Expression and Single-step Purification of GRA8 Antigen of Toxoplasma gondii in Escherichia coli

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Abstract
Diagnosis of Toxoplasma gondii (T.gondii) infection is of great medical importance especially for pregnant women and immunosuppressed patients. Numerous studies have shown that the recombinant production of several toxoplasma antigens, including dense granule antigens (GRAs) has a great potential as diagnostic reagents. Previous studies reported expression of amino terminal GRA8 protein in fusion with large tags. In the present study, we produced truncated GRA8 (GRA8), excluded from the signal peptide and C-terminal transmembrane domain, with a short fusion tag in Escherichia coli (E.coli). GRA8 was purified using an optimized single-step Immobilized Metal Ion Affinity Chromatography (IMAC). The purity and yield of GRA8 was highest at pH=9.25. At this pH, 13.6 mg of GRA8 was obtained with the purity of 97.97%. Immunogenicity of the protein was evaluated in Western blot analysis showing the serum sample from a rabbit immunized with GRA8 recognized a single antigen of T.gondii tachyzoite at the expected molecular weight of native GRA8. To diagnosis acute toxoplasma infection in pregnant women, an indirect immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) was developed using GRA8 resulting in a test specificity and sensitivity of 97.1% and 60.6%, respectively. These results demonstrated that immunogenic GRA8 can be produced in fusion with a short tag and purified near to homogeneity using an optimized IMAC. GRA8-IgM-ELISA was useful for detection of acute toxoplasma infection.

Keywords: Enzyme-linked immunosorbent assay, Gene expression, GRA8 protein, Immunoglobulin M, Toxoplasma gondii

Introduction
Toxoplasmosis is caused by the protozoan parasite Toxoplasma gondii in a large variety of domestic and wild mammals and birds including human being (1). The infection generally is asymptomatic in immunocompetent individuals. However, in immunocompromised patients, such as those with HIV/AIDS, infection can cause life-threatening encephalitis and can be fatal if not recognized and treated soon (2). Furthermore, since the parasite can cross the placenta, the primary maternal infection with T.gondii can be transmitted to the fetus and may lead to severe congenital defects such as hydrocephaly, mental retardation, chorioretinitis, visual impairment or even death in utero (2-5). In livestock, abortion of ewes causes considerable economic losses (6).

Accurate diagnosis of primary toxoplasma infection and subsequent chemotherapy re-
duce the risk of congenital infection (2,3). Detec-
tion of toxoplasma infection and distinc-
tion between acute and chronic phases of the
infection are mainly based on serological tests
enabling the detection of anti-toxoplasma
immunoglobulin G (IgG) and IgM antibodies
in blood (9).

The conventional serological tests are pri-
marily based on whole T.gondii antigens
which might have inconstant quality and ex-
ternal antigens from mouse peritoneal cavity
or cell culture. The use of these antigen prepa-
rations makes standardization of the assays
difficult and also results in insufficient speci-
ficities for differentiation between acute and
chronic infection (3,7,8). The recombinant toxo-
plasma antigens, which can be easily produc-
ed in large amounts and with constant quality,
are the good candidates for replacement of the
crude antigens. Development of serological
tests using recombinant toxoplasma antigens
which are representative of acute or chronic
infection is a present trend in discrimination
between the acute and chronic infections (7-10).

The dense granule proteins (the GRA anti-
gens) of T.gondii are secreted in abundance
and constitute an important fraction of the
antigens which circulate in the blood stream
during the first hours following infection (11).
These proteins are highly immunodominant
and induce strong antibody response (11). Nu-
umerous studies have shown the diagnostic
potential of several GRA antigens including
GRA2, GRA4, GRA6, GRA7 and GRA8 for
serodiagnosis of toxoplasma infection and
discriminating acute from chronic infections (12-20).

Previous studies introduced GRA8 as a
marker of acute infection and showed that
IgG and IgM ELISA with recombinant GRA8
was able to differentiate acute from chronic
infection (15,18-20). The sequence of GRA8 is
composed of 267 amino acids from which 64
are hydrophobic proline (24% overall). GRA8
contains an amino terminal signal peptide,
three degenerate proline-rich repeats in the
central region and a transmembrane domain
near the carboxyl terminus (21). The hydropho-
bic transmembrane domain greatly reduces
the bacterial expression level (22) and is usually
excluded from the recombinant GRA8 (15,16,19,20,23).
In the previous studies, GRA8 was expressed either in fusion with large fusion
tags such as glutathione S-transferase (GST)
(19,23) and the CMP-2-keto-3-deoxy octulo-
sonic acid synthetase (CKS) (15) or in fusion
with a relatively small tag encoded by the
pUET-1 plasmid (16).

Immobilized Metal Affinity Chromato-
graphy (IMAC) is one of the most popular
techniques for the purification of recombinant
proteins, which use the metal binding prop-
erty of certain amino acids, in particular histi-
dine (24-27). IMAC is usually applied as the
initial capture step in purification scheme
(26,28). Many studies, however, showed proteins
with high purity, e.g. >95%, can be obtained
through proper optimization of single-step
IMAC conditions (29,30).

In the present study, we sought to produce
the truncated GRA8, with a very small fusion
tag and optimize the expression and purifica-
tion of the protein. The pH optimized IMAC
was applied for highly efficient purification of
GRA8 and the purified protein was used in
IgM Enzyme-Linked Immunosorbent Assay
(ELISA) for distinguishing acute from chron-
ic infection.

Materials and Methods

Parasite, bacterial strains and plasmid
T.gondii RH strain was used for the extrac-
tion of genomic DNA. Tachyzoites were in-
jected into the peritoneal cavity of Swiss
mice. Three days later, they were harvested
from peritoneal fluid, washed with phosphate-
buffered saline (PBS) and stored at -70 °C.

The E.coli DH5α strain (Invitrogen) was
used for cloning and E.coli BL21 (DE3),
BL21 (DE3) plysS and Rosetta (DE3) strains
(Promega) were used for expression of the
recombinant antigen. The pET-28b(+) (Novagen)
plasmid was utilized for the construction of
the expression system.

Serum samples
A total number of 68 serum samples from
pregnant women, each sample corresponding
to a woman (provided from three medical
diagnostic laboratories in Tehran, Iran), were
divided into 2 groups according to the results
of the standard toxoplasma-specific IgG, IgM,
and IgG avidity ELISA (Euroimmun, Lubeck,
Germany). Group I (acute-phase sera) consist-
ed of 33 serum samples collected from preg-
nant women with clinical signs of suspected
acute toxoplasmosis. They were IgG and IgM
positive and have low avidity of toxoplasma-
specific IgG antibodies. Group II (chronic
sera) were from 35 women with the positive
IgG and negative IgM response.

Construction of the recombinant expression
plasmid
Genomic DNA was extracted from the
tachyzoites of T. gondii RH strain using the
DNA extraction kit (Bioneer) and used as the
template for the PCR amplification. A trunc-
cated fragment of gra8, encoding amino acid
23 to 169, was PCR amplified using a pair of
specific primers (Forward primer: 5′-GCG
GAT CCC GCC ATG AAC GGT CCT TTG
AGT TAT-3′ and Reverse primer: 5′-GCC
TCG AGA GCT GTC GTG GTT GTA ACG TAG G-3′) designed based on the pub-
lished sequence of gra8 on GenBank database
(AF150729). Forward and reverse primers
were designed to contain the BamHI and
XhoI restriction sequences, respectively. PCR
was performed as follows: 94°C for 5 min,
followed by 35 cycles at 94°C for 1 min, 57°C
(+ 0.1°C increment per cycle) for 1 min, and
72°C for 50 sec. The final extension was
7 min at 72°C.

The PCR product was digested with
BamHI and XhoI and inserted into the pET-
28b(+) expression vector digested with the
same enzymes. The resulting recombinant
plasmid, containing the truncated sequence
of gra8, was named pET-28b(+) GRA8. The
presence of the gra8 fragment in the recombi-
nant plasmid was investigated by the EcoRV
restriction enzyme digestion and DNA se-
quencing determination.

Production of GRA8
To obtain the recombinant GRA8, E. coli
BL21(DE3), BL21(DE3)plysS and Rosetta
(DE3) strains harboring the pET-28b(+)-
GRA8 plasmid was cultivated in Luria Bert-
tani (LB), 2XTY and Terrific broth (TB)
media supplemented with 25 μg/ml kana-
mycin and 35 μg/ml chloramphenicol, where
appropriate, at 37°C with shaking at 200 rpm.
Expression of GRA8 was induced at OD600 of
0.6 by addition of various concentration of
Isopropyl-β-D-Thiogalactopyranoside (IPTG)
ranged from 0.1-1 mM. Following incubation
at 30°C for an additional 5 hr, the induced
bacteria were harvested by centrifugation at
6000 × g for 15 min. The expression of GRA8
was analyzed by 12% SDS-Polyacrylamide
Gel Electrophoresis (SDS-PAGE).

To assess the solubility of GRA8, the pellet
of 1 ml of induced bacteria was incubated in
100 μl of the lysis buffer (500 mM NaCl, 50
mM Tris-Cl, pH=9.25, 1 mg/ml lysozyme) at
4°C for 30 min. The cells were lysed by
gentle vortexing and centrifuged at 16000 ×
g for 15 min at 4°C to separate the insoluble
proteins and cell debris from the soluble ones.
The pellet and supernatant were analyzed by
SDS-PAGE.

Purification of GRA8
The purification of GRA8 was performed by
IMAC using HisTrap FF columns (5 ml)
(GE Healthcare) and the Bio-Rad FPLC
instrument. The induced cells from 200 ml
culture were collected by centrifugation and
the resulting pellet was resuspended in 15 ml
of binding buffer (500 mM NaCl, 50 mM
Tris-Cl, 5 mM imidazole). The purification
process performed at four pH values of
binding buffer: 7.5, 8.1, 8.75 and 9.25. The
cells suspension was sonicated for 6 min
bursts at 200-300 W using an MSE ultrasonic
disintegrator (Fisons). The cell lysate was
subsequently centrifuged at 12000 × g at 4°C
for 20 min. The supernatant was passed
through a 0.2 μm (pore size) filter. The
cleared lysate was applied to the column
previously equilibrated with the binding buff-
er at a flow rate of 1 ml/min. The column was
then washed step-wised with 10 column vol-
umes of binding buffer until the absorbance at
Expression and Single-step Purification of GRA8 Antigen

280 nm of the effluent was minimal. Afterwards, the column was washed step-wise with 25 ml, 20 ml and 15 ml of the binding buffer containing 35, 60 and 90 mM of imidazole at 4 ml/min. Absorbed material was eluted at 1 ml/min with binding buffer containing 500 mM imidazole. The purified samples were analyzed on SDS-PAGE stained with Coomassie Blue G-250 as well as silver nitrate.

**Rabbit immunization with GRA8**

One rabbit was immunized i.m with 100 μg of the purified GRA8 emulsified in Freund’s complete adjuvant. Injection was repeated twice with two weeks intervals using Freund’s incomplete adjuvant. The serum sample was collected two weeks after the last immunization. The serum obtained from another rabbit injected with the adjuvant alone was taken as the control accordingly.

**Western blotting analyses**

To assess the immunogenicity of GRA8, Western blotting was performed using sera of the immunized and non-immunized rabbits. About 5×10⁶ tachyzoites of the RH strain were loaded onto SDS-PAGE. The antigens then transferred onto the nitrocellulose membrane, blocked with 2% Bovine Serum Albumin (BSA) in PBS containing 0.1% Tween 20 (PBS-T) and probed with rabbit sera diluted 1:500 in blocking buffer. The membrane was washed three times with PBS-T and incubated with Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Ray Bio-tech) diluted 1:500 in blocking buffer. The plates were then incubated at 37°C for 1 hr and washed three times with PBS-T. One hundred μl of the sample sera diluted 1:100 in blocking buffer were added to the plates. The plates were then incubated at 37°C for 1 hr and washed three times with PBS-T. One hundred μl of HRP conjugated goat anti-human IgM antibody (Sigma)-diluted 1:10000 in blocking buffer was added to each well. The plates were incubated at 37°C for 30 min and washed 3 times. One hundred μl of tetramethylene benzidine dihydrochloride (Kem-En-Tec) was then added as the substrate. After 15 min of incubation at room temperature, the reaction was stopped by addition of 100 μl of 1 M HCL. The optical density was measured at 450 nm by a BioTek ELISA reader (BioTek Instruments, Highland Park, VT) with a reference wave length of 630 nm. Each serum sample was screened in duplicate wells.

The result for each serum sample was determined by taking the mean value of the absorbency readings for the duplicate wells. A sample was considered positive if the final value was equal to or higher than the defined cutoff.

**Results**

**Expression of GRA8**

The gra8 gene fragment corresponding to amino acids 23 to 169 was PCR amplified yielding a single specific product of 458 bp. The PCR product was cloned into the pET-28b(+) vector. The presence of gra8 gene
fragment in the recombinant plasmid was suggested by restriction digestion using EcoRV enzyme. Sequence analysis of the correct clone revealed 100% homology with the published sequence of gra8 (data not shown). In the pET-28b(+) GRA8 construct, the sequences encoding the amino terminal signal peptide (amino acids 1 to 23) and the C-terminal transmembrane domain were excluded. Moreover, a short sequence encoding 35 aa including a T7 tag and a cluster of hexa-histidine residues were fused into the N-terminus of the gene. A second hexa-histidine tag was placed in C-terminal fusion of the target gene to further facilitate the subsequent purification of the recombinant protein. SDS-PAGE analysis of induced bacteria showed expression of a protein band of 27 kDa, which was absent in uninduced bacteria (Figure 1). The discrepancy observed between the calculated molecular weight of GRA8 (20.2 kDa with 189 amino acids) and the apparent band on SDS-PAGE is a common feature of GRA proteins (21). High proline content of GRA8 (53 of 189 residues) also likely contributes to the aberrant electrophoretic migration (21).

To optimize the bacterial production of GRA8, the expression was carried out in three E.coli expression hosts: BL21(DE3), BL21(DE3)plysS and Rosetta(DE3) (Figure 1A) and three culture media: LB, 2XTY and TB (Figure 1B). The expression level of GRA8 seemed to be equal in the three bacterial hosts, however, the highest expression level of GRA8 was achieved in E.coli cells cultured in LB medium (Figure 1). To evaluate the solubility of GRA8, the recombinant Rosetta(DE3) cultivated in different medium were lyzed and fractionated into the soluble and insoluble proteins. Figure 1B showed that most of GRA8 in induced bacteria is in insoluble form, however, densitometry analysis using ImageJ software showed soluble expression of GRA8 was enhanced by cultivating in LB medium, as compared to the two other culture media (data not shown).

**Optimization of immobilized metal affinity chromatography (IMAC) for purification of GRA8**

The soluble fraction of GRA8 was purified using IMAC. In the preliminary experiments, we observed marked variations in the purity and yield of GRA8 at different pH values of binding buffer. Therefore, IMAC was performed at four pH value of 7.5, 8.1, 8.75 and
9.25 as explained in the Materials and Methods. SDS-PAGE analysis of protein samples showed that the purity of GRA8 was improved when pH of binding buffer was increased from 7.5 to 9.25, and the highest purity of 97.97% was achieved at pH value of 9.25 (Figures 2A and 2B). Similarly, yield of GRA8 was increased from 10 mg/l to 13.6 mg/ml when pH reached to 9.25 (Figure 2A).

Figure 2. Purification of TGRA8. A) Chromatograms of the immobilized metal affinity chromatography (IMAC) purification of TGRA8. The purification was performed by using the HisTrap FF column (5 ml) and buffers made at four pH values of 7.5, 8.1, 8.75 and 9.25. Initially, the column was equilibrated with the binding buffer (5 mM imidazole, 50 mM Tris–HCl, 500 mM NaCl). The soluble *E. coli* lysate was passed though the column and the unbound proteins were collected (Flow Through). The column was then washed step-wised with the binding buffer containing 35 (Wash 1), 60 (Wash 2) and 90 (Wash 3) mM imidazole. Absorbed material was eluted with the binding buffer containing 500 mM imidazole (Elution). The purity and yield of TGRA8 was improved when pH was increased from 7.5 to 9.25. The star mark possibly represents shows the peak of impurities. FT) flow through, W1: wash 1, W2: wash 2, W3: wash 3 and E: Elution. B) SDS-PAGE analysis of TGRA8 purified by IMAC at four different pH. Protein samples of unbound proteins (FT), wash 1 (W) and elution (E) fractions were analyzed. The purity of TGRA8 was considerably improved with increasing the pH from 7.5 to 9.25. The arrows represent bacterial proteins co-purified with TGRA8. The 54 kDa protein band, marked with a star sign, might be the dimmer of TGRA8 as it reacts with *Toxoplasma*-specific antibody on 12 % SDS-PAGE stained with Coomassie Blue. C) Silver nitrate staining of the purified TGRA8. At pH 9.25 TGRA8 was purified near to homogeneity. On silver stained gel only 2 specific bands of 27 and 54 kDa could be observed. M: Protein molecular weight marker.
The improved purity and yield of GRA8 at higher pH values was also evident from the corresponding chromatograms (Figure 2A). The peak of some impurities seen at pH=7.5 and 8.1 was disappeared or weakened at higher pH. In addition, the area under the peak of GRA8, representing the yield of protein, was apparently higher at pH=8.75 and 9.25, compared to the lower pH values. Moreover, the bacterial impurities co-purified with GRA8 were also decreased from pH=7.5 to pH=9.25 (Figure 2B). The same results were obtained when Ni-NTA agarose resin was used for batch purification of GRA8 (data not shown).

The silver nitrate staining of the GRA8 purified at pH=9.25 showed that protein was purified near to homogeneity (Figure 2C). The higher band revealed by silver nitrate staining, about 54 kDa, might be attributed to the dimmerization of GRA8. In fact, oligomerization of GRA proteins has been suggested in the previous studies (32-34).

**Antigenicity and immunogenicity evaluation of GRA8**

Antigenicity of GRA8 was investigated in Western blotting using pooled human sera representing the acute and chronic infection. The purified antigen strongly reacted with IgG and IgM antibodies in acute sera. No specific band, however, was observed on the blot probed with chronic sera (Figure 3A). The results demonstrated the antigenicity of GRA8 and its superior reactivity towards acute sera in comparison to chronic ones.

Moreover, the immunoreactivity of GRA8 was confirmed by Western blotting using the serum sample from a GRA8-immunized rabbit. On the blot of RH tachyzoite probed with anti-GRA8 serum, a single protein band of 35 kDa, the same size of the native GRA8, was detected. In contrast, there was no specific band on the blot of tachyzoites probed with the serum from a rabbit injected with the adjuvant alone (Figure 3B).

**Detection of acute toxoplasma infection by GRA8-IgM-ELISA**

A total number of 68 sera from pregnant women with acute and chronic infection were
Expression and Single-step Purification of GRA8 Antigen

The truncated GRA8 antigen was expressed in fusion with a small tag and expression of the protein was analyzed in different expression hosts and culture media. The single-step purification of GRA8 was performed by IMAC at the optimized pH, and led to the highly pure protein. Western blot analyses showed proper antigenicity and immunogenicity of the protein.

IgM ELISA using GRA8 was shown to be useful to distinguish between the acute and chronic toxoplasma infection. GRA8, amino acids 23 to 169 was produced in fusion with a small tag to avoid possible nonspecific reactivities of long fusion partners in ELISA tests \(^{(35)}\). The full-length GRA8 was difficult to express \(^{(22)}\) and exclusion of the amino terminal signal peptide and the C-terminal transmembrane domain enhanced the expressivity and solubility of the hydrophobic protein. The major drawback of using small

Table 1. Results of the TGRA8-IgM-ELISA and standard serologic tests on sera from Group I (acute-phase sera) pregnant women

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>IgM-ELISA Euroimmun (index) (^a)</th>
<th>IgG-ELISA Euroimmun (index) (^b)</th>
<th>IgM-ELISA GRA8 (OD(_{450}) value) (^c)</th>
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<td>1</td>
<td>2.78</td>
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<td>2</td>
<td>6.55</td>
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<td>4</td>
<td>2.17</td>
<td>2.24</td>
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<tr>
<td>5</td>
<td>8.20</td>
<td>2.20</td>
<td>0.31</td>
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<tr>
<td>6</td>
<td>1.64</td>
<td>6.25</td>
<td>0.22</td>
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<td>33</td>
<td>1.62</td>
<td>1.27</td>
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Results printed in boldface show positive ones according to the corresponding cut-off

\(^a\) Positive results were defined as indexes above 1.1
\(^b\) Positive results were defined as indexes above 1.1
\(^c\) Positive values were defined as readings equal to or above 0.31.

Discussion

The truncated GRA8 antigen was examined individually by GRA8-IgM-ELISA. A cut-off value (0.31) was determined on the basis of the mean value plus two standard deviations of the OD values of chronic sera. One (2.8%) of 35 chronic serum samples scored above the cut-off (No. 19, OD: 0.36), resulting in a test specificity of 97.1% (Table 1). Twenty of 33 acute serum samples had OD values equal to or higher than the cut-off, leading to a sensitivity of 60.6% for the assay (Table 1). These results indicated that IgM ELISA using the purified GRA8 was useful in discriminating acute from chronic infection.
fusion tags is low expression of the recombinant protein (35). In attempt to increase expression of GRA8, a variety of related parameters were analyzed; among them the media culture was the most influential.

The soluble fraction of GRA8 was purified using single-step IMAC. The pI of histidine residue is 6 and it is usually recommended to perform the purification at pH = 7 to 8 to make sure that the histidine residues are negatively-charged and allow binding to Ni$^{2+}$ ions. Nevertheless, in our setting for IMAC purification we observed that the optimum purity and yield of GRA8 was achieved at 9.25. The greater purity and yield in pH = 8.75 and 9.25, compared to those of pH = 7.5 and 8.1, were probably due to the more specific binding of GRA8 to the Ni$^{2+}$ resin. It is usually suggested by the manufacturers to avoid pH higher than 8 as additional protein-metal-coordinating groups, presumably surface amines, might contribute to chromatographic retention in IMAC at alkaline pH values and result in nonspecific binding to the resin. In contrast, we observed the highest purity of GRA8 in IMAC performed at pH = 9.25. Several studies reported the influence of pH in the performance of IMAC and found that the optimum pH might be higher than 7 to 8 (36).

The strong reactivity of the purified GRA8 toward sera from T.gondii acute-phase sera, as compared to chronic sera, is in agreement with previous studies. Besides, GRA8 was capable of induction of specific immune response in a rabbit verifying the presence of the immunodominant epitopes of the native protein in GRA8.

The performance of IgM ELISA using GRA8 for detection of acute toxoplasma infection was comparable with the previously published studies. Aubert et al used GRA8, amino acid 1-135 produced in fusion with CKS (CMP-2-keto-3-deoxy octulosonic acid synthetase), in IgM ELISA and reported a sensitivity of 46.1% (41 of 89 sera) for detection of acute infection in individuals with acute infection (20). A double-sandwich IgM ELISA using GRA8, produced in fusion with GST was developed and showed a sensitivity of 90% (18 of 20 sera) and specificity of 100% for discriminating recent from past Toxoplasma infection (19). Pfrepper et al from Mikrogen Company applied GRA8, amino acid 22-165, in IgM ELISA and showed sensitivity of 64.7% (44 of 68 sera) and specificity of 100% for detection of acute infection (18). In the present study, IgM ELISA using GRA8 showed a sensitivity and specificity of 60.6% and 97.1% for detection of acute infection.

The differences between the sensitivities reported in different studies might be attributed to the methods of antibodies detection (direct ELISA and double sandwich ELISA (19), the variations in cloning strategy of the selected epitopes and using the different local infection serum samples (3). Recently, GRA8 has been used for the development of a commercial immunoassay kit for the detection of specific toxoplasma IgG antibodies (37).

### Conclusion

Taking together, soluble GRA8 was produced with a very small fusion tag and purified near to homogeneity using an optimized single-step IMAC. Our simplified extraction and purification protocols resulted in reduced losses and increased yield and purity of the recombinant protein. An IgM ELISA using GRA8 was useful in differentiation between chronic and acute toxoplasmosis.

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### References


