A cDNA encoding a Schistosoma japonicum aspartic proteinase was cloned, sequenced, and found to encode a zymogen of 380 amino acid residues, and its gene was shown to be present as a single copy in the S. japonicum genome. Identity comparisons showed that the enzyme (Sjpasp) was most closely related to the cathepsin Ds. The deduced amino acid sequence has four potential glycosylation sites, two of which are in identical positions to the two glycosylation sites of human kidney lysosomal cathepsin D. Furthermore, all four disulfide bonds found in mammalian cathepsin D sequences are present in Sjpasp, although the β-hairpin (loop 3), which is cleaved during maturation of vertebrate cathepsin Ds to yield light and heavy chain subunits, is absent from Sjpasp. While most residues involved in substrate specificity and catalysis of aspartic proteinases are preserved in Sjpasp, several residues in these regions exhibit changes that may result in a novel substrate specificity. Aspartic proteinase activity is present in extracts of adult S. japonicum and Schistosoma mansoni and in culture media in which schistosomes were maintained and was capable of digesting hemoglobin. The schistosome aspartic proteinase may play a pivotal role in the catabolism of hemoglobin obtained from host erythrocytes.

Blood flukes of the genus Schistosoma are the cause of schistosomiasis, which afflicts more than 250 million people in the tropics. Schistosomes feed on red blood cells, and it has been estimated that male and female adult Schistosoma mansoni ingest 39,000 and 330,000 red blood cells/h, respectively (1). Accordingly, catabolism of host hemoglobin is likely to be integrally involved in schistosome nutrition. It was suggested in the 1950s that acid proteinases were secreted by schistosomes (2). The precise enzyme activities involved in the catabolism of hemoglobin into readily absorbable peptides have still not been determined, although both cysteine and aspartyl proteinases have been implicated (3).

We have already described the characterization of a cathepsin L proteinase secreted by adult schistosomes, which we suggested was involved in the degradation of host hemoglobin (Hb), and we also reported the gene sequence for schistosome cathepsin L (4). Now we describe the isolation of a cDNA encoding the Schistosoma japonicum aspartic proteinase. Gene fragments were initially isolated from a cDNA library using a primer that we designed based on the consensus sequence adjacent to the active site Asp-32 of 16 eukaryotic aspartic proteinases. The larger fragment was used as a probe to screen a cDNA library to isolate a full-length clone. In addition, aspartic proteinase activity was detected using the synthetic substrate Phe-Ala-Ala-Phe(NO₂)-Phe-Val-Leu-OM4P (5) in extracts of adult schistosomes and in culture medium in which schistosomes had been maintained, and we demonstrate that this type of activity can degrade human Hb. Since Southern hybridization indicated that the gene encoding the aspartic proteinase was present as a single copy in the parasite genome, it is likely that the cDNA we characterize here encodes these proteinase activities. Therefore, adult schistosomes secrete both aspartic proteinase and cathepsin L cysteine proteinases, which may all be involved in the degradation of Hb in vivo (3).

MATERIALS AND METHODS

Consensus PCR—The amino acid sequences of aspartic proteinases are highly conserved (6). We aligned the regions around the active site Asp-32 residue of 16 aspartic proteinases (6, 7) and obtained the following consensus amino acid sequence DTGSSNLW where the D residue is Asp-32. We synthesized the 256-fold degenerate dinucleotide primer 5′-TTGAYACNGNCTACTAAAYCNTTGG (where Y is C or T) based on the consensus amino acid sequence and using the codon bias table of Caenorhabditis elegans (8). This consensus oligo was paired with a universal 3′-primer (Promega) and used in PCR to amplify an aspartic proteinase gene fragment from the S. japonicum (Philippine strain) cDNA library, constructed in λ UNI Zip-XR (Stratagene) library kindly provided by Drs. J. Kurtis and B. Ramirez. The PCR involved denaturation for 5 min at 94°C, followed by 35 cycles at 94°C for 40 s, 60°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 10 min, using a Perkin Elmer Cetus, model 480, thermal cycler.

cDNA Cloning and Analysis—PCR products were fractionated by electrophoresis through 2% low-melting point agarose (3,1 NuSieve GTG:Seakem ME, FMC Bioproducts, Rockland, ME) in Tris acetate-EDTA buffer and stained with ethidium bromide. Products were isolated from gels using Wizard DNA clean-up columns (Promega), ligated into plasmid pGEM-T (Promega) using T4 DNA ligase (Amersham Corp.) and were used to transform Escherichia coli strain XL1-Blue cells by electroporation. Transformed cells were cultured on LB-agar supplemented with ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and isopropyl-1-thio-β-D-galactopyranoside. Maxiplants of recombinant pGEM-T and pBlueScript (below) plasmids were prepared from bacterial cultures using QiaGene-500 columns (Qiagen Inc., Chatsworth, CA). Nucleotide sequences of both strands of plasmid DNA clones were determined using Perkin-Elmer/Cetus Sequenase DNA Sequencing Kit.

The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase pair(s); ES, excreto/secretory products; NHMec, 7-amino-4-methylcoumarin; AEBsF, 4-(aminoethyl)benzenesulfonyl fluoride hydrochloride.
DNA, both pGEM-T or pBluescript excised from λ UNI Zap-XR (below), were determined by the dye-dilution method using the ABI (Foster City, CA) Taq DyeDeoxy terminator cycle sequencing system and an automated DNA sequencer (ABI, model 373A). Oligomers were synthesized using the β-cyanoethyl phosphoramidite procedure on a ABI model 380B DNA synthesizer.

Results and Discussion

An Adult S. japonicum Transcript Encodes a Cathepsin D Aspartic Proteinase—When the aspartic proteinase gene-specific primer was used in tandem with the universal M13 primer in the PCR with the S. japonicum cDNA library as the template, two ethidium-stainable bands of ~1.3 and ~0.75 kb were amplified. When cloned and sequenced, both of these PCR products were found to represent fragments of a novel aspartic proteinase gene (not shown). The larger gene fragment was subcloned and used to probe the cDNA library. A positive clone (pSjasp) was obtained after screening 10,000 plaques. The insert in the clone was ~1.6 kb in length, and when sequenced it was found to include a 5'-untranslated stretch of 5 base pairs, a start codon, an open reading frame of ~400 base pairs terminating in a poly(A) stretch, which therefore represents the 3' terminus of the mRNA (GenBank L41346). The two PCR products, which differed in size by about ~550 base pairs, above, were generated as the result of annealing of our 5' aspartic acid consensus primer to the coding regions around active site Asp-33 and the second active site.

![Fig. 1. Dendrogram of multiple pairwise alignments of deduced amino acid sequences of aspartic proteinases from Schistosoma japonicum and other species producing clusters of similar sequences (GenBank accession numbers from the top: K75787, L10740, P10977, P07267, P07242, P03955, P04073, P25796, P14091, P03956, P03953, P07281, P07282, P00792, P00793, P18276, P16476, P27823, P06281, P00796, P08424, P00797, P18242, P24268, P07339, P00795, Q05744, Q03168, L41346 (Sjasp), P28871, P22929, P06026, P10602, P17946). The dendrogram predicts evolutionary relationships of Sjasp to other members of the aspartic proteinase class (9). Similarities (%) to Sjasp were obtained using the FASTA program. Boldface denotes the group of cathepsin Ds that includes Sjasp.](image-url)
Fig. 2. Alignment of deduced amino acid sequences of cathepsin D aspartic proteinases from S. japonicum (catD_pSjpasp, accession number L41346), mouse (catd_mouse, P18242), rat (tD_catd_rat, P24268), human (catd_human, P07339), chicken (catd_chick, Q57444), and the mosquito A. aegypti (aspp_aedae) (Q03168). The numbering system is based on the sequence of human kidney cathepsin D (catd_human); the presumed NH₂-terminal residue of the mature enzyme is Gly-1. The residues of the mature enzymes are counted in the positive from this residue, while the pre- and proenzyme regions are numbered in the negative from Gly-1. Blocks denote conserved residues, and gaps have been introduced to maximize alignment. Symbols on the schistosome sequence pSjpasp designate the aspartic proteinase catalytic Asp residues (solid triangles), S₂ subsite residues (solid circles), S₃ subsite residues (open circles), potential Asn glycosylation sites (diamonds), phosphorylation determinants (dotted lines), cleavage of signal peptide (solid arrow), beginning of mature proteinase (open arrow), and cysteine residues (open triangles). The eight loops characteristic of aspartic proteinases (15) are indicated by numbered bars.
residue Asp-231 (see below).

The open reading frame encodes an entire preproenzyme of 380 amino acid residues. Since Tang and Wong (6) consider that all aspartic proteinases have evolved from a common, primordial enzyme, we constructed a dendrogram of aspartic proteinase sequences in order to predict the phylogenetic relatedness of the S. japonicum aspartic proteinase (Sjasp) to 36 other aspartic proteinases (Fig. 1). Closest identities for Sjasp were with mammalian cathepsin Ds (52.5–55.1%) and to the aspartic proteinase of the mosquito Aedes aegypti (53.1%) (13), and accordingly we consider the schistosome aspartic proteinase can be characterized as cathepin D-like (EC 3.4.23). By contrast, Sjasp showed less identity to renins, pepsinogens, cathepsins E, or to several other aspartic proteinases including two putative hemoglobinases (pfaasphem and pfaaspprot) of the malaria parasite Plasmodium falciparum (14, 15).

Structural and Functional Motifs—Fig. 2 presents a multiple alignment of amino acid sequences of aspartic proteinases from mouse, rat, human, chicken, A. aegypti, and S. japonicum. The numbering system we have adopted in this alignment is based on the sequence of human kidney cathepsin D. All other sequences examined could be aligned with this sequence by introducing gaps without disruption of the human cathepsin D sequence. By comparison with the other aspartic proteinases, and according to von Heijne's algorithm (16), the incipient schistosomezymogen is apparently composed of a signal region of 14 residues, a proenzyme region of 37 amino acids, and the mature proteolytic sequence of 329 residues of predicted Mr 35,933 with pl 5.39 begins with Glu-1 (Gly-1 in human cathepsin D). The catalytic apparatus of aspartic proteinases consists primarily of two aspartic acid residues at positions 32 and 215 (6). The schistosome enzyme bears these catalytic active site aspartic acid residues at positions 33 and 231 in our alignment (Fig. 2). These two Asp residues are located within the substrate-binding cleft in the native molecule (6), which accounts for the strong conservation in primary amino acid sequence around both of these two active site residues in cathepsin Ds. However, the S. japonicum aspartic protease exhibits changes at positions 29, 30, 44, 227, and 230. Residue Gln-14 is pivotal in determining S2 substrate interactions with substrates (6), and is positioned within a block of other conserved residues. While Gln-14 is conserved in the S. japonicum enzyme, two adjacent residues at positions 16 and 20 differ. The sequence of residues 307–309 (Ile-Gly-Met, situated at the NH2 terminus of loop 8) in Sjasp, which is involved in the interaction between substrate and the S2 enzyme subsite, differs from the conserved mammalian sequence Met-Gly-Met. However, this particular site also shows variation within chicken (Ser-Gly-Leu) and mosquito (Met-Gly-Ile) aspartic proteinases. In addition, the Sjasp sequence has only one proline in contrast to four prolines within the polyproline loop 8 of mammalian cathepsin D (14). A tyrosine residue at position 78 and threonine (or serine) at 80, located in the β-hairpin loop (loop 2), which partially occludes the active site and which contribute to catalysis, are both conserved in Sjasp and other cathepsin Ds. However, His-77 in mammalian cathepsin D has been replaced by arginine in the schistosome proteinase (6, 15). Two residues of loop 4, which extends into the binding pocket and interacts with the S1 substrate site, differ between Sjasp and all other cathepsin Ds examined (Fig. 2). Overall, differences in a number of critical catalytic residues or in adjacent positions distinguish the schistosome aspartic proteinase from those previously reported and may relate to its specificity of substrate binding.

A major structural difference within cathepsin Ds is the sequence involved in the processing of the single chain form to the two-chain form (6). Processing of vertebrate cathepsin Ds involves the proteolytic cleavage of a β-hairpin (loop 3), which in human cathepsin D is formed by residues 94–107. This β-hairpin loops outside the molecular surface, where it is readily hydrolyzed to transform the enzyme from a single chain into a dimeric polypeptide. This proteolytic cleavage also stabilizes the tertiary structure of the cathepsin D (17). However, the schistosome aspartic acid, like those of chicken and A. aegypti, does not contain this kind of β-hairpin. Aspartic proteinases that lack the β-hairpin are processed in other ways; the aspartic proteinase of barley and that of A. aegypti are cleaved at other sites to generate hetero- and homodimeric enzymes, respectively (12). Whether the native schistosome aspartic proteinase exists as one of these kinds of bilobed enzymes requires further investigation.

The mature Sjasp enzyme has four potential Asn-linked glycosylation sites at positions Asn-70, Asn-172, Asn-199, and Asn-213, although only sites at Asn-70 and Asn-199 align with conserved, glycosylated asparagine residues of mammalian cathepsin Ds. Lys-203, a determinant for phosphorylation of high-mannose oligosaccharides (13), is conserved in cathepsin Ds and in Sjasp. However, Arg-202 and Ala-204, conserved residues on each side of Lys-203 in mammalian, avian, and mosquito cathepsin Ds, are exchanged for Glu-202 and Ser-204 in the schistosome enzyme. A second phosphorylation determinant spans 28 residues from Cys-265 to Leu-292 (13). 19 of these determinant residues are conserved in mammalian cathepsin Ds, whereas only 13 of these 19 are conserved in Sjasp. Elucidation of the extent of glycosylation/phosphorylation of the schistosome aspartic proteinase will provide insight into the mechanism by which adult schistosomes process this enzyme in the gastrodermal cells and secrete it into the cecal lumen (see below). Three disulfide linkages present in other cathepsin Ds are preserved in the schistosome enzyme, including Cys-46–Cys-53 (within loop 1), Cys-222–Cys-226 (within loop 5), and Cys-265–Cys-302 (6, 7, 15).

Aspartic Proteinase Gene Identified in the Genomes of S. japonicum and S. mansoni—Since the cDNA encoding Sjasp has one PstI in its sequence (not shown; GenBank L41346), only two bands of hybridization would be expected to the PstI-digested locus of the Sjasp gene if a single copy of the gene was present. Hybridization of a 32P-labeled BamHI/HindIII frag-
that an homologous gene is present in

Moreover, the Southern hybridization result demonstrated

of the aspartic proteinase gene in the schistosome genome.

with all three enzymes indicate that there is a single copy only

proteinase gene. Overall, the simple patterns of hybridization

these results suggest the presence of introns in the aspartic

OM4P, a peptide found to be an improved substrate for the

activity in Schistosoma Aspartic Proteinase (Fig. 3). We now show that the schistosome cathepsin L is active in the pH range 3.5–6.0, with an optimum pH of 5.5 (Fig. 4B). Therefore, we conclude that the aspartic proteinase and the cathepsin L have activity through overlapping ranges of pH. Similar aspartic proteinase and cathepsin L activities were detected in extracts of S. mansoni, and in ES from both S. japonicum and S. mansoni (not shown).

Hemoglobin Is Digested by Schistosome Proteinases—Hb was employed as a substrate for proteinase activity in schistosome extracts, and products of the digestion of Hb were analyzed by nonreducing SDS-polyacrylamide gel electrophoresis. Hb incubated without extract migrates as three bands that represent the whole molecule (64 kDa), a subunit dimer (32 kDa), and a subunit monomer (16 kDa) (Fig. 5A, lane 2). By contrast, Hb was completely degraded by proteinase activity in the schistosome extracts (4). Using Z-Phe-Arg-NHMe as substrate for proteinase activity in schistosome extracts against Phe-Ala-Ala-Phe(NO2)2-Phe-Val-Leu-OM4P, the pH optimum for activity against Phe-Ala-Ala-Phe(NO2)2-Phe-Val-Leu-OM4P was 3.5, a pH optimum typical of aspartic proteinases (6). The activity declined rapidly with increasing pH so that no activity was detectable at pH 5.5 (Fig. 4B).

We have previously observed cathepsin L activity in these schistosome extracts (4). Using Z-Phe-Arg-NHMe as substrate, we now show that the schistosome cathepsin L is active in the pH range 3.5–6.0, with an optimum pH of 5.5 (Fig. 4B) (4). Therefore, we conclude that the aspartic proteinase and the cathepsin L have activity through overlapping ranges of pH.

Aspartic Proteinase Activity in Schistosome Extracts and ES—A proteinase activity in the S. japonicum extracts deaved the peptide substrate Phe-Ala-Ala-Phe(NO2)2-Phe-Val-Leu-
whereas AEBSF and EDTA had no effect on its digestion (Fig. 5A, lanes 6 and 7). When Z-Phe-Ala-CHN2 was included in the assay, a band of ~10 kDa remained, indicating that this inhibitor partially blocked hemoglobinolysis (Fig. 5A, lane 5). The Hb-degrading activity showed a pH optimum of 3.5, which is the pH optimum of the aspartic proteinase, although cathepsin L-like activity also exhibits partial activity at this pH. However, degradation of Hb was still evident at pH 5.5, a pH close to the optimum of the cathepsin L but a pH at which the aspartic proteinase exhibits little or no activity (Fig. 5B). No degradation of Hb was observed at pH 6 or greater (not shown). Hb was digested by proteinases secreted in vitro into culture medium by adult S. japonicum (ES products). Addition of pepstatin or Z-Phe-Ala-CHN2 to the digestion assay totally blocked this partial digestion of Hb by ES, indicating the presence of both aspartic and cysteine proteinase activities in ES (Fig. 5C). Together, these Hb-digestion analyses indicate that S. japonicum extracts contain an aspartic proteinase activity that is capable of cleaving Hb, and the inhibition profile and pH optimum of the enzyme responsible is similar to the aspartic proteinase activity against Phe-Ala-Ala-Phe(NO2)-Phe-Val-Leu-OM4P, described above. In addition to this aspartic proteinase activity, the extracts appear to contain another activity against Hb, ascribable to a cysteine proteinase that was inhibited by Z-Phe-Ala-CHN2. Furthermore, ES also contained activities capable of digesting Hb, activities inhibited by pepstatin or Z-Phe-Ala-CHN2.

Bogitsh et al. (19) reported hemoglobinase activity in extracts of S. japonicum that was inhibited by pepstatin but was unaffected by leupeptin, an inhibitor of cysteine proteinases. In contrast, Chappell and Dresden (20) attributed the hemoglobinase activity in schistosome ES to a cysteine proteinase since it was inhibited by leupeptin. It is now apparent, based on our present results, that soluble extracts of S. japonicum contain both aspartic and cysteine proteinase activities, each of which can degrade Hb. In our inhibition analysis, the cathepsin L inhibitor Z-Phe-Ala-CHN2 partially blocked the digestion of Hb by activities in the crude parasite extract (as evidenced by the appearance of a 10-kDa fragment) and completely blocked Hb digestion by activities in the parasite ES. Our results are therefore consistent with the reports of both Bogitsh et al. (19) and Chappell and Dresden (20) and indicate that the cathepsin L-like proteinase may be comparatively more active or abundant in ES relative to crude extracts, suggesting that it functions in the schistosome gut.

In contrast to the cathepsin L-like activity, the aspartic proteinase may be more pervasive in schistosome tissue (18). Using anti-bovine cathepsin D sera, Bogitsh and Kirschner (21) localized a cathepsin D-like enzyme not only to the gastrodermis and cecal lumen but also to the dorsal tegument and tubercles of male schistosomes. Presumably, the aspartic proteinase activity that we have identified in ES is secreted from the gastrodermis or cecum. However, the distribution of aspartic proteinases in tissues distant from the gut (21) suggests that the aspartic proteinase may be expressed in diverse tissues, given that our Southern hybridization indicated that only a single copy of its gene was present in the schistosome genome. Thus, it is likely that the hemoglobinolytic activity we report here is encoded by the Sjasp transcript.

A Catalytic Pathway of Hemoglobin Degradation—It is likely that a series of proteolytic enzymes is involved in the digestion of Hb by schistosomes and that Hb is digested to short peptides in an ordered fashion (3, 22). Because other proteases besides aspartic proteinase are reputed to be involved in digestion of schistosomes, including the cathepsin B Sm31 and cathepsin L, and because these enzymes possess different pH optima, each of them may contribute to the catabolism of Hb (3, 23). Perhaps the falling pH of the blood meal, which is expected to occur during feeding by the schistosome (from the pH 7.3 of blood to the highly acidic pH of the schistosome gut (20)) might be the control mechanism for an ordered series of cleavages by these several proteinases. Initially, one proteinase may catalyze a defined (perhaps very restricted) scission, thereby exposing other sites to digestion. This situation obtains with Hb digestion in the malaria parasite Plasmodium falciparum; an aspartyl proteinase located in the acidic, digestive vacuole cleaves the α-chain of Hb between Phe-33 and Leu-34, after which other proteolytic activities digest the Hb fragments (24). If our hypothesis is correct, the schistosome aspartic proteinase would function in the latter stages of Hb-digestion rather than being responsible for the initial scission.

The aspartic proteinase described here, along with the cathepsin L-like activity that we reported previously (4), may comprise the acid proteinase activities originally described nearly four decades ago by Timms and Bueding (2) as being responsible for the digestion of Hb. Expressed recombinant active enzymes of both the aspartic proteinase and the cathepsin L can now be employed to define the precise mechanism of Hb digestion by schistosomes. In addition, these molecules may provide novel targets for drug and/or vaccine development.

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