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SHORT COMMUNICATION

Molecular characterization of a *Spirometra mansoni* antigenic polypeptide gene encoding a 28.7 kDa protein

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Abstract The *Spirometra mansoni* antigenic polypeptide (SmAP) gene was expressed in *Escherichia coli*, and its characteristics and value as an antigen for the serodiagnosis of sparganosis were investigated. The recombinant SmAP protein (rSmAP) has the molecular weight of 28.7 kDa. On Western blotting analysis, the rSmAP strongly reacted with the sera of mice infected with spargana, but not with normal sera; the anti-rSmAP serum obviously recognized the 28.7-kDa band in the crude antigens and excretory–secretory (ES) antigens of spargana. The immunofluorescence test (IFT) results showed that the positive staining was observed at different stages of spargana from the infected frogs and mice, but not adult worm of *S. mansoni.* An immunolocalization analysis identified SmAP in the teguments and parenchymal tissues of spargana. ELISA with rSmAP antigen or sparganum ES antigens were evaluated for the serodiagnosis of sparganosis. The results showed that the sensitivity of rSmAP-ELISA and ES-ELISA was 83.3 % (25/30) and 100 % (30/30), respectively, for the detection of anti-sparganum IgG antibodies in sera of the experimentally infected mice ($P>0.05$), the specificities of both ELISA were 100 % (67/67). It is suggested that the rSmAP might be a potential candidate antigen for serodiagnosis of sparganosis.

Keywords *Spirometra mansoni* · Sparganum · Sparganosis · Antigenic polypeptide · Serodiagnosis

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Introduction

Sparganosis is a serious parasitic zoonosis caused by invasion of the spargana, the plerocercoid larvae of various diphyllobothroid tapeworms belonging to the genus *Spirometra* (Nithiuthai et al. 2004). The most important species of the genus *Spirometra* include *Spirometra mansoni* (syn. *Spirometra erinaceieuropaei* or *Spirometra erinacei*) which is the most common in Asia, and *Spirometra mansonoides* is mainly distributed in North America (Roberts et al. 2009). Human infections are acquired by drinking water contaminated with cyclops harboring procercoids, ingesting raw or undercooked flesh of frogs and snakes infected with plerocercoids, or placing frog or snake flesh on open wound for treatment of skin ulcers or eye inflammation (Fukushima and Yamane 1999; Magnino et al. 2009). Sparganosis is distributed worldwide, but most cases occur in Southeast Asia, Japan, Korea, and Thailand (Anantaphruti et al. 2011). In the People's Republic of China, sparganosis is an important food-borne parasitic zoonosis and poses a serious threat to human health, with more than 1,000 human cases reported in 27 out of 34 provinces, autonomous regions, or municipal districts (Cui et al. 2011b).

The preoperative diagnosis of sparganosis is rather difficult and often misdiagnosed because the larvae have no predilection site in humans and the characteristic clinical manifestations are lacking. A definite diagnosis of subcutaneous sparganosis can be achieved by detection of the larvae in a biopsy specimen from the lesion, but the confirmative diagnosis is very difficult for visceral and cerebral sparganoses since the larva is found only by surgical removal (Murata et al. 2007). ELISA using the crude or excretory–secretory (ES) antigens of plerocercoids has high sensitivity for the detection of sparganum infection in humans, but the main disadvantage is the false negative results during the early stage of infection and the cross-reactions with sera from patients with other

parasitic diseases (viz. anisakiasis, cysticercosis, paragonimiasis and clonorchiasis) (Cui et al. 2011a; Hu et al. 2013; Nishiyama et al. 1994). In addition, the preparation of the crude or ES antigens of sparganum for ELISA requires collection of spargana from naturally infected intermediate hosts or experimentally infected laboratory animals, which is practically inconvenient in terms of cost, labor, and time.

Recombinant proteins are a good alternative to the crude or ES antigens as they can be produced easily in large amounts by using a bacterial expression system and can be used as an antigen in a sensitive, specific, and standardized ELISA for serodiagnosis of sparganosis (Rahman et al. 2014). Therefore, development of a sensitive and specific recombinant sparganum antigen will improve the early diagnosis and subsequent treatment of the disease.

The *S. mansoni* antigenic polypeptide (SmAP) gene (GenBank accession No. AB019222.1) was immunoscreened from the complementary (c)DNA library from *S. mansoni* spargana by the sera of sparganum-infected mice. The full-length cDNA sequence of the SmAP gene was 1,084 bp, contained an 828 bp open reading frame (ORF) encoding a protein of 276 amino acids (Liu et al. 1999). In the present study, the SmAP gene encoding a 28.7 kDa protein from *S. mansoni* spargana was cloned, expressed, and characterized, and the recombinant SmAP protein (rSmAP) as a potential candidate antigen for serodiagnosis of sparganosis was investigated.

Materials and methods

Parasites and experimental animals

S. mansoni spargana used in this study were obtained from naturally infected frogs in Henan Province. The spargana were maintained by serial passage in BALB/c mice every 10–12 months. Specific pathogen-free (SPF) female BALB/c mice aged 6 weeks were purchased from the Experimental Animal Center of Henan Province and used for the immunological studies and challenge infection. All of the animal experiments reported herein were approved by The Life Science Ethics Committee of Zhengzhou University.

Serum samples

Serum samples of mice infected with two spargana of *S. mansoni* were collected 35 days post-infection (dpi). Fifteen serum samples of mice infected with 300 muscle larvae of *Trichinella spiralis* were collected 42 days post-infection. Sixteen serum samples of mice infected with *Schistosoma japonicum* and six serum samples of mice infected with *Toxoplasma gondii* were gifted by Prof. JH Lei and Dr. T Wang (Tongji Medical College of Huazhong University of Science and Technology) and Prof. GR Yin (Shanxi Medical University), respectively. Thirty serum samples from normal mice were used as negative control.

Collection of worms and preparation of crude and ES antigens

The spargana were collected from the subcutaneous tissues and muscles of the experimentally infected mice. The sparganum crude antigens were prepared as described previously (Hu et al. 2014; Kong et al. 1994). Briefly, spargana were homogenized in sterile normal saline solution with a glass tissue grinder with for 1 h and then sonicated with an ultrasonic disintegrator at 4 °C for 5 min. The supernatant was obtained by centrifugation at 15,000×*g* for 1 h; the dialysis against deionized water was conducted at 4 °C for 2 days. The protein concentration (3.4 mg/ml) of the supernatant was assayed by the method determined by Bradford (1976). The preparation of sparganum ES antigens were performed as described previously (Cui et al. 2011a). In brief, after washing thoroughly in sterile normal saline solution and serum-free RPMI-1640 medium supplemented with 100 U penicillin per milliliter and 100 U streptomycin per milliliter, the spargana were incubated in a 75-cm^2 culture dish with the same medium at concentration of two worms per milliliter for 18 h at 37 °C in 5 % CO_2. After incubation, the media that contained the ES products were filtered through a 0.2-μm membrane into a 50-ml conical tube and then centrifuged at 4 °C, 15,000×*g* for 30 min. The supernatant was dialyzed and then lyophilized by a vacuum concentration and freeze-drying (Heto Mxi-Dry-Lyo, Denmark). The ES antigens were diluted to a concentration of 1 mg/ml and stored at −20 °C before use.

Cloning, expression, and identification of SmAP

The sequences of the SmAP was synthesized by the GENEWIZ Inc. (Beijing, China) and the *Bam*HI and H*ind*III restriction sites were added at the 3′ and 5′ ends of sequences and cloned into the pUC57-Kan vector (GENEWIZ Inc., Beijing, China). After preliminary identification, the target sequence was subsequently sub-cloned into the expression vector pMAL-c2X (New England Biolabs, USA). The recombinant plasmid was then transformed into *Escherichia coli* BL21 (Novagen, USA). The expression of the recombinant protein was induced with 0.2 mM IPTG for 4 h at 37 °C. The pellets of the bacterial culture were harvested following the induced incubation and disrupted by sonication in 20 mM Tris–HCl/0.2 mM NaCl buffer (pH 7.4), and the recombinant SmAP protein (rSmAP) was expressed in supernatant. The rSmAP was purified by Amylose Pre-packed Column (NEB Ltd, China). The purified recombinant proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) using a 5 % acrylamide stacking gel and 10 % acrylamide separating gel (83×73×1.0 mm) with a Mini-

PROTEAN 3 Cell electrophoresis unit (Bio-Rad, USA) at 120 V for 2.5 h (Wang et al. 2011). After electrophoresis, the gel was stained with 0.25 % Coomassie brilliant blue R-250 for 4 h and then destained (10 % acetic acid and 5 % ethanol). Another gel was prepared in the same way and used for the Western blotting described below.

Generation and determination of antibodies to rSmAP

Polyclonal antibodies to rSmAP were produced in ten female BALB/c mice injected subcutaneously with approximately 20 μg of the rSmAP with the equal volume of complete Freund's adjuvant, followed by three boosts with the same dose with the incomplete adjuvant at 10-day intervals. Seven days after the last boost, the mice were euthanized, and the sera were collected. The specific IgG antibodies to rSmAP in serum samples of immunized mice were determined by ELISA using rSmAP (2.5 μg/ml). The procedure of ELISA was performed as previously described (Cui et al. 2013).

Western blot analysis

Samples including crude and ES antigens of spargana and the rSmAP were separated by SDS–PAGE using a 5 % acrylamide stacking gel and 12 % acrylamide separating gel (83 × 73 × 1.0 mm) with a Mini-PROTEAN 3 Cell electrophoresis unit (Bio-Rad, USA) at 120 V for 2.5 h and then transferred onto nitrocellulose membranes (Millipore, USA) using a trans-blot SD transfer cell (Bio-Rad, USA) (Wang et al. 2011; Wang et al. 2013). The membranes were cut into strips, blocked with 5 % skimmed milk in Tris-Buffered Saline with Tween-20 (TBST) at 37 °C for 1 h, and incubated at 37 °C for 1 h with 1:100 dilutions of different mouse sera (anti-rSmAP serum, infection serum, and normal mouse serum). After being washed, the strips were incubated at 37 °C for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5,000 dilution; Southern Biotechnology, USA), and finally with 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma).

Immunofluorescence test

Immunofluorescence test (IFT) was used to observe the expression of SmAP at different developmental stages and immunolocalization in the parasite. The adults and spargana of *S. mansoni* were fixed respectively in 4 % paraformaldehyde and embedded in paraffin. Microtome-cut 4-μm sections were placed on slides, deparaffinized in xylene, and rehydrated. The parasite sections were blocked with 5 % normal goat serum in PBS and then incubated in a moist chamber at 37 °C for 1 h with a 1:10 dilution of immune, infection, and normal sera. After washing three times in PBS, the sections were incubated with a 1:50 dilution of FITC-labeled goat anti-mouse IgG (Santa Cruz, USA), washed five times in PBS, and examined under a fluorescent microscope (Olympus, Japan) (Zhang et al. 2013).

Enzyme-linked immunosorbent assay

The anti-sparganum IgG antibodies in serum samples of mice infected with spargana and other parasites were determined by ELISA using rSmAP protein or sparganum ES antigens. The procedure was performed as previously described (Cui et al. 2011a). Briefly, microtiter plates (Nunc) were coated with rSmAP proteins (6 μg/ml) or ES (2.5 μg/ml) in coating buffer overnight at 4 °C and blocked with 200 μl of PBS-0.1 % Tween 20 (PBST) containing 5 % skimmed milk. Then, 100 μl of serum samples with 1:100 dilutions in PBS were added to each well and incubated at 37 °C for 1 h. HRP-conjugated goat anti-mouse IgG antibodies (1:5,000; Southern Biotechnology, USA) were added and incubated for 1 h at 37 °C. The plates were developed with *o*-phenylenediamine dihydrochloride substrate (OPD; Sigma), and the absorbance was measured at 490 nm. All samples were run in duplicate. The ratio <2.1 of samples to be tested/negative sample (optical density (OD) values of the samples to be tested divided by OD of the negative, S/N <2.1) were regarded as negative and S/N ≥2.1 as positive. The cutoff values of rSmAP proteins and ES antigen ELISA for detection of experimentally infected mice were 0.22 and 0.20, respectively.

Statistical analysis

All of the statistical analyses of the data were performed using SPSS for Windows, version 17.0 (SPSS Inc., Chicago, IL). The OD values of ELISA were expressed as the mean value± standard deviation (SD). The detection rates of anti-sparganum IgG antibodies in the mice infected with sparganum and other parasites were compared using a chi-square test. The statistical significance was defined as $P<0.05$.

Results

Molecular cloning and expression of a cDNA encoding SmAP

The full coding sequence of SmAP gene was cloned into the prokaryotic expression plasmid pMAL-C2X. After induction with 0.2 mM IPTG, BL21 bacteria harboring pMAL-c2X-SmAP expressed a fusion protein. Using SDS–PAGE, the molecular size of the recombinant SmAP protein was 71.7 kDa and consistent with the predicted combined size of

the polypeptide encoded by the cDNA clone (28.7 kDa) and maltose-binding protein (MBP) tag from the vector (43 kDa) (Fig. 1).

Western blot analysis of the recombinant SmAP protein

The specific IgG antibodies to rSmAP in serum of immunized mice were determined by ELISA using rSmAP as antigen, and the IgG antibody titer of anti-SmAP serum was $1:10^4$. The Western blot analysis showed that the rSmAP was recognized by anti-rSmAP serum and sera of mice infected with spargana. The 28.7 kDa protein components of the crude antigens and ES antigens of spargana were recognized by the anti-rSmAP serum (Fig. 2), indicating that SmAP is one component of both the crude and ES antigens of *S. mansoni* spargana.

Expression and immunolocalization of SmAP at different developmental stages

The results of IFT with the sparganum sections showed that the intense staining using anti-rSmAP serum was found in the teguments and parenchymal tissues of different stages of spargana collected from the infected frogs and mice, but the section of *S. mansoni* adult worm was not recognized by anti-rSmAP serum (Fig. 3).

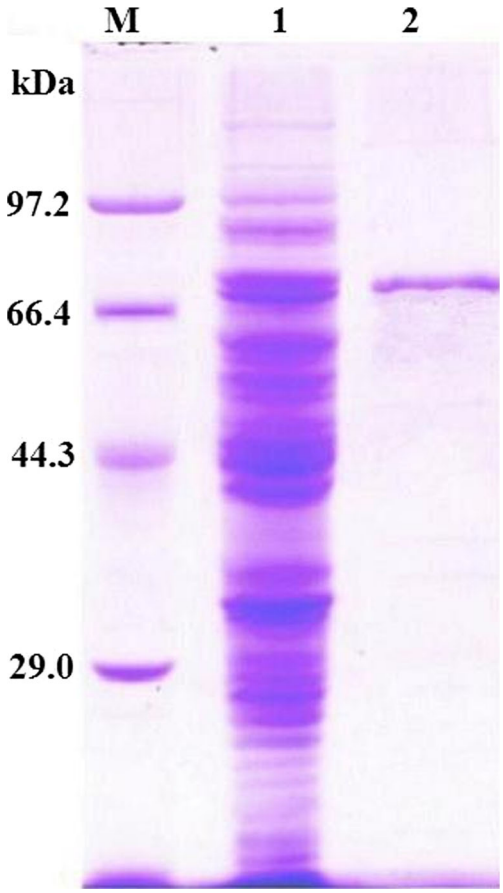

Fig. 1 SDS–PAGE analysis of recombinant SmAP protein. *M* protein molecular weight marker, *1* the lysis of the induced recombinant bacteria, *2* rSmAP purified by Amylose Pre-packed Column

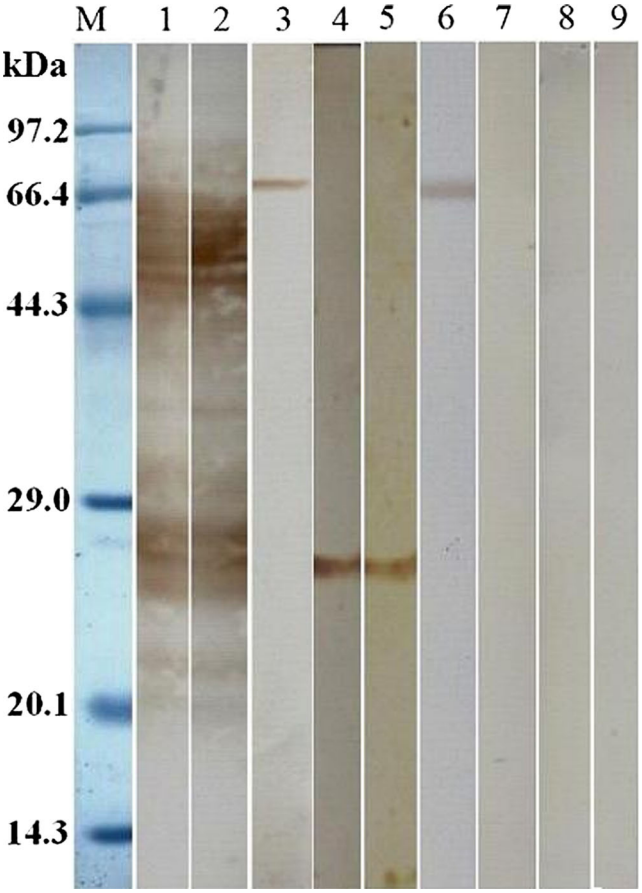

Fig. 2 Western blot analysis of rSmAP antigenicity. The sparganum crude antigens (*lane 1*), ES antigens (*lane 2*), and rSmAP (*lane 3*) were recognized by sera of mice infected with sparganum. The native SmAP protein in crude antigens (*lane 4*) and ES antigens (*lane 5*) and rSmAP protein (*lane 6*) were recognized by anti-rSmAP serum. The sparganum crude antigens (*lane 7*), ES antigens (*lane 8*), and rSmAP (*lane 9*) were not recognized by sera of normal mice

Detection of anti-sparganum IgG antibodies in sparganum-infected mice

The specific anti-sparganum IgG antibodies in serum samples of mice infected with sparganum and other parasites were determined, and the results are shown in Table 1. The detection rate of serum anti-sparganum antibodies in mice infected with sparganum was 83.3 % by rSmAP-ELISA and 100 % by ES-ELISA, respectively; the sensitivity of both ELISA was no significant difference (P=0.052). The specificities of both ELISA assays were 100 % (67/67) as they did not show any positive reaction with sera of mice infected with other parasites and non-infected normal mice.

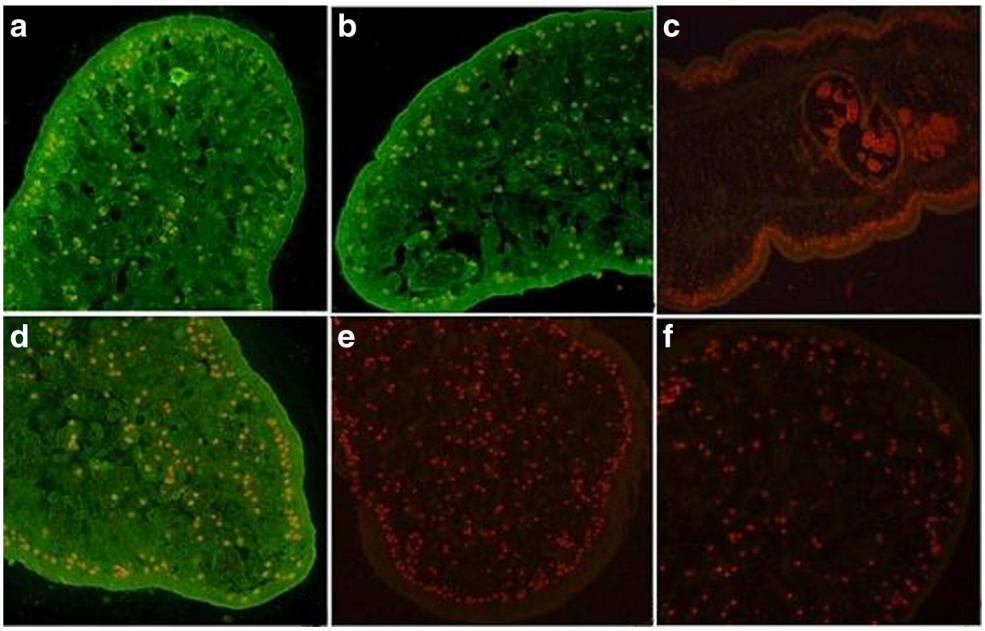

Fig. 3 Expression of SmAP at different developmental stages of *Spirometra mansoni* and immunolocalization by IFT with anti-rSmAP serum. The notable immunostaining is found in the teguments and parenchymal tissues of sparganum of naturally infected frogs (**a**) and experimentally infected mice (**b**); the section of *S. mansoni* adult worm was not recognized by anti-rSmAP serum (**c**). The sparganum from infected mice reacted with infection serum (**d**) as a positive control; sparganum did not show recognition by normal mouse serum (**e**) and PBS (**f**) as a negative control

Discussion

In the present study, the SmAP gene encoding a 28.7 kDa protein from *S. mansoni* spargana was successfully cloned and expressed using the MBP fusion-based pMAL-c2X vector system, which contained a mutation in the translocation signal and thus would yield only cytoplasm-associated recombinant proteins (Branco et al. 2008), and the resulting recombinant protein and immune serum were used to define some characteristics of the native 28.7 kDa protein of *S. mansoni*. The produced fusion protein was highly soluble in the cytoplasm of the bacteria; after being purified by affinity chromatography using amylose resin, such recombinant protein had a good immunogenicity in mice and could be used as an immunogen to produce antibodies (Harlow and Lane 1988). Our results showed that the BALB/c mice immunized with the purified rSmAP produced strong specific antibodies against the rSmAP, and the serum anti-rSmAP IgG antibody titer was $1{:}10^4$assayed by rSmAP-ELISA.

On Western blot analysis, the anti-rSmAP serum obviously recognized the 28.7 kDa band in the crude antigens and ES antigens of spargana. The results demonstrated that SmAP might be one component of both the crude and ES antigens of *S. mansoni* spargana. The characteristics of the SmAP gene were identified at the protein expression levels by using IFT. As shown in Fig. 3, the IFT results showed that the positive staining was widely distributed in the teguments and parenchymal tissues of different stages of spargana from the infected frogs and mice, but the section of *S. mansoni* adult worm was not recognized by anti-rSmAP serum. The results suggested that the SmAP was the sparganum-specific protein. The differential protein expression in different developmental stages of *S. mansoni* has been identified by the immunoproteomics (Kim et al. 2009).

In order to evaluate the sensitivity and specificity of rSmAP for detecting anti-sparganum IgG antibodies, rSmAP-ELISA was used for detection of sera of experimentally infected mice, and the results were compared with ES-

Table 1 Detection of anti-sparganum IgG antibodies in serum samples of experimentally infected mice by rSmAP-ELISA and ES-ELISA

Sera of mice infected with	No. of serum samples	rSmAP-ELISA		ES-ELISA	
		OD value ($\bar{x}\pm$SD)	No. of positive serum samples (%)	OD value ($\bar{x}\pm$SD)	No. of positive serum samples (%)
Sparganum	30	0.27±0.08	25 (83.3)	0.64±0.07	30 (100)
T. gondii	6	0.10±0.02	0 (0)	0.12±0.03	0 (0)
S. japanicum	16	0.10±0.02	0 (0)	0.13±0.03	0 (0)
T. spiralis	15	0.10±0.02	0 (0)	0.04±0.03	0 (0)
Normal mice	30	0.1±0.04	0 (0)	0.09±0.001	0 (0)

ELISA. The results showed that the sensitivity of rSmAP-ELISA and ES-ELISA was 83.3 % (25/30) and 100 % (30/30), respectively ($P>0.05$). The specificities of rSmAP-ELISA and ES-ELISA were 100 % (67/67), as no cross-reactions of both ELISA were observed with sera of mice infected with *T. gondii*, *S. japonicum*, *T. spiralis*, and normal mice. The results demonstrated that rSmAP might be a potential candidate antigen for serodiagnosis of sparganosis. However, the serum samples of the patients with sparganosis should be tested to confirm the sensitivity of rSmAP-ELISA. Additionally, specificity of the rSmAP also needs to be further investigated with sera of the patients with other helminthiasis including anisakiasis, cysticercosis, paragonimiosis, clonorchiosis, and so on.

In conclusion, the present study demonstrated that the SmAP is the sparganum stage-specific protein and located in the teguments and parenchymal tissues of spargana. The rSmAP was sensitive and specific for diagnosis of experimental sparganosis by ELISA and might be a potential candidate antigen for serodiagnosis of sparganosis. But it needs to be further investigated with sera of the patients with sparganosis and other helminthiasis

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