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DIFFERENCES IN CYSTEINE PROTEASE ACTIVITY IN *SCHISTOSOMA MANSONI*-RESISTANT AND -SUSCEPTIBLE *BIOMPHALARIA GLABRATA* AND CHARACTERIZATION OF THE HEPATOPANCREAS CATHEPSIN B FULL-LENGTH cDNA

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Abstract

Biomphalaria glabrata snails are known to display a wide range of susceptibility phenotypes to *Schistosoma mansoni* infection depending on the genetics of both the snail and the invading parasite. Evidence exists for a role of hydrolytic enzymes in the defense of molluscs against invading parasites. To elucidate the role of these enzymes in the outcome of infection in the snail, proteolysis was examined in parasite-resistant and -susceptible snails. Zymographs of extracts from the whole snail or hepatopancreas indicated higher proteolytic activity in resistant, compared with susceptible, snails. Lytic activity coincided with a high-molecular-weight smear (220 to 66 kDa) that was abrogated by the cysteine protease inhibitor *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane. Quantitative fluorimetric assays showed 3.5-fold higher activity in resistant than in susceptible snails. From a hepatopancreas cDNA library, several cysteine protease encoding expressed sequence tags including the full-length cDNA for cathepsin B were identified. Sequence analysis revealed that this cathepsin B belonged to the C1A family of peptidases characterized by the presence of the catalytic cysteine–histidine dyad, the “occluding loop,” signal sequence, and cleavage sites for the prepro and propeptides. Quantitative real-time reverse transcriptase-polymerase chain reaction showed higher up-regulation of cathepsin B transcript in resistant than in the susceptible snail after parasite exposure.

Biomphalaria glabrata, the major intermediate snail host of the parasitic trematode *Schistosoma mansoni* in the Western Hemisphere, displays variations in susceptibility to parasite infection (Lewis et al., 2001). Using different combinations of snail and parasite strains for infections, these variations were shown to be genetically controlled, with the genetics of both the snail host and parasite affecting the outcome of infection (Newton, 1955; Richards and Shade, 1987; Richards et al., 1992). From the observed complex compatibility between snail and parasite interactions, snails were categorized as either susceptible or nonsusceptible to parasite infection (Richards and Shade, 1987). In cases where an active defense response against the invading parasite was evident (manifested by encapsulation of the miracidia by hemocytes), snails were categorized as being resistant to infection (Paraense and Correa, 1963; Richards, 1970).

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Despite the complex nature of this snail–parasite relationship, the existence of pedigree stocks displaying different susceptibility phenotypes has enabled work toward elucidating mechanisms that determine the outcome of trematode infections in the *B. glabrata* snail host to progress (Lewis et al., 2001). In this context, studies with resistant and susceptible stocks have shown that in adult snails, resistance is controlled by a single gene trait that follows a Mendelian pattern of inheritance, with resistance being dominant (Richards, 1970). Juvenile snail resistance, in contrast, has been shown to be a polygenic trait involving the interaction of at least 4–5 genes (Richards and Merritt, 1972).

From molecular studies undertaken with snails that are either parasite resistant or susceptible, several factors that may affect parasite development have now been described (Adema et al., 1997; Knight et al., 1998; Davids et al., 1999; Miller et al., 2001; Raghavan et al., 2003; Lockyer et al., 2004, 2007; Vergote et al., 2005; Knight and Raghavan, 2006). Recently, using suppressive subtractive hybridization (SSH) strategies, differential regulation of transcripts showing significant hits to proteolytic enzymes (serine protease and cathepsin L) have been described in resistant *B. glabrata* snail hemocytes after trematode infection (Bouchut et al., 2007; Lockyer et al., 2007). Nonetheless, despite these advances, few studies have focused on how the snail's internal environment and biochemistry affect parasite development.

Earlier studies showed that hydrolytic lysosomal enzymes may play a role in the phagocytic innate defense response against pathogens (Cheng et al., 1977, 1978; Cheng, 1978; Kassim and Richards, 1978; Cheng and Dougherty, 1989). By analyzing the segregation of susceptibility phenotypes with different enzyme markers (aconitase, acid phosphatase, esterase, leucylglycylglycine peptidase, and 6-phosphogluconate dehydrogenase), Mulvey and Woodruff (1985) were unable to find linkage with these markers and the resistance phenotype. A recent study that showed the presence of serine protease activity in *B. glabrata* snails (hemocytes) also failed to find an association with this enzyme and variations in the snail's susceptibility phenotype (Bahgat et al., 2002). In the literature, certain snails have been described as being “unsuitable” for schistosome infection. Because these snails do not exhibit the obvious nonself cell-mediated encapsulation reaction against the parasite typically seen in resistant snails, the “unsuitability” phenotype is thought to reflect an unfavorable biochemical environment that curtails parasite development in these snails (Sullivan and Richards, 1981).

Most biochemical and molecular studies concerning proteolytic enzymes and their involvement in the schistosome life cycle in general, have been investigated within the context of the parasite's relationship with the vertebrate (mouse and human) rather than the snail host (Sajid and McKerrow, 2002; Stack et al., 2005; Donnelly et al., 2006). In the present study, we rationalized that because proteases have been shown to play several key roles in the biology and pathogenicity of schistosomes, these enzymes may also be relevant in the biology of the snail stage of the parasite's development. Specifically, because proteases, in particular cysteine proteases, have been shown to exist in the invading miracidia and primary sporocyst stages of the parasite (Yoshino et al., 1993; Fryer et al., 1996), we thought it plausible that the presence of similar enzymes, or their natural inhibitors (in the snail), or both, could interfere with development of the parasite when encountered by the invading miracidia.

We analyzed by gelatin gel zymographs the basal levels of protease activity in soluble extracts prepared from either the whole snail or various tissues of parasite resistant (BS-90) and susceptible (M-line, NMRI) snails, i.e., the hepatopancreas, ovotestis, albumen gland, and the cell-free plasma (hemolymph). From this analysis, we report here, for the first time, the occurrence of higher levels of enzyme activity corresponding to cysteine proteases in *B. glabrata* snails that are resistant to *S. mansoni* compared with those that are susceptible, suggesting that cysteine proteases in the snail host may be a contributing factor in the dynamics of the complex interaction between *B. glabrata* and *S. mansoni*. To facilitate the initiation of

molecular studies toward an understanding of the possible involvement of these enzymes in the snail–parasite relationship, we constructed a cDNA library from the hepatopancreas, in which the majority of proteolytic activity was found to reside, and we also report from this library the isolation and characterization of a full-length cDNA encoding the snail cysteine protease cathepsin B.

MATERIALS AND METHODS

Snails

Adult snails, 10–12 mm in diameter, that were either resistant (BS-90) or susceptible (M-line, NMRI) to infection by the NMRI strain of *S. mansoni*, were used in this study. The resistant snail stock used has been shown to be resistant both as adults and juveniles. The BS-90 stock, also known as the Salvador strain, was isolated in Brazil (Paraense and Correa, 1963), and it has been maintained in the laboratory since then. The susceptible M-line and NMRI stocks have been maintained in the laboratory since they were selected for this phenotype (Newton, 1955). Snails were kept overnight in sterile water containing 100 µg/ml ampicillin. Their shells were removed by crushing between 2 microscope slides. Tissues (headfoot, hepatopancreas, albumen gland, and ovotestis) were dissected from individual snails and processed immediately for soluble protein extraction. Individual snails were exposed to the parasite either as juveniles (4 mm) or adults (10 mm, 6 miracidia/juvenile and 25/adult).

Preparation of extracts and analysis

Soluble protein extracts were prepared from either the whole snail (without shell) or freshly dissected tissues after homogenization in phosphate-buffered saline, pH 7.0, containing 0.5% Triton X-100 (v/v) on ice by using a mechanized Kontes pestle (VWR, West Chester, Pennsylvania). Supernatants from the homogenates were recovered after centrifugation at 10,000 g for 15 min at 4 C. Extracts were either analyzed immediately or aliquoted and stored at –80 C until required. Long-term storage (up to 6 mo) at –80 C had no effect on enzyme activity. Protein concentrations of extracts were measured using the Bradford (1976) assay according to manufacturer's instructions (Bio-Rad, Hercules, California). Similar amounts of soluble protein extract (25 µg) from resistant and susceptible snail stocks (3 snails each) were analyzed under either reducing or nonreducing conditions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie Blue, and destained before subjecting the protein extracts to the gelatin zymograph assay described below.

Proteolytic activity in the soluble extracts prepared from resistant and susceptible snails was determined as described previously (Lockwood et al., 1987). Briefly, samples (100 µg/lane) were diluted (1:1) with 2.5× sample buffer (250 mM Tris-Cl, pH 6.8, 5% SDS, 0.25% bromphenol blue, and 25% glycerol) and loaded onto a 10% SDS-containing polyacrylamide gel copolymerized with 1% gelatin (300 Bloom) as a substrate. Polyacrylamide gel electrophoresis was performed overnight at a constant current of 30 mA in electrode buffer (25 mM Tris-HCl, pH 8.8, 192 mM glycine, and 0.1% SDS [w/v]). After electrophoresis, the gel was immersed in wash buffer (2.5% [v/v] Triton X-100), washed twice for 60 min at room temperature, and immersed for 3 hr in pre-heated (37 C) incubation buffer (0.1 M sodium acetate, pH 5.5, 1 mM dithiothreitol [DTT]). Proteolytic activity was also examined by immersing gels in incubation buffer at pH 7.0. Evidence of proteolytic activity, indicated by a clear region in the gel, was visualized by staining with Coomassie Blue and destaining in fixative containing 10% acetic acid (v/v) and 10% methanol (v/v).

Inhibition of proteolytic activity

Inhibition of proteolytic activity was performed with various inhibitors to block the development of lysis. Briefly, soluble protein extract (2 mg/ml) was subjected to SDS-gelatin

impregnated PAGE as described above but using, in this case, a preparative gel. After electrophoresis, gel strips (1 cm in width) were cut, and each strip was washed (3 times in wash buffer as described above) before incubation in the presence of the following protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM phenanthroline, 1 mM iodoacetic acid, 40 µg/ml leupeptin, 40 µg/ml pepstatin, 1 mM *N*- α -*p*-tosyl-L-lysine chloromethyl ketone, 1 mM ethylenediaminetetraacetic acid (EDTA), or 10 µM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64). All inhibitors were dissolved in water except for PMSF and pepstatin. PMSF was dissolved in ethanol, and pepstatin was dissolved in 1 mM dimethyl sulfoxide (DMSO). All reagents were purchased from Sigma-Aldrich (St. Louis, Missouri).

Quantitative assessment of proteolytic activity in snail protein extracts

Protease activity was assessed quantitatively on the same soluble extracts analyzed qualitatively by gelatin zymographs. The fluorogenic substrate Z-Phe-Arg-AMC (AMC is 7-amino-4-methyl-coumarin; Axxora, San Diego, California) dissolved in DMSO and stored at -20 °C as a 10 mM stock solution was used in an enzymatic assay. Before finalizing the assay conditions, varying concentrations of protein extracts, substrate, and the inhibitor E-64 were used in different assays to determine the optimal concentration of these reagents. Briefly, for the fluorimetric assay, soluble protein extracts (15 ng each) from either the resistant or susceptible snails were incubated at 37 °C for 60 min with the substrate (0.05 mM in 0.1% Brij 35 solution; J. T. Baker, Phillips-burg, New Jersey) in the incubation buffer containing 100 mM Na₂HPO₄, 1 mM disodium EDTA, and 2 mM DTT at pH 6.0 (Tchoupe et al., 1991). Reactions were performed in 96-well microtiter plates as described previously (Barrett, 1980). Individual wells containing 130 µl of assay stock buffer and 50 µl of extract of known concentration (diluted with 0.1% Brij 35 solution) were prewarmed at 40 °C. The substrate (20 µl from a 50 µM diluted stock solution) was added to each well with mixing. The total assay volume was 200 µl. Reactions were monitored continuously at 1-min time intervals, and the fluorescence intensity of AMC released into the supernatant was measured in real-time in a Biotek FL 600 fluorescence spectrophotometer set at excitation and emission wavelengths of 360 ± 5 and 460 ± 5 nm, respectively. The amount of AMC released was calculated from a standard curve. One unit of enzymatic activity was defined as the amount of enzyme required to release 1 µmol of AMC per min. Activity was measured in duplicate on protein extracts from individual snails for an incubation period of 60 min with a kinetic interval of 1 min. A similar assay was performed simultaneously in the presence of 1 µM of the inhibitor E-64. The background fluorescence was also calculated continuously for each 1-min interval in an assay reaction containing all components except the protein extract. The background from each time point was subtracted from the corresponding test samples before calculating the final fluorescence of each sample. Before deriving the specific activity, the initial velocity (*i*) of the enzyme reaction (fluorescence units released/minute) was computed using the formula $i = \Delta y / \Delta x$, where *i* is the initial velocity and Δy is rate of release of fluorescence units calculated as the difference in release of fluorescence units ($y_2 - y_1$) using the linear part of the slope at time periods x_2 and x_1 . Δx denotes the difference between the time periods ($x_2 - x_1$). Specific activity of the extract for each assay was then calculated as the amount of fluorescence units released per minute per microgram of protein.

Isolation of nucleic acids and construction of a hepatopancreas cDNA library

Genomic DNA and RNA from various tissues of either resistant or susceptible snails were prepared as described previously (Knight et al., 1998). The hepatopancreas cDNA library was constructed with poly(A)⁺ RNA from tissue samples dissected from normal adult BS-90 snails using the lambda ZAP vector (Stratagene, La Jolla, California) as described previously (Raghavan et al., 2003). Mass excision of nonamplified libraries (from 6 separate ligation reactions) with titers ranging between 0.8×10^6 and 2.7×10^6 plaque-forming units/ml were

performed as described previously (Raghavan et al., 2003) and individual phagemids, plated on X-Gal/IPTG plates, were picked and stored as glycerol stocks in 96-well format ordered arrays. Recombinant phagemids (insert sizes ranging from 0.4 to 1.5 kb) from 10 microtiter plates were sequenced, and expressed sequence tags (ESTs) were obtained for 861 clones as described previously (Knight et al., 1998). All 861 hepatopancreas EST sequences have been submitted to GenBank with accession numbers ES491296–EF492156. In addition, all EST sequences mentioned in this article are designated with BRI numbers and clone identification numbers.

Sequence analysis of the hepatopancreas cathepsin B full-length cDNA

The hepatopancreas EST single pass sequence with the accession ES491472 was sequenced completely from either end using M13 forward and reverse primers. The snail hepatopancreas cathepsin B gene sequence (CTSB) has been submitted to GenBank (EU035711). DNA sequences were analyzed using EMBOSS (Rice et al., 2000), and comparisons were made with sequences in the protein and nucleic acid public databases using the BLAST algorithm (Altschul et al., 1990). The deduced amino acid sequence of the snail cathepsin B protease was further analyzed using the National Center for Biotechnology Information conserved domain database (Marchler-Bauer et al., 2005) to classify and characterize the type of cathepsin and the family and clan to which it belongs. These data were further analyzed against the Interpro EMBL-EBI (Zdobnov and Apweiler, 2001), a database of protein families, domains, and functional sites in which identifiable features found in known proteins can be applied to unknown protein sequences and also against the Sanger MEROPS release 7.80 (Rawlings et al., 2006) databases. Signal sequence prediction was performed using the Signal P program version 3.0 (Bendtsen et al., 2004).

Assessment of differential regulation of snail cathepsin B transcript pre- and postparasite exposure by real-time reverse transcription-polymerase chain reaction (RT-PCR)

Real-time PCR analysis of the snail hepatopancreas cathepsin B gene (GenBank EU035711) was performed with gene-specific primers (forward [F]: 5'-AGCAACACCATTCACATC-3'; reverse [R]: 5'-ATAG CCTCCGTTACATCC-3'). RT-PCR of the housekeeping gene myoglobin (myoglobin primers F: GATGTTTCGCCAATGTTCCC; R: AGCG ATCAAGTTTCCCCAG) was used to assess the comparability of samples and to confirm that equivalent template cDNA was used in each amplification reaction. The RT-PCR reactions were performed using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Foster City, California). The reaction was performed in a 1-step format with 80 ng of DNase-treated total RNA. DNase treatment was done with RNase-free DNase (RQI) according to the manufacturer's suggested protocol (Promega, Madison, Wisconsin). First-stand cDNA reactions and PCR amplifications were performed in triplicate in a single tube with the Full Velocity SYBR Green QRT-PCR Master mix according to the manufacturer's instructions (Stratagene). Twenty-five microliters of the final reaction volume consisted of 200 nM primers (50 nM myoglobin primers used for adjusting the difference in concentration of the reverse-transcribed RNA starting material), 300 nM reference dye, 1× of Full Velocity SYBR Green QRT-PCR Master mix containing RT-PCR buffer, SYBR Green I dye, MgCl₂, and nucleotides (GAUC). The amplification protocol included an initial incubation at 48 C, 45 min for cDNA synthesis and a 95 C initial denaturation for 10 min followed by 40 cycles with 95 C denaturation for 10 sec, and annealing/amplification at 58 C for 1 min. Detection of the fluorescently labeled product was performed at the end of the amplification period. The -fold increase of gene expression was calculated by comparative Ct method with the formula indicated below (Livak and Schmittgen, 2001); standard error and 95% confidence intervals (CI) were calculated for each sample:

$$\begin{aligned}
 \text{-fold increase} &= 2^{-\Delta\Delta C_t} \\
 &= 2^{-\left[C_{t, \text{RT}, \text{exposed}} - C_{t, \text{myoglobin}, \text{exposed}} \right] - \left[C_{t, \text{RT}, \text{unexposed}} - C_{t, \text{myoglobin}, \text{unexposed}} \right]}
 \end{aligned}$$

To determine significant difference ($P < 0.05$) in gene expression for the different time points after exposure compared with nonexposed snails within each strain, the CI was subtracted from the mean of the -fold increase value. If this value was greater than 1 (1 = value for nonexposed snails), the -fold increase was considered to be significant.

RESULTS

Gelatin zymograph reveals higher proteolytic activity in resistant compared with susceptible snails and locates activity to posterior hepatopancreas region of the snail

Figure 1A shows destained gels representing soluble protein from individual snails (whole snail) that are either resistant (BS-90) or susceptible (M-line) to infection after subjecting to gelatin zymograph analysis. Results showed differences in proteolytic activity (clear region resulting from the degradation of impregnated gelatin substrate) between these stocks, with the individual resistant (BS-90) snail extracts showing more activity compared with the same amounts of extracts from individual susceptible (M-line) snails. Lytic activity in all snails corresponded to a high-molecular-weight smear ranging from approximately 66 to 22 kDa or higher. The size range of the smear remained the same regardless of whether the samples were examined under reducing (sample buffer containing β -mercaptoethanol; data not shown) or nonreducing conditions. To determine the location of the observed protease activity in the snail, extracts prepared from either the anterior (headfoot) or posterior (hepatopancreas and ovotestis) regions of 2 different stocks representing either resistant or susceptible snails were analyzed. Minor activity was detected in the anterior region of both resistant (BS-90) and susceptible (M-line and NMRI) stocks (Fig. 1B). The majority of proteolytic activity, however, was found in the posterior region, with the resistant stock showing higher levels of activity compared with the susceptible snails. Again, the clear region of lytic activity corresponded to a high-molecular-weight smear ranging from approximately 66 to 22 kDa or higher. Analysis of protein extracts from the hepatopancreas of resistant and susceptible snails showed that higher levels of lytic activity were again present in the resistant compared to the susceptible snail, with activity coinciding with the high-molecular-weight heterogeneous smear mentioned above. The clear region indicative of lytic activity of the gelatin substrate remained a complex smear irrespective of using diluted (titrated) extract to determine whether defined bands would then be visible (data not shown). Lytic activity represented by the high-molecular-weight smear only disappeared by incubating gels in buffer at pH 7.0 (data not shown).

Resistant (BS-90) snails possess higher activity levels of cysteine protease activity than susceptible (M-line) snails

Of all the protease inhibitors used to determine the class of proteolytic enzyme in extracts that was responsible for the proteolysis represented by the complex high-molecular-weight smear in the gelatin zymographs, only the generic cysteine protease inhibitor E-64 blocked this lytic activity (data not shown). To confirm that the lytic activity was due to cysteine protease(s) in the extract from resistant and susceptible snails, enzyme activity was examined quantitatively for the ability to cleave the cysteine protease synthetic substrate Z-Phe-Arg-AMC. Initial experiments were conducted to determine the optimal concentrations of protein extracts, substrate, and the cysteine protease inhibitor E-64 to be used in the fluorimetric assay. All assays were performed in duplicate and repeated. The final assay was then performed in duplicate using 15 ng of protein extract from both resistant (BS-90) and susceptible (M-line) snails. Figure 2A shows the results of the fluorimetric assay performed using 15 ng of resistant

(BS-90) snail extract in the presence (gray) and absence (black) of E-64. Figure 2B shows a similar assay using susceptible (M-line) extract in the presence (gray) and absence (black) of E-64. Based on these assays, the specific activities of the resistant snail (BS-90) extract was calculated as ~21,466 fluorescence units released/min/μg protein compared with the ~6,066 fluorescence units released/min/μg protein for the susceptible (M-line) snail extract. Both activities were completely abrogated (100%) in the presence of E-64 (Fig. 2A, B, shown in gray). This indicated that all protease activity observed in the presence of Z-phe-arg-AMC using the crude snails extracts could be specifically attributed to the presence of cysteine proteases in the extracts, with activity in resistant snails being 3.5× higher than that from susceptible snails.

ESTs generated from a hepatopancreas cDNA library reveal the presence of several transcripts encoding proteolytic enzymes, including cysteine proteases

From 861 recombinant clones obtained from the mass excision procedure performed using the nonamplified hepatopancreas library, ESTs were generated by single pass sequencing using the M13 universal reverse primer. Sequence identity/similarity (E-value < 10^{-4}) of the open reading frames of these ESTs to other homologs analyzed by the BLAST algorithm (Altschul et al., 1990) is shown in Table I. Clones showing significant sequence identity to proteolytic enzymes, in particular to cysteine proteases cathepsin B, cathepsin L, and legu-main, were identified from the library. In addition, several clones from this library also showed significant matches to other hydrolytic enzymes, including elastase, disintegrin, and metalloprotease, lysozyme, serine protease, hydrolase, cellulase, α -L-fucosidase, and β -1,3-glucanase.

Sequence analysis of the *B. glabrata* hepatopancreas cathepsin B sequence

The hepatopancreas EST single pass sequence (GenBank ES491472) showing similarity to cathepsin B that contained the initiation codon ATG was sequenced completely from either end using M13 forward and reverse primers. Sequence analysis of the DNA indicated that this clone contained the full-length cathepsin B sequence of 1183 nucleotides encoding 333 amino acids (Fig. 3). The sequence has been submitted to GenBank (EU035711) and shows the characteristic hallmark domains of a cysteine protease. Blast analysis of the nucleic acid and deduced amino acid sequence of this clone against the non-redundant databases showed >60% sequence similarity (E-value < e^{-70}) to other cathepsin B sequences. Preliminary analysis of the deduced amino acid sequence of the snail ortholog against the conserved domain database of NCBI indicated the sequence to represent a C1A peptidase cathepsin B. This was further confirmed by analyzing the deduced amino acid sequence against the Interpro EMBL-EBI and the Sanger MEROPS databases. The deduced amino acid sequence encodes the entire preproprotein of cathepsin B that includes the catalytic Cys 115 (C, bold and indicated by a star) and His 281 (H, bold and indicated by a star) that form the catalytic dyad typical of the C1 family of peptidases. The first 19 amino acids encompass the highly hydrophobic signal sequence that is present in secretory proteins. The arrow at amino acid position 19 denotes the putative cleavage site of signal peptide (amino acids 1–19 indicated by bold underline) of the cathepsin B prepropeptide and the arrow between amino acids 86 and 87 indicates the potential cleavage site of the cathepsin B propeptide (amino acids 20–86). Two other residues, although not a part of the catalytic dyad, play an important role in the catalytic mechanism: Gln 109 (Q, in bold and indicated by dark circle) preceding the catalytic Cys 115 forms an oxyanion hole, and Asn 301 (N, bold and indicated by dark circle) orients the imidazolium ring of the catalytic His 281. The “occluding loop” (italics and underlined) unique to cathepsins helps it to act also as an exopeptidase in removing C-terminal dipeptides. The polyadenylation signal AATAAA for the cathepsin B gene is shown in bold, underlined lowercase letters.

Real-time quantitative RT-PCR reveals the differential regulation of the cathepsin B encoding transcript in *B. glabrata* upon parasite exposure

To determine the differential regulation of the cathepsin B encoding gene in resistant or susceptible juvenile (4-mm) snails, the snails were exposed (0 to 48 hr) to 5–6 *S. mansoni* miracidia. RNA was prepared from 3 individual size-matched snail for each snail stock. Real-time RT-PCR analysis of the RNA samples showed (Fig. 4) that the early time point (5 hr after exposure) produced a 12.3-fold increase in the cathepsin B transcript in the resistant snail (BS-90), whereas in the susceptible snails (NMRI and M-line) only a 5.2-fold change was observed. Similarly, at the late time period (48 hr) after exposure, a dramatic 24.8-fold increase was observed in the cathepsin B transcript in the resistant snail, but either no increase or a relatively lower level of induction (7.1) was observed in the susceptible snails (NMRI and M-line).

DISCUSSION

The involvement of proteases in the intra-molluscan stage of parasite development has been well documented (Yoshino et al., 1993). Although investigations showed that several aspects of parasite development, e.g., snail penetration, nutrient acquisition, and suppression of the snail defense system, rely on the release of proteolytic enzymes (including cysteine proteases) in excretory-secretory products, reciprocal studies to determine whether the presence of similar enzymes in the snail can interfere with the progression of parasite development have not been as intensely investigated. This study shows that higher levels of cysteine protease activity occurs in parasite resistant *B. glabrata* than in susceptible snails. It is possible, therefore, that these enzymes may be important in determining the outcome of the *S. mansoni*–*B. glabrata* interaction. Although previous studies (Bahgat et al., 2002; Mitta et al., 2005) described the presence of several proteolytic enzymes in the snail (aminopeptidase, hydrolase, lysozymes, and genes encoding serine proteases, cathepsin L, and metalloproteases), none described differences (qualitative and quantitative) in activity of these enzymes between parasite resistant and susceptible snails as shown here for cysteine proteinases.

Proteolytic enzymes have been detected in both the humoral and cellular components of the snail's innate defense system, the hemolymph and hemocytes, respectively, with levels changing relative to either bacteria or schistosome infections (Cheng et al., 1977, 1978; Kassim and Richards, 1978). Granulocytes, a type of hemocyte involved in the cellular encapsulation reaction typically seen in the nonspecific reaction against incompatible parasites, were shown to express high levels of acid phosphatase activity in a resistant snail upon exposure to *S. mansoni* miracidia, and they were thus hypothesized by Cheng and Garra-brant (1977) to contribute to parasite destruction mediated by these cells. Other snail tissues, including the headfoot, hepatopancreas, and visceral mass (Cheng, 1978; Cheng and Rodrick, 1980), were also shown to express high levels of proteolytic enzymes. In the present study, although cysteine protease activity was detected in the hemolymph and ovotestis (data not shown), most of the enzyme activity was present in the hepatopancreas. Insignificant proteolytic activity was detected in the headfoot, and the activity in hemocytes remains to be tested. Experiments to test for enzyme activity in these cells by methods described here were hampered by the difficulty in isolating large numbers of hemocytes. However, in a recent study using the SSH approach, Bouchut et al. (2007) were able to show the up-regulation of cathepsin L-like transcripts in these cells from an unrelated resistant *B. glabrata* snail after exposure to the trematode *Echinostoma caproni*. Because it is thought that schistosome sporocysts are not easily destroyed by toxic material present in snail plasma (Bayne and Yoshino, 1989), we can only speculate that our results showing higher (qualitatively and quantitatively) activity of cysteine proteases in resistant compared with susceptible snails may indicate that these enzymes could be indirectly rather than directly involved in mechanisms relating to the

processing of molecules that are directly toxic for sporocysts. The presence of higher enzyme activity in the hepatopancreas (in both resistant and susceptible snails) relative to other tissues, also suggests that these enzymes may play a significant role in the snail's digestive process. Natural substrates of these cysteine proteases in the snail remain unknown.

Because of results showing elevated activity of these enzymes in the hepatopancreas, a cDNA library was constructed from this tissue and ESTs generated. As expected, several clones corresponding to *B. glabrata* hydrolytic enzymes (cellulase, elastase, disintegrin and metalloprotease, lysozyme, α -L-fucosidase, and serine protease), including cysteine proteases (cathepsin B and L, and legumain), were isolated. One of the ESTs (accession ES491472) encoding the full-length coding sequence of cathepsin B was sequenced in its entirety (accession EU035711). The snail cysteine protease cathepsin B encoding 333 amino acids has all the hallmark domains that are needed for a functional peptidase. Cysteine proteases have characteristic molecular topologies both in their 2- and 3-dimensional structures where the nucleophile is the sulfhydryl group of a cysteine residue. In addition, they are also divided into clans that are evolutionarily related, and further into families on the basis of the architecture of their catalytic dyad or triad (Barrett and Rawlings, 2001). Based on the above-mentioned criteria, the snail cathepsin B belongs to the MEROPS (accession MER00647; Rawlings and Barrett, 1993) cysteine peptidase family C1 and subfamily C1A similar to papain. The catalytic residues of family C1 have been identified as Cys and His, forming the catalytic dyad (Cys 115 and His 281). Two other active site residues are found, a Gln residue preceding the catalytic Cys and an Asn residue following the catalytic His (Gln 109 and Asn 301). The C1A cathepsin B family may contain both endo- and exopeptidase activities, which allows it to make internal cleavages and also remove the C-terminal dipeptide units from the substrate. E-64 is an irreversible inhibitor of peptidases in family C1 (Barrett et al., 1982). In cathepsin B, the presence of an approximately 20-residue "occluding loop" that carries the histidine residues is important for peptidyl-dipeptidase (exopeptidase) activity, and it is inserted between the catalytic Cys and His residues (Illy et al., 1997). Although we do not know the localization of the snail cathepsin B, the presence of the hydrophobic signal peptide at the amino terminus (residues 1–19) of the preprocathepsin B shows it may be a secreted molecule. In addition, results of proteolysis in the gel zymographs coinciding with a complex high-molecular-weight smear (220 to 66 kDa) is considerably higher than the expected size of cysteine proteases (approximately 30–36 kDa). The snail recombinant cathepsin B that has been deduced from the translated sequence has a potential *N*-glycosylation site ($n = 1$), protein kinase C ($n = 5$), casein kinase II ($n = 5$) phosphorylation sites, and *N*-myristoylation ($n = 13$) sites. It is, therefore, possible that posttranslational modifications accounts for the discrepancies in the sizes of the native enzyme and the deduced amino acid sequence.

The biological role of cathepsins in mechanism(s) relating to the antiparasite function of the snail innate defense system, especially encapsulation, remains unknown. With the availability of several cloned transcripts encoding *B. glabrata* cysteine proteases and at least 1 full-length cathepsin B, characterization of various activities of this enzyme at both biochemical and molecular levels can be achieved with the expression of the recombinant protein. We hope to express the full-length recombinant enzyme in a prokaryotic expression system to raise polyclonal antisera that will be used to purify the native snail cathepsin B by affinity column chromatography. Future physical and biochemical characterization of the purified enzyme should help to resolve the discrepancy between the sizes of the deduced translated sequence and the native enzyme. Antibodies against the recombinant protein will also be useful in the identification of homologs of cathepsin B from hemocytes and other tissues that are not easy to obtain in large quantities, but that are considered important regarding mechanisms involved in snail/parasite interactions, e.g., cerebral ganglia.

Aside from the identification of several ESTs encoding proteolytic enzymes from a hepatopancreas cDNA library (Table I), transcripts encoding a natural inhibitor (Kazal-like serine protease inhibitor) were also isolated. Previously, we identified the gene encoding cystatin, a known inhibitor of cysteine pro-tease from a resistant (BS-90) snail cDNA library (Knight et al., 1998). Several recent studies have now shown the quantitative increase of the cystatin transcript after trematode infection of *B. glabrata* snails (Guillou et al., 2007; Lockyer et al., 2007). The occurrence of the proteinase inhibitor α -macroglobulin has also been shown in the snail hemolymph (Bender and Bayne, 1996; Fryer et al., 1996).

In other studies where the effects of parasite infection on hydrolytic enzyme activity have been investigated, levels of glycosidases were shown to correlate with the progress of infection in schistosome infection of *B. glabrata* snails (Zelck, 1999). Likewise, in the American oyster, *Crassostrea virginica*, a significant increase in protease activity was observed after infection with the parasite *Perkinsus marinus* (Munoz et al., 2003). Together, it is clear that future investigations of the possible cytotoxicity of proteases and their natural inhibitors toward warding off trematode infection in the snail host are warranted. Results from our studies using real-time quantitative RTPCR showing a higher -fold increase of the corresponding cathepsin B transcript in resistant compared with susceptible snails upon parasite exposure is further evidence that proteolytic enzymes play a significant role in the host–parasite relationship.

In summary, qualitative and quantitative differences in the levels of protease activity have been shown to occur between snail stocks that are either resistant or susceptible to *S. mansoni* infection, with resistant snails consistently expressing higher protease activity than susceptible snails. The majority of enzyme activity detected corresponded to the presence of cysteine proteases in the hepatopancreas. With the availability of cloned cathepsin B and other cysteine proteases from *B. glabrata*, we anticipate that the molecular and biochemical pathways involving cysteine proteases in killing of schistosomes in the snail host will soon be unravelled.

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Figure 1.

(A) Gelatin zymograph analysis of soluble protein extracts from 3 individual *B. glabrata* snails that are either resistant (BS-90) or susceptible (M-line) to *Schistosoma mansoni* infection. Note clear region of proteolysis (from 66 to 22 kDa or higher) of the impregnated gelatin substrate visualized by Coomassie Blue staining and destaining of the extracts resolved by acrylamide gel electrophoresis. (B) Gelatin zymograph analysis of soluble extracts from parasite-resistant (BS-90) and -susceptible (M-line and NMRI) snails isolated either from the anterior or posterior regions of the snails. Note clear regions of proteolysis after Coomassie Blue staining and destaining extending from approximately 66 to 22 kDa or higher in the posterior hepatopancreas region, and it is more intense in the resistant than in the susceptible snails.

Figure 2.

Quantitative fluorimetric analyses of cysteine protease using the substrate Z-Phe-Arg-AMC. The fluorescence intensity of AMC released into the supernatant was measured in real-time in a Biotek FL 600 fluorescence spectrophotometer set at excitation and emission wavelengths of 360 ± 5 and 460 ± 5 nm, respectively. Activity was measured in duplicate on protein extracts from (A) individual resistant (BS-90, shown in black) and (B) susceptible (M-line, shown in black) snails or an incubation period of 60 min with a kinetic interval of 1 min.

Figure 3.

Nucleotide (1,183 bp) and deduced amino acid sequence (333 amino acids) of *B. glabrata* C1A cysteine peptidase cathepsin B. Arrow at amino acid position 19 denotes the putative cleavage site of signal peptide (amino acids 1–19 indicated by bold underline) of the cathepsin B prepropeptide, and the arrow between amino acids 86 and 87 indicates the cleavage site of the cathepsin B propeptide (amino acids 20–86). Gln 109 (Q, in bold and indicated by dark circle) preceding the catalytic Cys 115 (C, bold and indicated by a star) forms an oxyanion hole; Asn 301 (N, bold and indicated by dark circle) orients the imidazolium ring of the catalytic His 281 (H, bold and indicated by star). Cys 115 and His 281 form the catalytic dyad typical of the C1 family of peptidases. The “occluding loop” (italics and underlined, including 2 essential

histidines indicated in bold and dark circles) unique to cathepsins helps it to act also as an exopeptidase in removing C-terminal dipeptides. The poladenylation signal AATAAA for the cathepsin B gene is shown in bold underlined lowercase letters.

Figure 4.

Real-time quantitative RT-PCR analysis of the snail hepatopancreas cathepsin B transcript in resistant (BS-90) and susceptible (NMRI and M-line) pre- and postexposure to miracidia. RT-PCR was performed with the cathepsin B gene-specific primers using RNA from individual snails after various times postexposure. Gene-specific primers for the housekeeping gene myoglobin were used to assess sample uniformity and to confirm that the template cDNA was used in equivalent amounts for each amplification reaction. RT-PCR reactions were performed using an Applied Biosystems 7300 Real-Time PCR System, and detection of the fluorescently labeled product was performed at the end of the amplification period. The -fold increase of gene expression was calculated by comparative Ct method as described in Materials and Methods. A value of >1-fold increase was considered to be significant (1 = value for nonexposed snails).

Hydrolytic enzymes identified from the hepatopancreas EST library of the resistant (BS-90) *B. glabrata*. Each of the column headers represent the unique clone identifier, GenBank accession number (sequence similarity to a known gene in GenBank using Blastx analysis and the number of clones represented from each category in the subset of hepatopancreas EST library (861 clones).

Table 1

Clone Identifier	GenBank Accession No.	Sequence similarity	Best matched ortholog Genus and Species	No of clones
RBGM011TR	ES491393		<i>Haliotis discus</i>	10
RBGM029TR	ES491409		<i>Biomphalaria glabrata</i>	
RBGM034TR	ES491413		<i>Haliotis discus</i>	
RBGM246TR	ES491600		<i>Biomphalaria glabrata</i>	
RBGM346TR	ES491685		<i>Haliotis discus</i>	
RBGM347TR	ES491686		<i>Biomphalaria glabrata</i>	
RBGM726TR	ES492001		<i>Haliotis discus</i>	
RBGM736TR	ES492011		<i>Mytilus edulis</i>	
RBGM876TR	ES492134		<i>Haliotis discus</i>	
RBGMA77TR	ES491365		<i>Haliotis discus</i>	
RBGM016TR	ES491397	disintegrin metalloproteinase	<i>Gallus gallus</i>	1
RBGM170TR	ES491537		<i>Caenorhabditis elegans</i>	1
RBGM063TR	ES491441		<i>Gallus gallus</i>	2
RBGM825TR	ES492087		<i>Gallus gallus</i>	1
RBGM742TR	ES492017		<i>Bos taurus</i>	
RBGM070TR	ES491446		<i>Araeneus ventricosus</i>	3
RBGM102TR	ES491472		<i>Araeneus ventricosus</i>	
RBGM125TR	ES491495		<i>Araeneus ventricosus</i>	
RBGM073TR	ES491449	alpha-L-fucosidase	<i>Strongylocentrotus purpuratus</i>	1
RBGM108TR	ES491478	serine protease	<i>Biomphalaria glabrata</i>	3
RBGM648TR	ES491935	serine protease		
RBGM741TR	ES492016	serine protease		
RBGM173TR	ES491540	cathepsin L	<i>Tenebrio molitor</i>	3
RBGM715TR	ES491992	cathepsin L		
RBGM844TR	ES492105	cathepsin L		

Clone Identifier	GenBank Accession No.	Sequence similarity	Best matched ortholog Genus and Species	No of clones
RBGM330TR	ES491670	beta-1,3-glucanase	<i>Pseudocardium sachalinensis</i>	3
RBGM415TR	ES491744	beta-1,3-glucanase	<i>Chlamys albidus</i>	
RBGM642TR	ES491929	beta-1,3-glucanase	<i>Mizuhopecten yessoensis</i>	