Molecular & Biochemical Parasitology 219 (2018) 17–23

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

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

Expression, purification and characterization of two leucine aminopeptidases of the blood fluke, *Schistosoma mansoni*

Gabriela Maggioli[a,*], Gabriel Rinaldi[b,1], Ines Giaudrone[a], Patricia Berasain[a], José F. Tort[c], Paul J. Brindley[b], Carlos Carmona[a]

[a] Unidad de Biología Parasitaria, Instituto de Higiene, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay
[b] Department of Microbiology, Immunology & Tropical Medicine, Research Center for the Neglected Diseases of Poverty, School of Medicine and Health Sciences, George Washington University, Washington, DC, 20037, USA
[c] Dpto. Genética, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay

ARTICLE INFO

Keywords:
Schistosoma mansoni
Leucine aminopeptidase
Metalloprotease
Parasite nutrition
Host-parasite interface

ABSTRACT

Schistosomiasis is a major neglected tropical disease (NTD) and considered the most important of the human helminthiases in terms of morbidity and mortality. Whereas treatment with praziquantel has been effective since the 1980s, the potential for the emergence of drug resistance has propelled the search for new interventions. Studies have revealed key roles of proteases in parasitic helminths during establishment of infection, tissue invasion, immune evasion, parasite feeding and development throughout the different developmental stages, pinpointing them as possible candidates. The leucine aminopeptidases (LAPs), members of the M17 family of Zn-metalloproteases, preferentially cleave leucine (Leu) residues at the N-terminal end of proteins and short peptides. These enzymes display broad proteolytic activities beyond Leu hydrolysis and are involved in processing, maturation, activation and/or degradation of substrates. As a vaccine immunogen, LAP induces protection against infection with the liver fluke *Fasciola hepatica*. Herein, two LAPs, SmLAP1 (Smp_030000) and SmLAP2 (Smp_083870) of the human blood fluke *Schistosoma mansoni* were cloned, expressed, purified and biochemically characterized. The enzymes differed in activity against diagnostic substrates, including leucine, methionine and arginine, with an optimal pH of 8.0. The activity increased in the presence of Mg^{+2} and Mn^{+2}, and was inhibited by bestatin, a specific inhibitor of aminopeptidase. In addition, 1,10-phenanthroline and EDTA inhibited the enzymatic activity of SmLAP2. Finally, immunolocalization using antibodies specific for SmLAP1 and SmLAP2 identified the expression of these proteases in the egg and adult developmental stages of *S. mansoni*, and in intestinal epithelia, vitelline cells and sub-tegumental regions of the parasite. Characterization of schistosome proteases not only enhances understanding of the biology of schistosomes and schistosomiasis, but may also provide novel intervention approaches.

1. Introduction

Schistosomiasis, caused by the infection with blood flukes *Schistosoma mansoni, S. haematobium, S. japonicum, S. intercalatum,* and *S. mekongi*, is considered the most important of the human helminthiases in terms of morbidity and mortality [1,2]. Schistosomiasis affects almost 240 million people worldwide and more than 700 million people live in endemic areas [3]. Moreover, as many as 200,000 people die from this neglected tropical disease (NTD) every year [4,5]. Praziquantel is the most effective drug and is employed widely in mass drug administration programs [6]. However, drug therapy cannot sustain control of infection and transmission given that reinfection occurs rapidly after treatment. Evolution and spread of resistant parasites represents a realistic threat that could limit the utility and effectiveness of praziquantel [7,8].

Proteases are involved in key activities in the host-parasite interface performing critical molecular tasks that lead to a successful parasitism. These enzymes contribute to tissue invasion, parasite nutrition and evasion of the host immune response [9]. Given these critical roles, helminth proteases have been considered attractive drug and/or vaccine targets for novel control strategies. Within the widely diverse group of Zn-metalloproteases of the M17 family, leucine aminopeptidases (LAPs) are considered promising vaccine candidates. LAPs preferentially cleave a leucine residue at the N-terminus of the proteins and

* Corresponding author.
E-mail address: gmaggioli@gmail.com (G. Maggioli).
[1] Current address: Parasite Genomics, Wellcome Trust Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge, CB10 1SA, UK.

https://doi.org/10.1016/j.molbiopara.2017.11.006
Received 13 October 2017; Received in revised form 17 November 2017; Accepted 18 November 2017
Available online 21 November 2017
0166-6851/

peptides, although displaying a broad amidolytic activity towards other amino acid hydrolysis. In addition, activity of these enzymes is dependent of metal cofactors, and has been implicated in processing/maturation/activation and/or degradation of substrates [10,11]. The relevance of LAPs as vaccine candidates for control of trematodiases at large was highlighted by findings with sheep immunized with *F. hepatica* LAP1, which provides ~90% protection and reduced disease [12–14]. The liver fluke enzyme is expressed in the gut and also found in the excretory/secretory products recognized by infection sera [15,16]. Leucine aminopeptidases (CsLAP1 and CsLAP2) also have been reported from the carcinogenic liver fluke *Clonorchis sinensis* [17], where they are expressed throughout parasite development and co-localize in the epithelial cells lining the gut of the fluke. CsLAP1 and CsLAP2 may participate in terminal hydrolysis of peptides before absorption from the gut lumen into gastrodermal cells.

Related peptidases also are known from schistosomes. LAP1 of *S. japonicum* and *S. mansoni* are expressed in the gut and tegument [18]. Interestingly, LAP activity has been found in eggs, miracidia, cercariae and egg hatching fluid of *S. mansoni.* Moreover, the specific inhibitor of metalloproteinases bestatin inhibited egg hatching [19]. A second LAP of *S mansoni* (SmLAP2) displays a differential expression profile among developmental stages with higher expression in miracidia and sporocysts. Gene silencing mimicked the effects of the aminopeptidase inhibitor bestatin, which reveals that both enzymes participate in hatching of the schistosome egg to release of the miracidium [20]. Since up to now only studies of SmLAP1 are available [18], in the current study we decided to express, purify, characterize the biochemical properties of both recombinant SmLAPs of *S. mansoni* (SmLAP1 and SmLAP2), and immunulocalise within the schistosome. This study provides a solid foundation for subsequent studies on these enzymes as tentative targets for control of this neglected tropical disease.

2. Materials and methods

2.1. Parasite material and serum from experimentally infected mice

Adult worms and eggs of *S. mansoni* were collected as described [21]. In brief, adult worms were recovered from experimentally infected mice by portal perfusion, washed in 1X PBS pH 7.3 and stored as wet pellets at −20 °C. The eggs were isolated from livers collected from mice 7 weeks post-infection. In brief, livers were finely chopped, digested with clostridial collagenase (Sigma-Aldrich, St. Louis, MO) at 37 °C overnight, washed in 1X PBS and sequentially filtered through 250 and 150 μm sieves. The filtrate was centrifuged and applied to Percoll gradient. The schistosome eggs, which pelleted tightly at the bottom of the tube, were washed with 1X PBS and stored as wet pellet at −20 °C. Briefly, adult worms or eggs were collected in a 1.5 ml tube, washed with 500 ml of cold 1X PBS and sonicated in ice bath for 5 min with 60 s bursts at 20% power followed by 30 s pauses. The homogenates was centrifuged at 10,000*g* for 20 min at 4 °C and supernatant and pellet stored at −20 °C until used. Serum from *S. mansoni* experimentally-infected mice (five mice per group), were obtained by bleeding the tail vein 29 and 49 days post-infection, i.e. before and after the female worms start to lay eggs, respectively. Fifty to 100 μl of whole blood was collected from each mouse in glass tubes. The samples were incubated at 37 °C for 1 h, to allow them to clot, and at 4 °C for ~16 h, to allow the clot to contract. The supernatant was clarified by centrifugation at 4000 rpm for 20 min at 4 °C, after which serum was transferred to a new tube and stored at −20 °C.

2.2. Cloning, expression and purification of recombinants SmLAP1 and SmLAP2

Full length coding sequences of SmLAP1 (Smp_030000) and SmLAP2 (Smp_083870) [20], were amplified using gene specific primers [SmLAP1, 5′-ATGTCTACCAGCGTTGTCAC-3′ (fwd), 5′-TTACTTGAAACTTAATCGTG (rev), SmLAP2, 5′-ATGACACATCGCGAGTCAAT (fwd), 5′-TCACACCTTTCTATTAGGCC (rev)], and cloned in TOPO-TA vector (Invitrogen). The expression plasmids pET-SmLAP1 and pET-SmLAP2 were engineered by PCR amplification from pCRTOPO-SmLAP1 and pCRTOPO-SmLAP2 respectively, using the primers 5′-GGCCGCGAGCTCGCCCTTATG-3′ (fwd) and 5′ GGGACTAGTCGACC AGGTTTAAACC-3′ (rev). The PCR products were digested using *Sac* I-*Sal* I and the fragments inserted into the pET28a vector (Novagene) previously digested with the same restriction enzymes, cloned in *E. coli* strain (XL1-blue) and the recombinant constructs confirmed by sequencing. *E. coli* strain BL21 (DE3) cells were transformed with these plasmids for expression and purification of the recombinant proteins. Briefly, recombinant clones were grown in LB media with kanamycin (50 μg/mL), to an OD600 of 0.6, and recombinant protein production induced with 0.4 mM IPTG for 24 h at 25 °C. The bacterial cultures were centrifuged, the pellets resuspended in 50 mM Tris-HCl pH 8.5, 100 mM NaCl and 5 mM imidazole, containing 1 mg/mL lysozyme, and sonicated (10 pulses of 1 min with 1-min pauses). The lysates were centrifuged for 30 min at 20,000*g*, and supernatants were applied to a nickel–nitrilotriacetic acid column (GE Healthcare, USA), washed with 50 mM Tris-HC pH 8.5, 100 mM NaCl, 5 mM imidazole and eluted in imidazole at 20, 50, 200 and 400 mM. The fractions containing the recombinant protein were applied to PD10 desalting columns (GE Healthcare) using 50 mM Tris-HCl pH 8.5 and stored at 4 °C. The protein concentration was determined using the bicinchoninic acid assay (BCA kit, Pierce, USA). The purity of recombinant schistosome enzymes was examined using Coomassie-stained 10% SDS-PAGE gels, under reducing conditions. The identity of the recombinant proteins was confirmed by MALDI-TOF MS/MS at the Proteomic Unit of the Pasteur Institute of Montevideo.

2.3. Fluorogenic substrate assay for the detection of aminopeptidase activity

Aminopeptidase activity of SmLAPs were carried out by incubating samples in a reaction mix of 20 μM fluorogenic substrate (Sigma-Aldrich), 0.5 mM $MgCl_2$ and 50 mM Tris-HCl (pH 8.0) at 37 °C for 60 min. For activation studies, the enzymes were pre-incubated with the divalent cations $MgCl_2$, $MnCl_2$, $CaCl_2$ and $ZnCl_2$ prior to the addition of Leu-AMC to the assay mix. The inhibition studies were carried out by incubating samples with 1,10-phenanthroline (1 mM), EDTA (5 mM), the specific aminopeptidase inhibitor bestatin (10 μM), PMSF (1 mM), E64 (5 μM) and DTT (5 mM) prior to addition of the substrate. The pH profile was determined by incubating the enzymes in sodium phosphate (pH 6.0–6.5), Tris-HCl (pH 7.0–8.5), and glycine-NaOH (pH 9.0–10.5). One unit of enzyme activity (U) was defined as the amount of enzyme that catalyzes the release of 1 nmol of AMC per minute at 37 °C. Assays were performed in triplicate, and the mean and standard deviation were calculated.

2.4. Antiserum production

Rabbit immune serum was produced by subcutaneous immunization with 100 μg of the purified SmLAP1 or SmLAP2 recombinant enzymes in Adyuvac 50 (Virbac-Santa Elena, Uruguay), and boosted by intramuscular immunization on week 4 with 100 μg of the purified enzyme in Adyuvac 50. Serum was collected 15 d after the boost and stored aliquoted at −20 °C.

2.5. Western blot

Purified recombinant SmLAPs, egg extracts and adult worm extracts were assessed by 10% SDS–PAGE under reducing conditions (1 mM DTT final concentration). After electrophoresis, the proteins were transferred onto nitrocellulose membranes according to the method previously described, using a Tris Glycine SDS buffer [22]. Membranes were incubated in blocking solution (5% non-fat milk in 1X PBS, pH