RESEARCH NOTE

Biochemical Characterization of Cathepsin D from Adult *Schistosoma mansoni* Worms

Italo M Cesari+, Elizabeth Valdivieso, Joseph Schrével*

Laboratorio de Inmunoparasitología, Centro de Microbiología y Biología Celular, Instituto Venezolano de Investigaciones Científicas, Apartado 21827, Caracas 1020 A, Venezuela *Laboratoire de Parasitologie, Muséum Nationale d'Histoire Naturelle, Paris, France

Key words: cathepsin D - aspartyl proteinase - *Schistosoma* - haemoglobin - proteolytic activity

Schistosomes ingest and lyse host blood cells, releasing the haemoglobin (Hb) into their gut (MR Kasschau & MH Dresden 1986 *Exp Parasitol 61*: 201-209). AR Timms and E Bueding (1986 *Br J Pharmacol 14*: 68-73) found an acid protease activity in *Schistosoma mansoni* which was capable of hydrolysing Hb; they suggested that host Hb degradation provided the major amino acid source for the synthesis of parasite proteins. From 1979 on, Hb degradation by schistosomes was considered mostly due to cysteine proteinase (CP) activity (MH Dresden & AM Deelder 1979 *Exp Parasitol 48:* 190-197, JP Dalton et al. 1995 *Parasitol Today 11:* 299-303). Several *S. mansoni* and *S. japonicum* CPs have been reported to be possibly involved in the degradation of this substrate which includes cathepsin B (Sm31, Sj31 antigens) (M-Q Klinkert et al. 1989 *Mol Biochem Parasitol 33:* 113-122, B Götz & M-Q Klinkert 1993 *Biochem J 29:* 801-806), cathepsin L (MA Smith et al. 1994 *Mol Biochem Parasitol 67:* 11-19, SR Day et al. 1995 *Biochem Biophys Res Commun 217:* 1-9, A Michel et al. 1995 *Mol Biochem Parasitol 73:* 7-18) and an asparaginyl endopeptidase (Sm32, Sj32 antigens) (Klinkert et al. 1989 *loc. cit.*, A Merckelbach et al. 1994 *Trop Med Parasitol 45:* 193-198). However, a proteinase-processing, rather than a direct Hb-digesting role for the Sm32 have been suggested by JP Dalton and PJ Brindley (1996 *Parasitol Today 12:* 125). On the other hand, cathepsin L has been mainly located in the reproductive system of the worms and it is present in smaller amount than cathepsin B in the adult worm vomitus of several *Schistosoma* species, suggesting a minor role of this enzyme in the digestion of Hb (C Caffrey et al. 1996 *Parasitol Res 83:* 37-41). An important proportion of the Hb degradation exerted by *S. mansoni* extracts occurs in the absence of thiols between pH 3.5 and 4.5 (IM Cesari et al. 1981 *Acta Cient Venez 32:* 324-329, J Maki & T Yanagisawa, 1986 *J Helminthol 60*: 31-37, H Gogheim & M-Q Klinkert 1995 *Int J Parasitol 25*: 1515-1519) and this activity is inhibited by pepstatin A (a classic aspartyl proteinase inhibitor) but not by thiol-, serine- and metalloproteinase inhibitors (Maki & Yanagisawa 1986 *loc. cit.*). Using mercury-labeled pepstatin, BJ Bogitsh and KF Kirschner (1986 *Exp Parasitol 62:* 211-215) localized an aspartyl proteinase in the cecal lumen and to the gastrodermis of *S. japonicum.* Immunocytochemical studies using heterologous antiserum to bovine cathepsin D indicated that the *S. japonicum* cathepsin D-like enzyme is also localized to the dorsal and lateral surfaces of the tegument and tubercles of male worms (BJ Bogitsh & KF Kirschner 1987 *Exp Parasitol 64:* 213-218). A cDNA encoding this proteinase was isolated and the native enzyme biochemically characterized at pH 3.5 (MM Becker et al. 1995 *J Biol Chem 270:* 24496-24501).

In the present work, we report the capacity of different adult male and/or female worm extracts of the Venezuelan *S. mansoni* JL strain to digest human and bovine Hb at pH 3.8 in the absence of thiol groups. The extent of Hb proteolysis was also assessed electrophoretically by high density SDS-PAGE, some biochemical and immunological characteristics of the enzyme were preliminary studied and it was partially purified.

Approximately equal numbers of adult male and/or female *S. mansoni* worms obtained by perfusion from infected hamsters were washed and homogenized in 0.85% NaCl in the presence of 1 mM PMSF, 5.0 mg/ml of aprotinin, 1 mM EDTA and 10 mM E-64; the homogenate was centrifuged at 100,000 g for 2 hr at 4°C and the resulting supernatant dialyzed overnight against either 0.85% NaCl at 4°C (saline extract, SE) or 0.2 M Na-acetate buffer, pH 3.8 containing 1 M NaCl (acid extract, AE). Dialyzed samples were centrifuged

+Corresponding author. Fax:+58-2-504.1382.
Received 4 May 1998
Accepted 31 August 1998

at 14,000 g for 15 min at 4ºC and the corresponding supernatants used for the experiments. The protein content of the extracts was measured by the method of MJ Bradford (1976 *Anal Biochem 72:* 248-254), using the Coomassie Protein Assay Reagent of Pierce (Rockford, IL, USA) and BSA as a protein standard.

The Hb assay was done according to K Yamamoto and VT Marchesi (1984 *Biochem Biophys Acta 790:* 208-218) with slight modifications. Briefly, reactions were performed in triplicate in a total volume of 0.5 ml of 0.1 M Na-acetate buffer, pH 3.8 and 0.1% (w/v) Triton X-100, with an aqueous solution of human or bovine Hb (Sigma) added last to the reaction mixture at a final concentration of 1 mg/ml. The assay mixtures were incubated at 37°C for 90-120 min. Reactions were stopped by addition of 0.5 ml of 5% (w/v) trichloroacetic acid (TCA). After 10 min on ice, samples were centrifuged at 14,000 g for 5 min at 4°C and absorbance of acid-soluble peptides in the supernatants assessed at 280 nm. Absorbance values were corrected by subtracting the blank value done as described above except that the parasite extract was added immediately after addition of TCA. Haemoglobinase activity was estimated by interpolating the 280 nm absorbance values of samples in a standard tryptophan calibration curve and expressed as equivalents of nmoles/min. The hydrolysis products were directly proportional to time and extract concentrations under the present experimental conditions. Specific activities were related to mg of sample protein. Some assays were run in the presence of 7.0 µM pepstatin A or 12 mM diazoacetyl-DL-norleucine methyl ester (DAN). Inhibition was inferred from the percent residual activity.

Saline (SE) and acid (AE) adult *S. mansoni* extracts were able to hydrolyze human and bovine Hb in the absence of thiols at pH 3.8 and 37ºC (Table I). Results indicate that proteolysis mediated by SE and AE was higher on bovine than on human Hb and that AE showed higher activity than SE. A partial purification (3-4 fold) of the enzyme was achieved by the acid dialysis (Table I). The hemoglobinolytic activity shown by SE and AE was more than 60% inhibited by 7.0 mM Pepstatin A (data not shown), confirming the aspartyl proteinase nature of the activity. SE from separated male (M) or female (F) adult worms was tested with bovine Hb under the above experimental conditions to check for differences in proteolysis between sexes. Specific activity of SE from mixed M/F was 3.77 ± 0.71 (SD) nmol/min/mg protein (n = 3); from F, 3.1 nmol/min/mg protein (n = 2); and from M, 1.26 nmol/min/mg protein (n = 2). Specific activity was about 1.8 fold greater in F than in M, as already observed earlier (Cesari et al. 1981 *loc. cit.*).

TABLE I

Degradation of human and bovine haemoglobin (Hb) by adult *Schistosoma mansoni* extracts

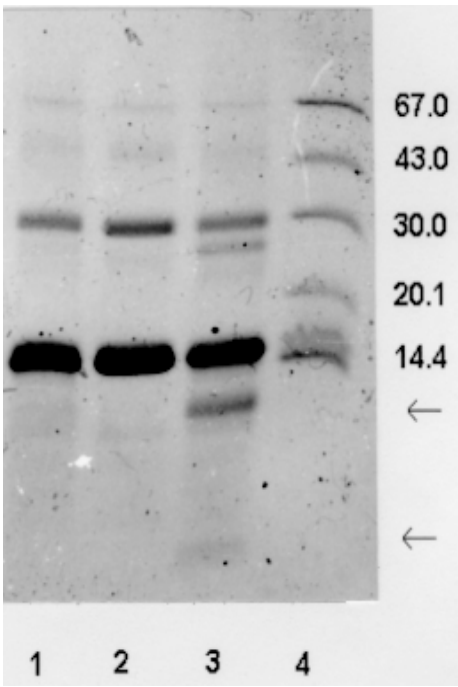
Hb species	Extracts	Specific activity[a]
Human	SE	1.98 ± 0.36
	AE	5.27 ± 0.48
Bovine	SE	3.23 ± 0.39
	AE	12.71 ± 0.62

a: nmol of tryptophan/min/mg protein. Mean values ± SD from three independent experiments (n = 3), each performed in triplicate in the absence of thiols; SE: saline; AE: acid.

The ability of the extracts to degrade Hb *in vitro* at pH 3.8 in the absence of thiols was also monitored on 20% homogeneous nonreducing SDS-PAGE as the decrease in the intensity of the Hb subunits. After 6 hr of incubation at 37ºC, digested and undigested (control) Hb samples (1-3 µg) were immersed for 3 min in a boiling water bath in 40 mM Tris-HCl buffer (pH 8.0), containing 2.5% (w/v) SDS, 1 mM EDTA, and 0.01% bromophenol blue. Samples were electrophoresed at 500 V for approximately 35 min at 15°C and peptides stained with Coomassie Blue according to standard PhastSystem protocols (Pharmacia LKB Biotechnology). Hb incubated without extract migrates as three bands that represent the whole molecule (64 kDa), a subunit dimer (32 kDa), and a subunit monomer (16 kDa). The Hb subunits were degraded by SE and AE (Fig.). The extracts cleaved the 16 kDa subunit monomer of bovine and human Hb at a specific site of the sequence, provoking the appearance of two peptide fragments of about 10 and 4-5 kDa (Fig.). This reaction was inhibited by 7.0 mM pepstatin (Fig.) or 12 mM DAN (data not shown). Incubation of extracts with bovine Hb coupled to agarose beads (Sigma) produced the release of the red pigment and of a soluble 10 kDa fragment, as detected after 20% high density SDS-PAGE and silver staining of the supernatant (data not shown); no 4-5 kDa fragment was seen in this case (presumably left bound to the agarose support). This result supported the limited proteolysis observed in Fig.

Preliminary work indicate that AE is able to hydrolyze the synthetic chromogenic cathepsin D substrate Boc-Phe-Ala-Ala-*p*-nitro-Phe-Phe-Val-Leu-4-hydroxymethyl pyridine (Bachem) at pH 3.8. Cleavage of the *p*-nitrophenylalanine-phenylalanine amide bond in this substrate was measured spectrophotometrically at 310 nm (N Agarwal & DH Rich 1983 *Anal Biochem 130:* 158-165). The

hydroysis of this substrate was inhibited by 7.0 mM pepstatin A. Agarwal and Rich (1983 *loc. cit.*) highlighted the importance of substituents in the P2-P4 substrate positions (the designation P2-P4 follows I Schecter & A Berger 1967 *Biochem Biophys Res Commun 27:* 157) where precise steric interactions between substrate and enzyme appear to contribute to rapid hydrolysis of substrates by cathepsin D. The P2-P4 (Phe-Ala-Ala) sequence of the above substrate is present at position 85-87 in the b-chain of bovine Hb (Phe-Ala-Thr is present in the b-chain of human Hb) (R Petruzzelli et al. 1991 *Biochim Biophys Acta 1076:* 221-224), representing a possible site of cleavage. A theoretical cleavage at the signaled position would produce an N-terminal peptide fragment of about 9-10 kDa and a C-terminal peptide of about 5-6 kDa, similar to those observed in the Figure. If experimentally confirmed, the Hb b-chain might be a putative target for cathepsin D in schistosomes, differing in this respect from the aspartyl proteinase activity of *Plasmodium falciparum* that cleaves the a-chain (DE Goldberg et al. 1991 *J Exp Med 173:* 961-969). At present, we do not know how important is the cathepsin D-type of activity in the Hb digestive pathway of schistosomes *in vivo*. From their experiments, Gogheim and Klinkert (1993 *loc. cit.*) implied that it is important. However, due also to its location on the dorsal tegument of males (BJ Bogitsh et al. 1992 *J Parasitol 78:* 454-459), it may have other protein processing activities.



High density (20%) SDS-PAGE analysis of bovine haemoglobin (Hb) incubated with adult *Schistosoma mansoni* worm extracts. Samples (1-3 mg per lane) were incubated with or without crude extracts. 1: bovine Hb incubated for 6 hr with the acid enzyme extract in the presence of 7.0 mM Pepstatin A; 2: undigested bovine Hb control; 3: bovine Hb incubated with the acid enzyme extracts. Arrows indicate main products of hydrolysis; 4: molecular weight markers.

AE was passed through a pepstatinyl-agarose (Sigma) column equilibrated with 50 mM Na-acetate buffer, pH 3.8; an affinity-bound material could be eluted with 50 mM Tris/HCl, pH 8.0 containing 1 M NaCl. The nonreducing 12% SDS-PAGE of this material showed a band of approximately 45 kDa after silver staining, readily degradable into polypeptides of lower molecular masses (data not shown).

Preliminary immunological experiments indicate that the non thiol Hb-digesting activity was precipitated from *S. mansoni* extracts by polyclonal rabbit anti-bovine cathepsin D antibodies raised in our laboratory according to Bogitsh and Kirschner (1987 *loc. cit.*). The IgG fraction of this antiserum was adsorbed on Protein A-Sepharose beads that were then incubated with the enzymatic extracts and later with Hb (M Damonneville et al. 1982 *Mol Biochem Parasitol 6:* 265-275, IM Cesari et al. 1987 *Mem Inst Oswaldo Cruz 82* Suppl. IV: 175-177). The immunoadsorbed material exhibited Hb digestion. The above antibodies recognized also the *S. mansoni* enzyme in western blots but contrary to what was found by Bogitsh et al. (1992 *loc. cit.*) with the *S. japonicum* enzyme, the digestion of Hb was not inhibited. On the other hand, the parasite enzyme was not detected in western blots using a few sera from *S. mansoni*-infected patients, suggesting that it may be poorly immunogenic and/or it may not be available to the host immune system as an abundant circulating antigen.

Data presented here support the existence in adult *S. mansoni* (Venezuelan JL strain) worm extracts of a non-thiol Hb-digesting enzyme of a cathepsin D-type acting through limited proteolysis on human and bovine Hb at pH 3.8. A summary of the properties of the *S. mansoni* enzyme under study is presented in Table II. Some of these properties are similar to those reported for the cathepsin D from *S. japonicum* (Becker et al. 1995 *loc. cit.*). Hb degradation is assumed to be essential for the parasitic way of life and cathepsin B seems to play the major role in this function (Caffrey et al. 1996 *loc. cit.*); the role of cathepsin D still remaining an open question. Further work is necessary to know its role(s) in the parasite physiology and in the host-parasite relationships to consider its potential target for chemotherapy and/or vaccine development.

TABLE II

Properties of cathepsin D from adult *Schistosoma mansoni* worms

Source	Adult male and female worms
Type	Aspartyl proteinase
Substrate susceptibility	Haemoglobin (bovine, human)
Synthetic substrate hydrolyzed	Boc-F-A-A-*p*-nitro-F-F-V-L-4-HM
Haemoblobin 16 kDa subunit digestion	Limited proteolysis (10 kDa, 4-5 kDa)
Experimental conditions for use	50 mM Na-acetate buffer, pH 3.8
Inhibitors	Pepstatin A, DAN
Isolation	Pepstatin A - agarose
Mol. Wt. (12% nonreducing SDS-PAGE)	Approx. 45 kDa
Crossed immune recognition	Rabbit anti-bovine cathepsin D
Enzyme antigenicity (western blot)	Undetected by *S. mansoni* - infected patient sera tested
Comments	Unstable