplasma* IgG and IgM antibodies. In addition, *in vivo* experiments suggested that recSAG2-C was sufficiently immunogenic to evoke antibody production against the native SAG2. Therefore, it would be worthwhile in future studies to examine the protective potential of recSAG2-C in immunized mice by challenging with live tachyzoites of *T. gondii*.

It was noted that the molecular mass of recSAG2-C was much larger than the expected truncated SAG2. However, this was not unexpected because some recombinant proteins that are produced in the *Pichia* expression

system are known to be hyperglycosylated, causing significant increase in the molecular mass (Cereghino and Cregg, 2000; Letourneur *et al*, 2001). An increase in molecular mass was also found in our previous work on *P. pastoris* expression of a *Toxocara canis* worm antigen (Fong and Lau, 2004). There is also concern that glycosylation by the *Pichia* system may introduce glycans that negatively affect the specificity of diagnostic tests (van Oort *et al*, 2004). However, from this study, we can suggest that the difference in the size and extent of glycosylation did not significantly affect the antigenicity and specificity of the recombinant SAG2. This was also observed in our work on *Pichia*-derived worm antigen (Fong and Lau, 2004).

As discussed above, we chose the *P. pastoris* expression system primarily for its high efficiency to produce recombinant proteins, and its ability to modify and fold the recombinant proteins into conformations that are similar to those of the native proteins. Another advantage of this expression system is the use of methanol as a component of the growth medium. Methanol plays two roles in this system. First, as a substrate for growth because *P. pastoris* is capable of utilizing methanol as the sole source of carbon. Second, methanol acts as an effective inducer in the expression of the foreign gene. This is unlike the *E. coli* expression system that requires expensive induction agents, such as IPTG or histidine. Therefore, the use of methanol can significantly reduce the cost of large-scale expression of the recombinant protein. In addition, the recombinant protein can be selectively secreted by *P. pastoris* into the growth medium. It has been estimated that recombinant protein makes up 80% of the secreted proteins, hence making harvesting and purification of the protein much easier.

In conclusion, our findings in this study have laid the groundwork for our further endeavor to produce a highly specific recombinant antigen that can be used for the development of an inexpensive seroassay kit for human toxoplasmosis.

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