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CHARACTERIZATION AND CLONING OF THE CATHEPSIN L PROTEINASES OF *SCHISTOSOMA JAPONICUM* †

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SUMMARY Adult *Schistosoma japonicum* parasites synthesize and secrete both cathepsin L and cathepsin B cysteine proteinases. The specific activities of cathepsin L were many-fold higher than that of cathepsin B. The cDNAs encoding two distinct cathepsin L proteinases, here termed cathepsin L1 and L2, were isolated. The deduced amino acid sequences of the mature cathepsin L1 and L2 were ~41% identical, and moreover, *S. japonicum* cathepsin L2 showed more similarity with human cathepsin L than with schistosome cathepsin L1. Schistosome cathepsin L proteinases may be involved in the digestion of hemoglobin obtained from host erythrocytes. However, since we detected their presence in schistosome eggs, the release of these enzymes by eggs trapped in the liver and other organs may be associated with the granulomatous responses which characterize the pathology of human schistosomiasis.

Cathepsin L activity in schistosomes was first described by Smith *et al.* (1) who suggested that it might be involved in the digestion of host hemoglobin, the main source of amino acids required by the parasite for its metabolism (2-5). Since then, two *Schistosoma mansoni* cathepsin L cDNAs, which we now term SmCL1 and SmCL2, have been isolated (1,6,7). The similarity of the deduced primary sequence of the two enzymes - 43% - was low, indicating that they are not the products of alleles of the same gene but that they are separate cathepsin L proteases. Immunolocalization studies showed that one of these enzymes is expressed in cells lining the parasite's gut while the other was present in the tegument, suggesting that the two cathepsin Ls perform separate functions, possibly hemoglobin digestion and surface tegument turnover (7).

Here we characterize the cathepsin L activity of the Asian schistosome *S. japonicum*. Furthermore, we have isolated cDNAs encoding *S. japonicum* homologs, here termed SjCL1 and

†Nucleotide sequences reported here have been submitted to the GenBank database with the Accession Nos. U38475 and U38476.

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SjCL2, of the two *S. mansoni* cathepsin Ls. Cathepsin L-like cysteine proteinases predominate in acidic extracts of adult schistosomes, are secreted *in vitro*, and are present in extracts of eggs. This latter observation indicates that cathepsin L may contribute to the pathogenesis associated with schistosomiasis.

MATERIALS and METHODS

Schistosomes, genomic DNAs, soluble extracts. Mixed sex, adult *S. japonicum* (Philippine strain) and *S. mansoni* (Puerto Rican strain) were perfused from rabbits or from BALB/c mice 7-8 weeks after infection and stored at -70°C for up to 7 months. Extracts of schistosomes were prepared in phosphate buffered saline pH 7.3, as previously described (1). *S. japonicum* eggs, obtained by trypsin digestion of infected rabbit livers, were extracted into PBS by 20 x 7 sec bursts of sonication with 3 min intervals. Protein concentration of extracts was measured by a modified Lowry method (DC Protein Assay, Bio-Rad, CA).

Proteinase substrates and inhibitors. The synthetic fluorogenic peptide substrates Z-Arg-AMC, Boc-Arg-Arg-AMC, Z-Phe-Val-Arg-AMC, Tos-Gly-Pro-Arg-AMC, Suc-Leu-Tyr-AMC, Boc-Val-Leu-Lys-AMC and the peptidyl diazomethylketones inhibitors, *N*-benzyloxycarbonyl-l-phenylalanyl-phenylalanine (Z-Phe-Phe-CHN_2) and benzyloxycarbonyl-l-phenylalanyl-l-alanine-diazomethylketone (Z-Phe-Ala-CHN_2) were from Bachem Biosciences (Philadelphia, PA). Dithiothreitol (DTT), L-cysteine, Z-Phe-Arg-AMC, H-Leu-Val-Tyr-AMC, Suc-Leu-Leu-Val-Tyr-AMC, Tos-Gly-Pro-Lys-AMC, and the cysteine proteinase inhibitor trans-epoxysuccinyl-l-leucylamido (4-guanidino)-butane (E64) were from Sigma Chemical Co., St. Louis, MO.

Fluorometric assays, zymography, and fluorography. Enzyme assays, zymography and direct visualization of proteinases within gels using fluorogenic peptide substrates (fluorography) were performed as described previously (7).

Screening of *S. japonicum* cDNA phage libraries. A mixed sex adult *S. japonicum* (Philippine strain) cDNA library, constructed in λ UNI Zap-XR (library kindly provided by Drs. J. Kurtis and B. Ramirez) and a mixed sex adult *S. japonicum* (Chinese strain) λ Zap II cDNA library constructed by standard procedures were screened by nucleic acid hybridization with the [^{32}P]-labeled fragments (~50ng) of the *S. mansoni* cathepsin L1 (1) and L2 genes (6,7) using nylon membranes (Amersham, Australia). (The gene encoding the *S. mansoni* cathepsin L2 was amplified by PCR from a *S. mansoni* cDNA library, and cloned as described (7)). The probes were radiolabeled using [^{32}P]dCTP, random oligomer priming, and Klenow polymerase (AMRAD-Pharmacia, Australia), and used with hybridization and washing conditions as described (1,8). Autoradiography was performed at -70°C using Fuji X-ray film. pBluescript phagemids in positive clones were excised using P408 helper phage (Promega) and *E. coli* strain XL1-Blue according to the UNI Zap XR manufacturer's instructions (Stratagene, San Diego, CA). Plasmid DNAs were isolated on Qiagen-100 columns (Qiagen Inc. Chatsworth, CA.) and nucleotide sequences determined using the Taq DyeDeoxy Terminator Cycle Sequencing System (Applied Biosystems Inc. [ABI], Foster City, CA) and an ABI automated DNA sequencer.

RESULTS

Substrate specificity of *S. japonicum* proteinase activity. Proteinase activity in extracts of adult schistosomes was investigated using a panel of fluorogenic substrates that could distinguish between different classes of cathepsin proteinases, namely Z-Arg-AMC (cathepsin H), Z-Arg-Arg-AMC (cathepsin B), Z-Phe-Arg-AMC (cathepsin B and L) and Z-Phe-Val-Arg-AMC (cathepsin S and L) (9,10). Assays were conducted using substrates at 2.5 μM, a concentration

much lower than the K_m for cathepsin proteinases (11), so that a comparison of the relative efficiency of cleavage could be made. The efficient cleavage of peptides used for the detection of cathepsin B and L (Z-Phe-Arg-AMC, Z-Phe-Val-Arg-AMC) demonstrated the predominance of these proteinases in extracts of adult *S. japonicum* at pH 4.5 (Fig. 1A). However, the low efficiency of Z-Arg-Arg-AMC cleavage indicates that the majority of cathepsin activity measured by Z-Phe-Arg-AMC cleavage can be attributed to cathepsin L. The Z-Phe-Arg-AMC cleaving activity was diminished in the absence of reducing agents (DTT or cysteine) and completely inhibited by addition of the cysteine proteinase-specific inhibitor E64 (Fig. 1A). A similar substrate profile was obtained using these synthetic substrates with the activity(s) in extracts of *S. japonicum* eggs (not shown). Cathepsin L-like activity against the substrate Z-Phe-Arg-AMC was optimal at pH 5.2 (Fig. 1B). Cathepsin B-like activity, measured using fluorogenic substrate

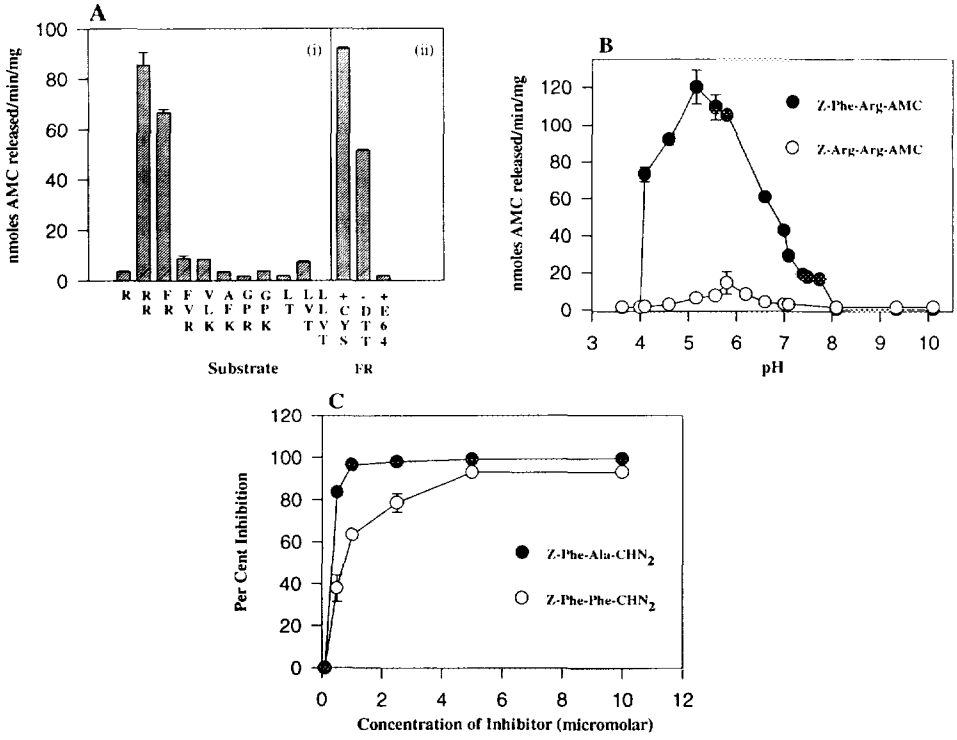

Fig. 1. Characterization of protease activity in adult *S. japonicum* extracts in 0.1 M sodium acetate, pH 4.5. **A(i)** Extracts were assayed for activity against Z-Arg-AMC (R), -Arg-Arg- (RR), -Phe-Arg- (FR), -Phe-Val-Arg- (FVR), -Val-Leu-Lys- (VLK), -Ala-Phe-Lys- (AFK), -Gly-Pro-Arg- (GPR), -Gly-Pro-Lys- (GPK), -Leu-Tyr- (LT), -Leu-Val-Tyr- (LVT), and -Leu-Leu-Val-Tyr- (LLVT); **(ii)** effect on the activity against Z-Phe-Arg-AMC of the cysteine proteinase inhibitor E64 and of the presence or absence of reducing agents cysteine (Cys) or DTT. **B**: The pH optima for activity of adult *S. japonicum* cathepsin L and cathepsin B were determined using Z-Phe-Arg-AMC and Z-Arg-Arg-AMC as substrates, respectively. **C**: Inhibition of the activity of *S. japonicum* cathepsin Ls against Z-Phe-Arg-AMC at pH 4.5 by the inhibitors Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2.

Z-Arg-Arg-AMC, was optimal at pH 5.8-6.0 (Fig. 1B). Similar pH profiles were obtained for *S. japonicum* egg extracts (not shown). The cathepsin L activity, when measured at pH 4.5 where cathepsin B activity is negligible, was inhibited by the diazomethylketones Z-Phe-Ala-CHN_2 and Z-Phe-Phe-CHN_2. Z-Phe-Ala-CHN_2 was the more potent inhibitor with an inhibition constant K_i of 0.05 μM whereas the K_i of Z-Phe-Phe-CHN_2 was 0.4μM (Fig. 1C).

The specific activities (mean nmol AMC released mg schistosome $protein^{-1}$ min^{-1} ± SD, triplicate assays) of cathepsin L measured against Z-Phe-Arg-AMC at pH 4.5 was 19.6 ± 1.09 for adult *S. mansoni* extract, 92 ± 3 for adult *S. japonicum* extract, 0.14 ± 0.004 for adult *S. japonicum* ES products, and 58.4 ± 0.03 for *S. japonicum* egg extract. The specific activities for cathepsin B measured using Z-Arg-Arg-AMC at pH 6.2 were 10.38 ± 0.79 for adult *S. mansoni* extract, 8.2 ± 0.03 for adult *S. japonicum* extract, 0.0021 ± 0.0001.5 for adult *S. japonicum* ES products, and 6.88 ± 2.1 for *S. japonicum* egg extract.

Analysis of schistosome proteinases by zymography and fluorography. *S. mansoni* and *S. japonicum* extracts were electrophoresed through 10% polyacrylamide gels containing 0.1% gelatin. Following electrophoresis, gels were probed with the fluorogenic substrate Z-Phe-Arg-AMC to detect cathepsin L activity (Fig. 2A) and then incubated overnight at 37°C to detect gelatinolytic activity (Fig. 2B). Consistent with our earlier report (7), *S. mansoni* worm extracts contained two gelatinolytic activities could cleave Z-Phe-Arg-AMC (Fig. 2A and B). Adult *S. japonicum* extracts contained gelatinolytic activity which appeared as a broad band that migrated more slowly than the *S. mansoni* proteases (Fig. 2B). This activity also cleaved the fluorogenic substrate Z-Phe-Arg-AMC (Fig 2A). Furthermore, *S. japonicum* egg extracts

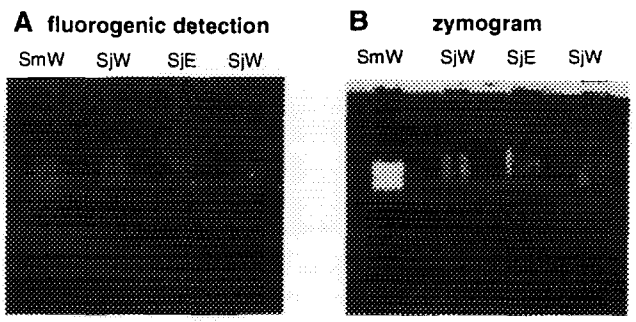

Fig. 2. Gel electrophoretic analysis of the cathepsin L activity in soluble extracts of adult *S. mansoni* (SmW), adult *S. japonicum* (SjW) and *S. japonicum eggs* (SjE). Extracts were separated in 10% polyacrylamide gels containing 0.1% gelatin. Following electrophoresis, the gels were incubated in 0.1 mM sodium citrate, pH 4.5, containing 1 mM DTT and the fluorogenic substrate Z-Phe-Arg-AMC (10 μM) for 10 min, then photographed while UV transilluminated at 253 nm (Panel A). Gels were subsequently incubated overnight in the same buffer after which gelatinolytic activity visualized by staining with Coomassie blue (Panel B).

contained Z-Phe-Arg-AMC-cleaving and gelatinolytic protease(s) which co-migrated with those in adult *S. japonicum* extracts (Fig. 2).

Deduced amino acid sequences of the SjCL1 and SjCL2 cathepsin Ls. Plasmid clone pSjP13 was isolated from the Philippine strain library, and was found after its nucleotide sequence was determined to encode part of the *S. japonicum* cathepsin L1 (SjCL1). Since pSjP13 did not encode the entire mature protease (not shown), its insert was employed to screen the *S. japonicum* cDNA libraries in order to isolate clones encoding the missing regions. Of the one hundred thousand plaques screened, eight hybridized to the probe. While still representing a truncated transcript, one of these (from the Chinese strain library) encoded the full 215 deduced amino acids of the mature SjCL1 protease, plus 26 amino acids of the proregion. The transcript also contained a stop codon, a 3'-untranslated region of 354 bp, and a poly (A) stretch (GenBank accession no. U38475). In like fashion, when the gene encoding *S. mansoni* cathepsin L2 was used to probe the *S. japonicum* cDNA libraries, a cDNA was isolated from the Chinese strain library which encoded for the entire *S. japonicum* cathepsin L2 zymogen (SjCL2). This transcript included a start codon, an open reading frame of 666 bp including a stop codon, a 3'-untranslated region of 78 bp, and a poly (A) tail (GenBank accession no. U38476). The deduced amino acid sequences of the SjCL1 and SjCL2 proteases are shown in Fig. 3 in a multiple alignment with each other and with SmCL1, SmCL2, and human cathepsin L. The mature enzyme sequences of SjCL1 and SjCL2 contain 215 and 216 residues, respectively, of which 41% were identical between the two enzymes. Both are predicted to encode mature proteases of ~24 kDa. Mature SjCL1 was 92% identical to mature SmCL1, whereas mature SjCL2 and SmCL2 were 78% identical. SjCL1 and SjCL2 were 45% and 50% identical, respectively, to human cathepsin L.

When the residues that comprise the S_2 subsites of these proteases were compared, the S_2 subsites of SjCL1 and SjCL2 shared only 3 of the 8 subsite residues. By contrast, the S_2 subsites of SjCL1 and SmCL1 were identical, and those of SjCL2 and SmCL2 shared 5 of the 8 residues. SjCL1 and SjCL2 each shared 4 of the 8 S_2 subsite residues with human cathepsin L (Table 1).

DISCUSSION

Alignment of the deduced amino acid sequences of SjCL1 and SjCL2 demonstrated that they were distinct cathepsin Ls. Therefore, adult *S. japonicum* express two cathepsin L, cysteine proteases, as in *S. mansoni* (1,6,7). SjCL2 showed a greater divergence from SmCL2 (78% similar) than did SjCL1 from SmCL1 (92% similar). In addition, the residues in the S_2 subsite, which determines the substrate specificity of these proteases (5), were identical in SjCL1 and SmCL1, but differed in three out of eight residues for SjCL2 and SmCL2. These observations indicate a divergence in the function of cathepsin L2 in *S. mansoni* and *S. japonicum*. Intriguingly, cathepsin L1 and L2 of these two schistosome species have dissimilar compositions of the S_2

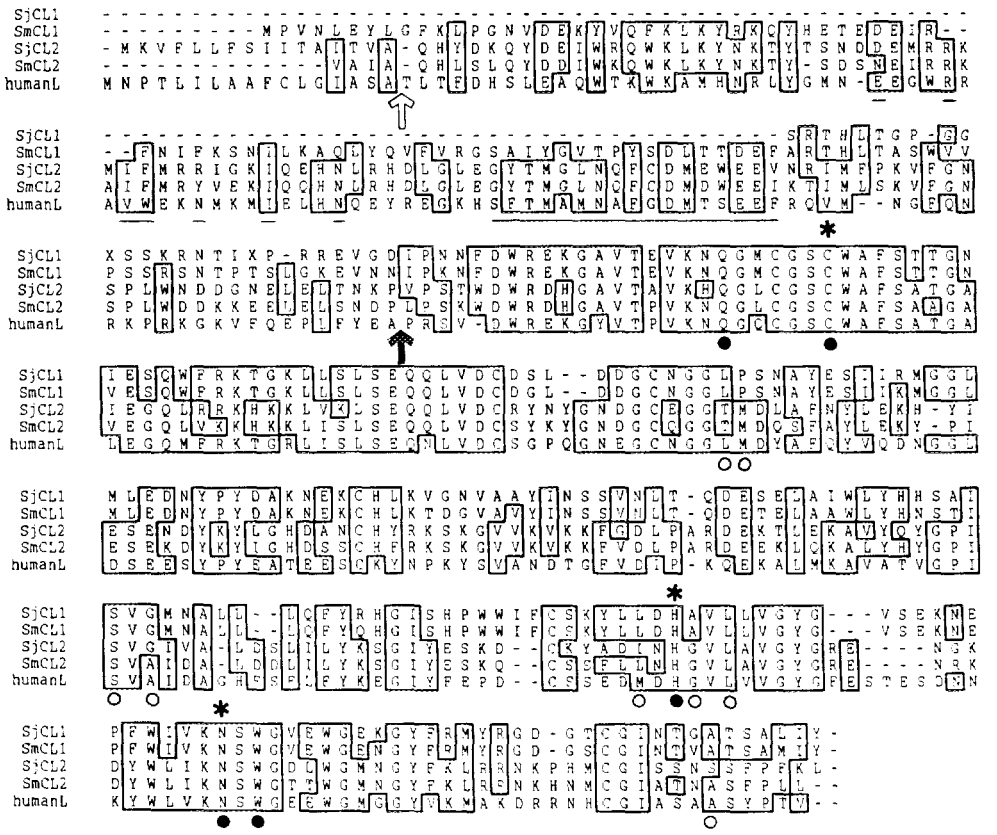

Fig. 3. Alignment of the deduced amino acid sequence of *S. japonicum* cathepsin L1 (GenBank accession no. U38475) (SjCL1) and cathepsin L2 (GenBank U38476) (SjCL2) with that of *S. mansoni* cathepsin L1 (GenBank U07345) (1) (SmCL1) and cathepsin L2 (GenBank Z32529) (6,7) (SmCL2) and human cathepsin L (GenBank M20496) (18). The boxes denote conserved residues and gaps have been introduced to maximize alignment. The closed arrow indicates the putative signal peptide cleavage site. The open arrow indicates the cleavage site between the propeptide and mature enzyme. The active site Cys, His, and Asn residues are marked with asterisks. The ERFNIN-like motif and the conserved block of amino acids in the propeptide are underlined (19). Residues contained within the S_1 subsite of the active site are indicated with solid circles and those contained within the S_2 subsite with open circles.

subsite, indicating that the two enzymes differ in their substrate specificities. Moreover, both SmCL1 and SjCL1 have three potential glycosylation sites whereas SmCL2 and SjCL2 have one; however, this latter site is located next to the S_2 subsite residue 205 and therefore is unlikely to be glycosylated. These different glycosylation patterns likewise suggest different functions for cathepsin L1s and L2s. SjCL1 may be glycosylated and trafficked to lysosomes whereas SjCL2, which is probably not glycosylated and therefore lacks sorting signals, may be secreted from cells. Similar differences exist between SmCL1 and SmCL2 (7).

Adult *S. japonicum* synthesize and secrete both cathepsin L and cathepsin B cysteine proteases, with the cathepsin L activity about eleven-fold higher than cathepsin B. In adult *S.*

Table 1. Comparison between the residues within the S_2 subsite of papain (Pap), human (Hm), *Schistosoma mansoni* (Sm), and *S. japonicum* (Sj) cathepsin Ls (CL)

Residue no.*	Pap	HmCL	SmCL1	SmCL2	SjCL1	SjCL2
67	Tyr	Leu	Leu	Thr	Leu	Thr
68	Pro	Met	Pro	Met	Pro	Met
131	Ser	Ser	Ser	Ser	Ser	Ser
133	Val	Ala	Gly	Ala	Gly	Gly
157	Val	Met	Leu	Leu	Leu	Ile
160	Ala	Gly	Ala	Gly	Ala	Gly
162	Ala	Leu	Leu	Leu	Leu	Leu
205	Ser	Ala	Ala	Ala	Ala	Ser

*See ref. 5 for numbering system.

mansoni the specific activity for cathepsin L was about double that of cathepsin B (7). The cathepsin B specific activities are similar in the two schistosomes, whereas the specific activity of the cathepsin L protease is over five-fold higher in extracts of *S. japonicum* than in *S. mansoni* extracts. This greater synthesis of cathepsin L activity in *S. japonicum* is interesting since females of this species produce 4-10 times more eggs than *S. mansoni* females (12) and would consequently require more nutrients. Perhaps the increased cathepsin L is needed to degrade a higher intake of hemoglobin. Alternatively, cathepsin L may be involved in egg synthesis as reported in the related parasite *Fasciola hepatica* (13). Zymographic analysis revealed that one major gelatinolytic activity was present in the *S. japonicum* worm extracts, and that the same kind of activity was present both in ES from adult worms and in extracts of *S. japonicum* eggs. Previously we have also observed cathepsin L activity in medium in which adult *S. japonicum* were maintained (14), that is in the ES products, which suggests that these parasites actively secrete cathepsin L, a requisite for hemoglobin-degrading proteases functioning in the lumen of the schistosome gut (5).

Chronic schistosomiasis is an immunopathological disease provoked by inflammatory responses to antigens liberated from eggs trapped in the liver of the host, resulting in the formation of granulomata around the eggs (12). Although It is not clear which antigens that induce this T cell dependant response, proteases have been implicated (15). A key function of these enzymes, which diffuse from the eggs, would be to facilitate the passage of the eggs through the intestinal wall into the gut lumen. Pino-Heiss *et al.* (16) showed that eggs of *S. mansoni* release enzymes that could degrade connective tissue macromolecules. However, while this activity was active against the glycoprotein portion of extracellular matrix it was not active against elastin or collagen.

Both Asch and Dresden (17) and Pino-Heiss *et al.* (16) found that the proteolytic activity released by eggs was enhanced by reducing agents. Whereas Pino-Heiss *et al.* (16) demonstrated that this activity on extracellular matrix proteins was active at neutral pH, the activity described by Asch and Dresden (17) was most active on azocoll and cartilage proteoglycan at pH 4.8 - 5.2. The activities observed by these workers can most likely be ascribed to both cathepsin L and cathepsin B since we have now demonstrated the presence of these enzymes in eggs. However, much higher (8-fold) cathepsin L activity than cathepsin B was present in eggs, as we founds in extracts of the adult parasites. This predominance of cathepsin L activity would account for the pH optimum for egg protease activity reported by Asch and Dresden (17). Therefore, we consider that we have identified two protease activities, cathepsin L and cathepsin B, that may be responsible for inducing the immunopathological responses against eggs. We showed previously that these activities are stable at 37°C and thereby would be capable of causing liver damage if secreted by entrapped eggs (7). It will be of interest to determine whether modulation of granuloma (10) (decrease in lesion size as infection proceeds) is related to immune responses to cathepsin proteinases, and whether vaccination with recombinant enzymes can inhibit their activity.

Cathepsin Ls are the predominant cysteine proteases synthesized and secreted by adult *S. japonicum*, as in *S. mansoni* (1). While cathepsin L activity is likely to be involved in hemoglobin digestion and membrane turnover, it is also present in schistosome eggs. Cathepsin L may contribute to the pathology associated with schistosomiasis.

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