Charles university
Faculty of Science

Study program: Parasitology

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Characterization of serine proteases of *Schistosoma mansoni* involved in interaction with host

Charakterizace serinových proteáz u motolice *Schistosoma mansoni* participujících na interakci s hostitelem

Doctoral Thesis

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Prague 2024

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Acknowledgments

My deepest gratitude goes to Martin Horn and Jan Dvořák who guided me during my scientific years and who introduced me to biochemistry and schistosomal world. I am grateful to Michael Mareš for providing the scientific background during my research. My dearest thanks go also to Lucie Marešová for supportive and secure environment she has been creating in the laboratory. I would be nothing without Pavel Ostašov who introduced me to the microscopy techniques and helped me whenever I needed. Many thanks to Marta Chanová for advice regarding to schistosoma breeding, tissue preparation and for intensive brainstorming over lunches. I am grateful for her time and encouragement whenever I needed. I also thanks to all my coworkers form the laboratory, you often cheered me up! And finally, I deeply thank to my family, that they have been standing by my site all the time. Especially, to Danka for babysitting, and to Pavel and Vojta for their patience over the years. I appreciate this more than you will ever know.

Fundings

SCIEX (PRENINS), grant No.: 11.222, Project: Proteolytic enzymes and inhibitors of trematode parasite *Schistosoma mansoni*

Grantová agentura UK, grant No. Grantová agentura UK: Tkáňová lokalizace serinových proteáz lidského parazita – motolice *Schistosoma mansoni*

Cestovní stipendia od Nadace „Nadaní Josefa, Marie a Zdeňky Hlávkových“ a „Nadace Český literární fond“

Abstract

Schistosomiasis is a serious parasitic disease, caused by blood flukes of the genus *Schistosoma*. It remains a global health problem in the 21st century, with more than 250 million people infected in 78 countries. Current therapy relies on the drugs praziquantel and oxamniquine, for which there are concerns of emerging drug resistance. Proteases of schistosomes are involved in critical steps of host-parasite interactions and are promising targets for the development of new therapeutic strategies against schistosomiasis. This work focuses on the characterization of *Schistosoma mansoni* serine proteases (SmSPs) and the determination of their role in the interaction with the human host using a variety of genomic, bioinformatic, RNA- and protein-based techniques. First, the major types of proteolytic activities secreted by the blood-dwelling developmental stages of *S. mansoni* were classified using functional proteomics. The analysis revealed the complexity of proteolytic activities secreted by the schistosome life stages parasitizing the human host. All stages secreted significant serine protease activities, and consequently their genes were retrieved from the genome database and annotated. Localization in adult worms determined by fluorescence *in situ* RNA hybridization revealed complex expression patterns of individual SmSPs in different tissues. Two SmSPs, serine protease 2 (SmSP2) and prolyl oligopeptidase (SmPOP), were biochemically and functionally characterized using recombinant proteins, and their biological roles in modulating host hemostasis were proposed. The work provides important new information on *S. mansoni* serine proteases and their potential roles in host-parasite interactions by modulating host physiology, which is relevant for the development of novel anti-schistosomal interventions.

Abstrakt

Schistosomóza je závažné parazitární onemocnění způsobené motolicemi rodu *Schistosoma*. I ve 21. století zůstává celosvětovým zdravotním problémem, neboť je jí nakaženo více než 250 milionů lidí v 78 zemích světa. Současná terapie je založena především na dvou lécích, praziquantelu a oxamniquinu. Vzrůstající riziko vzniku rezistence vyvolává potřebu nových alternativních chemoterapeutik nebo vakcín. Proteázy schistosom se podílejí na mnoha kritických interakcích parazita s hostitelem, a proto představují slibné cíle pro vývoj nových léčebných strategií proti schistosomóze. Tato dizertační práce se zaměřuje na charakterizaci serinových proteáz krevničky *Schistosoma mansoni* a na určení jejich role v interakci s lidským hostitelem za použití genomických a bioinformatických přístupů, včetně metod molekulární biologie RNA a proteinové biochemie. Nejprve byly pomocí funkční proteomiky klasifikovány hlavní typy proteolytických aktivit v exkrečně sekrečních produktech vylučovaných vývojovými stadii *S. mansoni* žijícími v krvi hostitele. Analýza odhalila komplexnost těchto proteolytických aktivit, které jsou jedinečné pro každé stádium parazitující v lidském hostiteli. U všech studovaných stádií byla v sekrečních produktech detekován významný podíl aktivit serinových proteáz. Následně byla v genomové databázi pro *S. mansoni* detekována a anotována sada genů pro serinové proteázy. Analýza jejich lokalizace v dospělých krevničkách provedená pomocí fluorescenční RNA *in situ* hybridizace ukázala komplexní expresní profil jednotlivých proteáz v různých tkáních. Dvě z nich, serinová proteáza 2 (SmSP2) a prolyl oligopeptidáza (SmPOP), byly biochemicky a funkčně analyzovány za použití rekombinantních proteinů a byla navržena jejich biologická role v modulaci hemostázy hostitele. Disertační práce přinesla nové důležité informace o serinových proteázách *S. mansoni* a jejich potenciální roli v interakci parazita s hostitelem prostřednictvím modulace jeho fyziologie. Tyto informace jsou důležité pro vývoj nových anti-schistosomálních léčiv.

Content

Abbreviations

Ca^{2+}	calcium cation
CE	cercarial elastase
CUB	Complement-Uegf-BMP-1 extracellular domain
DIG	digoxigenin
ES	excretory/secretory
EVs	extracellular vesicles
FISH	fluorescence *in situ* hybridization
HRP	horseradish peroxidase
kDa	kilodalton
LDLa	low-density lipoprotein binding receptor domain class A
mRNA	messenger ribonucleic acid
MSP-MS	multiplex-substrate profiling by mass spectrometry
NATs	natural anti-sense transcripts
NTS	newly transformed schistosomula
PZQ	praziquantel
RNA	ribonucleic acid
RNAseq	ribonucleic acid sequencing
RT-qPCR	quantitative reverse transcription PCR
Sm29	a surface protein of *Schistosoma mansoni*
SmCB1	*S. mansoni* cathepsin B1
SmCB2	*S. mansoni* cathepsin B2
SmPOP	*S. mansoni* prolyl oligopeptidase

SmSP1-5	*S. mansoni* serine protease 1-5
SmSP	*S. mansoni* serine protease
SmTSP-1	*S. mansoni* tetraspanin 1
SmTSP-2	*S. mansoni* tetraspanin 2
SP	serine protease
$TRPM_{PZQ}$	a transient receptor potential ion channel of the melastatin subfamily activated by praziquantel
T cell	a group of lymphocytes that can be distinguished from other lymphocytes by the presence of a T cell receptor on their cell surface
TSA	tyramide signal amplification system
TSR-1	thrombospondin type 1 repeat domain

1 Introduction

Schistosomiasis is a serious disease caused by blood flukes of the genus *Schistosoma*. According to the latest estimates, at least 250 million people required medical treatment for this disease in 2022 [1]. Schistosomiasis transmission has been reported in 78 countries, of which 52 are endemic countries with moderate to high transmission rates. Recently, autochthonous infections by human schistosomiasis have also been identified in southern Europe in Corsica and Spain [2, 3]. Due to a climate changes and international mobility of people and animals, increasing numbers of cases are expected in moderate climates.

Human infection occurs in water infested with schistosome larvae (cercariae). Cercariae actively penetrate the human skin, where they begin the transformation into larvae (schistosomula). When they enter the host's vascular system, schistosomula develop into adult worms. They can live in the host for years [4], producing large numbers of eggs each day [5]. Eggs pass through the intestinal or bladder wall and are excreted in the feces or urine to complete the parasite's life cycle. However, a portion of the laid eggs that do not successfully pass through the intestinal or bladder wall and become trapped in the tissue. Here, the eggs induce inflammation leading to the clinical manifestations of chronic schistosomiasis. The eggs are therefore the main pathogenic agent of schistosomiasis [6].

Two chemotherapeutic agents - praziquantel and oxamniquine - are recommended for the treatment and prophylaxis of all forms of schistosomiasis [7, 8]. However, there is increasing evidence that the provision of prophylaxis and control of the disease remains inadequate, because it does not lead to a situation where the number of infected individuals is consistently and significantly reduced [9]. One of the major limitations of schistosomiasis control, in addition to the limited availability of praziquantel, is the fact, that preventive treatment is recommended to be repeated over several years due to the high risk of re-infection [1, 10]. The second limitation is the increasing resistance to this drug in some endemic areas, as it has been already used for the last 40 years [11]. Third, schistosomes can survive in the definitive host for more than a decade while producing massive numbers of eggs. However, the disease manifests itself over a

longer period, resulting in late diagnosis and treatment [12]. These facts indicate that there is an urgent need for the development of alternative drugs or vaccines.

Proteolytic enzymes (proteases) are critical for successful parasitism of schistosomes [13]. They are important for the parasite invasion, intra-host migration, digestion, reproduction, modulation of host homeostasis and manipulation of the host immune system [13-16]. Therefore, proteases are increasingly being studied as potential therapeutic targets and vaccine candidates.

This thesis focuses on the serine proteases of the blood fluke *Schistosoma mansoni*. Compared to other classes of schistosome proteases, which are intensively studied, the serine proteases remain neglected. The main aim of the thesis is to identify *S. mansoni* serine proteases from the S1 and S9 protease families, to characterize them biochemically, and to determine their role in parasite-host interactions as potential targets for the development of novel anti-schistosomal therapies.

2 Literature review

2.1 Genus *Schistosoma* – blood flukes infecting human

The genus *Schistosoma* belongs to the class of trematodes and the subclass of digeneans [17]. Digeneans are parasitic flatworms of medical and veterinary importance [18, 19]. The life cycle of digenetic trematodes typically involves three hosts, but a significant number of species have reduced the number of hosts during development, most likely to increase their survival [18]. Compared to the large number of trematodes, about a hundred of digenetic flukes are known to cause serious human diseases including schistosomiasis, which affects more than 250 million people in tropical and subtropical countries [20, 21]. Recently, autochthonous human schistosomiasis infections have also been identified in southern Europe in Corsica and Spain [2, 3, 22]. Due to climate change and international mobility of people and animals, the number of cases in moderate climates is increasing.

Schistosomiasis is a zoonotic disease caused by blood flukes of the genus *Schistosoma*. This genus is very diverse with 23 described species [23]. Of these, at least 7 are medically important species that can infect humans and cause two major forms of human schistosomiasis - intestinal and urogenital. *S. mansoni*, *S. japonicum*, *S. mekongi*, *S. guineensis, S. malayensis*, and S. *intercalatum* are responsible for the intestinal form of the disease, and S*. haematobium* is the causative agent of urogenital schistosomiasis [24]. Adult schistosomes are approximately 1-2 cm long worms that live in the blood system surrounding the human intestine (intestinal schistosomiasis) or urogenital organs (urogenital schistosomiasis) [12].

2.1.1 The life cycle of *Schistosoma mansoni*

S. mansoni is the most common cause of intestinal schistosomiasis. The life cycle of this parasite is dependent on snails (as intermediate hosts, where the parasite reproduces asexually)

and mammals (as definitive hosts, where the parasite reproduces sexually) (Figure 1). The typical intermediate hosts are freshwater snails belonging to the genus *Biomphalaria.* The primary natural definitive hosts are humans, but *S. mansoni* can also infect other mammals, such as non-human primates or rodents [12]. Experimental models of *S. mansoni* include laboratory mice, rats, and hamsters as hosts.

In the nature, once *S. mansoni* eggs are deposited from the human host into the freshwater environment, free-swimming larvae called miracidia hatch immediately. The miracidia actively seek (following chemical cues) and invade an intermediate host [25]. Inside the snail, the miracidia transform into the mother sporocysts from which are later generated daughter sporocysts [26]. Within the daughter sporocysts, free-swimming larvae called cercariae develop. In approximately four weeks, cercariae are shed from the snail into the freshwater environment. Shedding is stimulated by light that in nature correlates with human circadian rhythms and by light [27]. Cercariae begin to seek a definitive host using thermal gradients and chemical cues on mammalian skin (such as linoleic acid) [28].

Once on the skin, cercariae release specific proteases (such as cercarial elastase or SjCB2 (*Schistosoma japonicum* cathepsin B2)) from the acetabular gland that help them to penetrate the human skin [29, 30]. Meanwhile, they lose their tails and transform into larvae called schistosomula. After 3-4 days [31, 32], schistosomula enter the blood vessels attracted by D-glucose, L-arginine, fibronectin, bradykinin and probably other components of human blood serum [33]. Once in the vasculature, the parasites are transported by the bloodstream into host´s internal organs, such as the heart, lungs, and the liver, where they develop into adult worms [34, 35].

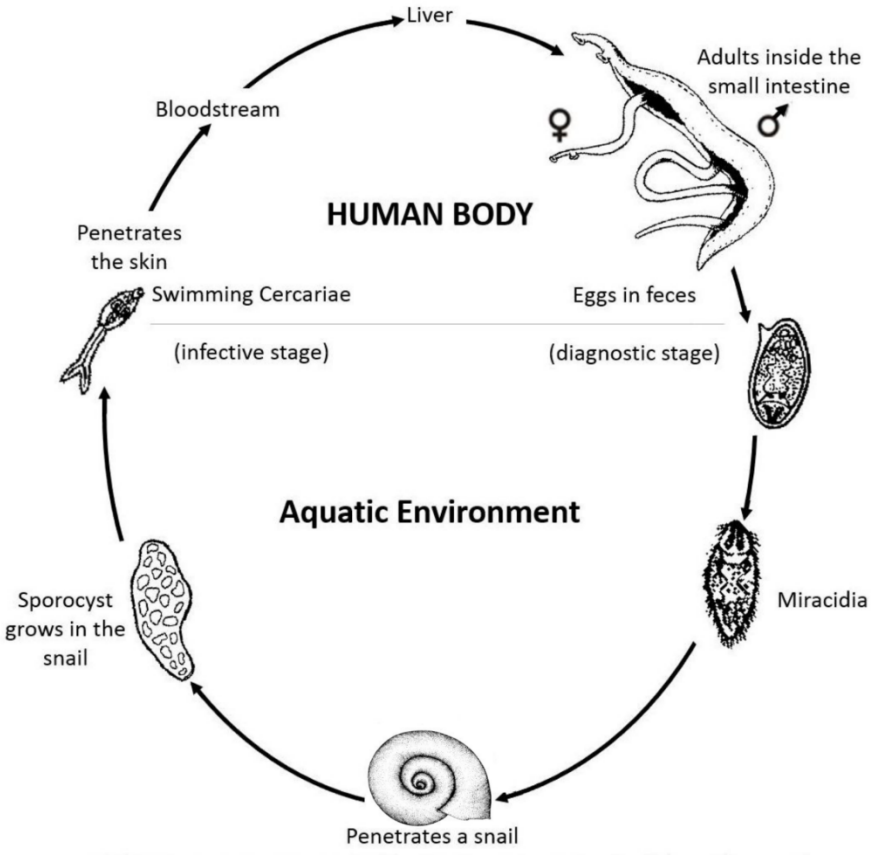

Figure 1. *Schistosoma mansoni* life cycle. Modified from [36, 37].

Schistosomes are unique among trematodes in that they exhibit sexual dimorphism between adult males and females. Adults reside in pairs in the hepatic portal venules in close proximity to the host intestine [34], where they mate [38]. Females lay an enormous number of fertilized eggs. Specifically, a *S. mansoni* female produces approximately 350 eggs daily [12]. The eggs circulate in the host's blood system for some time and then transverse to the lumen of the intestine. Later, they are shed into the outer environment with the fecal stream to continue the parasite's life cycle [12].

However, a proportion of *S. mansoni* eggs are unable to pass out from the human but are trapped in the host's internal tissues either at the oviposition site or are spread further by bloodstream, mainly to the liver, intestine, and spleen [39]. Here, the eggs induce inflammation

leading to progressive organ damage and the clinical manifestations of chronic schistosomiasis [12, 40].

2.1.2 Pathology and symptoms of schistosomiasis

Schistosomiasis has an acute and a chronic phase. The acute or invasive phase (also known as Katayama fever) is typical for travelers from non-endemic areas (non-immune individuals), whereas the chronic phase is more commonly diagnosed in residents of endemic areas (indigenous persons) [41]. People who are infected for the first time (usually migrants or tourists) may develop a skin rash that appears within a few hours or up to a week, and which arise at the site of percutaneous penetration by cercariae [42].

In many cases, acute schistosomiasis is initially asymptomatic [43], and is rarely reported in chronically exposed populations [44]. However, some individuals with severe reinfection or primary infection may present with non-specific clinical manifestations such as fever, fatigue, non-productive cough, eosinophilia, and visible pulmonary infiltrates on chest radiograph [45]. These symptoms are usually associated with parasite migration in the host tissue [44, 46]. Manifestations of acute schistosomiasis usually appear 2-12 weeks after parasite exposure and may persist for weeks [47, 48]. Such a delay (from weeks to months) and the non-specificity of early symptoms add to the diagnostic difficulty. In endemic areas with populations regularly exposed to *S. mansoni*, most infected individuals have chronic infection. Depending on the infection rate, symptoms may develop within months or years later [49].

Schistosomiasis mansoni

Clinical manifestations of the intestinal form of schistosomiasis are often characterized by granuloma formation and neoangiogenesis with subsequent periportal fibrosis causing portal hypertension and splenomegaly [50], and may lead to schistosomal colitis and polyp formation, often manifested by abdominal pain, diarrhea and tenesmus [50].

The pathogenesis of chronic schistosomiasis is related to the host's immune response to the schistosome eggs deposited in the internal tissues [50, 51]. Due to the relatively high antigenicity of the eggs and the continuous release of metabolites such as proteolytic enzymes [52], a variety of immunopathological processes are activated [53, 54]. The eggs induce a strong granulomatous immune response characterized by the infiltration of activated immune cells (macrophages, eosinophils and T-helper cells), with additional fibroblast proliferation and formation of extracellular matrix. This process protects host tissues from egg-derived toxins, but ultimately leads to the formation of fibrotic lesions [54]. Schistosome egg deposition can occur at different ectopic sites, resulting in specific clinical manifestations [40, 55].

A proportion of the population with severe infection and long duration of the disease develops a variety of clinical manifestations depending on the organ damage, the intensity of infection and the magnitude of immune response. In the advanced stage of the infection, significant morbidity with lifelong disability or severe complications may lead to death [40].

2.1.3 Diagnosis and therapy of schistosomiasis

The first diagnostic method for intestinal and hepatic schistosomiasis was the detection of schistosome eggs in host fecal samples [12]. The microscopy-based Kato-Katz stool smear technique (developed in 1972) is still widely used because of its high specificity and cost-effectiveness [68]. However, direct egg detection is not suitable for early diagnosis, because oviposition (egg laying) begins approximately 6 weeks after cercarial infection [40].

Compared to the Kato-Katz method, immunological tests have a higher sensitivity [56, 57]. Immunological diagnostics include the detection of anti-schistosomal antibodies, circulating schistosomal antigens or other biomarkers in plasma, serum, sputum or urine [58-62]. To date, an array of immunological tests (such as DLIA - dipstick with latex immunochromatographic assay; Dot-ELISA - dot enzyme-linked immunosorbent assay; ECISA - electrochemical immunosensor array; FA-ELISA - fraction antigen ELISA; Schistosoma ICT IgG-IgM - rapid

immunochromatographic test, etc.) have been formulated for the diagnosis of schistosomiasis, while new ones are still being developed [40, 57, 63].

Pathological processes in schistosomiasis that result in structural and biochemical changes in the host tissues can be detected by biopsy or imaging techniques. Ultrasound, computed tomography, and magnetic resonance imaging are the main methods used to assess the state of infection [64-66]. Especially in endemic areas, ultrasound is a routinely used technique [40, 65], which allows visualization of typical features of liver damage, such as periportal fibrosis and hepatic granulomas [67, 68].

Currently, the treatment relies on two chemotherapeutics: praziquantel (PZQ) and oxamniquine [69]. Oxamniquine is effective only against *S. mansoni*. It is administered orally and affects DNA and RNA of the parasite. However, cases of parasite resistance to this treatment have been reported [70]. PZQ is a chemical compound that has been used for the treatment of schistosomiasis since 1970 [71]. It is administered as a single dose, or in repeated two doses to treat juvenile worms (PZQ is unable to treat developing larvae) that were not eliminated during the initial administration [72].

Studies have shown that PZQ affects calcium channels, leading to the muscle paralysis and at the same time to the tegument destruction [73], although its exact target has remained unknown since its discovery. Recently, a transient receptor potential ion channel of the melastatin subfamily ($TRPM_{PZQ}$) has been identified as a target [74]. $TRPM_{PZQ}$ is a Ca^{2+}-permeable ion channel expressed in all parasitic flatworms (including some trematodes and cestodes) that are PZQ-sensitive [75]. PZQ non-selectively activates the $TRPM_{PZQ}$ channel, which allows the passage of various cations, including calcium ions, across the cell membrane [75]. In addition, PZQ likely interacts with other schistosome molecules, such as myosin regulatory light chain or glutathione S-transferase and may promote immunoregulatory pathways [76].

The advantage of this drug is effectiveness against all forms of human schistosomiasis with mild side effects and its low price [7], but the mass drug administration in endemic areas for more than forty years has led to a reduction in PZQ efficacy [77]. Resistance has also been successfully demonstrated in mice under laboratory conditions [8]. Therefore, current research

is intensively focused on the discovery of novel targets for new drug design and vaccine development [78]. A vaccine utilizing protein or mRNA mechanisms could significantly contribute to the control of schistosomiasis [79, 80]. But to date, no such vaccine has been successfully developed. The process of creating an effective vaccine is an ongoing and challenging endeavor [79, 80].

2.1.4 Schistosomiasis prevention and control

Currently, mass administration of PZQ is used as a key strategy recommended by the World Health Organization (WHO) for the control and elimination of schistosomiasis in endemic countries with moderate to high transmission rates [10]. However, the drug treatment alone is not sufficient [81]. The World Health Organization (WHO) suggests several methods to stop the spread of schistosomiasis: (a) Eliminate *Biomphalaria glabrata*, a key host in the disease's transmission found in contaminated water. (b) Remove and treat livestock, which can also be hosts for Schistosoma species, from areas with contaminated water. (c) Improve existing sanitation facilities and build new ones. (d) Educate people in affected areas, especially school-age children who are most at risk, about the dangers and how to avoid exposure to contaminated water. A combination of these approaches would successfully reduce morbidity and prevalence of infection [10].

2.2 Tegument – surface structure of schistosoma

The entire outer surface of schistosomula and adult worms consists of a syncytial layer called the tegument [82]. This highly dynamic structure provides a variety of needs for the parasite, including protection, excretion, nutrition (e.g., cholesterol, insulin) intake from the bloodstream, sensory, and signaling functions [82, 83]. On the apical site, the tegument is decorated with a number of subcellular surface structures such as spines, myofibrils, or sensory receptors and is rich in surface invaginations [84]. Only *S. mansoni* males have additional external

tegumental structures called tubercles [85]. The nucleated regions (also known as cell bodies or cytons) are located beneath the muscle layer. Cytons are connected to the syncytial surface part via cytoplasmic connections [86] (Figure 2) and represent centers of tegumental gene transcription and translation. Messenger RNAs and proteins are generated in cytons and can be transported via cytoplasmic connections to the apical site of the tegument [86], where they can be incorporated into the outer tegumental membrane or secreted directly into the host environment [87, 88]. In either case, these bioactive molecules are responsible for host-parasite interaction, providing protection against host immune system or altering host physiology to improve parasite life conditions [89-91].

In general, the localization of mRNA in multicellular organisms is enormously diverse [92, 93]. The endoplasmic reticulum and Golgi apparatus provide well-established membrane sorting machinery to shuttle transcripts to distant regions of the individual cell [92]. These mRNAs are typically stored in granules for immediate or emergency use in a variety of processes [92, 94]. They can (a) be reactivated and translated into proteins, and used in various rapid emergency processes including stress responses, repair of cell/tissue damage, adaptation to the changed condition; (b) repress or activate other mRNAs; (c) be sent directly to the decay [95-97]. Schistosomes may use similar mechanisms, especially in such a dynamic structure as the tegument (Figure 2).

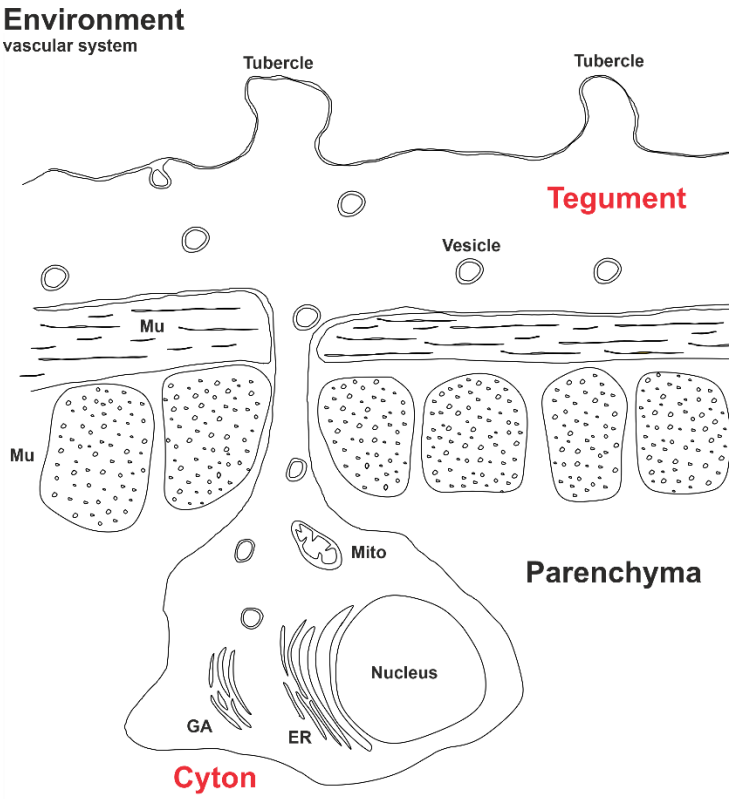

Figure 2. Schematic representation of the adult *S. mansoni* surface. Replication, transcription and translation apparatus are located exclusively in the tegumental cell body (called cyton), which is located beneath the muscle layers and surrounded by parenchyma. Cargoes such as proteins or RNAs are transported to through the cytoplasmatic connection to the tegumental surface in membranous vesicles. ER – endoplasmatic reticulum, GA – Golgi apparatus, Mito – mitochondria, Mu – muscle.

2.3 Host-parasite interaction of schistosomes

Parasitic helminths, employ a plethora of surface and secreted/excreted molecules (proteases, RNAs, lipid-binding proteins, glycans, etc.) that interact with the host environment [98-102]. During extensive evolutionary adaptation, schistosomes have achieved high defense efficiency. They release various substances into the mammalian vasculature, which enhance their survival chances. These substances are crucial for the host-parasite interaction, as they help to modulate the host's blood clotting processes [103, 104] and the immune system [105-107].

Hemostasis is the mechanism that stop bleeding from a blood vessel [108] by formation of clot consisting of platelets and fibrin polymer [109]. This clot seals the injured area, controlling and preventing further bleeding while the tissue regenerates. As the wound begins to heal, the clot slowly remodels and dissolves through fibrinolysis [110]. This process involves multiple interrelated steps [109] (Figure 3) and is mediated by a series of enzymatic activations in which serine proteases play crucial role. Function of these proteases is tightly regulated by endogenous inhibitors that are naturally present in the blood [110, 111].

The hemostasis network interacts directly with the complement system through protein-protein interactions (Figure 3) [112]. Complement consists of cascade of serine proteases and is an essential part of the innate immune system, providing immune surveillance and protection against infection [113].

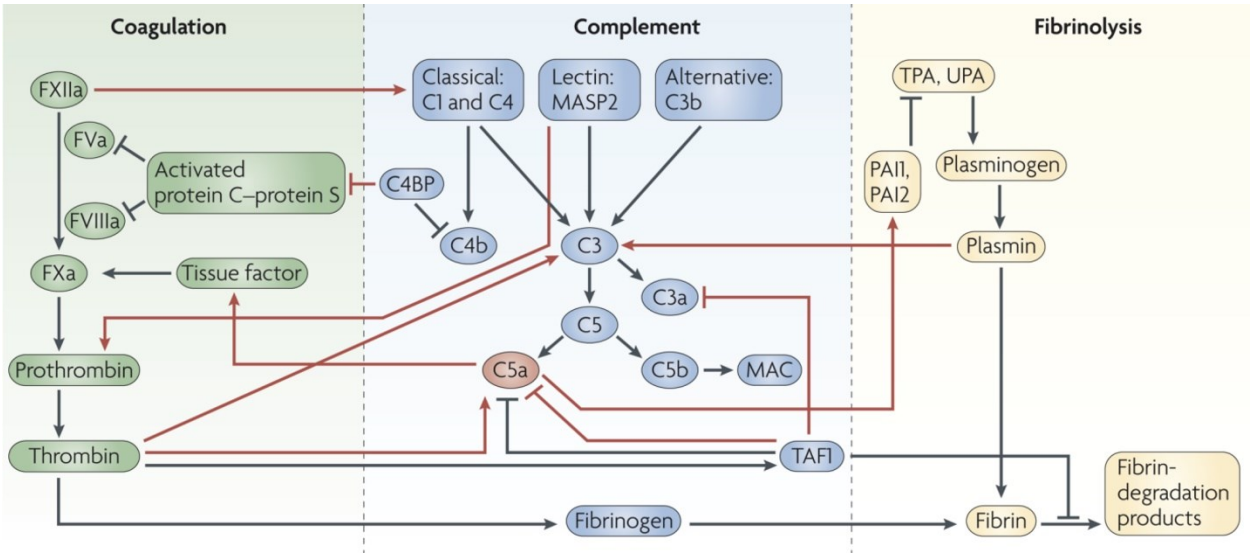

Figure 3. Scheme of the interaction of hemostatic network and complement system. Adapted from [114].

Adult schistosomes survive in the veins for many years and have evolved several mechanisms to evade and combat the inhospitable environment of the host. Worms incorporate some of host hemostatic and complement molecules onto their surface [115, 116], are able to

produce their own factors that interact with the hemostatic and complement cascades [102, 117], and mimic various host biomolecules [118, 119]. For example, schistosomes are known to release several antihemostatic, vasoregulatory, and anticoagulant factors that modulate coagulation, complement, and fibrinolysis [103, 120].

Depending on the phase of infection, schistosomes modulate the host immune system to respond in a pro-inflammatory or anti-inflammatory manner [121]. During the early stages of the acute phase (e.g., before the oviposition), there is a predominance of T helper type 1 (Th1) immune response [122]. Contrary, after oviposition, the immune response shifts strongly towards a Th2 profile [123]. *S. mansoni* and *S. japonicum* could also alter macrophage phenotypes (M1 and M2) [124], and thus influence the profile of infection [121, 125].

Factors released by schistosomes are not only represented by proteins, but also by RNA molecules [126, 127].The immune and hemostatic networks are modulated by the RNAs present in the extracellular vesicles (EVs; chapter 2.4.2) produced by the parasite. These RNAs are internalized into the host cells, such as T cells or vascular endothelia, where they regulate cellular processes through RNA silencing and post-transcriptional changes [128, 129]. *S. mansoni*, *S. japonicum*, and *S. haemotobium* are known to produce such RNAs [130-132].

However, the exact targets involved in remain poorly understood. Characterization of these key biomolecules may lead to a novel drug or vaccine [133, 134]. In addition, these helminth-derived products may have pharmacological potential for hemostatic and immunological disorders, including autoimmune and allergic diseases [135].

2.4 Schistosoma gene transcripts (mRNAs)

Transcriptomic analysis has been used to study how schistosoma parasites change their gene expression at different life stages [136-139]. It was described that gene expression varies not only during their life cycle but also between male and female parasites [140, 141], as well as between paired and unpaired adult parasites [142]. In addition, the gene expression patterns can vary depending on the geographic origin of the species [143]. Despite these findings, the

functions of many of these genes remain unknown. The transcriptomic data have helped to identify several genes encoding proteins such as tetraspanins, which are being investigated as potential targets for the development of new anti-schistosoma drugs or vaccines [144].

2.4.1 Gene transcript detection by RNA *in situ* hybridization

In situ hybridization (ISH) is a powerful molecular biology technique used to visualize and analyze the presence and distribution of specific nucleic acid sequences within cells or tissues [145]. This technique involves the use of labeled complementary probes that specifically bind to target sequences of interest. These probes can be labeled with fluorescent, radioactive, or enzymatic tags that enable detection of hybridized molecules by autoradiography or microscopy [146]. By examining spatial and temporal gene expression patterns in their native context, *in situ* hybridization provides valuable insights into developmental processes, disease mechanisms, genetic abnormalities and cellular interactions. The technique's versatility has led to its widespread adoption in fields such as developmental biology, neuroscience, oncology, and microbiology, making it an indispensable tool for unraveling the intricacies of genetic information within biological samples [145, 146].

Over the years, two main ISH approaches have been developed for schistosome tissues: (a) whole mount *in situ* hybridization (WISH), a protocol that allows gene transcript detection within the whole *S. mansoni* worms, and (b) ISH performed on their semi-thin sections [147, 148]. In our work, we improved existing methods for ISH suitable for *S. mansoni* tissues. The optimization included tissue preservation, sectioning, prehybridization tissue treatment, and amplification of fluorescent signal, which contribute to the improved sensitivity of the protocol. The modified method is sensitive enough to detect very low abundance targets, making it a powerful tool for studying gene expression in schistosomes.

Sense gene transcripts

Although the research of the RNA world of helminths is progressing, there are still large gaps in our understanding. Gene transcripts such as mRNAs (messenger RNAs) are important not just for making proteins, but also because they play other crucial roles. For example, parts of these mRNAs, known as introns, can be spliced into small RNAs. They then control the lifetime of mRNAs under normal and stress conditions, their stability, accessibility to other molecules, transport across the cell or into the extracellular space, etc. [149]. It has been shown that many organisms [150], including helminths [127, 151-153], transport their RNAs between cells and even between organisms via membranous structures called extracellular vesicles (EVs). Thus, RNAs are another important factor in host-helminth interaction [126, 127], and it would be intriguing to elucidate their biological function.

Anti-sense gene transcripts

Natural anti-sense transcripts (NATs) are abundantly transcribed in all eukaryotes but in many cases do not encode proteins [154]. Initially, NATs were considered to be transcriptional noise due to their heterogeneity, low expression levels, and unknown function. However, they are now increasingly being recognized as an important regulators of gene expression and post-transcriptional events [155, 156]. Furthermore, dysfunction of NATs can cause severe diseases [157].

Pathogens such as *Plasmodium*, *Toxoplasma*, *Leishmania*, and *Trypanosoma* have been described to use NATs in host-parasite communication. An increasing number of studies have shown that these parasites transfer RNA molecules to the host cells to modulate their functions [158]. This transfer is usually mediated by EVs (see chapter 2.4.2). NATs have also been detected in helminths. Our previous studies detected NATs in adult worms of *E. nipponicum* [159, 160]. And another study showed that NATs in *S. japonicum* may be a key regulator of reproduction. The authors believe that NATs may be another way leading to an effective anti-schistosomal drug development [161].

2.4.2 Extracellular vesicles in helminths

EVs represent a mechanism of intercellular communication between the parasite and the host. This system allows the exchange of molecular cargo between the two organisms [162]. EVs carry not only various RNA molecules (mRNAs, NATs, miRNAs), but also lipids and proteins [162-164]. The cargo of EVs can later be internalized by host cells [165-167].

Helminths release EVs to modulate the physiology of the host in order to increase the chances of survival [102, 168, 169]. EVs from the carcinogenic liver fluke *Opisthorchis viverrini* are known to be involved in the pathogenesis [170], similar to other trematodes such as *Echinostoma caproni*, *Fasciola hepatica*, *Dicrocoelium dendriticum* [127, 171], or in cestodes [152] and nematodes [153].

EV secretion has also been demonstrated in ***S. mansoni*** [172], *S. japonicum* [173], and S*. haematobium* [132]. Different schistosome life stages have been reported to generate and release EVs with overlapping but distinct compositions [174]. Such released molecules travel in the host bloodstream and have local or more distant effects on the host cells. EVs of *S. mansoni* and *S. japonicum* are internalized by immune cells and venous endothelial cells [128, 175] and can alter the expression of the host genes that regulate, for example, the immune system, cell proliferation and/or differentiation [129]. Unfortunately, the mechanisms of cargo sorting and packaging into EVs, intercellular trafficking, internalization into the host cells and further processing remain unknown [126].

Proteomic analysis of *S. mansoni* EVs revealed several vaccine candidates and potential virulence factors [87]. Among them, tetraspanin-1 (SmTSP-1, GeneDB Smp_155310.1), tetraspanin-2 (SmTSP-2, GeneDB Smp_181530), or the tegumental protein Sm29 (GeneDB Smp_072190) were detected, and also protease of our interest – *S. mansoni* serine protease 2 (SmSP2, GeneDB Smp_002150) [87, 176].

2.5 Proteolytic enzymes

Proteolytic enzymes (peptidases or proteases) cleave the peptide bonds of proteins and peptides. This process is known as proteolysis [177]. In typical mammalian and helminth genomes, genes encoding proteolytic enzymes represent 2-4%, making them one of the largest functional groups of proteins [178, 179].

They can be classified in several ways: (a) In the EC classification of enzymes, proteases belong to the class of hydrolases; they use a water molecule to cleave a peptide bond. (b) According to the localization of the cleavage site, proteases are divided into endopeptidases, which cleave within the polypeptide chain, and exopeptidases, which cleave from the N-terminus (aminopeptidases) or from the C-terminus of the peptide chain (carboxypeptidases). Dipeptidyl peptidases cleave the dipeptides from the N-terminus, peptidyl dipeptidases from the C-terminus. (c) Based on the catalytic mechanism (catalytic amino acid residue), proteolytic enzymes are divided into aspartic, asparagine, cysteine, glutamic, metallo-, serine, threonine proteases, and proteases with unknown mechanism [177]. (d) According to the MEROPS database [180], proteases are classified into clans and families. A clan contains proteases that are evolutionarily related and have similar tertiary structures. Clans are named by the letter of the catalytic type (A, C, G, M, N, P, S and T for aspartic, cysteine, glutamic, metallo, asparagine, mixed-type, serine, and threonine proteases, respectively) followed by a serial second consecutive capital letter. The clans are divided into families based on sequence similarity and identity of the enzymes. Each family is named either by a letter of the catalytic type followed by a sequential number, or by the exemplary protease that has been biochemically best characterized (e.g., chymotrypsin from S1 family) [179, 180].

2.5.1 Serine proteases

Serine proteases (SPs) are a large and ancient group of proteolytic enzymes found in prokaryotes and eukaryotes. Members of this protease class are abundant, and their function is highly diverse. They are involved in an enormous number of physiological and pathological

processes in higher organisms [180]. Serine proteases have emerged during evolution as the most abundant and functionally diverse group of proteases, as even the most primitive organisms use them to process external protein food sources and to cleave their own proteins [181].

SPs are predominantly extracellular in higher organisms and are generally active at neutral and weakly alkaline environments. They use a hydroxyl group of the catalytic serine residue in the active site to nucleophilically attack the peptide bond during protein cleavage, which occurs in two sequential steps. In the first step (acylation), a covalent tetrahedral acyl-enzyme intermediate is formed in which the carboxyl moiety of the peptide substrate forms an ester with the hydroxyl of the catalytic serine while the C-terminal portion of the cleaved peptide is released. In the second step (deacylation), a water molecule attacks the acyl-enzyme resulting in a second tetrahedral intermediate followed by release of the N-terminal portion of the peptide (Figure 4) [182]. SPs are classified into more than 50 families [180], and this thesis focuses on proteases from two families: the S1 chymotrypsin family and the S9 prolyl oligopeptidase family.

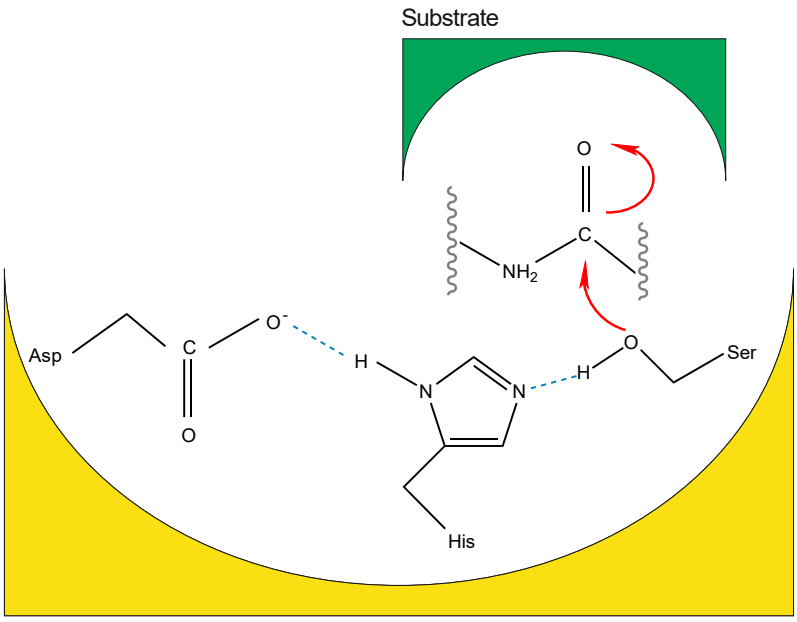

Figure 4. Arrangement of the serine protease active site. The catalytic triad consists of amino acid residues Asp, His and Ser. Hydrolysis of the peptide bond of the substrate is initiated by nucleophilic attack

(red arrows) of the hydroxyl group of serine on the carbonyl group of the cleaved peptide bond. Details are given in the text.

S1 family of serine proteases

SPs from clan PA, S1 chymotrypsin family are generally endopeptidases involved in many critical physiological processes [177, 180]. They play a crucial role in digestion, immunity, blood clotting, development, or pathogen invasion [177, 183]. They are characterized by a catalytic triad in the order His-Asp-Ser in the primary sequence [177]. Biologically important S1 family SPs include trypsin, chymotrypsin, subtilisin, pancreatic elastase, enteropeptidase, or myeloblastin [184].

S1 family SPs are synthesized as inactive precursors (zymogens, proenzymes) containing the N-terminal extension that acts as a pro-domain. Enzymatic activation is controlled by cleavage of this pro-domain, which results in a conformational change in the structure of the protease [180]. The length of the N-terminal extension varies widely from a few amino acids to hundreds of amino acids. The N-terminal extensions are usually connected to the protease domain by disulfide bridges and may have other non-proteolytic functions; they often act as binding domains [184].

According to the nomenclature of protease substrates [128] the amino acid residue of the substrate participating in the peptide bond with its carboxyl group is referred to as P1, the amino acid residue participating with its amino group is referred to as P1'. Amino acid residues in the substrate sequence are then numbered consecutively outward from the cleavage sites P1, P2, P3, P4, etc. in the N-terminal direction. Likewise, residues in the C-terminal direction are designated P1', P2', P3', P4' etc. (Figure 5). Based on the substrate specificity at the P1 position [180], the main types of S1 family protease activity can be classified as (a) chymotrypsin, with a preference for bulky hydrophobic amino acid residues (Trp, Tyr, Phe), (b) elastase, with a preference for small aliphatic residues (Val, Leu, Ala), and (c) trypsin, which prefers basic Arg or Lys residues [180, 184].

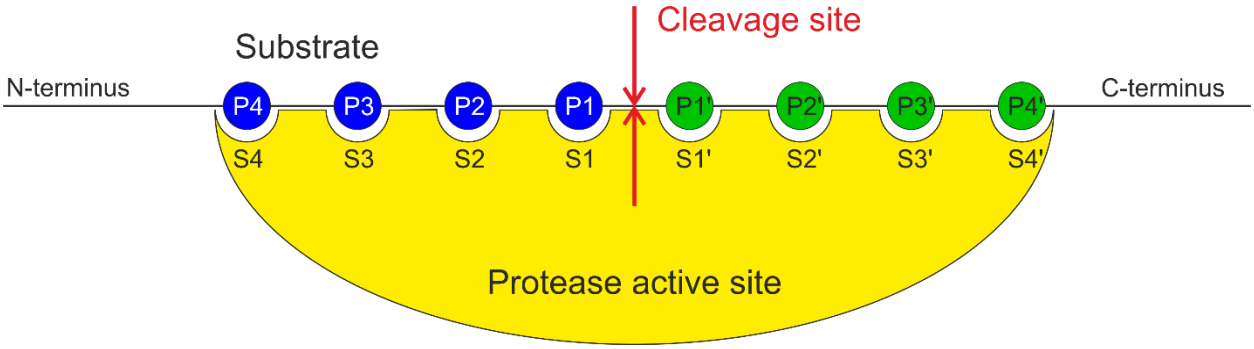

Figure 5. Schematic representation of the substrate binding to the specific binding subsites of the protease. The protease binding subsites are labeled S1 to Sn for substrate binding toward the N-terminus from the cleaved bond, and S1´ to Sn for substrate binding toward the C-terminus. Similarly, the amino acid residues of the substrate bound to the subsites are labeled P1-Pn and P1'-Pn'. The cleavage site of the substrate (red arrow) is located between two amino acid residues labeled P1 and P1'.

The structure of the S1 family proteases (Figure 6) consists of two six-stranded ß-barrel domains. The catalytic triad of His, Asp, and Ser residues is located at the junction between the β-barrels. The binding sites that determine the substrate specificity of the S1 family proteases have the same geometry but differ in the nature of the S1 binding site responsible for substrate binding at the P1 position. The negatively charged Asp182 at the base of the S1 binding substrate of trypsin binds positively charged Arg and Lys residues in the P1 position of the substrate (trypsin type), whereas the nonpolar Ser189 in chymotrypsin is responsible for binding hydrophobic amino acids (chymotrypsin type).

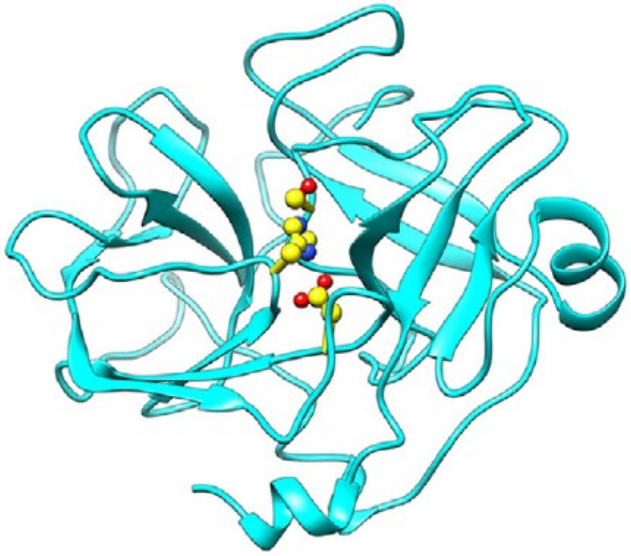

Figure 6. Three-dimensional structures of S1 family serine proteases . A homology model of the catalytic domain of *S. mansoni* serine protease 1 (SmSP1) from the family S1 clan PA. Family S1 catalytic domain with double barrel fold in cyan. The catalytic triad Ser, His, Asp residues are shown in yellow in ball-and-stick representation (heteroatoms are red and blue for O and N, respectively). Adapted from [179].

S9 family of serine proteases

The S9 family of serine proteases belongs to the SC clan, which is characterized by a catalytic triad in the order Ser-Asp-His in the primary sequence [177]. S9 family proteases have a molar mass of about 75 kDa, which is about three times higher than S1 family of proteases (e.g., trypsin and subtilisin (25-30 kDa)). Common features of this protease clan are an α/β-hydrolase fold of the catalytic domain and the ability to cleave Pro-Xaa peptide bonds (where Xaa is any amino acid residue). The S9 family proteases are multi-domain proteins typically composed of a C-terminal catalytic domain with an active site containing the catalytic triad, which is protected by an N-terminal cylinder-shaped domain called the β-propeller (Figure 7). The S9 family includes prolyl oligopeptidase (POP), oligopeptidase B, dipeptidyl peptidase IV etc. [180].

POP, also called prolyl endopeptidase, cleaves internal peptide bonds on the C-terminal side of proline residues and has been found throughout the living kingdom, including protozoa,

helminths and humans [185]. To date, three types of mammalian POPs have been described: in the cytoplasm [186], serum [187] and cell membranes [188]. The function of human POPs is not well characterized. POPs are known to cleave proline-containing hormones such as vasopressin and oxytocin [189], neuropeptides involved in memory and learning [190], and other substrates such as bradykinin, angiotensin, and endorphin [191].

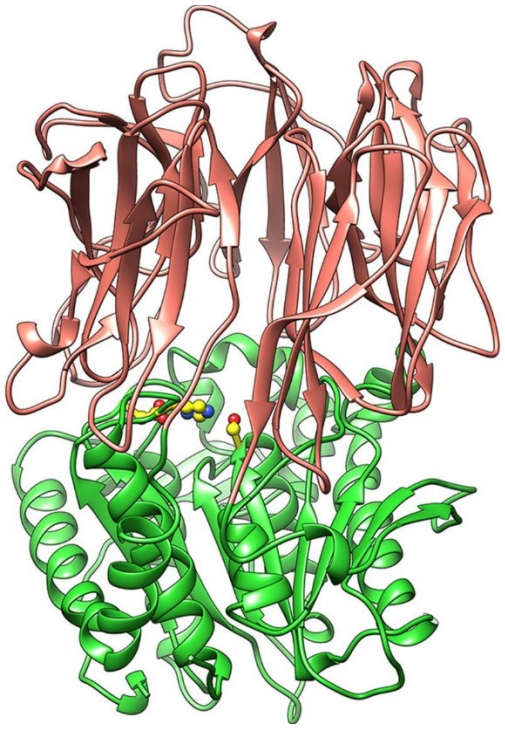

Figure 7. 3D homology model of the *S. mansoni* prolyl oligopeptidase (SmPOP) from the family S9 clan SC. A ribbon representation of the entire SmPOP structure showing the β-propeller domain (brown) and the catalytic domain (green). The catalytic triad Ser, His, Asp residues are depicted yellow in ball-and-stick representation (heteroatoms are red and blue for O and N, respectively). Adapted from [179].

2.6 Proteolytic systems of the blood fluke *S. mansoni*

Schistosome proteolytic enzymes (proteases) operate at the host-parasite interface and are essential for successful parasitism. They facilitate important functions such as parasite

invasion, migration, feeding, maturation and reproduction [192] and modulate the host physiology and immunity [179, 193].

In adult parasites, there are two major proteolytic systems that are intensively studied and that play a key role in the parasite-host relationship: a proteolytic system localized in the schistosome gut that is responsible for digesting ingested host blood proteins, and a proteolytic system on the surface of the parasite (tegument) (Figure 2) that plays a role in host-parasite interactions. Most studies of schistosome proteases have focused on cysteine and aspartic proteases from the digestive proteolytic system [180]. These enzymes are critical for blood digestion [14, 98]. Current research concentrates primarily on *S. mansoni* cathepsin B1 (SmCB1), a key digestive protease that has been validated as a molecular target for chemotherapy in a murine model of schistosome infection [78].

Proteases functioning in the tegument of schistosomes have not been extensively studied so far [194-196]. The tegument is a host-interactive outer surface of schistosomes that is critical for modulating host response and parasite survival. It is involved in host-parasite interactions such as nutrient uptake and immune evasion [84, 89]. Several proteomic studies have analyzed the tegumental proteome and aimed to identify tegument-specific and surface-exposed proteins [195], but, studies on the biological roles of tegumental proteases are rather scarce. To date, only a few proteases have been identified and biochemically characterized in the tegument, including the cysteine proteases cathepsin B2 [197] or calpain [198, 199] which is also considered a putative vaccine and drug target [133, 134, 200, 201]. The exact role of these enzymes is still unclear, but it's thought that they may be involved in the metabolism happening on the worm surface or play a specific role in the host-parasite interaction.

2.6.1 Serine proteases from *S. mansoni*

The genome of *S. mansoni* encodes more than 300 proteases [202-204], which is nearly 2.5% of the total genome. Out of these, about 25-30% of the genes have been functionally

annotated as SPs [205], but only a few of these SPs have been biochemically and biologically characterized.

SPs are the best studied group of proteases in the living kingdom, but they are much less studied in parasitic worms. Only a small number of SPs have been characterized in helminths (trematodes, cestodes, and nematodes). Their putative roles are estimated in parasite reproduction and development, nutritient intake, host tissue and cell invasion, anticoagulation, or immune evasion have been suggested [183]. The exceptions are the cercarial elastase (SmCE), which is used by cercariae to penetrate the definitive host and has been studied in detail [206, 207], and the partially described protease SmSP1, which is produced by the adult worms and eggs [16].

S1 family of serine proteases in *S. mansoni*

Among all SPs in the *S. mansoni* genome, only 16 unique sequences of serine proteases belong to the clan PA family S1 (chymotrypsin family). This is significantly less than the 135 family S1 proteases detected in the human genome [180, 202]. Of these 16 genes, two were identified as putative proteolytically inactive SmSPs (lacking the catalytic Ser or His residue in the catalytic triad), eight genes encode SmCE (*Schistosoma mansoni* cercarial elastase; encoding both – proteolytically active and inactive products), and five genes encode sequences of S1 family proteases that have not been previously characterized. The last five genes, designated SmSP1 to SmSP5, were identified in the gene database and were the focus of our investigation [208].

S. mansoni cercarial elastase, SmCE

Cercarial elastase (SmCE) is well-studied SPs of *S. mansoni*. SmCE is a key enzyme for the larval stage - cercariae. The enzyme is produced by the acetabular glands and released immediately after cercariae attach to the skin of the definitive host [29, 209]. SmCE is used by the parasite to degrade epidermal proteins, and thereby facilitating parasite infection [206]. SmCE has chymotrypsin-like activity - it cleaves the peptide chain behind large hydrophobic

amino acids [209]. It owes its name to its ability to degrade elastin. In addition, it can also cleave other connective tissue proteins such as collagen, fibronectin and laminin [210], or some immunoglobulins [211].

A total of 8 SmCE isoforms have been found in the *S. mansion* genome, but only two of them are responsible for most of the enzymatic activity [212]. The SmCE1c isoform was found only as a pseudogene (it is not transcribed into RNA), and only mRNA was found for the SmCE2b isoform, but no protein was detected [207].

SmCE expression occurs in pre- and post-acetabular glands at the sporocyst stage, when cercariae develop. In pre-acetabular glands, SmCE is found in vesicles containing large amounts of calcium, whereas post-acetabular glands contain SmCE together with mucopolysaccharides [212].

S. mansoni protease with kallikrein-like activity, sK1

Apart from SmCE, only two S1 family SPs have been partially characterized in *S. mansoni*: proteases designated sK1, and SmSP1. The protease sK1 was purified from the supernatant of *S. mansoni* adult worm homogenate. It is an enzyme with kallikrein-like activity that cleaves bradykinin from plasma kininogen [213]. Bradykinin is a potent vasodilator that increases vascular permeability. The activity of this enzyme was twenty times higher in males than in females [213, 214]. The primary structure of sK1 has not been characterized.

S. mansoni serine protease 1, SmSP1

Pivotal studies identified and cloned the gene encoding the S1 family protease named *S. mansoni* serine protease 1 (SmSP1), with sequence similarity to plasma kallikrein and human complement factor I-like protease [15, 16]. SmSP1 is highly expressed in the eggs and at lower levels in the adult worms. In adults, SmSP1 has been immunolocalized in the surface tubercles (in the tegument) of males and detected in ES products of adults and eggs [16]. SmSP1 has been

proposed to be the anticoagulant and vasodilatory factors that modulate blood hemostasis during the infection [214].

S9 Family of serine proteases in *S. mansoni*

A single gene encoding prolyl oligopeptidase (POP) was identified in the *S. mansoni* genome and was designated SmPOP (Smp_213240). The sequence was identified in the *S. mansoni* genome database [202, 215] by analysis with the BLASTp program using the human prolyl oligopeptidase sequence as a template. Orthologues have been identified in *S. japonicum*, *S. haematobium* and *Trypanosoma cruzi* genome databases [216].

For other parasites, the best characterized POP is Tc80 in the infective trypomastigote stage [217]. Inhibition of Tc80 prevents parasite entry into host cells suggesting that it may play role in the parasite invasion [216]. Accordingly, Tc80 has been investigated as a potential drug target [216, 218].

Other *S. mansoni* serine proteases

Other previous studies have shown that *S. mansoni* SPs may be associated with modulation of the host immune system. The activity of SPs is very often involved in the degradation of some types of immunoglobulins and some components of the complement system [219, 220]. For example, migrating larvae (cercariae, schistosomula) of *S. mansoni* secrete unknown SPs that can degrade immunoglobulin E [220]. Other study has shown that certain SPs secreted by *S. mansoni* are capable of degrading some members of complement, such as C3, C4, factor B, complement factor H, clusterin, or others [105]. The mechanisms and specific SPs involved in these processes remain to be elucidated.

3 Aims of the Thesis

The thesis focuses on proteolytic enzymes (proteases) of the blood fluke *S. mansoni*. This parasite causes schistosomiasis, a neglected tropical disease affecting more than 250 million people. Schistosome proteases are important for all blood-dwelling stages of the parasite in the human host. They play a key role in parasite-host interactions such as invasion of the host body, migration through tissues, digestion of proteins, suppression of immunity and modulation of host homeostasis. Excretory/secretory (ES) products of the schistosomes play a major role in these interactions. The main objective of this work is to analyze and characterize the proteolytic systems of the blood fluke *S. mansoni* with a focus on serine proteases.

The specific aims are as follows:

- Optimization of the method for preparation of ES products from different developmental stages of *S. mansoni*
- Analysis and classification of the main types of proteolytic activity in the ES products
- Bioinformatic analysis and annotation of SPs in the genome of *S. mansoni*, analysis of their expression profile in different life stages of *S. mansoni*
- Validation and optimization of fluorescence *in situ* hybridization method for detection of RNA transcripts in adult *S. mansoni*
- Localization of serine protease gene transcripts in adult *S. mansoni* using RNA *in situ* hybridization technique
- Biochemical and functional characterization of two *S. mansoni* serine proteases, SmSP2 and SmPOP, their immunolocalization and analysis of their potential role in host-parasite interactions

4 Results

4.1 Overview of thesis results

The results of this thesis are summarized in 7 publications, 5 papers in international peer-reviewed journals and two book chapters. These publications are presented below; each publication is preceded by a short summary that recapitulates the main results of the experimental work and summarizes the contribution of the researcher's work. These publications describe the identification, localization and functional characterization of serine proteases of the parasite *S. mansoni*.

The first publication describes the localization of gene transcripts (mRNAs) encoding serine proteases within *S. mansoni* adults using the fluorescence *in situ* hybridization (FISH). The second publication is dedicated to the identification and classification of proteolytic activities in ES products of *S. mansoni* developmental stages invading humans. The third publication deals with the annotation, expression analysis and characterization of the activity of five *S. mansoni* serine proteases (SmSPs), designated SmSP1 to SmSP5. Publications No. 4 and No. 5 provide detailed biochemical and functional characterization of two serine proteases, SmSP2 and SmPOP, from the *S. mansoni* parasite, and the publications No. 6 and No. 7 are book chapters describing in detail the methods for ES product collection from *S. mansoni* developmental stages and FISH in adult schistosomes.

List of Publications:

Publication No. 1: **Spatial expression pattern of serine proteases in the blood fluke *Schistosoma mansoni* determined by fluorescence RNA *in situ* hybridization.** Ulrychová L, Ostašov P, Chanová M, Mareš M, Horn M, Dvořák J, 2021, Parasit Vectors, 2021; 14:274. doi: 10.1186/s13071-021-04773-8.

Publication No. 2: **Excretion/secretion products from *Schistosoma mansoni* adults, eggs and schistosomula have unique peptidase specificity profiles.** Dvořák J, Fajtová P, Ulrychová L,

Leontovyč A, Rojo-Arreola L, Suzuki BM, Horn M, Mareš M, Craik CS, Caffrey CR, O'Donoghue AJ, Biochimie, 2016; 122:99-109. doi: 10.1016/j.biochi.2015.09.025.

Publication No. 3: **Trypsin- and chymotrypsin-like serine proteases in *Schistosoma mansoni*-'the undiscovered country'.** Horn M, Fajtová P, Rojo Arreola L, Ulrychová L, Bartošová-Sojková P, Franta Z, Protasio AV, Opavský D, Vondrášek J, McKerrow JH, Mareš M, Caffrey CR, Dvořák J, PLoS Negl Trop Dis., 2014; 8:e2766, doi: 10.1371/journal.pntd.0002766.

Publication No. 4: **SmSP2: A serine protease secreted by the blood fluke pathogen *Schistosoma mansoni* with anti-hemostatic properties**. Leontovyč A, Ulrychová L, O'Donoghue AJ, Vondrášek J, Marešová L, Hubálek M, Fajtová P, Chanová M, Jiang Z, Craik CS, Caffrey CR, Mareš M, Dvořák J, Horn M, PLoS Negl Trop Dis, 2018; 12:e0006446, doi: 10.1371/journal.pntd.0006446.

Publication No. 5: **Prolyl Oligopeptidase from the Blood Fluke *Schistosoma mansoni*: From Functional Analysis to Anti-schistosomal Inhibitors.** Fajtová P, Štefanić S, Hradílek M, Dvořák J, Vondrášek J, Jílková A, Ulrychová L, McKerrow JH, Caffrey CR, Mareš M, Horn M., PLoS Negl Trop Dis., 2015; 9:e0003827, doi: 10.1371/journal.pntd.0003827.

Publication No. 6: **Sensitive Fluorescence *in situ* Hybridization on Semithin Sections of Adult *Schistosoma mansoni* Using DIG-Labeled RNA Probes.** Ulrychová L, Horn M, Dvořák J, Methods Mol Biol., 2020;2151:43-53, doi: 10.1007/978-1-0716-0635-3_4.

Publication No. 7: **Collection of Excretory/Secretory Products from Individual Developmental Stages of the Blood Fluke *Schistosoma mansoni*.** Leontovyč A, Ulrychová L, Horn M, Dvořák J, Methods Mol Biol., 2020;2151:55-63, doi: 10.1007/978-1-0716-0635-3_5.

4.2 Publication No. 1: Spatial expression pattern of serine proteases in the blood fluke *Schistosoma mansoni* determined by fluorescence RNA *in situ* hybridization.

Proteases of schistosomes are involved in critical steps of host-parasite interactions and are promising therapeutic targets. The gene expression levels of some proteases are very low, and therefore are not detectable by standard genome sequencing technologies, they are marginally detectable at the method thresholds. In this publication, the highly sensitive fluorescence *in situ* hybridization (FISH) technique, based on the detection of digoxigenin-labeled RNA probes by anti-digoxigenin antibody conjugated to horseradish peroxidase, was used to study the distribution of RNA transcripts in adult male and female *S. mansoni*. A detailed protocol for this technique is described in publication No. 6.

The technique was optimized and validated by detecting a set of target RNA transcripts whose distribution in *S. mansoni* adults had previously been determined by other methods - digestive cathepsin B1 (SmCB1), surface-localized prolyl oligopeptidase (SmPOP), tegumental tetraspanin (SmTsp-2) and membrane-bound protein Sm29. The validated FISH technique was then used to determine the tissue distribution of transcripts of individual S1 family *S. mansoni* serine proteases (SmSPs) (their identification and characterization is described in publication No. 3). The analysis revealed complex expression patterns of all SmSPs which were found in multiple tissues (reproductive organs, parenchymal cells, esophagus, and the tegument surface). The expression patterns of individual proteases were distinct, but partially overlapping, and consistent with existing transcriptome sequencing data [221, 222] or our immunolocalization data (publications No. 4 and No. 5). The exceptions were genes with significantly low expression levels, which were also localized in tissues where they had not been previously detected by RNA sequencing methods. Interestingly, some of the schistosome genes are not only transcribed as sense mRNAs, but also as antisense transcripts that do not encode proteins.

The results suggest that SmSPs play a role in various biological processes of the parasite. Some of the surface-expressed SmSPs may be involved in host-parasite interactions.

PhD applicant contribution: RNA probe design and production, adult schistosome isolation, tissue preparation for microscopy, fluorescence *in situ* hybridization (FISH), microscopy techniques, data analysis and interpretation, image processing, manuscript preparation and writing and editing.

Ulrychová *et al. Parasites Vectors* (2021) 14:274
https://doi.org/10.1186/s13071-021-04773-8

Parasites & Vectors

RESEARCH Open Access

Spatial expression pattern of serine proteases in the blood fluke *Schistosoma mansoni* determined by fluorescence RNA in situ hybridization

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Abstract

Background: The blood flukes of genus *Schistosoma* are the causative agent of schistosomiasis, a parasitic disease that infects more than 200 million people worldwide. Proteases of schistosomes are involved in critical steps of host–parasite interactions and are promising therapeutic targets. We recently identified and characterized a group of S1 family *Schistosoma mansoni* serine proteases, including SmSP1 to SmSP5. Expression levels of some SmSPs in *S. mansoni* are low, and by standard genome sequencing technologies they are marginally detectable at the method threshold levels. Here, we report their spatial gene expression patterns in adult *S. mansoni* by the high-sensitivity localization assay.

Methodology: Highly sensitive fluorescence in situ RNA hybridization (FISH) was modified and used for the localization of mRNAs encoding individual SmSP proteases (including low-expressed SmSPs) in tissues of adult worms. High sensitivity was obtained due to specifically prepared tissue and probes in combination with the employment of a signal amplification approach. The assay method was validated by detecting the expression patterns of a set of relevant reference genes including SmCB1, SmPOP, SmTSP-2, and Sm29 with localization formerly determined by other techniques.

Results: FISH analysis revealed interesting expression patterns of SmSPs distributed in multiple tissues of *S. mansoni* adults. The expression patterns of individual SmSPs were distinct but in part overlapping and were consistent with existing transcriptome sequencing data. The exception were genes with significantly low expression, which were also localized in tissues where they had not previously been detected by RNA sequencing methods. In general, SmSPs were found in various tissues including reproductive organs, parenchymal cells, esophagus, and the tegumental surface.

Conclusions: The FISH-based assay provided spatial information about the expression of five SmSPs in adult *S. mansoni* females and males. This highly sensitive method allowed visualization of low-abundantly expressed genes that are below the detection limits of standard in situ hybridization or by RNA sequencing. Thus, this technical approach turned out to be suitable for sensitive localization studies and may also be applicable for other trematodes. The results

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suggest that SmSPs may play roles in diverse processes of the parasite. Certain SmSPs expressed at the surface may be involved in host–parasite interactions.

Keywords: Platyhelminthes, Blood fluke, *Schistosoma mansoni*, mRNA detection, Transcript, Fluorescence RNA in situ hybridization, Serine proteases

Background

Human schistosomiasis is a chronic infectious disease affecting more than 200 million people in 76 countries, mostly in tropical and subtropical areas, caused by flukes of the genus *Schistosoma* [1]. Current treatments rely on one drug, praziquantel, and no effective vaccine has yet been developed [2]. Several schistosome species infect humans, with three of the most abundant species being *Schistosoma haematobium*, which causes urinary schistosomiasis, and *Schistosoma japonicum* and *Schistosoma mansoni*, causing intestinal schistosomiasis [3]. Humans are infected by schistosome larvae called cercariae, which are released into freshwater by intermediate snail hosts. Cercariae penetrate human skin and subsequently develop into adult male or female worms in the host vascular system, where they produce hundreds of eggs per day [4]. Morbidity arises from immunopathological reactions to and entrapment of schistosome eggs in various tissues [5].

The genome of *S. mansoni* was sequenced, and numerous genes were identified or predicted [6]. In the post-genomic era, however, it is vital to elucidate functions of these genes. Quantification and spatial distribution of RNA transcripts in individual cells of the whole multicellular organism helps to narrow potential functions of individual genes [7–9]. RNA in situ hybridization represents a powerful tool to investigate the spatial distribution of gene transcripts [7, 10]. Hybridization methods are based on the binding of an individual RNA target with specifically designed complementary RNA probes labeled with a reporter molecule, such as digoxigenin (DIG). Such probes can then be detected by specific antibodies recognizing this molecule. These antibodies are conjugated with enzymes allowing visualization of the probe via fluorescent or colored staining. However, detection and visualization of low-abundantly expressed transcripts is challenging in multicellular organisms such as schistosomes with diverse cellular and tissue organization.

Blood flukes of the genus *Schistosoma* are unique among the trematodes because they have evolved separate sexes [11]. They are acoelomates, animals without a body cavity except for the gut and reproductive organs (testes, vitellaria, ovary, and oviduct). The interior body is filled with mesodermal tissue called parenchyma, which is composed of diverse cell subtypes such as stem cells [12], cells derived for example from neuro-excretory systems [13, 14], tegumental cell bodies [14, 15], and other yet undefined cell types with unknown functions. The entire surface of adult schistosomes is covered by the syncytial layer called the tegument [16].

Serine proteases (SPs) are key virulence factors for many parasitic helminths; they are critical for parasite invasion, migration, nutrition, and reproduction, and they facilitate adaption to and evasion from the host's physiological and immune responses [17–19]. Recently, we found that the S1 family of serine proteases of *S. mansoni* (SmSPs) significantly contributed to proteolytic activities detected in excretory/secretory (E/S) products of blood-dwelling developmental stages [20]. We uncovered a repertoire of SmSPs (designated SmSP1 to SmSP5) by performing a series of genomic, transcriptomic, proteolytic, and phylogenetic analyses [21], and described the major SmSP, SmSP2, at the protein level [22].

In this work, we used the fluorescence in situ hybridization (FISH) technique based on a detection of DIG-labeled RNA probes by anti-DIG antibody conjugated with horseradish peroxidase (HRP) [23] to investigate RNA distribution in adult male and female *S. mansoni*. The technique was validated by detecting a set of target RNA transcripts with known localization, the digestive protease cathepsin B1 of *S. mansoni* (SmCB1) [24–27], the surface-associated prolyl oligopeptidase (SmPOP) [28], the tegumental tetraspanin SmTsp-2 [29–31], and Sm29, a membrane-bound glycoprotein found at the *S. mansoni* tegument [31–34]. A probe targeting a bacterial neomycin gene (*neo*), which is absent in the *S. mansoni* genome, was used as a negative control. The validated FISH technique was then used to determine the tissue distribution of transcripts of individual SmSP genes (SmSP1 to SmSP5) [18, 21]. The analysis revealed complex expression patterns of all SmSPs generally consistent with existing transcriptome sequencing data [35, 36]. Moreover, the method also enabled us to localize the genes with significantly low expression in tissues where they had not previously been detected by RNA sequencing (RNAseq) methods.

Methods

Schistosome material

S. mansoni (a Puerto Rican LSHTM strain) was maintained in the laboratory by cycling between the

intermediate snail hosts, *Biomphalaria glabrata*, and outbred ICR (CD-1) mice as definitive hosts. Infective larvae (cercariae) were shed by light stimulation from infected snails placed in bottled drinking water. Adult female mice were infected by immersing their feet and tail into 50 mL of water containing approximately 300 cercariae for 45 min. Six weeks post-infection, mice were over-anesthetized by an intraperitoneal injection of ketamine (Narkamon 5%—1.2 mL/kg body weight) and xylazine (Rometar 2%—0.6 mL/kg body weight), and the worms were recovered from the hepatic portal system by transcardial perfusion with RPMI 1640 medium (Sigma-Aldrich) as described previously [20, 22, 37].

Isolation of mRNA and cDNA synthesis

Collected adult worms were washed three times with 50 mL phosphate-buffered saline (PBS) and re-suspended in 500 µL of the Trizol reagent (Thermo Fisher), and RNA was isolated as described previously [38]. Single-stranded cDNA was synthesized from total RNA by SuperScript III reverse transcriptase (Thermo Fisher) and an oligo$(dT)_{23}$ primer according to the manufacturer's protocol. The final cDNA product was purified using the QIAquick PCR purification kit (Qiagen) and stored at −20 °C.

Probe production

Probes (600–1500 nucleotides) were designed to hybridize with the catalytic domain sequences of selected target gene transcripts. DNA templates for probe synthesis were amplified by polymerase chain reaction (PCR) from *S. mansoni* cDNA using gene-specific primers (Additional file 1: Table S1). The PCR products were cloned into the pGEM-T Easy vector (Promega), and the cloned sequences were verified by DNA sequencing. Constructs were linearized by restriction enzymes (NEB) selected based on insert orientation within the construct. Linearized plasmid DNA (1 µg) was used as a template to generate digoxigenin (DIG)-labeled probes by in vitro transcription using the DIG RNA labeling kit (SP6/T7) (Roche). The probes were transcribe using either SP6 or T7 RNA polymerases (final concentration 1 U per 1 µL) at 37 °C for 2 h according to the manufacturer's protocol. DNA templates were removed by incubation with DNase I (final concentration 1 U per 1 µL) at 37 °C for 15 min. The reactions were terminated by the addition of EDTA (final concentration 0.02 M), and the probes were stored at −20 °C prior to use. DIG-labeled "antisense" Neo RNA, which is a component of the SP6/T7 DNA labeling kit (Roche), was used as a control probe. The DNA construct containing the Sm29 sequence was kindly provided by Christoph G. Grevelding (Justus Liebig University, Giessen, Germany). All hybridization probes were verified by the sequencing. The probe specificity was verified by BlastN analysis on the National Center for Biotechnology Information (NCBI) database. Sequences shared 100% identity with the studied genes and showed no significant similarities to other genes in the organism [35], including those used in our study.

Tissue preparation for fluorescence in situ hybridization

Following perfusion, *S. mansoni* couples were separated on ice by gentle prodding with a brush, washed three times in 50 mL of PBS, and fixed with boiling 4% formaldehyde solution (Sigma-Aldrich). Worms were left to cool to 25 °C, incubated at 25 °C for 90 min, and dehydrated by incubation in a series of increasing ethanol concentrations (25%, 50%, 70%, 90%, 96%, 100% v/v at 25 °C for 5 min each). Incubation with 100% ethanol was performed twice for 5 min. Subsequently, males were incubated in methyl benzoate (Sigma-Aldrich) at 25 °C for 45 min, females for 20 min. Worms were washed twice at 25 °C for 5 min each in benzene (Sigma-Aldrich), which was then exchanged three times with 60 °C hot paraffin (Paraplast X-TRA, Leica). All incubation and washing steps were performed in approximately 50 mL of appropriate solution. After the last wash, worms were incubated in 20 mL paraffin (Leica) at 60 °C for 2 h and then embedded in paraffin blocks. Sections (6 µm) were prepared on a microtome (Shandon Finesse® ME+) and applied to X-TRA adhesive glass slides (Leica).

Pre-hybridization tissue treatment

Slides with fixed tissue sections (6 µm) were de-paraffinized by two 5-min washes in xylene followed by two 5-min washes in 100% ethanol. Sections were rehydrated by incubation in a series of decreasing ethanol concentrations (100%, 96%, 90%, 70%, 50%, 25% v/v) at 25 °C for 5 min each, followed by a wash in diethyl-pyrocarbonate (DEPC, Sigma-Aldrich)-treated water for 5 min. To minimize background quench by endogenous peroxidases and improve cell permeability, slides were incubated in 0.2 N HCl at 25 °C for 20 min, followed by incubation in 0.01 M sodium citrate, pH 6.0, in a boiling water bath for 15 min. Slides were then cooled to 25 °C for 30 min, incubated in 0.2% glycine for 5 min, followed by incubation in ice-cold 20% acetic acid for 15 s and PBS at 25 °C for 5 min. Finally, the slides were incubated in 20% glycerol at 25 °C for 15 min and briefly rinsed with 2× saline sodium citrate buffer (SSC, Sigma-Aldrich). All incubation and washing steps were performed in 150 mL of appropriate solution.

RNA in situ hybridization

To denature secondary RNA structures within the tissues, sections were preheated to 70 °C for 10 min prior to hybridization and briefly cooled on an ice-cold metal

plate. One hundred microlitres of hybridization mixture A [5× SSC, 1x PBS, 0.1% torula yeast RNA (Sigma-Aldrich)] containing RNA probe (0.5–5 ng/mL) was heated to 70 °C to denature secondary RNA structures. The mixture was then briefly cooled on ice, immediately mixed with 160 µL of hybridization solution B [50% formamide, 10% dextran sulfate molecular weight 4000 (Sigma-Aldrich) and 1% Tween 20], applied to sections, and covered with a coverslip. All samples were then hybridized in a moisture chamber at 42 °C for 16 h.

Post-hybridization treatment

After hybridization, the slides were washed in 2x SSC with 0.1% Tween 20 at 42 °C for 15 min, followed by washes in 1x SSC, 0.5x SSC, and 0.1x SSC, each at 25 °C for 15 min. The slides were then washed twice for 5 min with MAB buffer (0.1 M maleic acid, pH 7.5, 0.15 M NaCl) at 25 °C and incubated in 4% blocking solution [4% heat-inactivated horse serum (Sigma-Aldrich) in MAB buffer] for 30 min. The DIG-labeled probes hybridized with tissue RNA were labeled by incubation with anti-digoxigenin antibody conjugated with horseradish peroxidase (anti-DIG-HRP antibody, Perkin Elmer) diluted 1:500 in 2% blocking solution (2% heat-inactivated horse serum in MAB buffer) at 37 °C for 2 h. Excess antibody was then removed by three washes in MAB at 25 °C for 10 min. All incubation and washing steps were performed in 150 mL of appropriate solution. Hybridized probes labeled with DIG-HRP antibodies were visualized by the Tyramide Signal Amplification (TSA) system with the Cyanine Plus 5 Tyramide Reagent fluorescence system (Perkin Elmer) according to the manufacturer's protocol. Briefly, the slides were washed three times with 150 mL TNT buffer (0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) for 5 min each, and incubated in a moisture chamber with Cyanine Plus 5 dye diluted 1:50 in 500 µL of 1× Plus Amplification Diluent for 10 min. After three washes with 150 mL TNT buffer for 5 min, sections were rinsed in DEPC-water and mounted in ProLong Diamond Antifade reagent containing DAPI (Thermo Fischer).

Microscope observation

Fluorescent signals were detected using an Olympus IX83 fluorescence microscope (Olympus) equipped with a pco. edge 5.5 camera. To define the background signal threshold, samples underwent FISH procedure without probe, or hybridization with the control probe carrying the sequence of a bacterial neomycin-resistance gene (*neo*) which is not present in the schistosome genome. Microscopy images were processed using Fiji software [39]. Images were collected from three parts of adult worms: the anterior head region (females: from ventral sucker to the anterior margin of ovaries; males: extending from the anterior extremity of the schistosome to the ventral sucker), the middle region (females: containing mature and immature oocytes and oviduct; males: containing testis), and the posterior hind region (females: containing vitellaria and gut; males: the body part located posterior to the margin of testes and focusing on the parenchyma, tegument, and gut). This division is very coarse and used for simplification; some organs may occur in several regions. Moreover, obtaining a representative image of the anterior region of the female (head with esophagus) is generally problematic because fixation always causes some degree of contraction and distortion, and sectioning is difficult. To verify reproducibility, all experiments including probe production, tissue preparation, and FISH procedure were carried out in at least two independent experiments.

Results

Validation of fluorescence RNA in situ hybridization (FISH) for the tissues of *S. mansoni* adults

We used FISH based on digoxigenin (DIG)-labeled RNA probes [23] to localize individual RNA molecules in *S. mansoni* adult males and females. Initially, the FISH assay was validated by localization of a set of relevant control genes with a known localization [determined using other localization techniques, e.g. immunolocalization or whole-mount in situ hybridization (WISH)]. The genes used for validation were digestive protease SmCB1 [40], surface-localized protease SmPOP [28], and tegumental proteins SmTsp-2 [41] and Sm29 [33].

The localization results obtained for individual target RNAs are summarized in Fig. 1. Generally, transcripts of the genes were localized by FISH in the same tissues of adult males or females as previously described. Due to the high sensitivity of the technique, additional new localizations of some transcripts were found. The transcript profiles for SmCB1, SmPOP, SmTsp-2, and Sm29 also corresponded to previous RNAseq data obtained for *S. mansoni* adults and their gonads (http://schisto.xyz/; [35, 36]).

Digestive SmCB1 was previously detected in the gastrodermis [40], and its activity was also demonstrated by fluorescence histochemistry in the vitellaria [42]. We confirmed localization of the transcripts coding SmCB1 in the gastrodermis of females and males and in the vitellaria (Figs. 2 and 3). Additionally, in females, a significant signal was present in the oviduct and a faint signal was detected in the parenchyma surrounding mature oocytes (Fig. 2). In males, faint fluorescence was present in testicular cells and in the region of the esophagus (Fig. 3).

SmPOP is a protease that was immunolocalized to the tegument (including tubercles) and the parenchyma of

♀

	tegumental surface	tegumental cytons	muscles	parenchyma	gastrodermis	mature oocytes	immature oocytes	oviduct	vitelline cells
SmSP1	-	-	-	+	-	+	-	+	+
SmSP2	+	+	-	+	-	+	+	-	+
SmSP3	-	-	-	+	-	+	-	-	+
SmSP4	+	+	-	+	-	+	+	+	+
SmSP5	-	-	-	+	-	+	+	-	+
SmCB1	-	-	-	-	+	-	-	+	+
SmPOP	-	+	-	+	-	+	+	+	+
SmTsp-2	-	+	-	+	-	+	+	+	+
Sm29	-	+	-	+	-	-	-	-	-
Neo	-	-	-	-	-	-	-	-	-
No probe	-	-	-	-	-	-	-	-	-

♂

	tegument surface	tegument tubercles	tegumental cytons	muscles	parenchyma	gastrodermis	testes	esophagus region
SmSP1	-	-	+	-	+	-	+	+
SmSP2	-	-	+	-	+	-	+	+
SmSP3	-	-	-	-	+	-	+	+
SmSP4	-	+	+	-	+	-	+	+
SmSP5	-	-	-	-	+	-	+	-
SmCB1	-	-	-	-	-	+	+	+
SmPOP	-	+	+	-	+	-	+	+
SmTsp-2	-	+	+	-	+	-	+	+
Sm29	-	-	+	-	+	-	+	+
Neo	-	-	-	-	-	-	-	-
No probe	-	-	-	-	-	-	-	-

Fig. 1 An overview of localization of gene sense transcripts in *S. mansoni* adult male and female tissues using FISH. Transcripts whose localizations were observed in the given tissue are highlighted in pink (females) or blue (males). Light shades represent a faint positive signal for a given transcript. Localization in the esophagus region was possible only in the sections of adult males; obtaining a representative section of the smaller females' head region (containing esophagus) is generally problematic

adult parasites [28]. In males, transcripts of SmPOP were detected in the tegumental cytons and, surprisingly, in tegumental tubercles—morphological structures above the muscle layer (Additional file 2: Figure S1a)—which are not expected to contain RNA molecules and where RNA translation should therefore not occur. Transcripts of SmPOP were also detected in parenchyma, around the esophagus, and in the testes (Fig. 3). In females, SmPOP was distributed in the parenchyma throughout the whole body, a strong signal was detected in the oviduct and the vitellaria, and a faint signal was also detected in oocytes (Fig. 2).

SmTsp-2 and Sm29 are known as tegumental markers [29, 30, 33, 41, 43, 44]. Transcripts of SmTsp-2 were detected in the tegument of both males and females and in the parenchyma (Figs. 2 and 3). In females, transcripts were also detected in the ovary, vitellaria, and oviduct, and in males, around the esophagus and in the testes. Surprisingly, but in accordance with previous findings [14], SmTsp-2 transcripts were also localized in the tegumental tubercles (Additional file 2: Figure S1).

Contrary to SmTsp-2, transcripts of Sm29 had a distinct expression pattern. In females (Fig. 2), strong fluorescence was found only in the parenchyma and tegumental cytons in the anterior regions. In males, Sm29 mRNA was found in the testes, some parenchymal cell subtypes, in the esophageal area, and in the tegumental cytons but not in the tegumental surface layer and tubercles (Fig. 3, Additional file 2: Figure S1a). No signal was detected in the ovary, oviduct, or gut of either gender (Figs. 2 and 3).

Also, sense probes hybridizing to antisense gene transcripts that do not encode proteins were designed for genes of all transcripts studied (Additional file 3: Figures S2 and S3) and used in the FISH procedure. Only antisense transcripts of SmPOP and SmCB1 were found solely in the female oviduct. No antisense transcripts of these genes were detected in the tissues of the males.

Non-specific bindings and backgrounds were analyzed using two different approaches: (i) hybridization with a probe targeting a sequence-coding bacterial gene (*neo*) which is not naturally present in genomes of schistosomes, and (ii) hybridization procedure without any probe. No signals were detected after the hybridization procedure in either case (Additional file 3: Figure S4).

The validated and optimized FISH therefore represents a robust method for detection of RNA in sections of adult *S. mansoni*. This was documented by the precise localization of selected transcripts, and the result corresponded to previous transcript profiling as determined by other methods such as immunolocalization [28, 33, 40, 43] or differential RNAseq [35, 36].

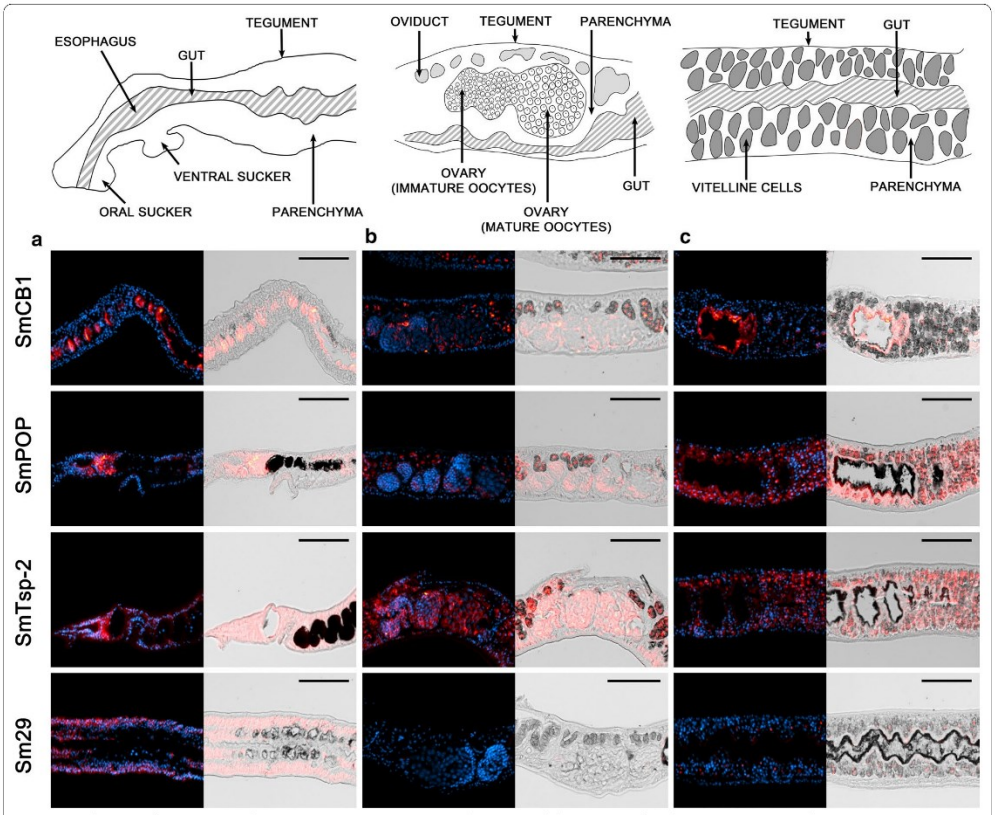

Fig. 2 Localization of mRNAs encoding SmCB1, SmPOP, SmTsp-2, and Sm29 in adult *S. mansoni* females using FISH. Semi-thin (6 µm) sections of adult *S. mansoni* female worms were probed with DIG-labelled RNA probes designed to detect SmCB1, SmPOP, SmTsp-2, or Sm29 mRNAs. The probes hybridized with transcripts were visualized by tyramide amplification assay (red). Adult females were monitored in three parts: **a** an anterior part, **b** oviduct, mature and immature oocytes, and **c** vitellaria and gut. DAPI was used to label nuclear DNA (blue). The left columns show merged fluorescent channels; in the right columns, the fluorescent red signal is merged with differential interference contrast. **a** A strong signal of SmPOP, SmTsp-2; and Sm29 was detected in parenchymal cells of the anterior part of the female. **b** A strong fluorescent signal of SmTsp-2 and faint scattered SmPOP signals were observed in mature and immature oocytes; the oviduct is rich in SmCB1, SmPOP, and SmTsp-2 transcripts. **c** SmPOP and SmTsp-2 mRNAs are strongly expressed in vitellaria, while mRNAs of SmCB1 and Sm29 are not shown. **a**–**c** Transcripts of SmCB1 are present in the gut throughout the whole worm. The scale bars represent 100 µm

FISH revealed a diverse distribution of serine proteases SmSP1 to SmSP5 in adult *S. mansoni*

The FISH technique was used to precisely localize transcripts encoding serine proteases SmSP1 to SmSP5 in adult *S. mansoni*. Results showing localization of SmSPs RNA are summarized in Fig. 1. Generally, transcripts of all SmSP1 to SmSP5 were detected in the parenchyma with different patterns. No expression of SmSPs was detected in the gastrodermis or the muscles. In other tissues, SmSPs showed diverse expression patterns that are described below (Figs. 4 and 5).

In females, SmSP1 transcripts were detected in the oviduct, vitellaria, and mature but not immature oocytes (Fig. 4). In males, SmSP1 genes were transcribed in the tegumental cytons, the area around the esophagus, and in the testes (Fig. 5). Low expression occurred in the parenchyma in both genders (Figs. 4 and 5).

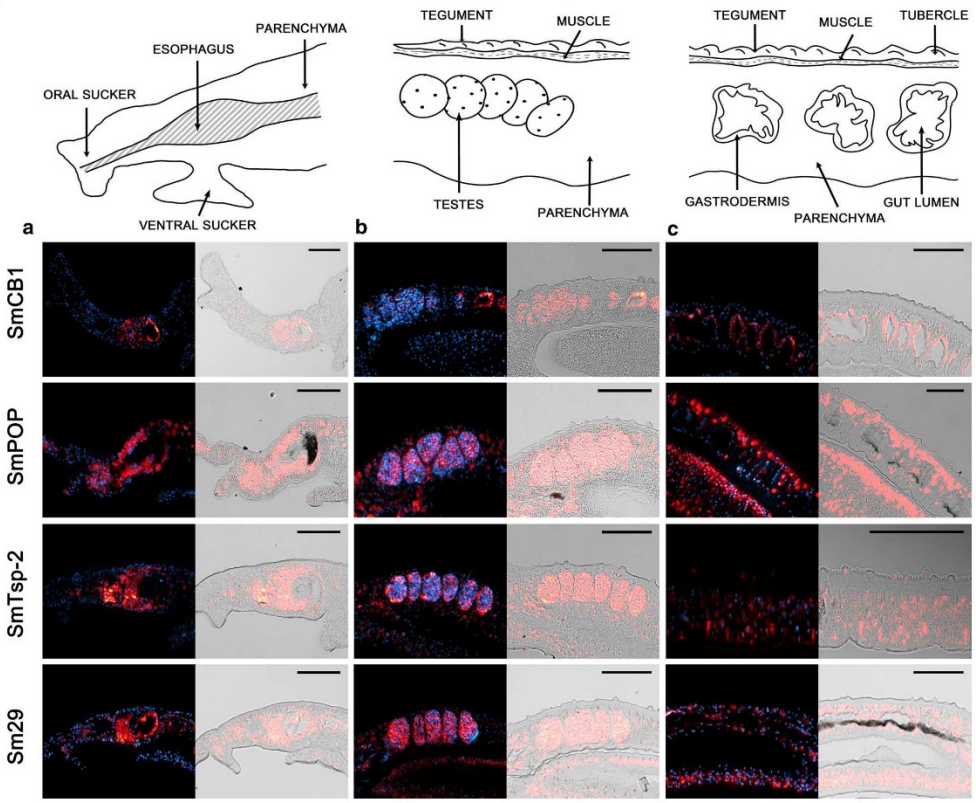

Fig. 3 Localization of mRNAs encoding SmCB1, SmPOP, SmTsp-2, and Sm29 in adult *S. mansoni* males using FISH. Semi-thin (6 µm) sections of adult *S. mansoni* male worms were probed with DIG-labelled RNA probes designed to detect SmCB1, SmPOP, SmTsp-2, or Sm29 mRNAs. The probes hybridized with transcripts were visualized by tyramide amplification assay (red). Adult males were monitored in three parts: **a** a head part, **b** testes, and **c** a posterior part with the focus on parenchyma, tegument and gut. DAPI was used to label nuclear DNA (blue). The left columns show merged fluorescent channels; in the right columns, the fluorescent red signal is merged with differential interference contrast. Transcripts of SmPOP, SmTsp-2, and Sm29 were detected in and around the esophagus, in the testes, and in parenchymal cell subtypes. SmPOP and SmTsp-2 were also detected in tegumental cells and in tegumental tubercles. SmCB1 is expressed strongly in the gut, weakly in parenchyma around the esophagus and testes. The scale bars represent 100 µm

SmSP2 transcripts were found in the esophagus and parenchyma throughout the bodies of both genders and also in the tegumental cytons, which agrees with previously described SmSP2 immunolocalization [22] (Figs. 4 and 5, Additional file 2: Figure S1a). In females, SmSP2 gene transcripts were also found in mature and immature oocytes, vitellaria, and in the tegumental surface above the muscle layer (Fig. 4); in males in testes (Fig. 5). No expression occurred in the tubercles or in the oviduct.

SmSP3 mRNA was found only in the parenchymal tissue surrounding the vitellaria in the posterior region of females, in the vitelline cells, and weakly in the mature oocytes (Fig. 4). Males expressed SmSP3 only in the esophagus region, parenchyma, and testes (Fig. 5). No fluorescence was detected in the immature oocytes, the oviduct, the tegumental surface, or the tegumental tubercles (Figs. 4 and 5).

SmSP4 was abundantly expressed in parenchymal cells and tegumental cytons in whole adult worms. SmSP4 transcripts were also detected in females in the tegumental surface, oviduct, mature and immature oocytes, and the vitelline cells (Fig. 4). Expression of SmSP4 in males

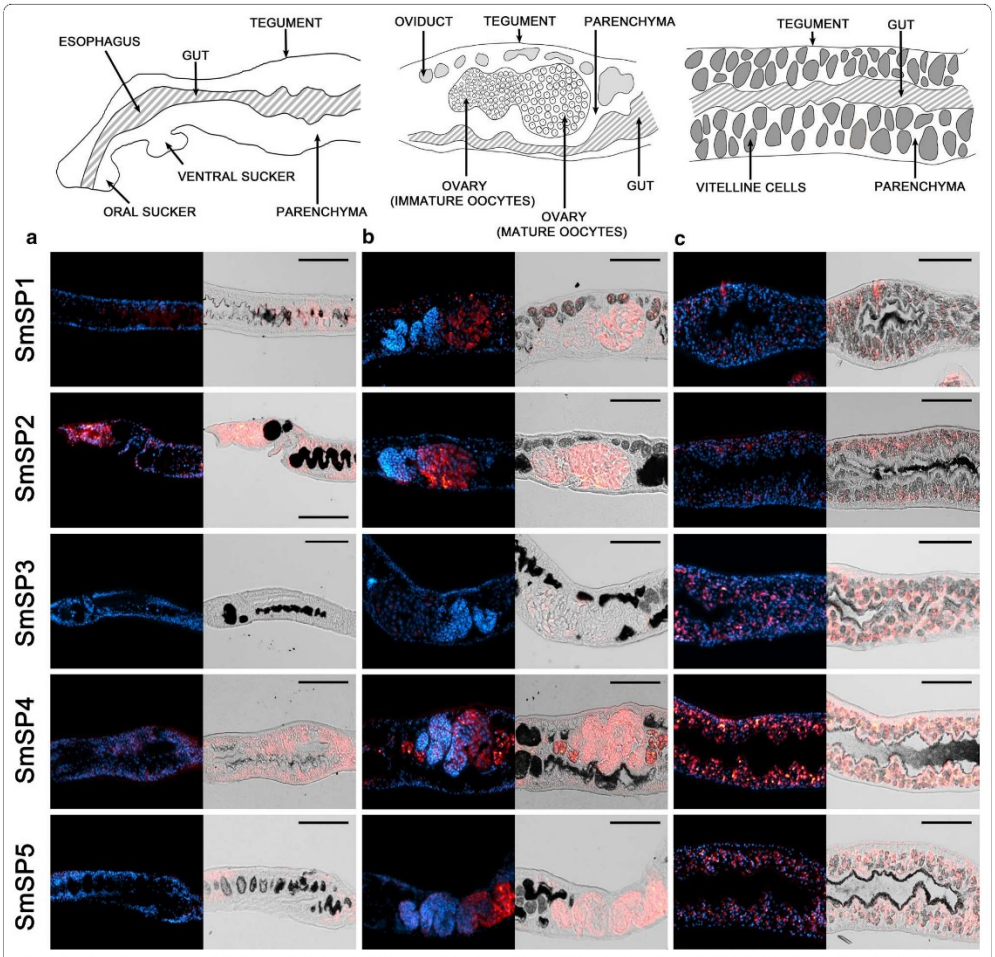

Fig. 4 Localization of mRNA encoding SmSP1 to SmSP5 in adult *S. mansoni* females using FISH. Semi-thin (6 µm) sections of adult *S. mansoni* female worms were probed with DIG-labelled RNA probes designed to detect mRNAs of serine proteases SmSP1 to SmSP5. Probes hybridized with transcripts were visualized by tyramide amplification assay (red). Adult females were monitored in three parts: **a** an anterior part, **b** oviduct, mature and immature oocytes, and **c** vitellaria and gut. DAPI was used to label nuclear DNA (blue). The left columns show merged fluorescent channels; in the right columns, the fluorescent red signal is merged with differential interference contrast. **a** A strong signal of transcripts (red) belonging to SmSP2 and SmSP4 genes and less abundant signal of SmSP1 and SmSP5 was detected in parenchymal cells of the anterior part of the females. SmSP3 was not detected in this part. **b** Strong fluorescence of SmSP2, SmSP4, and SmSP5 was observed in mature and immature oocytes, while SmSP1 sense transcript is expressed exclusively in mature oocytes, and faint signal of SmSP3 was detected in ovary. SmSP1 and SmSP4 are also expressed in the oviduct. **c** Transcripts of all studied SmSPs were present in vitellaria but with different patterns of expression. A faint fluorescent signal of SmSP2 and SmSP4 transcripts is also detected in tegument. The scale bars represent 100 µm

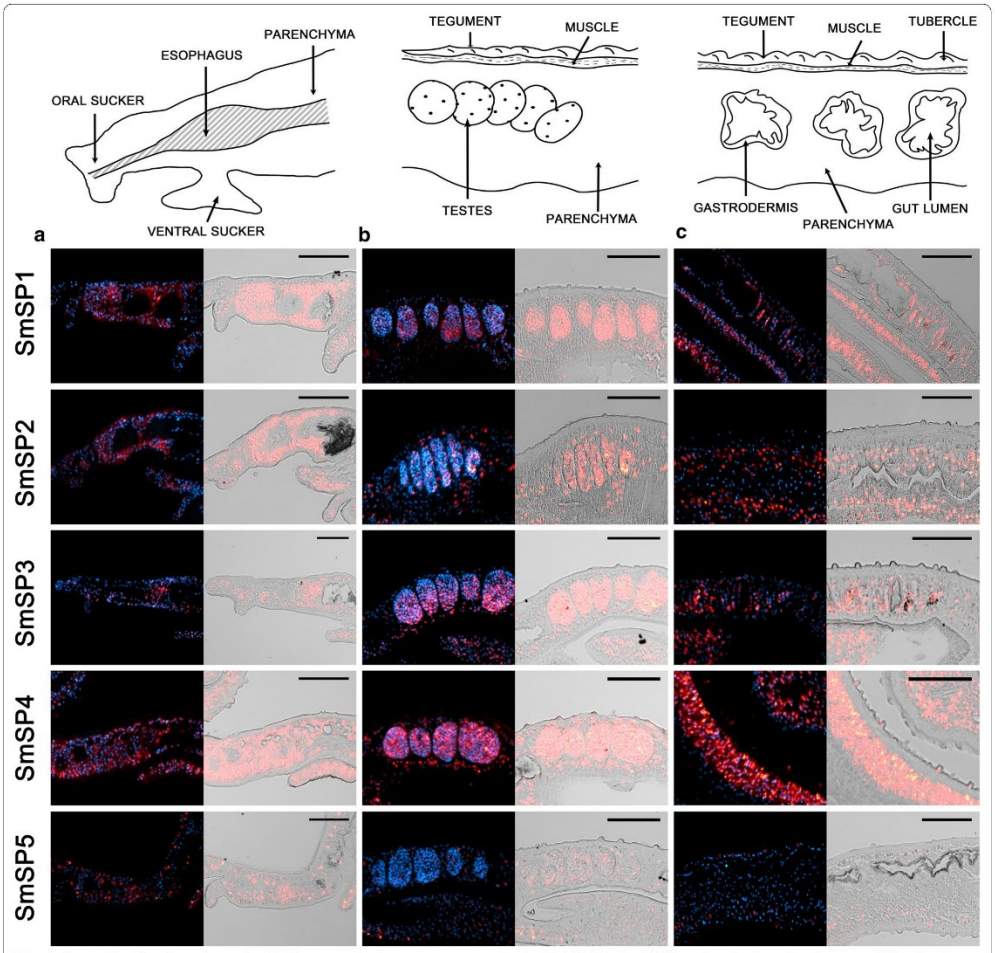

Fig. 5 Semi-thin (6 µm) sections of adult *S. mansoni* male worms were probed with DIG-labelled RNA probes designed to detect mRNAs of serine proteases SmSP1 to SmSP5. Probes hybridized with transcripts were visualized by tyramide amplification assay (red). Adult males were monitored in three parts: **a** a head part, **b** testes, and **c** a posterior part with the focus on parenchyma, tegument, and gut. DAPI was used to label nuclear DNA (blue). The left columns show merged fluorescent channels; in the right columns, the fluorescent red signal is merged with differential interference contrast. **a** Except for SmSP5, transcripts of all SmSPs were identified in parenchyma around the esophagus. **b** Strong signal of SmSP1 to SmSP4 transcripts was detected in testes where only weak signal of SmSP5 mRNA was observed. **c** All SmSPs are present in parenchymal cell subtypes, however each with a different pattern. Compared to others, SmSP4 is abundant in parenchymal cells, in tegumental cytons, and also in tegumental tubercles. The scale bars represent 100 µm

occurred additionally in the testes and the tegumental tubercles (Fig. 5).

SmSP5 expression was revealed in mature and immature oocytes, and in the vitelline cells. Weak fluoresce was detected in the parenchyma, none in the oviduct (Fig. 4). In males, SmSP5 showed a different expression pattern compared to other SmSPs: a small number of parenchymal cells expressed SmSP5. In comparison with other SmSPs, only faint fluorescence was detected

in the esophageal region and the testes, and no SmSP5 signal was detected in the tegument (Fig. 5).

As in the case of transcripts of proteins with known localizations (see above), sense probes for the detection of antisense transcripts of all SmSPs were designed and used to hybridize with the female and male dissected tissues. Only antisense transcripts of SmSP5 were found in the oviducts of females where no sense SmSP5 was expressed (Additional file 3: Figures S5 and S6).

FISH technology therefore allowed detection of the diverse localization of transcripts of all SmSP genes in adult schistosomes. The results showed their distinct distribution patterns within the tissues of males and females. A common feature for all SmSPs was the expression in parenchymal tissue; however, further expression patterns varied. None of these transcripts was detected in the gastrodermis or muscle layer.

Discussion

Family S1 serine proteases (SPs) are crucial for successful parasitism by facilitating invasion, nutrient intake, and evasion of the host immune system, and modulation of the host physiology [17, 19]. However, information about SPs in *S. mansoni* (SmSPs) remains limited despite current discoveries [18, 22]. Our previous study demonstrated that SmSPs, designated SmSP1 to SmSP5, were differentially expressed among *S. mansoni* developmental stages [21]. Here, we used fluorescence in situ hybridization (FISH) to localize individual RNA molecules in *S. mansoni* adults in tissues including the esophagus, testis, ovary, vitellarium, and parenchymal and tegumental cells, including tubercles.

The FISH method was validated by detecting expression patterns for a set of transcripts of formerly described genes. We employed the previously established FISH protocol [45] by using (i) gentle tissue fixation and optimization of several pre- and post-hybridization steps with antigen retrieval, (ii) background minimization procedures, and (iii) amplification of the fluorescent signal by TSA [23]. The obtained results corresponded well with previous findings reported using other techniques: transcripts of digestive protease SmCB1 were localized in the gut, the expression of surface-localized SmPOP was detected in the parenchyma and tegument, and the mRNAs of tegumental SmTsp-2 and Sm29 were found in tegumental cytons or tegument [28, 33, 40, 41]. SmPOP and Sm29 transcripts were also localized in the vitellarium, which agrees with previous studies [28, 30, 33]. Lastly, the transcription profiles for SmCB1, SmPOP, SmTsp-2, and Sm29 were congruent with sequencing data obtained for *S. mansoni* [35, 36].

In addition to previous data, we discovered new localizations of some transcripts due to the high sensitivity of the FISH method. SmCB1 transcripts were detected in the vitellarium, whereas SmCB1 activity was previously detected by fluorescence histology only in oviducts and testes [42]. Localization of SmCB1 in vitellaria is in the line with expression of orthologous proteases from other parasitic flukes, *Fasciola gigantica* and *Eudiplozoon nipponicum*, in which cathepsin B expression was also detected in testes and vitelline cells [46, 47]. The function of these proteases in reproductive organs is as yet unknown; however, the authors hypothesized that cathepsin B may process eggshell or yolk protein vitellogenin precursors, as described in other organisms [48–50]. In addition to localization in the tegument, SmTsp-2 and Sm29 transcripts were found in parenchyma and testes, and SmTsp-2 also in the oviduct (Figs. 2 and 3). Tetraspanins stabilize extracellular vesicles [51], and these types of vesicles are known to be secreted as well by the specific types of cells into the lumen of the oviduct [52], which may explain the abundance of tetraspanin SmTsp-2 transcripts in such a highly dynamic organ structure.

Our previous research [21] and transcriptome sequencing data [9, 35, 36, 53] revealed that SmSP2 and SmSP4 are highly abundantly transcribed in adult schistosomes and, contrarily, expression levels of SmSP1, SmSP3, and SmSP5 are low. Highly sensitive FISH analysis employed in this paper revealed expression patterns of all SmSPs (including low-abundantly expressed ones) distributed in multiple tissues of adult schistosomes, except for more specific localization identified for SmSP3 in females and SmSP5 in males. All SmSPs were commonly detected only in parenchyma of both genders (Figs. 4 and 5), but individual SmSPs showed distinct expression patterns in different subtypes of parenchymal cells (Figs. 4 and 5), indicating their unique functional roles. Distribution of SmSP1 and SmSP2 transcripts in parenchymal cells and tegument agrees with protein immunolocalization data [22, 54]. Except for SmSP5, all studied genes localized within the anterior part of males in the head area containing the esophagus. Previously, this area was shown to be highly dynamic in gene transcription; more than 1000 genes were detected. Among them, SmSP2 was found to be upregulated twofold or more in this region in comparison with the whole worm body [55]. This agrees with our results, because strong signals of SmSP2 transcripts were detected in the parenchyma of the anterior body of both genders (Figs. 4 and 5). Detection of SmSP2 transcripts in the esophageal region also fits with our previous immunolocalization data [22].

Additional new localizations of several SPs were found in some reproductive organs of male or female worms. These organs are highly dynamic, comprising 7000 transcripts of genes upregulated twofold or more in reproductive organs compared to whole worm controls [36,

55]. Expression levels of particular genes are upregulated twofold or more after mating, and RNA storage is also frequently observed in gonadal cells [36, 56]. Additionally, mRNA stored in spermatozoa can be transported from males to females during sexual reproduction via sperm fluid into the oocyte [36, 57]. FISH revealed the presence of all SmSPs in gonads of both genders (Figs. 4 and 5). Contrarily, RNAseq showed (with exception of SmSP2) low or no SmSP transcripts in schistosome gonads [36]. Such divergence is probably based on distinct methodologies. RNAseq has a limited number of reads, reflecting a limited number of molecules that can be detected. Thus, low-abundant transcripts may be below the detection limit. On the other hand, FISH employed in this study has the capacity to detect every single RNA in the tissue due to amplification of the fluorescent signal by TSA (signal amplification up to 200fold), which has the ability to detect low-abundant transcripts which under normal circumstances would be part of the threshold. SmSPs may have a similar function reported for serine proteases in the gonads of several nematodes or flies: *Caenorhabditis elegans* or *Ascaris suum* employ serine proteases for spermatogenesis and sperm activation in the uterus [58, 59]; serine proteases of *Drosophila melanogaster* are thought to process peptides and activate enzymes inside the female reproductive ducts and mediate critical postmating responses [60].

The entire surface of adult schistosomes is covered by the syncytial layer called the tegument [16]. In contrast to females, males have additional tegumental structures called tubercles [61]. The nucleated regions of tegumental cells are also known as cell bodies or tegumental cytons, where protein-synthesizing and sorting machinery (including endoplasmic reticulum and Golgi apparatus), is situated below the musculature and has connections to the syncytial surface part via cytoplasmic connections (Additional file 2: Figure S1a). Synthesized proteins, RNAs, and other cargos are transported to the cell surface by microtubule-lined cytoplasmic channels through cytoplasmic connections. The most common destination for mRNAs within the cell is in the immediate proximity of the site of translation, i.e. close to the endoplasmic reticulum. Therefore, mRNA would be expected in the tegumental cytons but not in the surface layer [62]. Nevertheless, mRNAs of SmPOP, SmTsp-2, and SmSP4 were localized not only in tegumental cytons, but a strong signal was also detected in tegumental tubercles (Figs. 3 and 5). These results suggest that a not fully understood transport mechanism exists for moving mRNA molecules from cytons to the schistosome surface. This cargo is usually transported through the tegument via extracellular vesicles sent to the outer environment. Recently, SmSP2, SmTsp-2, and Sm29 were identified in extracellular vesicles released from *Schistosoma* [63], which coincides with our localization in the tegumental tubercles.

The localization of mRNAs within eukaryotic cells is enormously diverse [64, 65]. The endoplasmic reticulum and Golgi apparatus provide well-established membrane-sorting machinery to shuttle mRNAs to distant regions within the cell [64]. These mRNAs are usually stored in granules and are ready for immediate or emergency use in a wide range of processes [64, 66]. They can (i) be reactivated, translated into proteins, and used in various rapid emergency processes including stress responses, metabolic reprogramming, repair of stress-induced damage, and adaptation to changed conditions; (ii) play a role in the repression of other mRNA species; or (iii) be directly sent for decay [67, 68]. We hypothesize that schistosomes may employ similar mechanisms, especially in such a dynamic structure as the tegument, which protects worms in the unfriendly blood environment of the host.

Antisense transcripts are frequently transcribed in eukaryotes and represent important regulators of gene expression; they control the state of chromatin or modulate the post-transcriptional fate of mRNAs [69, 70]. We identified antisense transcripts of SmPOP, SmSP5, and SmCB1 only, and they were localized exclusively in the oviduct (Additional file 3: Figures S2 and S5). As known from previous research, these antisense transcripts most likely do not encode proteins but may control gene expression [69].

Conclusions

We validated and optimized a FISH method [23] for the detection of RNA transcripts in adult *S. mansoni* tissues. We documented the efficacy of the method by precise localization of the transcripts of selected proteins (SmCB1, SmPOP, SmTsp-2, and Sm29), whose distribution in schistosome adults was previously determined by other methods. In addition, we provided new insights into the localization of transcripts of these genes. Compared to RNAseq, FISH, due to its high sensitivity, is able to detect mRNA with low expression potential. The FISH methodology was then successfully applied to localize transcripts encoding serine proteases SmSP1 to SmSP5. Transcripts were found in *S. mansoni* females and males in various organs (parenchyma, tegument, reproductive organs) but with distinct patterns. Furthermore, we detected transcripts in previously unknown locations such as the syncytial part of the tegument or in tegumental tubercles. Based on the evidence of different transcript locations and our previous research [21, 22], we hypothesize that SmSPs may play various physiological roles in host–parasite interaction, including regulation of the

host vascular system, repair of stress-induced damage, and/or adaptation to changed conditions in the external environment. However, elucidation of the precise function of individual SmSPs is a matter of future studies.

Abbreviations

DIG: Digoxigenin; FISH: Fluorescence in situ hybridization; HRP: Horseradish peroxidase; PBS: Phosphate-buffered saline; SSC: Standard saline citrate; SmSP1-5: *S. mansoni* serine protease 1–5; SmCB1: *S. mansoni* cathepsin B1; Sm29: Membrane-bound glycoprotein found on *S. mansoni* tegument, GenBank accession number AAC98911.1; SmPOP: *S. mansoni* prolyl oligopeptidase; SmTsp-2: *S. mansoni* tetraspanin 2.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13071-021-04773-8.

Additional file 1: Table S1. Primers used to generate DIG-labelled RNA probes.

Additional file 2: Figure S1. Schematic representation of the adult *S. mansoni* surface and detailed micrograph of SmTsp-2 localization in the tegument and parenchyma of *S. mansoni* adult males.

Additional file 3: Figure S2. Localization of antisense mRNA of SmCB1, SmPOP, SmTsp-2 and Sm29 in adult *S. mansoni* females using FISH. **Figure S3.** Localization of antisense mRNA of SmCB1, SmPOP, SmTsp-2 and Sm29 in adult *S. mansoni* males using FISH. **Figure S4.** FISH with the probe for bacterial (*neo*) gene and a negative control (with no probe) in *S. mansoni* males and females. **Figure S5.** Localization of antisense mRNA of SmSP1 to SmSP5 in adult *S. mansoni* females using FISH. **Figure S6.** Localization of antisense mRNA of SmSP1 to SmSP5 in adult *S. mansoni* males using FISH.

Acknowledgements

We would like to express our profound gratitude to Prof. John D. Brooker for critical proofreading of the manuscript and to Prof. Christoph G. Grevelding for providing plasmids for the Sm29 probe, for consultation, and for critical reading of the manuscript.

Authors' contributions

All authors contributed significantly to this study. JD and LU conceived and designed the experiments. JD, LU, and MH coordinated the study implementation. LU, MC, JD, and PO performed the experiments. LU, JD, and PO analyzed the data. LU, MH, and JD wrote and revised the paper. All authors read and approved the final manuscript.

Funding

JD was supported by the Operational Programme Research, Development and Education, the Call International Mobility of Researchers—MSCA– IF CZ.0 2.2.69/0.0/0.0/17_050/0008014 and by the Czech Science Foundation project 18-14167S and by the NutRisk Centre, CZ.02.1.01/0.0/0.0/16_019/0000845. MH was supported by the grants NV18-05-00345 from the Ministry of Health of the Czech Republic, 19-17269S from Czech Science Foundation, 8J19AT036 (OeAD CZ17/2019) from the Ministry of Education, Youth and Sports of the Czech Republic, and by the institutional project RVO 61388963. LU was supported by grant 1496214 from Charles University Grant Agency. PO was supported by the Charles University Research Fund (Progres Q39), by Charles University Research Centre program UNCE/MED/006 "University Center of Clinical and Experimental Liver Surgery" and by the National Sustainability Program I (NPU I) No. LO1503 provided by the Ministry of Education Youth and Sports of the Czech Republic. MC was supported by the Charles University Research Fund (Progres Q25).

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate

Research with experimental animals was performed in accordance with the animal welfare laws of the Czech Republic and under the European regulations for transport, housing and care of laboratory animals (Directive 2010/63/EU on the protection of animals used for scientific purposes). This project, including the use of experimental animals for the present study, was approved by the Ministry of Education, Youth and Sports of the Czech Republic (Approval Number MSMT-7063/2017-2). All animals used in the study were maintained by a certified person (Certificate Number CZ 02627) in specifically accredited laboratories of the Institute of Immunology and Microbiology of the First Faculty of Medicine, Charles University and the General University Hospital in Prague (Accreditation Numbers 70030/2013-MZE-17214 and 8615/2019-MZE-17214), both issued by the Ministry of Agriculture of the Czech Republic.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 28 January 2021 Accepted: 3 May 2021
Published online: 22 May 2021

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4.3 Publication No. 2: Excretion/secretion products from *Schistosoma mansoni* adults, eggs and schistosomula have unique peptidase specificity profiles.

Proteases are important for successful schistosome parasitism of allowing them to invade, grow, feed reproduce and help them to manipulate the host immune system and physiology [179]. To date, research has focused on annotation of their sequences as part of global genome and/or transcriptome studies [136, 202], proteomic analysis [178, 195], or the functional characterization of individual proteases [16, 91, 201, 223]. Proteomic analysis of ES products revealed the presence of a large number of proteases secreted by schistosomes into the environment where they play an essential role in parasite-host interactions, e.g., by modulating host physiology and immunology [174].

Publication 2 is devoted to the analysis and classification of the proteolytic activity of ES products of *S. mansoni* developmental stages parasitizing the human body; namely schistosomula (post-infective migratory larvae), adults and the eggs. *S. mansoni* ES products were collected (detailed method was described in publication No. 7) and proteolytic activity was analyzed using the Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) assay [224], and a kinetic assay with a panel of internally quenched, fluorescent peptidyl substrates. Both methods were used in combination with specific inhibitors of different protease classes allowing the identification of protease classes contributing to the global activity of the parasite.

The results revealed the complexity of proteolytic activities released by the schistosoma life stages parasitizing the human host. Each stage produced a different set of endo- and exo-peptidase activities. All stages secreted serine proteases, which predominated in the ES products of schistosomula. In contrast, three major classes of proteases - serine, cysteine and metalloproteases - are represented in both egg and adult ES products, with metalloproteases and serine proteases predominant in adult schistosomes, whereas the activity of serine and cysteine proteases, which are active at neutral pH, is pronounced in the egg. Identification and annotation of the serine proteases is described in publication No. 3, the detailed characterization of two serine proteases in the publications No. 4 and No. 5. Other proteases from ES products are awaiting further investigation.

PhD applicant contribution: Preparation of individual *S. mansoni* developmental stages parasitizing humans (schistosomula, adults, and eggs), and their cultivation, collection of ES products and their preparation for MSP-MS analysis.

Biochimie 122 (2016) 99–109

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Excretion/secretion products from *Schistosoma mansoni* adults, eggs and schistosomula have unique peptidase specificity profiles

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ARTICLE INFO

Article history:
Received 12 July 2015
Accepted 22 September 2015
Available online 26 September 2015

Keywords:
Parasite
Fluke
Secretion
Excretion
Protease
Inhibitor

ABSTRACT

Schistosomiasis is one of a number of chronic helminth diseases of poverty that severely impact personal and societal well-being and productivity. Peptidases (proteases) are vital to successful parasitism, and can modulate host physiology and immunology. Interference of peptidase action by specific drugs or vaccines can be therapeutically beneficial. To date, research on peptidases in the schistosome parasite has focused on either the functional characterization of individual peptidases or their annotation as part of global genome or transcriptome studies. We were interested in functionally characterizing the complexity of peptidase activity operating at the host–parasite interface, therefore the excretory-secretory products of key developmental stages of *Schistosoma mansoni* that parasitize the human were examined. Using class specific peptidase inhibitors in combination with a multiplex substrate profiling assay, a number of unique activities derived from endo- and exo-peptidases were revealed in the excretory-secretory products of schistosomula (larval migratory worms), adults and eggs. The data highlight the complexity of the functional degradome for each developmental stage of this parasite and facilitate further enquiry to establish peptidase identity, physiological and immunological function, and utility as drug or vaccine candidates.

1. Introduction

Schistosomiasis caused by the *Schistosoma* blood fluke is a chronic disease of poverty infecting more than 200 million people with as many as 800 million people at risk [1,2]. Schistosome larvae (cercariae), released into freshwater by intermediate snail hosts, penetrate human skin and subsequently develop into adult male or female worms in the host vascular system. Pairs of worms can survive for years, if not decades [3] and release many hundreds of eggs a day [4]. These eggs induce immune-inflammation and tissue damage that contribute to disease morbidity [5]. The disease hampers growth and development of children and severely impacts the ability of families to provide for themselves [6–8].

Proteolysis is a fundamental physiologic process contributing to both health and disease [9,10]. *Schistosoma* peptidases (a.k.a. proteases or proteolytic enzymes) are vital to successful parasitism, and facilitate invasion of the host, digestion of host proteins,

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http://dx.doi.org/10.1016/j.biochi.2015.09.025
0300-9084/

reproduction, modulation of the host's physiology [11–19] and immune response [14,20]. Interference with these mechanisms by specific vaccines [21,22] or drugs may provide therapeutic benefits. Indeed, peptidases are excellent druggable targets [23–25] and a large body of literature exists demonstrating the therapeutic benefits of small molecule inhibitors targeting peptidases of schistosomes [26–29] and other infectious organisms [30–36].

Research on parasite peptidases has traditionally been driven by investigator interest in a particular molecule or class of molecules. For example, much of the considerable research focused on schistosome cysteine cathepsin enzymes as drug or vaccine targets has been facilitated by often inexpensive, sensitive and easy-to-use tools that include peptidyl substrates, inhibitors and antibodies. Detailed molecular, structural and immunological characterizations of these enzymes [29,37,38] have been greatly aided by their straightforward 'expressability' in heterologous systems such as yeast [38–41]. Over the last 10 years, the increasing availability of accurately annotated genomic [42–47] and transcriptomic data [48–53] has expanded our view of the number and complexity of peptidases (the 'degradome') expressed by the schistosome and how their expression is regulated throughout the parasite's life cycle. In parallel, a number of studies have generated (sub)proteome data for schistosomes (for reviews see Refs. [54,55]), including for the parasite's tegument (surface) [54,56–58] and excretory/secretory (ES) products [59,60]. Though fundamentally informative, these studies do not provide information on which peptidases are functionally active, including at the host–parasite interface.

We performed a global and unbiased analysis of peptidase activity and specificity in the ES products of key *Schistosoma mansoni* developmental stages residing in the human host, namely schistosomula (post-infective migratory larvae), adults and their eggs. We chose to examine ES products as these would contain peptidases more likely to operate at the host–parasite interface rather than extracts which would also include less relevant somatic activities. The substrate specificity for peptidase activities was detected using Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS). This highly sensitive peptidase assay utilizes tandem mass spectrometry to monitor the degradation of a synthetic peptide library. MSP-MS can simultaneously detect endo- and exo–peptidase activities, and has been successfully employed to profile the proteolytic specificities of human neutrophil extracts [61], ES products from a pathogenic fungi [62] and *S. mansoni* cercariae [63]. In addition, we employed a panel of internally quenched, fluorescent peptidyl substrates in the presence and absence of peptidase-class-specific inhibitors to identify which peptidase classes were contributing to the global activity. Overall, we characterize a number of new proteolytic activities that sets the stage for their formal identification and exploration of their respective biological functions.

2. Materials and methods

2.1. Ethics statement

Maintenance and handling of vertebrate animals were carried out in accordance with a protocol (AN107779) approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California San Francisco.

2.2. Schistosome material

A Puerto Rican isolate of *S. mansoni* is maintained in the laboratory by cycling between Golden Syrian hamsters (*Mesocricetus auratus*) and the freshwater snail, *Biomphalaria glabrata*. Female 3–5 week-old hamsters were subcutaneously injected with 200 cercariae and sacrificed 6–7 weeks post-infection using an intraperitoneal injection of sodium pentobarbital (50 mg/kg). Adults and eggs were isolated as described [64]. Cercariae (infectious larvae) were obtained from infected snails induced to release the parasite under a light stimulus. Cercariae were chilled on ice, collected and mechanically transformed into schistosomula as described previously [65–67].

2.3. Collection of ES products

Fifty pairs of adult worms, 1000 eggs or 1000 schistosomula were washed five times in Basch medium 169 [68] supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 1% Fungizone (Gibco), and allowed to stand for 1 h at 37 °C in 5% CO_2. Samples were washed 10 times and then incubated at 37 °C in 5% CO_2 in the above medium supplemented with 5% fetal calf serum but in the absence of Fungizone. Adults and eggs were incubated overnight, and schistosomula were incubated for five days to allow for complete transformation from cercariae and remove contaminating cercarial peptidases. Parasite materials were washed three times in the above medium and then washed 10 times in M-199 medium containing 100 U/ml penicillin and 100 mg/ml streptomycin, but without serum. In 5 ml of the same medium, samples were evenly distributed into a 6-well cultivation dish and incubated for 16 h at 37 °C in 5% CO_2.

Medium containing ES products was removed, filtered over an Ultrafree-MC 0.22 mm filter (Millipore), and buffer exchanged into ice-cold Dulbecco's-Phosphate-Buffered Saline (D-PBS). The medium was then concentrated to 2 ml by centrifugation at 4000 *g* and 4 °C using an Amicon 10000 Ultra-15 Centrifugal Filter Unit (Millipore). The total volume of PBS used for buffer exchange was 40 ml. Sample materials were quickly frozen in liquid nitrogen and stored at −80 °C as 100 μL aliquots. Protein concentration was measured at 280 nm on a NanoDrop 2000c (Thermo Scientific).

2.4. Multiplex peptide cleavage assay

The Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) assay was performed as previously described [63] with minor modifications. Briefly, 20 μg/ml of protein from *S. mansoni* ES products of schistosomula, adults or eggs were pre-incubated for 15 min at room temperature with 0.2% DMSO, 1 mM AEBSF (Sigma–Aldrich 76307), 100 μM E-64 (Sigma–Aldrich E-3132) or 2 mM 1,10-Phenanthroline (Sigma–Aldrich 131377) in D-PBS containing 4 mM DTT. Each reaction was split into two tubes containing an equimolar mixture of 62 peptides in D-PBS (124 total). The final assay consisted of 500 nM of each peptide, 10 μg/ml protein, 2 mM DTT, 0.1% DMSO and either 500 μM AEBSF, 50 μM E-64, 1 mM 1,10-Phenanthroline or no inhibitor in D-PBS, in a total volume of 300 μl. Aliquots were removed at defined time intervals, adjusted to <pH 3.0 with formic acid and then desalted using C18 tips (Rainin).

Mass spectrometry was performed on a LTQ FT instrument (Thermo), equipped with a nanoACUITY (Waters) ultra-performance liquid chromatography. Reverse phase LC was performed using an EZ-Spray C18 column (Thermo, ES800, PepMap, 3 μm bead size, 75 μm × 15 cm) at 600 nL/min for loading and 300 nL/min for peptide separation over a linear 65 min gradient from 2% to 30% acetonitrile in 0.1% formic acid. The mass spectrometer was operated using identical acquisition parameters as reported previously [63]. Substrate specificity profiles were generated using iceLogo software [94].

2.5. Internally quenched peptide assays

All assays were performed at room temperature in D-PBS

containing 2 mM DTT and 0.01% Triton X-100. Assays were performed in triplicate in round-bottom 96-well plates in a spectrofluorimeter (Molecular Devices Flex Station) using a λ_{ex} 328 nm and λ_{em} 393 nm. Initial velocities in relative fluorescent units per second were calculated using Softmax Pro. Protein from *S. mansoni* conditioned media was assayed with a set of internally quenched fluorescent substrates (30 μM each). The total protein concentration in the internally quenched (IQ) substrate screen was 1.95 μg/ml, 18.7 μg/ml and 3.3 μg/ml for schistosomula, adults and eggs, respectively. Each IQ substrate consisted of a 7- or 8-mer peptide flanked with 2,4-dinitrophenyl-L-lysine on the carboxyl terminus and either 7-methoxycoumarin-4-acetic acid or 7-methoxycoumarin-4-yl-acetyl-L-lysine on the amino terminus. IQ substrates were synthesized using standard Fmoc chemistry and purified to >90% by reverse phase HPLC. Inhibition assays were performed using the same concentration of proteins as above. Assays contained 30 μM of IQ substrate, 1.5% DMSO, 100 μM of E-64, 500 μM of AEBSF or 1 mM of 1,10-Phenanthroline.

3. Results

3.1. Detection of cleavage sites derived from peptidases in S. mansoni ES products

We took a global and unbiased approach to characterizing the proteolytic components of the ES products of three *S. mansoni* life-stages that parasitize the mammalian host, namely schistosomula, adults and eggs. After extensive washing to remove blood and serum components, including contaminant proteases, each developmental stage was placed in serum-free medium overnight. The ES products (conditioned medium) were then concentrated and added to a mixture of 124 physicochemically diverse peptides that are each 14-residues in length. Cleavage of any one of the 1612 available peptide bonds within these peptides can be readily detected by LC-MS/MS sequencing. This assay is termed Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) and has been previously used with *S. mansoni* to characterize the specificity of peptidases in conditioned water from parasite-infected snails [63].

Assays on *S. mansoni* ES products were performed at pH 7.4 to mimic physiological pH of the mammalian host. Incubation of schistosomula, adults and eggs ES peptidases with the peptide library for 15 min resulted in 23, 7 and 15 cleavage sites, respectively (Fig. 1A). However, after the longest incubation of 1200 min, the number of cleavage sites increased to 107, 151 and 301, respectively. In addition, cleavage sites were assessed at the intermediate time intervals of 60 and 240 min (Supplementary File 1).

The complexity of these hydrolytic events is illustrated using a sample peptide, *AYNnWSLYRnIRQE*, from which multiple cleavage sites were detected at various time intervals (Fig. 1B). Peptidases secreted from schistosomula, cleave at the Trp–Ser and Arg-Nle site and products of this hydrolysis were evident after only 15 min. After 60 min, additional cleavage products were detected that corresponded to hydrolysis at the Tyr–Arg bond. Unlike the other two stages, schistosomula ES peptidases could not cleave the Nle-Ile bond. Adult peptidase activity also cleaved the Trp–Ser and Arg-Nle bonds but these products appeared at later time intervals compared to hydrolysis of the Nle-Ile bond. No cleavage of the Tyr–Arg bond was evident in the adult ES products even after 1200 min incubation, however, products derived from Tyr–Asn hydrolysis were found after 240 min incubation. Finally, ES peptidases from eggs preferentially cleaved at the Arg-Nle site within 15 min and later at the Trp–Ser and Nle-Ile sites. Like adults, egg ES peptidases did not cleave the Tyr–Arg bond. Thus, interrogation of just this single sample peptide from the mixture of 124, indicates that different peptidases are present in the ES products of each of the *S. mansoni* intra-mammalian stages.

The presence of different peptidases in the conditioned media from each of these developmental stages was investigated by directly comparing the cleavage sites. For this, the 145 sites identified after 240 min in the egg ES products was compared to the 107 and 151 sites identified from 1200 min incubation of schistosomula and adults peptidases, respectively. These assays correspond to the earliest incubation time required to digest at least 5% of the 1612 peptide bonds in the library (≥81 cleavage sites). Peptidases from schistosomula, adults and eggs cleaved at 35, 79 and 62 unique sites, respectively, whereas 45 sites were common to all three (Fig. 1C). This analysis confirms that unique peptidases are present in the ES products from each of the intra-mammalian life stages.

In our experience, exopeptidases that remove mono-, di- or tripeptides from the amino or carboxy terminus of proteins and oligopeptides are difficult to detect with standard reporter substrates due to the positioning of the fluorescent or colorimetric reporter group. For carboxypeptidases, the reporter group blocks the carboxyl terminus and therefore prevents cleavage. For aminopeptidases, substrates that are too long or too short, will not be hydrolyzed correctly between the canonical P1 residue and the reporter group. Our MSP-MS approach can simultaneously detect exo- and endo-peptidase activity because the peptides employed have free amino and carboxyl termini. The location of each cleavage site within the 14-mer peptides was compared for egg ES peptidases after 240 min incubation and adult and schistosomula ES peptidases after 1200 min incubation (Fig. 1D). In general, the majority of peptide bond hydrolysis occurred away from the termini indicating that endopeptidases are most active. However, enzymes in the adult ES products cleaved between position 2 and 3 of the 14-mer peptides at a higher frequency than egg and schistosomula peptidases indicating that an enzyme with di-aminopeptidase activity may be present. Conversely, there is a higher frequency of cleavage by egg ES products between position 12 and 13 which may represent a di-carboxypeptidase activity.

3.2. Comparison of the substrate specificity profiles between life cycle stages

For each of the cleavage sites generated by the ES peptidases, we obtained prime and non-prime site substrate specificity information (Fig. 2). Using iceLogo software, a P4 to P4′ substrate signature was generated for all cleavage sites that occur after a defined incubation time. Peptidases secreted by schistosomula preferentially degraded peptides on the C-terminal side of Arg or Lys residues. This type of substrate specificity is commonly called “trypsin-like”. Gln was most often found in the P2 position and Ser or Arg were frequently found at P1′. In addition, cleavage rarely or never occurred at the C-terminal side of Gly, Pro, Nle or Glu or at the N-terminal side of Pro or Asn (Fig. 2A).

In contrast to schistosomula, much greater cleavage promiscuity was apparent in the ES products of adults suggesting that there are multiple enzymes present (Fig. 2B).Adult peptidases also preferentially cleaved at sites when Arg or Trp were present in the P1 position and Ser or Arg at P1′. The degradation of peptides by the egg ES peptidases had a preference for Arg and Gln at P1, Arg at P1′ and bulky hydrophobic residues at P3 (Fig. 2C).

To complete the life cycle, we included the substrate signature generated from our previous study using water conditioned for 1200 min with *S. mansoni* infected and non-infected *B. glabrata* snails [63]. Non-infected snails secrete at least one peptidase with a trypsin-like specificity (Arg and Lys at P1; Fig. 2D). This is consistent with the previous biochemical characterization of a major tryptase enzyme in snail extracts [69]. The substrate signature generated from conditioned water from *S. mansoni* infected-snails presented a

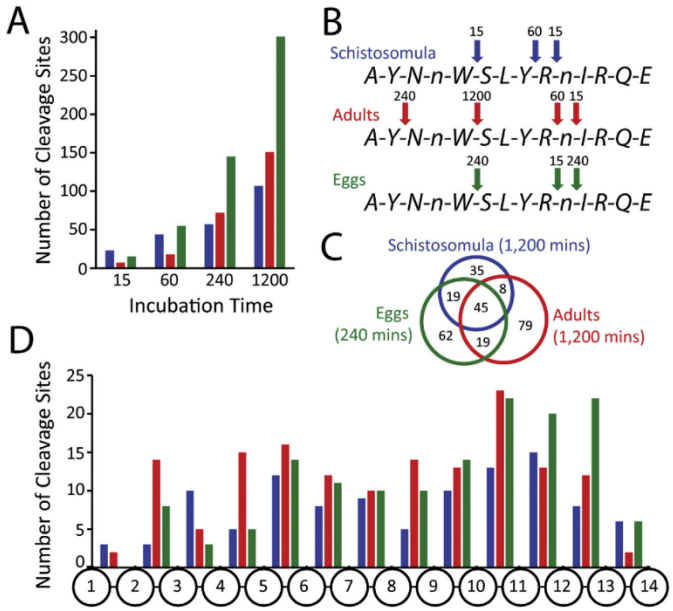

Fig. 1. Detection of proteolytic activity in the ES products of *S. mansoni*. A. ES products from schistosomula (blue), adult worms (red) and eggs (green) were incubated for 15 to 1200 min with a 14-mer peptide library. LC-MS/MS sequencing was used to detect the appearance of peptidase cleavage sites B. A sample 14-mer peptide illustrating the complexity of peptidase cleavage. The position and time (minutes) that cleavage products were first detected are indicated. Amino acids are shown in single letter code. Lowercase "n" corresponds to norleucine (Nle). C. Venn Diagram showing the number of unique and shared cleavage sites. D. Spatial distribution of cleavage sites within the 14-mer peptide scaffold.

different substrate specificity profile (Fig. 2E). Infected snails in water release cercariae which contain a number of chymotrypsin-like serine peptidases known collectively as 'cercarial elastase'. An in-depth characterization of these enzymes uncovered a P1 specificity for Phe and Tyr (Fig 2E). In addition, a preference for Pro at P2 is recorded and this is consistent with the known specificity of purified cercarial elastase using P1–P4 substrate positional scanning [70]. Schistosomula are generated from cercariae by mechanical shearing of tails followed by *in vitro* culture [65]. After 5 days of culture *in vitro*, it is clear that the chymotrypsin-like hydrolysis signature of the cercarial elastase is replaced by one that is trypsin-like.

3.3. Detection of peptidase activity in ES products using IQ substrates

As the majority of activity in ES products was derived from endopeptidases, we utilized a panel of internally quenched (IQ) fluorescent peptides with diverse sequences to quantify this activity. These substrates were previously synthesized by our group to detect aspartic acid and glutamic acid [71], cysteine [72] and serine [73] peptidases from a variety of microbial sources. These peptides were collectively used to detect proteolytic activity in conditioned media from the fungal pathogen, *Pseudogymnoascus destructans* [62]. Each IQ peptide is either seven or eight amino acids long and flanked on the N-terminus by a fluorophore and on the C-terminus by a fluorescent quenching group. Cleavage of any bond results in an increase in fluorescence due to the separation of the quencher from the fluorophore.

Peptidases in the ES products from each life cycle stage hydrolyzed three IQ substrates containing the sequences QCACSNHE, tQASSRS and GRFGVWKA (Fig. 3). No other peptides were cleaved by schistosomula ES products. Egg-conditioned medium cleaved 5 additional IQ substrates, and in general showed a higher specific activity relative to schistosomula and adult enzymes. This is consistent with egg peptidases cleaving at a greater number of sites in the MSP-MS assay. The conditioned medium from adults cleaved all IQ substrates but generally had low specific activity relative to the schistosomula and egg ES enzymes. tQASSRS was the commonly cleaved IQ substrate, and was therefore deemed to be a useful reporter substrate to quantitatively measure proteolytic activity in the presence and absence of class-specific inhibitors.

3.4. Investigating the contribution of serine, cysteine and metallo-peptidases using class-specific inhibitors

Using the tQASSRS substrate, ES products from each of the *S. mansoni* developmental stages were treated with class-specific inhibitors that target serine, cysteine and metallo-peptidases. All assays were performed at pH 7.4, at which aspartic acid peptidases are unlikely to be active. Therefore, pepstatin A that targets aspartic acid peptidases was not included in the inhibitor screen.

3.4.1. Schistosomula

Cleavage of tQASSRS was unchanged upon treatment with the metallopeptidase inhibitor 1,10-Phenanthroline when compared to the non-inhibited control (DMSO) while the cysteine peptidase inhibitor E-64 actually increased activity. However, in the presence of the serine peptidase inhibitor, AEBSF, turnover of the fluorescent

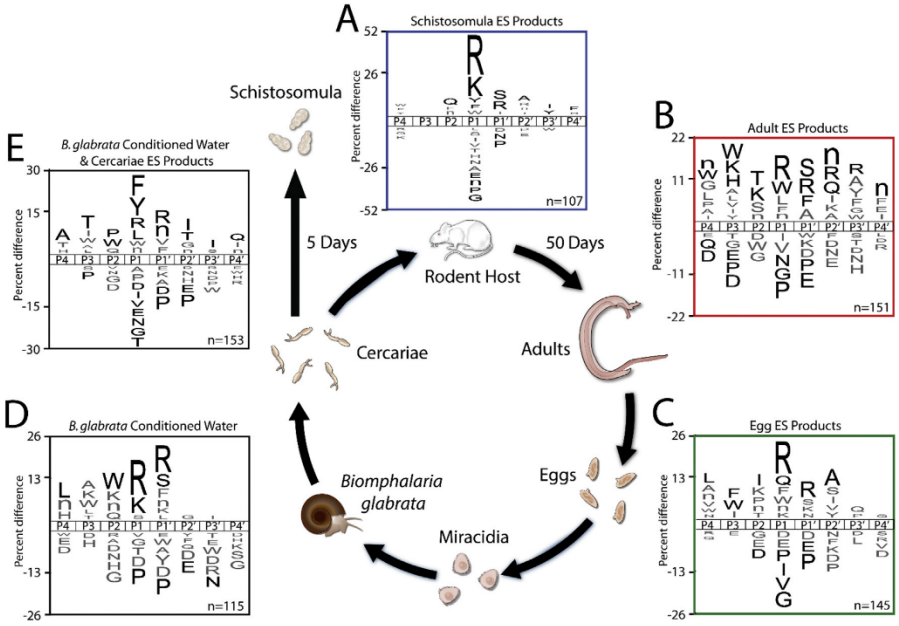

Fig. 2. Generation of a proteolytic specificity signature in the ES products of *S. mansoni* at different life cycle stages. Generation of a substrate specificity iceLogo signature for A. schistosomula, B. adult and C. egg secretions following a 240 or 1200 min incubation period with the 14-mer peptide library. Amino acids colored black are significantly increased ($p < 0.05$) in the position relative to a control dataset that corresponds to all possible cleavage sites. Amino acids above the X-axis are found at the given position with higher frequency than the control dataset while amino acids below the axis are rarely or never found at the given position. IceLogo signature showing the substrate specificity profiles of conditioned water from D. uninfected and E. *S. mansoni* infected *Biomphalaria glabrata*.

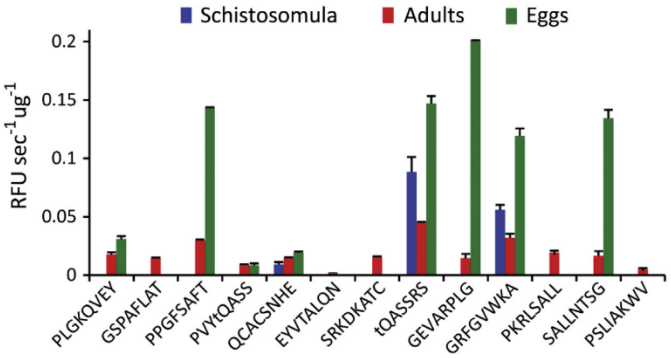

Fig. 3. Quantitation of proteolytic activity in ES products of *S. mansoni* at different life cycle stages. Proteolytic activity in the conditioned media was detected using internally quenched fluorescent substrates. Amino acids are described in single letter code and lowercase 't' corresponds to *tert*-butyl glycine.

substrate was abolished (Fig. 4A). These data are consistent with the presence of a predominant serine peptidase activity.

MSP-MS assays were performed with the same inhibitor treated ES products that were used in the IQ studies. Using the sample 14-mer peptide highlighted in Fig. 1B, we show that treatment of medium with E-64 results in a cleavage pattern that is identical to DMSO-treated control (Fig. 4B). In contrast, AEBSF prevented hydrolysis of the Trp–Ser and Tyr–Arg bonds and greatly delayed hydrolysis of the Arg-Nle bond, which was only recorded after 1200 min. Two new cleavage sites (Tyr–Asn and Nle-Ile) appeared after 1200 min that were never observed in the DMSO-treated control (Fig. 4B). The appearance of 'new' cleavage sites following treatment with an inhibitor has been observed previously [62]. These sites were not detected in the DMSO-treated control assay because the peptide was rapidly degraded into tri-, tetra- and pepta-peptides by the serine peptidase(s) and therefore any subsequent cleavage sites would not be readily detected by mass spectrometry. Finally, treatment with 1,10-Phenanthroline caused a delay in the processing of the Tyr–Arg bond from 60 to 240 min but otherwise the cleavage sites remained unchanged.

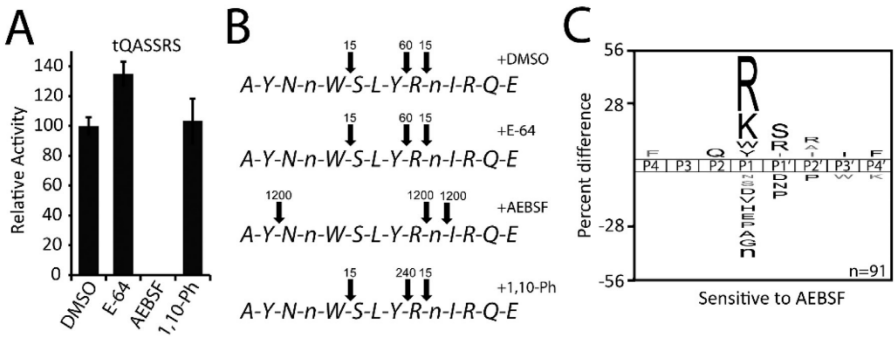

Fig. 4. Use of class-specific inhibitors to characterize peptidase activity and specificity in schistosomula secretion. A. ES products were assayed with the internally quenched tQASSRS substrate in the presence of class-specific inhibitors. B. Use of a sample 14-mer peptide to illustrate the time dependent changes in cleavage site accumulation in the presence of class-specific inhibitors compared to a non-inhibited DMSO control. C. An iceLogo substrate profile generated from all cleavage sites in the 14-mer peptide library that were sensitive to AEBSF.

Treatment of schistosomula ES products with AEBSF resulted in the delay in appearance of 91 of the 107 cleavage sites that were detected in the DMSO-treated control. These sites were termed "sensitive to AEBSF" and frequently had Arg and Lys in the P1 position (Fig. 4C). Based on this substrate profile it is reasonable to suggest that a single major peptidase with trypsin-type specificity is present in the ES products of schistosomula.

3.4.2. Adults

Treatment of adult ES products with either E-64 or AEBSF resulted in a 19% and 35% decrease in the overall cleavage of tQASSRS, respectively (Fig. 5A). However, when these compounds were combined, E-64 provided no additional inhibition, indicating that this inhibitor may be targeting the serine peptidase(s). Competitive inhibition of bovine trypsin by E-64 has been previously reported [74]. 1,10-Phenanthroline decreased activity by more than 80%, indicating that metallo-peptidases predominate in adult ES products under the conditions employed. Combining 1,10-Phenanthroline with E-64 and AEBSF completely inhibited cleavage of the tQASSRS substrate (Fig. 5A).

When AEBSF was added to the MSP-MS assay and compared to a DMSO-treated control, 59 of 151 cleavage sites were sensitive to this inhibitor (Fig. 5B). The specificity profile of the AEBSF-sensitive enzymes had a preference for Arg at P1, but a much lower preference for Lys compared to the schistosomula ES peptidase. This indicates that it is unlikely that the same serine peptidases are being measured in ES products of schistosomula and adults.

When the adult ES products were treated with 1,10-Phenanthroline, 107 of the total of 151 cleavage sites in the 14-mer peptide library were sensitive to this agent (Fig. 5C). The substrate specificity profile of the 1,10-Phenanthroline-sensitive enzymes had a preference for bulky residues at P3, Leu at P1 and Arg, Phe and Ser at P1′. In addition, Gln and Lys were most often found at P2′ whereas Arg, Ala and Tyr were frequently identified at P3′.

Interestingly, all cleavage sites that occurred between the 2nd and 3rd position of each 14-mer peptide were sensitive to 1,10-Phenanthroline but only two were sensitive to AEBSF (Fig. 5D). In some cases, AEBSF treatment resulted in an earlier appearance of the cleavage product, when compared to the DMSO control. For example, removal of AY from the sample tetradacapeptide, *AYNnWSLYRnIRQE* occurs after 240 min incubation in the DMSO-treated assay, but the dodecapeptide cleavage product can be detected after only 15 min incubation in the AEBSF treated assay. Thus, in addition to the metallo—endopeptidase activity sensitive to 1,10-Phenanthroline, there is a least one other metallo-peptidase in the ES products with a specificity for amino-terminal dipeptides.

3.4.3. Eggs

Treatment of egg ES products with AEBSF completely inhibited cleavage of tQASSRS indicating that serine peptidase activity is a major proteolytic component (Fig. 6A). Cysteine and metallo-peptidases are also present as E-64 and 1,10-Phenanthroline decreased activity by 61% and 15%, respectively. A substrate specificity profile was generated for both the AEBSF-sensitive (Fig. 6B) and E-64-sensitive (Fig. 6C) cleavage sites. Cleavage sites that were sensitive to AEBSF had a trypsin-like substrate specificity profile using the MSP-MS assay. Interestingly, the cleavage sites that were sensitive to E-64 also had a strong P1 preference for Arg and Gln and generally accepted hydrophobic residues at P2, consistent with that known for cysteine cathepsins [75–77].

The MSP-MS assay on the egg ES products indicated that not all of the cleavage sites were sensitive to either AEBSF or E-64 treatment. Indeed, 61 of the original 145 cleavages identified in the control-treated assay were resistant to both inhibitors suggesting that enzymes other than serine or cysteine peptidases are also present (Fig. 6D). To further probe the egg-conditioned media we utilized the lQ substrate, GRFGVWKA, identified in Fig. 3. This substrate was also cleaved by peptidases in the conditioned media from all developmental stages tested, although with lower activity than the tQASSRS substrate. Using the GRFGVWKA substrate, AEBSF treatment only caused a 35% decrease in overall activity, whereas 1,10-Phenanthroline abolished activity (Fig. 6E). Using these same conditions, the 1,10-Phenanthroline-treated ES products were interrogated with the MSP-MS assay. The resulting sensitive cleavage sites had a preference for Trp, Ile, Ser and Arg at P4, P3, P2 and P1, respectively. In addition, a P1′ preference for Arg was evident. Notably, this 1,10-Phenathroline sensitive peptidase differs in specificity from the metallo-peptidases in adults. Taken together, these studies indicate that serine, cysteine and metallo-peptidases are all active in the ES products of *S. mansoni* eggs.

4. Discussion

Understanding the biological function of a peptidase in any organism, including *Schistosoma*, requires orthogonal data inputs, including gene expression, protein localization, post-translational modifications and substrate specificity. Global 'omic' analyses

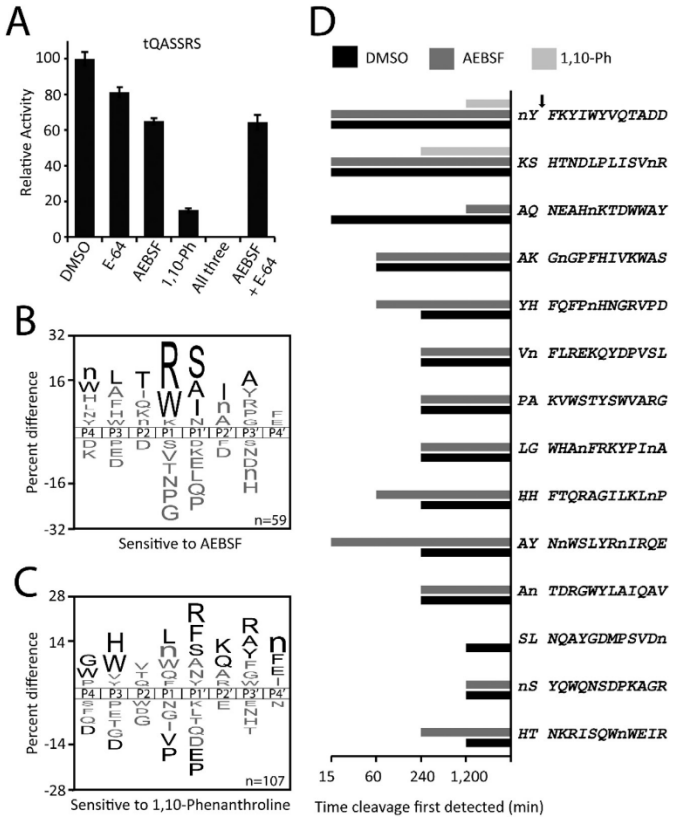

Fig. 5. Use of class-specific inhibitors to characterize peptidase activity and specificity in adult secretion. A. ES products were assayed with the internally quenched tQASSRS substrate in the presence of class-specific inhibitors. B. An iceLogo substrate profile consisting of all cleavage sites in 14-mer peptides that were sensitive to AEBSF treatment. C. An iceLogo substrate profile consisting of all cleavage sites in 14-mer peptides that were sensitive to 1,10-Phenanthroline treatment. D. Analysis of the di-aminopeptidase specificity detected in adult ES products. The bar graph represents the time interval at which each the cleavage product was first detected.

such as those have made key contributions in these respects. To date, however, there has been no global *functional* profiling (qualitative nor quantitative) of the peptidases across developmental stages. Our employment of peptidase-agnostic technologies in combination with class-specific peptidase inhibitors reveals previously unrecognized activities that are released by key schistosome developmental stages that parasitize the human host. The technologies applied to schistosomula, adults and eggs have been previously utilized in a number of different contexts [61,62] including to characterize *S. mansoni* cercarial secretions [63]. The present report is, therefore, a natural extension of that work. We employed ES products as these are likely to contain peptidases operating at the host parasite interface and that potentially influence host immunology and physiology. Finally, ES products were collected and processed at neutral (physiologic) pH to inactivate or at least mitigate the proteolytic contributions of aspartic and cysteine proteases [39,78,79], not least those adult gut proteases that have been extensively characterized [11,13,29,80,81] and are regurgitated by the worm into culture medium [11,78,82,83]. Thus, our conditions facilitate the identification of novel peptidolytic activities, specifically, metallo- and serine proteases.

We utilized two IQ substrates as reporters of activity and inhibitor sensitivity. The cleavage sites within these substrates were not investigated as the specificity profile was revealed in the subsequent MSP-MS assays. The proteolytic profile of schistosomular ES products is the simplest measured and centers on a single or predominant serine peptidase with a strict P1− P1′ specificity for charged amino acids and only minor amino acid engagements at the other prime and non-prime binding sites. The activity may be due to one or more of a number of schistosomular serine peptidases we previously measured by both gene expression profiling and functional activity analyses with small peptidyl substrates [19]. Importantly, the specificity profile of this serine peptidase activity differs markedly from the chymotrypsin-like cercarial elastase that is secreted by the *S. mansoni* cercariae and from which we derive schistosomula [63,70].

In contrast to schistosomula, the specificity profiles of adults and eggs are much more complex and different from one another with respect to the proteolytic contributions of serine, metallo- and cysteine proteases. Adults produce a serine peptidase that has a strong preference for P1-Arg but not Lys. Generally, trypsin-like serine peptidases cleave peptides with these P1-amino acids equally well. These data support our previous finding that a number of different trypsin peptidases are expressed in adult *S. mansoni*

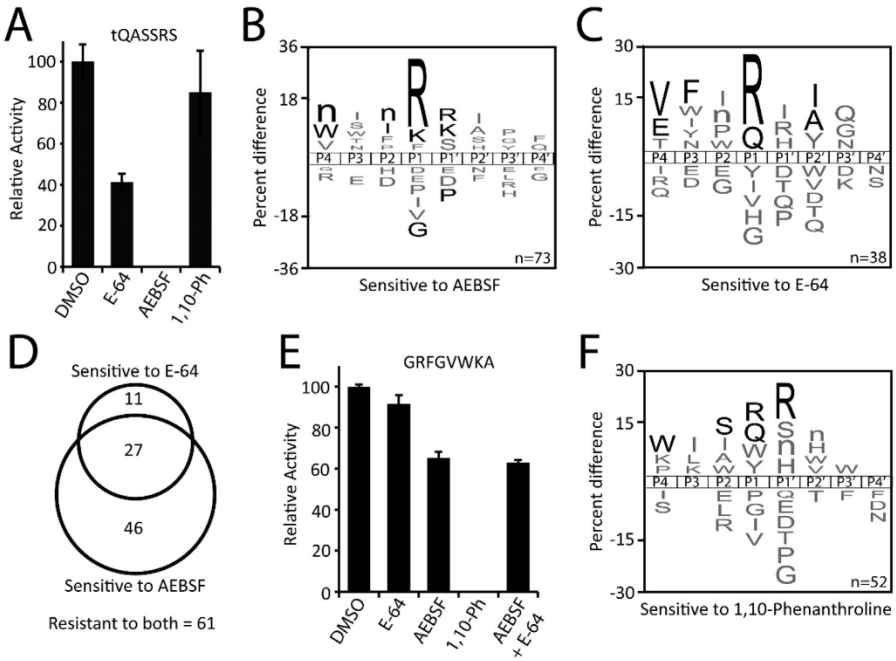

Fig. 6. Use of class-specific inhibitors to characterize peptidase activity and specificity in egg secretion A. ES products were assayed with the internally quenched tQASSRS substrate in the presence of class-specific inhibitors. B. An iceLogo substrate profile consisting of all cleavage sites in the 14-mer peptides that were sensitive to AEBSF treatment. C. An iceLogo substrate profile consisting of all cleavage sites in the 14-mer peptides that were sensitive to E-64 treatment. D. A Venn diagram showing that many of the cleavage sites are resistant to both AEBSF and E-64. E. ES products were assayed with the internally quenched GRFGVWKA substrate in the presence of class-specific inhibitors. F. An iceLogo substrate profile consisting of all cleavage sites in the 14-mer peptides that were sensitive to 1,10-Phenanthroline treatment.

[19]. In addition, a second serine peptidase activity is present that has chymotrypsin-like P1 specificity for Trp. However, this enzyme has little preference for P1-Tyr, -Phe and -Leu which are normally associated with chymotrypsin. Adults also produce at least two metallo-peptidases, one of which is a metallo di-aminopeptidase activity, thus underscoring the utility of the MSP-MS approach to not only detect endopeptidase activity but also exopeptidase activity. Neither of the metallo-peptidase specificity profiles identified are similar to those recorded for a M17 family leucine aminopeptidase activity previously characterized in *S. mansoni* [84,85].

Like adult ES products, those from eggs contain serine and metallo-peptidase activity profiles, however, these differ from the adult profiles and from each other suggesting that different enzymes are being measured. It's possible that the serine peptidase activity may be in part due to a PMSF-sensitive fibrinolytic activity previously described in egg extracts [18] although no protein identification for this activity was subsequently carried out. Regarding the metallo–endopeptidase activity, we are not aware of such being described in egg ES products but it's clear that a major 1,10-Phenanthroline-sensitive activity is present. Interestingly, egg conditioned medium also contains a cysteine protease activity that is robustly active under the neutral pH conditions employed. This is in contrast to adult ES products that predominantly contain neutral pH-labile cathepsins that arise from the gastrointestinal tract [78]. The presence of cysteine protease activity has been described in eggs [86,87] and miracidia (which eventually emerge from the egg) [88]. Peptidase activity has also been measured at neutral pH in egg ES products [89] and live eggs were shown to degrade the glycoprotein component of an artificial extracellular matrix at neutral pH. This activity was enhanced in the presence of reducing agent suggesting a role for cysteine peptidases [90]. Accordingly, it's possible that eggs release a specialized cathepsin–like activity that facilitates their passage through host tissues and their eventual escape into the environment via the urogenital or digestive tracts.

Our application of peptidase-agnostic technologies in combination with peptidase class-specific inhibitors reveals the heretofore unrecognized complexity of peptidolytic activities released by key schistosome life-stages parasitizing the human host. Further biochemical studies are required to identify the peptidases responsible but there are now well-annotated genomic and transcriptomic data (references cited in the Introduction) that can be brought to bear in such studies. Aided by knowledge of both substrate specificity and peptidase class, we can now develop tools to characterize the individual enzymes in greater detail, *e.g.*, activity-based probes that bind to the peptidase active site. Such probes require knowledge of both the substrate recognition sequence and the nature of the active-site nucleophile, and have been previously engineered to identify and characterize peptidases, and image disease as biomarkers [91–93].

5. Conclusion

This study describes the application of an unbiased and global technology to characterize *S. mansoni* excretion-secretion peptidase activity. Each intra-mammalian developmental stage

produces a different set of activities and all stages except cercariae secrete a trypsin-type serine peptidase. In addition, a metallopeptidase with dipeptidase specificity was observed in adult ES products and eggs release a cysteine protease that is active at neutral pH. These studies will facilitate the development of selective active-site directed affinity probes.

Acknowledgments

Research was funded by the University of California, San Francisco Program for Breakthrough Biomedical Research and the Sandler Foundation (AJO and CSC). JD, AL, MH and LU were supported by the grant P302/11/1481 from the Czech Science Foundation. JD was supported by a FP7 Marie Curie International Reintegration Grant 248642. MH, MM, AL and PF are supported by the project InterBioMed LO1302 from the Ministry of Education, Youth and Sports of the Czech Republic and by the institutional project RVO 61388963. CRC was supported in part by NIH-NIAID grants R01AI089896 and R21AI107390. We thank K.C. Lim for his help with the *S. mansoni* life cycle and Chris Franklin for the life cycle illustration. MS analysis was performed in the Bio-Organic Biomedical Mass Spectrometry Resource at University of California, San Francisco (A. L. Burlingame, Director), supported by the Biomedical Technology Research Centers program, National Institutes of Health, National Institute of General Medical Sciences 8P41GM103481.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2015.09.025.

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4.4 Publication No. 3: Trypsin- and chymotrypsin-like serine proteases in *Schistosoma mansoni* - 'the undiscovered country'.

Significant serine protease activity was found in ES products of all developmental stages of *S. mansoni* parasitizing humans (publication No. 2). According to the MEROPS database [180], the most abundant family of serine proteases is the S1 family of chymotrypsin. Therefore, publication No. 2 was dedicated to the identification, annotation, expression and phylogenetic analysis, and characterization of the activities of the *S. mansoni* S1 family serine proteases (SmSPs).

Five different genes encoding SmSPs, designated SmSP1 to SmSP5, were identified and annotated by bioinformatic analysis of the *S. mansoni* genome using BLAST against the sequences of vertebrate S1 family proteases. Individual SmSPs are distinct molecules with the same S1 family type catalytic protease domain at the C-terminus, but with a different domain structure at the N-terminus. qRT-PCR analysis revealed complex expression patterns for SmSPs in different schistosome developmental stages (eggs, miracidia, daughter sporocysts, cercariae, schistosomula, and adult worms). SmSP2 stood out as being massively expressed in schistosomula and adult stages. Phylogenetic analyses separated SmSPs into distinct clusters of family S1 proteases. Based on bioinformatics and cleavage preferences, SmSP1 to SmSP4 are trypsin-like proteases (with a preference for basic residues at the P1 subsite of the active site), whereas SmSP5 is chymotrypsin-like (with a P1 preference for bulky hydrophobic residues). Consistent with the number of trypsin-like sequences in all life stages examined, significant trypsin-like activity was detected using a panel of peptidyl fluorogenic substrates in protein homogenates and ES products from eggs, schistosomula and adults.

Our findings in this work provided a basis for further exploration of the functions of the individual SmSPs, including their potential contributions to influencing host physiology.

PhD applicant contribution: Implementation and optimization of the protocol for ES products collection and analysis, preparation and cultivation of the *S. mansoni* developmental stages, collection and preparation of their ES products for further analysis.

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Trypsin- and Chymotrypsin-Like Serine Proteases in *Schistosoma mansoni* – 'The Undiscovered Country'

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Abstract

Background: Blood flukes (*Schistosoma* spp.) are parasites that can survive for years or decades in the vasculature of permissive mammalian hosts, including humans. Proteolytic enzymes (proteases) are crucial for successful parasitism, including aspects of invasion, maturation and reproduction. Most attention has focused on the 'cercarial elastase' serine proteases that facilitate skin invasion by infective schistosome larvae, and the cysteine and aspartic proteases that worms use to digest the blood meal. Apart from the cercarial elastases, information regarding other *S. mansoni* serine proteases (SmSPs) is limited. To address this, we investigated SmSPs using genomic, transcriptomic, phylogenetic and functional proteomic approaches.

Methodology/Principal Findings: Genes encoding five distinct SmSPs, termed SmSP1 - SmSP5, some of which comprise disparate protein domains, were retrieved from the *S. mansoni* genome database and annotated. Reverse transcription quantitative PCR (RT- qPCR) in various schistosome developmental stages indicated complex expression patterns for SmSPs, including their constituent protein domains. SmSP2 stood apart as being massively expressed in schistosomula and adult stages. Phylogenetic analysis segregated SmSPs into diverse clusters of family S1 proteases. SmSP1 to SmSP4 are trypsin-like proteases, whereas SmSP5 is chymotrypsin-like. In agreement, trypsin-like activities were shown to predominate in eggs, schistosomula and adults using peptidyl fluorogenic substrates. SmSP5 is particularly novel in the phylogenetics of family S1 schistosome proteases, as it is part of a cluster of sequences that fill a gap between the highly divergent cercarial elastases and other family S1 proteases.

Conclusions/Significance: Our series of post-genomics analyses clarifies the complexity of schistosome family S1 serine proteases and highlights their interrelationships, including the cercarial elastases and, not least, the identification of a 'missing-link' protease cluster, represented by SmSP5. A framework is now in place to guide the characterization of individual proteases, their stage-specific expression and their contributions to parasitism, in particular, their possible modulation of host physiology.

Citation: Horn M, Fajtová P, Rojo Arreola L, Ulrychová L, Bartošová-Sojková P, et al. (2014) Trypsin- and Chymotrypsin-Like Serine Proteases in *Schistosoma mansoni* – 'The Undiscovered Country'. PLoS Negl Trop Dis 8(3): e2766. doi:10.1371/journal.pntd.0002766

Editor: Robin B. Gasser, University of Melbourne, Australia

Received December 16, 2013; **Accepted** February 16, 2014; **Published** March 27, 2014

Funding: JD and MH were jointly supported by Grant P302/11/1481 from Grant Agency of the Czech Republic. JD was supported by a FP7 Marie Curie - International Re-integration Grant (248642) and by a Czech Ministry of Education, Youth and Sports grant KONTAKT (ME10011). Institute of Molecular Genetics of the Academy of Sciences of the Czech Republic and the Institute of Organic Chemistry and Biochemistry of the Academy of Sciences of the Czech Republic were supported by RVO 68378050, and RVO 61388963, respectively. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Schistosomiasis caused by *Schistosoma* blood flukes is a chronic disease with more than 200 million people infected [1]. Schistosome larvae (cercariae), released into an aquatic environment from snail intermediate hosts, penetrate human skin and subsequently develop into adult worms. Adult worms reside in the host vascular system as male/female pairs, and survive for many years, if not decades [2], producing hundreds of eggs per day. Morbidity arises from the host immune responses to eggs in tissues [3]. Treatment relies on one drug, praziquantel, and no effective vaccine has yet been developed [4]. During its complex life cycle, the parasite survives in various environments by presenting or releasing bioactive molecules that aid survival and modulate host physiology [5,6]. Disruption of these potential mechanisms by specific drugs/vaccines may provide therapeutic benefits.

Proteolysis is a fundamental physiologic process [7,8]. Proteases (proteolytic enzymes) are crucial to parasitism, including by

Author Summary

Schistosomes are blood flukes that live in the blood system and cause chronic and debilitating infection in hundreds of millions of people. Proteolytic enzymes (proteases) produced by the parasite allow it to survive and reproduce. We focused on understanding the repertoire of trypsin- and chymotrypsin-like *Schistosoma mansoni* serine proteases (SmSPs) using a variety of genomic, bioinformatics, RNA- and protein-based techniques. We identified five SmSPs that are produced at different stages of the parasite's development. Based on bioinformatics and cleavage preferences for small peptide substrates, SmSP1 to SmSP4 are trypsin-like, whereas SmSP5 is chymotrypsin-like. Interestingly, SmSP5 forms part of a 'missing link' group of enzymes between the specialized chymotrypsin-like 'cercarial elastases' that help the parasite invade human skin and the more typical chymotrypsins and trypsins found in the nature. Our findings form a basis for further exploration of the functions of the individual enzymes, including their possible contributions to influencing host physiology.

schistosomes, in facilitating invasion, nutrient intake, hatching, excystment, immune evasion [9,10] and modulation of host physiology [10–15]. Most schistosome research has focused either on cysteine and aspartic proteases (MEROPS database Clans CA and AA, respectively [8]), which are responsible for digesting the blood meal [16,17] or on the serine proteases (SPs), known as cercarial elastases (CEs; Clan PA, family S1) that facilitate active penetration of the mammalian host [18–20].

Regarding the nomenclature for eukaryotic SPs, whereas members of the S1 or 'chymotrypsin' family of SPs share a similar tertiary structure, their substrate cleavage specificities differ [8]. Thus, substrate preferences at the P1 subsite [21] may be divided into trypsin-like (P1 preference for basic residues), chymotrypsin-like (bulky hydrophobic residues) and elastase-like (small aliphatic residues) [7].

Despite their name, which was derived from their ability to cleave insoluble elastin, the *S. mansoni* CEs have a chymotrypsin-like P1 specificity [22] due to preferences for phenylalanine and leucine. In contrast to these well-studied CEs [18–20], there are fewer descriptions of 'non-CE' Clan PA, family S1 serine proteases in *S. mansoni* (SmSPs) [6,12–15,23,24].

Among these, SmSP1 (*S. mansoni* serine protease 1, GenBank AJ011561), has been partially described [13,14]. The open reading frame (ORF) of SmSP1 comprises two non-proteolytic domains, followed by a C-terminal trypsin protease domain. Expression of the trypsin domain (mRNA and protein) was noted in adult worms with a significant accumulation in the tegument (surface) of males [13]. Another SmSP was identified (under TC16843 code) by microarray analysis with a remarkably elevated expression in post-infective larvae (schistosomula) that had been maintained *in vitro* [23]. Two additional biochemical studies support a function for schistosome SPs in modulating host physiology. Specifically, a protein fraction of *S. mansoni* adult worm extracts was shown to possess kallikrein-like protease activity [12]. The isolated native enzyme, termed sK1, cleaved kallikrein substrates and processed kininogen to bradykinin which induced strong vasodilatation and decreased arterial blood pressure in experimental rats; sK1 was found in higher abundance in males [12]. Both, sK1 and SmSP1, are proposed to regulate host vascular functions [6]. In the second study, SP activity in extracts of *S. mansoni* eggs induced significant fibrinolytic activity and was associated with a 27 kDa protein [15]. This protease activity had a similar cleavage pattern to human plasmin and it was hypothesized that the enzyme blocks the intravascular deposition of fibrin by platelets activated by schistosome eggs [15].

In the present study, we sought to understand the gene repertoire of non-cercarial elastase SmSPs by employing a series of genomic, transcriptomic, proteolytic and phylogenetic approaches. In addition to SmSP1, we identified and re-annotated four distinct SmSPs in the *S. mansoni* GeneDB genome database [25,26] and term them SmSP2 through SmSP5 according to a previous terminology [13]. The data reveal intriguing expression profiles and phylogenetic relationships that stimulate further study of the individual proteases involved, and their contributions to modulating host physiology.

Materials and Methods

Ethics statement

Mice are kept in the animal facility of the Biology Center (Academy of Sciences of the Czech Republic) in Ceske Budejovice and all animal experiments are carried out as approved by the Animal Rights Ethics Committee under protocol no. 068/2010 issued according to the national regulation 246/1992 Sb.

Schistosome material

A Liberian isolate of *S. mansoni* has been maintained in the laboratory by cycling between CD-1 mice and the freshwater snail, *Biomphalaria glabrata*. Mice were subcutaneously injected with 200 cercariae and sacrificed 6–7 weeks post-infection by intraperitoneal injection of thiopental (50 mg/kg). Adults, eggs and miracidia were isolated as described previously [27]. Cercariae were obtained from infected snails induced to release the parasite under a light stimulus. Cercariae were chilled on ice, collected and transformed mechanically to schistosomula [27,28], which were then cultured for five days under a 5% CO_2 atmosphere at 37°C in Basch Medium 169 [29] containing 5% fetal calf serum and 1% ABAM (antibiotics/antimycotics; Sigma-Aldrich). Daughter sporocyst material was isolated by excision of the hepato-pancreases from two month-infected *B. glabrata* snails. The hepato-pancreases from uninfected snails were used as a negative control when evaluation gene expression.

Isolation of mRNA and cDNA synthesis

Adult worms, eggs, miracidia, daughter sporocysts, cercariae and schistosomula were re-suspended in 500 µl of Trizol reagent (Life Sciences) and processed [30]. Single-stranded cDNA was synthesized from total RNA by SuperScript II reverse transcriptase (Life Sciences) and an oligo dT_{18} primer, and then stored at −20°C.

Gene annotation, domain expression evaluation and sequencing

Genes encoding complete SmSPs or their specific domains were retrieved from the *S. mansoni* genome database (*S. mansoni* GeneDB, available at http://www.genedb.org/Homepage/Smansoni) through BLAST searches. Amino acid sequences of vertebrate family S1 SPs were used as queries. Specific PCR primers were employed to amplify each of the sequences retrieved, and the respective amplicons cloned into the TOPO TA 2.1 vector (Life Technologies) for propagation in TOP10 *E. coli* cells. For SmSP4 and SmSP5, full-length sequences were obtained by 5′ and 3′ RACE (Rapid Amplification of cDNA Ends, Life Technologies).

Based on more recent annotations, the original sequence information for SmSP4 and SmSP5 (GenBank XM_002572739

and XM_002574902) were corrected in the *S. mansoni* GeneDB database. All newly described SmSP sequences were deposited in GenBank under the accession numbers listed in Table 1. For genes with multi-domain structures, PCR analysis was performed using domain-specific primers in order to detect possible differential expression.

Evaluation of gene expressions by RT-qPCR analysis

Gene expression of the SmSPs was assessed using RT-qPCR. For genes with multi-domain structures (SmSP1 and SmSP3), the expression levels of individual domains were evaluated separately. cDNA for various life stages was generated using the mRNA isolation protocol described above and previously [30]. For mRNA isolation, 3 infected *B. glabrata* hepatopancreases and approximately 20 adult pairs, 500 hundred eggs, cercariae and schistosomula were used. Primers for quantitative PCR analysis were designed using the Primer 3 software (http://frodo.wi.mit.edu/ [31],), in order to amplify 150–250 bp regions of the targeted genes or their domains. Primer efficiency was evaluated by serial dilutions of both the primers and the cDNA template as described [32,33]. Two to three primer pairs were generated per target from which one primer set with optimal efficiency and generating only a single dissociation peak was used (see Supporting Information Table S1).

Reactions, containing SYBR Green I Mastermix (Eurogentech), were prepared in final volumes of 25 µL in 96-well plates [30]. The amplification profile consisted of an initial hot start (95°C for 10 min), followed by 40 cycles comprising 95°C for 30 s, 55°C for 60 s and 72°C for 60 s, and ended with a single cycle of 95°C for 60 s, 55°C for 30 s and 95°C for 30 s. PCR reactions were performed in duplicate for each cDNA sample. At least one biological replicate, i.e., samples from a different RNA isolation was performed for each gene target. Analysis of the cycle threshold (C_T) for each target was carried out as described [30] and employed *S. mansoni* cytochrome C oxidase I (SmCOX I, GenBank AF216698, [33]) as the sample normalizing gene transcript [27]. Finally, the resulting transcript values were calculated as a percentage of the expression of the normalizing gene (SmCOX I) which was set as 100%. Transcript levels were expressed as log functions and as a percentage relative to that of SmCOX I in order to compare variable expression patterns. The threshold for significance of expression was set to 0.01% of the expression of SmCOX I.

Phylogenetic analyses of SmSPs

The amino acid sequences of 96 vertebrate and invertebrate members of the S1 serine protease family were aligned in MAFFT [34] using the E-INS-i method, and gap opening (–op) and extension penalties (–ep) of 5.0 and 0.0, respectively. The non-catalytic domains and N-terminal extensions were excluded from the resulting alignment in BioEdit (v7.0.5.2; [35]). The bacterial trypsin from *Streptomyces griseus* was used as an outgroup. The list of family S1 proteases (SPs sequences) used for the phylogenetic analysis is in the Supplementary Table S2. The Maximum Parsimony analysis was performed in PAUP* (v4.b10; [36]), using a heuristic search with random taxa addition, the ACCTRAN option, and the TBR swapping algorithm. All characters were treated as unordered whereas gaps were treated as missing data. Maximum Likelihood analysis was performed in RAxML under the WAG model [37]. Clade support values were calculated from 1000 bootstrap replicates with random sequence additions for both analyses. All trees were displayed using the TreeView32 program [38].

Table 1. List of studied serine proteases and their accession numbers.

Name	SchistoDB	GenBank
SmSP1	Smp_030350	KF535923
SmSP2	Smp_002150	KF510120
SmSP3	Smp_103680	KF510121
SmSP4	Smp_129230	KF510122
SmSP5	Smp_141450	KF939306

doi:10.1371/journal.pntd.0002766.t001

Collection of E/S products and soluble protein extracts

Fifty pairs of adult worms, 1 000 eggs or 1 000 schistosomula were washed five times in Basch Medium 169 containing 1% Fungizone (Gibco) and allowed to stand for 1 h at 37°C in 5% CO_2. Samples were washed 10 times and then incubated in the same Basch Medium overnight (adults and eggs) or for five days (schistosomula) at 37°C in 5% CO_2. Parasite material was then washed 10 times in M-199 medium (alternative medium for schistosoma cultivation without serum and proteins,Gibco) containing 1% ABAM and incubated in the same medium for 16 h at 37°C in 5% CO_2. Medium containing E/S products was removed and filtered using an Ultrafree-MC 0.22 µm filter (Millipore). Filtered medium was buffer exchanged into ice-cold 1× PBS (pH 7.4) and concentrated at 4°C to a 2 ml final volume by centrifugation at 4000 *g* using an Amicon 10000 Ultra-15 Centrifugal Filter Unit (Millipore). The total volume of PBS used for buffer exchange was 40 ml. Samples (0.04–0.37 mg protein/ml) were frozen in liquid nitrogen and stored at −80°C.

Soluble protein extracts (1–5 mg protein/ml) from *S. mansoni* adults, eggs and 5 day-old schistosomula were prepared by homogenization in 50 mM Tris-HCl buffer, pH 8.0, containing 1% CHAPS, 1 mM EDTA and 10 µM of the cysteine protease inhibitor, E-64, in an ice bath. The extracts were cleared by centrifugation (16,000 *g*, 10 min, 4°C), filtered with an Ultrafree-MC 0.22 µm and stored at −80°C.

Proteolytic activity measurement

Proteolytic activities were measured in a kinetic continuous assay using the following peptidyl fluorogenic, 7-amino-4-methylcoumarin (AMC) substrates (Bachem) at a 50 µM final concentration: Z-F-R-AMC (Z, Benzyloxycarbonyl), Bz-F-V-R-AMC (Bz, Benzoyl), Z-G-P-R-AMC, P-F-R-AMC, Boc-L-R-R-AMC (Boc, t-Butyloxycarbonyl), Boc-Q-A-R-AMC, Boc-V-L-K-AMC Suc-A-A-F-AMC (Suc, Succinyl), Suc-A-A-P-F-AMC, Suc-L-Y-AMC, MeOSuc-A-A-P-V-AMC (MeOSuc, 3-Methoxysuccinyl), Z-G-G-L-AMC and Z-V-K-M-AMC. Assays were performed at 37°C in 96-well black microplates in a total volume of 100 µl. Parasite extracts (1–3 µg) or E/S products (0.05–1 µg) were pre-incubated for 10 min in 150 mM Tris-HCl, pH 8.0, containing 10 µM E64, 1 mM EDTA in the presence or absence of 0.5 mM of the serine protease inhibitors, Pefabloc SC and PMSF. E64 was included routinely in extract preparations in order to inhibit Clan CA cysteine protease activity that is present in the life-stages examined [30,39,40]. Hydrolysis of substrate was measured continuously using an Infinite M1000 microplate reader (Tecan) at excitation and emission wavelengths of 360 and 465 nm, respectively. All measurements were performed in triplicate and results normalized to protein concentration.

Molecular modeling

A spatial model of SmSP1 was constructed using the template X-ray structure of bovine trypsin in complex with the peptidyl inhibitor leupeptin (PDB entry 1JRT) and utilizing a pairwise sequence alignment generated by the BLAST program (BLOSUM62 substitution matrix). The homology module of the MOE program was used for modeling the SmSP1 structure (MOE: Chemical Computing Group; http://www.chemcomp.com). The conformation of leupeptin was refined by applying the LigX module of the MOE. The final binding mode of the inhibitor was selected by the best fit model based on the London dG scoring function and the generalized Born method [41]. Molecular images were generated with UCSF Chimera (http://www.cgl.ucsf.edu/chimera/). The electrostatic surface potential was calculated using the APBS software [42] and input data were prepared using PDB2PQR [43].

Results

Gene annotation and sequence analysis reveals complex domain organizations for some SmSPs

Genes were selected *in silico* based on a proteolytic domain organization that matched with family S1 serine proteases: cercarial elastases were excluded because of their detailed studies previously [20,22]. The five remaining SmSP genes, including the previously sequenced and partially characterized SmSP1 [13,14], were cloned and sequenced. The other four gene sequences named SmSP2 through SmSP5 (Table 1) were significantly corrected and re-annotated in the primary database (*S. mansoni* GeneDB) due to various sequence inaccuracies. The sequences of SmSP2 through SmSP5 were deposited into the GenBank as KF510120, KF510121, KF510122, KF939306, respectively. The sequence of SmSP1 defined here was also deposited (KF535923) because of sequence differences from the original description (CAA09691 [13]) and from the information in *S. mansoni* GeneDB (Smp_030350; Figure S1). A search of the *Schistosoma japonicum* genome [44] indicates that orthologs for each of the SmSPs are present; SjSP1 (GeneDB Sjp_0012180, GenBank N/A), SjSP2 (Sjp_0100980, CAX74751), SjSP3 (Sjp_0023390, CAX73257), SjSP4 (Sjp_0047680, N/A) and SjSP5 (Sjp_0114710, CAX73292).

The sequence domain organization for the particular proteases is represented in Figure 1. Based on sequence homology analysis, we describe SmSP1 as a multi-domain protein comprising a matriptase-like structure made up of **C**omplement-**U**egf-**B**MP-1 (CUB) extracellular and plasma membrane-associated domains, a LDL-binding receptor domain class A (LDLa domain) and a S1 family serine protease domain. However, the full gene product has been detected only in the eggs, whereas in other parasite stages, the CUB and protease domains are expressed as separate spliced products, as demonstrated by PCR and sequencing (Figure S2).

Primary sequence homology analysis shows that SmSP2 to SmSP5 are distinct molecules with the same family S1 type catalytic protease domain at the C-terminus, but with different N-terminal extensions which include a potential pro-peptide, i.e., a peptide that is removed during zymogen activation. The N-terminal extensions vary from 201 residues in SmSP2 to just a seven residues in SmSP5 (Figure 1). SmSP1, SmSP3 and SmSP5 do not contain a predicted signal sequence for the secretory pathway as identified by the SignalP program [45]. In contrast, SmSP2 and SmSP4 are synthesized as pre-pro-proteins with a typical N-terminal signal peptide preceding an N-terminal extension region containing a putative pro-peptide ('activation peptide') that is then followed by the protease domain (Figure 1). The pro-peptide is separated from the protease domain of SmSPs by a basic residue, Arg or Lys (Figure 2) which constitutes a potential activating cleavage site, i.e., is hydrolyzed during protease maturation as is known for other S1 family proteases [7]. For SmSP3, the N-terminal extension contains an incomplete CUB domain. PCR and sequencing revealed that, as found for SmSP1, the CUB and the protease domains of SmSP3 are only co-expressed in eggs whereas they are separate spliced gene products in the other stages (Figure S2). SmSP5 contains a Thr/Asn rich C-terminal sequence extension not present in orthologous SPs from other trematodes (Figure S4).

The catalytic protease domains of SmSP1 to SmSP4 share significantly greater sequence identity (about 30%) with each other than with SmSP5 (about 20%; Figure S3). All five SmSPs have a catalytic triad in the order of His, Asp and Ser that is typical for S1 family proteases; also, the regions surrounding the catalytic triad residues have the most notable sequence identity (Figure 2). The protease domains of SmSP1 to SmSP4 contain cysteine residues at positions 28, 44, 130, 160, 173, 184, 194, and 212 (SmSP1 protease domain numbering), which are conserved in other trypsin-like proteases. They form four disulfide bonds that can be predicted from the alignment with the crystal structures of bovine trypsin and bovine chymotrypsin (Figure 2). Moreover, the protease domain of SmSP2 through SmSP4 contains an additional cysteine residue, Cys112. By comparison with bovine chymotrypsin, this residue in SmSP2 and SmSP3 is likely to form a disulfide bond with a Cys in the N-terminal extension region (at the positions -p13 and -p9, respectively), whereas in SmSP4 a similar Cys in the N-terminal extension region is lacking (Figure 2).

SmSP5 diverges from the other four SPs in that it contains only six cysteine residues that likely form three disulfide bonds. The first two bonds, Cys28-Cys44 and Cys160-Cys173, are identical to those in trypsin, chymotrypsin and other SmSPs. The remaining cysteine residues (Cys46 and Cys72) are absent, but correspond to Cys46 and Cys77 in SmCE that were predicted to form a disulfide bond by homology modeling [46] (Figure 2). Moreover, both SmSP5 and SmCEs lack the disulfides Cys130-Cys194 and Cys184-Cys212, which are conserved in SmSP1 to SmSP4. Taken together, SmSP5 clearly differs in its disulfide pattern from the other investigated SmSPs. This close structural relationship between SmSP5 and the SmCEs is confirmed for the other analyses performed (see below). In addition, two other splice variants of SmSP5 were detected. Compared to the full-length SmSP5, both are C-terminally truncated and one is missing the crucial His residue from the catalytic triad (Figure S4).

Asp182 determines the trypsin-like specificity of serine proteases for substrates with Arg/Lys in the P1 position [47], and this residue is conserved in all of the SmSPs except SmSP5 (Figure 2), which has Gly. Therefore, it might be the case that SmSP5 displays a substrate specificity similar to that of chymotrypsin/elastase-type proteases which also contain a hydrophobic/uncharged residue in the position 182. The calcium binding site in mammalian trypsins is formed mainly by Glu70 and Glu80 (trypsin numbering, corresponding to Glu60 and Glu70 in SmSP1) [48]. This motif is not strictly conserved in the analyzed SmSP sequences; however, it might be present in a modified functional form in SmSP2, SmSP3 and SmSP4 that contain acidic residues in the close proximity of those locations (Figure 2).

SmSPs, including their domains, are differentially expressed across developmental stages

Messenger RNA transcript levels for the five SmSPs were evaluated in eggs, miracidia, daughter sporocysts, cercariae, schistosomula and adults using RT- qPCR (Figure 3). For SmSP1

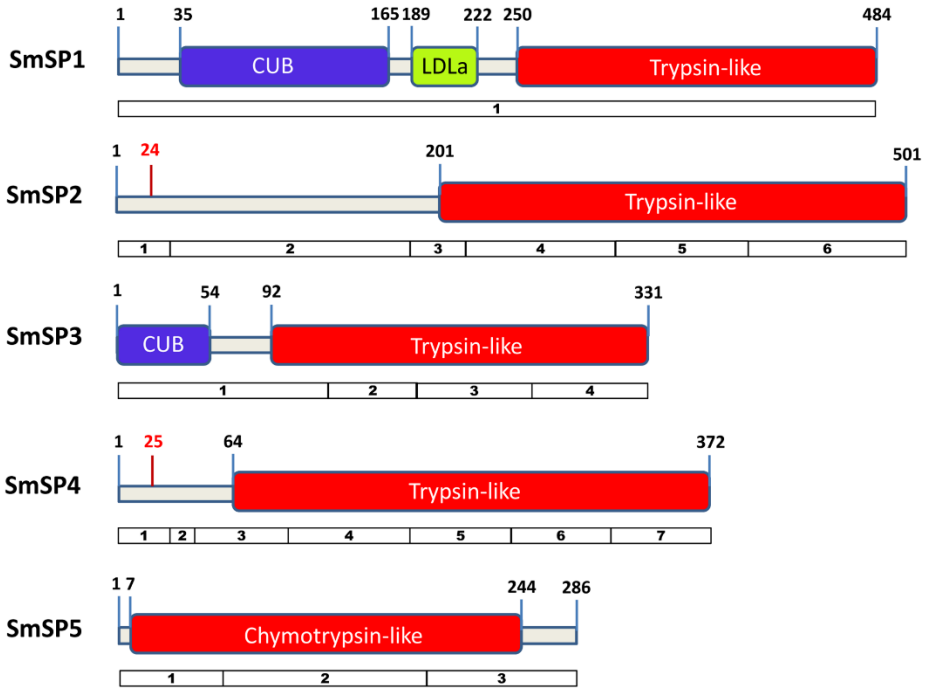

Figure 1. Predicted domain organization and open reading frames of SmSP proteases. CUB domains are depicted in blue, an LDLa domain in yellow and protease domains from the S1 family in red. In SmSP2 and SmSP4, N-terminal signal peptides are separated by red bars from the rest of N-terminal extensions with putative pro-peptides (protease activation peptides). Numbering indicates amino acid positions. Exon structure of the genes encoding SmSPs are shown as numbered boxes below each SmSP protein.
doi:10.1371/journal.pntd.0002766.g001

and SmSP3, we determined gene expression for both the protease and non-protease domains (Figure 4).

For SmSP1, the greatest expression was recorded in eggs at 2.5% of the expression level of the reference gene, SmCOX I. Low expression was recorded in adult worms, five-day old schistosomula and daughter sporocysts at around 0.1% or below relative to SmCOX I. Expression in the other stages was below significance, i.e., less than 0.01% of SmCOX I. As described above, the ORF of SmSP1 consists of 3 domains and their individual expression was evaluated by RT-qPCR and PCR (Figure 4A; Figure S2). The data show a differential expression pattern for the CUB, LDLa and protease domains of SmSP1: expression of the CUB domain is mostly in eggs and sporocysts, whereas LDLa is only expressed in eggs with an expression level about 20-fold lower than that of the protease domain (Figure 4A). As stated above, only in eggs is the whole ORF amplified by PCR suggesting that some SmSP1 is expressed as the full-length multi-domain protein (Figure S2).

Among the SmSPs, SmSP2 is the most abundantly expressed SmSP (Figure 3). In fact, expression in schistosomula and adults is on a similar level to that previously measured for the well-characterized *S. mansoni* cysteine and aspartic proteases [27]. In adults, SmSP2 expression is equivalent to that of SmCOX I, whereas in five-day old schistosomula expression is even greater - 150% that of SmCOX I. Significant expression, i.e., 10% that of SmCOX I, is also detected in eggs. In the other stages, expression is close to or below 1% of the SmCOX I level.

The expression pattern of SmSP3 across all life stages is similar to that of SmSP1 (Figure 3), with minor variations regarding expression in cercariae and schistosomula. Most expression is found in eggs at 2.5% of the SmCOX I expression level. Interestingly, the CUB and protease domains are only co-expressed in eggs and adults (Figure 4B), whereas differential expression is seen for the other developmental stages (Figure S2). SmSP4 is expressed predominantly in eggs (around 10% of SmCOX I level). For the other stages, approximately 1–2% of the SmCOX I level is detectable in cercariae, adults and five-day old schistosomula. Finally, SmSP5 is expressed predominantly in the eggs (2% of the level of SmCOX I) with low expression in the other life stages (0.02–0.05% of SmCOX).

Phylogenetic position of SmSPs: SmSP5 as 'a missing-link' chymotrypsin-like protease

The maximum likelihood analysis of a wide spectrum of vertebrate and invertebrate S1 family SPs based on amino acid sequences revealed that SmSPs clustered with related trematode proteases into five distinct and well-supported clades (Figure 5). Identical results were obtained using maximum parsimony analysis (data not shown). The clades did not create a monophyletic group.

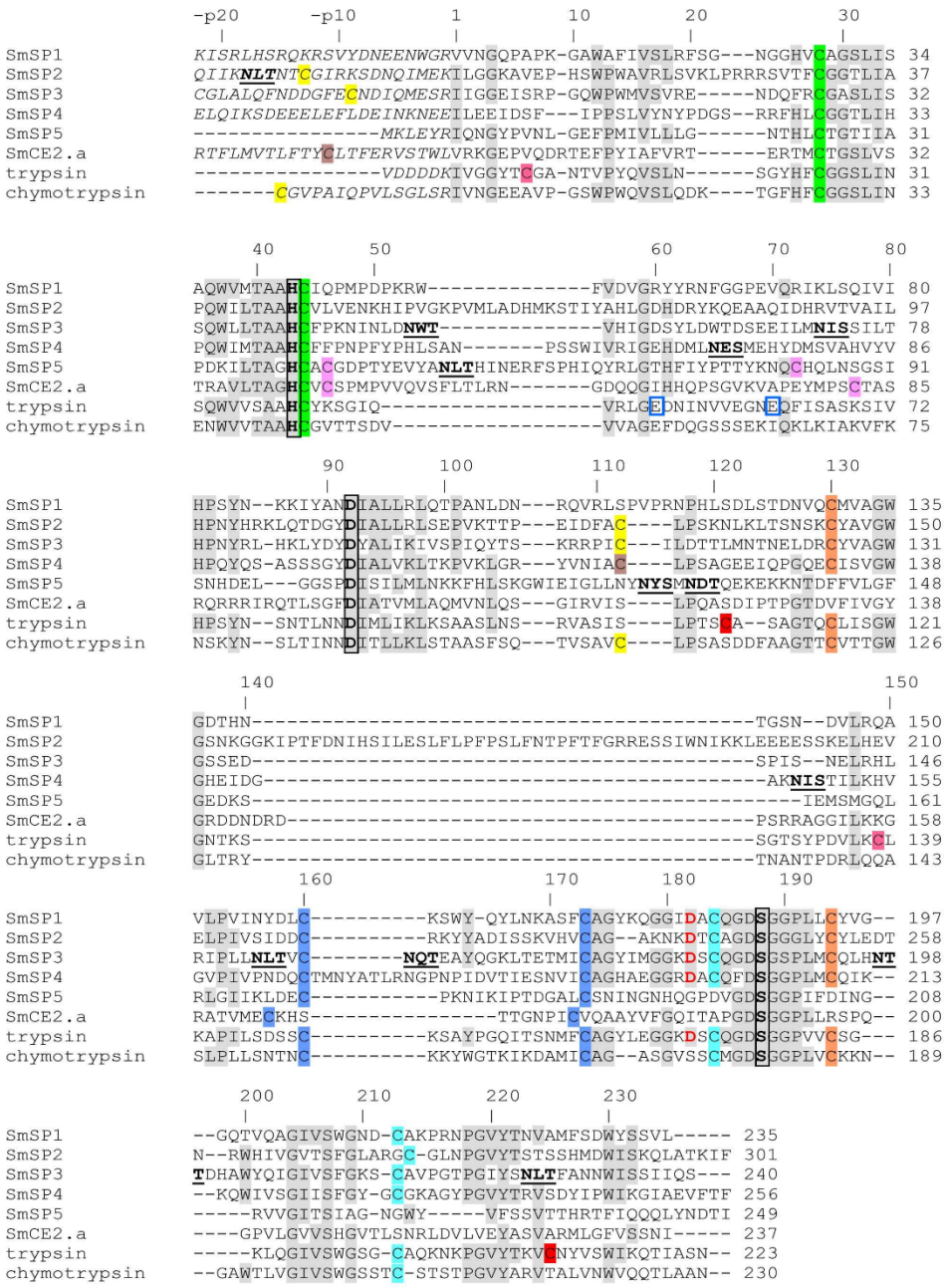
-p20 -p10 1 10 20 30
SmSP1 KISRLHSRQKRSVYDNEENWGRVVNGQPAPK-GAWAFIVSLRFSG---NGGHVCAGSLIS 34
SmSP2 QIIKNLTNTCGIRKSDNQIMEKILGGKAVEP-HSWPWAVRLSVKLPRRRSVTFCGGTLIA 37
SmSP3 CGLALQFNDDGFECNDIQMESRIIGGEISRP-GQWPWMVSVRE-----NDQFRCGASLIS 32
SmSP4 ELQIKSDEEELEFLDEINKNEEILEEIDSF---IPPSLVYNYPDGS--RRFHLCGGTLIH 33
SmSP5 ----------MKLEYRIQNGYPVNL-GEFPMIVLLLG------NTHLCTGTIIA 31
SmCE2.a RTFLMVTLFTYCLTFERVSTWLVRKGEPVQDRTEFPYIAFVRT------ERTMCTGSLVS 32
trypsin ----------VDDDDKIVGGYTCGA-NTVPYQVSLN------SGYHFCGGSLIN 31
chymotrypsin -------CGVPAIQPVLSGLSRIVNGEEAVP-GSWPWQVSLQDK----TGFHFCGGSLIN 33
40 50 60 70 80
SmSP1 AQWVMTAAHCIQPMPDPKRW----------FVDVGRYYRNFGGPEVQRIKLSQIVI 80
SmSP2 PQWILTAAHCVLVENKHIPVGKPVMLADHMKSTIYAHLGDHDRYKQEAAQIDHRVTVAIL 97
SmSP3 SQWLLTAAHCFPKNINLDNWT----------VHIGDSYLDWTDSEEILMNISSILT 78
SmSP4 PQWIMTAAHCFFPNPFYPHLSAN-------PSSWIVRIGEHDMLNESMEHYDMSVAHVYV 86
SmSP5 PDKILTAGHCACGDPTYEVYANLTHINERFSPHIQYRLGTHFIYPTTYKNQCHQLNSGSI 91
SmCE2.a TRAVLTAGHCVCSPMPVVQVSFLTLRN-------GDQQGIHHQPSGVKVAPEYMPSCTAS 85
trypsin SQWVVSAAHCYKSGIQ----------VRLGEDNINVVEGNEQFISASKSIV 72
chymotrypsin ENWVVTAAHCGVTTSDV----------VVAGEFDQGSSSEKIQKLKIAKVFK 75
90 100 110 120 130
SmSP1 HPSYN--KKIYANDIALLRLQTPANLDN---RQVRLSPVPRNPHLSDLSTDNVQCMVAGW 135
SmSP2 HPNYHRKLQTDGYDIALLRLSEPVKTTP---EIDFAC----LPSKNLKLTSNSKCYAVGW 150
SmSP3 HPNYRL-HKLYDYDYALIKIVSPIQYTS---KRRPIC---ILDTTLMNTNELDRCYVAGW 131
SmSP4 HPQYQS-ASSSGYDIALVKLTKPVKLGR---YVNIAC----LPSAGEEIQPGQECISVGW 138
SmSP5 SNHDEL---GGSPDISILMLNKKFHLSKGWIEIGLLNYNYSMNDTQEKEKKNTDFFVLGF 148
SmCE2.a RQRRRIRQTLSGFDIATVMLAQMVNLQS---GIRVIS----LPQASDIPTPGTDVFIVGY 138
trypsin HPSYN--SNTLNNDIMLIKLKSAASLNS---RVASIS----LPTSCA--SAGTQCLISGW 121
chymotrypsin NSKYN--SLTINNDITLLKLSTAASFSQ---TVSAVC----LPSASDDFAAGTTCVTTGW 126
140 150
SmSP1 GDTHN----------TGSN--DVLRQA 150
SmSP2 GSNKGGKIPTFDNIHSILESLFLPFPSLFNTPFTFGRRESSIWNIKKLEEEESSKELHEV 210
SmSP3 GSSED----------SPIS--NELRHL 146
SmSP4 GHEIDG----------AKNISTILKHV 155
SmSP5 GEDKS----------IEMSMGQL 161
SmCE2.a GRDDNDRD----------PSRRAGGILKKG 158
trypsin GNTKS----------SGTSYPDVLKCL 139
chymotrypsin GLTRY----------TNANTPDRLQQA 143
160 170 180 190
SmSP1 VLPVINYDLC----------KSWY-QYLNKASFCAGYKQGGIDACQGDSGGPLLCYVG-- 197
SmSP2 ELPIVSIDDC----------RKYYADISSKVHVCAG--AKNKDTCAGDSGGGLYCYLEDT 258
SmSP3 RIPLLNLTVC--------NQTEAYQGKLTETMICAGYIMGGKDSCQGDSGSPLMCQLHNT 198
SmSP4 GVPIVPNDQCTMNYATLRNGPNPIDVTIESNVICAGHAEGGRDACQFDSGGPLMCQIK-- 213
SmSP5 RLGIIKLDEC----------PKNIKIPTDGALQSNINGNHQGPDVGDSGGPIFDING-- 208
SmCE2.a RATVMECKHS----------TTGNPICVQAAYVFGQITAPGDSGGPLLRSPQ-- 200
trypsin KAPILSDSSC----------KSAYPGQITSNMFCAGYLEGGKDSCQGDSGGPVVCSG--- 186
chymotrypsin SLPLLSNTNC----------KKYWGTKIKDAMICAG--ASGVSSCMGDSGGPLVCKKN-- 189
200 210 220 230
SmSP1 --GQTVQAGIVSWGND-CAKPRNPGVYTNVAMFSDWYSSVL----- 235
SmSP2 N--RWHIVGVTSFGLARGC-GLNPGVYTSTSSHMDWISKQLATKIF 301
SmSP3 TDHAWYQIGIVSFGKS-CAVPGTPGIYSNLTFANNWISSIIQS--- 240
SmSP4 --KQWIVSGIISFGY-GCGKAGYPGVYTRVSDYIPWIKGIAEVFTF 256
SmSP5 -----RVVGITSIAG-NGWY-----VFSSVTTHRTFIQQQLYNDTI 249
SmCE2.a ----GPVLGVVSHGVTLSNRLDVLVEYASVARMLGFVSSNI----- 237
trypsin -----KLQGIVSWGSG-CAQKNKPGVYTKVSNYVSWIKQTIASN-- 223
chymotrypsin --GAWTLVGIVSWGSSTC-STSTPGVYARVTALVNWVQQTLAAN-- 230

Figure 2. Primary sequence alignment of SmSP1 through SmSP5 with *S. mansoni* cercarial elastase 2a (SmCE2.a), bovine trypsin and bovine chymotrypsin. For SmSP1 to SmSP4, only the protease domains are shown; the upstream sequences (except a short sequence stretch) forming N-terminal extensions and non-proteolytic domains are not included in the alignment. Also, a downstream C-terminal extension of SmSP5 is not included. The catalytic residues His, Asp and Ser are highlighted in bold and black-boxed; critical Asp residues in the S1 subsite that account for trypsin-like activity are in bold red; Cys residues that are predicted to form disulfide bonds are indicated by the same color; putative unpaired Cys residues are highlighted in olive, and predicted N-glycosylation signals are in bold and underlined. Glu residues binding a Ca^{2+} ion in the trypsin molecule are blue-boxed. The upper line numbering is according to SmSP1; the predicted mature protease domain starts with 1, the suffix p indicates pro-peptide/N-terminal extension numbering. GenBank accession numbers are as follows: SmSP1 (KF535923), SmSP2 (KF510120), SmSP3 (KF510121), SmSP4 (KF510122), SmSP5 (KF939306), SmCE2a (AAM43941), bovine trypsinogen (XP_871686) and bovine chymotrypsinogen A (XP_003583409).
doi:10.1371/journal.pntd.0002766.g002

Thus, SmSP1 and SmSP3 were placed as two closely related but independent clades (trematode SP clade 1 and 3) and clustered with a large group of vertebrate SPs, including regulatory- and epithelial-derived effector trypsin-like proteases such as plasminogens, plasma kallikreins, tryptases, matriptases and transmembrane SPs (Figure 5). SmSP2 and SmSP4 also segregated into two separate but related trematode clades (numbers 2 and 4), which clustered with cestode SPs and a group of insect plasminogen-like and trans-membrane SPs (Figure 5). Finally, SmSP5 clustered with *S. japonicum* and *Clonorchis sinensis* (Chinese liver fluke) orthologs and created a sub-clade that grouped with a sub-clade of CEs within the trematode SP clade 5. This clade also clustered with chymotrypsin-like proteases from invertebrates. Accordingly, SmSP5 and its trematode orthologs associate more with the divergent schistosome CEs [22] than with other S1 family proteases [18].

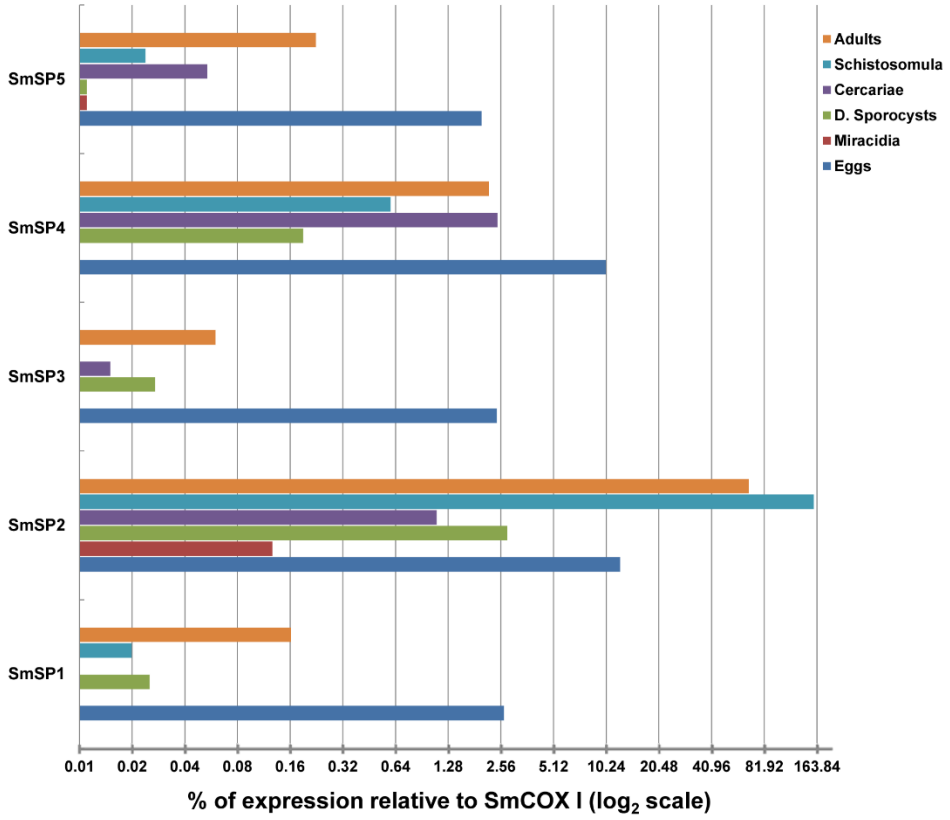

Figure 3. RT-qPCR to evaluate the expression of SmSP genes among *S. mansoni* developmental stages. mRNA levels are displayed as the percentage of expression compared to the constitutively expressed *S. mansoni* cytochrome oxidase I (SmCOX I). The value 0.01% was used as a significance threshold. The gene expression analysis of the protease domains of SmSPs. Each unit represents the -fold change in the transcription level using the $\log_2$ scale.
doi:10.1371/journal.pntd.0002766.g003

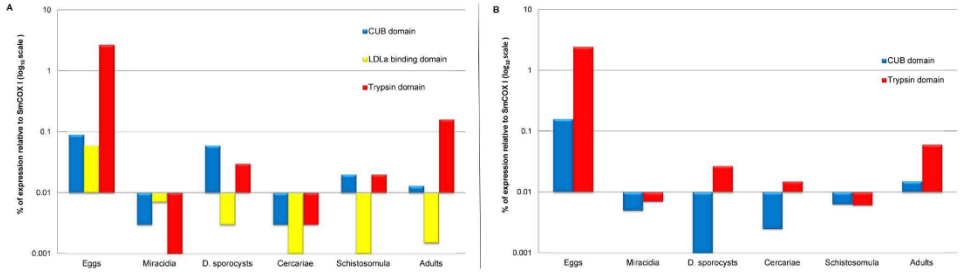

Figure 4. Comparisons of mRNA levels for the separate domains of SmSP1 (A) and SmSP3 (B) displayed in the log_{10} scale and as a percentage of SmCOX I expression level. D. sporocyst = daughter sporocyst.
doi:10.1371/journal.pntd.0002766.g004

Activity profiling demonstrates trypsin-like proteases in *S. mansoni* developmental stages

S1 family SP activities in soluble extracts of *S. mansoni* adults, five day-old schistosomula and eggs were profiled for proteolytic specificity using peptidyl fluorogenic substrates. Two sets of specific protease substrates were used; (i) substrates with a basic amino acid residue (Arg, Lys) in the P1 position that are cleaved by trypsin-like SPs, and (ii) substrates containing bulky hydrophobic (Phe, Tyr) or aliphatic residues (Val, Leu, Met) at P1 that are cleaved by chymotrypsin- or elastase-like SPs [49]. The measured activities were further authenticated as S1 family SPs by their sensitivity to the small molecule inhibitors, Pefabloc SC and PMSF.

The results indicate that trypsin-like activities predominate over chymotrypsin/elastase-like activities in the analyzed extracts (Figure 6). The trypsin substrates were hydrolyzed with variable efficiencies giving distinct cleavage patterns for the individual life stages. The prominent activity in all extracts was best measured with the Boc-L-R-R-AMC substrate, hence making this substrate a useful probe to detect and measure SmSPs. Extracts of eggs displayed a particularly complex profile by cleaving an additional two substrates, Bz-F-V-R-AMC, and Z-G-P-R-AMC. This suggests that this life-stage possesses additional, possibly stage-specific, trypsin-like proteases. In contrast to the major trypsin-like activities, chymotrypsin/elastase-like activity was relatively weak being measured only in schistosomula and adults.

Subsequently, we tested whether SmSPs is measurable in the E/S products from eggs, schistosomula and adults. For this purpose, we used the substrate Boc-L-R-R-AMC, which was identified as the most efficient substrate for homogenates of all the life stages (Figure 6). The specific activities of the E/S products, which were inhibited by the SP inhibitors, Pefabloc SC and PMSF, were 1.05 ± 0.10, 1.38 ± 0.05, and 0.11 ± 0.01 RFU/μg protein for eggs, schistosomula and adults, respectively.

Spatial structure modeling predicts a trypsin-like substrate specificity of SmSP1

A spatial homology model of the protease domain of SmSP1 was constructed to analyze its binding pocket and substrate specificity. The X-ray structure of bovine trypsin in complex with the small-molecule inhibitor, leupeptin (PDB code 1jrt), was used as a template. We used SmSP1 as representative of SmSP1 to SmSP4, which have substantial sequence identity, a similar disulfide pattern and homology in active site regions (Figures 2 and S3). Figure S5 shows that the SmSP1 protease domain displays the conserved architecture of S1 family proteases which consists of two six-stranded β-barrel domains packed against each other. The catalytic amino acid residues are located at the junction between the domains. The major insertion/deletion variations between SmSP1 to SmSP4 (such as the SmSP2 insertion at residue 140, Figure 2) are located at surface-exposed loops.

The primary substrate specificity determinant of S1 family proteases is the S1 binding subsite. In SmSP1, this subsite forms a deep and narrow negatively charged pocket that contains Asp182 at the bottom (Figures 7A and 7B). Leupeptin, the transition state analog protease inhibitor, was docked into the active site of SmSP1. The arginal residue of leupeptin forms a covalent linkage with the catalytic Ser188, a salt bridge with Asp182 in the S1 subsite and hydrogen bonds with the carbonyl oxygen of Ala183 and Asp211 (Figure 7C). An additional hydrogen bond is formed between the side chain nitrogen of Gln185 and the carbonyl oxygen Leu2 residue of leupeptin. The putative interaction pattern of leupeptin at the S1 subsite of SmSP1 is similar to that found in bovine trypsin [50]. This demonstrates that SmSP1 has a substrate binding preference for basic residues at the P1 position, the positive charge of which compliments the negatively charged Asp182, i.e., trypsin-like activity. This conclusion can be generalized to SmSP2 to SmSP4 which also contain the critical Asp182 residue.

Discussion

Much has been reported on the genetic, biochemical and functional characterization of cysteine and aspartic protease activities in schistosomes [16,17] and flatworms in general [16,51], and of the schistosome CE SPs [20] that putatively facilitate parasite invasion of the mammalian host [18–20]. By comparison, relatively little detail is available for non-CE SPs. There are, however, indications that non-CE S1 family SPs contribute to successful infection [6]. Thus, kallikrein-like protease activity from *S. mansoni* adults [12] and plasmin-like fibrinolytic activity from *S. mansoni* eggs [15] have been recorded previously. Both activities displayed trypsin type cleavage specificities and both may contribute to the phenomenon, whereby large occlusions of veins by schistosomes are not associated with intravascular deposition of fibrin and thrombus formation [52–54]. At the gene and primary sequence levels, however, only two SmSPs, namely SmSP1 [13,14] and another [23,24], which we term SmSP2, have been described.

The *S. mansoni* GeneDB currently contains 16 unique sequences that belong to Clan PA family S1 SPs. This number is significantly

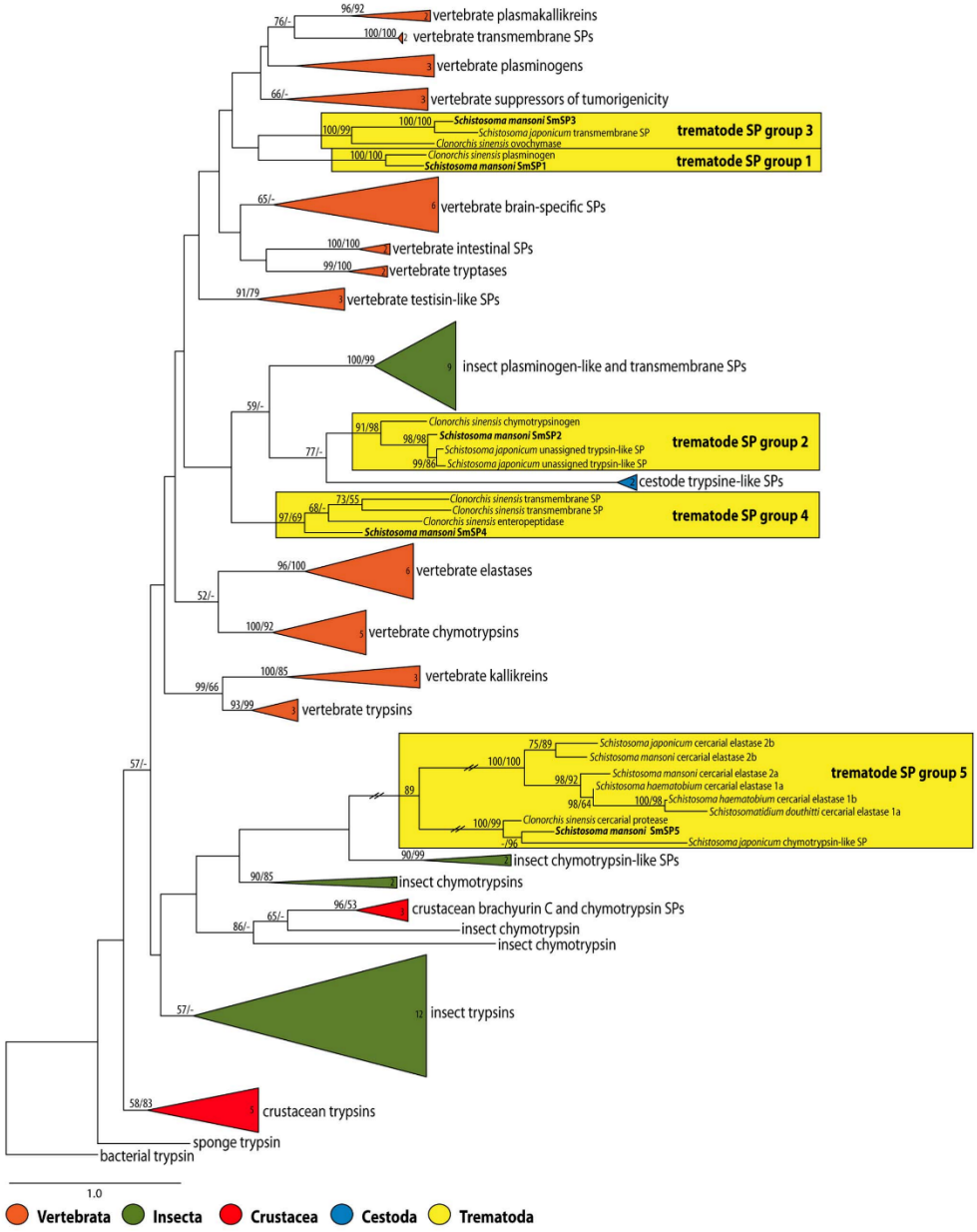

Figure 5. Maximum likelihood phylogenetic tree of 101 selected members of the S1 family of serine proteases with emphasis on trematode SPs. Numbers in the collapsed branches (triangles) indicate the number of taxa included in the branch. Maximum likelihood and maximum parsimony bootstrap supports are shown at nodes, bootstrap percentages with <50% support are not shown. Branches in the trematode clade 5 SPs are shortened to one third of their original length as indicated by the two diagonal lines. For clades 1 and 4, two *S. japonicum* orthologs

are missing due to their absence in the GenBank nr database. However, both sequences can be retrieved from the SchistoDB database under the identifiers Sjp_0012180 (SjSP1) and Sjp_0047680 (Sj SP4).
doi:10.1371/journal.pntd.0002766.g005

lower than the 135 family S1 proteases found in the human genome [8,25] and may be due to the lack of need to regulate the more complex and expanded physiological processes found in vertebrates [55]. In our study and apart from SmSP1 [13,14], we identified four additional SmSP genes encoding typical sequence features of the S1 family [7,8] and which we term SmSP2 through SmSP5. Two further genes (Smp_194090 and Smp_06530 in GeneDB) were identified in the *S. mansoni* GeneDB as putative proteolytically inactive SmSPs as they lack the catalytic serine or histidine residue in the catalytic triad. The remaining nine of the 16 family S1 SPs comprise eight CEs (encoding both putative proteolytically active and inactive products) and a gene (Smp_174530) that encodes an S1 family SP ORF fused downstream of an M01 family metallo-protease. This protease that was not known to us at the beginning of our study and because of its domain complexity and sequence size was not described further.

Our phylogenetic analyses of trematode SPs displayed interesting evolutionary trends. The SmSPs segregate into five clusters of family S1 proteases. The protease domains of SmSP1 and SmSP3, forming clades 1 and 3, respectively, cluster with a large group of vertebrate trypsin-like SPs including regulatory and effector epithelial-derived proteases. In addition to a protease domain, the ORFs for SmSP1 and SmSP3 include non-catalytic CUB domains and SmSP1 LDLa domain. Several vertebrate matriptases that also contain CUB domains are present in our phylogenetic analysis including those belonging to the 'suppressor of tumorigenicity' group. As judged by the domain organization, SmSP1 resembles mammalian matriptases (a.k.a. epithin, MT-SP); however unlike conventional matriptases with multiple CUB and LDLa domains, SmSP1 has only one of each. CUB domains were first described in the complement proteins C1r and C1s and are modules of approximately 110 amino acids with four conserved cysteine residues [56]. These domains mediate protein-protein interactions and are generally associated with proteins that have diverse, usually regulatory, functions in the extracellular space and/or plasma membrane [56]. CUB domains can also interact with heparin and glycoproteins [56] and are often associated with metallo-proteases, in addition to serine proteases [8].

Based on the RT-qPCR analysis, the complete ORFs of SmSP1 and SmSP3 molecules share a similar expression profile (quantitatively and, to a smaller degree, qualitatively) across the developmental stages tested. However, it is also clear that the individual protease, CUB and/or LDLa domains are differentially expressed across the developmental stages tested being only co-expressed in eggs and, for SmSP3, adults. The particular functions of these enzymes and their component domains are unknown and their importance to parasite vitality and/or survival might be tested via specific RNA interference (RNAi), which has been shown to operate in schistosomes [30,57,58]. According to our phylogenetic analysis, the closest vertebrate orthologs to SmSP1 and SmSP3 are those associated with regulatory cascades such as fibrinolysis and vasodilation. This, together with the fact that SmSP1 was detected apparently on the surface area of worms and secreted into the cultivation media [13], suggests a possible function at the host-parasite interface.

The presence in the ORF of SmSP1 of an LDLa domain (positioned between the CUB and catalytic domains) deserves a note. Schistosomes and other flatworms do not synthesize cholesterol (found within LDL) and must therefore scavenge it from the environment, particularly for the energy-intensive work of producing eggs [59,60]. There is also a report that the presence of *S. mansoni* eggs is connected with decreased circulating levels of cholesterol in the host [61], however, we can only speculate about the real function of the SmSP1 LDLa domain.

SmSP2 and SmSP4 form two other separate clades and cluster with trypsin SPs from insect and other invertebrates. Both proteases are characterized by their longer but different N-terminal extensions

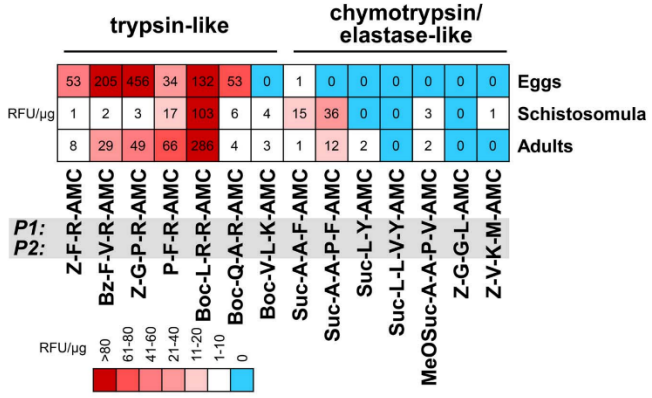

Figure 6. Profiling SP activities in extracts of *S. mansoni* developmental stages. The kinetic assays, performed at pH 8.0, employed 50 µM fluorogenic substrates (P1 and P2 positions are highlighted) that are specific for trypsin- and chymotrypsin/elastase-type proteases. SP activities (sensitive to inhibition by PMSF and Pefabloc SC) are expressed as relative fluorescence units (RFU/s) and normalized to the protein content of extracts. Data are displayed in a heat map.
doi:10.1371/journal.pntd.0002766.g006

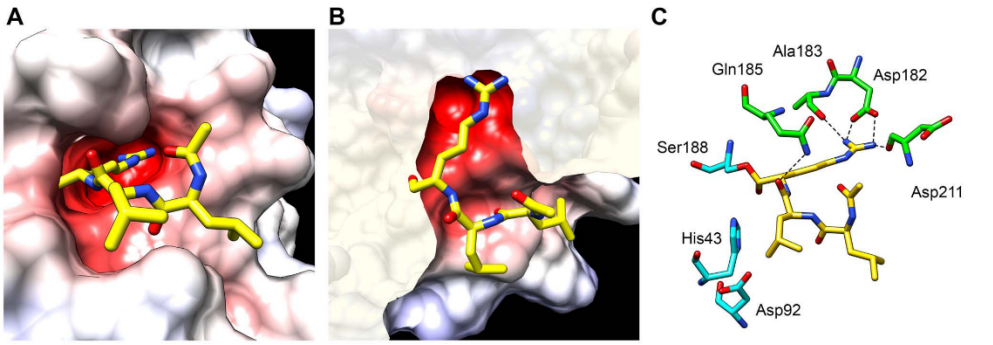

Figure 7. Homology model of the SmSP1 protease domain in complex with leupeptin. The model was built using the template X-ray structure of bovine trypsin in complex with substrate-like inhibitor leupeptin (N-acetyl-L-leucyl-L-leucyl-L-argininal; PDB code 1jrt). (**A**) Surface representation of the SmSP1 active site colored by electrostatic potential (at a scale from −10 kT/e (red) to +10 kT/e (blue)). Carbon atoms of leupeptin are yellow; heteroatoms have a standard color coding (O, red; N, blue). (**B**) The same detail as (A) but viewed from above (the surface display was clipped for a better view). (**C**) Schematic view of the active site residues of SmSP1 (green) forming hydrogen bonds (dashed lines) with leupeptin (yellow). Note the interactions between Asp182 (in the S1 protease subsite) and the basic P1 residue of leupeptin that mimic the S1-P1 salt bridge that is critical for trypsin-like substrate specificity. Catalytic residues (cyan) are shown, including the covalent linkage of leupeptin with the catalytic Ser188.
doi:10.1371/journal.pntd.0002766.g007

that lack homologies to known proteins but which are shared in orthologous SPs from *S. japonicum* [44] and *C. sinensis* [62]. Functions as yet are unknown, however, it is certainly remarkable that SmSP2 is massively expressed in schistosomula and adults (150% and 60% of SmCOX I expression levels, respectively) and, therefore, conceivably contributes significantly to host and/or parasite protein hydrolysis, perhaps in modulating of host physiologic processes [6,12]. The presence also of close orthologs of SmSP2 in *Fasciola gigantica* [63] and *C. sinensis* [62] suggests a general role for SP2 during infection in the mammalian host. The impressive expression levels for SmSP2 are consistent with high levels of SmSP2 expression from microarray [23] and transcriptome data [24]. Also, the expression levels are close to those for the gut-associated, digestive cysteine and aspartic proteases, SmCB1 and SmCD, respectively, for which expression is close to that of SmCOX I [27].

Finally, for SmSP5, phylogenetic analysis identified its relative position in what we term clade number 5. This clade is most closely related to chymotrypsins from invertebrates and comprises SP5 orthologs in *S. japonicum* [44] and *C. sinensis* [62], and the CE genes in *S. mansoni, S. haematobium* [20,22], *S. japonicum* [44] and *Schistosomatium douthitti* [20]. Clade 5 is particularly significant for phylogenetic relationship studies of schistosome proteolytic enzymes as it contains sequences that bridge the outlier CE group and other 'more typical' S1 family SPs. Specifically, our previous phylogenetic work [18] had highlighted that the CEs coalesce as a tight group that diverges from other family S1 protease sequences. At that time the SmSP5 sequence was incomplete and not amenable to analysis [18]. The current sequence analysis suggests that SmSP5 and its trematode orthologs are 'a missing link' between the outlier CE group and the common ancestor. CEs initially evolved from chymotrypsin regulatory proteases and may provide an evolutionary advantage in contributing to host invasion [22].

For the SmSP protease domains, we investigated the structure-function relationships using primary structure analysis, homology modeling and protease activity profiling with peptidyl substrates. The sequence alignment shows that all the SmSPs except SmSP5 share a conserved Asp182 residue. This residue defines the specificity for the S1 binding site and drives a strong preference for Arg and Lys residues at the P1 position of protein/peptide substrates, as demonstrated for vertebrate trypsins [47]. The homology model of SmSP1 reveals that the S1 pocket with its critical Asp182 residue has an architecture analogous to vertebrate trypsins. In contrast, the S1 binding pocket of SmSP5 has a Gly182. Also, SmSP5 lacks the disulfide Cys184-Cys212 which is present in the other four SmSPs and known to stabilize the S1 binding site in vertebrate trypsins. Interestingly, this disulfide is also absent in schistosome CEs, which contain non-polar residues (Ile or Leu) at the bottom of the S1 binding pocket resulting in elastase and chymotrypsin-like activities [22].

Consistent with the number of trypsin-like sequences in all of the life-stages studied, major trypsin-like activities could also be measured using peptidyl fluorogenic substrates in eggs, schistosomula and adult extracts. Eggs, in particular, presented the most diverse and active profile compared to adults and schistosomula suggesting they express more than one highly active SP. Schistosomula, in contrast, displayed an activity profile restricted to one substrate, and one might suppose that this activity is in fact due to SmSP2 which was, expressed at higher levels than other SPs as measured by RT-qPCR (see above). Finally, the finding that significant trypsin-like activity was found in the E/S products of the three life stages tested suggests that one or more SmSPs are secreted into the host environment where they may interfere with relevant proteolytic cascades such as blood coagulation, complement or blood pressure regulation [6,12].

In contrast to the trypsin-like activities measured, chymotrypsin/elastase-like activities were absent in eggs, and in schistosomula were at least one order of magnitude weaker. It is possible that the activity in schistosomula is, in whole or part, due to residual CE activity carried forward after mechanical transformation of cercariae and *in vitro* culture of schistosomula. In adults, however, this possibility seems remote and the minor activities measured may be contributed to by SP5.

To conclude, the present study provides a comprehensive phylogenetic, transcriptomic and functional framework illustrating the heretofore unknown complexity of schistosome S1 family SPs,

other than the well-studied CEs [20,22]. The individual enzymes underlying the activities measured remain 'undiscovered country' both in terms of their functional characterization and, not least, their possible contributions to successful parasitism by the schistosome, including at the host-parasite interface.

Supporting Information

Figure S1 Amino acid sequence alignment of three versions of SmSP1. Original description (CAA09691), the *S. mansoni* GeneDB (SchistoDB, Smp_030350) description (both Puerto Rican isolates) and our current version (KF535923) sequenced from a Liberian *S. mansoni* isolate. Sequence variations are highlighted in green, turquoise and grey for KF535923, Smp_030350 and CAA0969, respectively. The CUB, LDLa and trypsin domains are underlined in blue, yellow and red, respectively.
(TIF)

Figure S2 Expression of SmSP1 (CUB, LDLa and protease domains) and SmSP3 (CUB and protease domains) using PCR. Primers were designed to amplify particular domains, partial or whole ORF fragments from cDNA of various *S. mansoni* developmental stages. The lanes are as follows: 1, SmSP1CUB; 2, SmSP1CUB-LDLa; 3, SmSP1trypsin; 4, SmSP1trypsin-LDLa, 5, whole ORF SmSP1CUB-LDLa-trypsin; 6, SmSP3CUB; 7, SmSP3 trypsin and 8, whole ORF SmSP3 CUB-trypsin.
(TIF)

Figure S3 Matrix of amino acid sequence identities used in Figure 2.
(TIF)

Figure S4 Amino acid sequence alignment of three splice versions of SmSP5. The catalytic residues His, Asp and Ser are highlighted.
(TIF)

Figure S5 Comparison of the structures of SmSP1 and bovine trypsin. A stereo image displaying a superimposition of Cα traces of the homology model of SmSP1 (cyan) and the crystal structure of trypsin (PDB code 1JRT; magenta). The catalytic residues are shown as ball and sticks (SmSP1 in green, trypsin in yellow). Disulfide bonds are depicted in blue (SmSP1) and orange (trypsin).
(TIF)

Table S1 List of primers used for RT-qPCR analysis.
(PDF)

Table S2 The list of family S1 proteases (SPs sequences) used for the phylogenetic analysis.
(PDF)

Acknowledgments

The team at the CDIPD-UCSF thanks Brian Suzuki and KC Lim for vital technical support.

Author Contributions

Conceived and designed the experiments: MH PF LRA LU PBS MM JD. Performed the experiments: MH PF LRA LU PBS ZF AVP DO JV JD. Analyzed the data: MH PF LRA PBS AVP JV MM CRC JD. Contributed reagents/materials/analysis tools: MH PBS AVP JV JHM MM CRC JD. Wrote the paper: MH JHM MM CRC JD.

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4.5 Publication No. 4: SmSP2: A serine protease secreted by the blood fluke pathogen *Schistosoma mansoni* with anti-hemostatic properties.

This publication focuses on the detailed functional and biochemical characterization, including immunolocalization, of *S. mansoni* serine protease 2, SmSP2, the most highly expressed SmSP by blood-dwelling stages of schistosomes. SmSP2 has been described as a multi-domain protein consisting of an N-terminal region with of a stretch of histidine residues, a thrombospondin-1 type 1 repeat domain, and an S1 family serine protease domain at the C-terminus. The trypsin-like cleavage specificity of recombinant SmSP2 was demonstrated using positional scanning and multiplex combinatorial libraries. The protease was localized to the tegmental cells of the adult schistosome surface, from where it is secreted into the host environment. SmSP2 exhibited limited proteolysis of protein substrates. It effectively processed host blood bioactive peptides and proteins involved in host proteolytic cascades i.e., blood coagulation, fibrinolysis, and blood pressure regulation. Thus, SmSP2 may interfere with critical host hemostatic processes and contribute to the parasite survival in the host. Accordingly, SmSP2 may be an attractive drug target or vaccine candidate.

PhD applicant contribution: parasite cultivation, preparation of adult schistosome tissues for microscopy, immunofluorescence microscopy and subsequent microscopy data analysis and interpretation.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

SmSP2: A serine protease secreted by the blood fluke pathogen *Schistosoma mansoni* with anti-hemostatic properties

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OPEN ACCESS

Citation: Leontovyč A, Ulrychová L, O'Donoghue AJ, Vondrášek J, Marešová L, Hubálek M, et al. (2018) SmSP2: A serine protease secreted by the blood fluke pathogen *Schistosoma mansoni* with anti-hemostatic properties. PLoS Negl Trop Dis 12 (4): e0006446. https://doi.org/10.1371/journal.pntd.0006446

Editor: Robert M. Greenberg, University of Pennsylvania, UNITED STATES

Received: December 13, 2017

Accepted: April 11, 2018

Published: April 20, 2018

Data Availability Statement: All relevant data are within the paper and its Supporting Information files. Mass spectrometry data for Multiplex assay can be accessed here: ftp://massive.ucsd.edu/MSV000081747.

Funding: This work was supported by the grants LD15101, LH15040 and the project InterBioMed LO1302 from the Ministry of Education, Youth and Sports of the Czech Republic and by the institutional project RVO 61388963 (www.msmt.

Abstract

Background

Serine proteases are important virulence factors for many pathogens. Recently, we discovered a group of trypsin-like serine proteases with domain organization unique to flatworm parasites and containing a thrombospondin type 1 repeat (TSR-1). These proteases are recognized as antigens during host infection and may prove useful as anthelminthic vaccines, however their molecular characteristics are under-studied. Here, we characterize the structural and proteolytic attributes of serine protease 2 (SmSP2) from *Schistosoma mansoni*, one of the major species responsible for the tropical infectious disease, schistosomiasis.

Methodology/Principal findings

SmSP2 comprises three domains: a histidine stretch, TSR-1 and a serine protease domain. The cleavage specificity of recombinant SmSP2 was determined using positional scanning and multiplex combinatorial libraries and the determinants of specificity were identified with 3D homology models, demonstrating a trypsin-like endopeptidase mode of action. SmSP2 displayed restricted proteolysis on protein substrates. It activated tissue plasminogen activator and plasminogen as key components of the fibrinolytic system, and released the vasoregulatory peptide, kinin, from kininogen. SmSP2 was detected in the surface tegument, esophageal glands and reproductive organs of the adult parasite by immunofluorescence microscopy, and in the excretory/secretory products by immunoblotting.

cz). MC was supported by the Charles University in Prague (PROGRES Q25/LF1 and UNCE 204017, www.cuni.cz). AL was supported by grant 1404317 (Charles University Grant Agency, Czech Republic, www.cuni.cz). Research by CRC is supported in part by a grant from the US National Institute of Allergy and Infectious Diseases of the National Institutes of Health (www.niaid.nih.gov) under the award number R21AI126296. CSC was supported by NIH R01 GM104659 (www.nih.gov). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Conclusions/Significance

The data suggest that SmSP2 is secreted, functions at the host-parasite interface and contributes to the survival of the parasite by manipulating host vasodilatation and fibrinolysis. SmSP2 may be, therefore, a potential target for anti-schistosomal therapy.

Author summary

Schistosomiasis (bilharziasis) is a global parasitic infection with more than 240 million people infected. It is caused by *Schistosoma* flatworms that live in the bloodstream. Current treatment relies on one drug, and no effective vaccine has yet been developed. Proteolytic enzymes (proteases) help the parasite survive in the mammalian host, allowing the schistosome to invade, feed, grow, reproduce and, manipulate the immune system. Thus, proteases are considered potential drug and vaccine targets. We previously described, and herein, investigate at the protein level, SmSP2, the major serine protease produced by the blood-dwelling stages of *S. mansoni*. We show that SmSP2 is secreted by the parasite and effectively processes host blood bioactive peptides and proteins that are involved in fibrinolysis and regulation of vascular tone. We propose, therefore, that SmSP2 modulates host hemostasis to promote parasite infection and survival. Thus, SmSP2 and similar proteins in other parasitic flatworms represent potential targets for novel drug or vaccine interventions.

Introduction

Schistosomiasis (bilharziasis) is a chronic infectious disease caused by a trematode blood flukes of the genus *Schistosoma*. A public health problem in 74 developing countries, the parasite infects over 240 million people with up to 700 million people at risk [1, 2]. Adult schistosomes can reside for decades in the host vascular system as male-female pairs producing hundreds of eggs per day [3]. Morbidity arises from immuno-pathological reactions to and entrapment of schistosome eggs in various tissues [4]. In the absence of a vaccine, treatment and control of schistosomiasis relies on a single drug, praziquantel (PZQ) [5, 6]. During its complex life cycle, the parasite survives in various environments by presenting or releasing bioactive molecules that aid survival and modulate host physiology [7–10]. Disruption of these potential mechanisms by specific drugs or vaccines may provide therapeutic benefits.

Proteolytic enzymes (proteases) of schistosomes [11] are attractive drug targets as they frequently operate at the host–parasite interface, and facilitating parasite invasion, migration, nutrition and immune evasion [12–14]. Most studies on schistosome proteases, to date, have focused on cysteine and aspartic proteases that contribute to the digestion of the blood meal [15, 16]. Among them, the cathepsin B1 of *S. mansoni* (SmCB1) has been validated in a murine model of schistosomiasis as a chemotherapeutic target [17], and small molecule inhibitors of SmCB1 are under investigation as potential drug leads [18–20].

Schistosome serine proteases (SPs) are less studied with the exception of cercarial elastase, which facilitates penetration of the human host by infective larvae [21, 22]. Recently, we uncovered a repertoire of *S. mansoni* trypsin- and chymotrypsin-type S1 family serine proteases (SmSPs) by employing a series of genomic, transcriptomic, proteolytic and phylogenetic investigational strategies [23]. Among these, SmSP2 is the most abundantly expressed in blood-dwelling stages [23]. Interestingly, SmSP2 ortholog (mastin) was identified as potential

vaccine targets in *Schistosoma haematobium* based on IgG1 immune response of individuals with drug-induced resistance [24].

In this study, we report the first detailed biochemical and enzymatic characterization of SmSP2. This enzyme processes several proteins and peptides that are involved in host proteolytic cascades, *i.e.*, blood coagulation, fibrinolysis and blood pressure regulation, and, thus, may interfere with critical vascular hemostatic processes. Accordingly, SmSP2 may be a target for novel anti-schistosomal therapeutics.

Materials and methods

Ethics statement

Research with experimental animals was performed in accordance with the animal welfare laws of the Czech Republic and in accordance with European regulations for transport, housing and care of laboratory animals (Directive 2010/63/EU on the protection of animals used for scientific purposes). The project of experiments including the use of experimental animals for present study was approved by Ministry of education, youth and sports of the Czech Republic (approval number MSMT—7063/2017-2). All the animals used in the study were maintained by certified person (certificate number CZ 02627) in specifically accredited laboratories of Institute of Immunology and Microbiology of the First Faculty of Medicine, Charles University and the General University Hospital in Prague (accreditation number 70030/2013-MZE-17214), both issued by the Ministry of Agriculture of the Czech Republic.

Schistosome material

Schistosoma mansoni (a Puerto Rican isolate) was routinely maintained in the laboratory by cycling between the intermediate snail host, *Biomphalaria glabrata*, and outbred ICR mice as definitive hosts. Infective larvae (cercariae) were released after light stimulation from infected snails placed in fresh water. Adult mice were infected by immersing the feet and tails for 45 min in 50 ml of water containing approximately 500 cercariae. Six weeks post infection, mice were euthanized by an intra-peritoneal injection of ketamine (Narkamon 5% - 1.2 mL/kg bw) and xylazine (Rometar 2% - 0.6 mL/kg bw) and the worms recovered from the portal vein by transcardial perfusion with RPMI 1640 medium as described previously [25–27]. Newly transformed schistosomula (NTS) were prepared by mechanically transforming cercariae and cultivated in a Basch Medium 169 [28] containing 5% fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin for 5 days at 37˚C under a 5% CO2 atmosphere.

Preparation of schistosome extract, excretory/secretory products collection and purification of native SmSP2

Soluble protein extracts (1–3 mg protein/mL) from *S. mansoni* adults were prepared by homogenization in 50 mM Tris-HCl, pH 8.0, containing 1% CHAPS, 1 mM EDTA, 1 μM pepstatin and 1 μM E-64 on an ice bath. The extract was cleared by centrifugation (16,000 *g* at 4˚C for 10 min), ultra-filtered using a 0.22 μm Ultrafree-MC device (Millipore) and stored at -80˚C. Excretory/secretory products (ESP) of adult worms were collected as described previously [29]. Specifically, fifty pairs of adult worms were washed five times in Basch medium 169 containing 5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 1% Fungizone (Gibco), incubated for 1h at 37˚C under a 5% CO_2 atmosphere, washed five times and then incubated overnight at 37˚C in 5% CO_2 in the above medium supplemented with 5% fetal calf serum but in the absence of Fungizone. Parasites were washed three times in the above medium and then washed 10 times in M-199 medium containing 100 U/ml penicillin and

100 μg/ml streptomycin, but without serum. Adults were evenly distributed in 5 ml of the same medium in a 6-well cultivation dish and incubated for 16 h at 37˚C in 5% CO_2. Medium containing ESP was removed, filtered over an Ultrafree-MC 0.22 μm filter (Millipore), buffer exchanged into ice-cold PBS, concentrated to 2 mL using Amicon Ultracel-10K filters (Millipore) and aliquots stored at -80˚C.

Native SmSP2 was purified from the adult schistosome extract using Ni^{2+} chelating chromatography (Hi-Trap IMAC FF column, GE Healthcare Life Sciences) under native conditions. The bound material was eluted using a 0.5 M imidazole and the purified proteins analyzed by immunoblotting with anti-SmSP2 IgG.

Expression and purification of recombinant SmSP2 in *Pichia pastoris*

The full-length SmSP2 gene (GenBank KF510120; GeneDB Smp_002150) without the N-terminal signal sequence predicted by SignalP [30] was codon-optimized for expression in *P. pastoris* and cloned into the pUC57 vector (GenScript) with an incorporated C-terminal His-tag (GPHHHHHH). The SmSP2 protease domain (residues 201 to 501, Fig 1) containing a short N-terminal propeptide (residues 183 to 200, Fig 1) was prepared by PCR amplification of the synthetic SmSP2 gene using the forward primer, 5´-AAGAGAGGCTGAAGC**TGCA**AACTT GACAAACACCTGTGGTATCAG-3´ that contained a Pst I restriction site, and the reverse primer, 5´-GGCCACGTGAATTCCTTAGTGATGGTGATGGTGATGAGGACC-3. Both primers contained 15 nucleotide extensions (underlined) homologous to the ends of Pst I-linearized pPICZαB vector (Thermo Fisher). The PCR product was cloned into this expression vector using the In-Fusion HD Cloning Kit according to manufacturer protocol (Clontech) and verified by DNA sequencing. Transformation of *P. pastoris* X-33 cells (Thermo Fisher) and protein expression were carried out as described previously [31, 32].

The yeast medium containing recombinant SmSP2 was centrifuged (3,000 *g* for 10 min), and the supernatant filtered (0.45 μm), lyophilized and dissolved in 20 mM MES buffer, pH 6.0 (to 10% of the original volume). The solution was then desalted over a Sephadex G-25 column equilibrated with the same buffer. SmSP2 was purified using chromatography on a HiTrap Benzamidine FF column (GE Healthcare Life Sciences) equilibrated with 50 mM Tris-HCl, pH 8.0, containing 0.5 M NaCl, and eluted with 50 mM glycine, pH 3.0. The pH of the eluted fractions containing SmSP2 was adjusted to 6.0 by addition of 1M Tris-HCl, pH 8.0, and enzyme fractions were stored in -80˚C. The purification process was monitored with a kinetic activity assay using the fluorogenic substrate, P-F-R-AMC (AMC, 7-amino-4-methylcoumarin), and by SDS-PAGE and Western blotting.

Expression, refolding, purification of recombinant SmSP2 in *E. coli*

The Champion pET directional expression kit (Thermo Fisher) was selected for bacterial expression of the SmSP2 protease domain (residues 201–501) that contained a short N-terminal propeptide (residues 183 to 200, Fig 1). The 957 bp ORF was PCR amplified from a synthetic SmSP2 gene using specific forward (5´- caccATGAATCTAACTAATACATGTGGAA TACGTAAATCA-3´) and reverse (5´- AAATATTTTTGTTGCTAATTGTTTTGATATCC AA-3´) primers. The PCR product was cloned into the expression vector pET100/D-TOPO (Thermo Fisher) incorporating an N-terminal His tag and verified by DNA sequencing. Recombinant SmSP2 was produced in *E. coli* BL21(DE3) (Thermo Fisher), purified from inclusion bodies using Ni^{2+} chelating chromatography under denaturing conditions and the purified protein was refolded using the dialysis protocol described in [33].

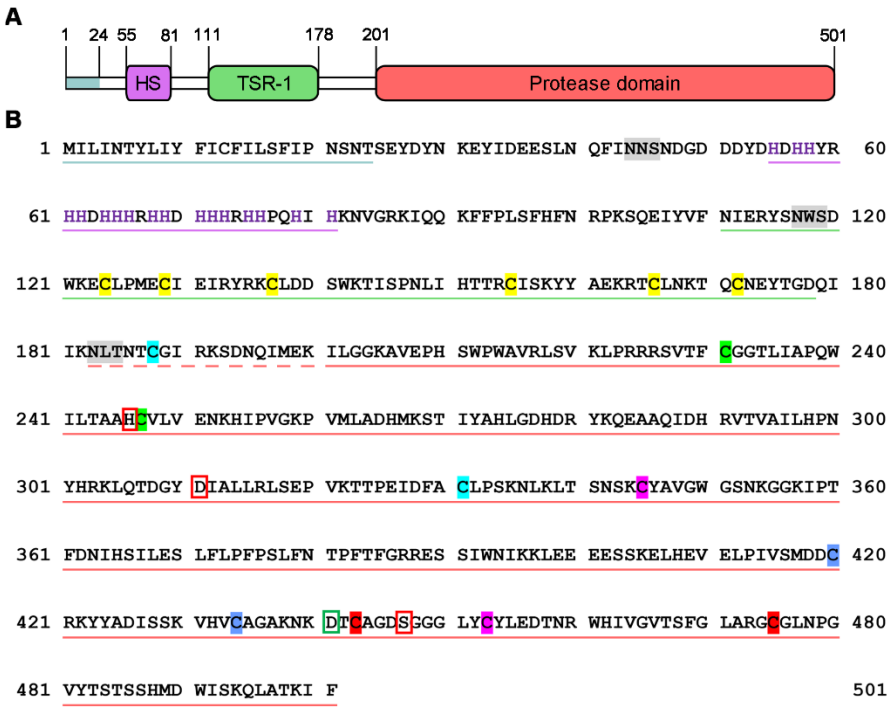

Fig 1. Domain organization and amino acid sequence of SmSP2. (A) Schematic diagram of the domain layout. The N-terminal signal peptide, "His stretch' (HS), thrombospondin type 1 repeat (TSR-1) and S1 family protease domains are depicted in blue, purple, green, and red, respectively. Amino acid residue numbers are indicated. (B) The amino acid sequence of SmSP2 with the various domains color-coded by underlining as in (A). Predicted N-glycosylation sites are highlighted in grey, and His residues in the His stretch are in purple. The catalytic residues, His246, Asp311 and Ser447 are red-boxed; and Asp441 in the S1 subsite that accounts for trypsin-like activity is green-boxed. Cys residues of the protease domain that are predicted to form a disulfide are indicated by the same color; Cys residues of the TSR-1 domain are colored yellow. The propeptide is underlined with dashed red line.

https://doi.org/10.1371/journal.pntd.0006446.g001

Preparation of antibodies and immunoblotting

Specific polyclonal antibodies were generated in rabbits (Moravian Biotechnology) against the bacterially-expressed SmSP2 protease domain. Antigen (50 μg in Freund's incomplete adjuvant) was administered three times each three weeks apart. IgG was isolated from the serum by affinity chromatography with a HiTrap Protein A column (GE Healthcare Life Sciences) according to the manufacturer's protocol. Immunoselection of SmSP2-specific IgG was carried out using a standard methodology [34]. Briefly, 800 μg of recombinant SmSP2 expressed in *E. coli* was resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Excised strips containing SmSP2 were blocked with 3% BSA in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.1% Tween (TTBS) for 1 h, washed with TTBS, and incubated with the protein-A purified anti-SmSP2 IgG overnight at 4˚C. After washing, bound antibodies were eluted with 100 mM glycine, pH 2.5 and the elution was immediately adjusted to pH 7.5 using 1 M Tris-HCl.

For immunoblotting, adult schistosome homogenate (30 μg protein) and rSmSP2 (1 μg) were resolved by SDS-PAGE (4–12% NuPAGE gel, Thermo Fisher) under reducing conditions and transferred onto a PVDF membrane. The membrane was blocked for 1 h with 3% BSA in TTBS, washed with TTBS and incubated overnight with anti-SmSP2 polyclonal IgG diluted 1:200 in TTBS. After washing with TTBS, the membrane was incubated for 1 h with goat horseradish peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich) at a dilution of 1:10,000. After washing with TTBS, the membrane was developed with Luminata Crescendo Western HRP substrate (Merck) and imaged using an ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences).

Immunofluorescence microscopy

Adult *S. mansoni* males and females were carefully separated over ice, washed three times in 100 mM sodium phosphate, pH 7.4, containing 154 mM NaCl (PBS), fixed in Bouin's solution (Sigma-Aldrich) for 45 min at 25˚C, and embedded in paraffin blocks. Sections (6–8 μm each) were deparaffinized in xylol, rehydrated in an ethanol series of decreasing ethanol concentration and boiled in a water bath in 10 mM sodium citrate, pH 6.0, for 15 min. Cooled sections were rinsed with PBS, permeabilized in PBS containing 0.25% Triton X-100 (PBS/Triton) for 20 min, and blocked overnight at 4˚C with 2% BSA in PBS/Triton. After washing in PBS/Triton, the sections were incubated for 60 min at 4˚C with rabbit polyclonal anti-SmSP2 IgG diluted 1:25 in PBS/Triton containing 1% BSA. Sections were then washed three times in PBS/Triton and incubated for 30 min at 25˚C with anti-rabbit IgG Alexa 647-conjugated secondary antibody (Thermo Fisher) diluted 1:500 in PBS/Triton. After washing with PBS/Triton, the sections were mounted in Prolong Diamond antifade reagent containing DAPI (Thermo Fischer). The fluorescence was visualized using an Olympus IX83 microscope equipped with PCO edge 5.5 camera and CoolLED pE-4000 LED illumination system. DAPI signal was detected using excitation diode 365 nm and emission filter 440/40 nm, Alexa 647 using diode 635 nm and emission filter 700/75. Appropriate lightning settings were determined using control slides probed with pre-immune serum to define the background signal threshold. Image stacks of optical sections were processed using the Fiji software.

Active site labeling of SmSP2

Recombinant SmSP2 expressed in *P. pastoris* (1 μg) was incubated 1 h at 37˚C with 1 μM activity-based probe BoRC [35] in 50 mM Tris-HCl, pH 8.0. The competitive labeling reaction was also performed after treatment of SmSP2 for 15 min at 37˚C with 1 mM serine protease inhibitor Pefabloc SC (Sigma-Aldrich). The labeled SmSP2 was precipitated with acetone, resolved by SDS-PAGE, and visualized in-gel using a Typhoon 9410 imager (GE Healthcare) as described previously [36].

Kinetic SmSP2 activity and inhibition assays

SmSP2 activity was measured in continuous kinetic assays using the following peptidyl fluorogenic AMC substrates (Bachem): Z-F-R-AMC (Z, Benzyloxycarbonyl), Bz-F-V-R-AMC (Bz, Benzoyl), P-F-R-AMC, Boc-L-R-R-AMC (Boc, t-Butyloxycarbonyl), Boc-Q-A-R-AMC, Boc-V-L-K-AMC, Suc-A-A-F-AMC (Suc, Succinyl), Suc-A-A-P-F-AMC, MeOSuc-A-A-P-V-AMC (MeOSuc, 3-Methoxysuccinyl), Z-G-G-L-AMC, Z-V-K-M-AMC, Boc-L-G-R-AMC, Z-V-V-R-AMC, V-P-R-AMC, Z-R-R-AMC, R-R-AMC, R-AMC and Boc-L-L-V-Y-AMC. Assays were performed at 37˚C in white 96-well microplates in a total volume of 100 μl. Recombinant SmSP2 expressed in *P. pastoris* (10 ng) was pre-incubated for 10 min at 37˚C in 80 μl of 0.1 M Tris-HCl, pH 8.0. The reaction was initiated by adding 20 μl of substrate

Table 1. Sensitivity of rSmSP2 to protease inhibitors.

Inhibitor[a]	Target protease[b]	Concentration (μM)	Inhibition (%)[c]
Pefabloc SC	SP	1000	100
Benzamidine	SP	1000	100
3,4-dichlorcoumarin	SP	100	71.4 ± 3.3
Antipain	SP, CP	20	100
Leupeptin	SP, CP	20	100
BPTI (Aprotinin)	SP	10	87.0 ± 2.6
STI	SP	10	91.8 ± 0.9
α-1-antichymotrypsin	SP	1	13.2 ± 2.4
α-1-antitrypsin	SP	1	79.0 ± 0.9
Antithrombin III	SP	1	99.1 ± 0.3
PAI-1	SP	1	100
E-64	CP	10	7.2 ± 0.9
Pepstatin A	AP	1	0
EDTA	MP	1000	3.2 ± 0.9
Bestatin	MP	1	4.5 ± 2.0
Phenanthroline	MP	1000	26.9 ± 4.7
α-2-macroglobulin	all types	0.1	61.6 ± 0.4

[a] Abbreviations: BPTI (bovine pancreatic trypsin inhibitor), STI (soybean trypsin inhibitor), E64 (trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane), PAI-1 (plasminogen activator inhibitor-1).

[b] The target proteases are classified based on catalytic type into aspartic (AP), cysteine (CP), serine (SP) proteases and metalloproteases (MP).

[c] rSmSP2 was pre-incubated with the given inhibitor and remaining activity was measured in a kinetic assay with the fluorogenic substrate P-F-R-AMC. The mean values ± S.D. of three replicates are expressed as the percentage inhibition relative to uninhibited controls.

https://doi.org/10.1371/journal.pntd.0006446.t001

solution (50 μM final concentration) in the same buffer. Proteolytic activity was measured continuously in an Infinite M1000 microplate reader (Tecan) at excitation and emission wavelengths of 360 and 465 nm, respectively. The pH activity profile was determined in 50 mM citrate, 100mM phosphate (pH range 3.0–7.5), and 0.1M glycine (pH range 8.0–11.0) using the P-F-R-AMC substrate. The pH profile of SmSP2 activity in schistosome homogenates (1–5 μg of protein) was measured analogously, but in the presence of 10 μM E-64 and 1 mM EDTA to prevent undesired proteolysis by cysteine proteases and metalloproteases, respectively. Protease activity was expressed as the remaining portion that was sensitive to 1 mM Pefabloc SC. For inhibition measurements, inhibitors were added to the 80 μL pre-incubation solution at the final concentrations listed in Table 1. After 10 min at 37˚C, reactions were initiated by the addition of substrate. For pH stability determinations, SmSP2 was incubated in 50 mM citrate, 100mM phosphate (pH range from 3.0 to 7.0) or 0.1M glycine (pH range from 8.0 to 11.0). After 1, 4 and 20 h, aliquots containing 10 ng SmSP2 were taken and SmSP2 activity was measured using P-F-R-AMC as described above. All measurements were done in triplicate.

Interaction of SmSP2 with protein substrates

Recombinant SmSP2 expressed in *P. pastoris* (300 ng) was incubated at 37˚C with 1–20 μg of human plasminogen (hPLG, R&D Systems), high molecular weight human plasma kininogen (HMWK; Merck), human tissue plasminogen activator (tPA), human serum albumin (HSA), human hemoglobin, calf collagen type I, human fibronectin and rabbit immunoglobulin G (all Sigma-Aldrich) in 25 μL 100 mM Tris-HCl, pH 8.0. After incubation (between 1 and 48 h), the reaction was stopped by adding Pefabloc SC (final concentration 1 mM) and 20 μl of the

reaction was resolved by SDS-PAGE (4-12% Nupage gel) and stained with Coomassie Brilliant Blue G250. In control experiments, protein was incubated in the absence of SmSP2 and analyzed under identical conditions. Processing products generated during HMWK degradation were identified by mass spectrometry, the reaction mixture was analyzed using LC-MS/MS as described previously [32, 37]. To analyze the activation of hPLG and tPA by SmSP2, aliquots of the reaction mixtures containing 100 ng of hPLG or tPA were withdrawn at different time intervals and activity was measured in a kinetic assay using 50 μM Boc-V-L-K-AMC or Z-G-G-R-AMC, respectively, in 100 mM Tris-HCl, pH 7.5, containing 100 mM NaCl. Proteolytic activity was measured continuously as described above. In control experiments, SmSP2, hPLG or tPA alone were analyzed under identical conditions.

Hydrolysis of peptide hormones by SmSP2

Extended bradykinin (Ac-SLMKRPPGFSPFRSSR-amide, Ac, acetyl) was synthesized as a peptidyl amide by Fmoc solid phase chemistry in an ABI 433A peptide synthesizer (Applied Biosystems), as described previously [19, 38]. Recombinant SmSP2 expressed in *P. pastoris* (200 ng) was incubated at 37˚C for 1 to 16 h with 25 nmol of bradykinin, lysyl-bradikinin (kallidin), vasopresin (all Sigma-Aldrich) or extended bradykinin in 0.1 M Tris-HCl, pH 8.0, in a total volume of 50 μL. The reaction was stopped by adding TFA to a final concentration of 1%. The resulting fragments were purified by reverse-phase HPLC using a Luna C18 column (25 x 0.46 cm, Phenomenex) and the TFA/acetonitrile system, and characterized by MS/MS [32, 37].

Hydrolysis of peptides by cultured schistosomes

Adult worms were washed and treated as described above (section Preparation of schistosome extract, excretory/secretory products collection and purification of native SmSP2). Five adult schistosome pairs were then placed into clear, 24-well, flat-bottom plates (Costar) containing 500 μL Basch Medium 199 [28], supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin (without fetal bovine serum). Human vasopressin or extended bradykinin (10 μM) was added and the incubation continued for 16 h at 37˚C under a 5% CO2 atmosphere. In control experiments, the peptides were incubated in the same system but in the absence of schistosomes. After incubation, the samples were ZipTiped and the resulting fragments were analyzed using MALDI-TOF performed on an UltrafleXtreme (Bruker Daltonik) operated in reflectron mode with an acceleration voltage of 25 kV and a pulsed ion extraction of 120 ns. Desorption and ionization were achieved using a Smartbeam II laser. α-Cyano-4-hydroxycinnamic acid was used as a matrix. The data were acquired from m/z 220 to 3700 and analyzed with the FlexAnalysis 3.3 software (Bruker Daltonik). The mass spectra were externally calibrated using a Peptide Calibration Standard I (Bruker Daltonik) and averaged from 3000 laser shots.

Subsite profiling of SmSP2 by a positional scanning synthetic combinatorial library (PS-SCL) and by multiplex cleavage assays

Synthesis of the PS-SCL has been previously described [39]. The assays were carried out in black 96-well microplates in 0.1 M Tris-HCl, pH 8.0, containing 0.01% Tween 20 and initiated by addition of recombinant SmSP2 (10 ng). Release of 7-amino-4-carbamoylmethylcoumarin (ACC) was measured in an SpectraMax Gemini fluorescence spectrometer (Molecular Devices) with excitation and emission wavelengths set to 380 and 460 nm, respectively.

The Multiplex Substrate Profiling by Mass Spectrometry (MSP-MS) assay was performed as previously described [29]. SmSP2, human plasma kallikrein (Sigma-Aldrich) and bovine trypsin Sigma-Aldrich) (all 17 nM) were incubated in triplicate with a mixture of 228 synthetic

tetradecapeptides (0.5 uM each) in 10 mM Tris-HCl, pH 8.0. After 15, 60, and 240 minutes, 20 μL aliquots were removed, quenched with 6.4 M guanidinium chloride, immediately frozen at -80˚C. Control reactions were performed by treating enzymes with guanidinium chloride prior to peptide exposure. Samples were acidified to < pH 3.0 with 1% formic acid, desalted with C18 LTS tips (Rainin), and injected into a Q Exactive Mass Spectrometer (Thermo) equipped with an Ultimate 3000 HPLC (Thermo). Peptides separated by reverse phase chromatography on a C18 column (1.7 um bead size, 75 um x 25 cm, heated to 65˚C) at a flow rate of 300 nL min^{-1} using a 55-min. linear gradient from 5% B to 30% B, with solvent A: 0.1% formic acid in water and solvent B: 0.1% formic acid in acetonitrile. Survey scans were recorded over a 150–2000 m/z range at 70000 resolutions (AGC target 1×106, 75 ms maximum). MS/MS was performed in data-dependent acquisition mode with HCD fragmentation (30 normalized collision energy) on the 10 most intense precursor ions at 17500 resolutions (AGC target 5×104, 120 ms maximum, dynamic exclusion 15 s).

Peak integration and peptide identification were performed using Peaks software (Bioinformatics Solutions Inc.). Quantification data are normalized by LOWESS and filtered by 0.3 peptide quality. Missing and zero values are imputed with random numbers in the range of the average of smallest 5% of the data ± sd. Differences between each time point and no-enzyme control were analyzed for statistical significance by multiple t-test. When compared to the control reaction, peptide cleavage products with >10-fold change in peak area intensity and p-value <0.05 were identified and the peptide sequence corresponding to the P4 to P4' subsite positions were used to make IceLogo frequency plots [40]. Mass spectrometry data and results can be accessed here: ftp://massive.ucsd.edu/MSV000081747.

Molecular modeling of SmSP2

A spatial model of the SmSP2 protease domain was constructed by homology modeling, as described previously [37]. Briefly, the X-ray structures of human mannan-binding lectin serine protease 1 (MASP-1) and bovine trypsin ((Protein Data Bank (PDB) entries: 3GOV and 1JRT, respectively) were used as templates. The homology module generated by the MOE program (Chemical Computing Group, Canada) was used to model the SmSP2 structure. The inhibitor conformation was refined by applying the LigX module of the MOE and the final binding mode of the inhibitor was selected by the best-fit model based on the London dG scoring function and the generalized Born method [37]. Molecular images were generated with UCSF Chimera (http://www.cgl.ucsf.edu/chimera/).

Results

SmSP2 is a S1 family protease with a unique domain organization

The SmSP2 open reading frame consists of 1,506 bp that encode a protein of 501 amino acid residues with a theoretical molecular mass of 58 kDa. The predicted signal sequence is 24 residues long and the amino acid sequence contains three potential N-glycosylation sites (Fig 1). Based on sequence homology analysis, we describe SmSP2 as a multi-domain protein made up of an N-terminal region (residues 25 to 110), a thrombospondin-1 type 1 repeat (TSR-1) domain (residues 111 to 178) and a S1 family serine protease domain at the C-terminus (residues 201 to 501). The N-terminal region lacks significant similarity with other published protein sequences in databases. The striking feature of this region is the presence of a stretch of histidine residues (the 'His stretch'—residues 55 to 81) which suggests that SmSP2 may bind metal ions.

The TSR-1 domain has been identified in multiple protein families and occurs in more than 40 human proteins, *e. g.*, thrombospondins, ADAMTS (A Disintegrin And

Metalloproteinase with Thrombospondin Motif), properdin and some complement factors [41]. It is known to mediate cell adhesion, protein-protein interactions, glycosaminoglycan binding and inhibit angiogenesis [42, 43]. The TSR-1 of SmSP2 consists of about 60 residues and its sequence contains all of the important conserved features of TSR-1 domains (S1 Fig): a cysteine residue pattern and conservation of tryptophan and arginine residues forming so called W and R layers [44].

The catalytic protease domain of SmSP2 belongs to the S1 family of serine proteases and has about 30% identity with other members of this family (S2 Fig). The protease domain of SmSP2 possesses a catalytic triad of His246, Asp311 and Ser447 (corresponding to amino acid residues 57, 102 and 195 by standard chymotrypsinogen numbering), which is typical of S1 family proteases. In addition, the amino acids surrounding the catalytic-triad residues have the highest sequence identity with other S1 family enzymes (S2 Fig). Cysteine residues in the catalytic domain form four conserved disulfide bonds that can be predicted by the alignment with the solved crystal structures of S1 family proteases (S2 Fig). Moreover, an additional cysteine residue, Cys311, is likely to form a disulfide bond with a Cys188 in the N-terminal region. An analogous disulfide bond is described, for example, in bovine chymotrypsinogen, human plasmin, urokinase (uPA), tissue plasminogen activators (tPA) and MASP-1. In bovine trypsin, Asp189 is located at the bottom of the S1 binding site and determines the trypsin-like specificity for substrates with Arg/Lys in the P1 position [45]. This residue is conserved in the sequence of SmSP2 (Asp441). When compared to bovine chymotrypsin, the S1 family holotype protease [46], SmSP2 has three insertions (S2 Fig) located between residues 222 and 226 (insertion-222), 251 and 268 (insertion-251), and between residues 358 and 400 (insertion-358). Whereas protein sequences corresponding to the short insertions-222 and -251 are found in uPA/tPA and MASP1, respectively, the long insertion-358 is unique to SmSP2.

SmSP2 orthologs are found in: (i) other schistosome species—*S. japonicum* (GenBank: AAW24683.1) and *S. haematobium* (XP_012796372.1) sharing 80% and 78% sequence identity, respectively; (ii) other trematodes, including, *Fasciola hepatica* (identified in the transcriptome database [47] - 53% identity), *Opisthorchis viverrini* (XP_009167273.1–49% identity), *Clonorchis sinensis* (GAA32831.2–47% identity); and (iii) the sequences of cestodes such as *Hymenolepis microstoma* (CDS25513.1), *Echinococcus multilocularis* (CDI97096.1), *Echinococcus granulosus* (CDI97096.1), and *Taenia solium* (ADP89566.1) sharing 27–39% identities (S3 Fig). All of the ortholog sequences contain the TSR-1 and protease domains, however, they differ in the N-terminal region in length and the presence of the His stretch that is unique to trematodes. Moreover, the insertion-358 in the cestode sequences is shorter and contains two additional Cys residues that might potentially form a disulfide stabilizing this structure (S3 Fig). To conclude, SmSP2 and its orthologs are serine proteases with unique domain organization found exclusively in the phylum Platyhelminthes.

Homology model of SmSP2 reveals the trypsin-like active site pocket shielded by additional loops

A spatial model of the SmSP2 protease domain was constructed by homology modelling to provide a structural framework to analyze structure-activity relationships. The X-ray structure of bovine trypsin (PDB code 1JRT) and human MASP-1 (PDB code 3GOV) were used as templates. Fig 2A shows that the SmSP2 protease domain has the conserved architecture of S1 family proteases which consists of two six-stranded β-barrel domains packed against each other. The catalytic amino acid residues, His246, Asp311 and Ser447 are located at the junction between these β-barrel domains. The major sequence insertions in SmSP2 compared to trypsin are located at surface-exposed loops surrounding the substrate binding region (Fig 2B).

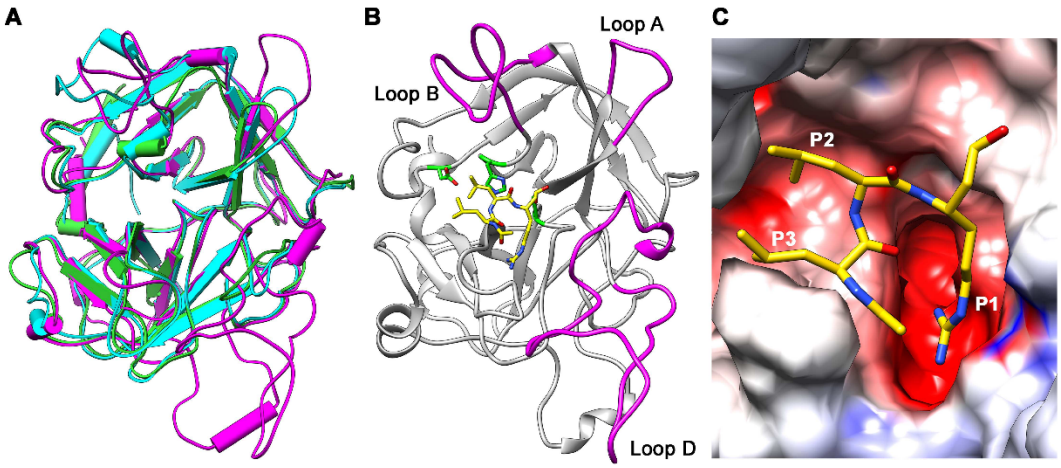

Fig 2. Homology model of the SmSP2 protease domain. The model was built using as a template the X-ray structures of bovine trypsin (PDB 1JRT) and human MASP-1 (PDB 3GOV). (A) A superposition of the SmSP2 model (magenta), the bovine trypsin (green) and MASP-1 (cyan) crystal structures in a cylinder representation. (B) A view from the top on the SmSP2 active site with covalently bound substrate-like inhibitor leupeptin (N-acetyl-L-leucyl-L-leucyl-L-argininal). Carbon atoms of leupeptin are yellow; heteroatoms have the standard color coding (N, blue; O, red). SmSP2 catalytic residues are green. The active site is partially blocked by loops A, B and D (magenta) that are formed by insertions in SmSP2 sequence compared to trypsin sequence. (C) A surface representation of the SmSP2 active site colored by electrostatic potential (at a scale from -10 kT/e (red) to +10 kT/e (blue)). Inhibitor leupeptin is colored as in (B).

https://doi.org/10.1371/journal.pntd.0006446.g002

SmSP2 insertions-222 and -251 are located on the top of the loops B and A, respectively (Perona and Craik nomenclature [48]). Their structural analogs can be found in the structure of the MASP-1 catalytic domain where they putatively interact with substrates [49]. The insertion-358 containing 43 residues are located at loop D; however, it has no structural analog in the PDB database (www.rcsb.org) to serve as a template for modeling. The secondary structure prediction did not reveal any conformational element in the loop, suggesting an unstructured character and flexibility that might be involved in interactions during substrate binding.

The ligand binding mode of the SmSP2 protease domain was further analyzed using leupeptin (N-acetyl-L-leucyl-L-leucyl-L-arginal), a transition-state analog protease inhibitor that inhibits SmSP2 (Table 1). Leupeptin was docked into the SmSP2 active site based on the crystallographic complex of this inhibitor with trypsin (PDB code 1JRT). The docking model (Fig 2) suggests that the arginal residue of the inhibitor forms a covalent hemi-acetal linkage with the catalytic Ser447 whereas leupeptin's side chain binds to a deep negatively charged S1 subsite pocket containing Asp441 at the bottom. This type of S1 binding subsite is the primary substrate specificity determinant of trypsin-type proteases and responsible for a substrate/inhibitor preference for basic residues at the P1 position [45].

To conclude, the model of the SmSP2 protease domain indicates that it is a S1 family protease with an trypsin-like substrate binding groove; shielded by surface exposed loops that surround the active site, namely, insertions-222 and -358, may modulate SmSP2's selectivity.

Recombinant expression of SmSP2 and identification of native SmSP2

The protease domain of SmSP2 (rSmSP2, residues 183–501) was expressed in *P. pastoris* and purified. The active enzyme cleaved Z-F-R-AMC and migrated on SDS-PAGE as a single band of approximately 28 kDa (Fig 3A), consistent with the expected molecular mass of 30 kDa.

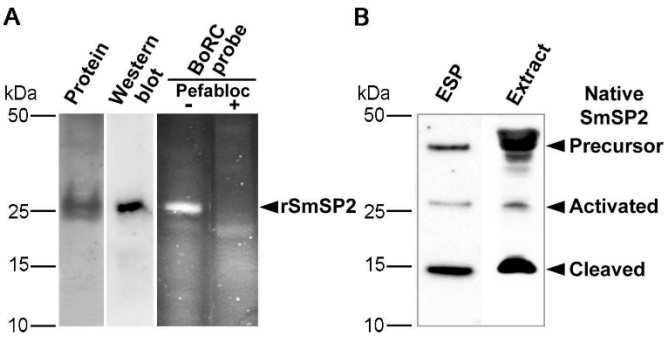

Fig 3. Preparation of recombinant SmSP2 and identification of native SmSP2. (A) The recombinant protease domain of SmSP2 (rSmSP2) expressed in *P. pastoris* was resolved by SDS-PAGE and protein-stained or visualized by polyclonal anti-rSmSP2 IgG. For in-gel activity-based labeling, rSmSP2 was incubated with the fluorescent active site probe, BoRC, resolved by SDS-PAGE and visualized using a fluorescence scanner. The competitive labeling was performed with the serine protease inhibitor, Pefabloc SC. (B) Protein extracts of *S. mansoni* adult worms and their ESP were resolved by SDS-PAGE and visualized by the anti-rSmSP2 IgG.

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rSmSP2 was visualized using the activity-based probe Bodipy-F-P-R-Cmk (BoRC) (Fig 3A) [35]; labeling by the probe was prevented by pre-incubation of the enzyme with the serine protease inhibitor, Pefabloc SC. Rabbit polyclonal antibodies, raised against the SmSP2 protease domain expressed in *E. coli*, reacted with the rSmSP2 by immunoblotting (Fig 3A). In homogenates of adult schistosomes, the antibody recognized three bands of approximately 45, 28 and 15 kDa (Fig 3B), which correspond to the predicted mass of the SmSP2 precursor, the activated SmSP2 protease domain without N-terminal domains and the two-chain form derived by further processing, respectively. Based on its molecular size, we hypothesize that this form was clipped in the insertion-358 loop D. Analogous immunoreactive bands were also demonstrated in the ESP of adult schistosomes (Fig 3B), indicating that SmSP2 is released into the host environment.

The full length SmSP2 sequence contains a stretch of histidine residues that may interact with metal ions. Indeed, native SmSP2 from schistosome extracts bound to a Ni^{2+}- affinity chromatography column and eluted using imidazole in solution (S4 Fig). The eluate contained the three forms of SmSP2 described above. The data also indicate that the activated and clipped protease domains (28 and 15 kDa bands) are disulfide-linked to the N-terminal portion of the molecule that contains the histidine stretch.

Substrate and inhibitor specificity classifies SmSP2 as a trypsin-like enzyme

The substrate specificity of rSmSP2 expressed in *P. pastoris* was initially explored using a panel of specific peptidyl fluorogenic substrates. Two sets of diagnostic protease substrates were used: (i) substrates with a basic amino acid residue (Arg and Lys) at the P1 position that are cleaved by trypsin-like serine proteases, and (ii) substrates containing bulky hydrophobic (Phe and Tyr) or aliphatic residues (Val, Leu and Met) at P1 that are cleaved by chymotrypsin- or elastase-like serine proteases, respectively [50]. The data show that rSmSP2 predominantly hydrolyzed trypsin substrates (Fig 4A). Activity was greatest with Bz-F-V-R-AMC and Z-R-R-AMC, whereas less activity was measured with Z-V-V-R-AMC, Z-V-P-R-AMC and Z-R-R-AMC. The cleavage of related short substrates with free N-termini occurs only very

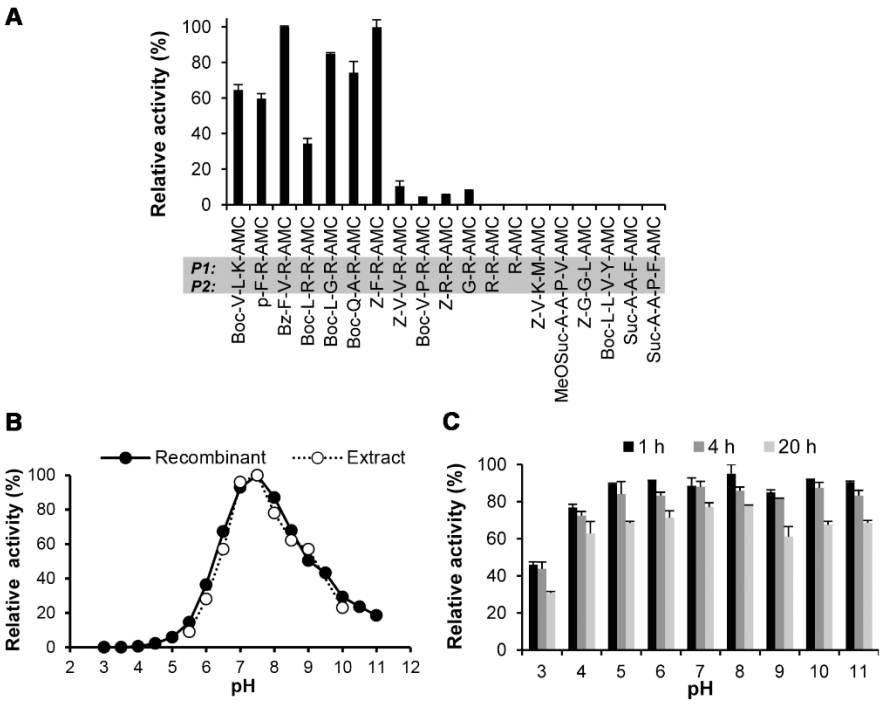

Fig 4. Substrate specificity and pH profile of SmSP2. (A) Activity of rSmSP2 was probed using a panel of peptidyl fluorogenic substrates used to assay trypsin-like and chymotrypsin/elastase-like serine proteases. Substrate hydrolysis was measured in a kinetic assay at pH 8.0. The mean values ± S.D. of three replicates are normalized to the maximum value. Amino acid residues at P1 and P2 positions are highlighted by the grey bar. (B) The pH profiles of rSmSP2 and native SmSP2 activity in extracts of adult worms. Activity was measured in a kinetic assay using the fluorogenic substrate P-F-R-AMC. The native activity (sensitive to the serine protease inhibitor Pefabloc SC) was measured in the presence of 10 μM E-64 and 1 mM EDTA to prevent undesired proteolysis of the substrate by cysteine proteases and metalloproteases, respectively. The mean values of three replicates, expressed as a percentage normalized to the highest value, are shown (standard deviation values are within 5% of the mean). (C) The pH stability of rSmSP2. Activity of rSmSP2 was measured at pH 8.0 in a kinetic assay as in (B) after incubation of the enzyme at pH 3 to 11 for different times. The mean values of three replicates, expressed as a percentage normalized to activity of non-incubated rSmSP2, are shown.

https://doi.org/10.1371/journal.pntd.0006446.g004

slowly (G-R-AMC) or not at all (R-R-AMC and R-AMC), suggesting that SmSP2 is not an aminopeptidase trimming N-terminal residues of substrates. The chymotrypsin/elastase substrates were not effectively hydrolyzed.

The pH activity profile of rSmSP2 was determined using the P-F-R-AMC and was similar to that of the serine protease activity in the schistosome adult homogenate (Fig 4B). Effective hydrolysis was observed between pH 6.0 and 10.0 with optimal activity around pH 8.0. No SmSP2 activity was detected below pH 5.0, although rSmSP2 is stable above pH 4.0 (Fig 4C).

To detail the cleavage specificity of rSmSP2, two distinct methods for unbiased substrate profiling were employed. First, a positional scanning-synthetic combinatorial library (PS-SCL) [39] was used to analyze specificity at the substrate positions P1 to P4 (Fig 5A). The cleavage map shows that rSmSP2 prefers basic residues (Lys and Arg) at the P1 position. The P2 and P3 positions display promiscuous specificity, although basic residues at the P2 position, and acidic

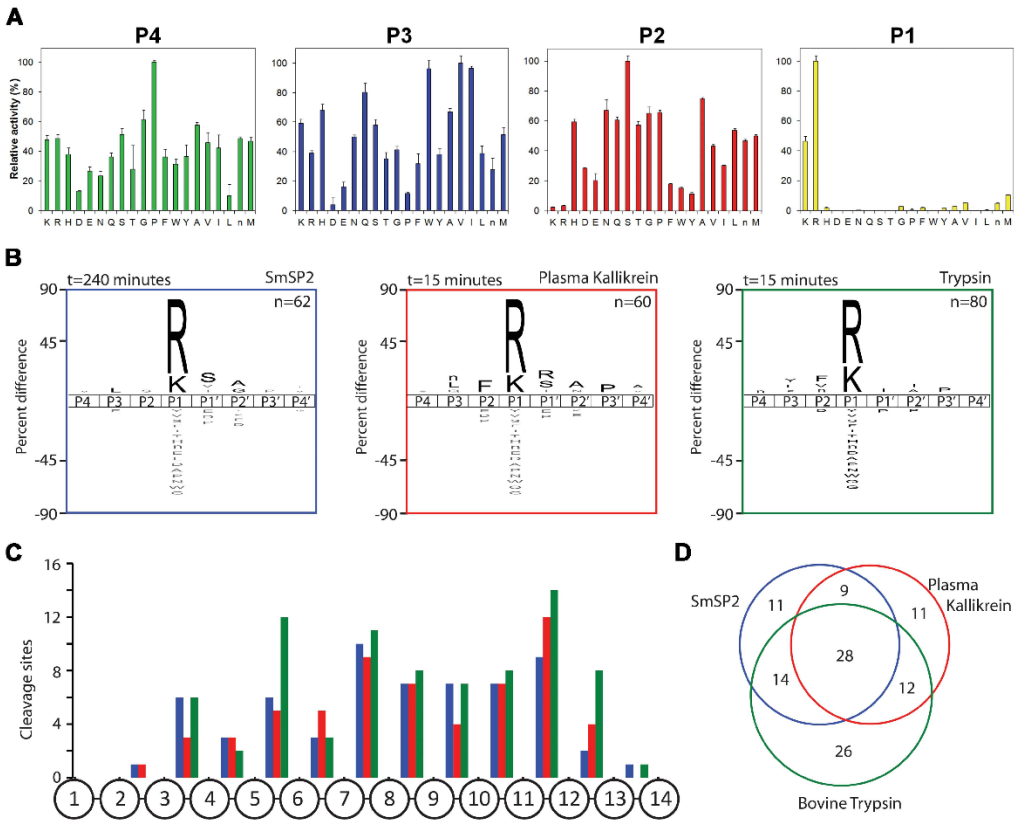

Fig 5. Substrate specificity of rSmSP2. (A) The P1 to P4 specificity of rSmSP2 was determined by a positional scanning-synthetic combinatorial library. The X-axis indicates 20 amino acids held constant at each position (n is norleucine). The Y-axis represents activity related to the most preferred amino acid (100%). (B) The P4 to P4′ specificity profiles of rSmSP2, human plasma kallikrein, and bovine trypsin were obtained using a multiplex combinatorial library (MSP-MS). The iceLogo substrate profiles were generated from the pattern of cleavage events after incubation with the 14-mer library. Amino acids that are most frequently found at each position are shown above the horizontal line, whereas amino acids that less frequently observed are below. (C) Spatial distribution of cleavage sites within the 14-mer peptide scaffold. (D) The Venn diagram shows the number of unique and shared cleavage sites.

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residues and Pro at the P3 position are unfavorable. Some degree of selectivity was measured for Pro at P4. Second, a sensitive multiplex substrate profiling by mass spectrometry (MSP-MS) was performed with a library of 228 tetradecapeptides [51] to analyze the subsites on both sides of the scissile peptide bond (Fig 5B). The MSP-MS profile confirmed the strong preference for Arg and Lys residues at the P1 position. At the primed sites, the preferred residues were Ser at the P1′, Ala and Gly at P2′ positions, respectively. Compared to human plasma kallikrein and bovine trypsin, the MSP-MS cleavage profile of SmSP2 were similar (Fig 5D) with 28 identical cleavage sites. However, trypsin produced 26 unique cleavages compared to 11 cleavages unique for rSmSP2 or plasma kallikrein. In general, cleaved peptide bonds

occurred away from the amino and carboxyl termini confirming endopeptidase mode of action of all three analyzed enzymes (Fig 5C).

The inhibitor specificity of rSmSP2 was investigated using a panel of small molecule and protein inhibitors (Table 1). rSmSP2 was highly sensitive to inhibitors of S1 family serine proteases, including the small-molecules, Pefabloc SC, benzamidine, leupeptin, antipain, and 3,4-dichlorcoumarin, and the proteinaceous soybean trypsin (STI) and bovine pancreatic trypsin inhibitors (BPTI). rSmSP2 was not affected by inhibitors of aspartic, cysteine and metalloproteases. Protein inhibitors of the serpin family inhibited rSmSP2 with variable efficiencies: PAI-I, α-1-antitrypsin and anti-thrombin III, which interact with trypsin-like proteases, inhibited rSmSP2 activity strongly, whereas the chymotrypsin protease inhibitor, α-1-antichymotrypsin, was weakly effective.

To summarize, SmSP2 hydrolyzes substrates as an endopeptidase and has a strict specificity for basic residues at P1.

SmSP2 releases bradykinin from kininogen and activates plasmin

The activity of rSmSP2 towards host-derived macromolecular substrates was tested with several human and bovine proteins. After incubation, the mixtures were subsequently analyzed by SDS-PAGE (Fig 6A). No hydrolysis was observed for hemoglobin and serum albumin, the two major protein components of vertebrate host blood. Also, neither immunoglobulin G nor collagen was cleaved by rSmSP2. On the other hand, rSmSP2 completely hydrolyzed the blood clotting protein, fibronectin. We also analyzed the processing of three blood proteins: HMWK, tPA and human plasminogen.

Amino acid sequencing showed that HMWK was processed to the kininogen light chain (Fig 6A), and that the heavy chain was completely fragmented. LC-MS/MS analysis of the reaction mixture revealed the release of the HMWK-derived vasodilatory nonapeptide, bradykinin (Fig 6B). To simulate the bradykinin release from HMWK precursor, we designed a synthetic hexadecapeptide (Ac-SLMKRPPGFSPFRSSR-amide) designated extended bradykinin. This peptide was derived from the HMWK sequence to contain processing sites; *i.e.*, the bradykinin sequence (RPPGFSPFR) was extended at the N- terminus by four additional HMWK residues (SLMK) and three residues on C-terminus (SSR). After incubation of this peptide with rSmSP2, the resulting fragments were separated by HPLC and the cleavage positions identified by mass spectrometry. The precursor was cleaved between Lys-Arg and Arg-Ser bonds thereby releasing bradykinin (Fig 6C). Synthetic bradykinin or kallidin (lysyl-bradykinin) were not cleaved by rSmSP2. Also, rSmSP2 degraded vasopressin, a nonapeptide hormone that increases arterial blood pressure by inducing vasoconstriction.

Next, we investigated whether living schistosomes produce a cleavage specificity similar to that of SmSP2 when incubating with vasopressin or extended bradykinin in culture. Adult schistosomes were incubated in the presence of the peptides and cleavage positions analyzed by mass spectrometry (Fig 6C). Both peptides were cleaved when added to the cultivation medium: vasopressin was inactivated by cleavage after penultimate Arg residue; extended bradykinin was cleaved to release bradykinin, however, cleavage precursors extended in N- or C-terminal direction were also identified. The fragmentation was significantly abolished in the presence of a serine protease specific inhibitor Pefabloc. The identified cleavage positions in the hormone sequences were identical with those obtained by *in vitro* fragmentation using rSmSP2.

The processing of human plasminogen, the precursor of the main fibrinolytic protease plasmin, by rSmSP2 was analyzed by SDS-PAGE and kinetic assay. Single chain plasminogen was completely converted into plasmin, consisting of a heavy chain and light chain). This

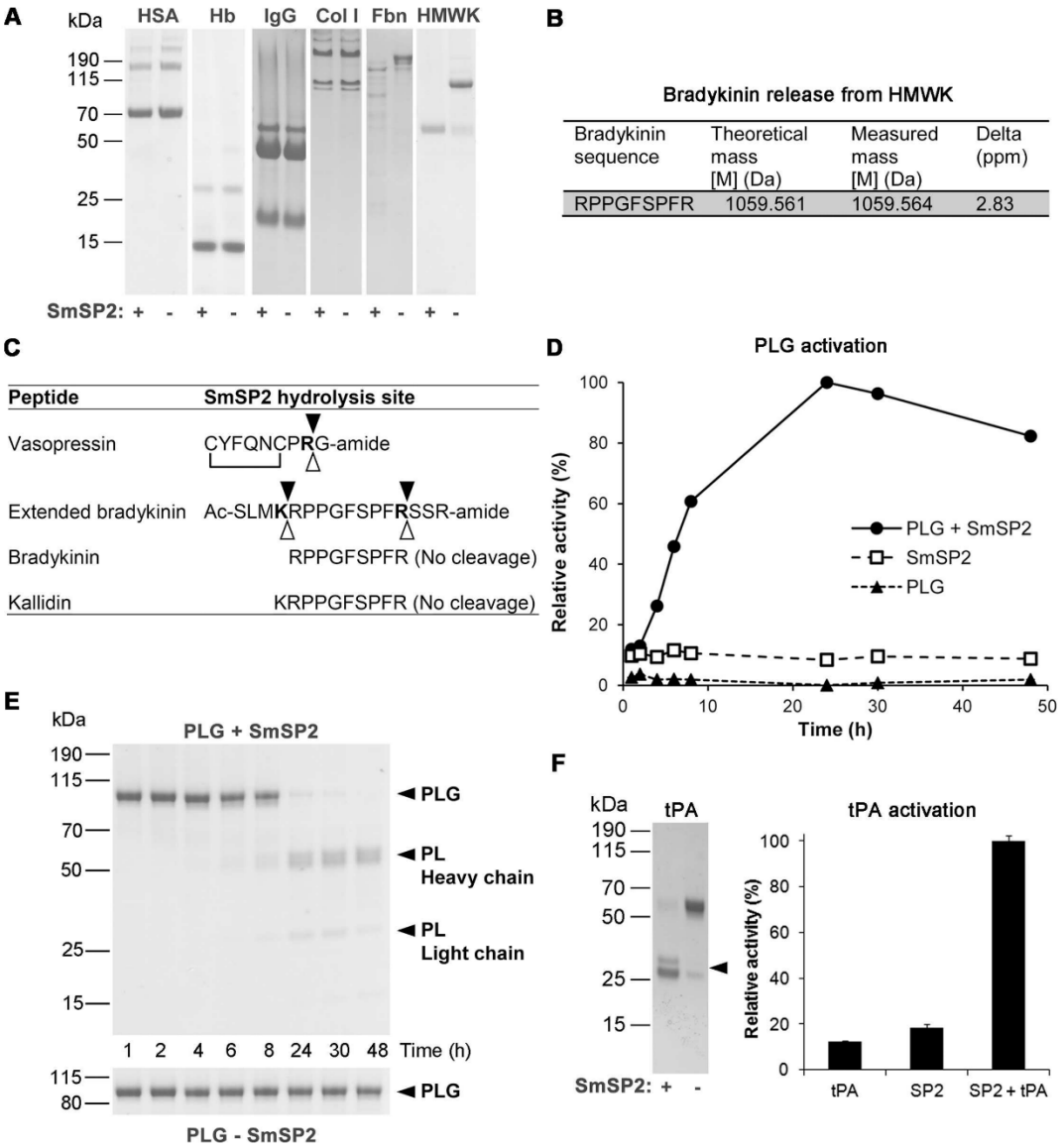

Fig 6. Processing of host-derived proteins and peptides by rSmSP2. (A) Human serum albumin (HSA), hemoglobin (Hb), immunoglobulin G (IgG), collagen type I (Col I), fibronectin (Fbn) and high molecular weight kininogen (HMWK) were incubated for 16 h at pH 8.0 in the presence (+) or absence (-) of rSmSP2. The reaction mixtures were subjected to SDS-PAGE and protein stained. (B) HMWK was incubated with rSmSP2 and the reaction mixture was subjected to LC-MS/MS analysis to identify bradykinin peptide released from HMWK. (C) Peptide hormones were incubated with rSmSP2 or with live adults maintained in culture and the cleavage positions (full triangles for rSmSP2, open triangles for adult schistosomes) were identified by mass spectrometry. Residues at the P1 position are in bold and the disulfide

connectivity of vasopressin is indicated. (D) Human plasminogen (PLG) was incubated in the presence or absence of rSmSP2 and the reaction mixture was analyzed at different time points. Plasmin proteolytic activity generated during plasminogen processing by rSmSP2 was determined in a kinetic assay with Boc-V-L-K-AMC. Mean values of triplicates are expressed relative to the maximum value (100%). The S.D. values of three replicates are within 10% of the mean. All experiments were performed at least twice with similar results. (E) The processed forms were resolved by SDS-PAGE and visualized by protein staining. The positions for PLG, and plasmin (PL) heavy and light chains are indicated. (F) Human tissue plasminogen activator (tPA) was incubated for 16 h at pH 8.0 in the presence/absence of rSmSP2 and analyzed by SDS-PAGE with protein staining; proteolytic activity generated during tPA processing was monitored in a kinetic assay using Z-G-G-R-AMC. Mean values ± s.d. of triplicates are expressed relative to the maximum value (100%). Two chain tPA is indicated with an arrow.

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processing was not the result of the self-activation as it only occurred in the presence of rSMSP2 (Fig 6D and 6E). SDS-PAGE analysis showed that SmSP2 processes single-chain tPA into its two-chain form; the cleavage was associated with a ten-fold increase in tPA activity (Fig 6F). tPA is the major physiological activator of plasminogen, thus the SmSP2 cleavage of single chain tPA into its fully active two-chain form would result in more efficient plasmin activation and in turn more effective fibrinolysis.

To conclude, SmSP2 cleaves several physiologically important blood proteins. Specifically, it processes the extracellular matrix and blood clot component, fibronectin, activates the major fibrinolytic enzyme, plasmin, and its activator, tPA, releases the vasodilatory peptide bradykinin from its HMWK precursor and processes the vasoconstrictory peptide vasopressin.

SmSP2 is localized in the tegument, parenchyma and reproductive organs of adult schistosomes

Indirect immunofluorescence microscopy on semi-thin sections using affinity purified polyclonal antibodies raised against rSmSP2 demonstrated that SmSP2 is expressed in distinct tissues of adult worms (Fig 7 and S5 Fig). In males, SmSP2 was observed in the parenchyma, tegument and in the tegumental surface membranes except the tubercles (for a detailed tegumental localization, see S5 Fig). In females, SmSP2 was observed in parenchyma but not in the tegument. In addition, intense staining was seen in the esophageal region of both genders, in the testes of males and in the ovaria and vitellaria of females (Fig 7). SmSP2 was absent from muscle, the tegumental tubercles, gastrodermis and gut lumen. Pre-immune serum was used as a negative control (S6 Fig) and only faint background fluorescence was detected.

Discussion

Serine proteases of the S1 family are key factors of virulence for many parasitic helminths. They contribute to parasite invasion, migration, nutrition and reproduction, and facilitate adaption to and evasion of the host's physiological and immune responses (for reviews see [12, 52]). Among serine proteases, most attention has been focused on *S. mansoni* cercarial elastases as these enzymes are implicated in tissue invasion and migration into the definitive mammalian hosts [13, 53]; however, information regarding other SmSPs is limited. Recently, we described the repertoire of non-cercarial elastase SmSPs by employing a series of genomic, transcriptomic, functional proteomic and phylogenetic approaches [23].

Here, we focus on SmSP2 as it is the most abundantly expressed SP in the *Schistosoma* blood-dwelling developmental stages [23]. The domain organization of SmSP2 is distantly reminiscent of the modular architecture of host blood-clotting serine proteases. Specifically, these enzymes contain a catalytic trypsin-like serine protease domain linked by disulfide bonds to an N-terminal multi-domain region that is involved in ligand-binding and protein-protein interactions [54]. SmSP2 also has unique features—an N-terminal region histidine stretch and a TSR-1 domain. TSR-1 domains mediate cell adhesion, protein-protein interactions and glycosaminoglycans binding [42, 43]. The histidine stretch may act as a metal binding site for divalent metal ions, as we demonstrated *in vitro* using metal-affinity

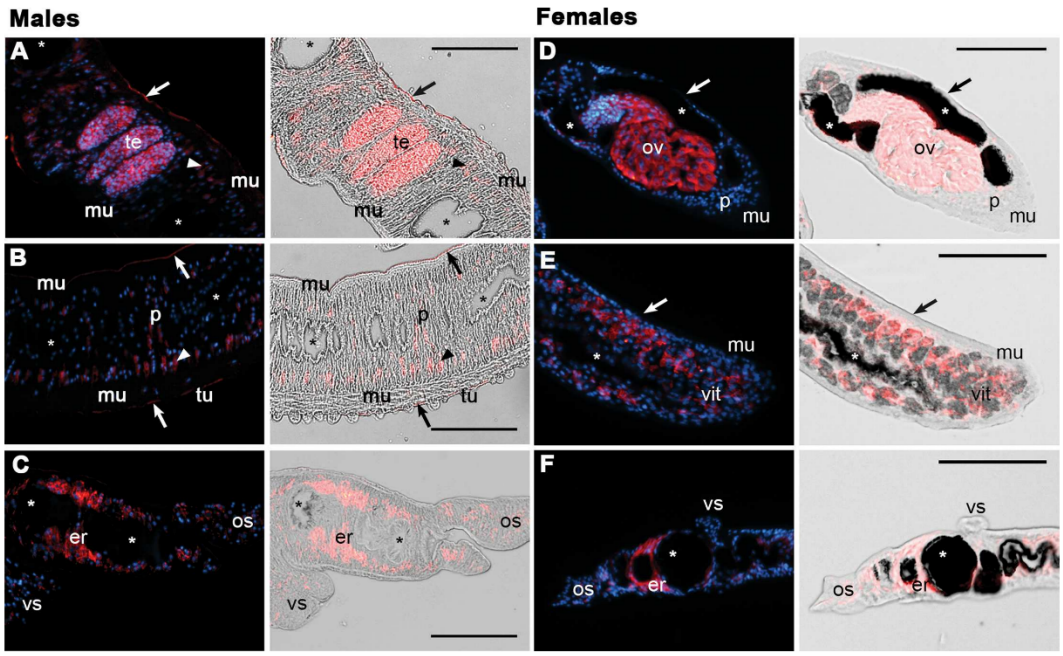

Fig 7. SmSP2 immunolocalization in sections of adult *S. mansoni*. Semi-thin sections of adult *S. mansoni* males (A to C) and females (D to F) were probed with an anti-SmSP2 IgG followed by reaction with an anti-rabbit Alexa 647-labeled secondary antibody (red). DAPI was used to label nuclear DNA (blue). The left columns show merged fluorescent channels; in the right columns, the signal is merged with differential interference contrast. A strong SmSP2 signal (red) was detected in both sexes in the parenchyma (p) and the esophageal region (er). A faint signal was noted in the ventral (vs) and oral suckers (os). No signal was detected in the gut (asterisks), muscle (mu) or tegumental tubercles (tu). In males, SmSP2 signal also appears in the tegument (arrowhead) in the tegumental membrane surface (arrow) and in the testes (te). In females, the signal is noted in the ovaries (ov) and vitellaria (vit). The scale bar represents 100 μm. A and D, reproductive organs; B and E, tegumental cells; C and F, head.

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chromatography to purify native SmSP2 from the adult schistosome homogenate. The TSR-1 domain is also present in the sequences of SmSP2 orthologs in other trematodes; cestodes retain only the TSR-1 domain while the histidine stretch is unique to trematodes (S3 Fig). Orthologous genes were not found in other organisms. Further research is needed to evaluate the exact function of the N-terminal domains of SmSP2.

SmSP2 orthologs (termed mastins) are present in S. *haematobium* and *S. japonicum*. For *S. haematobium* infections, mastin was identified in a protein array as an antigen targeted by a protective IgG1 immune response in those individuals with acquired resistance and, therefore, suggested as an attractive vaccine candidate [24]. This resistance is acquired after treatment with PZQ and the consequent exposure of parasite antigens to the host immune system [55]. SmSP2 orthologs are also present in the larval stage (cysticercus) of the cestodes, *Echinococcus granulosus* [56] and *Taenia solium* [57], and were identified as Antigen 5 excretory proteins (Ag5). Ag5 proteins are major components of cyst fluid and are used in a serodiagnostic test for cysticercosis [57, 58]. In comparison with SmSP2, the catalytic serine residue is replaced by threonine and Ag5 proteins show only marginal proteolytic activity [56, 57].

In adult *S. mansoni*, we demonstrate that SmSP2 is localized in the tegument. Treatment with PZQ causes tegumental damage [59, 60] and thereby exposes schistosome antigens, including SmSP2, to the host immune system [55]. The tegumental location may explain the recognition of SmSP2 by sera of PZQ-treated individuals [24]. Also, the localization of SmSP2 to the esophagus may indicate that the enzyme facilitates some aspect of nutrition during the ingestion of host proteins or is secreted. Indeed, we observed that SmSP2 is found in the ESP as was noted in *E. granulosus* previously for Ag5 [58, 61]. In addition, SmSP2 and its *S. japonicum* mastin ortholog were recently proteomically identified in exosome-like vesicles that are secreted by parasite and putatively modulate host-parasite interactions [62–64]. Finally, apart from a potential extracorporeal function, the protease domain of SmSP2 was localized to a number of internal tissues, including the ovaries, testes, muscle and parenchyma, suggesting a variety of functional roles.

To enzymatically characterize SmSP2, the protease domain was heterologously expressed in *P. pastoris* and purified as an active protease. SmSP2 was subjected to a range of biochemical analyses to determine its substrate and inhibitory specificity. SmSP2 was classified as a trypsin-like enzyme as it cleaves various peptide substrates in an endopeptidolytic mode at the carboxyl terminus of Arg or Lys residues and was inhibited by inhibitors targeting trypsin-like proteases such as leupeptin, benzamidine and antipain [65]. Consistent with this, systematic cleavage specificity analysis with the positional-scanning and multiplex substrate libraries revealed a preference for basic amino acids at P1. In agreement with the cleavage specificities, the homology model of SmSP2 reveals that the S1 binding pocket has an architecture analogous to vertebrate trypsins, including the critical Asp441 residue that defines the preference for basic P1 residues [45].

Based on homology modeling, the SmSP2 protease domain contains a large 43 residue-long insertion (at position 358) which is a unique structural feature of SmSP2 and its trematode orthologs not present in other S1 family proteases. This insertion is localized in the vicinity of the active site on the loop D (nomenclature according to Perona and Craik [48]). We demonstrate that SmSP2 performs limited proteolysis to process a number of physiologically- relevant host proteins: it degrades fibronectin, activates plasmin and tPA and releases bradykinin from its precursor HMWK. SmSP2 is incapable of cleaving macromolecular substrates such as Hb, BSA and IgG, which, for example, are cleaved by the gut-associated cysteine and aspartic proteases [15, 16]. SmSP2 has a more narrow substrate specificity than trypsin, the S1 family archetypal protease. In multiplex cleavage assays, trypsin cleaved more substrates than SmSP2. It resulted in similar number of cleavages produced by trypsin in 15 min whereas 4 h was required by SmSP2. A plausible explanation is that the B and D loop-insertions on SmSP2 limit the access of substrates to the active site resulting in the selective processing of conformationally compatible peptides. However, the insertion-D does not directly occlude the active site subsites as this would confer an exopeptidase activity that is not observed for SmSP2.

Adult schistosomes can survive for decades in the host [66]. It is thought that these large intravascular parasites manipulate the complex hemostatic system of the host at different levels via bioactive molecules in the ESP or on the tegument [67]. However, the detailed molecular processes underlying these survival mechanisms are not fully understood. Our work demonstrates that SmSP2 is present in both the ESP and tegument. We show that SmSP2 possesses a kallikrein-like activity as it cleaves the plasma protein kininogen to generate the peptide hormone, bradykinin. Bradykinin is a potent vasodilator that decreases blood pressure and increases vascular permeability [68]. Bradykinin also exerts anti-thrombogenic, anti-proliferative and anti-fibrogenic effects [69]. In a recent report, we demonstrated that living schistosomes cleave bradykinin and angiotensin I (converting this vasoconstrictor to the vasodilatory angiotensin-(1–7)), and that the tegumental, S9 serine protease, SmPOP, is involved in that

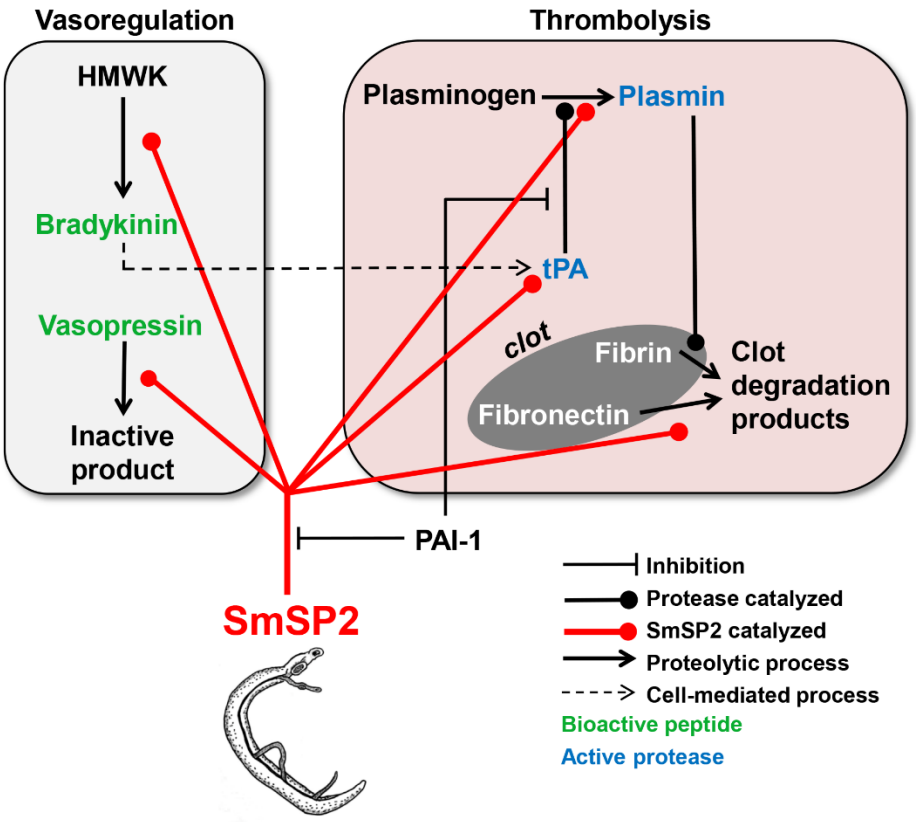

Fig 8. Model of how SmSP2 may interfere with the host's hemostatic system. SmSP2, secreted from adult schistosomes or localized at the surface, stimulates the degradation of blood clots (thrombolysis panel) by (i) activation of two critical components of the fibrinolytic system, tissue plasminogen activator (tPA) and plasminogen, and (ii) direct degradation of the blood-clot component, fibronectin. SmSP2 modulates vascular tone (vasoregulation panel) by processing bioactive peptide hormones. (i) It releases the vasodilatory bradykinin from kininogen (HMWK) and (ii) degrades the vasoconstrictory peptide, vasopressin. Bradykinin may stimulate the release of tPA from vascular endothelial cells (dashed line) which would promote fibrinolysis. SmSP2 may be regulated by plasminogen activator inhibitor-1 (PAI-1) that inhibits SmSP2 (Table 1).

https://doi.org/10.1371/journal.pntd.0006446.g008

processing [37]. Moreover, SmSP2 inactivates vasopressin, a hormone that increases arterial blood pressure by inducing vasoconstriction. It is, therefore, possible that both SmPOP and SmSP2 modulate the parasite's local vascular environment to the parasite's benefit during its residence and movement in the host's blood vessels. Additionally, we show that the whole schistosome parasite can cleave the vasopressin and release bradykinin from its synthetic precursor, when co-incubated *in vitro*. The activity is due to a serine protease(s) (possibly SmSP2) that is most abundantly expressed serine protease in adult schistosome [23] as indicated by mRNA expression levels, mass spectrometry and specific inhibition by a serine protease inhibitor.

Due to their large size, adult schistosomes may alter or disrupt normal blood flow, and damage the endothelium, which could lead to platelet activation and subsequently blood coagulation [67]. However, blood clots are not observed around the parasites residing in the host blood vessels and several mechanisms have been proposed with which schistosomes inhibit blood clot formation and/or promote blood clot lysis [70]. Physiologically, the latter process involves proteolytic degradation of fibrin that is mediated by plasmin. This central protease in the fibrinolytic system is generated from its zymogen, plasminogen (PLG), by, for example, tissue plasminogen activator (tPA). Based on our *in vitro* results, we propose here a new and complex mechanism with which schistosomes employ SmSP2 to promote fibrinolysis at multiple levels (Fig 8): 1) SmSP2 activates PLG to plasmin; this action can be accelerated by presentation of PLG on tegumental receptors (*e.g.*, enolase [71] of schistosomes); 2) SmSP2 processes the single-chain tPA to the more potent double-chain tPA form [72] which would then cause enhanced plasmin activation, 3) SmSP2 produces bradykinin that is known to stimulate the release of tPA from the vascular endothelium [73]; and 4) SmSP2 directly degrades the blood clot component fibronectin, which has also been recently shown for a tegumental calpain [74].

To conclude, we have expressed, and biochemical and functionally characterized the multidomain serine protease, SmSP2. The protease is a putative anti-hemostatic peculiar to platyhelminths, which are the only pathogens to express SmSP2 orthologs. Further research is needed to evaluate the role of SmSP2 (and orthologs) in modulating host-parasite interactions and its potential as a drug or vaccine candidate.

Supporting information

S1 Fig. Multiple sequence alignment of the TSR-1 domain of SmSP2 with selected TSR-1 domains of human proteins. Sequences are: TSP-1-1-3—thrombospondin-1 (TSP) type-1 domains 1, 2 and 3 (Uniprot accession number: P07996), properdin-TSR1-6—properdin thrombospondin type-1 domains 1–6 (P27918), ADAMTS13 (Q76LX8), and spondin-TSP-1—spondin-1 thrombospondin type-1 domain 1 (Q9HCB6). Cys residues are highlighted in yellow, tryptophan substituents forming the W layer are in blue, and amino acid residues forming the R layers are in green.
(TIF)

S2 Fig. Multiple sequence alignment of the SmSP2 protease domain with catalytic domains of selected human and bovine S1 family proteases. Human proteases: mannan-binding lectin serine protease 1 (MASP-1, Uniprot accession number: P48740), tissue plasminogen activator (tPA, P00750), urokinase plasminogen activator (uPA, P00749), plasmin (P00747), kallikrein 1 (P06870) and matriptase (MTSP-1, Q9Y5Y6). Bovine proteases: cationic trypsin (P00760) and chymotrypsin A (P00766). The catalytic triad residues (His, Asp, Ser) are red-boxed; the critical Asp residue in the S1 subsite that accounts for trypsin-like activity is green-boxed. Cys residues that are predicted to form disulfide bonds are indicated by the same color, cyan Cys form interchain disulfide bond with domains not included in the alignment. Residues that are shared between sequences are shaded in grey. Residues forming SmSP2 insertion-222, 251, and 358 are underlined. The upper line numbering is according to SmSP2, the lower line numbering according to bovine chymotrypsinogen.
(TIF)

S3 Fig. Multiple sequence alignment of SmSP2 with orthologs from other platyhelminth parasites. Trematode sequences: *Schistosoma. japonicum* (GenBank: AAW24683.1), *Schistosoma haematobium* (XP_012796372.1), *Fasciola hepatica* (sequence identified in the transcriptome database (Young et al. (2010), Biotechnol Adv 28, 222–231), *Opisthorchis viverrini*

(XP_009167273.1) and *Clonorchis sinensis* (GAA32831.2). Cestode sequences: *Hymenolepis microstoma* (CDS25513.1), *Echinococcus multiocularis* (CDI97096.1), *Echinococcus granulosus* (EUB58856.1) and *Taenia solium* (ADP89566.1). Predicted signal sequences are in blue, histidine residues in the N-terminal region are in purple and the TSR-1 domain is in green. The catalytic triad residues (His, Asp, Ser) are red-boxed, the critical Asp residue in S1 subsite that accounts for trypsin-like activity is green-boxed. Cys residues in the TSR-1 domain are highlighted in yellow and Cys residues in the protease domain are in cyan.
(TIF)

S4 Fig. Binding of native SmSP2 to a Ni^{2+}-ion affinity column. A protein extract of adult schistosomes (Extract) was applied to a HiTrap IMAC FF column containing immobilized Ni^{2+} ions and native SmSP2 eluted using 0.5 M imidazole. The extract, unbound material (FT) and eluted material (Elution) were resolved by SDS-PAGE, electrophoretically transferred onto a PVDF membrane and visualized by anti-rSmSP2 IgG.
(TIF)

S5 Fig. Detailed micrograph of SmSP2 localization in the tegument of adult male *S. mansoni*. The tissue section was probed with anti-SmSP2 IgG followed by an anti-rabbit IgG Alexa 594-labeled secondary antibody (red). DAPI was used to label the nuclear DNA (blue). The left image shows merged fluorescent channels; on the right, schematic depiction of the adult schistosome surface; sm—surface membrane, tg—tegument, mu—muscle, tc—tegumental cell (cyton), pa—parenchym.
(TIF)

S6 Fig. Pre-immune serum is not reactive. As a negative control, semi-thin sections of adult S. mansoni males and females were probed with a pre-immune serum (A-F) followed by reaction with an anti-rabbit IgG Alexa 647-labeled secondary antibody (red). DAPI was used to label nuclear DNA (blue). The first and third columns show merged fluorescent channels; in the second and fourth columns, the signal is merged with differential interference contrast.
(TIF)

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4.6 Publication No. 5: Prolyl oligopeptidase from the blood fluke *Schistosoma mansoni*: from functional analysis to anti-schistosomal inhibitors.

Publication No. 5 focuses on the localization, functional and biochemical characterization of *S. mansoni* prolyl oligopeptidase (SmPOP), the serine protease of the S9 family. Oligopeptidases of this family are known virulence factors for protozoan trypanosomatids but have not been studied in parasitic flukes.

The gene encoding SmPOP was identified in the *S. mansoni* genome database by a protein BLAST analysis using human prolyl oligopeptidases as a query. SmPOP has a characteristic domain composition similar to mammalian POPs, consisting of N-terminal, β-propeller and protease domains. SmPOP was shown to be expressed in an enzymatically active form in the developmental stages infecting humans (eggs, adult worms and schistosomula). By immunofluorescence microscopy, SmPOP was localized in the parenchyma and tegument (including tubercles) of adult worms and schistosomula. The active site specificity of SmPOP was investigated using synthetic substrate and inhibitor libraries, and by homology modeling. SmPOP is a true oligopeptidase that hydrolyzes peptide (but not protein) substrates with a strict specificity for Pro at P1.

Both recombinant and native enzymes in live adult worms cleave host vasoregulatory, proline-containing peptidic hormones such as bradykinin or angiotensin I, suggesting a role for SmPOP in the regulation of host vascular tone, thereby contributing to parasite survival in the host. In addition, designed nanomolar inhibitors of SmPOP induce deleterious phenotypes in schistosomes maintained in culture. The data suggest that SmPOP is important for parasite survival and thus a potential target for the development of anti-schistosomal therapeutics.

PhD applicant contribution: parasite cultivation, preparation of adult schistosome tissue preparations for microscopy, immunofluorescence microscopy, data analysis and interpretation.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Prolyl Oligopeptidase from the Blood Fluke *Schistosoma mansoni*: From Functional Analysis to Anti-schistosomal Inhibitors

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OPEN ACCESS

Citation: Fajtová P, Štefanić S, Hradilek M, Dvořák J, Vondrášek J, Jílková A, et al. (2015) Prolyl Oligopeptidase from the Blood Fluke *Schistosoma mansoni*: From Functional Analysis to Anti-schistosomal Inhibitors. PLoS Negl Trop Dis 9(6): e0003827. doi:10.1371/journal.pntd.0003827

Editor: Rodrigo Correa-Oliveira, René Rachou Research Center, BRAZIL

Received: January 8, 2015

Accepted: May 12, 2015

Published: June 3, 2015

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by the grant P302/11/1481 from Czech Science Foundation (www.gacr.cz), the project InterBioMed LO1302 from the Ministry of Education, Youth and Sports of the Czech Republic and by the institutional project RVO 61388963 (www.msmt.cz). LU was partially supported by the Sciex-NMS grant 11.222 from CRUS Switzerland. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Background

Blood flukes of the genus *Schistosoma* cause schistosomiasis, a parasitic disease that infects over 240 million people worldwide, and for which there is a need to identify new targets for chemotherapeutic interventions. Our research is focused on *Schistosoma mansoni* prolyl oligopeptidase (SmPOP) from the serine peptidase family S9, which has not been investigated in detail in trematodes.

Methodology/Principal Findings

We demonstrate that SmPOP is expressed in adult worms and schistosomula in an enzymatically active form. By immunofluorescence microscopy, SmPOP is localized in the tegument and parenchyma of both developmental stages. Recombinant SmPOP was produced in *Escherichia coli* and its active site specificity investigated using synthetic substrate and inhibitor libraries, and by homology modeling. SmPOP is a true oligopeptidase that hydrolyzes peptide (but not protein) substrates with a strict specificity for Pro at P1. The inhibition profile is analogous to those for mammalian POPs. Both the recombinant enzyme and live worms cleave host vasoregulatory, proline-containing hormones such as angiotensin I and bradykinin. Finally, we designed nanomolar inhibitors of SmPOP that induce deleterious phenotypes in cultured schistosomes.

Competing Interests: The authors have declared that no competing interests exist.

Conclusions/Significance

We provide the first localization and functional analysis of SmPOP together with chemical tools for measuring its activity. We briefly discuss the notion that SmPOP, operating at the host-parasite interface to cleave host bioactive peptides, may contribute to the survival of the parasite. If substantiated, SmPOP could be a new target for the development of anti-schistosomal drugs.

Author Summary

Schistosomiasis (bilharzia) is a major global health problem caused by the schistosome flatworm which lives in the bloodstream. Treatment and control of the disease relies on a single drug, and should resistance emerge, there would be increased pressure to discover new drug targets. Proteolytic enzymes are fundamental to the survival of parasites, and, hence, are attractive targets for drug intervention. Oligopeptidases from the S9 family are known virulence factors for protozoan trypanosomatids but have yet to be studied in parasitic flukes. We, therefore, investigated prolyl oligopeptidase in *Schistosoma mansoni* (SmPOP) and found that it is expressed in those developmental stages that infect humans. We provide a comprehensive analysis of the peptidase's expression, localization and functional biochemical properties. Interestingly, SmPOP, which is found in the tegument and parenchyma of the parasite, can cleave blood peptides involved in vasoregulation and we discuss how this ability may aid the parasite's survival. Finally, we designed potent inhibitors of SmPOP that cause deleterious changes in cultured parasites. We conclude that SmPOP is important for parasite survival and may be a potential target for the development of anti-schistosomal drugs.

Introduction

Schistosomiasis, also known as bilharzia, is caused by blood flukes of the genus *Schistosoma* with approximately 240 million people infected [1]. Three species of schistosome principally infect humans: *Schistosoma haematobium*, which causes urinary schistosomiasis, and *S. japonicum* and *S. mansoni*, which cause intestinal schistosomiasis [2]. Adult schistosomes can reside for decades as pairs in the veins surrounding the bladder or in mesenteric and the portal veins, and produce hundreds of eggs per day [3]. Morbidity arises from immuno-pathological reactions to and entrapment of schistosome eggs in various tissues [4]. Disease symptoms include spleno- and hepatomegaly, periportal fibrosis and hypertension, and urinary obstruction. Bladder carcinoma, sterility, malnutrition, and developmental retardation are common [3]. Infections can last a lifetime [5].

In the absence of a vaccine [6], control and treatment of schistosomiasis rely on a single drug, praziquantel, and the possibility of emergent drug resistance is a constant concern [7,8]. Accordingly, there is a continued impetus to identify new schistosome drug targets and chemotherapeutically active anti-schistosomals [8,9].

Proteolytic enzymes (peptidases) of schistosomes are attractive drug targets as they operate at the host–parasite interface, where they may facilitate parasite invasion, migration, nutrition and immune evasion [10–12]. Most studies concerning schistosome peptidases have focused on either the serine peptidase called cercarial elastase that facilitates penetration of the human

host by some schistosome species [13] or on those cysteine and aspartic peptidases that contribute to the digestion of the blood meal [14,15]. Among the latter, the digestive cathepsin B of *S. mansoni*, known as SmCB1, has been validated in a murine model of *S. mansoni* infection as a molecular target for therapy [9,16] and small molecule inhibitors of SmCB1 are under consideration as potential drug leads [16–19]. Other peptidase groups of schistosomes are less studied [12], including post-proline cleaving peptidases. This work focused on a *S. mansoni* prolyl oligopeptidase.

Prolyl oligopeptidases (POPs, also called prolyl endopeptidases) are approximately 70–80 kDa and belong to the S9 family of serine peptidases [20]. POPs cleave internal peptide bonds on the C-terminal side of proline residues and are found in plants, bacteria, fungi, protozoa, invertebrates and vertebrates [21]. For parasites, the best characterized POP is Tc80 in the infective trypomastigote stage of *Trypanosoma cruzi*, the causative agent of Chagas disease [22]. Tc80 seems to be involved in the parasite invasion as inhibition of Tc80 prevents parasite entry into host cells [23]. Accordingly, Tc80 is under investigation as a potential drug target [23,24].

In this report, we identified and functionally characterized the prolyl oligopeptidase from *S. mansoni* (SmPOP). We demonstrate that enzymatically active SmPOP is produced in several developmental stages and localized to the tegument and parenchyma of the parasite. We characterized in detail the biochemical activity of recombinant and native SmPOP, and designed nanomolar inhibitors of SmPOP that derange schistosomes maintained in culture. The data suggest that SmPOP is important to parasite survival and is, thus, a potential target for the development of anti-schistosomal therapeutics.

Materials and Methods

Ethics statement

All animal procedures were performed at the UCSF, USA, in accordance with protocol (AN107779–01) approved by the UCSF Institutional Animal Care and Use Committee (IACUC) as required by the Federal Animal Welfare Act and the National Institutes of Health Public Health Service Policy on Humane Care and Use of Laboratory Animals (http://grants.nih.gov/grants/olaw/references/phspol.htm).

Schistosome material

S. mansoni (a Puerto Rican isolate) was kept in the University of California San Francisco (UCSF) laboratory by cycling between the intermediate snail host, *Biomphalaria glabrata*, and female golden Syrian hamsters (infected at 3–5 weeks old), as the definitive host. Hamsters are infected by subcutaneous injection of 800 cercariae and sacrificed 6–7 weeks post-infection by intra-peritoneal injection of pentobarbital (50 mg/kg). Adults, eggs and miracidia were then isolated as described previously [25,26]. Free-swimming cercariae were obtained from water containing infection-patent *Biomphalaria* to 'shed' under a bright light. Cercariae were chilled over ice. Newly transformed schistosomula (NTS) were prepared by mechanically transforming cercariae [26,27] and cultivated in a Basch Medium 169 [28] containing 5% fetal calf serum, 100 units/mL penicillin and 100 μg/mL streptomycin for 5 days at 37°C under a 5% CO_2 atmosphere. Daughter sporocysts were isolated by excision of hepato-pancreases from infected *B. glabrata* snails.

Isolation of mRNA, cDNA synthesis and qRT-PCR

Adult worms, eggs, miracidia, daughter sporocysts, cercariae and NTS were collected, washed three times in 1.5 mL PBS, re-suspended in 500 μL Trizol reagent (Invitrogen) and processed

as described previously [26]. Single-stranded cDNA was synthesized from total RNA by SuperScript III reverse transcriptase (Life Technologies) and an oligo dT_{18} primer. The final cDNA product was purified and stored at -20°C.

The gene expression profile of the SmPOP was assessed using reverse transcription- quantitative PCR (RT-qPCR). The following primers were used: forward 5'-CATTCGTGGTGGAGGAGAAT-3' and reverse 5'- CGCATACTGGAACTTGAGCA -3'. The primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/) and their efficiency was evaluated as described previously [25,26]. The reactions, containing SYBR Green I Mastermix (Eurogentech), were prepared in a final volume of 25 μL in 96-well plates (Roche). The amplification profile consisted of an initial hot start (95°C for 10 min) followed by 40 cycles comprising 95°C for 30 s, 55°C for 60 s and 72°C for 60 s, and ending with a single cycle of 95°C for 60 s, 55°C for 30 s and 95°C for 30 s. The PCR reactions were performed in duplicate for each cDNA sample. At least one biological replicate, *i.e.*, samples from a different RNA isolation, was performed. The analysis of the cycle threshold for each target was carried out as described [25,26] employing *S. mansoni* cytochrome c oxidase I (SmCOX I, GenBank AF216698) [29] as the sample-normalizing gene transcript. Transcript levels were expressed as log functions and as a percentage relative to that of SmCOX I in order to compare expression patterns.

Expression and purification of recombinant SmPOP

The single gene encoding SmPOP (SchistoDB code: Smp_213240) was identified in the *S. mansoni* genome database [12] (*S. mansoni* GeneDB available at http://www.genedb.org/Homepage/Smansoni) via a protein BLAST search with the amino acid sequences of human and porcine prolyl oligopeptidases (GenBank accession numbers P48147 and P23687, respectively) as queries. The same search in the *S. japonicum* and *S. haematobium* genome databases [30,31] identified SmPOP orthologs with 88% and 95% identity, respectively (*S. japonicum*: GeneDB Sjp_0080730.1, GenBank AAX26405; *S. haematobium*: HelmDB Shae8836338, GenBank KGB33720).

The Champion pET directional expression kit (Life Technologies) was selected for expression of the SmPOP gene. The 2139 bp ORF was amplified using Phusion High-Fidelity DNA Polymerase (New England Biolabs) from adult schistosome cDNA using specific forward (5´-caccATGGAGCATACCAGTATCAACTATCC-3´) and reverse (5´-TTCTTTCCATGTGAGTGACATT-3´) primers. The PCR product was cloned into the expression vector pET101/D-TOPO (Invitrogen) and verified by DNA sequencing. Recombinant SmPOP (rSmPOP) with a C-terminal His_6-tag was produced in *E. coli* BL21(DE3) by induction in LB broth medium containing 0.5 mM IPTG for 16 h at 16°C. Soluble rSmPOP was purified from the bacterial lysate using Ni^{2+} chelating chromatography (Hi-Trap IMAC FF column, GE Healthcare Life Sciences) under native conditions. The bound rSmPOP was eluted using a linear gradient of 0.01–0.5 M imidazole. The preparation was buffer-exchanged into 20 mM Tris-HCl, pH 8.0, using an Amicon Ultracel-30k ultrafiltration device (Millipore). rSmPOP was subsequently purified by FPLC over a Mono Q HR 5/5 column (GE Healthcare Life Sciences) equilibrated in 20 mM Tris-HCl, pH 8.0, and eluted using a linear gradient of 0–1 M NaCl in the same buffer. The purification process was monitored by a kinetic assay incorporating the peptidyl fluorogenic substrate, benzyloxycarbonyl (Z)-Gly-Pro-7-amino-4-methylcoumarin (AMC), and by SDS-PAGE. The preparation was concentrated and desalted into 20 mM Tris-HCl, pH 8.0, using an Amicon Ultracel-30k. The typical yield was approximately 3 mg of rSmPOP from 1 L of culture medium.

Preparation of schistosome extracts

Soluble protein extracts (0.2–3 mg protein/mL) from *S. mansoni* adults, miracidia, cercariae, eggs and NTS were prepared by homogenization in 50 mM Tris-HCl, pH 8.0, containing 1%

CHAPS, 1 mM EDTA, 1 μM pepstatin and 10 μM E-64 in an ice bath. The extracts were cleared by centrifugation (16000 *g* at 4°C for 10 min,), ultra-filtered using a 0.22 μm Ultrafree-MC device (Millipore) and stored at -80°C.

Preparation of antibodies and immunoblotting

Specific polyclonal antibodies (Moravian Biotechnology) were generated in rabbits against the purified rSmPOP antigen using 50 μg of rSmPOP in Freund's incomplete adjuvant and applied three times three weeks apart. IgG was isolated from the serum by affinity chromatography with a HiTrap Protein A column (GE Healthcare Life Sciences) according to the manufacturer's protocol.

For immunoblotting, adult schistosome homogenate (30 μg protein) and rSmPOP (1 μg) were resolved by SDS-PAGE (15% polyacrylamide gel) under reducing conditions and transferred onto a PVDF membrane. The membrane was blocked 16 h in 10% non-fat milk in 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 0.1% Tween (TTBS). The membrane was then washed three times in TTBS and incubated for 1 h with anti-SmPOP polyclonal IgG diluted 1:1000 in TTBS. After washing in TTBS, the membrane was incubated for 1 h with goat horseradish peroxidase-conjugated anti-rabbit IgG (Sigma-Aldrich, catalog number A6154) at a dilution of 1:20000. After washing in TTBS, the membrane was developed with SuperSignal West Femto Chemiluminescent Substrate (Pierce) and imaged using an ImageQuant LAS 4000 biomolecular imager (GE Healthcare Life Sciences).

Immunofluorescence microscopy

For sample preparation, adult *S. mansoni* worms were washed three times in PBS and fixed either in acetone (75% acetone in ethanol) at -20°C for 10 min or 4% formaldehyde in PBS at 25°C for 45 min. The samples were then rinsed with PBS and incubated in a 30% sucrose solution at 4°C for 16 h. The worms were placed in cryofixation molds and the sucrose solution was replaced with Optimal Cutting Temperature (OCT) medium (CellPath Ltd). The molds were placed over dry ice to freeze and the frozen blocks then stored at -80°C. The OCT-embedded worm samples were sectioned with a cryotome (Cryostat 2800 Frigocout, Cambridge Instruments). Sections of ~7 μm were air-dried and further processed for immunostaining.

Sections were rehydrated in PBS and fixed again either with formaldehyde or cold acetone as described above. The formaldehyde-fixed samples were further blocked in 100 mM glycine at 22°C for 20 min, followed by 2% BSA in PBS at 4°C for 16 h. Working solutions of primary and secondary antibodies were prepared in PBS containing 2% BSA; rabbit polyclonal anti-SmPOP IgG was diluted 1:900 and anti-rabbit IgG Alexa 594-conjugated secondary antibody (Molecular Probes) was diluted 1:200. The antibodies were incubated at 25°C on the sections for 45 min with three washes between the primary and secondary antibody incubations, and four washes after the secondary-antibody incubation (the fourth wash contained DAPI at 1 μg/mL for nuclear staining).

NTS samples were fixed in 4% formaldehyde in PBS at 4°C for 16 h. After fixation, they were washed 3 times in PBS at 25°C for 10 min and subsequently blocked with 100 mM glycine at 25°C for 20 min. Samples were permeabilized with 0.2% Triton X-100 in PBS for 40 min at 25°C and blocked with 2% BSA in PBS for 16 h at 4°C. The antibody diluent contained 0.1% Triton X-100, 0.1% BSA and 0.2% NaN_3. Primary and secondary antibody solutions were incubated for 24 h with four washes of diluent over a 10 h period (the fourth wash contained DAPI at 1 μg/mL for nuclear staining).

Sections of adults and whole-worm preparations of NTS were embedded in Mowiol (Sigma-Aldrich) and visualized using a Leica SP2 AOBS confocal laser scanning microscope (Leica

Microsystems) and a 20x oil immersion objective. Appropriate lighting settings were determined using control slides probed with preimmune serum to define the background signal threshold. Image stacks of optical sections were further processed using the Huygens deconvolution software package version 2.7 (Scientific Volume Imaging).

Preparation of substrates and inhibitors

Fluorescence resonance energy transfer (FRET) substrates containing o-aminobenzoic acid (Abz) as the fluorescent group and p-nitro-phenylalanine (NPh) as the quencher acceptor were synthesized as peptidyl amides by Fmoc solid phase chemistry in an ABI 433A peptide synthesizer (Applied Biosystems) as described previously[16,32].

Substrates containing the fluorogenic group, 7-amino-4-carbamoylmethylcoumarin (ACC), were synthesized in the format Z-Xaa-Pro-ACC, with proteinogenic amino acids (except for cysteine) at the Xaa position, as described previously [33].

The inhibitors Z-Ala-Pro-chloromethyl ketone (CMK) and Z-Arg-Pro-CMK were prepared from the peptides Z-Ala-Pro-OH and Z-Arg(Pbf)-Pro-OH, respectively, according to the described procedure [34]. Z-Ala-Pro-OH and Z-Arg(Pbf)-Pro-OH were synthesized on solid phase using 2-chlorotritylchloride resin (Iris Biotech). Z-Xaa-Pro-CHO (CHO, aldehyde) inhibitors, where Xaa is Gly, Ala, Tyr, Arg or Lys, were synthesized on solid phase using H-Thr-Gly-NovaSyn TG resin (Merck) as described [35]. All of the substrates and inhibitors were purified by reverse-phase (RP)-HPLC over a C18 column using a TFA/acetonitrile system and characterized by electrospray ionization mass spectrometry on an LCQ Classic Finnigan Mat device (Thermo Finnigan).

The substrates Z-Gly-Pro-AMC, Succinyl (Suc)-Gly-Pro-Leu-Gly-Pro-AMC, Lys-Pro-AMC, Gly-Pro-AMC and Pro-AMC were purchased from Bachem. The POP inhibitors Y-29794 oxalate and Z-Pro-Pro-CHO were purchased from Santa Cruz Biotechnology, and SUAM 14746 from PeptaNova.

Kinetic POP activity and inhibition assays

Assays were performed in triplicate in black, flat-bottomed, 96-well microplates (Nunc) in a total volume of 100 μL at 37°C. Z-Gly-Pro-AMC was used as substrate at a 50 μM final concentration. rSmPOP (50–100 ng), human POP (25–50 ng; Sigma-Aldrich, catalog number O9515) or schistosome homogenates (1–5 μg of protein) were pre-incubated for 10 min at 37°C in 80 μL of 0.1 M sodium phosphate, pH 8.0, containing 0.1% PEG 6000. Substrate (20 μL in the same buffer) was added to a final concentration of 50 μM. Hydrolytic activity was measured continuously in an Infinite M1000 microplate reader (Tecan) at the excitation and emission wavelengths of 360 and 465 nm, respectively. The pH profile of the activity was determined in 100 mM citrate phosphate (pH range 5.5–8.0), 100 mM Tris-HCl (pH range 8.0–9.0) and 100 mM sodium borate (pH range 9.0–10.0). For inhibition measurements, inhibitors were added to the 80 μL pre-incubation solution at a final concentrations of 0 to125 μM for 10 min and the reaction was initiated by the addition of the substrate. IC_{50} values were determined by nonlinear regression using the GraFit software (Erithacus Software). SmPOP activity in homogenates was measured in the presence of 10 μM E-64 to prevent undesired proteolysis by cysteine peptidases that contribute significant proteolytic activity in worm extracts [36]. POP activity was also measured with ACC and FRET substrates at excitation/emission wavelengths of 380/460 nm and 320/420 nm, respectively. Stock solutions of substrates and inhibitors (10 mM) were prepared in DMSO and the final assays concentration of DMSO was 1.5%.

Interaction of rSmPOP with protein substrates

rSmPOP (0.7 μg) was incubated at 37°C for 16 h with 100 μg of human hemoglobin, human serum albumin, human collagens type I and IV (Sigma-Aldrich, catalog numbers H7379, A3782, C7774 and C7521 respectively) in 100 mM Tris-HCl, pH 8.0, in a final volume of 50 μL. After incubation, a 10 μL sample was resolved by 15% SDS-PAGE or Tricine-SDS-PAGE and stained with Coomassie Brilliant Blue G250.

Hydrolysis of peptide hormones and neuropeptides by rSmPOP

The following synthetic analogues of human bioactive peptides were analyzed: angiotensin II (Sigma, catalog number A9525), angiotensin I, bradykinin, luteinizing-hormone-releasing hormone (LHRH), α-melanocyte-stimulating hormone (α-MSH), neurotensin, oxytocin, substance P, and vassopresin (all Bachem, catalog numbers H-1680, H-1970, H-6728, H-1075, H-4435, H-2510, H-1890 and H-1780, respectively). Stock solutions of peptides (10 mM) were prepared in water. rSmPOP (0.7 μg) was incubated at 37°C for 16 h with 25 nmol of peptide in 0.1 M Tris-HCl, pH 8.0, in a total volume of 50 μL. The reaction was stopped by adding TFA to a final concentration of 1%. The resulting fragments were purified by RP-HPLC over a C18 column (Vydac, 25 x 0.46 cm) using a TFA/acetonitrile system and characterized by electrospray ionization mass spectrometry on an LCQ Classic Finnigan Mat device (Thermo Finnigan).

Hydrolysis of peptide hormones and neuropeptides by cultured schistosomes

Five adult schistosome pairs were placed into clear, 24-well, flat-bottom plates (Costar) containing 500 μL Basch Medium 199 [28], supplemented with 2.5% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin. Human angiotensin I or bradykinin in 5 μL water was added to a final concentration of 100 μM and the incubation continued for 16 h at 37°C under a 5% CO_2 atmosphere. In control experiments, the peptides were cultivated in the same system in the absence of schistosomes. After incubation, the samples were ZipTiped and the resulting fragments were analyzed using MALDI-TOF performed on an UltrafleXtreme (Bruker Daltonik) operated in reflectron mode with an acceleration voltage of 25 kV and a pulsed ion extraction of 120 ns. Desorption and ionization were achieved using a Smartbeam II laser. α-Cyano-4-hydroxycinnamic acid was used as a matrix. The data were acquired from m/z 220 to 3700 and analyzed with the FlexAnalysis 3.3 software (Bruker Daltonik). The mass spectra were externally calibrated using a Peptide Calibration Standard I (Bruker Daltonik) and averaged from 3000 laser shots.

Fluorescence SmPOP activity assay with cultured schistosomes

Adult worms (3 pairs) or approximately 150 NTS were incubated at 37°C and 5% CO_2 for 2 days in 200 μL of Basch Medium 169 containing 5% FBS, 100 units/mL of penicillin and 100 μg/mL using black clear bottomed 96-well microplates (Costar). After incubation, half of the medium (100 μl) was transferred to an empty well leaving the parasites in the remaining half. Then SmPOP activity was measured in both wells upon the addition of 20 μL of Z-Gly-Pro-AMC (prepared as a 250 μM stock in Basch Medium 169) and in the presence or absence of 1 μM of the POP inhibitor, Z-Ala-Pro-CMK. Controls contained medium alone.

Molecular modeling of SmPOP

A spatial model of SmPOP was constructed by homology modeling as described previously [17]. Briefly, the X-ray structure of porcine POP in complex with the inhibitor Z-Pro-Pro-CHO (PDB

entry: 1QFS) was used as a template. The homology module of the MOE program (Chemical Computing Group) was used for the modeling of the SmPOP structure. The inhibitor conformation was refined by applying the LigX module of the MOE for the optimization procedure and its final binding mode was selected by the best-fit model based on the London dG scoring function and the generalized Born method [37]. Molecular images were generated with UCSF Chimera (http://www.cgl.ucsf.edu/chimera/).

Parasite assay and phenotype scoring

NTS (200–300 parasites) were incubated in 200 μL of Basch Medium 169 and supplements, as described above. Inhibitors were added at final concentrations of 1 or 10 μM (0.5% DMSO final) and the incubations continued for 4 days. Grading of phenotypic responses arising as a function of time and concentration was modified after Jilkova *et al.* [16]: Grade I, dead NTS by 2 days of culture at 10 μM and dying/dead NTS by 3 days at 1 μM. Grade II, dead NTS by 3 days at 10 μM and round/dark/dying by 3 days at 1 μM; Grade III, round/dark by 3 days at 1 and 10 μM concentrations (S1 Fig). 'Dead' was adjudicated as the loss of normal shape and the lack of movement often accompanied by obvious internal disruptions. 'Dying' was similar to death except that movement was detectable. Otherwise, the terms 'round/dark' were used to indicate less severe but obvious changes in the parasites relative to DMSO controls.

Results

SmPOP is homologous to prolyl oligopeptidases from various parasites

A protein BLAST analysis of the *S. mansoni* genome database [12,38] using mammalian prolyl oligopeptidases as queries identified a gene ortholog (SmPOP), Smp_213240, located on the sex-determining Z/W chromosomal pair. SmPOP cDNA was cloned, sequenced, and the sequence was deposited into the GenBank as KF956809. The blast analysis did not identify other gene isoforms. The SmPOP open reading frame consists of 2,139 bp that encodes a protein of 712 amino acid residues with a calculated molecular mass of 82 kDa. No signal/leader peptide was predicted for the sequence. SmPOP has about 50% identity with human and porcine POPs (S1 Table) and belongs to the S9 family of serine peptidases (S2 Fig). SmPOP has the characteristic domain composition of mammalian POPs, consisting of N-terminal, β-propeller and peptidase domains. The peptidase domain of SmPOP has a catalytic triad in the order of Ser556, Asp643 and His682, which is typical of POPs and other S9 family peptidases [39]. In addition, the regions surrounding the catalytic-triad residues have the most notable sequence identity. A phylogenetic tree constructed for prolyl oligopeptidases of animal, plant, protozoan and bacterial origin (S3 Fig) demonstrates that SmPOP clusters with other trematode and nematode POPs. This monophyletic group is well separated from other clades.

S. mansoni developmental stages express active POP

Messenger RNA transcript levels for SmPOP were evaluated in eggs, miracidia, daughter sporocysts, cercariae, NTS and adults using qRT-PCR (Fig 1A). The expression of SmPOP was recorded in eggs, daughter sporocysts, NTS and adult schistosomes (in the range of 4–12% of the expression of the validated reference gene, SmCOX I [26]). In miracidia and cercariae, expression was below 1% of the SmCOX I level (Fig 1A).

At the protein level, SmPOP enzymatic activity in soluble extracts of various developmental stages was determined in a kinetic assay using the fluorogenic substrate, Z-Gly-Pro-AMC, which is specific for prolyl oligopeptidases. The measured activities were further authenticated as being due to a prolyl oligopeptidase by their sensitivity to Z-Pro-Pro-CHO, a selective

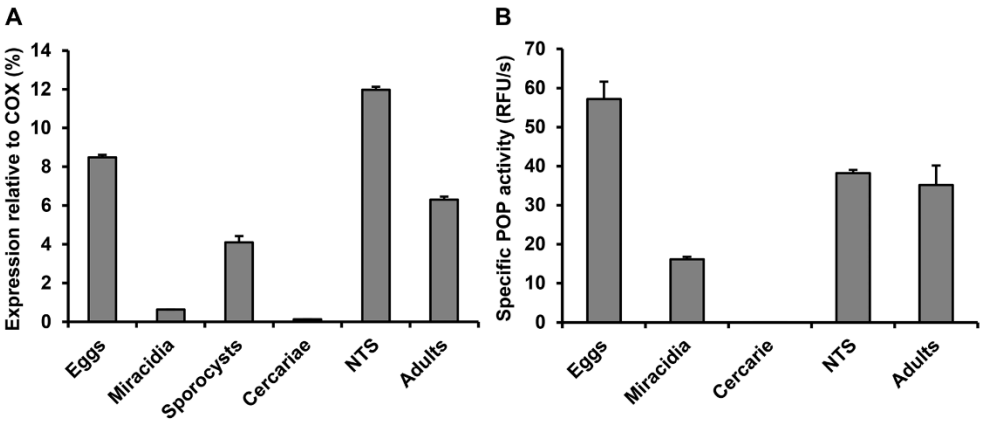

Fig 1. Activity and transcriptional profiling of SmPOP in the developmental stages of *S. mansoni*. (A) The expression of SmPOP was evaluated by quantitative RT-PCR. mRNA transcriptional levels are presented as the percentage of expression relative to the constitutively expressed *S. mansoni* cytochrome oxidase I (SmCOX I). The mean values ± S.D. of three replicates are given. (B) SmPOP activities were measured in protein extracts of the developmental stages (except sporocysts not available in sufficient amount and purity) using a kinetic assay with the fluorogenic substrate Z-Gly-Pro-AMC at pH 8.0. POP activities (sensitive to inhibition by the specific POP inhibitor Z-Pro-Pro-CHO) are expressed in relative fluorescence units (RFU/s) and normalized to protein content.

doi:10.1371/journal.pntd.0003827.g001

small-molecule inhibitor of prolyl oligopeptidases [40]. Prominent SmPOP activity was measured in the homogenates of eggs, NTS and adults, whereas weak activity was measured in miracidial homogenates; no activity was detected in cercariae (Fig 1B).

Overall, active SmPOP is expressed in the *S. mansoni* developmental stages that live in the human host and the activity profile is consistent with that for mRNA expression. In addition, the presence of SmPOP was confirmed in the protein homogenate of adult *S. mansoni* by mass spectrometry proteomics (S2 Table).

SmPOP cleaves proline-containing neuropeptides and oligopeptide hormones of the host

Recombinant SmPOP (rSmPOP) was expressed in *E. coli* as a soluble and catalytically active enzyme. rSmPOP was purified to homogeneity by a combination of metal-affinity chromatography and ion-exchange chromatography, and subsequently migrated on SDS-PAGE as a single band of approximately 80 kDa (Fig 2A). Rabbit polyclonal antibodies raised against rSmPOP reacted with the original rSmPOP antigen by immunoblotting and recognized a single band in the homogenates from schistosome adults (Fig 2A). The molecular mass of both the native SmPOP and rSmPOP is in good agreement with the theoretical mass of SmPOP predicted from the amino-acid sequence (82 kDa).

The pH activity profile of rSmPOP was determined using the fluorogenic substrate Z-Gly-Pro-AMC and compared with that of the native SmPOP in schistosome adult homogenates (Fig 2B). For both protein sources, the substrate was cleaved between pH 6.0 and 10.0 with optimal activity around pH 8.0. No POP activity was detected below pH 5.0.

Prolyl oligopeptidases perform specific post-proline cleavages of various peptides [39,41]. Accordingly, using a broad panel of proline-containing bioactive peptides, we asked whether SmPOP cleaves human peptide hormones and neuropeptides (Fig 3). After incubation of the

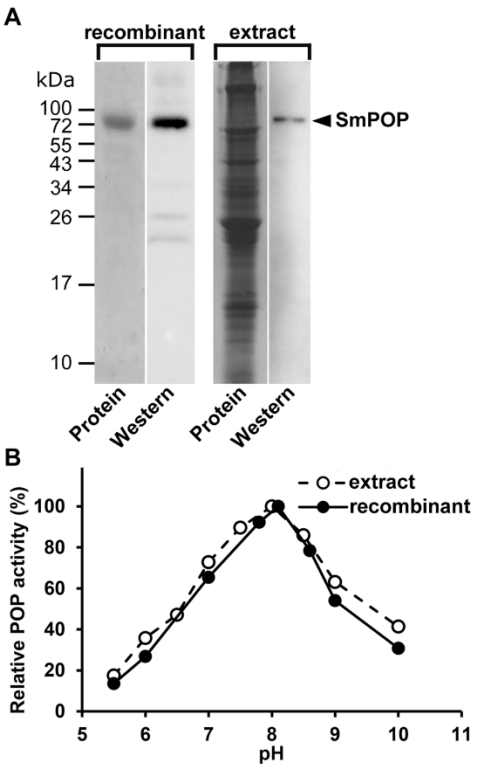

Fig 2. A comparison of recombinant SmPOP and native SmPOP. (A) Recombinant SmPOP expressed in *E. coli* (two left lanes) and *S. mansoni* protein extract (two right lanes) were resolved by SDS-PAGE, blotted onto a membrane, and visualized by protein staining or by anti-SmPOP IgG (polyclonal antibodies raised against recombinant SmPOP). (B) The pH profile of recombinant SmPOP and native SmPOP (in *S. mansoni* extracts). Activity was measured in a kinetic assay with the fluorogenic substrate Z-Gly-Pro-AMC. Mean values, expressed as a percentage are shown (the S.D. values of three replicates are within 10% of the mean).

doi:10.1371/journal.pntd.0003827.g002

tested peptides with SmPOP, the resulting fragments were separated by HPLC and the cleavage positions identified by mass spectrometry. All substrates were cleaved specifically at the carboxyl terminus of proline residues with the exception of the Pro-Lys bond in Substance P and the Pro-Pro bond in bradykinin (Fig 3). The substrate specificity resembles that of mammalian prolyl oligopeptidases, which cleave a Pro-Xaa bond in peptides, where Xaa is not a Pro residue. Also, like mammalian prolyl oligopeptidases, SmPOP does not cleave after a penultimate N-terminal Pro residue [42].

The activity of rSmPOP towards host-derived macromolecular substrates was tested with several human proteins, including hemoglobin, serum albumin and collagens I and IV. No hydrolysis was observed even after prolonged incubation (S4 Fig), indicating that SmPOP is a true oligopeptidase with an action restricted to oligopeptide substrates.

Peptide	SmPOP hydrolysis site
Angiotensin I	D R V Y I H **P** F H L
Angiotensin II	D R V Y I H **P** F
Bradykinin	R **P** **P** G F S **P** F R
LHRH	pE H W S Y G L R **P** G-amide
α-MSH	ac-S Y S M E H F R W G K **P** V-amide
Neurotensin	pE L Y E N K **P** R R **P** Y I L
Oxytocin	C Y I Q N C **P** L G-amide [Disulfide 1-6]
Substance P	R **P** K **P** Q Q F F G L M-amide
Vasopressin	C Y F Q N C **P** R G-amide [Disulfide 1-6]

Fig 3. SmPOP cleaves human, proline–containing peptide hormones and neuropeptides. The peptides were incubated with recombinant SmPOP at pH 8.0 and the cleavage positions (the red triangles) identified by mass spectrometry. Proline residues are indicated in bold; the disulfide connectivity is indicated in parentheses.

doi:10.1371/journal.pntd.0003827.g003

A fluorogenic substrate library was used to determine the SmPOP cleavage specificity at the substrate P2 position (Fig 4A). The greatest preference was recorded for basic residues (Arg and Lys), but a variety of other amino acid residues was also acceptable at this position, including hydrophobic, aliphatic and polar residues. Substrates with acidic residues and Pro at P2 were least preferred.

The substrate specificity of rSmPOP was further investigated using FRET synthetic substrates which had been designed based on the aminobenzoyl (Abz)-nitrophenylalanine (NPh) donor–acceptor pair and contained a Pro residue at P1 (Fig 4B). We prepared a set of substrates with variations in the P2 position (Abz-Ala-Pro-NPh, Abz-Gly-Pro-NPh, Abz-Lys-Pro-NPh, and Abz-Pro-Pro-NPh) and which were lengthened to include the P3 (Abz-Ala-Ala-Pro-NPh and Abz-Gly-Gly-Pro-NPh) or P1' positions (Abz-Ala-Pro-Ala-NPh and Abz-Ala-Pro-Gly-NPh). The greatest rSmPOP activity was measured with the substrates Abz-Ala-Pro-NPh and Abz-Lys-Pro-NPh, whereas the substrate Abz-Pro-Pro-NPh was not digested; increasing the substrate length to P3 and P1' positions did not increase its affinity (Fig 4B).

Finally, rSmPOP was tested for its ability to hydrolyze substrates with Pro in the P1 position that allows for cleavage by other post-proline cleaving enzymes, including collagenase-like peptidases (Suc-Gly-Pro-Leu-Gly-Pro-AMC), dipeptidyl aminopeptidase II (Lys-Pro-AMC), dipeptidyl aminopeptidase IV (Gly-Pro-AMC) and prolyl aminopeptidase (Pro-AMC; Fig 4C). Only Suc-Gly-Pro-Leu-Gly-Pro-AMC, suitable for the endopeptidase mode of cleavage, was digested by rSmPOP with the same efficiency as found for the classical and minimal POP substrate, Z-Gly-Pro-AMC. The cleavage of exopeptidase substrates with free N-termini occurs only very slowly (Lys-Pro-AMC) or not at all (Gly-Pro-AMC and Pro-AMC).

To summarize, SmPOP is a true oligopeptidase that hydrolyzes peptide but not protein substrates in the endopeptidase mode with a strict specificity for Pro at P1.

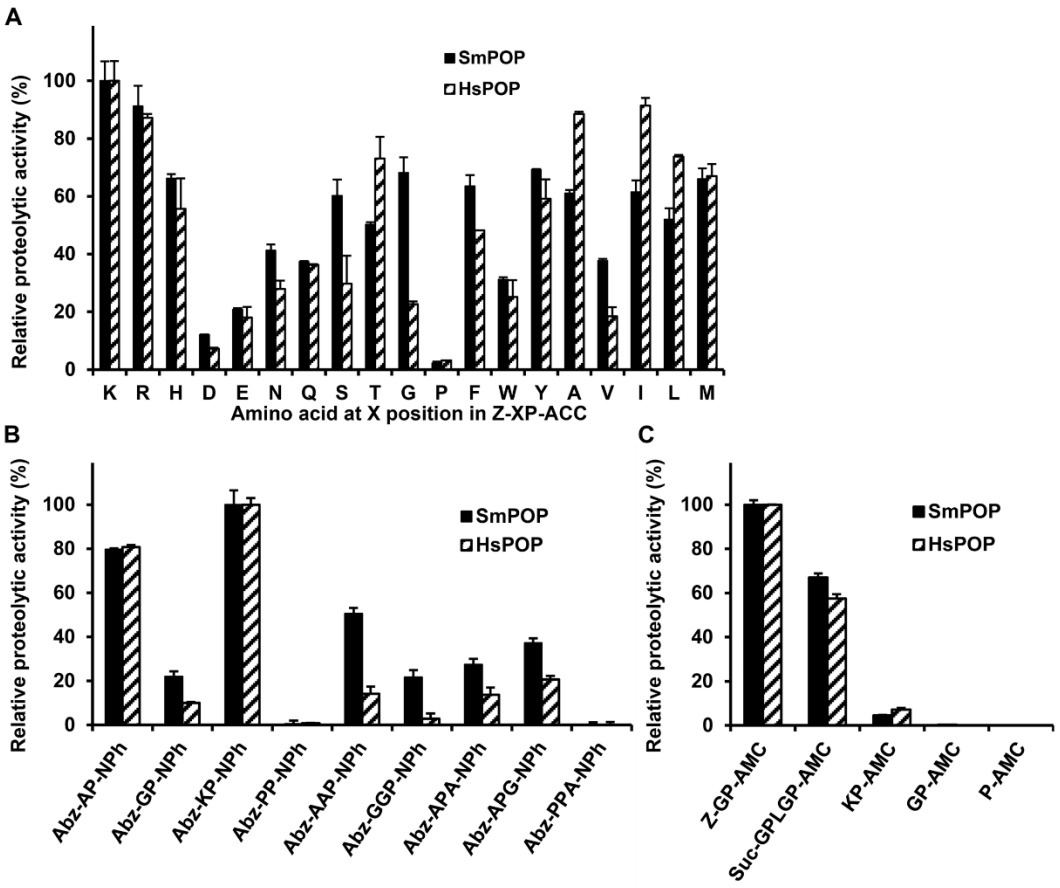

Fig 4. Substrate specificity of recombinant SmPOP. The peptidolytic activity of SmPOP was probed using a series of synthetic substrate libraries: (A) fluorogenic substrates Z-XP-ACC with proline in the P1 position and the indicated amino acids in the P2 position; (B) FRET-based peptide substrates with-XP- (in the P2 and P1 positions) extended to occupy the P3 and P1' positions; (C) fluorogenic substrates with proline in the P1 position, which w used to assay the following peptidases: collagenase-like peptidases (Suc-GPLGP-AMC), dipeptidyl aminopeptidase II (KP-AMC), dipeptidyl aminopeptidase IV (GP-AMC) and prolyl aminopeptidase (P-AMC). The substrate hydrolysis was measured in a kinetic assay at pH 8.0 using recombinant SmPOP or human POP (HsPOP). The mean values ± S.D. of three replicates are normalized to the maximum value in each series.

doi:10.1371/journal.pntd.0003827.g004

Specificity of SmPOP inhibition and the design of specific inhibitors

The general inhibition specificity of rSmPOP was analyzed using a panel of peptidase class/type-selective small-molecule inhibitors as listed in Table 1. rSmPOP activity was completely inhibited by selective prolyl-oligopeptidase inhibitors with chloromethyl (CMK) and aldehyde (CHO) warheads (Z-Ala-Pro-CMK and Z-Pro-CHO), and by the general serine peptidase inhibitor, diisopropyl fluorophosphate. Partial inhibition was observed with Pefabloc SC, PMSF

Table 1. Inhibition of recombinant SmPOP by protease inhibitors.

Inhibitor[a]	Target protease[b]	Concentration (μM)	Inhibition (%)[c]
Pefabloc SC	SP	1000	12.0±3.1
PMSF	SP	1000	47.6±1.6
Benzamidine	SP (trypsin type)	10	3.7±1.1
TLCK	SP (trypsin type)	1	38.3±1.2
TPCK	SP (chymotrypsin type)	1	67.2±6.2
3,4-dichloroisocoumarin	SP	100	77.3±0.6
BPTI (Aprotinin)	SP	50	1.4±1.1
STI	SP	10	12.3±3.2
Diisopropyl fluorophosphate	SP	100	100±1
Leupeptin	SP, CP	20	2.3±1.2
Antipain	SP, CP	20	32.4±1.4
E64	CP	10	6.5±6.1
Pepstatin A	AP	1	7.3±3.5
EDTA	MP	1000	3.8±2.3
Bestatin	MP (leucin aminopeptidase)	1	2.3±2.1
Z-Ala-Pro-CMK[d]	SP (prolyl oligopeptidase)	1	100±3
Z-Pro-Pro-CHO[d]	SP (prolyl oligopeptidase)	1	100±1
Z-Pro-Pro-OH[d]	SP (prolyl oligopeptidase)	100	37.6±2.1
Z-Pro-OH[d]	SP (prolidase)	100	41.1±1.8

[a] Abbreviations: PMSF (phenylmethylsulfonyl fluoride), TLCK (Nα-Tosyl-L-lysine chloromethyl ketone), TPCK (N-p-Tosyl-L-phenylalanine chloromethyl ketone), BPTI (bovine pancreatic trypsin inhibitor), STI (soybean trypsin inhibitor), E64 (trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane)

[b] The target proteases are classified based on catalytic type into aspartic (AP), cysteine (CP) and serine (SP) proteases, and metalloproteases (MP).

[c] The recombinant SmPOP was pre-incubated with the given inhibitor and remaining activity was measured in a kinetic assay with the fluorogenic substrate Z-Gly-Pro-AMC. The mean values ± S.D. of three replicates are expressed as percentage inhibition relative to the uninhibited control.

[d] CMK: chloromethyl ketone; CHO: aldehyde; OH: free carboxyl.

doi:10.1371/journal.pntd.0003827.t001

(phenylmethylsulfonyl fluoride), TLCK (Nα-tosyl-L-lysine chloromethyl ketone), TPCK (N-p-tosyl-L-phenylalanine chloromethyl ketone) and 3,4-dichloroisocoumarin, all of which target the serine peptidases of the chymotrypsin S1 family. SmPOP activity was neither affected by protein inhibitors of serine peptidases (soybean trypsin inhibitor (STI) and bovine pancreatic trypsin inhibitor (BPTI)) nor by the inhibitors of cysteine, aspartic and metallo-peptidases. This overall inhibition profile shows that SmPOP has the ligand-binding characteristics analogous to those of mammalian POPs [41,42].

A more detailed inhibitor specificity profile for rSmPOP was investigated using a panel of synthetic peptidic inhibitors with the structure Z-Xaa-Pro-CHO/CMK, which included aldehyde (CHO) or chloromethylketone (CMK) reactive warheads (Table 2). The amino-acid residues for the Xaa position were selected based on the S2 substrate specificity of rSmPOP (Fig 4A). Table 2 shows that the synthesized aldehyde derivatives inhibit SmPOP with IC_{50} values in the low micromolar concentration range (1.3 to 6.1 μM); the inhibitory specificity at the binding subsite S2 corresponds to the substrate specificity profile (Fig 4A) and shows that inhibitors with the basic amino acids in the P2 have position have the lowest IC_{50} values.

The introduction of an irreversible covalent CMK warhead to the inhibitor scaffold improved the IC_{50} value by three orders of magnitude (IC_{50} from 2.9 to 3.2 nM) in comparison with inhibitors containing reversible covalent CHO warhead (Table 2). Furthermore, we tested the sensitivity of rSmPOP to three commercially available inhibitors developed for human POP, namely Y-29794 oxalate [43], SUAM 14746 [44], and Z-Pro-Pro-CHO [40]. Whereas the

Table 2. Inhibition of SmPOP activity and anti-schistosomal effect of synthetic SmPOP inhibitors.

Inhibitor	IC_{50} (μM)[a]		Severity of phenotype against parasite[b]
	SmPOP	HsPOP	Grade
Y-29794 oxalate[c]	8.6±0.4	0.49±0.03	II
SUAM 14746[d]	0.092±0.005	0.083±0.007	no effect
Z-Pro-Pro-CHO[e]	0.16±0.03	0.012±0.005	no effect
Z-Ala-Pro-CHO[e]	3.1±0.2	6.1±0.3	III
Z-Gly-Pro-CHO[e]	6.1±0.4	7.6±0.9	II
Z-Tyr-Pro-CHO[e]	4.4±0.4	11.4±0.7	II
Z-Arg-Pro-CHO[e]	1.3±0.3	2.4±0.2	II
Z-Lys-Pro-CHO[e]	3.0±0.6	7.2±0.6	I
Z-Ala-Pro-CMK[e]	0.0032±0.0004	0.0168±0.0046	II
Z-Arg-Pro-CMK[e]	0.0029±0.0001	0.0048±0.0006	III

[a] The IC_{50} values were determined in a kinetic activity assay with recombinant SmPOP or HsPOP and the fluorogenic substrate Z-Gly-Pro-AMC at pH 8.0. The mean values ± S.D. of three replicates are given.
[b] Induction of phenotype alterations by the inhibitors was determined with NTS in culture. The inhibitors were tested at 10 μM and 1 μM concentrations, and the resulting phenotypes, arising as a function of time and concentration, were graded I to III, with grade I being the most severe (see Materials and Methods).
[c] Y-29794 oxalate: 2-[[8-(Dimethylamino)octyl]thio]-6-(1-methylethyl)-3-pyridinyl-2-thienylmethanone oxalate
[d] SUAM 14746: 3-([4-[2-(E)-styrylphenoxy]butanoyl]-L-4-hydroxyprolyl)thiazolidine).
[e] Peptidic inhibitors with reactive aldehyde (CHO) or chloromethyl ketone (CMK) warheads (see Materials and Methods).

doi:10.1371/journal.pntd.0003827.t002

inhibition by SUAM 14746 was similar for both the human and schistosomal enzymes (IC_{50} values of 83 nM and 92 nM, respectively), Y-29794 oxalate and Z-Pro-Pro-CHO inhibited SmPOP with IC_{50} values that were about one order of magnitude greater than those for human POP (IC_{50} values of 8.6 μM and 0.49 μM, respectively, for Y-29794 oxalate, and 0.16 μM and 0.01 μM, respectively, for Z-Pro-Pro-CHO).

A spatial model of SmPOP was constructed by homology modeling to study the structure-activity/inhibition relationship. The X-ray structure of porcine POP (PDB code 1QFS) was used as a template. Fig 5 shows that SmPOP has the conserved architecture of the mammalian POP comprising both the β-propeller and peptidase domains [45]. The peptidase domain (residues 430–712) has a characteristic α/β-hydrolase fold [46,47] which consists of a central eight-stranded β-sheet flanked on both sides by eight α helices. The catalytic amino-acid residues Ser556, Asp643 and His682 are located in a large cavity at the interface between the domains. The disk-shaped β-propeller domain (residues 76–429) is composed of seven repeats of four-stranded antiparallel β-sheets which are arranged around a central tunnel.

The binding mode of SmPOP was analyzed using the transition-state analog POP inhibitor Z-Pro-Pro-CHO (benzyloxycarbonyl-L-prolyl-L-prolinal) which was docked into the SmPOP active site based on the crystallographic complex of this inhibitor with porcine POP (PDB code 1QFS). The docking model (Fig 5) shows that the prolinal residue of the inhibitor forms a covalent hemi-acetal linkage with the catalytic Ser556. The P1 Pro ring binds to the hydrophobic S1 binding pocket (defined by Phe478, Trp597, Tyr601 and Val646 residues) and is stacked against a Trp597 side chain. The backbone of both the P1 and P2 proline residues forms three

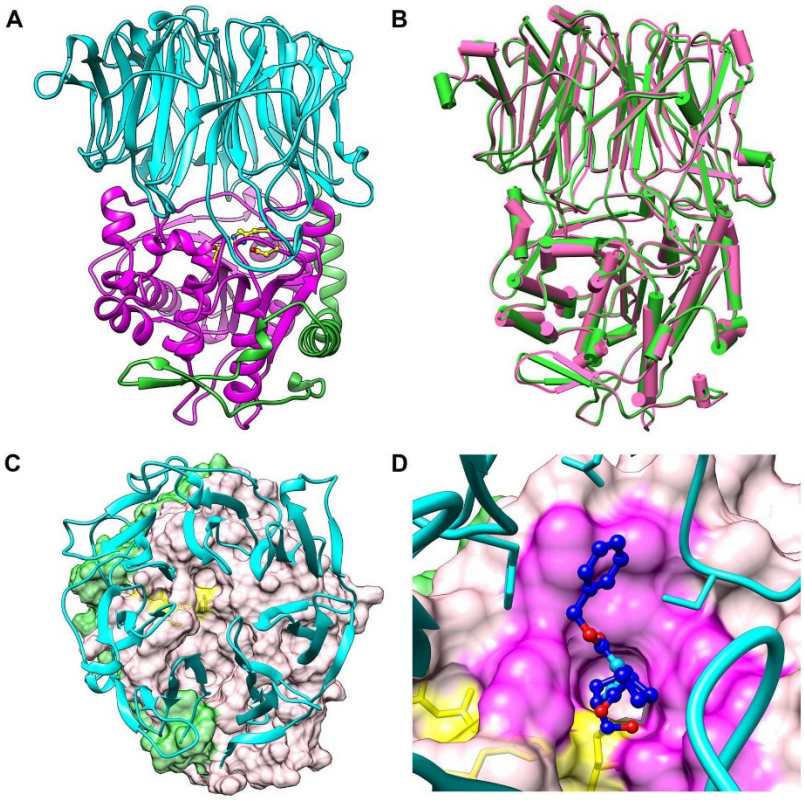

Fig 5. A three-dimensional homology model of SmPOP. (A) A ribbon representation of the overall SmPOP structure showing the β-propeller domain (cyan) and the catalytic domain (pink); the N-terminal segment is colored green. The active site containing the catalytic triad Ser556, Asp643 and His682 (yellow) is located at the interface of the two domains. (B) A superposition of the SmPOP model (green) and the porcine POP crystal structure (pink with the PDB code 1QFS) in a cylinder representation. (C) A view from the top of the SmPOP model (the β-propeller domain (cyan, ribbon representation) controls access to the active site of the catalytic domain (the pink surface) indicated by the catalytic triad residues (the yellow surface and sticks); the N-terminal segment is shown as the green surface. (D) A surface representation of the SmPOP active site located in the catalytic domain (the pink surface). The covalently-bound inhibitor Z-Pro-Pro-CHO is depicted in the ball-and-stick representation (carbon atoms in blue, oxygen in red and nitrogen in light blue). The catalytic-domain residues forming contacts with the inhibitor are highlighted as the magenta surface; the catalytic triad residues are represented by the yellow surface/sticks. The β-propeller domain is shown as a cyan ribbon, the residues interacting with the inhibitor as cyan sticks.

doi:10.1371/journal.pntd.0003827.g005

hydrogen bonds to the SmPOP active site. Additionally, the P3 benzyloxycarbonyl group binds to the hydrophobic S3 binding site (residues Phe175, Cys257, Asn273, Ile593 and Ala596).

SmPOP is localized in the tegument and parenchyma of adult schistosomes

Indirect immunofluorescence microscopy on semi-thin sections using affinity-purified antibodies against rSmPOP demonstrate that SmPOP is expressed in the parenchyma and tegument of adult schistosomes (Fig 6; for a high-resolution micrograph, see Fig 7). The intensity

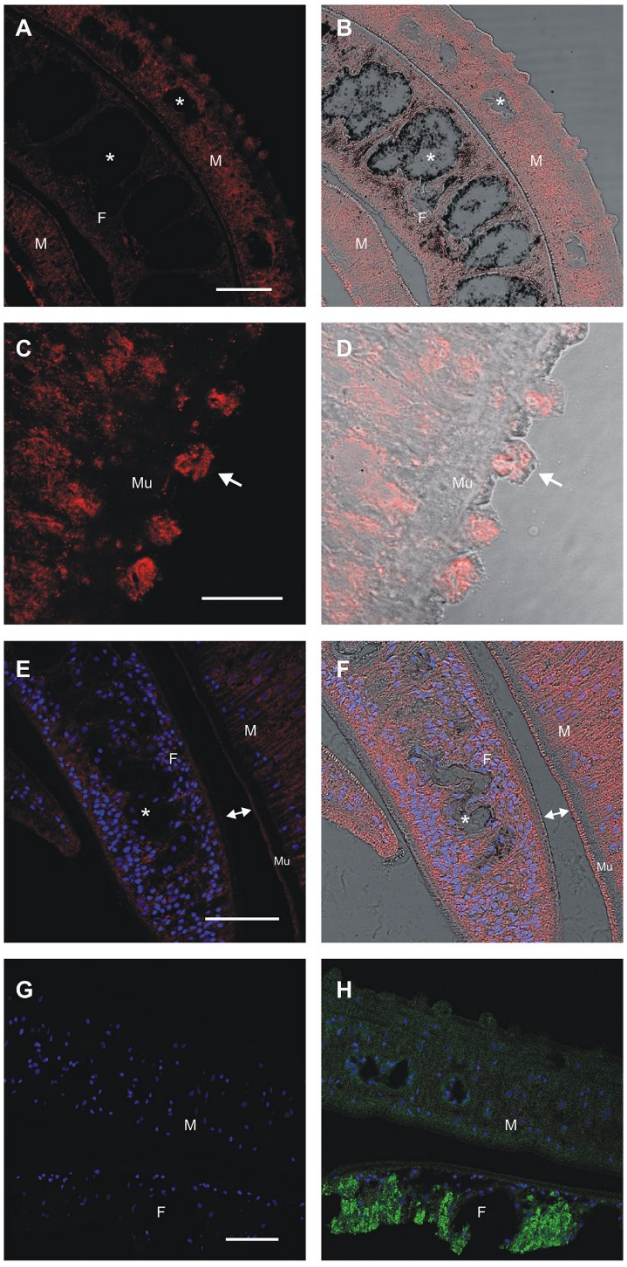
A
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Fig 6. SmPOP is localized to the tegument and parenchyma of adult *S. mansoni*. Semi-thin sections of adult male and female *S. mansoni* were probed with an anti-SmPOP IgG (A–F) or a pre-immune IgG (G, H) followed by reaction with an anti-rabbit IgG Alexa 594-labeled secondary antibody (red). DAPI was used to label the nuclear DNA (blue); female vitellaria are characterized by strong autofluorescence in the green spectrum (H). The left column shows merged fluorescent channels; in the right column, the signal is merged with differential interference contrast (except in H). Male worms (M) incubated with anti-SmPOP show a stronger immune-reactivity than female worms (F) (micrographs A and B). A red fluorescent signal is found in the parenchyma and tegument, but it is absent from the gut (the asterisks in A, B, E and F) and muscular tissue (Mu, micrographs C-F). In male worms the signal is found accumulated in the tubercles of the dorsal tegument (the arrows in C and D) and also outlines the gynaecophoral canal. Note the difference in signal intensity between the male and female tegument (the connected arrowheads in E and F). Only faint background fluorescence could be detected in the red spectrum in the negative control probed with pre-immune IgG (the micrographs G and H). The scale bar in C and D represents 20 μm; in A, B, E–H, 50 μm.

doi:10.1371/journal.pntd.0003827.g006

of the signal was greater in the tegument of the male compared to the female (Fig 6C and 6D). Labeling was not observed in the gastrodermis, gut lumen and muscular tissues (Fig 7). Intense staining was seen in the male tegumental tubercles (Fig 6C and 6D). Pre-immune serum was applied as a negative control and only faint background fluorescence was detected (Fig 6G and 6H). Similar results were obtained in immuno-histochemical studies with NTS (S5 Fig). With this developmental stage, SmPOP was localized at or close to the surface; a low diffuse signal was also seen in the parenchyma whereas the gut exhibited no specific fluorescence. No reaction was observed with pre-immune serum (S5 Fig).

SmPOP on living parasites cleave host peptides containing proline

We investigated whether SmPOP can interact with peptidic substrates in the environment surrounding the schistosome. NTS or adult schistosomes were incubated in the presence of the

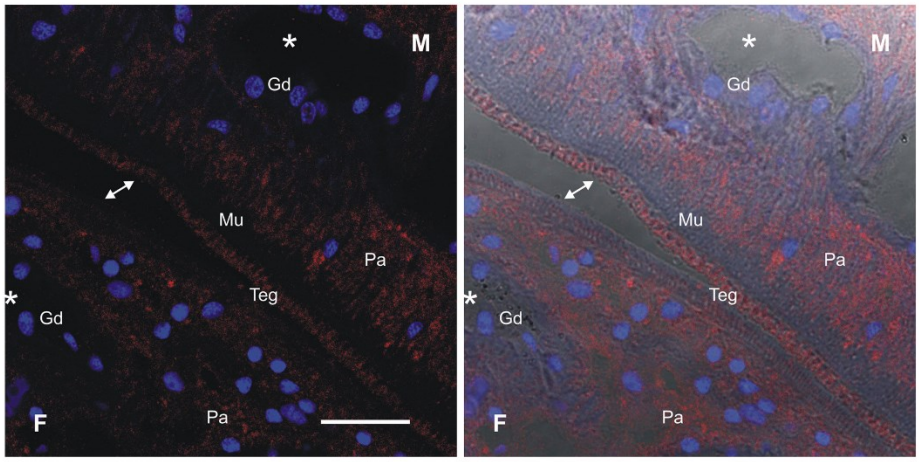

Fig 7. Detailed micrograph of SmPOP localization in the tegument of adult *S. mansoni*. The tissue section was probed with anti-SmPOP IgG followed by an anti-rabbit IgG Alexa 594-labeled secondary antibody (red). DAPI was used to label the nuclear DNA (blue). The left image shows merged fluorescent channels; on the right, the fluorescent signal is merged with differential interference contrast imaging. A red fluorescent signal is found in the parenchyma (Pa) and tegument (Teg), but is absent from the gastrodermis (Gd), gut lumen (the asterisks) and muscular tissue (Mu). Male worms (M) show a stronger immuno-reactivity than female worms (F). Note the difference in the signal intensity on the tegument of the male compared to the female (the connected arrowheads). Scale bar = 20 μm.

doi:10.1371/journal.pntd.0003827.g007

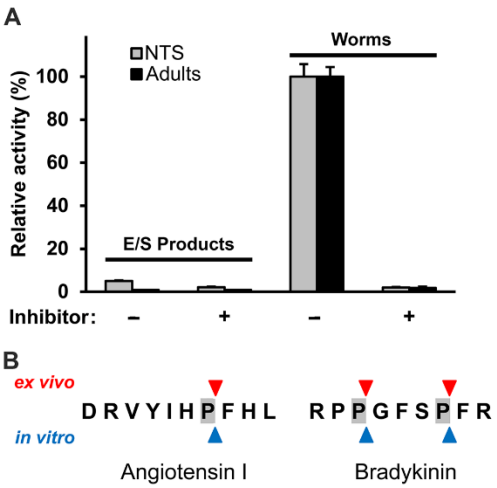

Fig 8. SmPOP in live *S. mansoni* cleaves vasoregulatory hormones. (A) SmPOP activity detected in the excretory-secretory products of or associated with live NTS and adults (worms) maintained in culture was determined using the fluorogenic substrate Z-GP-AMC. The inhibitor, Z-Ala-Pro-CMK, was added in the control experiments to specifically block SmPOP activity. The mean values ± S`.D. of three replicates are normalized to the maximum value in each series. (B) The peptide hormones angiotensin I and bradykinin were incubated with recombinant SmPOP (*in vitro*) or with live adults maintained in culture (*ex vivo*). The reaction mixture and cultivation medium, respectively, were analyzed by mass spectrometry and cleavage positions (triangles) in the hormones were identified (see also S3 Table).

doi:10.1371/journal.pntd.0003827.g008

fluorogenic peptide substrate Z-Gly-Pro-AMC. Cleavage of the substrate was measured in a microplate reader and was abolished in the presence of the specific POP inhibitor Z-Ala-Pro-CMK (Fig 8A). We also tested whether SmPOP activity is measurable in the excretory/secretory (E/S) products of NTS and adults. For this purpose, E/S products were collected after a two-day cultivation of parasites and SmPOP activity was measured using the same fluorogenic substrate. No significant POP activity was detected in E/S products, demonstrating that SmPOP is not secreted into the cultivation media.

In the next step, we used the above culture assay to measure cleavage by adult parasites of two vasoregulatory proline-containing hormones from the human host, namely angiotensin I and bradykinin. Both hormones were cleaved when added to the cultivation medium and the cleavage occurred specifically after Pro residues as demonstrated by mass spectrometry (Fig 8B). Again, the fragmentation was abolished in the presence of a POP-specific inhibitor Z-Ala-Pro-CMK (but not in the presence of the cysteine peptidase inhibitor E-64; S3 Table). The identified cleavage positions in the hormone sequences were identical with those obtained by *in vitro* fragmentation using rSmPOP.

To conclude, SmPOP, although not secreted from the parasite, can nonetheless interact with physiologically relevant host peptides in the environment.

SmPOP inhibitors induce deleterious phenotypes in cultured schistosomula

A panel of SmPOP inhibitors was tested at 1 and 10 μM against NTS and the phenotypic responses graded I through III from the most to the least severe (Table 2). The CHO inhibitor, Z-Lys-Pro-CHO, induced a grade I response. Grade II phenotypes were induced by Z-Gly-Pro-CHO, Z-Tyr-Pro-CHO, Z-Arg-Pro-CHO and the CMK inhibitor, Z-Arg-Pro-CMK. The inhibitors Z-Ala-Pro-CHO and Z-Ala-Pro-CMK induced the least severe grade III phenotype. The commercial inhibitors of human POP, Y-29794 and SUAM 14746, induced a grade II response or had no effect, respectively (Table 2).

Discussion

We identified and functionally characterized a S9-family serine peptidase from the human blood fluke, *S. mansoni*. It was denoted SmPOP, *S. mansoni* prolyl oligopeptidase, based on its 51% primary sequence identity to human and porcine prolyl oligopeptidases. Also, homology modeling of SmPOP using porcine POP as a structural template revealed that both enzymes share the same spatial architecture and domain structure; specifically, a catalytic peptidase domain with an α/β hydrolase fold and a catalytic triad, and a cylindrical β-propeller domain that covers the active site and defines prolyl oligopeptidase as an oligopeptidase [48].

SmPOP was heterologously expressed in *E. coli*, purified as an active peptidase and subjected to a series of biochemical analyses to determine its substrate and inhibitory specificity. Consistent with its classification as a S9-family prolyl oligopeptidase, the enzyme cleaves various oligopeptide substrates in an endopeptidolytic mode at the carboxyl terminus of Pro residues [45]. Cleavage specificity analysis with the positional-scanning substrate library revealed a preference for basic amino acids over hydrophobic or aliphatic amino acids at P2; a Pro residue at P2 was unfavorable. A similar S2 subsite specificity profile was obtained for human POP (Fig 4).

rSmPOP was effectively inhibited by the general serine peptidase inhibitor, diisopropylfluorophosphate [49], but only weakly by inhibitors targeting the S1 family of serine peptidases such as Pefabloc, benzamidine, and BPTI. These data are consistent with the inhibitory specificities of mammalian and trypanosomal POPs [50,51]. The inhibitor specificity of rSmPOP was investigated further using a panel of synthetic prolinal inhibitors that vary at the P2 amino-acid residue (Z-Xaa-Pro-CHO, Table 2). The inhibitor specificity profile mirrored that determined with the positional- scanning substrate library, with the exception of the Pro residue in the P2 position, which generates a good inhibitor but a poor substrate (Z-Pro-Pro-CHO vs. Z-Pro-Pro-ACC, respectively, Table 2 and Fig 4A). Note that Z-Pro-Pro-ACC substrate does not bind effectively in the active site neither as the uncleaved form nor as the hypothetical cleavage product Z-Pro-Pro-OH (as they do not compete with Z-Gly-Pro- substrate). A similar discrepancy was observed for human POP (Table 2 and Fig 4A). Based on the assembled biochemical and structural data, therefore, it is clear that SmPOP and its mammalian orthologs are almost identical in their catalytic specificity profiles suggesting a strong evolutionary conservation of function and structure.

The panel of SmPOP inhibitors was further evaluated for their anti-schistosomal effects against NTS in culture. These tests demonstrated that some of the investigated inhibitors induced deleterious phenotypes or death. Although, interactions other than with the specific target protein cannot be excluded, the data encourage the search for small molecule inhibitors of SmPOP. Inhibitors of human POP are currently being examined as drug leads in several neurological disorders such as depression, Alzheimer's disease and amnesia, and a number are in preclinical and clinical trials as nootropics (for review see [44]). POP is also of interest for the treatment of celiac sprue, an inflammatory disease of the small intestine caused by ingesting proline-rich gluten [39].

The prolyl oligopeptidases Tc80 and Tb80 from the protozoan parasites *Trypanosoma cruzi* and *T. brucei*, respectively, are secreted and can degrade host extracellular-matrix (ECM) proteins such as proline-rich collagens I and IV [23,51]. Tc80's ability to ability to degrade of ECM components contributes directly to the invasion of mammalian cells by *T. cruzi* trypomastigotes [22]. In contrast, SmPOP cannot degrade protein substrates, including collagens, even though it has about 40% identity with trypanosomal POPs (S1 Table). Further, SmPOP is not found in *S. mansoni* E/S products suggesting that it is not secreted by schistosomes, a finding consistent with the absence of the signal peptide in the SmPOP sequence. The data would therefore indicate that the trypanosomal POPs possess different physiological functions from those postulated below for the schistosomal enzyme.

By RT-qPCR and substrate analysis, SmPOP is expressed in those developmental stages parasitizing the definitive mammalian host (adults, NTS and eggs). By immunolocalization with a monospecific rabbit antibody SmPOP is distributed in the tegument (males) and parenchyma of NTS and adult schistosomes. The enzyme is absent from the gastrodermis and gut lumen suggesting that the enzyme does not contribute to the digestion of ingested blood proteins. The antibody signal was significantly greater in male worms in accordance with the activity profiling of worm extracts, whereby male worm extracts displayed 5–6 times greater SmPOP specific enzymatic activity than females (S6 Fig). Intriguingly, SmPOP is found in the male tegument, not least in the tubercles, but is apparently absent from the female tegument. This suggests that SmPOP may have male-specific peptidolytic functions at the host–parasite interface and/or at the male-female interface. As noted above, the enzyme seems not to be secreted by the parasite yet, via contact with the endothelium of the host vasculature, may exert localized effects on vascular physiology, including the degradation of vasoactive peptides (see below). A similar localization in the tegument and parenchyma was previously noted for the *S. mansoni* cysteine peptidase cathepsin B2 for which physiological function(s) are not yet known [52].

We demonstrate that the schistosome parasite can cleave the vasoregulatory peptides, angiotensin I and bradykinin, when co-incubated *in vitro* and that the activity is due to SmPOP as indicated by mass spectrometry and specific inhibition by a POP inhibitor. Angiotensin I is produced by the renin-angiotensin system which is the primary physiological regulator of blood pressure, sodium balance and fluid volume [53]. SmPOP converts angiotensin I (precursor of the main vasoconstrictor angiotensin II) to the vasodilatory angiotensin-(1–7). Angiotensin-(1–7) also inhibits cell proliferation, angiogenesis, fibrosis, and inflammation [54,55]. Bradykinin is generated by the kallikrein-kinin system which also participates in the regulation of blood pressure [53]. Bradykinin is a potent vasodilator, promotes natriuresis, diuresis and inflammation. Proteolytic cleavage by SmPOP inactivates this hormone. The possible contribution, therefore, by a tegument-localized SmPOP to the modulation or dysregulation of both these, and possibly, other, homeostatic systems is conceivable whereby cleavage of the pro-inflammatory and vasoconstrictory angiotensin I and pro-inflammatory bradykinin may provide a survival benefit to the schistosome during its residence in and movement through the venous blood system. Follow-up *in vivo* studies will examine these possibilities in more detail.

Supporting Information

S1 Fig. Examples of phenotypes induced in cultured NTS by POP inhibitors listed in Table 2. NTS were incubated up to four days in Basch Medium 169 in the presence of inhibitors (for details see Methods). Images were captured using a Zeiss Axiovert 40 C inverted microscope (10x objective) and a Zeiss AxioCam MRc digital camera controlled by AxioVision 40 (version 4.8.1.0) software. Scale bar = 150 μm.
(TIF)

S2 Fig. A multiple sequence alignment of *S. mansoni* prolyl oligopeptidase (SmPOP) with selected POPs from other blood-feeding parasites and mammalian POPs. Parasite POPs: SmPOP (S. mansoni, GenBank accession number KF956809), *Pediculus humanus* (P. humanus, XP_002430998), *Aedes aegypti* (A. aegypti, Q16WP2), *Ixodes scapularis* (I. scapularis, B7PDF5), *Toxoplasma gondi* (T. gondi, XP_002369249), *Trypanosoma cruzi* (T. cruzi, AAQ04681) and *Leishmania infantum* (L. infantum, CAM72491.1). Mammalian POPs: human (H. sapiens, P48147) and porcine (S. scrofa, P23687). Catalytic-triad residues (Ser, Asp and His) are indicated in red; those residues identical with those of SmPOP are shaded in gray. The residue numbering corresponds to the SmPOP sequence and its color coding refers to the domain structure of POPs consisting of the N-terminal segment (green), the β-propeller domain (cyan) and the peptidase catalytic domain (magenta).
(TIF)

S3 Fig. The maximum-likelihood phylogenetic tree displaying the evolutionary relationship of SmPOP to selected POPs from other organisms. A multiple alignment of SmPOP with 35 other POP protein sequences was performed using Clustal X 2.0 and the default parameters. The resulting alignment was edited to exclude ambiguous regions by the BioEdit 7.0 editing software. The phylogenetic analysis of the multiple alignment was performed using the maximum likelihood method in PAUP 4.0. The tree was visualized using the Treeview 1.6.6. program. Bootstrap values with 100 repeats are shown at the nodes. GenBank or HelmDB accession numbers of the aligned sequences are indicated. SmPOP is underlined in red and bold type faces. **GenBank accession numbers: Kinetoplastida**—CAM72491 *Leishmania infantum*, *AAQ04681 Trypanosoma cruzi*, CAD42967 *Trypanosoma brucei; Apicomplexa*—XP_002369249 *Toxoplasma gondii*; **Bacteria—**WP_007903716 *Ktedonobacter racemifer*, WP_008080757 *Vibrio sinaloensis*, WP_012088900 *Shewanella baltica*, WP_013626821 *Planctomyces brasiliensis*, WP_011140683 *Gloeobacter violaceus*, WP_011612632 *Trichodesmium erythraeum*, EHJ13806 *Crocosphaera watsonii*, WP_012411436 *Nostoc punctiforme*, WP_006634458 *Microcoleus vaginatus*; **Arthropoda**—XP_002430998 *Pediculus humanus*, B7PDF5 *Ixodes scapularis*, Q16WP2 *Aedes aegypti*, B0W4N7 *Culex quinquefasciatus*, EFN76622 *Harpegnathos saltator*, EFN66352 *Camponotus floridanus;* **Plantae—**A9SA32 *Physcomitrella patens*, XP_002285910 *Vitis vinifera*, ACG43067 *Zea mays*; **Vertebrata**—C0HBI8 *Salmo salar*, Q503E2 *Danio rerio*, Q6P4W3 *Xenopus tropicalis*, F1NUS2 *Gallus gallus*, O70196 *Rattus norvegicus*, Q9QUR6 *Mus musculus*, F1PHX2 *Canis familiaris*, P48147 *Homo sapiens*, Q9XTA2 *Bos taurus*, P23687 *Sus scrofa;* **Trematoda—**KF956809 Schistosoma mansoni. **HelmDB accession numbers: Nematoda—**Asuu161668 *Ascaris suum;* **Trematoda**—Fhep110926 *Fasciola hepatica*, Shae172866 *Schistosoma haematobium*.
(TIF)

S4 Fig. Recombinant SmPOP does not digest host-derived macromolecular protein substrates. Human serum albumin (HSA), human collagens type I and IV (Col I and Col IV) and human hemoglobin (Hb) were incubated for 12 h in the presence or absence of rSmPOP. The reaction mixtures were subjected to SDS-PAGE (HSA, Col I and Col IV) or Tricine-SDS-PAGE (Hb) and protein stained. For details, see Methods.
(TIF)

S5 Fig. The localization of SmPOP in *S. mansoni* newly transformed schistosomula (NTS). Parasites were fixed and probed with anti-SmPOP IgG (A) or a pre-immune IgG from the same rabbit (B). Anti-rabbit IgG Alexa 594-was used as the secondary antibody (red). DAPI was used to label the nuclear DNA (blue) and the fluorescent signals were merged with differential interference contrast (DIC). The greatest red fluorescence is localized to the surface (tegument) with a low diffuse signal in the parenchyma (SmPOP in A). The gut is negative for the

SmPOP signal (the asterisk). NTS probed with pre-immune IgG lack any visible fluorescence in the red channel (SmPOP in B). Scale bar = 50 μm.
(TIF)

S6 Fig. The SmPOP activity in *S. mansoni* adult males and females. SmPOP activities were measured in protein extracts of adult males and females (green bars) or in cultivation medium post incubation with live parasites (red bars). Z-Gly-Pro-AMC was used as the fluorogenic substrate. SmPOP activity (which was sensitive to inhibition by the specific POP inhibitor, Z-Pro-Pro-CHO) was normalized to protein content of extracts or number of worms used.
(TIF)

S1 Table. Identity matrix of POP amino acid sequences aligned in S1 Fig.
(PDF)

S2 Table. Identification of native SmPOP by mass spectrometry.
(PDF)

S3 Table. The fragmentation of peptide hormones by live *S. mansoni* adults.
(PDF)

Acknowledgments

We thank Brian M. Suzuki and KC Lim (CDIPD UCSF, San Francisco) for technical support, Zuzana Demianova for mass spectrometry, and Jan Paces for phylogenetic analysis (IOCB ASCR, Prague).

Author Contributions

Conceived and designed the experiments: SS JD JV CRC MM MHo. Performed the experiments: PF SS MHr JD JV AJ LU CRC MHo. Analyzed the data: PF SS MHr JD JV CRC MM MHo. Contributed reagents/materials/analysis tools: SS MHr JD JV JHM CRC MM MHo. Wrote the paper: PF SS JD JHM CRC MM MHo.

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4.7 Publication No. 6: Sensitive fluorescence *in situ* hybridization on semithin sections of adult *Schistosoma mansoni* using DIG-labeled RNA probes.

RNA *in situ* hybridization is a technique that allows the analysis of the spatial distribution of gene expression within tissues or single cells. This publication describes an optimized protocol for sensitive fluorescence detection of gene transcripts (mRNAs) on semi-thin sections of adult *S. mansoni*. It is based on the annealing of digoxigenin-labeled RNA probes to complementary sequences in the sample. These probes are then detected using anti-digoxigenin antibody coupled to fluorescent tyramide signal amplification, which amplifies the signal and allows visualization of the probe. Improved methods of tissue preservation, sectioning, amplification of fluorescent signal, and prehybridization tissue treatment, were specifically tailored for the detection of gene transcripts in fine structures. The protocol was sensitive enough to detect very low abundance targets. The procedure was optimized for adult *S. mansoni* tissues; however, it can be successfully applied to other trematode species [159, 160].

PhD applicant contribution: parasite collection and cultivation, tissue preparation (fixation, sectioning, paraffin embedding), microscopy techniques, co-author of probe design (cloning, plasmid validation, probe synthesis, probe labeling), *in situ* hybridization protocol optimization (all hybridization steps, signal amplification), data interpretation, image preparation, manuscript writing and drafting.

The full text of this book chapter cannot be included in the thesis due to copyright restrictions. Therefore, only the title page is included.

Chapter 4

Sensitive Fluorescence In Situ Hybridization on Semithin Sections of Adult *Schistosoma mansoni* Using DIG-Labeled RNA Probes

Lenka Ulrychová, Martin Horn, and Jan Dvořák

Abstract

In situ hybridization is a tool for evaluation of gene expression within tissues or single cells. This protocol describes optimized sensitive fluorescence detection of gene transcripts (mRNAs) in semithin sections of *Schistosoma mansoni* adult worms using specifically designed and labeled RNA probes. Due to improved methodologies in tissue preservation, sectioning, amplification of fluorescent signal, and prehybridization tissue treatment, it is possible to detect transcripts in the fine structures of schistosomes. The protocol is sensitive enough to detect very low abundance targets. This procedure is optimized for tissues derived from *S. mansoni* adult worms; however, it can be successfully applied to other trematode species.

Key words *Schistosoma mansoni*, Adult worm, Tissue section, In situ hybridization, RNA probe, Digoxigenin labeling, Fluorescence RNA detection

1 Introduction

In situ hybridization (ISH) is a technique that enables the localization and visualization of specific nucleic acids in the cells, tissues, or whole organisms. It is based on annealing of labelled RNA probes to complementary sequences in the sample. ISH was introduced more than 50 years ago [1]; however, this technique underwent enormous development during the time. It is mostly because different types of tissue and/or cells require optimization not only for the hybridization itself but also for subsequent visualization. Critical steps include the tissue pretreatment that is highly specific for different tissue types. Therefore, the effective in situ hybridization protocol must be always optimized.

Several ISH protocols have been developed for schistosome tissues—whole mount in situ hybridization (WISH), a protocol that enables to detect gene transcripts within the whole *S. mansoni* adults [2] and ISH performed on their semithin sections

David J. Timson (ed.), *Schistosoma mansoni: Methods and Protocols*, Methods in Molecular Biology, vol. 2151,
https://doi.org/10.1007/978-1-0716-0635-3_4,

4.8 Publication No. 7: Collection of excretory/secretory products from individual developmental stages of the blood fluke *Schistosoma mansoni*

Individual developmental stages of the blood fluke *S. mansoni* excrete or secrete a wide variety of biomolecules from their bodies into the surrounding host environment. These excretory/secretory (ES) products enable successful schistosome parasitism. They function at the host-parasite interface where they facilitate host invasion and contribute to the parasite survival by modulating host immune and homeostatic responses. To study these processes in detail, it is necessary to collect sufficient amounts of ES products. This publication describes optimized protocols for the collection of ES products from *S. mansoni* developmental stages that infect or reside in the human host: cercariae, schistosomula, adults, and eggs.

PhD applicant contribution: Preparation of individual developmental stages of *S. mansoni* and their cultivation, optimization of protocols for collection of ES products and their processing for further analysis.

The full text of this book chapter cannot be included in the thesis due to copyright restrictions. Therefore, only the title page is included.

Chapter 5

Collection of Excretory/Secretory Products from Individual Developmental Stages of the Blood Fluke *Schistosoma mansoni*

Adrian Leontovyč, Lenka Ulrychová, Martin Horn, and Jan Dvořák

Abstract

Individual developmental stages of blood fluke *Schistosoma mansoni* excrete or secrete a different set of molecules. Here we describe optimized protocols for collection of excretory/secretory products (E/S products) from cercariae, schistosomula, adult worms, and eggs. These E/S products are essential for successful parasitism functioning at the host-parasite interface, enabling invasion into the host and contributing to the survival of the parasite by modulation of host physiology and immune responses. Collection of sufficient amounts of E/S products is required for detailed research of these processes.

Key words *Schistosoma mansoni*, Developmental stages, Excretory/secretory products

1 Introduction

Blood flukes of the genus *Schistosoma* are highly adapted to the human host. Their larvae (cercariae) invade the host via skin, transform into schistosomula (larval migratory worms), and based on schistosome species migrate through the lungs to the blood vessels of mesentery or urinary bladder where they reach maturity. Adult schistosomes can reside for decades in the host vascular system as male-female pairs producing hundreds of eggs per day. Entrapped eggs in the host tissues induce inflammatory processes that significantly contribute to disease morbidity [1].

Schistosomes excrete or secrete a broad range of molecules from their bodies into the surrounding environment of the host. Composition of these excretory/secretory products (E/S products) greatly differs among individual developmental stages [2–5]. E/S products of cercariae are mostly produced by pre- and post-acetabular glands, and their main function is to penetrate the host skin and to downregulate the host's immune response during the invasion [6–9]. The molecules secreted by schistosomula and adults

David J. Timson (ed.), *Schistosoma mansoni: Methods and Protocols*, Methods in Molecular Biology, vol. 2151,
https://doi.org/10.1007/978-1-0716-0635-3_5,

5 Discussion

Schistosoma spp. are blood flukes that can survive for many years or decades in the vascular system of the definitive host, including humans [12]. Proteolytic enzymes (proteases, peptidases) are key players in successful schistosome parasitism. These enzymes are essential for the survival and vital functions of the parasite and are also released into the surrounding environment where they interact with the host. They play key roles in processes such as reproduction and development, nutrient uptake, invasion of ng the host, and alternation of the host's body functions. They can also interfere with blood clotting, dilate blood vessels, or modulate the host's immune response [183, 213, 214].

This thesis focuses on the serine proteases of the blood fluke *S. mansoni*, with an emphasis on the S1 family serine proteases (SmSPs). Previous research has mainly concentrated on cysteine and aspartic proteases from the schistosome digestive network, a physiological cascade related to the nutrient intake [14, 98, 206]. Serine proteases have long been neglected and little information is available about them. The exception is a serine protease known as cercarial elastase, which is used by cercariae to penetrate the host skin and has been well studied and characterized [207, 212].

To survive, parasitic helminths manipulate the host's complex homeostatic systems through surface (tegument) molecules or through various biomolecules released into their environment, which are generally referred to as excretory/secretory (ES) products. In publication No. 2, the proteolytic activities of ES products from *S. mansoni* developmental stages (eggs, schistosomula and adults) were analyzed using a panel of substrates and class-specific protease inhibitors. The protocols for the preparation of these ES products are described in detail in publication No. 7. Each of the developmental stages produced a distinct profile of proteolytic activities, indicating a different set and ratio of proteases. The proteolytic profile of schistosomula ES products is the simplest with a predominant activity of serine proteases. More complex profiles were identified for adult and egg ES products, with serine, cysteine and metalloproteases contributing to the proteolytic activities. Such differences in ES production may

be related to the fact that different life stages reside in a different types of host tissues, and therefore have different conditions and strategies for survival and further development.

Significant serine protease activity was detected in ES products from all analyzed stages. The vast majority of publications on SmSPs deal with cercarial elastase (an enzyme produced by infective larvae cercariae to facilitate penetration into the human body) [206, 207, 209-212, 224]. Of the other SmSPs, only two representatives, SmSP1 and sK1, have been partially characterized previously [16, 213]. For both proteases, a role in the regulation of vascular tone has been suggested. In publication No. 3, a group of five serine proteases of the S1 family [180], designated SmSP1-5, was identified and described using a variety of genomic, transcriptomic, phylogenetic and functional proteomic approaches. All five SmSPs have a catalytic domain typical for S1 family of proteases with a catalytic triad in the order of His, Asp and Ser. The catalytic domains of SmSP1 to SmSP4 share significantly greater sequence identity (about 30%) with each other than with SmSP5 (about 20%) [179, 208].

The sequence alignment of SmSPs revealed that all members, except SmSP5, share a conserved Asp182 residue, which determines the specificity of the S1 binding site, favoring Arg and Lys residues at the P1 position. This specificity is analogous to that of vertebrate trypsins [225]. In contrast, the S1 binding pocket of SmSP5 has a Gly182. In addition, SmSP5 lacks the disulfide Cys184-Cys212, which is present in the other four SmSPs and is known to stabilize the S1 binding site in vertebrate trypsins. Interestingly, this disulfide is also absent in schistosome cercarial elastases, which contain non-polar residues (Ile or Leu) at the bottom of the S1 binding pocket, resulting in elastase and chymotrypsin-like activities [207]. In summary, SmSP5 is likely to have a substrate specificity similar to that of chymotrypsin-type proteases and is therefore structurally closer to the cercarial elastase and invertebrate chymotrypsin-like proteases than other S1 family proteases. Orthologs of all SmSPs have been found in other Platyhelminthes such as *Opistorchis* spp., *Clonorchis* spp., *Echinococcus* spp. or *Taenia solium* [176, 208].

SmSP1 and SmSP3 are multi-domain enzymes. Their domain organization resembles that of host proteases involved in blood coagulation or fibrinolysis. In addition to the catalytic protease domain, SmSP1 contains a Complement-Uegf-BMP-1 (CUB) extracellular and plasma membrane-associated domain, an LDL-binding receptor domain class A (LDLa) domain, and

SmSP3 contains an incomplete CUB domain. The LDLa domain of SmSP1 may be responsible for cholesterol uptake. Schistosomes and other trematodes do not synthesize cholesterol and they must scavenge it from the host. A 2009 study showed that *S. mansoni* eggs release factors that mediate cholesterol lowering in mouse sera, while the presence of adult worms appeared to have little or no effect [226]. The CUB domain of SmSPs (SmSP1 and SmSP3) has an unknown function. In vertebrates, it is associated with regulatory proteases incorporated into the plasma membranes or with proteins in the extracellular space [227]. Accordingly, SmSP1 has been detected at the surface of the adult males and in the ES products [16, 228].

In publications No. 4 and No. 5, two *S. mansoni serine* proteases, SmSP2 and SmPOP, were biochemically characterized, and their biological roles were proposed. The SmSP2 sequence contains an N-terminal multidomain region, consisting of two unique structural features, a histidine stretch, a thrombospondin type 1 repeat (TSR-1) domain, and an S1 family catalytic protease domain at the C-terminus. The histidine stretch may act as a metal binding site, as we have shown *in vitro* using metal affinity chromatography with SmSP2 (publication No. 4). The TRS-1 domain has been found in multiple protein families and is present in more than 40 human proteins e.g. thrombospondins and some complement factors [229]. It is known to mediate cell adhesion, protein-protein interactions, or angiogenesis inhibition [176, 230]. SmPOP is a serine protease from the S9 family and its orthologues have been also found in other trematodes and nematodes [231, 232]. It is a true oligopeptidase that hydrolyzes peptide (but not protein) substrates with a strict specificity for Pro at P1. SmPOP cleaves host vasoregulatory, proline-containing hormones such as angiotensin I and bradykinin.

SmSP2 and SmPOP contribute to the parasite survival by modulating the local host vascular environment in favor of the parasite. During the residence of adult schistosomes in the host´s blood vessels, the worms can alter or disrupt normal blood flow and damage the endothelium of the veins. This could lead to the platelet activation and subsequent blood clotting. However, no blood clots were found on the surface of the parasite residing in the host. Our *in vitro* results showed that SmSP2 prevents blood clotting, is responsible for fibrinolysis and simultaneously adjusts vasoconstriction and vasodilation as needed [176]. At the same time, SmPOP possess important vasodilatory and anti-inflammatory effects [231]. Thus, SmSP2 and

SmPOP provide a survival benefit to the schistosomes during their residence and movement within the venous blood system. We hypothesize that the serine proteases SmSP2 and SmPOP may be a novel target for the development of anti-schistosomal treatment.

In publication No. 1, fluorescence *in situ* hybridization (FISH) was used to localize individual RNA molecules in adult *S. mansoni* in tissues including the esophagus, testis, ovary, vitellarium, and parenchymal and tegumental cells, including tubercles. The method is based on the detection of digoxigenin-labeled RNA probes by a horseradish peroxidase-conjugated anti-digoxigenin antibody. Probes hybridizing to transcripts were visualized by tyramide amplification assay [233]. The protocol is described in detail in publication No. 6.

The FISH method was validated by detecting expression patterns for a set of transcripts of previously described genes. The obtained results were in good agreement with previous findings reported using other techniques: transcripts of the digestive protease SmCB1 were localized in the gut, the expression of the surface-localized SmPOP was detected in the parenchyma and tegument, and the mRNAs of the tegumental SmTsp-2 and Sm29 were found in the tegumental cytons or tegument.

The transcriptome sequencing data [208, 221, 222, 234, 235] revealed that SmSP2 and SmSP4 are abundantly expressed in adult schistosomes, while SmSP1, SmSP3, and SmSP5 are significantly less expressed. The highly-sensitive FISH described in publication No. 1 revealed the expression pattern of all SmSPs, including the low-abundantly expressed ones, distributed in multiple tissues of adult schistosomes. All SmSPs were commonly detected only in the parenchyma of both sexes, but individual SmSPs showed distinct expression patterns in different subtypes of parenchyma cells, indicating their unique functional roles. Several SmSPs were localized in the esophagus and in some reproductive organs of male or female worms, suggesting their involvement in vital functions and reproduction. SmSPs may have there a function similar to that of SPs in the gonads of *Caenorhabditis elegans* or *Ascaris suum*, where they are involved in spermatogenesis and sperm activation in the uterus [236, 237], or SPs in the *Drosophila melanogaster* reproductive duct, which mediate post-mating responses [238].

Transcripts of some genes (SmSP4, SmPOP, and SmTsp-2) were localized on the surface of the male worms in tegumental cell structures known as tegumental tubercles (Figure 2). Protein synthesizing and sorting machinery (including the endoplasmic reticulum and Golgi apparatus) are located in the nucleated regions of the tegumental cells known as cell bodies or tegumental cytons (Figure 2). They are located beneath the musculature and are connected to the syncytial surface portion by cytoplasmic junctions. The most common destination for mRNAs within the cell is in the close proximity to the site of translation, i.e. near to the endoplasmic reticulum. Therefore, mRNA would be expected in the tegumental cytons, but not in the surface layer, in the tegumental tubercles. The unexpected localization in the tegument and tubercles suggests the existence of a yet to be understood transport mechanism that moves mRNA molecules from the cell bodies to the tegument and may play a role in the interactions between the worm and its host, e.g. via extracellular vesicles released by schistosomes.

The last interesting result of the localization study is the detection of natural antisense transcripts (NATs) (see chapter 2.4) exclusively in the female oviduct. This suggests their potential regulatory function in the reproductive processes of *S. mansoni*. Understanding the precise role of NATs in these processes warrants further investigation.

6 Conclusion

The thesis focused on proteolytic enzymes from the blood flukes *S. mansoni*, which cause schistosomiasis, one of the most important parasitic diseases. The results are summarized in five publications in international journals and two book chapters. The work provides a new comprehensive description of the proteases secreted by the blood flukes into the host environment. Newly discovered serine proteases have been characterized in this thesis and their potential function in parasite-host interactions has been described.

The thesis fulfilled its aims and the main results achieved are as follows:

- Excretion-secretion (ES) products of selected developmental stages of *S. mansoni* were prepared and the procedures for their collection were optimized.
- Proteolytic activities of ES products of *S. mansoni* developmental stages potentially interacting with the human host were identified. Each developmental stage secretes a unique spectrum of proteases, which invariably includes a significant proportion of serine proteases.
- Using bioinformatic analysis, genes for five different serine proteases (SmSPs) were identified and annotated in the *S. mansoni* genome. RT-qPCR analysis of their expression at different developmental stages revealed a complex expression profile for each SmSP, with the SmSP2 protease being the most abundant. The activities of SmSPs were also detected using selective fluorogenic substrates in homogenates and ES products of the studied stages.
- A novel protocol for fluorescent *in situ* hybridization (FISH) was optimized and validated. This protocol is highly sensitive for the detection of low abundance targets and is applicable to other trematodes such as *Eudiplozoon* spp.
- Gene transcripts (mRNAs) of all studied SmSPs were detected in adult *S. mansoni* males and females by FISH method. Transcripts of SmSP2 and SmSP4 were found in the tegument and its tubercles. Anti-sense transcripts were detected exclusively for SmSP5 in the oviduct.

- Protease SmSP2 and SmPOP were biochemically characterized and immunolocalized in adult worms. Their potential role in host-parasite interaction was described.

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