

Lab Note

Identification of a linear IgE inducing epitope on the SmATPDase1 surface

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The Immunoglobulin E (IgE) response to *Schistosoma mansoni* constitutes a relevant host defense mechanism and is associated with resistance to reinfection and parasite expulsion [1]. Intriguingly, the IgE response raised against worms resembles an allergic reaction, with both being characterized by an intense Th2 response and increased levels of IgE antibodies and IL-4, IL-5, and IL-13 cytokines [2]. There is strong evidence that this similarity does not occur by chance and that the allergic response might have evolved as a defense against metazoan parasites [3,4], an hypothesis supported by the cross-reactivity between parasite antigens and highly similar proteins in allergens [5,6]. The enzyme SmATPDase1, expressed on the tegument of *S. mansoni*, is involved in the regulation of several physiological processes of the host immune system in the worm [7–9]. Thus, this enzyme constitutes an interesting target for the development of new vaccines/therapies against *S. mansoni*.

The search for putative IgE epitopes in this enzyme was performed using a tool in the Structural Database of Allergenic Proteins (SDAP; <https://fermi.utmb.edu/>) following a guideline from the FAO/WHO (Food and Agriculture Organization of the United Nations and World Health Organization) for the assessment of potential protein allergenicity. This guideline evaluates allergenicity based on sequence homology and suggests that cross-reactivity should be considered when there is an exact match of six contiguous and identical amino acid residues in the query protein and proven allergens [10]. The potential IgE-binding peptides identified (Supplementary Table S1) in this search were highlighted in the predicted SmATPDase1 3D structure and FASTA sequence with the same color as shown in Supplementary Fig. S1A. Interestingly, many of these peptides are exposed in the extracellular domain of the

protein and, therefore, accessible for IgE antibodies binding in the native structure of the enzyme (Supplementary Fig. S1B).

We selected the peptide-KDVAKI to further characterize it as a SmATPDase1 IgE epitope. This sequence was chosen as it is present in a higher number of known allergens compared to the other peptides. Hence, the peptide-KDVAKI was obtained by solid phase peptide synthesis and used as the coating antigen (1 µg/ml) in the ELISA assays to analyze the IgE and IgG1 levels in serum samples from experimentally *S. mansoni*-infected mice. Levels of IgG1 against the peptide-KDVAKI (Fig. 1A) in pooled serum samples of *S. mansoni*-infected animals were found to be higher in the infected group than in the control group (dilutions 1:40 to 1:640). This result is consistent with the well-known competition between IgE and IgG for similar epitopes. Considering this competition and that IgG is present in higher levels than IgE, we previously depleted IgG antibodies from animal sera to improve the detection of specific IgE. As shown in Fig. 1B, significant reactivity of IgE antibody (dilutions 1:40 to 1:80) against the sequence KDVAKI was observed, suggesting that this peptide elicits humoral immune response during murine schistosomiasis, therefore, representing an IgE epitope in the SmATPDase1 structure.

To further characterize the KDVAKI sequence as an SmATPDase1 epitope, mouse anti-KDVAKI antibodies were used for precipitating proteins from detergent-solubilized worm homogenate. The antibodies were able to precipitate a protein of 63 kDa that was recognized by rabbit anti-CD39 antibodies (due to cross-immunoreactivity since SmATPDase1 and CD39 belong to the same protein family) in the western blot assay (Fig. 2A). Additionally, a band exhibiting ATPase and ADPase activity in a

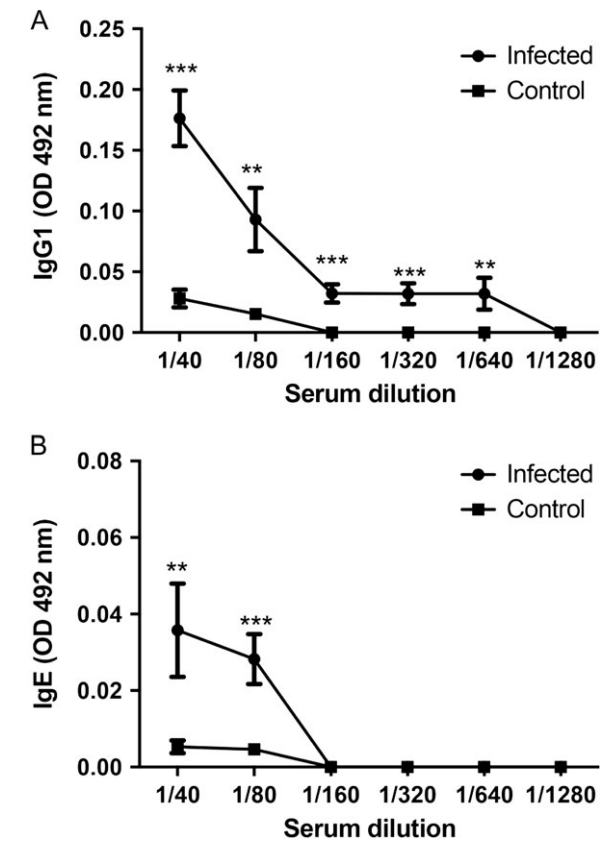


Figure 1. Cross-reactivity between antibodies in the sera from *Schistosoma mansoni*-infected animals and the synthetic peptide-KDVAKI In the ELISA assays, the peptide was used as the coating antigen (1 µg/ml) and sera from non-infected animals were used as a control. The sera were diluted from 1:40 to 1:1280 in quadruplicate. (A, B) Peptide-specific IgG1 (A) and peptide-specific IgE (B) were detected using peroxidase-conjugated, isotype-specific, anti-mouse IgG1 (1:1000), anti-mouse IgE (1:10,000), and o-phenylenediamine-dihydrochloride/H₂O₂ as substrate. Data are reported as the mean ± standard deviation. ***P* < 0.01, ****P* < 0.001.

non-denaturing gel electrophoresis was excised and submitted to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by electroblotting. This active band was recognized in western blot using anti-KDVAKI antibodies as two bands of 63 and 57 kDa, the latter probably resulting from proteolysis of the 63 kDa band as a consequence of the experimental conditions (Fig. 2B). Together, these results strongly suggest the recognition of the SmATPDase1 by anti-KDVAKI antibodies.

The definitive proof was obtained by mass spectrometry analysis of the 63 kDa band isolated from SDS-PAGE and revealed in western blot assays using both immune sera anti-CD39 and anti-KDVAKI. The mass spectrometry analysis revealed 13 unique tryptic peptides covering 30% of the SmATPDase1 sequence (Fig. 2C), confirming the identity of the enzyme and, thus, ensuring that the amino acid sequence KDVAKI belonging to the SmATPDase1 is an IgE-inductor. Noteworthy, detailed description of the methods used in this study is available as Supplementary data.

In summary, we demonstrated that the KDVAKI sequence within the SmATPDase1 is an IgE epitope. The potential immunotherapeutic application of this new peptide identified herein is currently being investigated in our research group.

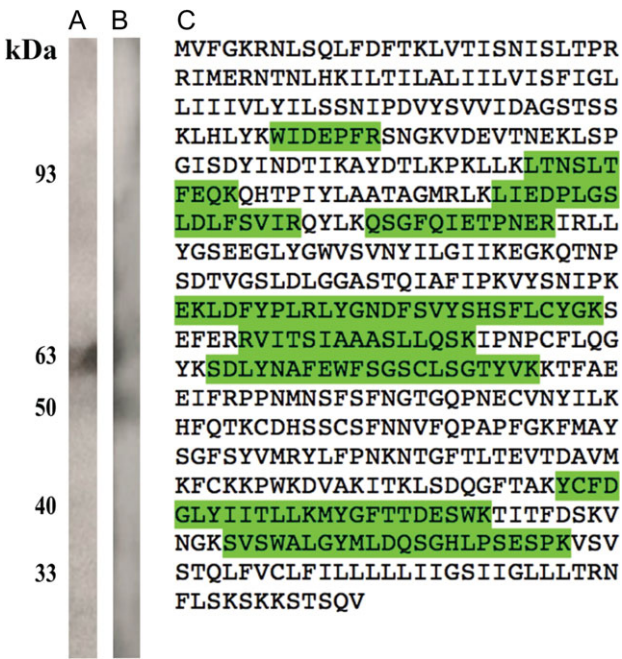


Figure 2. Anti-peptide antibodies recognize the SmATPDase1 in worm homogenates (A) Immunoprecipitation of SmATPDase1 (63 kDa) by antibodies in the sera from mice immunized with the peptide-KDVAKI was confirmed by western blot assay using rabbit antibody anti-CD39 and secondary anti-IgG antibody. (B) Active band of SmATPDase1 excised from the non-denaturing gel electrophoresis was recognized by mouse IgE anti-KDVAKI antibodies, as revealed by secondary-specific anti-IgE antibodies. Signals were revealed by chemiluminescence using secondary antibody coupled to horseradish peroxidase and luminol as the substrate, and exposed to X-ray film. (C) Tryptic peptides identified by mass spectrometry in the band of 63 kDa confirmed the identity of the protein. The mass spectrometry analysis after protein digestion found 13 unique tryptic peptides from the SmATPDase1, covering 30% of the protein sequence.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

Funding

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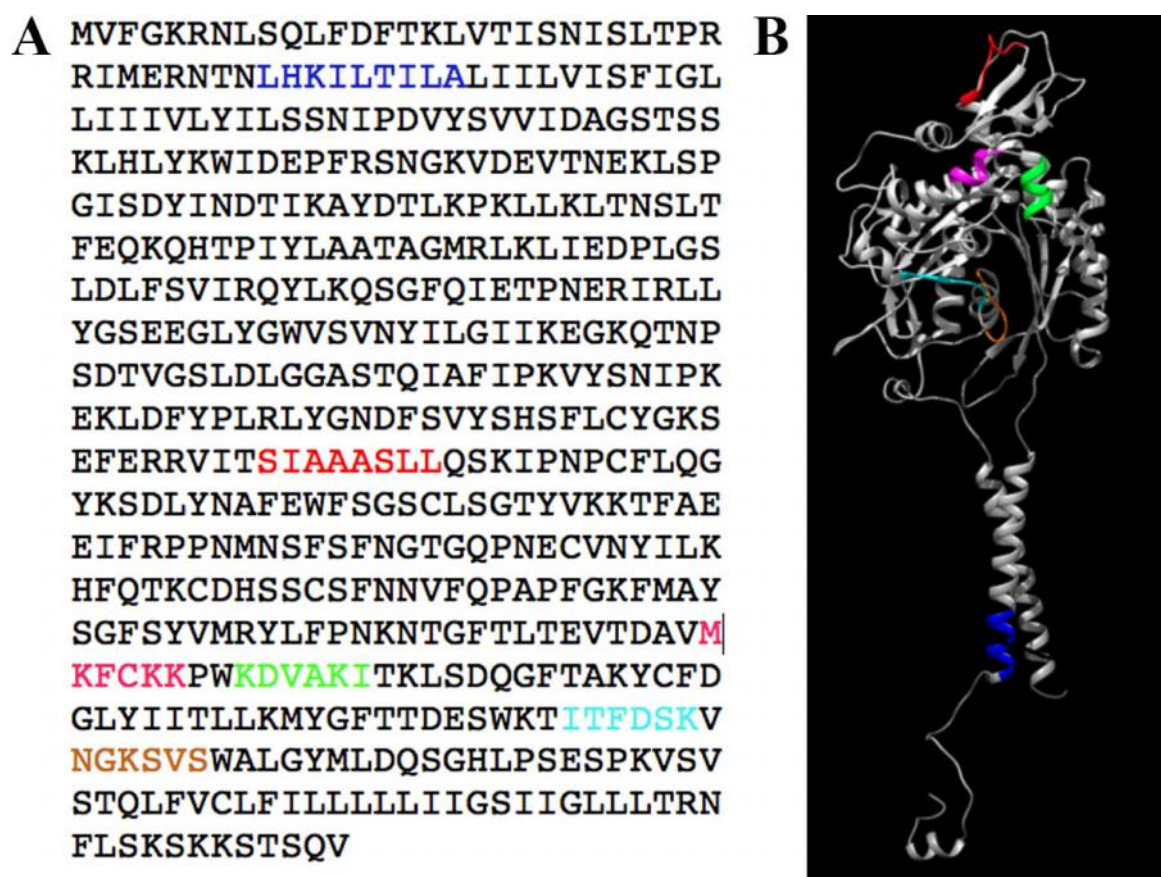
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Supplementary Table S1. Matches of 6 contiguous identical amino acids within FASTA sequence of SmATPDase1 and proven allergen proteins from SDAP database

Peptide sequence	Allergen	Uniprotkb entry name	Peptide allergen
³⁷ LHKILT	Calcium-binding allergen Ole e 8	ALL8_OLEEU	¹¹⁷ LHKILT
⁴⁰ ILTILA	Gamma Gliadin Gamma Gliadin B Gamma Gliadin Gamma Gliadin	GDBX_WHEAT GDBB_WHEAT GDB0_WHEAT GDB2_WHEAT	⁶ ILTILA ⁶ ILTILA ⁶ ILTILA ⁶ ILTILA
²⁸⁹ SIAAAS	Ovalbumin	OVAL_CHICK	³ SIAAAS
²⁹¹ AAASLL	BW10 kDa allergen protein	Q8W3Y9_FAGES	⁸ AAASLL
⁴²⁰ MKFCKK	Salivary antigen 1	CTF1_CTEFE	⁷² MKFCKK
⁴²⁸ KDVAKI	Polcalcin Syr v 3 PolcalcinBra n 2 PolcalcinBra r 2 PolcalcinAln g 4 PolcalcinOle e 3 PolcalcinBet v 4	POLC3_SYRVU POLC2_BRANA POLC2_BRARA POLC4_ALNGL ALL3_OLEEU POLC4_BETPN	⁷⁵ KDVAKI ⁷⁷ KDVAKI ⁷⁷ KDVAKI ⁷⁹ KDVAKI ⁷⁸ KDVAKI ⁷⁹ KDVAKI
⁴⁷⁰ ITFDSK	Pathogenesis-related protein 1 Stress-induced protein SAM22	PR1_PHAVU SAM22_SOYBN	⁹⁹ ITFDSK ⁹⁸ ITFDSK
⁴⁷⁷ NGKSVS	Kiwellin	KIWEL_ACTDE	¹³⁰ NGKSVS



Supplementary Figure S1. Potential IgE epitopes in the SmATPDase1 sequence (A) Exact matches of six contiguous amino acids in the SmATPDase1 sequence and allergens from SDAP database. (B) The predicted IgE-binding peptides are highlighted in the modelled three-dimensional structure of the SmATPDase1.

Supplementary Material

Materials and Methods

In silico analysis

SmATPDase1 sequence (UniprotID: AAP94734) was retrieved from Universal Protein Resource database (UNIPROT; <http://www.uniprot.org/>) and submitted to the Structural Database of Allergenic Proteins (SDAP; <http://fermi.utmb.edu/SDAP/sdapwho>), a web server that integrates a database of experimentally proven allergen sequences and computational tools to assess the potential allergenicity of novel proteins [1, 2].

SmATPDase1 three dimensional model (3D) was built by comparative modelling as described by Nunes et al. [3]. Briefly, the model was based on two templates available in the Protein Data Bank (PDB; <http://www.rcsb.org/pdb/home>): 3CJA (*Rattus norvegicus* NTPDase 2) and 3ZX3 (Rat NTPDase1). The alignment between amino acid sequences of SmATPDase 1 and templates was performed using Clustal Omega software and 3D structure was created in Modeller software version 9.10. Procheck [4] was used for additional analysis of stereochemical quality.

Peptide synthesis, purification and antibodies production

The peptide ⁴²⁸KDVAKI⁴³³ was synthesized manually accordingly to standard Boc protocol as described by NAKAIE *et al.* [5] and purified by preparative HPLC. Peptide homogeneity was determined through analytical HPLC, mass spectrometry (LC/MS-electrospray; Micromass, Manchester, UK) and amino acid analysis (Biochrom 20 Plus; Amersham Biosciences, Uppsala, Sweden).

Seven-week-old BALB/c mice (n=3) were inoculated with the synthetic peptide (5µg) by peritoneal route three times at intervals of 15 days. The first injection was emulsified in Freund's complete adjuvant and the second and third injections were delivered in Freund's incomplete adjuvant. The sera were obtained 15 days after the third injection, pooled and stored at -20°C.

Sera of *S. mansoni* infected animals and total worm homogenate

Approximately 100 *S. mansoni* cercariae (LE strain) obtained from infected *Biomphalaria glabrata* snails were subcutaneously inoculated in eight-week-old male Swiss mice as described by Smithers and Terry [6]. After seven weeks, blood samples were collected and

adult worms were recovered by means of perfusion of hepatic and mesenteric veins [7]. To obtain the total worm homogenate, adult worms of *S. mansoni* were submitted to a combination of 3 freeze-thaw cycles in liquid nitrogen followed by grinding and sonication for 10 min. These studies were approved by the Ethical Committee of the Federal University of Juiz de Fora, Juiz de Fora, state of Minas Gerais, Brazil (protocol 25/2012 and 26/2012). These experimental procedures were conducted in adherence to the institution's guidelines for animal husbandry.

Enzyme-linked immunosorbent assays (ELISA)

Antigen optimal concentration was determined by testing various dilutions (from 1 µg/ml to 6 µg/ml) and 1 µg/ml was found to be enough to saturate the ELISA plate (data not shown). The synthetic peptide diluted in 0.1 M NaHCO₃, pH 9.6 (1 µg/ml) was adsorbed on flat-bottom microtiter plates (COSTAR) overnight at 4° C. Then, the plates were blocked (0.15 M PBS, pH 7.2, 0.05% Tween-20, 1% casein, 10% fetal bovine serum) for 2 h and incubated with four pooled serum samples for 4 h. IgG1 (diluted from 1:40 to 1:1280) was assessed by using untreated sera obtained from the *S. mansoni* infected-mice. For IgE (diluted from 1:40 to 1:1280) analysis, serum samples were previously depleted from their IgG content: the sera were pre-incubated with anti-IgG (1:1; v/v) for 20 min and complexes formed were removed by centrifugation at 5000 rpm for 5 min, as adapted from Souza-Atta *et al.* [8]. Non-infected animal sera were used as control. Peptide-specific IgE and IgG1 antibody levels were detected by using peroxidase-conjugated, isotype-specific, anti-mouse IgG1 (1:1000; Zymed), anti-mouse IgE (1:10,000; Bethyl) and o-phenylenediaminedihydrochloride/H₂O₂ (Sigma Chemical Co, USA) as substrate. Subsequent color reaction was read at 492 nm wavelength on microplate reader (Molecular Devices Corp., Menlo Park, USA). Statistical analysis was performed using GraphPad Prism Software (version 5). *P* values <0.05 were considered significant.

Immunoprecipitation and western blotting

An aliquot of total worm homogenate was prepared in 50 mM MOPS buffer, pH 7.4, 1 mM CaCl₂ and C12E9 (1mg/ml) and centrifuged at 14,000 *g* for 10 min. The soluble supernatant was then incubated with mouse serum anti-peptide (final dilution 1:50) for 3 h at room temperature with gentle agitation. Protein G-Sepharose was added and incubated for additional 2 h. The resin-antibody-antigen complex was sedimented by centrifugation for 5

min, washed 3 times in 50 mM MOPS buffer, pH 7.4, solubilized in gel loading buffer and submitted to SDS-PAGE on 10% polyacrylamide gels using Mini-Protean III Cell (Bio-Rad). The proteins were electroblotted onto nitrocellulose membranes, followed by a blocking step (0.15 M phosphate buffer solution, pH 7.4, plus 0.1% Tween-20 and 1% casein) using standard procedures. The Western blots of immunoprecipitated protein was developed with rabbit antibody produced against a CD39 peptide (1:250; Santa Cruz, Santa Cruz, USA), a mammalian protein homologue to the SmATPDase1 [3]. Signals were revealed by chemiluminescence using anti-IgG antibody coupled to horseradish peroxidase and Luminol as substrate (ECL Western Blotting System; GE Healthcare, Brazil), and exposed to X-ray film following the manufacturer's instructions.

Non-denaturing polyacrilamide gel electrophoresis and western blotting

Non-denaturing polyacrylamide gel electrophoresis was performed as described by Vasconcelos et al. [9]. Aliquots of total worm homogenate (100 µg) were solubilized in sample buffer supplemented with 0.2% Triton X-100 plus 0.4% sodium deoxycholate and subjected to electrophoresis for approximately 80 min at 150 V at 4°C in a 6% polyacrylamide gel and 0.1% (v/v) Triton X-100 plus 0.1% (w/v) sodium deoxycholate in a Mini-Protean III Cell (BioRad). The gel was washed for 40 min in standard reaction medium without nucleotides. Fresh standard reaction medium containing 10 mM CaCl₂ and 5 mM ADP or ATP were added. The gel was incubated at 37°C until white calcium phosphate precipitates appear. Bands containing ATPase or ADPase activity were cut out, sliced into small pieces and electroeluted for 15 h and submitted to electrophoresis in SDS-PAGE/10% gel. The gel was electroblotted onto nitrocellulose membrane. The membrane was blocked (0.15 M phosphate buffer solution, pH 7.4, plus 0.1% Tween-20 and 1% casein) overnight and then incubated with mouse anti-peptide antibodies (1:50) for 3 h at room temperature. Signals were revealed by chemiluminescence using specific anti-IgE antibody (1:5,000/Bethyl) coupled to horseradish peroxidase and Luminol as substrate (ECL Western Blotting System; GE Healthcare, Brazil), and exposed to X-ray film following the manufacturer's instructions.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), western blotting and mass spectrometry

Total worm homogenates (60 µg/lane) aliquots were loaded onto 8% to 16% polyacryamide gel (Amersham ECL Gels) and the electrophoresis carried out at a constant voltage of 160 V.

One part of the gel was stained with Coomassie Blue and its duplicate electrotransferred onto nitrocellulose membrane. The membranes were blocked with 1% casein overnight at 4°C, incubated with anti-CD39 antibodies (1:250; Santa Cruz) and pooled serum anti-peptide (1:50) for 3 h and revealed by ECL solution. The band of 63 kDa recognized by both anti-CD39 and anti-KDVAKI antibodies was excised from gel, diced into small pieces, destained with 25 mM NH_4HCO_3 in 50% ACN, and proteins were reduced and alkylated with 50 mM DTT and 100 mM iodoacetamide respectively.

After three washings with 20 mM NH_4HCO_3 in 50% ACN, the gel pieces were dried and rehydrated with 20 ng/mL trypsin solution in 25 mM ammonium bicarbonate at room temperature for 20 min. The digestion was continued at 37°C for 17 h. The tryptic peptides were extracted with 0.1% trifluoroacetic acid in 50% ACN for 30 min, concentrated and desalted by C-18 ziptip (Millipore). The tryptic peptides were loaded onto a UltiMate® 3000 UHPLC system (Thermo Scientific, Bremen, Germany) equipped with an Acclaim PepMap100 C18 Nano-Trap Column (100 μm i.d. $\times$ 2 cm, 5 μm , 100 Å; Thermo Scientific) in line with an Acclaim PepMap100 C18 RSLC (75 μm i.d. $\times$ 15 cm, 2 μm , 100 Å; Thermo Scientific) capillary column. First, the peptides were trapped and washed for 3 min in 2% acetonitrile and 0.05% trifluoroacetic acid, at a flow rate of 3 $\mu\text{l}\cdot\text{min}^{-1}$ and then separated on the capilar column using as mobile phase: (A) formic acid 0.1% and (B) acetonitrile 80% and formic acid 0.1% with a gradient of B changing from 4 to 90% over 61 minutes, flow rate of 0.300 $\mu\text{l}\cdot\text{min}^{-1}$ and 40°C. LC eluent was sprayed into Q-Exactive (Thermo Scientific). Survey scans were acquired at a resolution of 70,000 with maximum injection time of 100 ms and target value of 3×10^6 ions. The 12 most abundant ions scanned in the range of 300-2000 m/z and charge state ≥ 2 and ≤ 5 were isolated over a window of 2 m/z before fragmentation via higher-energy collisional dissociation (HCD) with normalized collision energy of 30 V. MS/MS spectra were acquired at a 17,500 resolution with maximum injection time of 150 ms and target value of 2×10^5 ions. Dynamic exclusion of ions was set to 40 s. The acquired MS/MS data were searched against *S. mansoni* data base obtained from Uniprot using Proteome Discoverer software (versão 1.4; Thermo Scientific) and SequestHT as the search engine. Search parameters were set as follows: (i) enzyme: trypsin/P; (ii) maximum missed cleavage allowed 2; (iii) fixed modification: carbamidomethyl; (iv) dynamic modifications: methionine oxidation and N-terminal acetylation; (v) tolerance of 10 ppm to parental ions and 0.1 Da to fragments. Only proteins with at least 5% false discovery rate and two unique peptides were accepted.

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