Schistosome invasion of human skin and degradation of dermal elastin is mediated by a single serine protease

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Running Title: ELASTINOLYTIC PARASITE SERINE PROTEASE
SUMMARY

Aquatic larvae (cercariae) of the trematode parasite, *Schistosoma mansoni*, rapidly penetrate human skin by degrading host proteins including elastin. Two serine proteases, one chymotrypsin-like and the second trypsin-like, have been proposed to be involved. To evaluate the relative roles of these two proteases in larval invasion both were purified, identified by sequence, and then biochemically characterized. The trypsin-like activity was resolved into two distinct serine proteases 76% similar in predicted amino acid sequence. Southern blot analysis, genomic PCR, and immunolocalization demonstrated that the trypsin-like proteases are in fact not from the schistosome, but released with larvae from the snail host *Biomphalaria glabrata*. Invasion inhibition assays using selective inhibitors confirmed that the chymotrypsin-like protease is the enzyme involved in skin penetration. Its ability to degrade skin elastin was confirmed and the three sites of cleavage within elastin help define a new family of elastases.
INTRODUCTION

Infection of a human host by the trematode parasite *Schistosoma mansoni* begins with invasion of intact skin by an aquatic larva, the cercariae (1). Exiting the freshwater snail *Biomphalaria glabrata*, cercariae locate a human host by thermal (2) and chemical signals (3), and rapidly penetrate the skin, entering the vascular system in the dermis (4). *S. mansoni* cercariae are approximately 150 microns long and 70 microns wide and require lysis of skin tissues to migrate into blood vessels. Host macromolecules representing barriers to cercarial invasion are known to be cleaved by proteolytic activities present in cercarial secretions. These include elastin (5), chondromucoprotein (6), keratin (7), fibronectin, laminin, and collagen IV and VIII (8). Two distinct serine proteases have been reported in extracts of cercariae or in secretions from cercariae induced upon contact with skin lipids. One is a "chymotrypsin-like" protease with a preference for large hydrophobic side chains at P1 (9). The second is a "trypsin-like" protease, with preference for positively charged side chains at P1 (10). The class of proteases responsible for host protein degradation has been demonstrated by several independent studies to be serine proteases (6) but the relative contributions to invasion of the trypsin-like or chymotrypsin-like proteases is not known.

To analyze the relative contributions of each of these proteases to the degradation of host proteins, cercarial secretions were fractionated and the two proteases purified. The trypsin-like activity, that had not been previously purified or sequenced, was purified and a cDNA was cloned by RT-PCR based on amino-terminal amino acid sequence. Specific inhibitors were identified to evaluate the role of each protease in skin invasion. Proteases were localized by immunohistochemistry and specific sites of cleavage in elastin, the most protease-resistant target in host skin, were analyzed.
The abbreviations used are: AAPF-pNA, N-Succinyl-alanine-alanine-proline-phenylalanine \( p \)-nitroanilide; LGR-pNA, N-t-BOC-leucine-glycine-arginine \( p \)-nitroanilide; AAPF-CMK Succinyl-alanine-alanine-proline-phenylalanine chloro-methyl ketone; FPR-CMK, Carbobenzoxy-phenylalanine-proline-arginine chloro-methyl ketone; BgSP, *Biomphalaria glabrata* serine protease; SmCE *Schistosoma mansoni* cercarial elastase; RT-PCR, Reverse Transcription-Polymerase Chain Reaction; LB, Luria-Bertani Medium; IPTG, isopropylthio-B-D-galactoside; Ni-ATA, nickel(II)-nitrilotriacetic acid; DMSO, Dimethyl sulfoxide
EXPERIMENTAL PROCEDURES

Chemical Materials

AAPF-pNA and LGR-pNA were purchased from Sigma. FPR-CMK and AAPL-CMK were purchased from ESP (Enzyme Systems Products, Livermore, CA), p32-α-dCTP was purchased from NEN Research Products, IPTG was purchased from Amersham Pharmacia Biotech, Piscataway, NJ.

Biologic Materials

Schistosoma mansoni -- Approximately 5x10^5 cercariae (Puerto Rican strain) were collected in 750ml of distilled water from 200-300 infected Biomphalaria glabrata snails using a light induction method previously reported (11). Cercarial secretions were collected using a modification of a previously reported technique (12). The cercariae were placed in petri dishes coated with linoleic acid (to simulate skin contact) and floated in a 37°C water bath to produce a thermal gradient. After 2 hours the cercariae had released the majority of their gland contents and the conditioned water was collected and filtered to remove cercarial bodies and debris. The secretion sample was then lyophilized and stored at +4°C until further purification.

Biomphalaria glabrata -- Snails were maintained with a diet of organic lettuce and school chalk as a calcium supplement (13). All snails were housed in the absence of light to increase yields of cercariae during light induction.

E. coli -- BL21 cells (Novagen, Madison, WI) were electroporated with the appropriate plasmid construct and selected overnight using LB 50 ug/ml ampicillin plates. A single colony was picked and grown overnight in 4ml of LB with 100 ug/ml ampicillin. 1 ml of overnight
culture was added to 1000 ml LB with 100 ug/ml ampicillin and grown to an OD$_{600}$ = 0.6. IPTG was then added to 100 uM and after four hours of continued incubation the cells were pelleted and frozen at -70°C.

*Gel filtration chromatography* -- Lyophilized secretions from 4x10$^6$ cercariae were resuspended in 6ml of running buffer (200mM Na-Acetate pH6.8) and loaded onto a SR 16/100 column (Amersham Pharmacia Biotech, Piscataway, NJ) packed with Sephacryl 200 (Amersham Pharmacia Biotech, Piscataway, NJ). Fractions were eluted at a pumping rate of 25ml / hour. 4ml fractions were collected at +4°C.

*Benzamidine affinity chromatography* -- Lyophilized secretions from 4x10$^6$ cercariae were resuspended in 8ml of running buffer (100mM Tris pH 8.0, 500mM NaCl) and pumped at 1ml / min through a HR 5/5 column packed with benzamidine-sepharose (Amersham Pharmacia Biotech, Piscataway, NJ). The column was washed with 6ml of washing buffer (10mM Tris pH 8.0, 50 mM NaCl) and eluted with 6ml of elution buffer (20 mM Na-Acetate pH 4.5, 50mM NaCl). 300ul fractions were collected.

*Ion exchange chromatography* -- Fractions from the benzamidine affinity column were diluted 10 fold into running buffer (20mM Tris pH 8.0) and pumped through a HR5/5 Mono-Q column (Amersham Pharmacia Biotech, Piscataway, NJ) at 1ml / min. The column was then washed with an additional 10ml of running buffer. Elution was performed over 30 1ml fractions using running buffer and a linear salt gradient from 0 to 1.0M NaCl.

*Ni-ATA affinity chromatography* -- *E. coli* pellets from 330ml of inducted culture were thawed and resuspended in 5ml of binding buffer (8M urea, 50mM Tris pH8.0). Cell disruption
was completed with 6 rounds of 20 second sonications on ice. Histidine tagged proteins were then purified according to the manufacturers instructions (Qiagen, Valencia, CA) using 1ml spin columns.

Enzyme and Inhibitor Assays-- All assays were performed with native enzymes in buffer with 100mM glycine pH 9.0 (the pH optima with small synthetic substrates) with 100uM of pNA substrate at room temperature unless otherwise noted. Inhibitors were tested in the same buffer system under the same conditions. A 15 minute pre-incubation of inhibitor with enzyme was carried out before substrate was added. A standard assay consisted of 10ul of sample and 100ul of assay buffer in nylon 96 well plates (Falcon PVC plates, Becton Dickinson, Franklin Lakes, NJ). Optical density was monitored using a UV-Max spectrophotometer and SoftMax v2.02 software (Molecular Devices, Sunnyvale, CA).

Skin Invasion Assays -- Skin invasion assays were performed as previously described (11). Briefly, human skin samples were fixed on plastic wells over warm media (RPMI-1640) while 200µl of inhibitor solution (2 mM in DMSO) or controls, including DMSO, were applied to the skin surface and allowed to permeate and dry for 30 minutes. Approximately 3000 cercariae in 3ml of water were then applied using 15 mm plastic cylinders on the skin. After 120 minutes the skin was fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned at 7 uM, and stained with hematoxylin and eosin. Cercariae penetrating the skin were counted as described previously (11). Multiple sections of three separate skin samples were counted for each inhibitor and control.

Protein Blotting and N-terminal Peptide Sequencing -- NU-PAGE gradient gels (Invitrogen, Carlsbad, CA) were used according to the manufactures instructions. Proteins were
blotted onto PVDF membranes using the Novex transfer system. Peptide sequencing was carried out using an ABI Procise 491 Protein Sequencer (Applied Biosystems, Foster City, CA) at the UCSF Biomolecular Resource Center.

*Nucleic Acid Purification and cDNA Synthesis* -- Poly(A)$^+$ mRNA was isolated by poly(T) affinity chromatography (Amersham Pharmacia Biotech, Piscataway, NJ) from the hepatopancreases of 5 infected snails. RNA was converted to cDNA using AMV Reverse Transcriptase as described by the manufacturers (Life Technologies, Rockville, MD). DNA isolation from snails required CsCl purification because of the high proportion of glycoproteins. DNA was isolated from *Schistosoma mansoni* by standard detergent lysis and phenol / chloroform extraction.

*Cloning and Plasmid Construction* -- A nested PCR strategy was used to clone both BgSP's. Amplification conditions for the $1\degree$ PCR were 2 cycles of 94°C, 40°C, and 72°C each for 1 minute then 35 cycles of 94°C, 50°C, and 72°C each for 1 minute. $2\degree$ PCR conditions were 25 cycles of 94°C, 50°C, and 72°C each for 1 minute. Primers used for these reactions: BgSP-alpha $1\degree$ forward 5'-ATCGTCGGNGGNAARGARTCNATGC-3', BgSP-beta $1\degree$ forward 5'-ATGGTCGGWGGWCARGARGCNGTNC-3', BgSP-alpha $2\degree$ forward 5'-CCNAAYAAYCAYAWNTGYGG-3', BgSP-beta $2\degree$ forward 5'-CCGCCNACNCAYCAYTGYGG-3', the reverse primer for both $1\degree$ and $2\degree$ reactions was p(dT)$_{15}$. The *E. coli* expression construct pET-21a-BgSP-beta was assembled by inserting the active portion of BgSP-beta into the restriction sites NdeI and XhoI of the vector pET21-a (Invitrogen, Carlsbad, CA). The forward primer 5'-CGCCATATGGTCGGWGGWCARGARGCNGTNC-3' created a NdeI site (underlined) and
changed the 1st amino acid to a start codon (I→M). This substitution creates an inactive protease. The reverse primer 5’-GCGCTCGAGTCTGTTGATGACGGTGTTA-3’ added an XhoI site (underlined) and deleted the stop codon creating an open reading frame into the pET21-a vector that added a 6 x histidine tag to the C-terminus of the protein.

Genomic PCR was performed using S. mansoni or B. glabrata DNA. Forward primers were targeted to an internal region overlapping the catalytic serine: BgSP-alpha 5’-ACGCGGGATGCCGGCCTTCGTC-3’ and BgSP-beta 5’-CAGGGTGATGCCGGTGGCC-3’. Reverse primers were targeted to unique regions within the 3’ UTR of each gene: BgSP-alpha 5’-TTGGTCTACCGACAGACTT-3’ and BgSP-beta 5’-CGCCCTTTACTATTCGCAT-3’.

Southern Blot -- Genomic DNA (10ug) was digested overnight with the individual restriction enzymes EcoRI, PstI, XbaI, and XhoI (New England Biolabs, Beverly, MA). The DNA was transferred to a Hybond-N+ charged nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ) using alkaline capillary transfer. The DNA was fixed to the membrane by UV cross linking and pre-hybridized for 45 minutes in Rapid-Hybe solution (Amersham Pharmacia Biotech, Piscataway, NJ). 40ng of probe (full active length PCR product) was labeled with P32 (NEN Research Products, Wilmington, DE) using a High Prime nick translation kit (Roche, Alameda, CA) according to the manufacturers instructions. Hybridization was carried out at 65°C for one hour. 2x SSC stringency washing was sufficient for the removal of non-specific probe. The membrane was exposed for 3 days at -70°C to Hyperfilm (Amersham Pharmacia Biotech, Piscataway, NJ) using intensifying screens. The membrane was then stripped for 30 minutes in 0.4M NaOH at 45°C, washed twice for 10 minutes at room temperature in strip solution (200mM Tris-HCl pH7.0, 0.1 SSC, 0.1 (w/v) SDS), and reused.
Antibody Production, Immunohistochemistry, and Floresent Microscopy -- Recombinant

His-tagged protein from *E. coli* was used to produce rabbit antisera using standard commercial procedures (Corvance, Richmond, CA). Materials prepared for immunohistochemistry were incubated in fixative (2% paraformaldehyde, 1% gluteraldehyde, 0.1M phosphate buffer pH7.4) for > 2 hours and then embedded in JB4 plastic, 7 micron sections were cut, and antibody localization was visualized using an ABC kit (Vector Labs, Burlingame, CA).
RESULTS

Identification of trypsin-like activity within cercarial secretions -- In order to identify and isolate a previously reported trypsin-like(10) activity from cercariae, gel filtration was chosen as the first chromatography step to fractionate the complex protein mixture of cercarial secretions (Fig. 1). The chromogenic substrate LGR-pNA was used to detect the trypsin activity, and AAPF-pNA was used to detect the chymotrypsin-like activity (SmCE--Schistosoma mansoni Cercarial Elastase). The trypsin activity was identified and resolved from the SmCE activity and while it was 30-fold more active than SmCE against its small tri-peptide substrate, it had no significant activity against macromolecular elastin.

Using molecular weight standards as a reference, the trypsin-like activity had an apparent molecular weight of 25 kD while the SmCE activity peak eluted at an apparent molecular weight of 15kD. SmCE has a pI greater than 9.0 and may interact with the column resin, retarding its migration relative to other proteins. At higher salt concentrations the peak of SmCE is shifted to the left (data not shown).

Purification of native trypsin activity -- To further characterize this native trypsin activity, benzamidine conjugated to sepharose was employed as a first step affinity column yielding a 1000-fold purification (Table I). Ion exchange chromatography was employed as the second and final step. The trypsin activity was resolved into two distinct proteases. Sufficient material was generated to obtain unambiguous N-terminal sequences (Fig. 2) for both peaks of activity. These two activities were designated BgSP-alpha (Biomphalaria glabrata Serine Protease) and BgSP-beta corresponding to the order in which they eluted from the column.
Biochemical characterization of BgSP-alpha and BgSP-beta -- As shown in Table II both native BgSP-alpha and native BgSP-beta have a very similar pH optimum and calcium optimum. BgSP-beta is significantly more effective at degrading Azocoll (denatured collagen), than BgSP-alpha. BgSP-beta will cleave several different peptide substrates that have a charged amino acid in the P1 position, while BgSP-alpha prefers the substrate LGR-pNA.

Cloning of BgSP-alpha and BgSP-beta cDNAs by RT-PCR -- Based on N-terminal sequence data, a set of nested degenerate PCR primers were designed for each protease. RT-PCR was performed on mRNA isolated from the hepatopancreas of infected snails. This is the location where the parasite replicates and develops. A SmCE cDNA had previously been cloned from this source (14). Once the cercaria leaves the host snail there is relatively little transcription or translation until after it enters the human host.

The nested primers in conjunction with a polyA reverse primer yielded two bands of the predicted size. Cloning and sequencing of these two bands revealed two serine proteases of the chymotrypsin-fold family that are 76% similar (Fig. 3). The amino termini matched those of the purified proteases and the S1 subsites were consistent with trypsin-like serine proteases. Both S1 pockets contained an aspartic acid at the position corresponding to trypsin 195D. A BLAST (15) search confirmed that both sequences are unique but similar to other serine proteases with a chymotrypsin tertiary structure. BgSP-alpha has a putative glycosylation site at position N51. The utilization of this glycosylation site is consistent with the observed molecular weight determined by SDS-PAGE. BgSP-alpha has an apparent molecular weight 2-3 kD larger than BgSP-beta yet their calculated MW's are 26,241 and 26,337 daltons respectively.

* The nucleotide sequence for the BgSP-alpha and BgSP-beta genes have been deposited in the GenBank database under GenBank Accession Number AF302259 and GenBank Accession Number AF302260 respectively.
**BgSP-alpha and BgSP-beta are produced by the host snail Biomphalaria glabrata** --

Southern blot analysis shows that both protease sequences are present only in the genome of the intermediate host snail (Fig. 4). Additionally, genomic PCR using *Biomphalaria glabrata* DNA produced a fragment that contained both the protease sequence and the sequence of an intron with the consensus sequence for an acceptor site at the predicted junction (data not shown).

**Localization of BgSP-beta and SmCE by microscopy** -- Polyclonal rabbit antisera was generated against recombinant BgSP-beta produced in *E. coli* and purified using a 6X histidine tag. Specific localization of the antisera was visualized in granules produced by the epithelial cells in the hepatopancreas of the snail. No staining was seen in any stage of cercarial development or in or on free swimming cercariae. In contrast polyclonal anti-sera reactive against SmCE, generated with recombinant SmCE produced in *E.coli*, only localizes within the secretory glands of the cercaria and does not stain the adjoining snail tissue (Fig. 5).

**Similarity of BgSP's to other serine proteases of Mollusca**-- A search of the gene database reveals only five proteases that have been identified within the phylum Mollusca [NCBI:txid6447]. Three of these proteases belong to be Furin/Kex family of serine proteases (AF140362-*Lymnaea stagnalis*, AF140361-*Lymnaea stagnalis*, AF107213-*Helix aspersa*) a structurally unrelated serine protease. The two remaining proteases belong to the chymotrypsin family (AA547777-*Biomphalaria glabrata*, X71438-*Haliotis rufescens*). The amino acid similarities between BgSP-alpha, BgSP-beta and these two family members range from 35% to 43%.

**BgSP production under various conditions** – Two conditions were tested to determine what factors may influence the production of BgSP’s within the snail. The total activity of
BgSP’s within the hepatopancreas of the snail was not effected by feeding. No significant differences were seen over an eight hour time course of feeding following 24 hours of starvation. Total activity within the hepatopancreas was effected by schistosome infection. The displacement and diminution of the snail hepatopancreas by the dividing parasite causes a “crowding out” effect and reduces the total activity of the BgSP’s on average 10 fold (data not shown).

*Identification of inhibitors for SmCE and BgSP’s for use in "chemical knockout"* -- The tri-peptide inhibitor FPR-CMK was highly effective against the pooled native BgSP’s (0.9 nM IC50) and also had some activity against native SmCE (1700 nM IC50). In contrast AAPF-CMK was effective against SmCE (1200 nM IC50) but had no measurable activity against the BgSP’s (>10,000 nM IC50) (Table III). To evaluate the role of these proteases in the invasion process an *in vitro* human skin invasion assay was used(9,11). AAPF-CMK was effective in reducing the number of cercariae penetrating by over 80% (Fig. 7), while FPR-CMK produced a 50% reduction in cercarial penetration.

*Relative rates of elastin degradation by BgSP, SmCE, and chymotrypsin* -- Elastin is the skin protein most resistant to proteolysis. It is a major component of the dermal barrier to cercarial invasion (16,17). Native BgSP, native SmCE, and sequence grade bovine chymotrypsin were compared on a molar basis against insoluble native bovine elastin. Only SmCE demonstrated significant elastase activity (Fig. 7).
Identification of elastin cleavage sites by SmCE -- Three cleavage sites localizing to exons 12 (amino acids 202..225) and 13 (226..239) of elastin, were identified (Fig. 8). Exon 12 is the same location where macrophage elastase cleaves (18)∗

∗ R. P. Mecham (Washington University School of Medicine), personal communication
DISCUSSION

_Schistosoma mansoni_ cercariae directly penetrate human skin within five to 10 minutes after contact (19). During this process 10 glands that comprise approximately 30 percent of the volume of a cercaria release their contents. Previous studies have shown these glands contain and release proteolytic activity (12,14) and that cercarial invasion is a lytic, not solely a mechanical event (20).

Two proteases were proposed to be involved in skin invasion. The first, a chymotrypsin-like serine protease also known as SmCE (_Schistosoma mansoni_ Cercarial Elastase), cleaves a variety of human skin macromolecules and has been studied in detail (9,14,21,22). The second, a trypsin-like serine protease, has only recently been identified through the use of small synthetic substrates (10). Secretions from cercariae were collected and assayed for trypsin-like activity using the substrate LGR-pNA. A purification strategy was developed and the activity was found to result from two similar serine proteases designated BgSP-alpha (_Biomphalaria glabrata_ Serine Protease) and BgSP-beta.

Southern blot analysis and genomic DNA PCR both demonstrate that the trypsin-like proteases are of snail host origin. The predominance of single bands and their similar arrangements between the two blots suggest that both genes are present in single copies and may be contiguous. Immunohistochemistry localized the protease to secretory vesicles within the epithelium of the snail hepatopancreas. The function of the trypsins for the snail is likely digestion. The trypsin-like activity is released by the snail during the release of cercariae and would therefore contaminate preparations of cercariae or their secretions. Expression of protease activity in the snail was "constitutive" without induction or enhanced release during feeding. However less activity was found in infected snails due to replacement of the snail tissue by the developing parasites.
Elastin is one of the most difficult host macromolecules to degrade and few proteases are "true" elastases capable of degrading native insoluble elastin (23). To gain insight into the mechanism of elastin degradation by parasite larvae, the site of cleavage by SmCE was determined. Solubilized fragments from native bovine elastin degraded by SmCE were purified and sequenced. Three cleavage sites localizing to two exons (12 and 13 / amino acids 202..239) within bovine elastin were discovered.

To confirm the role of SmCE in the invasion process, and rule out any possible contribution of the BgSP (e.g. being passively carried to skin by cercariae) a human skin invasion assay was used in combination with selective protease inhibitors. One inhibitor (AAPF-CMK) inhibited SmCE alone while the other (LGR-CMK) inhibited both the proteases but was 1900-fold more effective against the BgSP. If BgSP contributed to invasion FPR-CMK should have the predominant effect. If SmCE alone was responsible both inhibitors should be effective but AAPF-CMK would be more active. AAPF-CMK inhibited 80% of cercariae invading, while LGR-CMK inhibited 50%. This is the expected result if the SmCE was acting alone to facilitate cercarial invasion. SmCE is a biologically potent histolytic protease with activity against many of the macromolecular barriers of skin (5-8). It is one of the few "true elastases" capable of degrading insoluble elastin itself. Its cleavage pattern on elastin suggests exons 12 and 13 (amino acids 202-239) are solvent accessible domains. Exon 12 is also targeted by macrophage elastase and the observations reported here support the conclusion of Mecham et. al. (23) that "elastases" are a more diverse family of proteases than originally thought. Insoluble elastin can be degraded by proteases acting outside the alanine-rich regions targeted by mammalian pancreatic elastases.
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REFERENCES

1. Stirewalt, M. (1974) *Advances in Parasitology* **12**, 115-182
2. Cohen, L. M., Neimark, H., and Eveland, L. K. (1980) *J Parasitol* **66**(2), 362-4
3. Haas, W., Diekhoff, D., Koch, K., Schmalfuss, G., and Loy, C. (1997) *J Parasitol* **83**(6), 1079-85
4. Gordon, R., and Griffiths, R. (1951) *Ann. Trop. Med. and Parasitol.* **45**, 227-243
5. Gazzinelli, G., and Pellegrino, J. (1964) *Journal of Parasitology* **50**(4), 591-592
6. Dresden, M. H., and Asch, H. L. (1972) *Biochim Biophys Acta* **289**(2), 378-84
7. Tzeng, S., McKerrow, J. H., Fukuyama, K., Jeong, K., and Epstein, W. L. (1983) *J Parasitol* **69**(5), 992-4
8. McKerrow, J. H., Jones, P., Sage, H., and Pino-Heiss, S. (1985) *Biochem J* **231**(1), 47-51
9. Cohen, F. E., Gregoret, L. M., Amiri, P., Aldape, K., Railey, J., and McKerrow, J. H. (1991) *Biochemistry* **30**(47), 11221-9
10. Dalton, J. P., Clough, K. A., Jones, M. K., and Brindley, P. J. (1997) *Parasitology* **114**(Pt 2), 105-12
11. Lim, K. C., Sun, E., Bahgat, M., Bucks, D., Guy, R., Hinz, R. S., Cullander, C., and McKerrow, J. H. (1999) *Am J Trop Med Hyg* **60**(3), 487-92
12. Stirewalt, M. A., and Austin, B. E. (1973) *J Parasitol* **59**(4), 741-3
13. Mishkin, E. M., and Jokinen, E. H. (1986) *J Parasitol* **72**(6), 885-90
14. Newport, G. R., McKerrow, J. H., Hedstrom, R., Petitt, M., McGarrigle, L., Barr, P. J., and Agabian, N. (1988) *J Biol Chem* **263**(26), 13179-84
15. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res* **25**(17), 3389-402
16. Lewert, R. M., and Lee, C. L. (1956) *J. Infect. Dis* **99**(1), 1-14
17. Fukuyama, K., Tzeng, S., McKerrow, J., and Epstein, W. L. (1983) *Curr Probl Dermatol* **11**, 185-93
18. Mecham, R. P. (2000)
19. Stirewalt, M. A., and Hackey, J. R. (1956) *Journal of Parasitology* **42**, 565-580
20. Stirewalt, M. A., and Dorsey, C. H. (1974) *Exp Parasitol* **35**(1), 1-15
21. McKerrow, J. H., Pino-Heiss, S., Lindquist, R., and Werb, Z. (1985) *J Biol Chem* **260**(6), 3703-7
22. Ring, C. S., Sun, E., McKerrow, J. H., Lee, G. K., Rosenthal, P. J., Kuntz, I. D., and Cohen, F. E. (1993) *Proc Natl Acad Sci U S A* **90**(8), 3583-7

23. Mecham, R. P., Broekelmann, T. J., Fliszar, C. J., Shapiro, S. D., Welgus, H. G., and Senior, R. M. (1997) *J Biol Chem* **272**(29), 18071-6

24. Raju, K., and Anwar, R. A. (1987) *J Biol Chem* **262**(12), 5755-62
FIGURE LEGENDS

FIG. 1 Identification of trypsin activity within cercarial secretions fractionated by gel filtration chromatography. Trypsin activity elutes from the column at its monomeric molecular weight (MW) of 25kD. Chymotrypsin activity (cercarial elastase) interacts with the Sephacryl 100 resin and has an apparent MW of 15kD vs. an actual MW of 25kD. The bar under the chymotrypsin-like activity peak indicates the fractions with activity against native elastin and the bar above the first peak indicates the void volume.

FIG. 2 Purification of native trypsin activity by affinity and ion exchange chromatography. Benzamidine sepharose yields over 1000x purification in the first step with a single peak of activity (top graph). Peak is diluted into ion exchange running buffer and fractionated with a mono-S ion exchange column (lower graph). Two enzymes are revealed using ion exchange and both N-terminally sequences are shown. These enzymes are referred to as BgSP-α and BgSP-β.

FIG. 3 Comparison of BgSP-α and BgSP-β. Bold sequence represents N-terminal sequence data collected from native purification. Underlined sequence indicates regions where degenerate primers were designed for cloning by RT-PCR. Bold and shaded amino acids represent the catalytic core of the serine protease. Solid vertical lines represent identity and dots represent similarities. Inverted triangle indicates the location of a possible glycosylation site for BgSP-α.

FIG 4. Southern blot of *S. mansoni*, *B. glabrata*, and *B. glabrata* infected with *S. mansoni*. cDNA probes of the mature proteases were labeled and hybridized to the same blot (BgSP-α top image/ BgSP-β bottom image) containing four different digestions of genomic DNA purified
from parasite, intermediate host, or infected intermediate. Plasmids containing the mature cDNA sequences were used as positive controls. BgSP-α has an XhoI site within its cDNA.

FIG 5. **Immunolocalization of proteases within the infected host *Biomphalaria glabrata***. Image A shows anti-cercarial elastase serum (identified by orange-brown color of peroxidase reaction) localizing within the pre-acetabular glands (PG) of a cercaria (SC with hashed bar) developing within a daughter sporocyst that is within the host snail. Image B shows anti-BgCT serum reacting within vesicles of host snail secretory epithelium (HE) but not within parasitic cercaria (SC with hashed bar). Solid bars are 50µm.

FIG. 6 **Inhibition of cercarial penetration into human skin by topical application of selective protease inhibitors.** Inhibition of cercarial elastase by the selective inhibitor AAPF-CMK (ala-ala-pro-phe chloromethyl ketone) or inhibition of BgSP-α and β with FPR-CMK (phe-pro-arg chloromethyl ketone) as described under "Materials and Methods".

FIG. 7 **Elastin degradation by native SmCE, Human Chymotrypsin, native BgSP-α, and native BgSP-β.** Insoluble elastin labeled with congo red dye was incubated with each enzyme for 17 hours. The amount of dye released into the supernatant was quantified and normalized to a scale of 100.

FIG. 8 **Locations of proteolytic cleavage within elastin by cercarial elastase.** Arrows mark location of proteolytic cleavage as determined from sequence analysis of fragments (F206/G207, Y228/G229, and Y232/K232). Single letter amino acid sequence only depicts exons 12 and 13 from the 36 exons of elastin (24). Lower case letters represent the sequenced portions of the peptide fragments generated (described in Material and Methods).
### TABLE I

*Purification of native snail trypsins*

| Step              | Volume (ml) | Protein Concentration (mg/ml) | Total Protein (mg) | Enzyme Concentration (mU/ml) | Total Activity (TA) (mU) | % Yield (TA/TA) | Specific Activity (SA) (mU/mg) | Purification (SA/SA) |
|-------------------|-------------|-------------------------------|--------------------|------------------------------|--------------------------|-----------------|-------------------------------|---------------------|
| Starting material | 4.5         | 6.4                           | 29.0               | 2.0                          | 8.9                      | --              | 0.3                           | --                  |
| Benzamidine       | 0.3         | 0.10                          | 0.031              | 39                           | 12                       | 130             | 380                           | 1300                |
| Mono-Q            | 1.0         | 0.001                         | 0.001              | 1.1                          | 1.1                      | 10              | 1100                          | 3600                |
Table II  
Biochemical characterization of BgSP’s and relative rates of cleavage normalized to preferred substrate

| Enzyme characteristics                  | BgSP-alpha | BgSP-beta |
|----------------------------------------|------------|-----------|
| pH optimum with LGR-pNA               | 9.0        | 9.0       |
| pH optimum with Azocoll               | 8.5        | 8.5       |
| Kcat (LGR-pNA, sec⁻¹)                 | 14         | 12        |
| Km (LGR-pNA, µM⁻¹)                    | 110        | 18        |
| Kcat / Km (sec⁻¹M⁻¹)                  | 1.2 x 10⁵   | 6.5 x 10⁵ |

Small Substrate Hydrolysis Normalized to LGR activity (mOD/min)

| Substrate   | BgSP-alpha | BgSP-beta |
|-------------|------------|-----------|
| LGR-pNA     | 100        | 100       |
| FR-pNA      | 1.3        | 61        |
| AFK-pNA     | 1.3        | 12        |
| RR-pNA      | 0.8        | 7.4       |

LGR=Leucine-Glycine-Arginine, FR=Phenylalanine-Arginine, AFK=Alanine-Phenylalanine-Arginine, RR=Arginine-Arginine, pNA=p-nitroanilide
**TABLE III**

*IC50s for inhibitors of native SmCE and pooled native BgSP's and their predicted effect on cercariae invading human skin*

| IC50 (nM) | Expected Inhibition |
|-----------|---------------------|
|           | AAPF-pNA | LGR-pNA | (if SmCE is required) | (if BgSP's are required) |
| AAPF-CMK  | 1200     | >10000  | ++                    | None                     |
| FPR-CMK   | 1700     | 0.9     | +                     | +++                      |

+++ = >95% inhibition, ++ = >75% inhibition, = + >50% inhibition
FIG. 2

Benzamidine Affinity Column

Ion Exchange Column

BgSP-α
N-term = IVGGKE...

BgSP-β
N-term = IVGGQE...

Flow Through
Wash 1
Wash 2
Elute 1
Elute 2

Flow Through

0 50 100 150 200 250 300 350 400 450 500

mOD/min

mM NaCl

LGR
NaCl
FIG. 3.

BgSP-α  IVGGKESMPYTWPAILCSLRFVQEPNNHICGSNLVKNLAGEYYLITAAHCL 50  
 | | | . . || | | . | | | | : | | | |  

BgSP-β  IVGGOEAVPYPYSHPSICSRLYTTAPTHHFCGGLTVKNLAGEYYVFVTAAHCV 50  
 ▼  

51 . NDTRASRYEAHCGIHRADESEPHRIIVHFNNLYIHSGYNSWTMDSDIA 99  
  : | | | | | | | | | | | | : | | | |  
51 YGEPRASRYEAHCGIHDRSDLREPHERVIVHFSALTSHPLYDDWTIDYDIA 100  

100 IFKIVTSLPTNMFISAVCIPNEGWTDEISIVAGWGALSSGSSPYKLHQ 149  
  | | : | . | | | | | | : | | : | | | |  
101 IFKVSTALPTNNYISAVCIPNEGWFEGEKGLVAGWGETSTGGDSPYKLHQ 150  

150 VNKPIKPRSIICEQRYGVAITPRMLCAGLPNGGVACTGDSSGGPLYTYRE 199  
  | | | | : . | | | | | | | | | | | | | |  
151 VQKPIKSKATCELRYGAGSISLRMLCAGLPQGGVDSCQGDSSGGPLYTLRE 200  

200 NRWTLTGIVSWGHGCGEVKPGVYSDVIELKDWINTVLNVL 240  
  | | | | | | | | | : | | : | | | | | | : | |  
201 NRWTLTGIVSWGYGCAEAGRPGVYADVIELKDWINTVINRL 241
| Digestion | EcoRI | PstI | XbaI | XhoI | 1kb ladder |
|-----------|-------|------|------|------|------------|
| *S. mansoni* | +     | +    | +    | +    | +          |
| *B. glabrata* | +     | +    | +    | +    | +          |

**FIG 4.**

*BgSP-α probe*

*BgSP-β probe*
FIG 5.
FIG. 6.

Number of cercariae penetrating human skin

|          | No Inhibitor | AAPF-CMK | FPR-CMK |
|----------|--------------|----------|---------|
|          | 175          | 75       | 75      |
FIG. 7.

- SmCE
- Human Chymotrypsin
- BgSP-alpha
- BgSP-beta
