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Potato Apyrase: A New Tool for Parasitic Disease Research

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ABSTRACT

Recent investigations on *Solanum tuberosum* (potato) apyrase, a protein of biomedical interest, are presented along with a discussion on the possible biological application for the study of interactions between parasites and the host immune system. Potato apyrase (PA), one of the first proteins of the ATP diphosphohydrolase family to be purified, has immunostimulatory properties. Polyclonal antibodies against PA show strong cross-immunoreactivity with native ATP diphosphohydrolase isoforms isolated from either *Schistosoma mansoni* egg and worm or *Leishmania* (*Leishmania*) *amazonensis* promastigotes. These results were confirmed by immunoprecipitation assays in which antibodies against different PA isoforms, immobilized on Sepharose-Protein A, depleted the ATPase and ADPase activities from parasite preparations. The data suggested that potato and parasite proteins share specific epitopes. Furthermore, sera from both experimentally infected-mice and patients showed cross-immunoreactivity with PA, which also suggests that the antigenicity of these conserved epitopes exits. Distinct humoral immune response profiles of IgG antibodies from American cutaneous leishmaniasis, schistosomiasis and Chagas disease patients, associated with the distinct life-cycles of the parasites. Presumably, these antigens are processed and presented to effectors cells from the host immune system by different pathways. *In silico* studies demonstrated evolutionary and close structural relationships between PA and parasites ATP diphosphohydrolases. Specific protein domains were suggested to be potentially involved in the host immune response. Schistosomes may live for several years in the host, evading hemostatic and immune responses. Leishmanias can persist at the scar after clinical cure for long time. Further studies of both parasite and PA conserved domains could contribute to a better understanding of host-parasite interactions, and may to be explored as a new tool in parasitic disease research.

Keywords: apyrase, ATP diphosphohydrolase, *Leishmania*, parasite, plant, *Schistosoma*, *Solanum tuberosum*, *Trypanosoma cruzi*

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INTRODUCTION

Biomedical interest in potato (*Solanum tuberosum*) apyrase (PA) activity goes back more than 30 years, to the time when this protein was found to possess remarkable adenosine triphosphatase (ATPase) and adenosine diphosphatase (ADPase) activities. The soluble apyrase isoform is obtained in high yield and purity using conventional methods assuring significance and reproducibility in pharmacological and immunological assays (Vasconcelos *et al.* 1996; Faria-Pinto *et al.* 2004; Kettlun *et al.* 2005; Penido *et al.* 2007). The native PA is commercially available in various degrees of purity, and the production of a recombinant apyrase from potato tubers was recently reported (Nourizad *et al.* 2003).

The PA activity is useful in many different applications.

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Invited Review

It has been employed for improving methods requiring monitoring of ATP or measurements of ATPase activity (Nyrén 1994; Karamohamed and Guidotti 2001) and is a valuable tool for *in vitro* assays on the depletion of ATP or pro-aggregating ADP (Whigham *et al.* 1976; Mrowiec *et al.* 1997; Langston *et al.* 2003; Cattaneo *et al.* 2007).

PA activity is also used in *in vivo* studies. Adenosine nucleotides have the potential for regulating platelet activation and vascular inflammatory processes through the activation of specific purinergic receptors (Marcus and Safier 1993), and ATP is an important signaling molecule in the peripheral and central nervous system (Morrone *et al.* 2006). After intravenous inoculation of a single dose of soluble PA in an animal model, followed by its continuous infusion directly into the graft, this protein prevented platelet microthrombic formation by inhibiting ADP-stimulated platelet aggregation and promoted significant prolongation of xenograft survival in both hyperacute and delayed xenograft rejection (Koyamada *et al.* 1996). Recently, PA activity was tested in the treatment of glioma, a type of primary brain tumor. Using a rat model, the investigators demonstrated that the presence of an ATP-depleting enzyme at the moment of glioma implantation caused a significant reduction in microvascular proliferation in the tumor and in its growth (Morrone *et al*. 2006).

In addition, it has been demonstrated that PA has immunostimulatory properties and polyclonal antibodies against this vegetable protein cross-react with parasites ATP diphosphohydrolase isoforms (Vasconcelos *et al.* 1996; Faria-Pinto *et al.* 2004, 2006; Coimbra *et al.* 2008; Faria-Pinto *et al.* 2008). In this review, recent investigations on PA and parasite ATP diphosphohydrolases are presented, along with a discussion of the possible biological applications of this relationship to the study of interactions between parasites and the host immune system.

A NEW PROTEIN FAMILY – THE ATP DIPHOSPHOHYDROLASE FAMILY - EMERGED FROM THE POTATO APYRASE STUDIES

Apyrase (ATP diphosphohydrolase, EC 3.6.1.5) has been characterized in plants, mammals, bacteria, fungi and parasites. These ubiquitous enzymes share several common features, such as ability to hydrolyze nucleoside di- and triphosphates to the corresponding nucleoside monophosphates upon bivalent metal ion activation. Several isoforms have been described, and they differ in their catalytic properties, solubilization, subcellular locations and/or functions (Vasconcelos *et al*. 1996; Gendron *et al.* 2002; Kettlun *et al*. 2005; Robson *et al*. 2006).

Although the nucleoside di- and triphosphate-hydrolyzing activity of apyrase in plants has been recognized for several decades, the first description of the ATP diphosphohydrolase family is recent (Handa and Guidotti 1996; Vasconcelos *et al.* 1996). The amino acid sequence deduced by cloning of the gene of a PA isoform from a commercial strain of *S. tuberosum* (Handa and Guidotti 1996) and the amino acid sequences of three peptides obtained from purified apyrase from the 'Desirée' clonal variety of *S. tuberosum* (Vasconcelos *et al*. 1996) were used in a search of the protein data bank of the National Center for Biotechnology Information (NCBI). Considerable homology was found with a nucleoside triphosphatase from garden pea (*Pisum sativum*), two NTPase isoforms from protozoan parasite, *Toxoplasma gondii*, a guanosine diphosphatase from yeast, *Saccharomyces cerevisiae*, a hypothetical protein from *Caenorhabditis elegans* and a mammalian lymphoid cell activation antigen (Handa and Guidotti 1996; Vasconcelos *et al*. 1996). The alignment of the amino acid sequences showed two domains similar to the phosphate binding site motif of the actin-hsp70-hexokinase family, indicating a possible role in nucleotide binding, and three additional conserved domains. These five motifs were named "apyrase conserved regions" or ACR, and a new family of so-far-unnoticed nucleoside triphosphatases and apyrases emerged from these studies (Handa and Guidotti 1996; Vasconcelos *et al*. 1996).

The apyrase isoforms isolated from *S. tuberosum* tubers, the first to be purified, are among the most extensively studied (Traverso-Cori *et al*. 1965; Valenzuela *et al*. 1973; Kettlun *et al*. 1981, 1982; Mancilla *et al*. 1984; Kettlun *et al*. 1992a, 1992b; Espinosa *et al*. 2000; Kettlun *et al*. 2000, 2005). Different isoforms have been characterized in the 'Pimpernel' and 'Desirée' varieties of *S. tuberosum*, which have the same molecular weight (49,000) but differ in their isoelectric points (p*I*) and ATPase/ADPase ratios (Kettlun *et al*. 1982; Mancilla *et al*. 1984). 'Pimpernel' apyrase has a p*I* of 8.74 and hydrolyzes ATP ten times faster than ADP, while the 'Desirée' enzyme has a p*I* of 6.69 and splits both nucleotides at the same rate (Kettlun *et al*. 1982; Mancilla *et al*. 1984). In the 'Ultimus' variety, at least two isoforms coexist, which have molecular weights 48,000 and 46,500 and ATPase/ADPase ratios of 1 and 10, respectively (Kettlun *et al*. 1992a, 1992b). Chemical modification and intrinsic fluorescence experiments identified the possible amino acids involved in the apyrase activity (Kettlun *et al*. 1981; Espinosa *et al*. 2000; Kettlun *et al*. 2000). Modeling of an isoform from *S. tuberosum* suggested that the single polypeptide chain is folded into two domains, and the putative catalytic site, composed by "apyrase conserved regions" is located between them (Kozakiewicz *et al*. 2008). Studies concerning the physiological role suggested that PA activity is related to the hydrolysis of nucleoside diphosphate produced during sucrose and starch biosynthesis (Anich *et al.* 1990). *S. tuberosum* was recently reported to contain at least three different apoplastically localized apyrase isoforms involved in the regulation of the synthesis of cell wall proteins and in energy transfer and starch synthesis (Riewe *et al*. 2008).

Significant progress has been made over the past 12 years in the structural studies of apyrases, but there is no uniformly accepted nomenclature for the various members of this new family. The members are being classified in their order of discovery and catalytic properties (Robson *et al*. 2006). The family members are named as either ATP diphosphohydrolase or NTPase (nucleoside triphosphate hydrolase) in parasites, NTPDase (nucleoside triphosphate diphosphohydrolase) in mammals, and apyrase in plants.

Apyrase-like genes have been identified in various plant species such as *P. sativum* (pea), *Glycine soja* (soybean), *Medicago truncatula*, *Dolichos biflorus* (cowpea), *Arabidopsis thaliana* (mouse-ear cress) and *Lotus japonicus* and their predicted amino acid sequences have been deposited in the protein data bank. In plant tissues, apyrase can be a cytosolic, membrane-associated or nuclear protein, and it has been implicated in playing functional roles in a variety of different systems (Kettlun *et al*. 2005). *P. sativum* apyrase mediates the uptake of phosphate from the extracellular matrix (Thomas *et al*. 1999). Apyrases from *D. biflorus*, *G. soja* and *M. truncatula* were associated with the nodulation signals produced by rhizobial infection (Etzler *et al*. 1999; Day *et al*. 2000; Cohn *et al*. 2001).

The first member of the mammalian NTPDase family to be cloned and sequenced was a lymphocyte cell activation antigen named CD39. The expression and characterization of catalytic properties of CD39 stimulated the molecular study of other NTPDases, and eight different genes encoding members of the mammalian NTPDase family were subsequently described (reviewed in Robson *et al*. 2006). The NTPDases 1, 2, 3 and 8 are typical cell surface-located enzymes with an extracellularly-facing catalytic site. NTPDases 5 and 6 exhibit intracellular localization and undergo secretion after heterologous expression. NTPDases 4 and 7 are entirely intracellularly located, facing the lumen of the cytoplasmic organelles (Robson *et al*. 2006). Mutagenesis data obtained from mammalian NTPDases showed that the apyrase-conserved regions are involved in substrate recognition, binding and hydrolysis (Robson *et al*. 2006). The functional role of NTPDases in mammalian animals has been intensively studied, and numerous works have de-

monstrated their participation in physiological processes involving the modulation of signals mediated by cell-surface purinergic receptors, such as the inhibition of ADP-induced platelet aggregation to prevent thrombosis (Gendron *et al*. 2002; Robson *et al*. 2006).

ATP diphosphohydrolases have been characterized in parasites such as *T. gondii*, *Schistosoma mansoni*, *Leishmania* (*L.*) *amazonensis*, *Trichomonas vaginalis*, *Entamoeba hystolytica* and *Trypanosoma cruzi* (Bermudes *et al*. 1994; Vasconcelos *et al*. 1996; Barros *et al*. 2000; Coimbra *et al*. 2002; Fietto *et al*. 2004; Tasca *et al*. 2004). Among the pathogenic parasites, only *T. gondii*, *T. cruzi*, and *S. mansoni* ATP diphosphohydrolase coding-genes have been reported (Bermudes *et al.* 1994; De Marco *et al*. 2003; Fietto *et al.* 2004; Levano-Garcia *et al.* 2007). Analyses of the databases of the NCBI using known sequences of ATP diphosphohydrolases revealed homologous putative proteins in the genomes of *Schistosoma japonicum*, a zoonotic helminth that causes schistosomiasis japonica; *Plasmodium falciparum*, an ethiologic agent of malaria; and in *L. major*, *L. Infantum* and *L. braziliensis* protozoan parasites, which cause leishmaniasis. It has been further suggested that their enzymatic activity could be associated with the purine recuperation and/or as a protective mechanism against the host organism that involves ATP or ADP, such as platelet activation cytotoxicity and cytolytic T-lymphocyte reactivity (Vasconcelos *et al.* 1993; Bermudes *et al.* 1994; Vasconcelos *et al.* 1996; Coimbra *et al.* 2002; Fietto *et al.* 2004; Tasca *et al.* 2004). Although a large number of ATP diphosphohydrolases from various sources have now been described, their precise functions in parasites are yet not fully understood.

STRUCTURAL RELATIONSHIP BETWEEN POTATO APYRASE AND OTHER MEMBERS OF THE ATP DIPHOSPHOHYDROLASE FAMILY

In silico analyses were recently performed (Faria-Pinto *et al*. 2006, 2008) in an attempt to determine the structural relationship between PA and other members of the ATP diphosphohydrolase family. The alignment of 32 members of the ATP diphosphohydrolase family, that included mammals, helminths and protozoan parasites, mosquitoes and plant proteins, was performed. Strong homology was observed among plant apyrases. The sequence identity among PA isoforms already deposited in the protein data bank is 92 to 98%, with 96 to 99% similarity. The identity between PA and other plant apyrases varied from 44 to 56%, with 70 to 75% similarity.

Among PA and mammalian proteins, the highest degree of identity (29-31%) was encountered with NTPDase 5 from mouse and human and NTPDase 6 from humans, while the human NTPDases1-4, 7 and 8, which are membrane-associated proteins, showed a lower degree of identity (22-29%).

Among parasites, the proteins having a higher homology with PA were the *Leishmania* NDPases (30-33% identity), followed by *S. mansoni* SmATPDase 2 and *S. japonicum* NTPDase6-like protein (26-28% identity), which

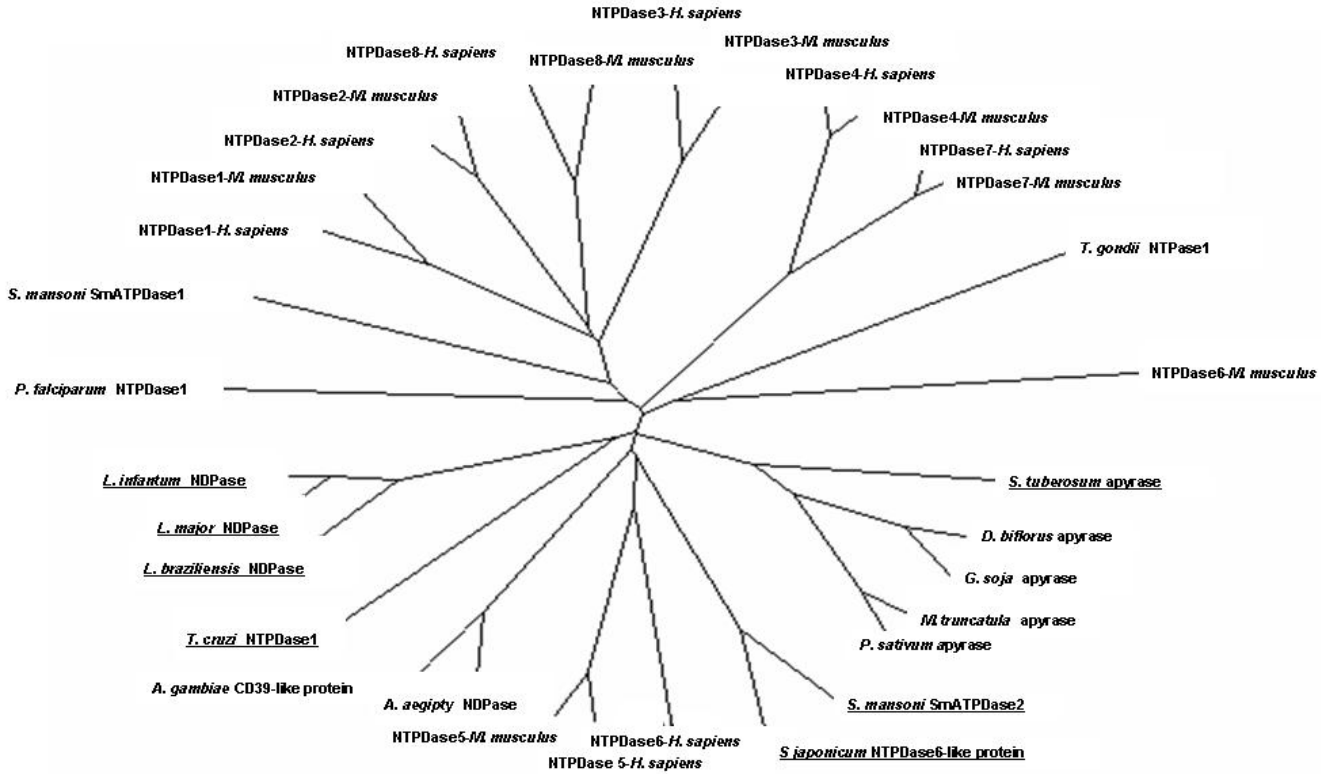

Fig. 1 Phylogenetic tree of several ATP diphosphohydrolases from different organisms. This tree was constructed using T-Coffee, excluding positions with gaps. GeneBank accession numbers of the sequences are: *Solanum tuberosum* apyrase, P80595; *Dolichos biflorus* apyrase, AF156781; *Glycine soja* apyrase, AAG32959; *Medicago truncatula* apyrase, AAO23007; *Pisum sativum* apyrase, BAB85978; *Schistosoma mansoni* SmATPDase2, DQ868522; *Schistosoma japonicum* NTPDase6-like protein, AAW26231; NTPDase6-*Homo sapiens*, AAP92131; NTPDase5-*Homo sapiens*, NP_001240; NTPDase5-*Mus musculus*; NP_001021385; *Aedes aegipty* NDPase, EAT42846; *Anopheles gambiae* CD39-like protein, XP_320057; *Trypanosoma cruzi* NTPDase1, AAS75599; *Leishmania braziliensis* NDPase, CAM42020; *Leishmania major* NDPase, CAJ03227; *Leishmania infantum* NDPase, CAM66723; *Plasmodium falciparum* NTPDase1, XP_00138471; *Schistosoma mansoni* SmATPDase1, AAP94734; NTPDase1-*Homo sapiens*, NP_001767; NTPDase1-*Mus musculus*, AAAH11278; NTPDase2-*Homo sapiens*, NP_982293; NTPDase2-*Mus musculus*, O55026; NTPDase8-*Homo sapiens*, AAR04374; NTPDase8-*Mus musculus*, NP_082369; NTPDase3-*Homo sapiens*, NP_001239; NTPDase3-*Mus musculus*, NP_848791; NTPDase4-*Homo sapiens*, NP_004892; NTPDase4-*Mus musculus*, NP_080450; NTPDase7-*Homo sapiens*, NP_065087; NTPDase7-*Mus musculus*, NP_444333; *Toxoplasma gondii* NTPase1, Q27893; NTPDase6-*Mus musculus*; NP_742115. Reprinted from **Faria-Pinto P, Rezende-Soares FA, Molica AM, Montesano MA, Marques MJ, Rocha MOC, Gomes JAS, Enk MJ, Correa-Oliveira R, Coelho PMZ, Neto SM, Franco OL, Vasconcelos EG** (2008) Mapping of the conserved antigenic domains shared between potato apyrase and parasites ATP diphosphohydrolases: potential application in human parasitic diseases. *Parasitology* **135**, 943-953, with kind permission from Cambridge University Press, ©2008.

showed particular domains of high identity among them.

The phylogenetic tree showed two main branches. The first clade is clearly composed of membrane-associated human NTPDases 1-4, 7-8, mouse NTPDases 1-4 and 6-8, *S. mansoni* SmATPDase 1, *P. falciparum* NTPDase 1 and *T. gondii* NTPase 1. The second is composed of human and mouse NTPDases 5 and human NTPDase 6, plant apyrases, *S. mansoni* SmATPDase 2, *S. japonicum* NTPDase6-like protein and *Leishmania* NDPases (**Fig. 1**; Faria-Pinto *et al*. 2008). Phylogenetic analysis, therefore, indicated two different evolutionary pathways, possibly a result of ancient divergence. Of these analyses, a closer structural relationship was observed between PA and ATP diphosphohydrolases from schistosomes and leishmanias (Faria-Pinto *et al*. 2008).

Hosts and parasites have co-evolved over millions of years, and these parasites live for years in mammals using a wide range of mechanisms to evade and manipulate the host's immune response (Requena *et al.* 2000; Dunne and Cooke 2005). We speculate that the existence of ATPase and ADPase activities on the surface of parasites, with kinetic characteristics similar to those of the mammalian NTPDases, raises the possibility that a common mechanism might exist that permits the parasites to mimic some of the conditions on the surface of host cells, thereby escaping the hemostatic or immune responses that are mediated by ADP or ATP. These include platelet activation cytotoxicity and cytolytic T-lymphocyte reactivity. In addition, it is possible that specific antigenic regions were conserved among the different parasites species and are related to the success of parasitism through disease immunomodulation.

POTATO APYRASE HAS IMMUNOSTIMULATORY PROPERTIES THAT INDUCE HUMORAL IMMUNE RESPONSE IN ANIMAL MODELS

Studies showed that PA purified from either 'Desirée' or 'Ultimus' variety of *S. tuberosum* or from a commercial strain of *S. tuberosum* presents remarkable immunostimulatory properties, activating humoral immune responses in Swiss mice and rabbits (Vasconcelos *et al*. 1996; Faria-Pinto *et al*. 2004). Recently, healthy BALB/c mice inoculated per via peritoneal with PA, a soluble isoform purified from a commercial strain of *S. tuberosum*, exhibited significant increase of their IgG1 and IgG2a antibody serum levels against this protein. Given that IgG isotypes represent markers for the Th1/Th2 dichotomous immune response (Hoffmann *et al*. 1999), these results suggested that this vegetable protein has distinct epitopes capable of inducing Th1- and Th2-type cytokine secretion profiles (unpublished data), information that could be applied in disease immunomodulation studies.

POLYCLONAL ANTI-POTATO APYRASE ANTIBODIES IDENTIFIED PARASITES ATP DIPHOSPHOHYDROLASES

The characterization of parasite proteins is essential to the understanding of the host-parasite relationship and/or for improving methods for the control of parasitic diseases such as diagnostic methods and implementation of vaccines. Investigations in our laboratory showed cross-immunoreactivity between rabbit polyclonal anti-PA antibodies and native ATP diphosphohydrolases from either *S. mansoni* worm and egg (Vasconcelos *et al*. 1996; Faria-Pinto *et al*. 2004) or *Leishmania* promastigote form (Coimbra *et al.* 2008), but not from mammalian NTPDases (Faria-Pinto *et al*. 2006).

Schistosoma mansoni ATP diphosphohydrolase isoforms have antigenic domains shared with potato apyrase

Schistosoma mansoni is one of the major etiological agents of schistosomiasis, a debilitating human infection. Infection

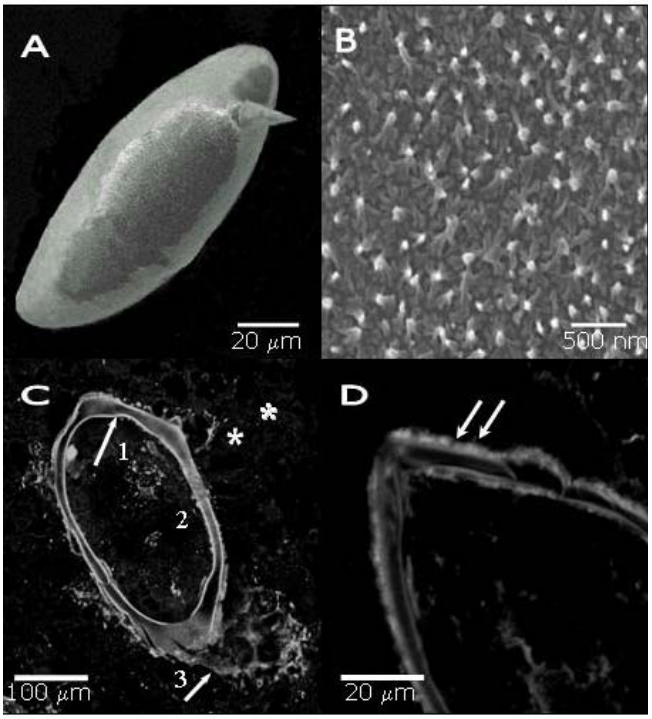

Fig. 2 (**A**) general features of *S. mansoni* egg seen by scanning electron microscope; (**B**) enlargement of a region of the egg-shell (A) showing surface microspines located outside the egg-shell; (**C**) Immunocytochemical localization of ATP diphosphohydrolase from the *S. mansoni* egg by immunofluorescence confocal microscopy. Anti-PA antibodies and secondary antibody coupled to TRITC (Tetramethyl Rhodamine Iso-thiocyanate) were used for fluorescence detection of ATP diphosphohydrolase on cryostat sections (10 μm thick) of infected mouse liver. General features of *S. mansoni* egg showing fluorescence homogeneously distributed on the external surface of miracidium (arrow 1), in the region between the miracidium and the inner side of the egg-shell (arrow 2) and immediately outside and spreading away from the egg-shell (arrow 3); (**D**) enlargement of a region of the egg-shell surface showing granular material entrapped by the surface microspines (arrow). * Periovular area of granulomatous inflammation. (**C**) and (**D**) were reprinted from **Faria-Pinto P, Meirelles MN, Lenzi HL, Mota EM, Penido MLO, Coelho PMZ, Vasconcelos EG** (2004) ATP diphosphohydrolase from *Schistosoma mansoni* egg: characterization of a new antigen. *Parasitology* **129**, 51-57, with kind permission from Cambridge University Press, ©2004.

by *S. mansoni* occurs following penetration of the skin by cercariae. After penetration, cercariae transform into schistosomules, sexually immature worms, that migrate to the lungs. Development of the adult parasite and sexual maturation are complete within 6-8 weeks after infection. Adult worms then migrate to the portal and mesenteric vasculature, where they initiate intense ovoposition. Immunopathology in schistosomiasis mansoni is a result of granulomatous inflammation around parasite eggs (**Fig. 2**) in the host liver and intestines, mediated by major histocompatibility complex (MHC) class II-restricted $CD4^+$ T lymphocytes specific for schistosome egg antigens (Kusel *et al*. 2007).

There is broad expression of ATP diphosphohydrolase during all stages of the *S. mansoni* life cycle (Vasconcelos *et al*. 1993, 1996; Torres *et al*. 1998; De Marco *et al*. 2003; Faria-Pinto *et al*. 2004; Levano-Garcia *et al*. 2007). The first demonstration of an ATP diphosphohydrolase activity on the external surface of *Schistosoma mansoni* was reported by Vasconcelos *et al*. (1993). Furthermore, two *S. mansoni* ATP diphosphohydrolase isoforms of approximately 63 kDa, differing in their catalytic properties and their solubilities and sensitivities to non-ionic detergents, were partially purified and characterized in both adult worm tegument and homogenized egg preparations (Vasconcelos *et al*. 1996; Faria-Pinto *et al*. 2004).

The two isoforms were identified by cross-immunoreac-

tivity with different polyclonal antibodies against native or denatured PA of different origins, suggesting that the parasite and vegetable proteins share conserved epitopes (Vasconcelos *et al.* 1996; Faria-Pinto *et al*. 2004). These data were confirmed by immunoprecipitation assays, since antibodies against PA isoforms, immobilized on Sepharose-Protein A, depleted the ATPase and ADPase activities from detergent-solubilized worm tegument (Vasconcelos *et al.* 1996). By confocal fluorescence microscopy using anti-PA antibodies, strong labeling was seen on the external surface of *S. mansoni* adult worms (Vasconcelos *et al*. 1996). Very strong fluorescence was seen on the external surface of miracidium in the *S. mansoni* egg, possibly involving the labeling of a membrane-associated isoform (**Fig. 2**, panel C; Faria-Pinto *et al*. 2004). Intense fluorescence was also seen in von Lichtenberg's envelope and on the outer side of the egg-shell, entrapped by surface microspines, suggesting that a soluble isoform is secreted (**Fig. 2**, panels C and D; Faria-Pinto *et al*. 2004). Furthermore, the cloning of two *S. mansoni* ATP diphosphohydrolase genes from the adult worm was reported (De Marco *et al*. 2003; Levano-Garcia *et al*. 2007). *In silico* protein analyses showed that *S. mansoni* SmATPDase 1, with a predicted MW 61,353, has two transmembrane domains with a great similarity to mammalian NTPDase 1, a mammalian membrane-associated enzyme (De Marco *et al*. 2003), while SmATPDase 2, of predicted MW 63,785, shows similarity to intracellular and soluble mammalian NTPDases 5 and 6, with one amino-terminal transmembrane region possibly subject to proteolytic post-translational processing that results in a secreted protein of 55 kDa (Levano-Garcia *et al*. 2007).

Due to this cross-immunoreactivity observed between PA and *S. mansoni* ATP diphosphohydrolase isoforms, the vegetable protein was used as an antigen. High levels of IgG-recognizing PA were observed in sera from Swiss Webster mice experimentally infected with *S. mansoni* (Faria-Pinto *et al*. 2004). In addition, spleen cells from chronically *S. mansoni* infected BALB/c mice showed significantly higher *in vitro* Interferon- γ (IFN-γ), Interleucin-4 (IL-4) and Interleucin-10 (IL-10) cytokine levels in response to PA (unpublished data). As recently reviewed, T helper type 1 (Th1) cells produce IFN-γ during the early phase of the parasitic diseases, while T helper type 2 (Th2) cells, which produce IL-4, are important for the regulation of the immune response to helminths. Interleukin-10, an immunoregulatory cytokine, has been associated with the function of regulatory T cells during a parasitic infection (Kusel *et al*. 2007). Therefore, it is possible that different domains shared between PA and *S. mansoni* ATP diphosphohydrolases isoforms are capable of stimulating cytokine production by distinct populations of T cells. This possibility motivates the exploration of this hypothesis in human cell culture.

The presence of an active ATP diphosphohydrolase on the surface of parasites might be a potential target for therapeutic drug action. In addition, the study of the effects of drugs on their ATPase and ADPase activities may be of help in assessing its functional significance (Gendron *et al*. 2002; Penido *et al*. 2007). Thus, the effects of the alkylaminoalkanethiosulfuric acids, new schistosomicidal drugs, were tested on *S. mansoni* ATP diphosphohydrolase isoforms (Penido *et al*. 2007). The results showed that these compounds are reversible inhibitors of the *S. mansoni* ATP diphosphohydrolase activity from the adult worm tegument, and their primary mechanism of action depends on the thiosulfate group (Penido *et al*. 2007). Since soluble *S. mansoni* ATP diphosphohydrolase isoform and PA share structural similarities, the pattern of PA inhibition was also analyzed. ADPase activity from soluble PA showed a higher sensitivity to alkylaminoalkanethiosulfuric acids than that shown by ADPase activity from the adult worm tegument, while the ATPase activities from both samples showed similar inhibition levels (Penido *et al*. 2007). Therefore, from these studies, one can conclude that both soluble and membrane-associated isoforms of *S. mansoni* ATP diphosphohydrolase are possible targets for the alkylaminoalkanethiosulfuric acids, and the variations observed for ATPase and ADPase activities suggested that the two isoforms are differently regulated (Penido *et al*. 2007).

Leishmania ATP diphosphohydrolase isoforms possess antigenic domains shared with potato apyrase

Leishmaniasis is a complex of diseases caused by different species of intracellular protozoan parasites belonging to the genus *Leishmania*. For most *Leishmania* species, humans are accidental hosts and leishmaniasis is primarily a zoonotic disease (Kedzierski *et al*. 2006). Leishmaniasis is transmitted through the bite of phlebotomine sandflies. In vertebrate hosts, *Leishmania* survive and multiply as non-motile amastigotes, primarily in macrophages. Amastigotes are ingested when a female sandfly sucks blood from an infected host. Amastigotes develop in the sandfly culminating in the generation of infective metacyclic flagellated promastigotes in the anterior parts of the digestive tract. The promastigotes are introduced into the skin of the host with the sandfly saliva at the next meal (Kedzierski *et al*. 2006). Clinical manifestations of leishmaniasis, i.e., cutaneous, mucocutaneous or visceral, depend on a close interaction between the genetic backgrounds of the parasite and the host (Kedzierski *et al*. 2006). *L.* (*L.*) *amazonensis* infection in humans presents a severe polar form, which is recognized as diffuse cutaneous leishmaniasis, sharing many features with lepromatous leprosy. *L.* (*L.*) *amazonensis* has been isolated from various clinical forms of leishmaniasis, and causes cutaneous, diffuse, mucocutaneous and visceral clinical forms (Almeida *et al.* 1996; Gonçalves da Costa 2005). *L.* (*V.*) *braziliensis* has been isolated from mucocutaneous leishmaniasis, while *L.* (*L.*) *infantum* is one of the species responsible for visceral leishmaniasis in the New World (Gonçalves da Costa 2005). The role of the host's genetic background, virulence factors of *Leishmania* strains and the immunological response, especially the Th1/Th2 pattern of immunity, in the outcome of the *Leishmania* infection has been extensively studied and implicated in some instances in susceptibility or resistance to infection (Calabrese and Gonçalves da Costa 1992; Abreu-Silva *et al.* 2003, 2004; Gonçalves da Costa 2005; Kedzierski *et al*. 2006).

The first demonstration of ATP diphosphohydrolase activity in the *Leishmania* genus was reported by Coimbra *et al.* (2002), who identified this protein on the external surface of *L.* (*L.*) *amazonensis* promastigote forms by ultrastructural cytochemical techniques (Coimbra *et al*. 2002). Ecto-localization of *L.* (*L.*) *amazonensis* ATP diphosphohydrolase confirmed that this parasite possesses mechanisms capable of hydrolyzing nucleoside di- and triphosphates and that the expression of ATP diphosphohydrolase is associated with the outer surface of the parasite's plasma membrane (Coimbra *et al*. 2002).

Furthermore, a *L.* (*L.*) *amazonensis* ATP diphosphohydrolase isoform from the plasma membrane of promastigote form was partially purified. The cross-immunoreactivity between polyclonal anti-PA antibodies and diffuse bands of about 58-63 kDa, possibly corresponding to glycosylated forms, permitted the identification of this parasite's protein as a true ATP diphosphohydrolase (Coimbra *et al*. 2008). High total IgG antibody level-recognizing PA was found in serum from promastigote-infected mice, thereby confirming both the existence of epitopes shared between the parasite and vegetable proteins, and the parasite ATP diphosphohydrolase antigenicity. According to Western blots, serum from amastigote-infected BALB/c mice recognized both PA and this antigenic ATP diphosphohydrolase isoform isolated from promastigotes, suggesting that it is also expressed in the amastigote stage (Coimbra *et al*. 2008).

Using PA as a coating antigen in the ELISA technique, the serum reactivity of amastigote-infected mice was monitored over a 90-day period. Reactivity of the IgG2a antibody was detected in the early stages of infection. During

the progression of leishmaniasis, the disappearance of the IgG2a response and an increase in the IgG1 antibody serum levels against the shared epitopes occurred (Coimbra *et al*. 2008). The humoral response of experimentally infected BALB/c mice against the epitopes shared between parasite ATP diphosphohydrolase and PA revealed that the antigenicity of this parasite's protein and, in addition, the ability of cross-immunoreactivity with PA to serologically differentiate stages of leishmaniasis in infected mice. The data again suggested that the shared epitopes of ATP diphosphohydrolase and PA are able to stimulate the host's cellular immune response.

An ATP diphosphohydrolase isoform was recently identified in *L.* (*V.*) *braziliensis* promastigote forms, and similar results were obtained in another *Leishmania* strain, belonging to the *Leishmania braziliensis* complex. By immunodepletion assays of ATPase and ADPase activities and Western blots, mouse and rabbit polyclonal anti-PA antibodies identified a 48 kDa-band in the two promastigote strains. These results confirmed that parasite and vegetable proteins share conserved epitopes, and that homologous proteins are present in several strains of *Leishmania* (unpublished data).

RABBIT POLYCLONAL ANTI-POTATO APYRASE ANTIBODIES DO NOT REACT WITH NTPDases ON THE MOUSE CELL SURFACE

NTPDases have been described in hepatocytes, epithelial cells of the bile duct system, vascular cells, and in immune cells, such as platelets, lymphocytes, monocytes and macrophages (Gendron *et al*. 2002; Robson *et al*. 2006). We showed that purified PA, when inoculated in the rabbit, generates polyclonal sera containing anti-apyrase antibodies that are capable of recognizing ATP diphosphohydrolase isoforms from *S. mansoni* egg (Faria-Pinto *et al*. 2004). Since the surrounding granulomatous tissues are rich in inflammatory cells, such as macrophages, eosinophils, neutrophils, lymphocytes, mast cells and fibroblastic-like cells (Lenzi *et al*. 1998), experimental protocols were developed to verify whether antibodies produced in the rabbit against PA would cross-react with mouse NTPDases. Using confocal immunofluorescence microscopy and rabbit polyclonal anti-PA antibodies, cryostat mouse liver sections presenting exudative-productive granulomas were analyzed. The results showed that the anti-PA antibody reacted only with miracidial and egg structures, and not with granulomatous cells (**Fig. 2**, panel C). Negative reactivity was also observed to hepatic parenchyma, blood vessels, and bile ducts (Faria-Pinto *et al*. 2006). Cross-immunoreactivity between anti-PA antibody and intracellular NTPDases, possibly not accessible under experimental conditions, were not discarded in these experiments (Faria-Pinto *et al*. 2006). However, the high identity between PA and *S. mansoni* ATP diphosphohydrolase and the lower identity with mammalian NTPDases observed by *in silico* analysis, suggested the presence of unique motifs shared between parasite and vegetable proteins, and that autoantibodies are not induced by immunization with PA (Faria-Pinto *et al.* 2006).

MAPPING OF THE CONSERVED DOMAINS SHARED BETWEEN POTATO APYRASE AND PARASITE ATP DIPHOSPHOHYDROLASES

By evaluation of the cross-immunoreactivity with PA and *in silico* analyses, the possible occurrence of conserved domains as functional regions in parasite ATP diphosphohydrolases and the associated antigenicity of these proteins in human parasitic diseases was demonstrated (Faria-Pinto *et al*. 2008). The highest identities between PA and leishmanias NDPases or SmATPDase 2 were observed in the regions that include the characteristic conserved domains of the ATP diphosphohydrolase family (ACR), and in three others sites (**Fig. 3**, arrows B, F and G). Predictions of putative epitopes and MHC Class-II binding peptides showed that these conserved shared domains have high scores for

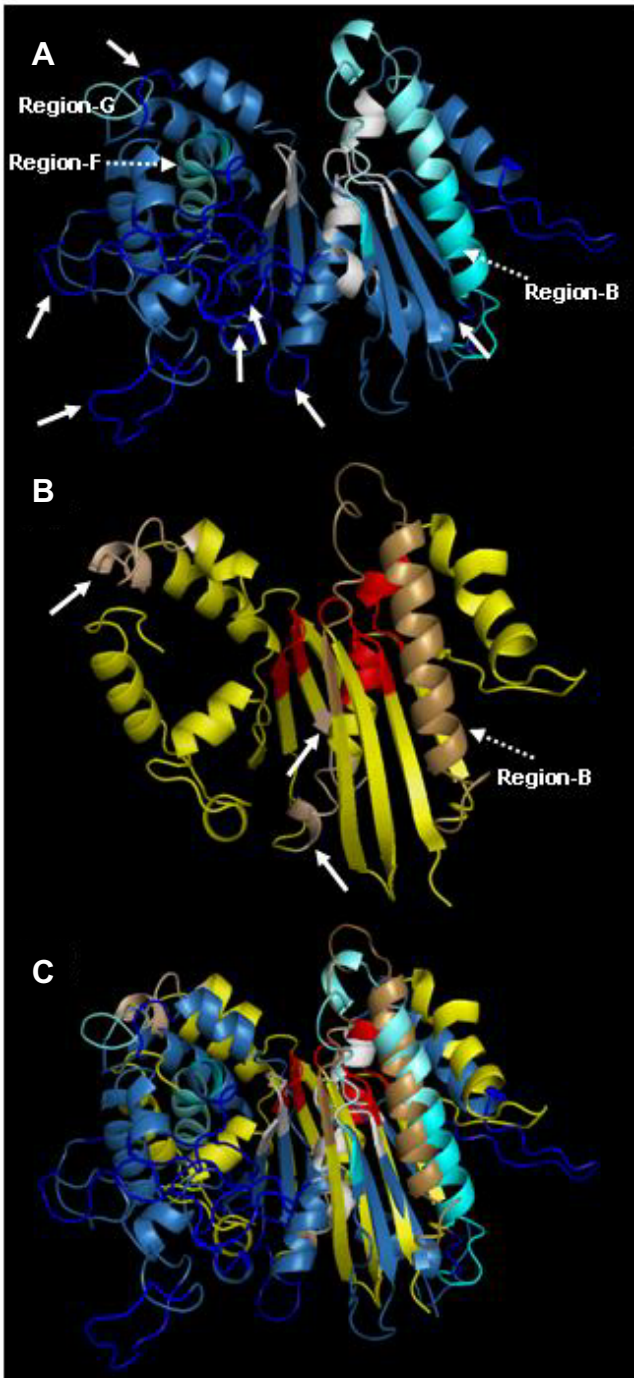

Fig. 3 Molecular models for isolated PA (**A**) and *L. braziliensis* NDPase (**B**), and after superposition of the two proteins (**C**) showing the high structural homology between them. The apyrase conserved regions (ACR) are represented in white (A) or red (B) and the exposed regions that bind antibodies are indicated by white arrows (A, B). Conserved region B is indicated by another tone of blue (A, C) or brown (B, C). Reprinted from **Faria-Pinto P, Rezende-Soares FA, Molica AM, Montesano MA, Marques MJ, Rocha MOC, Gomes JAS, Enk MJ, Correa-Oliveira R, Coelho PMZ, Neto SM, Franco OL, Vasconcelos EG** (2008) Mapping of the conserved antigenic domains shared between potato apyrase and parasites ATP diphosphohydrolases: potential application in human parasitic diseases. *Parasitology* **135**, 943-953, with kind permission from Cambridge University Press, ©2008.

reactivity by antibodies and/or high probability of binding human leukocyte antigen (HLA)-DR molecules and are theoretically capable of inducing a T cell immune response (Faria-Pinto *et al*. 2008). Antigenic loops in putative three-dimensional models of the *L.* (*V.*) *braziliensis* NDPase (**Fig. 3**) and *S. mansoni* SmATPDase 2 were shown to be conserved functional regions, indicating a clear association between structure and antigenicity (Faria-Pinto *et al*. 2008).

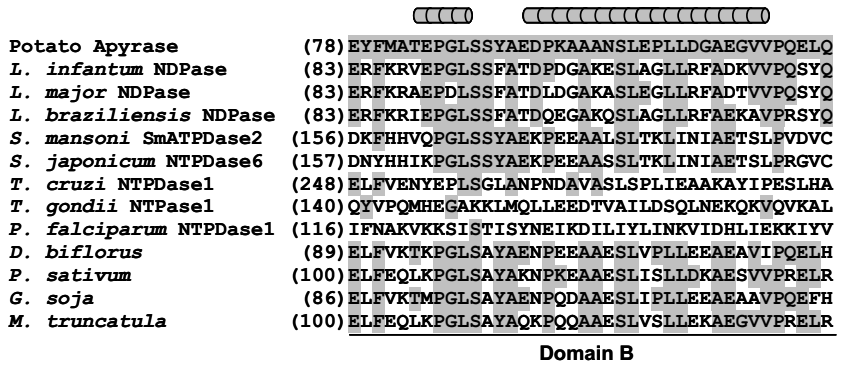

Fig. 4 Primary structure alignment of the domain B from plants and parasites apyrases. The identical amino acid residues of PA are shown as grey columns. Grey cylinders correspond to α-helices in the tri-dimensional structure of PA (**Fig. 3**). GeneBank accession numbers of the amino acid sequences are indicated in **Fig. 1**. Proteins from leishmanias (50-55%) and schistosomes (43-45%) showed a higher degree of identity to PA. No significant identity in linear sequence was observed between PA and either *T. cruzi*, *T. gondii* or *P. falciparum* proteins. The regions B from other plants share 58-60% identity with PA.

The domain B, which did not include "apyrase conserved region" from ATP diphosphohydrolase family, is shared between PA, *D. biflorus*, *P. sativum*, *G. soja*, *M. truncatula*, leishmanias NDPases, SmATPDase 2 and *S. japonicum* NTPDase6 like-protein, and it did not share significant identity with *P. falciparum* NTPDase1, *T. gondii* NTPase 1 and *T. cruzi* NTPDase 1 counterparts (**Fig. 4**). This particular domain is likely responsible for the cross-immunoreactivity between PA and *S. mansoni* or Leishmania proteins.

REACTIVITY OF HUMAN SERUM WITH POTATO APYRASE

Theoretical analyses showed a high degree of identity between PA and the soluble isoform of the *S. mansoni* ATP diphosphohydrolase or leishmanias NDPase-like proteins, and, to a lower degree, with *T. cruzi* NTPDase (Faria-Pinto *et al*. 2008). These studies stimulated the evaluation of the reactivity between PA and sera from American cutaneous leishmaniasis (ACL), schistosomiasis or Chagas diseased patients (Faria-Pinto *et al.* 2008). Significantly higher IgG antibody reactivity was found in sera from ACL patients, followed by antibodies from individuals with schistosomiasis. Sera from patients with Chagas disease had low IgG antibody reactivity against PA, significantly lower than that found in schistosomiasis patients (Faria-Pinto *et al*. 2008). Therefore, one can conclude that ATP diphosphohydrolase epitopes of the parasite, shared with PA, are promptly recognized by IgGs from schistosomiasis and leishmaniasis patients.

T. cruzi NTPDase was localized on the external surface of all life-cycle forms of *T. cruzi* (Fietto *et al*. 2004). *T. cruzi* is the etiological agent of Chagas disease that causes chronic infection in humans and a large number of other mammalian species. Transmission to host vertebrates is achieved by insects through feces contaminated with the metacyclic trypomastigote, an infective stage of the parasite that occurs in the lumen of the rectum of the insect. In the vertebrate host, *T. cruzi* is an obligate intracellular parasite that completes its life cycle in different nucleated cell types, reaches the cytoplasm where it replicates into amastigote forms (Fietto *et al.* 2004; Gomes *et al*. 2005). According to *in silico* analysis, *T. cruzi* NTPDase 1 shares a low identity with PA (Faria-Pinto *et al*. 2008), but showed domains that include "apyrase conserved regions" with a high predictive probability to bind antibody and human leukocyte antigen (HLA)-DR molecules. *T. cruzi* has antigens associated with autoimmune responses (Requena *et al*. 2000; Gomes *et al*. 2005), and it was concluded that the low reactivity with PA should not be neglected, since these conserved domains are interesting targets for further investigation into the role in the immune response against this parasitic infection (Faria-Pinto *et al*. 2008).

CONCLUDING REMARKS AND PERSPECTIVES

Parasites may live for several years in the host, and thus must possess mechanisms to evade hemostatic and immune responses, which are still poorly understood. We believe that immunological studies on parasites' ATP diphosphohydrolases could contribute to the elucidation of these mechanisms and a better understanding of host-parasite interactions. A highly distinct human humoral immune response profile of IgG antibody against PA was observed in patients with American cutaneous leishmaniasis, schistosomiasis or Chagas disease. This distinct immunoglobulin profile, associated with the different parasites' life-cycles, suggested that the parasite ATP diphosphohydrolases are processed and presented to effector cells from the host immune system by different pathways, warrants further study. Comparative studies of the immune response elicited by native or recombinant forms of parasite ATP diphosphohydrolases in different parasitic diseases will be of interest for evaluation of their immunogenicity, and for determining whether the shared epitopes between parasites and vegetable proteins are immunodominant. The conserved domains could be tested in proliferation and *in vitro* human leukocyte antigen (HLA)-DR binding assays in larger populations with a high genetic variability to determine the ability of those epitopes to elicit an immune response and to be presented by a wide range of human leukocyte antigen (HLA) molecules.

New approaches are required to supplement current disease control methods such as diagnosis and treatment, which are difficult and costly. Crude antigens used in epidemiological studies are valuable for the detection of general patterns in infected populations but, for comparative studies, the use of serological surveys on single antigens permits better definition, not only of the humoral response, but also of an analysis of the role of the antigen in inducing an effective cellular response (Mutapi 2001). It is possible that immunoglobulin subtype analysis, using vegetal protein or their derivative epitopes as antigens, can differentiate the parasitic diseases and these new tools could be considered for formulation of specific and sensitive methods for the diagnosis and prognosis of human parasitic diseases.

We can state that the PA has the potential to stimulate the production of antibodies and significant *in vitro* quantities of cytokines by immune cells of *S. mansoni*-infected mice. Novel immunostimulatory molecules from plants that enhance or direct an appropriate immune response against target immunogens are being investigated for treatment and prevention of several diseases (Spelman *et al*. 2006; Lee *et al*. 2007). The observations that both Th1 and Th2 responses are involved in either amelioration of the fibrous pathology of the granulomatous response in schistosomiasis, or in immunomodulation, resistance, and protective immune response against parasites (Hoffmann *et al*. 1999; Zouain *et al*. 2001; Kedzierski *et al.* 2006; Leenstra *et al*. 2006), and that

molecules produced by *S. mansoni* or *S. japonicum* contribute to the prevention of a range of immune disorders (Araújo *et al*. 2004; Yang *et al*. 2007) have been described. Therefore, further studies on the conserved domains of both parasite and PA, obtained by peptide synthesis or by cloning and heterologous expression, could be explored as molecular markers for the study of the parasitic diseases or for implementation of either vaccine or immunotherapy.

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