INFECTION AND IMMUNITY, Apr. 1996, p. 1328–1334 Vol. 64, No. 4
0019-9567/96/$04.00+0

Characterization of the Cathepsin-Like Cysteine Proteinases of *Schistosoma mansoni*

JOHN P. DALTON,[1,2] KAREN A. CLOUGH,[1] MALCOLM K. JONES,[3] AND PAUL J. BRINDLEY[1]*

Molecular Parasitology Unit, Queensland Institute of Medical Research, and Australian Centre for International & Tropical Health & Nutrition, Post Office, Royal Brisbane Hospital, Queensland 4029,[1] and Centre for Microscopy and Microanalysis, University of Queensland, St. Lucia, Queensland 4072,[3] Australia, and School of Biological Sciences, Dublin City University, Dublin 9, Republic of Ireland[2]

Received 5 September 1995/Returned for modification 29 November 1995/Accepted 15 January 1996

Adult *Schistosoma mansoni* parasites synthesize and secrete both cathepsin L and cathepsin B cysteine proteinases. These cysteine proteinase activities, believed to be involved in hemoglobin digestion by adult schistosomes, were characterized by using specific fluorogenic peptide substrates and zymography. Both cathepsin L- and B-like activities with pH optima of 5.2 and 6.2, respectively, predominated in soluble extracts of worms, and both these activities were secreted by adult worms into the culture medium. The specific activity of cathepsin L was about double that of cathepsin B when each was assayed at its pH optimum, and moreover, the specific activities of cathepsins L and B in extracts of female schistosomes were 50 to 100% higher than in extracts of male schistosomes. Analysis of the primary structure of two cloned *S. mansoni* cathepsins L, here termed cathepsin L1 and cathepsin L2, revealed that they are only 44% similar and that cathepsin L2 showed more identity (52%) with human cathepsin L than with schistosome cathepsin L1. Moreover, differences in their active site, propeptide region, and potential for glycosylation suggest separate functions for schistosome cathepsin L1 and cathepsin L2.

Cathepsin L (EC 3.4.22.15) and cathepsin B (EC 3.4.22.1) are lysosomal cysteine proteinases of the papain superfamily (23). These enzymes are attracting increasing interest not only because they are involved in the catabolism of proteins of mammalian cells (1) but also because of their role in the degradation and invasion of tissues by tumor cells (14, 15). Cathepsin proteinases have also been identified in many parasitic organisms and are involved in roles as diverse as tissue and skin penetration, virulence, and immune evasion (18, 19, 21, 25). Involvement in these kinds of key functions renders them potential targets at which to direct novel antiparasite chemotherapy and immunoprophylaxis.

Schistosomes cause the chronic and debilitating disease schistosomiasis, which afflicts more than 250 million people in tropical regions. Infection is initiated following skin penetration by an aquatic larva, the cercaria. After larval migration through the lungs and liver, the schistosomes mature in the vasculature of the intestines (*Schistosoma mansoni* and *S. japonicum*) or bladder (*S. haematobium*). Ingested host erythrocytes are lysed in the gut of the schistosome, and the hemoglobin is degraded to absorbable peptides that are used as a major source of amino acids (2, 3, 8, 16, 27). Cysteine proteinases, including cathepsin B-like and cathepsin L-like proteinases, have been detected in schistosomes and are suggested to be involved in this hemoglobin degradation (8). We have now characterized these activities with respect to substrate specificity, pH optima for activity, and stability with class-specific fluorogenic peptide substrates. In addition, we show by gelatin-substrate polyacrylamide gel electrophoresis (PAGE) that two cathepsin L proteinases are expressed and secreted by adult schistosomes, and we reveal that the two *S. mansoni* cathepsin L genes previously reported (20, 25) are substantially different in their primary structure, which strongly suggests that the products of these two genes perform discrete functions.

* Corresponding author. Phone: 61-7-3362 0413. Fax: 61-7-3362 0104. Electronic mail: paulB@qimr.edu.au.

MATERIALS AND METHODS

Schistosome extracts and ES products. Adult *S. mansoni* (Puerto Rican strain) of both sexes were perfused from BALB/c mice 7 to 10 weeks after infection with 100 to 150 cercariae and used immediately or stored at −70°C for up to 6 months. Parasite soluble extracts were prepared in phosphate-buffered saline (PBS), pH 7.3, by two freeze-thaw and sonication cycles followed by centrifugation for 30 min at 14,000 × *g* (25). Protein concentration was measured by a modified Lowry method (DC Protein Assay; Bio-Rad, Richmond, Calif.). Adult *S. mansoni* were maintained in vitro in Dulbecco's modified Eagle's medium (Gibco Laboratories, Life Technologies, Inc.) containing 10% fetal calf serum, penicillin (300 U ml^{-1}), streptomycin (300 μg ml^{-1}), and 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) for 16 h (20). Following this incubation, the medium containing excretory-secretory (ES) products was removed and centrifuged at 14,000 × *g* for 15 min, and the supernatant was concentrated 10-fold with a Centricon 10-kDa-cutoff cartridge (Amicon, Beverley, Mass.) and stored at −20°C.

Proteinase substrates and inhibitors. The synthetic peptides Z-Arg-NHMec, Boc-Arg-Arg-NHMec, Z-Phe-Val-Arg-NHMec, Suc-Ala-Phe-Lys-NHMec, Tos-Gly-Pro-Arg-NHMec, Suc-Gly-Pro-Leu-Gly-Pro-NHMec, Suc-Leu-Tyr-NHMec, and Boc-Val-Leu-Lys-NHMec and the peptidyl diazomethylketones benzyloxycarbonyl-L-phenylalanyl-L-alanine-diazomethylketone (Z-Phe-Ala-CHN_2) and *N*-benzyloxycarbonyl-L-phenylalanyl-phenylalanine-diazomethylketone (Z-Phe-Phe-CHN_2) were from Bachem Biosciences (Philadelphia, Pa.), where NHMec is 7-amino-4-methylcoumarin. Z-Phe-Arg-NHMec, H-Leu-Val-Tyr-NHMec, Suc-Leu-Lu-Val-Tyr-NHMec, Tos-Gly-Pro-Lys-NHMec, and the cysteine proteinase inhibitor *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64) were from Sigma Chemical Co. (St. Louis, Mo.). The serine proteinase inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) was purchased from ICN (Costa Mesa, Calif.).

Proteinase assays with fluorogenic peptide substrates. Proteinase activity was measured fluorometrically with peptide-NHMec substrates at a concentration of 2.5 μM. From the enzyme kinetic data for both mammalian (5) and trematode (9) cathepsin L in these assays, $[S] << K_m$, where the initial rate v_0 is directly proportional to the k_{cat}/K_m. Assays were carried out in triplicate in the presence of 1 mM dithiothreitol (DTT) in a volume of 1 ml at 37°C for 30 min, and the

amount of NHMec released was measured as described before (9, 25). Each assay was repeated at least twice. Three buffers, 0.1 M sodium acetate (pH 4.0 to 5.5), 0.1 M sodium phosphate (pH 5.8 to 7.0), and 0.1 M Tris-HCl (pH 7.0 to 7.75), were used for the pH activity profiles, and their ionic strength was equalized with NaCl.

Assays of enzyme stability were carried out by incubating soluble extracts at 37°C in PBS, pH 7.3, for 24 h. Aliquots were removed at intervals and stored at −20°C. Cathepsin L-like activity in the aliquots was assayed with the substrate Z-Phe-Arg-NHMec in 0.1 M sodium acetate, pH 4.5, and cathepsin B-like activity was assayed with Z-Arg-Arg-NHMec in 0.1 M sodium phosphate, pH 6.2. Inhibition assays were carried out with Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2. Inhibitor and schistosome preparation were added to the assay 10 min prior to the addition of substrate.

Zymography. Samples of mature *S. mansoni* soluble extract (20 to 40 μg) were separated by electrophoresis through nondenaturing 8% polyacrylamide gels containing 0.1% gelatin. Following electrophoresis, the gels were washed for 15 min in 0.1 M sodium citrate, pH 4.5, or 0.1 M sodium phosphate, pH 6.2, and then transferred into the same buffer containing 10 μM fluorogenic peptide substrate and 1 mM DTT. After an incubation period of 15 min at 37°C, fluorescent bands locating the substrate-cleaving enzymes were visualized in the gels by UV transillumination and recorded on Polaroid 667 film. The fluorogenic substrates used for the detection of proteinases within the gels included Z-Arg-Arg-NHMec, Z-Phe-Arg-NHMec, Z-Phe-Val-Arg-NHMec, and Z-Val-Leu-Lys-NHMec. Subsequently, gels were incubated in buffer containing 1 mM DTT overnight to allow digestion of the polymerized gelatin, after which gelatinolytic activity was visualized by staining the gels with Coomassie blue R250 (9). For inhibition studies, E-64 (10 μM) or Z-Phe-Ala-CHN_2 (10 μM) was added to the sample prior to application to gels and to all subsequent buffers.

PCR amplification and cloning of cathepsin L and B genes. mRNA was isolated directly from 200 mixed-sex (100 male and 100 female) and 200 single-sex (female) mature *S. mansoni* parasites by chromatography on oligo(dT)-cellulose (QuickPrep mRNA Purification Kit; Promega, Madison, Wis.). Double-stranded cDNA was synthesized from the mRNA with Moloney murine leukemia virus reverse transcriptase, RNase H, and DNA polymerase I (Riboclone Synthesis Kit; Promega). Oligonucleotide primers specific for the 5′ and 3′ ends of the mature cathepsin L gene reported by Smith et al. (25) (hereafter termed cathepsin L1), the mature cathepsin L gene reported by Michel et al. (20) (hereafter termed cathepsin L2), and the mature cathepsin B gene reported by Klinkert et al. (16) were synthesized. Target sequences for a restriction endonuclease that did not cleave within the gene were incorporated into the primers. The enzymes chosen were *Hin*dIII, *Bam*HI, and *Pst*I, and their target sequences are underlined in the following primer sequences: cathepsin L1 sense primer, 5′ CCG GGA TCC ATA CCA AAA AAC TTC GAT TGG, and antisense primer, 5′ CCG CTG CAG CGG AAT TGT ATT GAG ATT CTG; cathepsin L2 sense primer, 5′ AAC AAG CTT TTG CCA TCA AAA TGG GAT, and antisense primer, 5′ TTA AAG CTT CTA AAA TGT AAT GAA TAG; and cathepsin B sense primer, 5′ AAT GGA TCC ATT CCA TCC AAT TTC GAT TCC, and antisense primer, 5′ CCG CTG CAG TGA TGA TGT TCA AAG ATT. Primers were used in PCR to amplify the *S. mansoni* cathepsin L and B genes; 50 ng of cDNA was used as the template. Thermal cycling was as follows: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min for 30 cycles. PCR products were digested with restriction enzyme, purified by chromatography (Wizard columns; Promega), and ligated into the linearized plasmid expression vector pQE30 (Qiagen, Chatsworth, Calif.), and the ligation products were used to transform *Escherichia coli* M15 by electroporation. Plasmid DNAs were isolated on Qiagen-100 columns, and nucleotide sequences were determined in both directions with the Taq DyeDeoxy Terminator Cycle Sequencing System (Applied Biosystems Inc. [ABI], Foster City, Calif.) and an ABI automated DNA sequencer. The nucleotide sequences of the inserts of plasmids encoding the recombinant cathepsins were found to be identical to the published sequences (16, 20, 25).

Southern blot analysis. Genomic DNAs (3 μg) from mixed-sex adult *S. mansoni*, digested with *Eco*RI, *Hin*dIII, *Bam*HI, or *Pst*I (Biolabs, Beverley, Mass.), and PCR products were separated by electrophoresis through 0.8% agarose–Tris-acetate–EDTA and Southern transferred to nylon membranes (Bio-Rad). The cathepsin L1, L2, and B genes were amplified by PCR from recombinant pQE30 plasmids and purified by chromatography (Wizard columns; Promega). The inserts (50 ng) were radiolabeled with [α-^{32}P]dCTP (NEN-DuPont) by random oligomer priming and Klenow polymerase. Labeled insert DNA was hybridized to Southern blots at 65°C, with hybridization and wash conditions as described before (25).

Recombinant cathepsins L1, L2, and B. *E. coli* M15 transformed with the cathepsin L1, L2, or B gene was grown at 37°C with shaking until the optical density at 600 nm (OD_{600}) reached 0.8. Expression of recombinant proteins was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to 1 mM. After another 3 to 5 h, the cells were harvested by centrifugation at 2,000 × *g* for 15 min and stored at −70°C. Frozen pellets from 50 ml of bacterial cultures were thawed and resuspended in 0.1 M sodium phosphate–0.01 M Tris-HCl (pH 8.0) containing 6 M guanidine hydrochloride and agitated on a shaker for 2 h. The extract was centrifuged at 14,000 × *g* for 30 min, and the supernatant was mixed with 400 μl of Ni-NTA resin (Qiagen) for 1 h. The resin was then washed sequentially with 10 volumes of the 0.1 M sodium phosphate–0.01 Tris-HCl (pH 8.0) containing 6 M guanidine hydrochloride, 10 volumes of the same buffer containing 8 M urea, and 10 volumes of 0.1 M sodium phosphate–0.01 M Tris-HCl, pH 6.3, containing 8 M urea. Recombinant proteins were eluted with a further 10 volumes of the last buffer containing 125 mM imidazole. Protein concentrations in eluates were estimated by comparison with known quantities of bovine serum albumin on reducing sodium dodecyl sulfate (SDS)-PAGE. These estimations also showed that the recombinant cathepsin preparations were ≥90% pure. The proteolytic enzyme activity of recombinant cathepsins was examined as for schistosome extracts and ES products (above) by using fluorogenic peptide substrates and zymography.

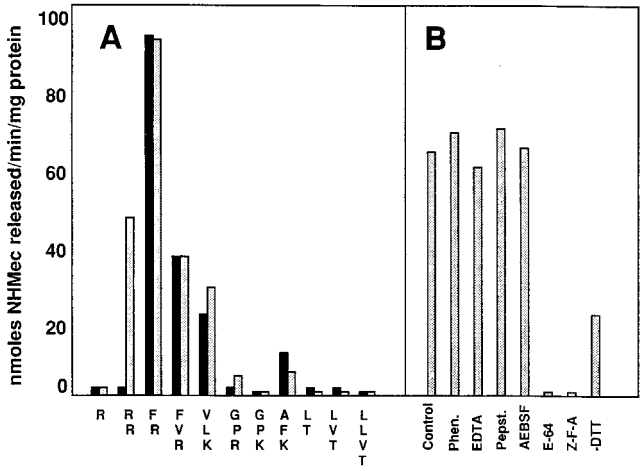

FIG. 1. (A) Analysis of proteinase activity in soluble extracts of adult *S. mansoni* with fluorogenic peptide substrates. The assays were performed with substrate concentrations of 2.5 μM, a concentration far below the K_m, and therefore v values can be considered proportional to k_{cat}/K_m. Assays were carried out in 0.1 M sodium acetate, pH 4.5 (solid bars), and 0.1 M sodium phosphate, pH 6.2 (shaded bars). R, Arg; RR, Arg-Arg; FR, Phe-Arg; FVR, Phe-Val-Arg; VLK, Val-Leu-Lys; GPR, Gly-Pro-Arg; GPK, Gly-Pro-Lys; AFK, Ala-Phe-Lys; LT, Leu-Tyr; LVT, Leu-Val-Tyr; LLVT, Leu-Leu-Val-Tyr. (B) Effect of class-specific inhibitor reagents on Z-Arg-Arg-NHMec- and Z-Phe-Arg-NHMec-cleaving activity of *S. mansoni* proteinases. The reagents used were *o*-phenanthroline (Phen., 1 mM), EDTA (5 mM), pepstatin (Pepst., 1 μM), AEBSF (2 mM), E-64 (5 μM), and Z-Phe-Ala-CHN_2 (Z-F-A, 5 μM). Assays were performed with the fluorogenic peptide substrate Z-Phe-Arg-NHMec in 0.1 M sodium acetate, pH 4.5. The reducing agent DTT (1 mM) was included in all reactions except in those testing the effect of its absence (−DTT). Means of triplicate assays are presented.

RESULTS

Proteinase activity in schistosome extracts. To investigate the relative expression of the cathepsin L and cathepsin B cysteine proteinases, activities in soluble extracts of adult *S. mansoni* were examined with a panel of fluorogenic peptide substrates, including substrates that could distinguish between classes of cathepsin proteinases, namely, Z-Arg-NHMec (cathepsin H), Z-Arg-Arg-NHMec (cathepsin B), Z-Phe-Arg-NHMec (cathepsins B and L), and Z-Phe-Val-Arg-NHMec (cathepsins L and S) (1, 5). Substrates were assayed at 2.5 μM, a concentration much lower than the K_m for cathepsin proteinases (5, 9), so that a comparison of the relative efficiency of cleavages could be made. The peptides used for the analysis of cathepsins B and L (Z-Arg-Arg-NHMec, Z-Phe-Arg-NHMec, and Z-Phe-Val-Arg-NHMec) were the most efficiently cleaved substrates among those examined, which indicated the predominance of these enzymes in the soluble extracts of adult *S. mansoni* (Fig. 1A). We have previously shown that these substrates are not cleaved by two other enzymes implicated in hemoglobin digestion, the cathepsin D-like aspartic proteinase (2) and the asparaginyl endopeptidase Sm32 (7). The activity against these substrates was completely inhibited by the cysteine proteinase inhibitor E-64 and diminished in the absence

of the reducing agent DTT. In contrast, the activity against these substrates was unaffected by the serine proteinase inhibitor AEBSF (1 mM), the metalloproteinase inhibitors *o*-phenanthroline (2 mM) and EDTA (1 mM), and the aspartic proteinase inhibitor pepstatin (1 mM) (Fig. 1B) (data for Z-Phe-Arg-NHMec are shown; those for other substrates are not shown).

Boc-Val-Leu-Lys-NHMec was also cleaved by activities in the *S. mansoni* extracts. This activity was inhibited by E-64 and Z-Phe-Ala-CHN_2 (data not shown). Although Boc-Val-Leu-Lys-NHMec has not been generally reported as a substrate for cathepsin proteinases, Dowd et al. (11) have shown that the cathepsin L proteinases of *Fasciola hepatica* have a high affinity for this substrate. We did not detect significant chymotrypsin-like activity in extracts of *S. mansoni*, and since the low activity observed for the chymotrypsin substrates Suc-Leu-Leu-Val-Tyr-NHMec, H-Leu-Val-Tyr-NHMec, and Suc-Leu-Tyr-NHMec was inhibited by E-64 (data not shown), we attribute the cleavage of Boc-Val-Leu-Lys-NHMec to the cathepsin B and/or L cysteine proteinase.

pH optima and stability of cathepsin L- and B-like proteinase activities. A comparison of the pH optimum for activity and the temperature stability of the cathepsins L and cathepsin B in the *S. mansoni* extracts revealed a marked difference in the physicochemical properties of the two enzymes. Cathepsin B activity measured against Z-Arg-Arg-NHMec showed an optimal pH for activity at pH 6.2 (Fig. 2A). The activity against the substrate Z-Phe-Arg-NHMec was optimal at a lower pH, peaking at pH 5.2. Since this substrate can be cleaved by both cathepsin B and cathepsin L, the profile shows a shoulder were it overlaps the Z-Arg-Arg-NHMec profile. Nevertheless, it is evident that the activity at lower pH, particularly below pH 4.5, was predominantly due to cathepsin L, since cathepsin B activity at these pHs is less significant. The specific activity of the cathepsin L-like proteinases, measured with the fluorogenic peptide substrate Z-Phe-Arg-NHMec at pH 4.5, was 12.1 ± 1.2 nmol of NHMec released (mg of protein)$^{-1}$) min^{-1} (± standard error, $n = 3$) in soluble extracts of male worms and 17.3 ± 1.1 in extracts of female worms. The specific activity of the cathepsin B-like proteinase, measured with Z-Arg-Arg-NHMec at pH 6.2 was 5.8 ± 0.7 nmol mg^{-1} min^{-1} in extracts of male worms and 13.3 ± 0.4 nmol mg^{-1} min^{-1} in extracts of female worms.

Figure 2B presents a comparison of the stability of the cathepsin L and cathepsin B activities at 37°C in PBS, pH 7.3, over 24 h. Both enzymes were stable for 1 h, although their activity decreased after this time, with the activity of cathepsin B decreasing at a faster rate than that of the cathepsin L. Less than 10% of the cathepsin B activity remained after 8 h of incubation, and the enzyme was completely inactive after 24 h. In contrast, the cathepsin L retained 33% of its activity after 8 h and 16.5% after 24 h.

Inhibition with diazomethylketones. The cathepsin L and cathepsin B activities in adult *S. mansoni* extracts were inhibited by the diazomethylketones Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2, with the former being the more potent inhibitor. The inhibition constants, K_i, for Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 were 0.2 and 5 μM, respectively, in assays with the cathepsin B-specific substrate Z-Arg-Arg-NHMec performed at pH 6.2, the optimal pH for this enzyme. In assays with the fluorogenic peptide substrate Z-Phe-Arg-NHMec performed at pH 6.2 the K_is for Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 were 0.1 and 5 μM, respectively. However, these inhibitors were more effective in assays performed at pH 4.5, at which cathepsin B activity is negligible. The K_is for Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2 at pH 4.5 were 0.05 and 0.4 μM, respectively.

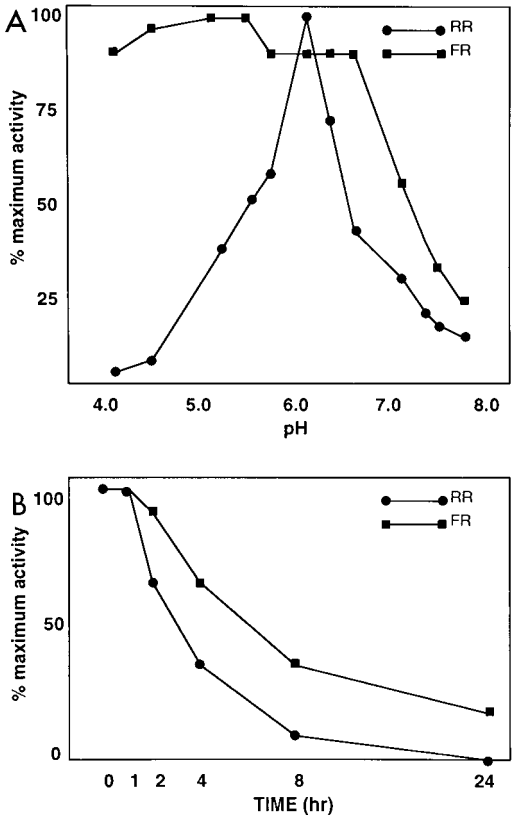

FIG. 2. (A) pH profile of cathepsin L- and cathepsin B-like activities. Activities in soluble extracts of *S. mansoni* were assayed with the substrate Z-Phe-Arg-NHMec (FR), which is cleaved by cathepsin L and cathepsin B proteinases, and with the cathepsin B-specific substrate Z-Arg-Arg-NHMec (RR) under the conditions described in the text. (B) Stability of cathepsin L- and cathepsin B-like activities in extracts of adult *S. mansoni* at 37°C. Cathepsin L-like activity was assayed at pH 4.5 with the substrate Z-Phe-Arg-NHMec and cathepsin B-like activity was assayed at pH 6.2 with the substrate Z-Arg-Arg-NHMec in aliquots removed at specific intervals. The values reported represent means of triplicate assays of single enzyme preparations.

Zymogram analysis of adult *S. mansoni* proteinases. *S. mansoni* extracts were separated by electrophoresis in 8% native polyacrylamide gels containing 0.1% gelatin, and the gels were incubated overnight to allow the detection of gelatinolytic activity. Two bands of activity were detected (Fig. 3A). In these gels, the activity of the two proteinases was higher at pH 4.5 than at pH 6.2. Since the activity of both bands was inhibited by E-64 and Z-Phe-Ala-CHN_2, the activities can be attributed to cathepsin-like cysteine proteinases (Fig. 3A). The substrate specificity of the two gelatinolytic bands was explored by probing native polyacrylamide gels with fluorogenic peptide substrates. At pH 4.5 the two proteinases cleaved the substrate Z-Phe-Arg-NHMec with greatest efficiency, followed by Z-Phe-Val-Arg-NHMec and Z-Val-Leu-Lys-NHMec (Fig. 3B). Taken together, these results indicate that the two proteinase activities are cathepsin L-like. No adult *S. mansoni* proteinase with cathepsin B-like activity was identified by zymography or by probing gels with the substrate Z-Arg-Arg-NHMec at either pH 4.5 or 6.2 (Fig. 3B and data not shown).

Analysis of proteinase activities in schistosome ES products and in recombinant cathepsin preparations. Cathepsin L- and cathepsin B-like activity was detected in the media in which

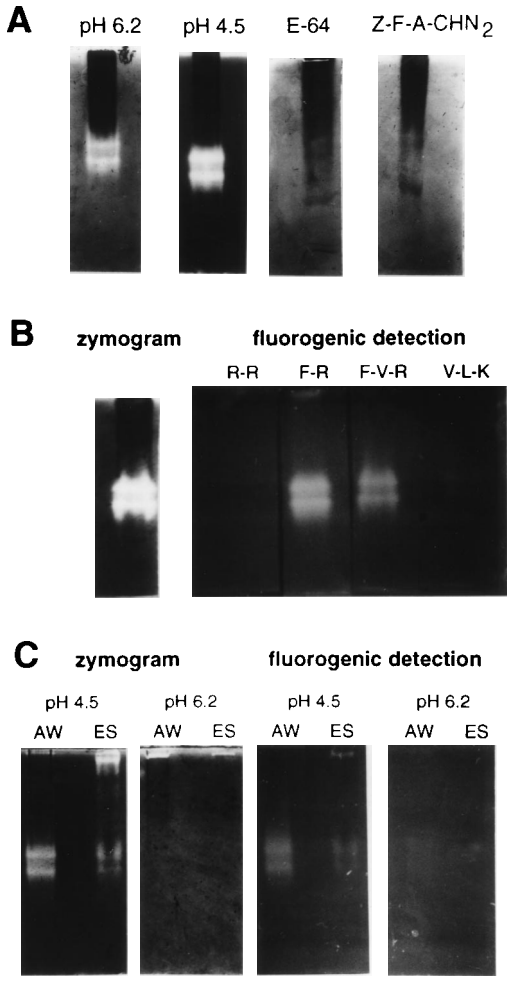

FIG. 3. (A) Analysis of proteinase activity in soluble extracts of adult *S. mansoni* by zymography. Extracts (20 μg) were applied to native 10% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were incubated in 0.1 M sodium phosphate, pH 6.2, or sodium acetate, pH 4.5, overnight and then stained with Coomassie. For inhibitor studies, gels were incubated in 0.1 M sodium acetate, pH 4.5, containing either E-64 (10 μM) or Z-Phe-Ala-CHN_2 (Z-F-A-CHN_2, 10 μM). (B) Direct detection of cysteine proteinases in native polyacrylamide gels. *S. mansoni* extracts (20 μg) were applied to native polyacrylamide gels containing gelatin. While one gel shown was processed for zymography, others were washed following electrophoresis in 0.1 M sodium acetate, pH 4.5, and then incubated in the same buffer containing 10 μM Boc-Arg-Arg-NHMec (R-R), Z-Phe-Arg-NHMec (F-R), Z-Phe-Val-Arg-NHMec (F-V-R), or Boc-Val-Leu-Lys-NHMec (V-L-K). (C) Comparison of proteinase activity in adult *S. mansoni* extracts and ES products. The proteinases in adult *S. mansoni* extracts (AW) and ES products were compared by using native polyacrylamide gel electrophoresis. Proteinases were identified by zymography or fluorography and detected by incubating the gels in 10 μM Z-Phe-Arg-NHMec in 0.1 M sodium acetate, pH 4.5, or 0.1 M sodium phosphate, pH 6.2.

adult *S. mansoni* were maintained for 16 h (i.e., in the ES products). The respective specific activities were 16.6 ± 2.0 nmol mg^{-1} min^{-1} with Z-Phe-Arg-NHMec at pH 4.5 and 19.3 ± 3.1 nmol mg^{-1} min^{-1} with Z-Arg-Arg-NHMec at pH 6.2. In zymograms, two ES proteinases comigrated with the two adult worm cathepsin L proteinases, were more active at pH 4.5 than at pH 6.2, and were visualized in gels probed with the substrate Z-Phe-Arg-NHMec (Fig. 3C). A slower-migrating proteinase was visualized in the ES products with Z-Phe-Arg-NHMec only and was more active at pH 4.5 than at pH 6.2 (Fig. 3C). This proteinase may represent an enzyme not detectable in adult worm extracts, or it may result from an aggregate of the cathepsin L-like activity. A second, slow-migrating proteinase was detected in ES and adult worm extracts; this activity was more active at pH 6.2 than at pH 4.5 and did not cleave the substrates Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec (Fig. 3C and data not shown). No cathepsin B-like activity was detected in ES in gels probed with fluorogenic substrates (not shown).

None of the three recombinant proteinases exhibited detectable proteolytic activity, as determined by gelatin-substrate PAGE or in test tube assays with Z-Phe-Arg-NHMec or Z-Arg-Arg-NHMec. Absence of activity is probably not surprising, given that these enzymes possess many cysteine residues which would have to refold properly within the reducing milieu of the *E. coli* cytoplasm to form approximately six disulfide bonds. Difficulty in obtaining active recombinant cysteine proteinases is well documented (17).

Analysis of cathepsin L gene sequences. A comparison of the putative amino acid sequences in the mature region of *S. mansoni* cathepsin L1 and cathepsin L2 revealed that the two proteinases had only 43.8% identity (Fig. 4). Comparison of these amino acid sequences with those of several mammalian cathepsin L's confirmed that both schistosome enzymes belong to the cathepsin L class (see Fig. 4 for comparison with human cathepsin L). The sequence of cathepsin L2 is more similar to rat (53.4%), mouse (53.4%), human (52.3%), and chicken (51.6%) liver cathepsin L sequences than is that of cathepsin L1 (46.4, 46.8, 44.3, and 46.4%, respectively). In contrast, the cathepsin L1 amino acid sequence is 41.3% similar to that of papain, whereas that of cathepsin L2 is 38.8%.

Schistosome cathepsin L1 and cathepsin L2 both contain the conserved amino acids Gln-19, Cys-25, His-159, Asn-175, and Trp-177 that are contained in the S_1 subsite of the enzyme active site and are involved in the catalytic cleavage of the scissile bond. However, cathepsins L1 and L2 differ in four of the eight residues that constitute the S_2 subsite of the active site, which determines the specificity of the proteinase for its substrate (8); cathepsin L1 contains Leu-67, Pro-68, Gly-133, and Ala-160, whereas cathepsin L2 contains Thr-67, Met-68, Ala-133, and Gly-160. Cathepsin L1 contains three potential N-linked glycosylation sites. By contrast, the cathepsin L2 sequence has a single potential glycosylation site at Asn-204, although its proximity to the S_2 subsite Ala-205, which interacts with the substrate P_2 amino acid, suggests that this site would not be glycosylated. A consensus alanine (Ala-17 in the human cathepsin L sequence) which terminates the putative signal sequence of all human cathepsins is absent from the schistosome cathepsins L (Fig. 4). Cathepsins L1 and L2 are only 22.2% similar in the propeptide region. Nevertheless, the propeptide regions of both possess ERFNIN-like sequence motifs and a block of conserved amino acids carboxyl to this motif that are characteristic of cathepsin L or H cysteine proteinases and are thought to be involved in the regulation of the proteinase activity (15) (Fig. 4).

Southern blot analysis. ^{32}P-labeled fragments of the cathepsin L1, cathepsin L2, and cathepsin B genes were employed to probe Southern blots of restriction enzyme-digested *S. mansoni* DNA. Each probe produced a discrete hybridization pattern (Fig. 5). Other Southern blot analyses demonstrated that these probes do not cross-hybridize under the same stringent conditions (not shown). Both the cathepsin L1 and cathepsin L2 probes hybridized to bands at >12 kb in all four digests (Fig. 4, compare panels A and B). The hybridization patterns were in some cases complex; for example, all probes hybridized to a number DNA fragments in the *Xba*I digests, although no site for this restriction enzyme exists within any of the cathepsin genes (Fig. 5).

FIG. 4. Alignment of the deduced amino acid sequence of *S. mansoni* cathepsin L1 (smL1) and cathepsin L2 (smL2) with that of human cathepsin L (humL) (accession number M20496). Boxes denote conserved residues, and gaps have been introduced to maximize alignment. The putative signal peptide cleavage site is indicated by an open arrow. The solid arrow indicates the cleavage site between propeptide and mature enzyme. The ERFNIN-like motif and the conserved block in the propeptide are underlined. Residues contained within the S_1 subsite of the active site are indicated with solid circles, and those contained within the S_2 subsite are indicated with open circles. Cathepsin L1 potential N-glycosylation sites are indicated with solid triangles.

DISCUSSION

Tissues and secretions of adult *S. mansoni* contain both cathepsin L- and cathepsin B-like cysteine proteinase activities. Using synthetic fluorogenic peptide substrates, we determined the pH optima for the cathepsin L and cathepsin B activities as pH 5.2 and 6.2, respectively. *S. mansoni* cathepsin L activity can be specifically measured by using Z-Phe-Arg-NHMec at pH 4.5, since at this pH cathepsin B-like activity is negligible. On the other hand, cathepsin B-like activity can be specifically assayed with Z-Arg-Arg-NHMec at pH 6.2. Both cathepsin L and cathepsin B activities were inhibited by diazomethylketones, although Z-Phe-Ala-CHN_2 was a much more potent inhibitor (8- to 50-fold) than Z-Phe-Phe-CHN_2 for both enzymes. In contrast, Z-Phe-Phe-CHN_2 is a poor inhibitor of mammalian cathepsin B but is a better inhibitor of cathepsin L than is Z-Phe-Ala-CHN_2 (1). Differences in the susceptibility of the *S. mansoni* and mammalian cathepsins to diazomethylketones may be exploitable in the search for specific antischistosomal agents.

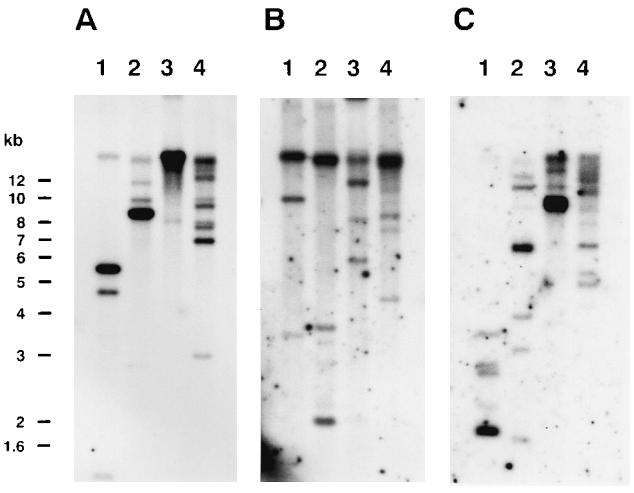

FIG. 5. Southern blot analysis of genomic DNA of *S. mansoni* digested with *Eco*RI (lanes 1), *Hin*dIII (lanes 2), *Pst*I (lanes 3), and *Xba*I (lanes 4) and hybridized with ^{32}P-labeled cathepsin L1 (A), cathepsin L2 (B), and cathepsin B (C) genes.

The specific activity of cathepsin L-like proteinases in these extracts was about double that of cathepsin B when each enzyme was assayed at its pH optimum. We previously reported that the cathepsin L-like activity was 60-fold greater than cathepsin B-like activity based on assays performed at pH 4.5 (25). Nonetheless, in extracts of both male and female worms, the cathepsin L activity predominated over cathepsin B-like activity. Both cathepsin L-like and cathepsin B-like activities were higher in extracts of female worms than in those of males worms but only on the order of 50 and 100%, respectively. Given the role postulated for these enzymes in hemoglobin digestion (8), this difference in specific activity does not correlate well with the estimated intake of 39,000 and 330,000 erythrocytes per h by male and female schistosomes, respectively (18). Synthesis and turnover of cathepsin

proteinases may be more efficient in females than in male worms.

Cathepsin L- and cathepsin B-like activities in extracts of adult *S. mansoni* were stable at 37°C, and both enzymes retained activity even after incubation for 8 h at physiological pH. The cathepsin L-like activity was more stable than the cathepsin B-like activity and retained substantial activity even after 1 day. In contrast, mammalian cathepsin L and cathepsin B are quickly and completely inactivated under these conditions (5). Mammalian cathepsins are usually located in the acidic lysosomes, where they are involved in protein catabolism. The instability of these enzymes at physiological pH is likely to protect the cell against leakage from the lysosomes (5, 14). By contrast, cathepsin S cysteine proteinases are stable and highly active at neutral pH and may be involved in pathological processes (5). Therefore, while the stability of the schistosome cathepsins at physiological pH likely reflects their postulated roles in extracellular digestion of hemoglobin, this property may also implicate them in the pathogenesis of schistosomiasis, since we have shown the presence of high levels of cathepsin L-like activity in schistosome eggs (10).

Zymographic analysis revealed that two major gelatinolytic activities were present in *S. mansoni* extracts. Both of these activities were characterized as cathepsin L, since they were more active at pH 4.5 than at pH 6.2 and preferentially cleaved the Z-Phe-Arg-NHMec substrate. It is not clear why the *S. mansoni* cathepsin B activity was not detected by zymography or by probing the gels with Z-Arg-Arg-NHMec. This enzyme may not migrate into gels, or its activity may be inactivated by electrophoresis. We also detected cathepsin L and cathepsin B activities in *S. mansoni* ES products. These results are of interest because they suggest that adult schistosome actively secrete these enzymes, a prerequisite for hemoglobin-degrading enzymes functioning in the lumen of the schistosome gut. Furthermore, we detected a slow-migrating gelatinolytic activity in extracts of adult worms and ES which did not exhibit cathepsin-like activity and was more active at pH 6.2 than at pH 4.5. This proteinase may represent calpain (24), the asparaginyl endopeptidase Sm32 (6–8), or a novel enzyme.

Alignment of the deduced amino acid sequence of the cathepsin L1 gene with that of the cathepsin L2 gene demonstrated that these were distinct cathepsins L. This observation was confirmed by Southern hybridization, which showed a different genomic organization for each gene. The cathepsin L1 and cathepsin L2 sequences differ in four of the eight residues that constitute the S_2 subsite of the active site, which determines the substrate specificity of the enzymes, indicating that the two enzymes have discrete substrate specificities. We did not observe any differences in the substrate specificity of the two cathepsin L bands of activity detected by zymography, although our analysis was restricted to a few peptide substrates. Apart from their potential to cleave different substrates, several additional observations indicate separate functions for cathepsin L1 and cathepsin L2. First, the two proteinases show very low similarity within the propeptide region, which influences enzyme trafficking, folding, and activity (15). Second, cathepsin L1 has three potential sites for glycosylation, whereas cathepsin L2 does not have any. Glycosylation of cathepsins and, more specifically, expression of the mannose 6-phosphate marker provide signals to direct zymogens to lysosomes (14, 15, 17). Cathepsin L1 may therefore be retained intracellularly, whereas cathepsin L2, which lacks glycosylation and hence sorting signals, may enter the secretory pathway.

The complex patterns observed in the Southern hybridizations indicated that introns were present on each cathepsin L gene and/or that each gene belonged to a different gene family. However, since only two cathepsin L genes have been reported, even after an extensive analysis of the transcripts of adult schistosomes by the expressed-sequence tag approach (12), it is likely that the two bands of activity detected in our gels represent the products of the cathepsin L1 and L2 genes. The demonstrations that cathepsin B and cathepsin L cysteine proteinases are major proteinases in extracts and secretions of adult *S. mansoni* and that these proteases are active at acidic pH support their postulated roles in digestion of hemoglobin (2, 8, 10, 13, 25). A cathepsin D aspartic proteinase is expressed in the cells lining the gut (3, 4) and likely also plays a key role in the degradation of hemoglobin (2). By contrast, we have recently shown that Sm32—originally touted as the schistosome hemoglobinase (9, 13)—is an asparaginyl endopeptidase with such low specific activity that it is unlikely to be directly involved in hemoglobin digestion. Rather, we have suggested that Sm32 posttranslationally modifies other proteinases (6). Since the schistosome cathepsins exhibit different pH optima, the gradual decline in pH of the blood meal from physiological pH to the acidic pH of the schistosome gut may be a mechanism by which worms regulate the activity of each of these enzymes and hence the systematic degradation of hemoglobin to diffusible peptides.

ACKNOWLEDGMENTS

We thank Mary Duke for maintenance of the schistosome life cycle.

We acknowledge financial assistance from the Australian National Health and Medical Research Council, ACITHN, and Dublin City University.

REFERENCES

1. **Barrett, A. J., and H. Kirschke.** 1981. Cathepsin B, cathepsin H and cathepsin L. Methods Enzymol. **80:**535–561.
2. **Becker, M. M., S. A. Harrop, J. P. Dalton, B. H. Kalinna, D. P. McManus, and P. J. Brindley.** 1995. Cloning and characterization of the *Schistosoma japonicum* aspartic proteinase involved in hemoglobin degradation. J. Biol. Chem. **240:**24496–24501.
3. **Bogitsh, B. J., and K. F. Kirschner.** 1986. *Schistosoma japonicum*: ultrastructural localization of a hemoglobinase using mercury labeled pepstatin. Exp. Parasitol. **62:**211–215.
4. **Bogitsh, B. J., and K. F. Kirshner.** 1987. *Schistosoma japonicum*: immunocytochemistry of adults using heterologous antiserum to bovine cathepsin D. Exp. Parasitol. **64:**213–218.
5. **Bromme, D., P. R. Bonneau, P. Lachance, B. Wiederanders, H. Kirschke, C. Peters, D. Thomas, A. Storer, and T. Vernet.** 1993. Functional expression of human cathepsin S in *Saccharomyces cerevisiae*: purification and characterization of the recombinant enzyme. J. Biol. Chem. **268:**4832–4838.
6. **Dalton, J. P., and P. J. Brindley.** Schistosome asparaginyl endopeptidase Sm32 in hemoglobin digestion. Parasitol. Today, in press.
7. **Dalton, J. P., L. Hola-Jamriska, and P. J. Brindley.** 1995. Asparaginyl endopeptidase activity in adult *Schistosoma mansoni*. Parasitology **111:**575–580.
8. **Dalton, J. P., A. M. Smith, K. A. Clough, and P. J. Brindley.** 1995. Digestion of haemoglobin by schistosomes: 35 years on. Parasitol. Today **11:**299–303.
9. **Davis, A. H., J. Nanduri, and D. C. Watson.** 1987. Cloning and gene expression of *Schistosoma mansoni* protease. J. Biol. Chem. **262:**12851–12855.
10. **Day, S. R., J. P. Dalton, K. A. Clough, L. Leonardo, W. U. Tiu, and P. J. Brindley.** 1995. Characterization and cloning of the cathepsin L proteinases of *Schistosoma japonicum*. Biochem. Biophys. Res. Commun. **217:**1–9.
11. **Dowd, A. J., A. M. Smith, S. McGonigle, and J. P. Dalton.** 1994. Purification and characterisation of a second cathepsin L proteinase secreted by the parasitic trematode *Fasciola hepatica*. Eur. J. Biochem. **223:**91–98.
12. **Franco, G. R., M. D. Adams, M. B. Soares, A. J. G. Simpson, J. C. Venter, and S. D. J. Pena.** 1995. Identification of new *Schistosoma mansoni* genes by the EST strategy using a directional cDNA library. Gene **152:**141–147.
13. **Gotz, B., and M.-Q. Klinkert.** 1993. Expression and partial characterization of a cathepsin B-like enzyme (Sm31) and a proposed 'haemoglobinase' (Sm32) from *Schistosoma mansoni*. Biochem. J. **290:**801–806.
14. **Joseph, L. J., L. C. Chang, D. Stamenkovich, and V. P. Sukhatme.** 1988. Complete nucleotide and deduced amino acid sequences of human and murine preprocathepsin L: an abundant transcript induced by transformation of fibroblasts. J. Clin. Invest. **81:**1621–1629.
15. **Karrer, M. K., S. L. Peiffer, and M. E. DiTomas.** 1993. Two distinct gene

subfamilies within the family of cysteine protease genes. Proc. Natl. Acad. Sci. USA **90:**3063–3067.
16. **Klinkert, M.-Q., R. Felleisen, G. Link, A. Ruppel, and E. Beck.** 1989. Primary structures of Sm31/32 diagnostic proteins of *Schistosoma mansoni* and their identification as proteases. Mol. Biochem. Parasitol. **33:**113–122.
17. **Kuhelj, R., M. Dolinar, J. Pungercar, and V. Turk.** 1995. The preparation of catalytically active human cathepsin B from its precursor expressed in *Escherichia coli* in the form of inclusion bodies. Eur. J. Biochem. **229:**533–539.
18. **Lawrence, J. D.** 1973. The ingestion of red blood cells by *Schistosoma mansoni*. J. Parasitol. **59:**60–63.
19. **McKerrow, J. H., and M. J. Doenhoff.** 1988. Schistosome proteases. Parasitol. Today **4:**334–340.
20. **Michel, A., H. Ghoneim, M. Resto, M.-Q. Klinkert, and W. Kunz.** 1995. Sequence, characterization and localization of a cysteine proteinase cathepsin L in Schistosoma mansoni. Mol. Biochem. Parasitol. **73:**7–18.
21. **North, M. J., J. C. Mottram, and G. H. Coombs.** 1990. Cysteine proteinases of parasitic protozoa. Parasitol. Today **6:**270–276.
22. **Pearce, E. J., P. F. Basch, and A. Sher.** 1986. Evidence that the reduced surface antigenicity of developing *Schistosoma mansoni* schistosomula is due to antigen shedding rather than host molecule acquisition. Parasite Immunol. **8:**79–94.
23. **Rawlings, N. D., and A. J. Barrett.** 1993. Evolutionary families of peptidases. Biochem. J. **290:**205–218.
24. **Siddiqui, A. A., Y. Zhou, R. B. Podesta, S. R. Karcz, C. E. Tognon, G. H. Strejan, G. A. Dekaban, and M. W. Clarke.** 1993. Characterization of Ca^{2+} dependent neutral protease (calpain) from human blood flukes, *Schistosoma mansoni*. Biochim. Biophys. Acta **1181:**37–44.
25. **Smith, A. M., J. P. Dalton, K. A. Clough, C. L. Kilbane, S. A. Harrop, N. Hole, and P. J. Brindley.** 1994. Adult *Schistosoma mansoni* express cathepsin L proteinase activity. Mol. Biochem. Parasitol. **67:**11–19.
26. **Smith, A. M., A. J. Dowd, S. McGonigle, P. S. Keegan, G. Brennan, A. Trudgett, and J. P. Dalton.** 1993. Purification of a cathepsin L-like proteinase secreted by adult *Fasciola hepatica*. Mol. Biochem. Parasitol. **62:**1–8.
27. **Timms, A. R., and E. Bueding.** 1959. Studies of a proteolytic enzyme from *Schistosoma mansoni*. Br. J. Pharmacol. **14:**68–73.

Editor: J. M. Mansfield