Evaluation of *Pichia pastoris*-Expressed Recombinant Rhoptry Protein 2 of *Toxoplasma gondii* for Its Application in Diagnosis of Toxoplasmosis

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Abstract. Rhoptry protein 2 (ROP2) of *Toxoplasma gondii* is a rhoptry-secreted protein that plays a critical role in parasitophorous vacuole membrane formation during invasion. In previous studies, ROP2 has been shown to be efficient in triggering humoral and cell-mediated responses. High immunogenicity of ROP2 makes it a potential candidate for diagnosis and vaccination against toxoplasmosis. In this study, the ROP2 gene was cloned into pPICZα A expression vector and extracellularly expressed in the yeast *Pichia pastoris*, which has numerous advantages over other expression systems for eukaryotic proteins expression. The effectiveness of the secreted recombinant ROP2 as a diagnosis agent was assessed by Western Blot with 200 human serum samples. Recombinant ROP2 reacted with toxoplasmosis-positive human serum samples and yielded an overall sensitivity of 90% and specificity of 95%. However, recombinant ROP2 is a better marker for detection of IgG (91.7%) rather than IgM (80%).

INTRODUCTION

Toxoplasma gondii is an obligate intracellular parasite that causes toxoplasmosis in warm-blooded animals, including humans. Nearly one-third of the human population has been exposed to this parasite.^{1,2} Immunocompetent patients with acute toxoplasmosis are usually asymptomatic.³ However, the infection causes severe problems in pregnant women and immunocompromised persons (e.g., acquired immunodeficiency syndrome patients, organ transplant recipients, and patients with malignancies). Primary infection in pregnant women leads to abortion, stillbirth, or neonatal malformations.^{3–5} Chronic infection in immunocompromised persons can reactivate and cause life-threatening toxoplasmic encephalitis.⁶

Current diagnosis tests against toxoplasmosis are mostly serologic assays. These assays involve the use of *T. gondii* that is grown either in mice or *in vitro* culture, which can be hazardous to laboratory staff handling the samples. The use of recombinant antigens could be an alternative source of antigen for diagnosis purposes. Numerous antigens of *T. gondii* have been identified and cloned into suitable expression vectors to produce recombinant antigens. Most of these proteins are surface antigens, rhoptry proteins (ROPs), and dense granule proteins. Among these proteins is ROP2, which is involved in the formation of parasitophorous membranes during parasite invasion.⁷

In 1996, epitopes from ROP2, which was recognized by a high proportion of the infected human population, was identified.⁸ Expression of ROP2 in *Escherichia coli* was conducted in because of its immunogenicity. Recombinant ROP2 expressed in *E. coli* has been shown to be efficient in detecting IgA, IgM, and IgG in human toxoplasmosis.⁹ Moreover, DNA vaccination with recombinant ROP2 in a mouse model is able to induce immune responses and provide partial protection against *T. gondii* infection in mice.^{10,11} These findings provide evidence that ROP2 is frequently recognized by host immune systems and may play an important role in generating protective immune response against toxoplasmosis.

In this study, the *ROP2* gene was cloned and expressed in *Pichia pastoris*. The *Pichia* expression system is capable of performing eukaryote-specific post-translational modifications and producing recombinant proteins that are highly similar to native proteins.¹² This yeast expression system is useful for large-scale and laboratory production of recombinant protein. It can be easily grown in simple and inexpensive media by using methanol as its sole carbon source.¹³ In addition, it has a fast growth rate and can produce large amounts of recombinant protein.

In this study, the gene encoding ROP2 was ligated into a *P. pastoris* expression vector (pPICZα A) and expressed extracellularly. The sensitivity and specificity of the recombinant ROP2 was tested against toxoplasmosis-positive serum samples by Western blotting.

MATERIALS AND METHODS

Generation of *ROP2* synthetic gene. The codon-optimized *ROP2* gene was designed on the basis of the published sequence (GenBank accession no. Z36906) with preferred codon usage of *P. pastoris* without modifying the protein sequence. The optimized *ROP2* gene was synthesized by GenScript (Piscataway, NJ).

Plasmid construction and transformation. The synthetic *ROP2* gene was digested with *Xho*I and *Xba*I restriction enzymes and inserted into the plasmid vector pPICZα A. The recombinant plasmid was transformed into competent *E. coli* TOP10F' by using chemical methods.¹⁴ It was grown in either Luria-Bertani broth or on Luria-Bertani agar, supplemented with zeocin (50 µg/mL) when required. Several positive clones were selected and sent to a commercial laboratory (GenScript) for sequencing to confirm the complete nucleotide sequence of the gene insert. Transformation of *P. pastoris* with the recombinant pPICZα A-*ROP2* was performed according to the manufacturer's protocol of the EasySelect™ *Pichia* Expression Kit (Invitrogen, Carlsbad, CA). Positive recombinant *P. pastoris* clones were selected for expression.

Expression of recombinant protein. A single colony of the recombinant clone was picked and inoculated into 10 mL of buffered complex medium containing glycerol (0.1 M potassium phosphate, pH 6.0, 1.34% yeast nitrogen base,

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0.0004% biotin, 2% dextrose, and 1% glycerol). The culture was grown overnight at 28°C. The cells were harvested and resuspended in 50 mL of buffered complex medium containing methanol (0.1 M potassium phosphate, pH 6.0, 1.34% yeast nitrogen base, 0.0004% biotin, 2% dextrose, and 1% methanol) (optical density = 1.0 at $\lambda = 600$ nm). The culture was incubated for 72 hours. Methanol was added every 24 hours to a final concentration of 1% (v/v) to induce expression. *Pichia pastoris* cells were collected every 24 hours after methanol induction for protein extraction and analysis. *Pichia pastoris* host cells (X-33 strain) transformed with pPICZ α A vector (without insert) were similarly treated and used as negative controls. To enhance the expression level of recombinant protein in *P. pastoris*, the concentration of methanol induction (0.5%, 1%, 1.5%, and 2%) was optimized.

Protein harvesting from buffered complex medium containing methanol. The recombinant *P. pastoris* cells were removed from the growth medium by centrifugation at 5,000 rpm for 5 minutes. Two methods were used to precipitate the recombinant protein in growth medium: precipitation with trichloroacetic acid and precipitation with ammonium sulfate. An equal volume of 20% (v/v) ice-cold trichloroacetic acid was added to the growth medium. The mixture was incubated overnight at -20°C. The mixture was then centrifuged at 12,000 $\times$ g for 30 minutes to harvest the protein precipitate. The pellet was washed two times with ice-cold acetone by centrifugation at 12,000 $\times$ g for 10 minutes, followed by air-drying at room temperature for 10 minutes. The protein was resuspended in phosphate-buffered saline (PBS). For ammonium sulfate precipitation, 80% ammonium sulfate powder was added slowly into the growth medium and stirred until the powder dissolved completely. The mixture was then centrifuged at 10,000 $\times$ g for 30 minutes to harvest the protein precipitate. The proteins were resuspended in PBS and dialyzed against PBS overnight at 4°C with four changes of buffer.

Purification of recombinant ROP2. ProBond™ nickel-chelating resin (Invitrogen) was used for single-step purification of recombinant ROP2. The native protein purification method was used as described by the manufacturer. Briefly, the total proteins secreted from *P. pastoris* (after ammonium sulfate precipitation) were loaded onto ProBond™ column containing resin. After binding for 60 minutes, the resin was washed four times with native wash buffer (50 mM sodium phosphate [monobasic], 0.5 M sodium chloride, and 20 mM imidazole), and recombinant protein was eluted with native elution buffer (50 mM sodium phosphate [monobasic], 0.5 M sodium chloride, and 250 mM imidazole). The eluted protein was dialyzed against PBS overnight at 4°C. Quantification of protein was carried out by using the Bradford Assay Kit (Bio-Rad, Hercules, CA).

Gel electrophoresis and Western blotting. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred by electroblotting onto polyvinylidene difluoride membranes (Bio-Rad). Blocking of the membranes was achieved by incubation in Tris-buffered saline (TBS) containing 5% skimmed milk overnight at 4°C. The membranes were washed three times with Tris-buffered saline containing 0.01% Tween 20 and then probed with 1:200 diluted anti-*Toxoplasma*-positive human serum samples. Bound antibodies were detected with 1:2,500 diluted biotin-labeled goat anti-human IgM/IgG (Kirkegaard and

Perry Laboratories, Gaithersburg, MD) and streptavidin-AP (Invitrogen). The protein band was identified by using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma Chemical Co., St. Louis, MO) as the chromogenic substrate.

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The band was excised from the Coomassie blue-stained sodium dodecyl sulfate–polyacrylamide gel and destained in 50% acetonitrile (ACN). The solution was discarded and the gel piece was incubated for 30 minutes in 10 mM of dithiothreitol at 60°C followed by a 20-minute incubation in the dark in 55 mM iodoacetamide. After washing three times with 50% ACN, the gel piece was dehydrated with 100% ACN and then dried in a speed vacuum for 15 minutes. Digestion of the protein was carried out by incubation of the gel piece with trypsin overnight at 37°C. Protein extraction was then performed by adding 50 μ L of 100% ACN and shaking for 15 min. The solution was transferred to a new 1.5-mL tube, and the digested sample was dried in a speed vacuum. The pellet was then dissolved in 0.1% formic acid and desalted by using ZipTip (Millipore, Billerica, MA). The eluted protein was mixed in at a 1:1 ratio with the matrix and loaded onto a plate for MALDI–time of flight analysis. MALDI–time of flight analysis was performed at the University of Malaya Center for Proteomics Research (Kuala Lumpur, Malaysia).

Sensitivity and specificity of recombinant ROP2. The purified recombinant ROP2 was further evaluated by using Western blotting with 100 human *T. gondii*-seropositive serum samples and 100 *T. gondii*-seronegative serum samples, including serum samples from healthy donors (84 samples), patients diagnosed with amebiasis (2 samples), cysticercosis (4 samples), filariasis (3 samples), malaria (6 samples), and toxocariasis (1 sample), which were obtained from the Diagnostic Laboratory (Para: SEAD), Department of Parasitology, University of Malaya. These serum samples were categorized into four groups: I, IgG negative, IgM positive ($n = 15$); II, IgG positive, IgM negative ($n = 51$); III, IgG positive, IgM positive ($n = 34$), and IV, IgG negative, IgM negative ($n = 100$) on the basis of Captia™ *T. gondii* IgG and *T. gondii* IgM Kits (Trinity Biotech, Bray, Ireland). Sensitivity was calculated as (number of true positive results)/(number of true positive results + number of false-negative results), and specificity was calculated as (number of true negative results)/(number of true negative results + number of false-positive results).

RESULTS

Cloning and expression of *T. gondii* ROP2 in *P. pastoris*.

The codon-optimized *ROP2* gene was cloned in frame into pPICZ α A vector. A positive recombinant pPICZ α A plasmid harboring the *ROP2* gene was selected, and sequence analysis showed complete similarity with the published amino acid sequence of Becker and others.⁷ This positive recombinant plasmid was then transformed into *P. pastoris*. ROP2 was extracellularly expressed in *P. pastoris* where the protein can be obtained from the culture medium. Total protein was harvested from the cell culture every 24 hours after methanol induction. Protein with apparent size of approximately 50 kD was observed after a 24-hour induction, which was not found in the negative control (Figure 1).

FIGURE 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of expression of recombinant rhoptry protein 2 (ROP2) in *Pichia pastoris*. **A**, Coomassie blue stained and **B**, Western blot probed with serum of a toxoplasmosis patient. Lane 1, prestained broad range protein marker (Bio-Rad, Hercules, CA); lanes 2–5, negative control consisting of supernatant of pPICZ α A clone (24, 48, 72, and 96 hours); lanes 6–9, supernatant of recombinant ROP2 clone (24, 48, 72, and 96 hours). The 50-kD recombinant ROP2 was detected (arrow).

Optimization of expression conditions. Qualitative analysis of the intensity of the bands at different hours after induction showed the appearance of a 50-kD protein at 24 hours post-induction, and its intensity peaked after 48 hours. Expression of the ROP2 was also determined at four methanol concentrations (0.5%, 1%, 1.5%, and 2%). A methanol concentration of 1% was optimal for yeast growth and expression.

Western blot assay of recombinant ROP2 with human serum samples. The purified recombinant ROP2 was detected by Western blot using anti-toxoplasmosis human serum, and a novel 50-kD protein was observed (Figure 2). The purified recombinant ROP2 was further evaluated by Western blot assay with human serum samples from categories I (IgG negative, IgM positive), II (IgG positive, IgM negative), III (IgG positive, IgM positive), and IV (IgG negative, IgM negative) (Figure 3). Ninety of 100 *T. gondii*-seropositive serum samples reacted with the recombinant ROP2 and showed a band of approximately 50 kD. For *T. gondii*-seronegative serum samples, 5 of 100 healthy donor serum samples reacted with the recombinant protein (Table 1).

DISCUSSION

Antigens used in most serologic assays for detection of toxoplasmosis are usually derived from *T. gondii* grown in animal models or *in vitro* culture. Growing and maintaining this parasite is expensive, laborious, time-consuming, and hazardous. Therefore, using recombinant DNA technology to produce recombinant antigens may be the alternative source of antigens.^{15,16} Several recombinant protein have been produced by *E. coli* and evaluated for their potential as antigens for toxoplasmosis diagnosis. These antigens include surface antigens, dense granule proteins, rhoptry proteins, and microneme proteins.

Van Gelder and others evaluated an *E. coli*-expressed ROP2 by enzyme-linked immunosorbent assay to detect specific *T. gondii* antibodies in infected patients.¹⁷ The *E. coli* recombinant ROP2 detected IgG in 87 (89%) of 98 toxoplasmosis-positive human serum samples. This recombinant protein was expressed as a fusion protein containing a 330-residue carboxy-terminal antigenic fragment of ROP2, followed by six histidyl residues and a 48-amino-acid sequence derived from bacteriophage λ protein *Cro* and the *E. coli* protein *LacI*.¹⁷ A similar study by Martin and others using 77 *T. gondii*-positive serum samples found that truncated ROP2 expressed in *E. coli* (amino acids 196–561) showed a sensitivity of 91% in detecting IgG.⁹

In an attempt to provide recombinant protein that is similar to the native protein, we produced recombinant ROP2 by using the methylotrophic yeast *P. pastoris*. *Pichia pastoris* is non-pathogenic and can be grown in simple and reasonably priced media. Thus, exploiting the biological features of this yeast and its high efficiency in heterologous gene expression is a practical approach for generating large quantities of inexpensive and safe recombinant antigens for use in serodiagnosis of toxoplasmosis.¹⁸ In this study, recombinant ROP2 with an approximate size of 50 kD was optimally expressed by induction with 1% methanol. Methanol taken up by *P. pastoris* cells is oxidized to formaldehyde in peroxisomes. This reaction is catalyzed by alcohol oxidase (AOX). It is important to keep the methanol level within a relatively narrow range for expression of protein using the AOX promoter. Excess methanol can be toxic to the cell and reduce the activity of promoter AOX, which may lead to cell death.^{19,20} A methanol concentration of 1% was optimal for yeast growth and expression of recombinant ROP2. Reduction of growth and expression was observed when the methanol concentration increased to 2%. This finding might be caused by the cytotoxic effects of accumulated methanol.²¹

FIGURE 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified recombinant rhoptry protein 2 (ROP2). **A**, Coomassie blue stained purified recombinant ROP2. **B**, Western blot probed with serum of a toxoplasmosis patient. The 50-kD purified recombinant ROP2 was detected (lane 2). Lane 1, prestained broad range protein marker (Bio-Rad, Hercules, CA).

FIGURE 3. Western blots of purified recombinant rhopty protein 2 (ROP2) with toxoplasmosis-positive patient serum samples. Lane 1, prestained broad range protein marker (Bio-Rad, Hercules, CA). Lanes 2–6, results of five serum samples from group I (IgG–, IgM+), lanes 7–11, results of five serum samples from group II (IgG+, IgM–); lanes 12–16, five serum samples from group III (IgG+, IgM+). The 50-kD purified recombinant ROP2 was detected. Lanes 17–21, results of five serum samples from negative control (group IV).

Recombinant ROP2 was then purified and the identity of the recombinant ROP2 was further confirmed by MALDI mass spectrometry analysis. The molecular mass of the recombinant ROP2 is slightly smaller than that of the mature native form of ROP2 (55 kD). This discrepancy in size may be caused by cleavage of expressed ROP2 by proteinases of *Pichia*, as reported in previous studies.²² Despite the differences in size, the antigenicity of the recombinant antigen was not significantly affected.

The ability of the recombinant ROP2 to detect specific *T. gondii* antibodies was evaluated by using 100 *T. gondii*-seropositive serum samples. Western blotting showed that purified recombinant ROP2 had a specificity of 95% for diagnosis of toxoplasmosis. The sensitivity of recombinant ROP2 in detecting IgG is 91.7%, which is slightly higher than results reported by Vangelder and others¹⁷ and Martin and others,⁹ in which the sensitivities of *E. coli*-expressed recombinant ROP2 in detecting IgG were 89% and 91% respectively. However, the sensitivity of recombinant ROP2 in detecting *T. gondii*-specific IgM alone (Group I) was low (80%). Previous studies have indicated that during the early stage of infection, antibodies against the surface antigens of *T. gondii* are detected but antibodies against ROP2 are rare.¹⁷ Gatkowska and others reported that levels of IgM against ROP2 were low during the early phase of infection (one week after infection) in infected mice.²³ The level of IgM against ROP2 increased during the transition period from the acute phase to the chronic phase (three weeks after infection).

In conclusion, recombinant ROP2 was successfully expressed in *P. pastoris* under controlled conditions. Recombinant ROP2 was able to detect IgG and IgM in *T. gondii* infection by Western blotting. Recombinant ROP2 also appears to be a more sensitive marker for detection of IgG rather than IgM. The findings in this study have laid the foundation for further endeavors in producing a highly specific recombinant antigen for use in immunodiagnosis assays, and possibly as a vaccine for toxoplasmosis.

TABLE 1

Summary of results obtained in Western blot of recombinant rhopty protein 2 detected with human serum samples

Group	No. human serum samples	Western blot			
		Positive		Negative	
		No.	%	No.	%
I (IgG negative, IgM positive)	15	12	80	3	20
II (IgG positive, IgM negative)	51	47	92.2	4	7.8
III (IgG positive, IgM positive)	34	31	91.2	3	8.8
IV (IgG negative, IgM negative)	100	5	5	95	95

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