Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Insect-cell expression, crystallization and X-ray data collection of the bradyzoite-specific antigen BSR4 from *Toxoplasma gondii*

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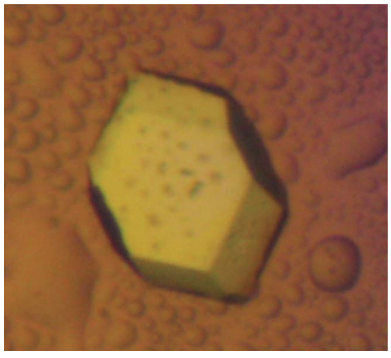
Received 12 March 2008
Accepted 18 April 2008



Toxoplasma gondii is an important global pathogen that infects nearly one third of the world's adult population. A family of developmentally expressed structurally related surface-glycoprotein adhesins (SRSs) mediate attachment to and are utilized for entry into host cells. The latent bradyzoite form of *T. gondii* persists for the life of the host and expresses a distinct family of SRS proteins, of which the bradyzoite-specific antigen BSR4 is a prototypical member. Structural studies of BSR4 were initiated by first recombinantly expressing BSR4 in insect cells, which was followed by crystallization and preliminary X-ray data collection to 1.95 Å resolution. Data processing showed that BSR4 crystallized with one molecule in the asymmetric unit of the $P4_12_12$ or $P4_32_12$ space group, with a solvent content of 60% and a corresponding Matthews coefficient of 2.98 Å^3 Da^{-1}.

1. Introduction

The protozoan parasite *Toxoplasma gondii* is an important global pathogen that infects nearly one third of the adult human population (Jackson *et al.*, 1987; McCabe & Remington, 1983). *T. gondii* infections can be fatal to the developing foetus and immunocompromised cancer, AIDS and organ-transplant patients and can cause severe ocular infections in both children and adults (Grigg *et al.*, 2001; Jackson *et al.*, 1987; Luft *et al.*, 1984; Luft & Remington, 1992; McDonald *et al.*, 1990; Nussenblatt & Belfort, 1994). In the agricultural industry, *T. gondii* infections are of considerable economic importance and are responsible for premature abortion in a wide range of animals destined for human consumption (Dubey, Brake *et al.*, 1986; Dubey *et al.*, 1984; Dubey, Murrell, Fayer *et al.*, 1986; Dubey, Murrell, Hanbury *et al.*, 1986).

The success of *T. gondii* largely arises from its ability to infect a broad range of host cells (Dubey *et al.*, 1998), which is in part mediated by a family of developmentally expressed antigenically distinct surface-antigen glycoproteins (SAGs). Prior to the release of the *Toxoplasma* genome, 21 SAG-related sequence (SRS) adhesins had been defined through gene discovery and mining of the *Toxoplasma*-expressed sequence tag (EST) database of 110 000 sequences (Lekutis *et al.*, 2000; Manger *et al.*, 1998). With the release of the *Toxoplasma* genome, more than 160 unique sequences belonging to the SRS superfamily have been identified (Jung *et al.*, 2004). In this latter study, classification as paralogous required that each sequence possessed either eight or 12 conserved cysteine residues, four conserved proline residues and a conserved tryptophan residue and shared at least 20% homology with the prototypical SAG1 surface antigen.

Detailed functional studies have identified at least eight members (including SAG1, BSR4, SAG2A, SRS2, SRS9 and SAG2C/D) of the SRS superfamily that are essential for successful infection, replication and persistence of *T. gondii* (Dzierszinski *et al.*, 2000; Grimwood & Smith, 1996; He *et al.*, 2002; Kim & Boothroyd, 2005; Mineo & Kasper, 1994; Mineo *et al.*, 1993). These SRS proteins are expressed either on the tachyzoite (lytic form, causing acute disease) or bradyzoite (highly infectious and tissue-cyst forming) form of *T. gondii*. To date, however, only the tachyzoite-specific SAG1

antigen has been structurally characterized and only in the apo form (He *et al.*, 2002). As a result, many structural details of the SRS superfamily, including the nature of the binding partner on the host cell, remain undefined.

To characterize the molecular strategies used by *T. gondii* to infect virtually any nucleated cell, a thorough structural characterization of the SRS-family members is required. As a first step toward this goal, the prototypical bradyzoite-specific antigen BSR4, which shows approximately 26.5% sequence identity to SAG1, has been expressed in insect cells, purified to homogeneity and crystallized, and preliminary X-ray diffraction data have been collected. This work represents the first reported characterization of a bradyzoite-specific antigen and is a key step in establishing a structural paradigm that defines the mode of action of the SRS superfamily.

2. Materials and methods

2.1. Cloning and production of recombinant baculovirus

The two tandem SAG domains of *bsr4* were amplified (forward primer, 5′-ATCGGATCCCGTGGAGGTGACTTCAAGGC-3′; reverse primer, 5′-AGCTCTAGAGTGATGGTGATGGTGATGGGCTTTGACAGTTACCAGC-3′) from *T. gondii* RH genomic DNA and cloned into *Bam*HI- and *Xba*I-cut pAcGP67A (Pharmingen). Sequencing confirmed that no mutations were introduced during amplification. Expression of BSR4 was carried out using the baculovirus insect-cell system. In the first step, a primary virus was generated by transfecting linear baculovirus DNA with the *bsr4*-pAcGP67A clone into *Spodoptera frugiperda* (sf9) insect cells. The recombinant primary virus encoding the *bsr4* gene was subsequently amplified to generate a high-titre secondary virus for use in expression studies.

2.2. Expression and purification

In total, 4 l of Hi5 insect cells (1.5×10^6) were infected with 1 ml of amplified *bsr4* virus per litre of culture (ExpressFive, Gibco) in the presence of tunicamycin (0.3 µg ml^{-1}), a potent inhibitor of N-linked glycosylation, for 60 h at 300 K. The supernatant was subsequently harvested using a two-step centrifugation process to remove cellular material and reduced in volume using tangential flow concentration. Ni–NTA resin was added to the concentrated supernatant and allowed to batch bind at 277 K. Fractions were eluted from the Ni–NTA resin in batch mode in buffer *A* (20 m*M* HEPES pH 7.5, 150 m*M* NaCl) supplemented with 150 m*M* imidazole and analyzed by SDS–PAGE and those that contained BSR4 were pooled and concentrated using Centricon (Millipore, Bedford, Massachusetts, USA) spin concentrators and injected onto an FPLC Superdex 75 Hi-Load 16/60 size-exclusion column in buffer *A*. Fractions were again analyzed by SDS–PAGE analysis, pooled based on purity and concentrated to 10 mg ml^{-1}. The final yield of purified BSR4 from the 4 l experiment was approximately 20 mg as determined by A_{280}. The final BSR4 protein extends from amino acids GSVEV (GS derived from the *Bam*HI site) at the N-terminus through VKAHHHHHH at the C-terminus to give a total molecular weight of 34 153 Da.

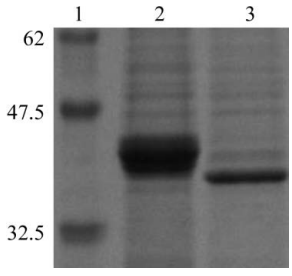
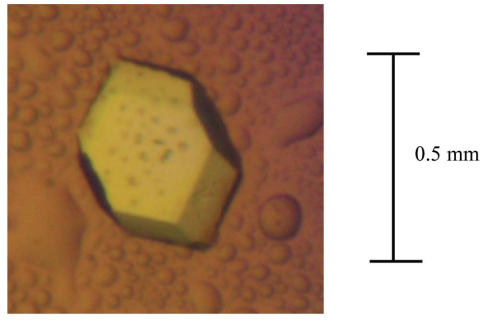


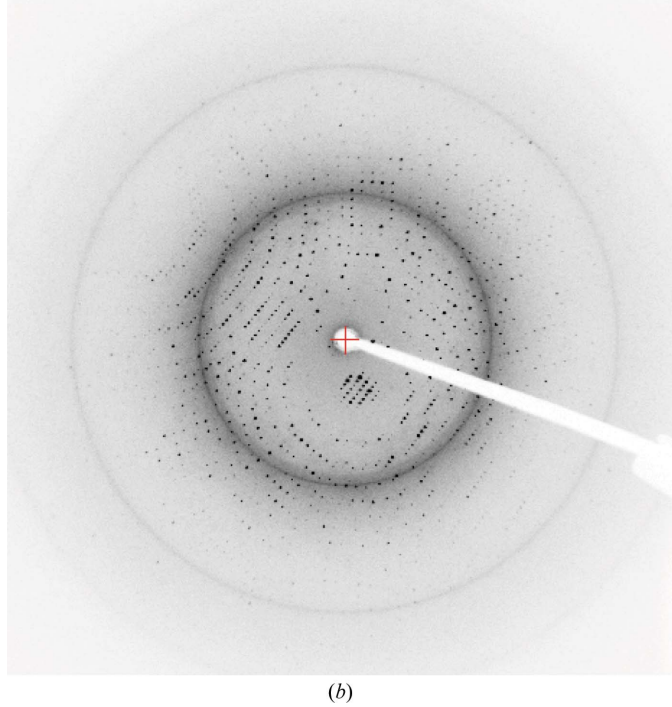
Figure 1
SDS–PAGE analysis of BSR4 expressed recombinantly in insect cells. Lane 1, molecular-weight markers (kDa). Lane 2 shows the fully glycosylated form of BSR4, which migrates as a diffuse band owing to the heterogeneity imparted by the N-linked glycosylation. Lane 3 shows the unglycosylated form of BSR4 expressed in the presence of tunicamycin, which migrates as a single band of lower molecular weight.

2.3. Crystallization

Crystallization trials were set up with Crystal Screens I and II and Index Screen (Hampton Research) in 96-well plates (Emerald Biosystems). The final drops consisted of 1.5 µl protein (at 10 mg ml^{-1}) in buffer *A* and 1.5 µl reservoir solution and were equilibrated against 100 µl reservoir solution. Crystals of BSR4 were observed after 2 d in 18% PEG 8000, 100 m*M* sodium cacodylate pH 6.5 and 100 m*M* zinc acetate. Crystals of BSR4 grew to maximum dimensions of 0.5 × 0.4 × 0.2 mm after 7 d at 293 K.



(*a*)



(*b*)

Figure 2
(*a*) BSR4 crystal grown in 18% PEG 8000, 100 m*M* sodium cacodylate pH 6.5 and 100 m*M* zinc acetate. (*b*) X-ray diffraction image of BSR4 to 1.95 Å resolution.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell (2.02–1.95 Å).

Space group	$P4_12_12$ or $P4_32_12$
Unit-cell parameters (Å, °)	$a = b = 92.05$, $c = 98.31$, $\alpha = \beta = \gamma = 90$
Wavelength (Å)	1.54
Resolution (Å)	33.60–1.95
Measured reflections	366686
Unique reflections	31408
Redundancy	11.67 (11.39)
Completeness	100 (100)
$I/\sigma(I)$	7.1 (2.2)
$R_{\rm merge}$† (%)	0.061 (0.434)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the average over all observations of reflection hkl.

2.4. Data collection and processing

A single BSR4 crystal (Fig. 2*a*) was looped into cryoprotectant consisting of mother liquor supplemented with a mixture of 10% glycerol and 10% ethylene glycol for 10 s and flash-cooled directly in the cryostream (100 K). Diffraction data were collected on a Rigaku R-AXIS IV^{++} area detector coupled to an MM-002 X-ray generator with Osmic 'blue' optics and an Oxford Cryostream 700. Each of the 500 images was exposed for 120 s over an oscillation range of 0.5°. Diffraction data to 1.95 Å (Fig. 2*b*) were processed using *CrystalClear*/*d*TREK* (Pflugrath, 1999). Data-collection statistics are presented in Table 1.

3. Results and discussion

The two tandem SAG domains of the bradyzoite-specific antigen BSR4 from *T. gondii* encode a total of 12 cysteines that participate in six disulfide bonds. Despite attempts to express BSR4 in the soluble fraction in *E. coli*, no soluble protein was observed. In this study, however, soluble BSR4 was successfully expressed using the baculovirus insect-cell system. SDS–PAGE analysis of the purified BSR4 showed a diffuse band that migrated with a larger molecular weight than the predicted from the sequence, which is consistent with the incorporation of N-linked glycosylation at one or more of the three predicted sites. While glycosylation can increase protein solubility, leading to greater expression levels, the heterogeneity imparted by these glycosylations can often result in poorly formed crystal lattices that show low-resolution X-ray diffraction. To eliminate the N-linked glycosylation, BSR4 was expressed in the presence of tunicamycin, a chemical that uncouples the N-linked glycosylation pathway. This approach proved successful, with the deglycosylated BSR4 migrating as a single band of lower molecular weight on SDS–PAGE (Fig. 1). Crystallization trials with BSR4 lacking N-linked glycans resulted in crystals that grew to dimensions of 0.5 × 0.4 × 0.2 mm within one week and diffracted to 1.95 Å resolution. Processing of the data (Table 1) showed that BSR4 crystallized with one monomer in the asymmetric unit of the $P4_12_12$ or $P4_32_12$ unit cell, with a solvent content of 47% and a Matthews coefficient of 2.32 Å^3 Da^{-1} (Matthews, 1968). Scaling and merging of the data resulted in an overall $R_{\rm merge}$ of 6.1%, with a value of 43.4% in the highest resolution shell (2.02–1.95 Å).

This work was supported by a research grant from the Canadian Institutes for Health Research (CIHR) to MJB and by the Intramural Research Program of the NIH and NIAID (MEG). MJB is a CIHR New Investigator award and a Michael Smith Foundation for Health Research (MSFHR) scholar. MEG is a scholar of the Canadian Institute for Advanced Research (CIFAR) Program for Integrated Microbial Biodiversity.

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