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Crystallization and preliminary X-ray analysis of Na-SAA-2 from the human hookworm parasite *Necator americanus*

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Human hookworms are among the most pathogenic soil-transmitted helminths. These parasitic nematodes have co-evolved with the host and are able to maintain a high worm burden for decades without killing the human host. However, it is possible to develop vaccines against laboratory-challenge hookworm infections using either irradiated third-state infective larvae (L3) or enzymes from the adult parasites. In an effort to control hookworm infection globally, the Human Hookworm Vaccine Initiative, a product-development partnership with the Sabin Vaccine Institute to develop new control tools including vaccines, has identified a battery of protein antigens, including surface-associated antigens (SAAs) from L3. SAA proteins are characterized by a 13 kDa conserved domain of unknown function. SAA proteins are found on the surface of infective L3 stages (and some adult stages) of different nematode parasites, suggesting that they may play important roles in these organisms. The atomic structures and function of SAA proteins remain undetermined and in an effort to remedy this situation recombinant Na-SAA-2 from the most prevalent human hookworm parasite *Necator americanus* has been expressed, purified and crystallized. Useful X-ray data have been collected to 2.3 Å resolution from a crystal that belonged to the monoclinic space group $C2$ with unit-cell parameters $a = 73.88$, $b = 35.58$, $c = 42.75$ Å, $\beta = 116.1^\circ$.

1. Introduction

Human hookworm infection affects an estimated 576–740 million people in tropical and subtropical regions of the developing world (de Silva *et al.*, 2003; Bethony *et al.*, 2006). Human hookworm infection is a leading global cause of anemia and malnutrition, and is one of the three major soil-transmitted helminth (STH) infections (Hotez *et al.*, 2004). The life cycle of hookworms involves a free-living or soil-based stage. Currently, the major approach to STH infection control worldwide relies on antihelminthic chemotherapy programs to reduce the number of worms in the gastrointestinal tract of humans, especially children. School-based antihelminthic chemotherapy programs may not effectively control hookworm infection because of (i) the high rates of hookworm re-infection following drug treatment (Albonico *et al.*, 1995), (ii) high rates of drug failure with mebendazole and the diminishing efficacy of the drug with repeated use (Albonico *et al.*, 2003; Keiser & Utzinger, 2008), possibly because of drug resistance (Albonico, 2003), and (iii) the high prevalence and intensity of hookworm infection among both pediatric and adult populations, most notably women of reproductive age (Bundy *et al.*, 1995).

The Human Hookworm Vaccine Initiative (http://www.sabin.org), a non-profit product-development partnership based at the Sabin Vaccine Institute, has a mission to develop an antihookworm vaccine with the aim of reducing worm burdens, parasite intensity and blood loss (Hotez *et al.*, 2003). While one major important approach to developing vaccines relies on inhibiting blood feeding at the site of adult parasite attachment (Loukas *et al.*, 2006), a second strategy is based on the selection of antigens, based partly on the early obser-

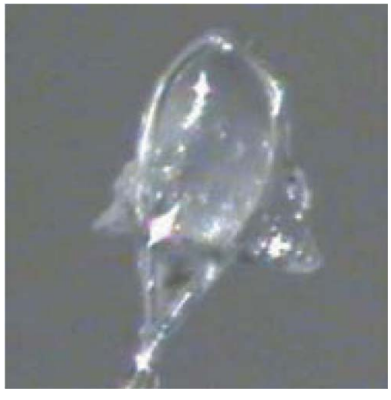


Table 1
Sequence identity of SAA proteins.

Ac16, *Ancylostoma caninum* immunodominant hypodermal antigen (GenBank accession No. ABD98404.1); As14, *Ascaris suum* antigen (BAB67769.1); Ani9, *Anisakis simplex* allergen precursor (ABV55106.1); Ral-2, *Acanthocheilonema viteae* homolog antigen (AAB53809.1); Ov17, *Onchocerca ochengi* rainforest immunodominant hypodermal antigen (ACB70199.1); SXP-1, *Brugia malayi* antigen (AAX18863.1); WB14, *Wuchereria bancrofti* antigen (AAC17637.1). The sequences are numbered according to their order in the alignment in Fig. 1.

Sequence *A*	Name	Length (amino acids)	Sequence *B*	Name	Length (amino acids)	Score (%)
1	Na-SAA-2	146	2	Ac16	147	73
1	Na-SAA-2	146	3	As14	146	46
1	Na-SAA-2	146	4	Ani9	147	36
1	Na-SAA-2	146	5	Ral-2	154	32
1	Na-SAA-2	146	6	Ov17	164	34
1	Na-SAA-2	146	7	SXP-1	148	34
1	Na-SAA-2	146	8	WB-14	153	28

vation that the infective stages of the parasite, the third-stage larvae (L3), are immunogenic (Miller, 1971) and that radiation-attenuated L3 have successfully been used as a veterinary vaccine for canine hookworm infection (Miller, 1978). An early objective of human hookworm vaccine development was to reproduce irradiated larval vaccines with genetically engineered, recombinant, secreted antigens (Hotez *et al.*, 2003). The two major antigens secreted by L3 upon host entry were initially identified as *Ancylostoma* secreted protein 1 (ASP-1) and *Ancylostoma* secreted protein 2 (ASP-2) (Hotez *et al.*, 2003). We recently solved the structure of both ASP-1 (work to be published) and ASP-2 (Asojo *et al.*, 2005) from *Necator americanus*. Furthermore, this ASP-2 is undergoing clinical trials as a hookworm vaccine candidate (Hotez *et al.*, 2003; Loukas *et al.*, 2006).

Recently, other vaccine candidates have been identified, including the surface-associated antigen (SAA) family of proteins. Ac-SAA-1 and Ac-SAA-2 both immunodominant 16 kDa surface-associated antigens of *Ancyclostoma caninum* infective larvae and adults, have shown promise as canine vaccine antigens (Fujiwara *et al.*, 2007). Consequently, studies are under way to characterize the potential of the SAA from the major human hookworm *N. americanus* (Na-SAA-2) as a vaccine candidate. While homologues of this protein have been found in other parasites including *Oncocerca*, *Ascaris*, *Loa loa*, *Wuchereria bancrofti*, *Brugia malayi*, *Globodera rostochiensis* and *Anisakis simplex*, the basic biology of these proteins has yet to be elucidated. SAA2 proteins from different parasites share considerable sequence identity (Fig. 1; Table 1) and are characterized by a 13 kDa conserved domain of unknown function, DUF148. SAA proteins are released by the infective L3 stages of these parasites, suggesting that they may play important roles in these organisms. In order to clarify the structures and functions of SAAs, we have expressed, purified and crystallized recombinant Na-SAA-2. Here, we present these preliminary studies.

2. Materials and methods

2.1. Cloning

Rabbit antiserum against Ac16, a 16 kDa surface-associated antigen of the canine hookworm *A. caninum* (Fujiwara *et al.*, 2007), was used to immunoscreen a cDNA expression library of adult *N. americanus*. A positive clone with 72% amino-acid identity to Ac16 was identified and dubbed Na-SAA-2 (surface-associated antigen 2 of *N. americanus*). The entire coding sequence without the N-terminal signal peptides of *Na-saa*-2 was PCR-amplified from the first-strand cDNA of adult *N. americanus* with *Na-saa*-2 gene-specific primers Na16-F2, 5′-CTCTCGAGAAAAGACAGGGACCACAAGGACCCCC-3′, and Na16-R2, 5′-TCTCTAGATTATCCTTTCATGGCGTTTTCGA-3′. The PCR product was subcloned into the *Pichia* expression vector pPICZαA (Invitrogen) through *Xho*I and *Xba*I sites. The *Na-saa-2* gene was fused to the pre-pro-leader sequence of *Saccharomyces cerevisiae* α-mating factor under the control of the AOX1 promoter. The construct sequence was confirmed by sequencing the insert. *Na-saa*-2/pPICZaA recombinant DNA was linearized with digestion of *Sac*I before transformation into *P. pastoris* X-33. Transformants were selected on YPD plates containing Zeocin (100 mg l^{-1}). The highest expression clone was

Figure 1
Na-SAA-2 has homologues in other parasites. The multiple sequence alignment was produced using *Clustal* v.2.0.11. Identical residues are marked with asterisks. Residues marked ':' are more highly conserved than those marked '.'. Abbreviations are defined and sequence identities are given in Table 1.

cultured in YPD medium to an OD_{600} of 10 to generate the Research Cell Bank (RCB) in YPD and 20% glycerol.

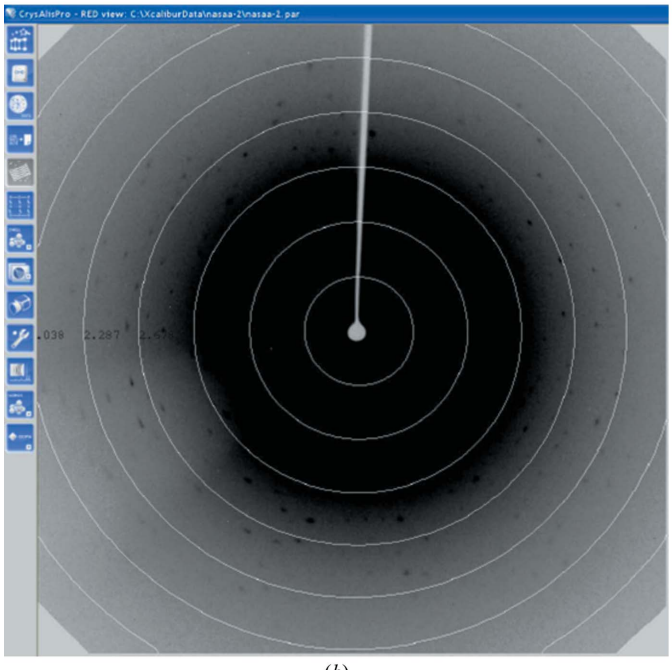
2.2. Fermentation

2 ml of the seed culture (RCB) was aseptically transferred to a 2.5 l Tunair shake flask containing 1 l BMG (buffered minimal glycerol medium; 1.34% yeast nitrogen base and 1% glycerol, 4×10^{-5}% D-biotin and 0.1 *M* potassium phosphate buffer pH 6.0). This starter culture was incubated at 303 ± 2 K for 27 h to a final OD_{600} of 8. Approximately 650 ml of this culture was used to inoculate 5 l of heat-sterilized basal salt media (BSM) containing 3.5 ml of a filter-sterilized trace-element solution per litre. Fermentations were performed in a BioFlo 3000 fermentor (New Brunswick Scientific, Edison, Georgia, USA). The pH of the BSM was adjusted to and maintained at 5.0 with a 14% ammonium hydroxide feed. The dissolved oxygen was maintained at 30% throughout the fermentation. Cells were grown at 303 ± 1 K with an agitation speed of 500 rev min^{-1}. Approximately 17 h into the glycerol phase and after a sharp increase in the percentage of dissolved oxygen, 50%(*v*/*v*) glycerol was introduced into the cell-culture medium at a set flow rate of 15 g l^{-1} h^{-1} for 6 h. The pH of the cell-culture media was then increased linearly from 5.0 to 6.0 by adding 14% ammonium hydroxide over a 2 h period before the completion of the fed-batch glycerol phase. The temperature was also decreased linearly from 303 to 289 K over a 2 h period before the completion of the fed-batch glycerol phase. Excessive foaming was controlled with a feed-on demand of 10%(*v*/*v*) antifoam KFO673 in deionized water (KABO Chemicals Inc). The methanol induction phase was initiated when the wet cell weight (WCW) reached approximately 210 g l^{-1}. The agitation speed increased to 700 rev min^{-1} and methanol was added at an initial flow rate of 1.5 ml l^{-1} h^{-1}, increasing to 11.0 ml l^{-1} h^{-1} over an 8 h period. Subsequently, a steady flow of methanol was continued at a flow rate of 11 ml l^{-1} h^{-1} for another 57 h. The final volume of cell culture harvested was 9.25 l and the measured WCW was 355 g l^{-1}. The fermentation cell culture was diluted with 4 l buffer *A* (20 m*M* Tris–HCl buffer pH 8.5) and concentrated to 6 l. The concentrated *P. pastoris* cells were washed four times with 6 l buffer *A* using a 0.075 µm microfiltration hollow-fiber cartridge and a Masterflex pump (Cole–Parmer Instrument Co.). Approximately 36.3 l of supernatant was collected. This supernatant was concentrated to 2 l of concentrated fermentation supernatant (CFS) by ultrafiltration using an Ultrafiltration unit consisting of Masterflex Pump and a 3 kDa hollow-fiber cartridge (GE Healthcare, Piscataway, New Jersey, USA). The CFS was flash-frozen and stored at 193 K until purification.

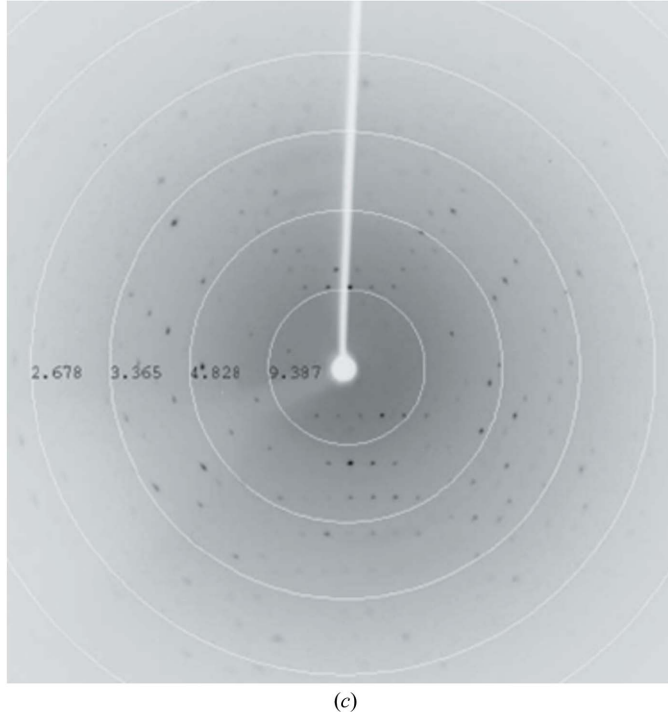
2.3. Purification

Na-SAA-2 was purified by sequential steps of anion-exchange chromatography followed by gel filtration on an ÄKTA Explorer Air 100 with *UNICORN* software (GE Healthcare, Piscataway, New Jersey, USA). Prior to anion-exchange chromatography, 500 ml CFS (equivalent to 2.3 l of fermentation cell culture) was thawed and diluted to 970 ml by adding 470 ml buffer *A* (20 m*M* Tris–HCl pH 8.5)

(*a*)



(*b*)



(*c*)

Figure 2
Diffraction quality of Na-SAA-2 crystals. (*a*) A typical Na-SAA-2 crystal mounted in a 0.3 mm cryo-loop. The crystal is 0.5 mm in the longest dimension and less than 0.1 mm in the smallest dimension. (*b*) The crystal diffracted to beyond 2.3 Å resolution. (*c*) The diffraction pattern from the image in (*b*) indicates that our crystal is of a protein and not of a salt.

to obtain dilute CFS at 2.5 mS cm^{-1}. 190 ml of this sample was loaded onto a 160 ml Q Sepharose XL column anion-exchange column prepared according to the manufacturer's instructions and equilibrated with buffer *B* (20 m*M* Tris–HCl, 17 m*M* NaCl pH 8.5) at room temperature at a flow rate of 15 ml min^{-1}. After sample loading, the column was washed with buffer *B* and the flowthrough containing partially purified Na-SAA-2 was collected. The purification step was repeated three times, regenerating the column each time. The flowthrough fractions containing Na-SAA-2 from three cycles of purification were pooled and concentrated to volume of 31 ml and a concentration of 2.1 mg ml^{-1} using a 3 kDa nominal molecular-weight cutoff hollow-fiber cartridge (GE Healthcare, Piscataway, New Jersey, USA).

Gel filtration was carried out using a 330 ml Superdex 75 column that was prepared according to the manufacturer's instructions and equilibrated with buffer *C* (50 m*M* sodium acetate pH 6.0) at a flow rate of 2.7 ml min^{-1}. For each run, 9 ml concentrated sample from anion-exchange chromatography was loaded onto the Superdex 75 column and eluted (isocratically) with buffer *C*. The peak fraction containing pure Na-SAA-2 was collected. Two further cycles of purification were carried out with the remaining fractions containing Na-SAA-2 from anion-exchange chromatography, re-equilibrating the Superdex 75 column each time. Fractions containing pure Na-SAA-2 from three cycles of purification were combined. A total of 113 mg Na-SAA-2 was obtained from 2.3 l *Pichia* cell culture (72 ml at a concentration of 1.57 mg ml^{-1}).

2.4. Crystallization

Initial crystallization screens were carried out on samples of Na-SAA-2 using sparse-matrix screens from Qiagen and Hampton Research, including Classic Screens I and II, Index Screen II, Natrix, MbClassic, Crystal Screens I and II and all Cryo Screens. These experiments were carried out at three different temperatures: 293, 285 and 277 K. Crystal growth was independent of growth temperature. Crystals were grown by vapor diffusion in hanging drops, which were equilibrated against 1 ml crystallization solution. The crystallization plate used was a Nextal Easy Xtal tool (Qiagen USA). Drops were prepared by mixing 2 µl protein solution with an equal volume of crystallization solution. All protein solutions for crystallization experiments consisted of 5–60 mg ml^{-1} Na-SAA-2 in 50 m*M* acetate pH 6.1. The initial protein concentration was confirmed spectrophotometrically prior to setting up crystallization experiments. Only one condition, 2.1 *M* DL-malic acid, gave diffraction-quality crystals from over 1000 screened conditions. The best crystals were obtained in three weeks by vapor diffusion from hanging drops containing a mixture of 3 µl (50 mg ml^{-1}) recombinant Na-SAA-2 and 1.5 µl DL-malic acid. The largest of these crystals were 0.5 × 0.3 × 0.05 mm in size and an example is illustrated in Fig. 2(*a*).

2.5. Diffraction experiments

Since crystals grew in solutions that contained adequate cryoprotectant, the crystals were flash-cooled in a stream of N_2 gas at 113 K prior to collecting diffraction data. Data sets were collected using an Xcalibur PX Ultra four-circle kappa platform with a 165 mm diagonal Onyx CCD detector and a high-brilliance sealed-tube Cu Enhance Ultra X-ray source (Oxford Diffraction, Oxford, England) operating at 40 kV and 40 mA. A diffraction image showing the crystal quality is illustrated in Fig. 2. Complete data sets were collected from single crystals using a crystal-to-detector distance of 65 mm and exposure times of 5 min per 0.5° oscillation. X-ray data sets were processed using the program *CrysAlis^{Pro}* (Oxford Diffraction). Crystallographic data are shown in Table 2. Based on the estimated Matthews probability and solvent-content prediction (http://www.ruppweb.org/Mattprob), we expect a monomer to be present in the asymmetric unit (Matthews, 1968; Kantardjieff & Rupp, 2003). The presence of a monomer in the asymmetric unit is based on the volume of the unit cell (*C*2) being 101 647 Å^3; this would correspond to a Matthews coefficient of 1.86 Å^3 Da^{-1} (33.9% solvent).

Table 2
Data-collection and reduction statistics.

Values in parentheses are for the highest resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 73.88, b = 35.58, c = 42.75, β = 116.1
Resolution limits (Å)	50–2.29 (2.38–2.29)
$I/\sigma(I)$	17.2 (2.62)
No. of reflections	62340
No. of unique reflections	10785
Redundancy	5.8 (5.8)
$R_{\rm merge}$† (%)	6.4 (34.6)
Completeness (%)	99.1 (98.9)
Unit-cell volume (Å^3)	101647
Matthews coefficient (Å^3 Da^{-1})	1.86

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the intensity of the ith observation and the mean intensity of the reflection with indices hkl, respectively.

3. Results and discussion

We have successfully cloned, expressed and purified Na-SAA-2, the surface-associated antigen from the major human hookworm *N. americanus*, using the *P. pastoris* expression system, yielding a 13 kDa protein which we have crystallized in space group *C*2. Sample crystals permitted the collection of data to 2.29 Å resolution in the home laboratory. Since there are no known homologous structures, we cannot generate phases by molecular replacement, so cocrystallization, soaking and cloning efforts are under way to generate heavy-atom derivatives for direct phasing. SAA proteins are released by the infective larval stages of parasites, suggesting that they may play important roles in these organisms, and we hope that our structure determination will facilitate the characterization of these roles as well as aid in the development of this family of proteins as potential vaccines.

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