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# *Fasciola hepatica:* Characterization and Cloning of the Major Cathepsin B Protease Secreted by Newly Excysted Juvenile Liver Fluke

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Wilson, L. R., Good, R. T., Panaccio, M., Wijffels, G. L., Sandeman, R. M., and Spithill, T. W. 1998. *Fasciola hepatica:* Characterization and cloning of the major cathepsin B protease secreted by newly excysted juvenile liver fluke. *Experimental Parasitology* **88,** 85–94. Proteolytic activity present in the excreted/secreted (ES) material of newly excysted juvenile (NEJ) *Fasciola hepatica* was biochemically analyzed. By gelatin substrate SDS–PAGE, only one region of activity was observed in the NEJ ES material at a molecular mass of 29 kDa. Both the secreted cathepsin L from adult fluke and the 29-kDa proteolytic activity of NEJ ES show a common pH optimum of 7.5, a cysteine protease inhibition profile, and preference for the *N*-benzyloxycarbonyl (Z)-Phe-Arg-NHMec fluorogenic substrate over Z-Arg-Arg-NHMec and Z-Arg-NHMec. *In vitro* analysis revealed that the NEJ protease activity digested sheep immunoglobulin heavy chain and bovine serum albumin but not bovine hemoglobin. Amino-terminal protein sequence analysis of the 29-kDa NEJ protease band revealed two sequences with homology to the cathepsin B family of proteases. Using degenerate oligonucleotides designed from the N-terminal sequence, reverse transcriptase polymerase chain reaction with NEJ RNA amplified a cDNA sequence encoding the first 236 amino acids of mature cathepsin B. Using this cDNA fragment an overlapping cDNA was isolated from a LambdaZAP cDNA library constructed with poly(A)$^+$ RNA from immature 5-week-old liver fluke. Together with the N-terminal sequence, these cDNAs predict a mature cathepsin B sequence of 254 amino acids which shows 48–51% sequence identity to mammalian and *Schistosoma mansoni* cathepsin B. We conclude that, in contrast to the major proteases released by adult fluke, the major secreted protease of NEJ of *F. hepatica* is of the cathepsin B class. 

*Index Descriptors and Abbreviations: Fasciola hepatica;* cathepsin B; protease; juvenile fluke; excretory/secretory; trematode; FhCAT-B, *Fasciola hepatica* cathepsin-B; BoCAT-B, bovine cathepsin-B; *Z-N*-benzyloxycarbonyl; NHMec, 7-amido-4-methylcoumarin; PCR, polymerase chain reaction; ES, excretory/secretory; SSC, standard saline citrate; Hb, hemoglobin; Ig, immunoglobulin; BSA, bovine serum albumin; SDS–PAGE, sodium dodecyl sulfate–polycrylamide gel electrophores; DTT, dithiothreitol.

## INTRODUCTION

The activity of proteases secreted by adult *Fasciola hepatica* has been known since at least 1965 when Thorsell and Bjorkman showed the lytic affect of fluke secretions on gelatin (Thorsell and Bjorkman 1965). Since then, *F. hepatica* proteases of both secreted and somatic origin have been shown to have activity against a variety of natural substrates such as gelatin (Thorsell and Bjorkman 1965; Dalton and Heffernan 1989), hemoglobin (Howell 1966; Hadju *et al.* 1979; Rupova and Keilova 1979; Klimenko 1980; Aoki *et al.* 1983, Coles and Rubano 1988), collagen (Howell 1966, Simpkin *et al.* 1980), immunoglobulin (Howell 1966, Locatelli and Paoletti 1969; Chapman and Mitchell 1982; Heffernan *et al.* 1991; Smith *et al.* 1993a), globin, and albumin (Locatelli and Paoletti 1969). Most *F. hepatica* proteases

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belong to the cathepsin L group of the cysteine protease family, based on a requirement for cysteine or reducing conditions to maintain activity (Halton 1967; Hadju *et al.* 1979; Chapman and Mitchell 1982; Dalton and Heffernan 1989; Smith *et al.* 1993a), inhibition by cysteine protease inhibitors (Aoki *et al.* 1983; Chapman and Mitchell 1982; Dalton and Heffernan 1989; Rege *et al.* 1989; Yamasaki *et al.* 1989; McGinty *et al.* 1993; Wijffels *et al.* 1994a), and amino acid and nucleotide sequence homology (Dowd *et al.* 1993; Yamasaki and Aoki 1993; Smith *et al.* 1993b; Wijffels *et al.* 1994a; Heussler and Dobbelaere 1994). Cathepsin B fragments have previously been amplified from adult *F. hepatica* RNA (Heussler and Dobbelaere 1994) and a protein with N-terminal sequence homology to cathepsin B has been identified in somatic extracts of NEJ (Tkalcevic *et al.* 1995). McGinty *et al.* (1993), using active site affinity labels, suggested that the adult and immature fluke ES cathepsin activity was cathepsin B-like. Creaney *et al.* (1996) have localized cathepsin B to the gut of NEJ.

Protease activity of the immature mammalian stage of *F. hepatica* has generally been demonstrated to be similar to the activity of the adult fluke stage, although some differences have been observed (Howell 1966; Dalton and Heffernan 1989; McGinty *et al.* 1993; Carmona *et al.* 1993). Activity of a 40-kDa protein on gelatin substrate SDS–PAGE is upregulated in the ES extracts of 5-week-old liver flukes (Dalton and Heffernan 1989). Carmona *et al.* (1993) showed further that protease(s) secreted by NEJ *in vitro* has activity at 52, 58, 76, 87 kDa on gelatin-substrate SDS–PAGE. Here we report the biochemical characterization of the major protease activity in the ES material of NEJ of *F. hepatica* and show by amino-terminal sequencing and cDNA cloning that this protease is a cathepsin B.

## MATERIALS AND METHODS

*Production of NEJ and NEJ ES.* *F. hepatica* metacercariae were obtained from Compton Paddock Laboratories, UK (VIAS strain), and Baldwin Aquatics, USA (Oregon strain) and stored at 4°C. Prior to excystment metacercariae were incubated at room temperature overnight and then gently removed from cellophane sheets with a fine brush and centrifuged at 1000*g* for 5 min. Metacercariae were resuspended in 1% (w/v) pepsin A (from porcine stomach mucosa, Sigma) in 0.2% (v/v) HCl and incubated for 1 h at 37°C. Between this and subsequent incubations metacercariae were centrifuged at 1000*g* for 5 min, washed with $H_2O$, and recentrifuged at 1000*g* for 5 min. The metacercariae were incubated for 1 h at 37°C in a solution of 0.02 M sodium hydrosulfite (sodium dithionite, Sigma) in $H_2O$, which had been bubbled with $CO_2$ for 30 s. Metacercariae were washed and incubated for 2 h at 37°C on a rocking platform in 10 ml RPMI 1640 medium (Sigma) supplemented with 2 $\mu$g ml$^{-1}$ amphotericin B (Squibb, Princeton, NJ) 10 $\mu$g ml$^{-1}$ gentamycin (Sigma), and either 10% (v/v) filter-sterilized sheep bile or 0.2% (w/v) taurocholic acid (Sigma). NEJ were separated from cysts and debris according to the method of Tielens *et al.* (1981). NEJ ES was collected by incubating NEJ in RPMI 1640 medium supplemented with amphotericin B (2 $\mu$g ml$^{-1}$) (Squibb) and gentamycin (10 $\mu$gml$^{-1}$) (Sigma) at 37°C in an atmosphere containing 5% (v/v) $CO_2$. NEJ were cultured in 96-well plates (Nunc) at a density of 3 $\mu$l$^{-1}$ and the medium was replaced every 2 h for 60 h.

*Collection of 5-week-old and adult liver fluke.* Adult fluke (14-week-old) and adult fluke ES were collected as previously described (Wijffels *et al.* 1994b). Five-week-old immature fluke were obtained from sheep after intraruminal infection with metacercariae suspended in 0.4% (w/v) carboxymethyl cellulose (BDH) or from rats infected by intragastric inoculation of metecercariae in water. Following slaughter of the sheep or rats, the livers were recovered and sliced, and the tissue was homogenized for 3 min at 4°C in PBS in a Colworth 400 Stomacher. Liver tissue was then washed through two sieves of 2 mm and 250 $\mu$m mesh. The 5-week-old fluke were between 2 and 5 mm long and were caught by the 250-$\mu$m screen.

*Fluorogenic protease assays.* Fluorogenic protease assays were performed essentially as described by Barrett (1980) with the reaction volume reduced to 20 $\mu$l and the substrate concentration increased to 80 $\mu$M. Assays were performed on a Leitz MPV DIAVERT inverted microscope photometer, equipped for incident light fluorescent measurements, coupled to a Hewlett Packard HP68B computer and a motorized platform. Fluorogenic protease assays were performed in triplicate in a total volume of 20 $\mu$l (2 $\mu$l substrate, 18 $\mu$l enzyme and buffer) in 60-well Terasaki plates (Nunc) and incubated at 37°C. The xenon light source was filtered with a Leica A2 filter (270–380 nm) and the emission light was filtered with a 437$\pm$23-nm filter. To determine the optimal pH of enzymatic activity NEJ ES ($\approx$20 ng protease), purified adult fluke secreted cathepsin L (40 ng) (Wijffels *et al.* 1994a) and papain (150 ng) (Sigma) were assayed using the fluorogenic substrates Z-Phe-Arg-NHMec, Z-Arg-Arg-NHMec, and Z-Arg-NHMec. A phosphate buffer (pH 5.8–8.2) was used as described (Wijffels *et al.* 1994a).

*Inhibition of protease activity with class specific inhibitors.* Inhibition of the protease activity of the ES material of NEJ and adult fluke was examined using 2.7 U ml$^{-1}$ aprotinin (Sigma), 1 $\mu$M pepstatin (Sigma), 1 $\mu$M L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E64) (Sigma), 1 mM ethylenediaminetetraacetic acid (EDTA) as described by Beynon and Bond (1989). Papain (Sigma) and trypsin (Sigma) were also assayed at final concentrations of 1 and 0.1 $\mu$g ml$^{-1}$, respectively. Protease inhibitors (2 $\mu$l) were added to 16 $\mu$l of enzyme/buffer and incubated at room temperature for 30 min prior to the addition of substrate (2 $\mu$l).

*Cleavage of naturally occuring substrates.* Equal molar concentrations (0.5 nM) of bovine hemoglobin (Hb, Calbiochem), bovine serum albumin (BSA, Sigma), or protein-G purified sheep Ig were incubated with a constant amount of ES material of NEJ and adult *F. hepatica.* All assays were made up to 0.18 ml with PBS (pH 7.4) and included 10 mM dithiothreitol (DTT). The amount of ES material used was adjusted empirically to give equivalent partial digestion of BSA after a pilot experiment using several dilutions of ES. Cleavage reactions were incubated at 37°C for 10 min and then rapidly frozen to $-70$°C to prevent further activity. Samples were thawed in the presence of an equal volume of reducing sample buffer, heated to 100°C for 3 min,

and loaded immediately onto a 16% (w/v) tricine-buffered SDS–PAGE gel. After electrophoresis, proteins were stained with Coomassie blue.

*SDS–PAGE and gelatin substrate gels.* SDS–PAGE was based on the method described by Laemmli (1970) and tricine-buffered SDS–PAGE was employed following the method of Schagger and von Jadow (1987). Gels were silver stained according to the method of Morrissey (1981). Gelatin substrate gels were performed essentially as described (Wijffels *et al.* 1994b) at pH 4.5 in 15% (w/v) SDS–PAGE gels using gelatin (0.1% (w/v), type A from porcine skin, 300 bloom, Sigma Catalog No. G2500). Samples for gelatin substrate gels were mixed with an equal volume of nonreducing SDS–PAGE sample buffer (0.0625 mM Tris, pH 6.8 [HCl], 2.3% (w/v) SDS, 10% (v/v) glycerol) and loaded onto the gel without boiling.

*Amino terminal sequencing of NEJ protease.* Six thousand NEJ were cultured in RPMI 1640 at 37°C in a humidified atmosphere of 5% (v/v) $CO_2$. The culture medium was collected and concentrated by microdialysis with the MicroProDicon Model 120 system (Spectrum, TX). ES proteins were separated by 15% SDS–PAGE and transferred for 1 h at 100 V to polyvinylidene difluoride membrane (Immobilon-P, Millipore) (Ploug *et al.* 1989). The membrane was stained with Coomassie blue for 2 min and destained in 50% (v/v) methanol. After the membrane had dried the 29-kDa protein band was excised and sequenced at the Baker Medical Research Institute Protein Sequencing Facility (Melbourne, Australia).

*RNA preparation and reverse transcriptase PCR (RT-PCR) of* F. hepatica *cathepsin B.* Total RNA was extracted from 35,000 NEJ (Oregon Strain) and from one-half of an adult liver fluke, freshly recovered from an infected sheep liver, using the Ultraspec RNA reagent as described by the manufacturer (Biotecx Laboratories, TX). Total RNA was extracted from 5-week-old liver fluke by the method of Chirgwin *et al.* (1979). For RT-PCR, the following reagents were added to a 0.5-ml tube (numbers in brackets indicate stock concentrations): 10 μl dNTPs (10 mM dATP, dCTP, dGTP, dTTP) (Promega), 2.5 μl $H_2O$, 0.25 μl rRNasin (40 U $\mu l^{-1}$) (Promega), 0.25 μl murine leukemia virus (M-MLV) reverse transcriptase (200 U $\mu l^{-1}$) (Promega), 4 μl 5× M-MLV reverse transcriptase buffer (Promega), 1 μl 3′oligonucleotide (oligo(dT)) (1.0 μM), 2 μl total RNA (200 ng). This mixture was heated to 42°C for reverse transcription for 15 min and then to 99°C for 5 min to inactivate the reverse transcriptase. The following were then added: 8 μl of 10× PCR buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris, pH 9 (HCl), 71 μl $H_2O$, 1 μl (5 units) Tth DNA polymerase (Tyobo), 1 μl 5′oligonucleotide (1.5 mM). This was then incubated on a Perkin–Elmer 9600 thermal cycler with one cycle of 95°C for 30 sec, 30°C for 30 sec, 72°C for 30 sec, one cycle of 95°C for 30 sec, 40°C for 30 sec, 72°C for 30 sec and 40 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 30 sec. In order to amplify the transcript encoding the 29-kDa NEJ cathepsin B, degenerate oligonucleotide primers were designed to target the amino-terminal sequence of cathepsin B: (NEJNTERMF1, C(ACGT)GA(AG)TC(ACGT) TT(TC)GA(TC)GC; NEJNTERMF2, CC(ACGT)GA(AG)AG(CT) TT(TC)GA(TC)GC).

*pCR-script blunt end cloning of PCR product.* RT-PCR products were separated by electrophoresis on low-melting-temperature agarose gels and the band of interest was excised and extracted using the Wizard DNA binding resin (Promega). RT-PCR products were cloned into the pCR-Script plasmid vector and transformed into Epicurian Coli XL1-blue cell using the pCR-Script kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The insert cDNA was sequenced using the T7 DNA polymerase kit (Pharmacia).

*Construction and screening of λZAP II cDNA library with cathepsin B cDNA fragment.* A lambdaZAP II phage library was constructed (Clontech, USA) using RNA from 5-week-old liver fluke. Phage were plated out at 25,000 PFU per plate. Filter lifts were made with nylon filters (Hybond-N, Amersham). The adherent nucleic acids were then denatured for 5 min (0.5 M NaOH, 1.5 M NaCl), bound to the filters by exposure to UV light for 2 min, neutralized in 0.5 M Tris, pH 8.0 (HCl), 1.5 M NaCl for 5 min, and washed in 2× SSC. Filters were hybridized at 42°C in hybridization buffer (50% (v/v) formamide, 5× SSC, 0.5% (w/v) SDS, 0.125% (w/v) skim milk powder) for 2 h prior to the addition of the random primer-labeled cathepsin B insert cDNA probe (pNPOH2) and hybridized overnight at 42°C. Filters were then washed at 42°C in 1× SSC and autoradiographed. Positive plaques were identified and phagemids were rescued using the protocol described in the Stratagene LambdaZAP II kit.

## RESULTS

*Biochemical analysis of protease activity.* NEJ ES and purified cathepsin L protease from adult fluke ES (Wijffels *et al.* 1994a) were assayed for protease activity using the synthetic substrate Z-Phe-Arg-NHMec. Using phosphate buffer over the range pH 5.8 to 8.2, the activity of the NEJ protease and adult liver fluke cathepsin L was optimal at approximately pH 7.5 (data not shown). In order to determine the classification of the protease activity in NEJ ES, the effect of several class specific inhibitors on protease activity was studied. The proteolytic activities of papain, adult ES, and NEJ ES material were inhibited >98% by the cysteine protease inhibitor E64 and in the absence of the reducing agent DTT. The protease activity of NEJ ES material was not inhibited by the serine class inhibitor, aprotinin, the aspartic protease inhibitor, pepstatin, or the metallo class inhibitor, EDTA. The substrate specificity of the adult liver fluke ES and NEJ ES proteases was assayed using the fluorogenic substrates Z-Phe-Arg-NMec, Z-Arg-Arg-NMec, and Z-Arg-NMec. The protease activity of both adult liver fluke ES and NEJ ES was significantly greater for the Z-Phe-Arg-NMec substrate over the Z-Arg-Arg-NMec or Z-Arg-NMec substrates (data not shown).

Having shown that NEJ ES and adult liver fluke ES proteases had similar activities with peptide substrates, it was of interest to characterize the cleavage of several natural substrates. BSA, bovine Hb, and sheep Ig were chosen as representative of substrates that would be encountered by the fluke during invasion. Tricine-buffered SDS–PAGE analysis of the cleavage profiles revealed that the cleavage of BSA by NEJ ES was different than that achieved with adult liver fluke ES (Fig. 1A). Cleavage of BSA (67 kDa) by NEJ ES resulted in a unique protein fragment of 43 kDa, whereas

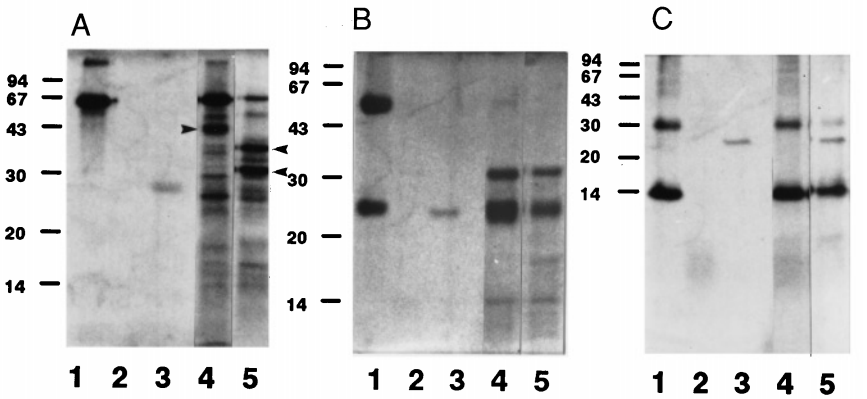



**FIG. 1.** Cleavage of natural substrates by the secreted proteases of adult and NEJ *F. hepatica.* (A) Bovine serum albumin. (B) Immunoglobulin. (C) Hemoglobin. Lanes 1, substrate alone; 2, NEJ ES alone; 3, adult ES alone; 4, NEJ ES + substrate; 5, adult ES + substrate. Arrowheads indicate unique cleavage products. The mobility of molecular mass standards is indicated beside each panel.

the cleavage of BSA with adult ES gave unique protein fragments of 32 and 38 kDa (Fig. 1A, lanes 4 and 5). The breakdown of Ig was similar using ES of both liver fluke life-cycle stages, with cleavage activity being primarily directed at the Ig heavy chain (Fig. 1B). Under the conditions of this assay neither adult nor NEJ ES protease showed detectable activity against bovine Hb (14 kDa) (Fig. 1C). This result was confirmed in three separate experiments.

*Gel analysis of ES material.* NEJ and adult fluke ES proteins were separated by 15% SDS–PAGE and silver stained. Under reducing conditions, major protein bands of 29 and 34 kDa were present in NEJ ES (Fig. 2A, lane 1). In adult fluke ES the 28-kDa cathepsin L protease doublet is a prominent component among a number of proteins, as previously described (Wijffels *et al.* 1994a; Smith *et al.* 1993b). Gelatin substrate SDS–PAGE also revealed differences between the NEJ and the adult ES material. A region of protease activity at about 28 kDa was present in NEJ ES which corresponded with the 29-kDa protein seen on the reduced silver-stained material. In contrast, the gelatin-digestion activity of adult fluke ES was a 26- to 27-kDa doublet and a ladder between 35 and 67 kDa, as previously described (Wijffels *et al.* 1994a, b; Smith *et al.* 1993b). In order to confirm the parasite origin of the 29-kDa NEJ protease, metacercariae were excysted using taurocholic acid in place of sheep bile. These NEJ were cultured and the ES material was analyzed by gelatin-substrate SDS–PAGE. The profile was identical to that shown in Fig. 2A (data not shown).

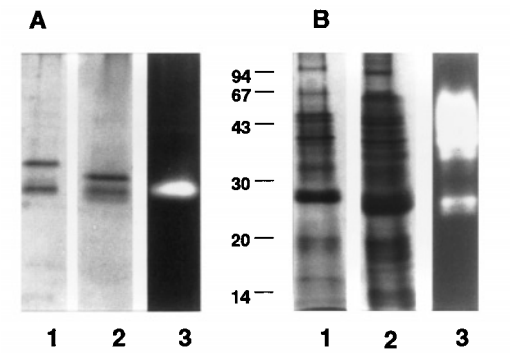



**FIG. 2.** SDS–PAGE analysis of NEJ and adult ES material. (A) NEJ ES. (B) Adult ES. Lanes 1, silver stained SDS–PAGE (reducing); 2, silver stained SDS–PAGE (nonreducing); 3, gelatin substrate SDS–PAGE (nonreducing). The mobility of molecular mass standards is indicated.

*Protein sequencing and cloning of NEJ ES protease.* The sequence of the first eight amino-terminal residues of the 29-kDa protease secreted by NEJ liver fluke was obtained. Two polymorphisms were revealed at residues 1 and 3. Comparison of the two amino-terminal sequences with the databases showed that the protease found in NEJ ES belongs to the cathepsin B family (Fig. 3). The 29-kDa NEJ ES protein sequence shows 100% identity to bovine and rat cathepsin B, 87% identity to mouse, human, and *Leishmania* cathepsin B, 50% identity to the cathepsin B of *Schistosoma mansoni* and *Schistosoma japonicum,* and only 37–50% identity to the cathepsin L of *F. hepatica* (Fig. 3). No cathepsin L-like N-terminal sequence was obtained from this 29-kDa band, suggesting that the band is homogeneous cathepsin B. The major 29-kDa amino-terminal sequence is identical to that described for a somatic cathepsin B of *F. hepatica* (Tkalcevic *et al.* 1995).

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **NEJ Protease** | | L | P | E | S | F | D | A | R | |
| | | V | | A | | | | | | |
| Bovine cathepsin B (L06075) | | L | P | E | S | F | D | A | R | 8 |
| Rat cathepsin B (M11305) | | L | P | E | S | F | D | A | R | 8 |
| Mouse cathepsin B (M14222) | | L | P | E | T | F | D | A | R | 7 |
| Human cathepsin B(L16510) | | L | P | A | S | F | D | A | R | 7 |
| *Leishmania mexicana* cathepsin B | | L | P | E | S | F | D | A | S | 7 |
| papain (M15203) | | I | P | E | Y | V | D | W | R | 4 |
| *Schistosoma mansoni* Cat B (M21309) | E | I | P | S | N | F | D | S | R | 4 |
| *Schistosoma japonicum* Cat B (X70968) | E | I | P | S | Q | F | D | S | R | 4 |
| Human cathepsin L (M20496) | | A | P | R | S | V | D | W | R | 4 |
| Rat cathepsin L (Y00697) | | I | P | K | T | V | D | W | R | 3 |
| Adult *F. hepatica* 1 | | V | P | E | D | I | D | W | R | 4 |
| Adult *F. hepatica* 2 | A | V | P | D | K | I | D | P | R | 3 |

**FIG. 3.** Amino terminal sequence of the NEJ protease and alignment with homologous proteases from other species. Identical residues are shaded. Numbers in brackets indicate the GenBank accession numbers. Other sequences are taken from Robertson and Coombs (1993), *L. mexicana;* Wijffels *et al.* (1994a), adult *F. hepatica* 1; Smith *et al.* (1993b), adult *F. hepatica* 2.

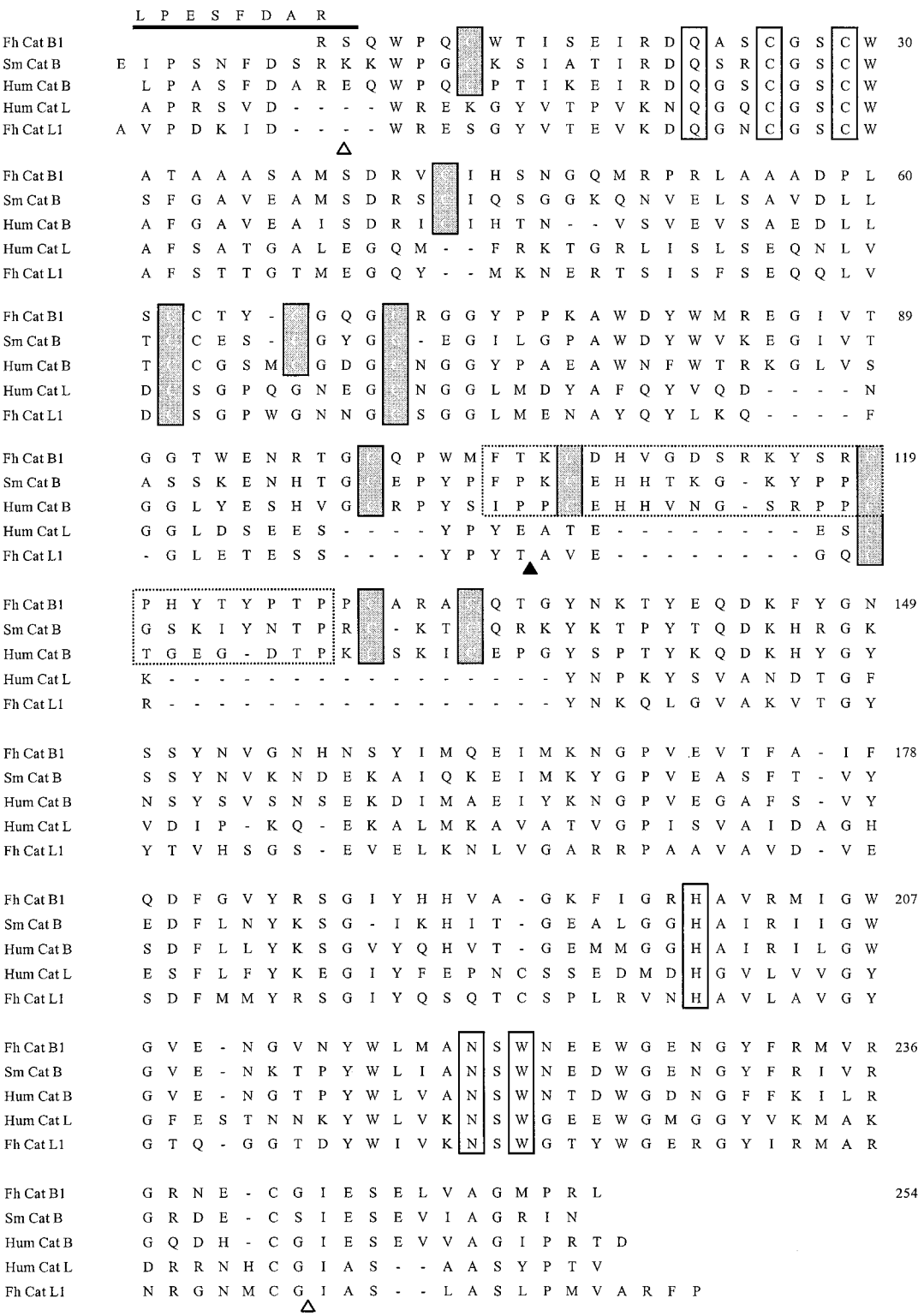
A *F. hepatica* NEJ cathepsin B transcript was amplified by RT-PCR on NEJ total RNA (Oregon strain) using oligonucleotide primers designed to target DNA encoding the amino-terminal sequence of cathepsin B in conjunction with an oligo(dT) primer. Analysis of the RT-PCR products by agarose electrophoresis revealed a band of approximately 700 bp which was cloned into the pCR-script plasmid and designated pNPOH2. We believe that the N-terminal oligo fortuitously bound to a complementary sequence on the antisense strand and therefore primed both the forward and the reverse reactions of the PCR. DNA sequencing of the pNPOH2 clone predicted an open reading frame of 708 nucleotides encoding 236 amino acid residues (data not shown). The deduced amino acid sequence showed high similarity with cathepsin B proteases of *S. mansoni* and mammals.

*Cloning of cDNA encoding the mature cathepsin B of* F. hepatica. In order to obtain a full-length cathepsin B transcript a cDNA library was constructed from 5-week-old liver fluke RNA (VIAS strain); RNA of 5-week-old fluke was more readily attainable than NEJ RNA. Moreover, we have found that the 29-kDa cathepsin B band is expressed by immature fluke until 5 weeks postinfection (data not shown, Wilson 1994). The cathepsin B-encoding insert of plasmid pNPOH2 was excised, labeled with $^{32}$P, and used to screen 150,000 PFU of the 5-week-old fluke cDNA library. One clone encoding cathepsin B sequences (pTPZA4) was obtained. The sequence of the pTPZA4 insert overlapped by 409 bp with the previously obtained pNPOH2 sequence and extended in the 3′ direction into the untranslated region to the poly(A)tail. The 3′ untranslated region consists of 84 bp and included a polyadenylation signal motif (data not shown, see GenBank Accession No. U58000).

A combination of the amino terminal protein sequence of the NEJ ES protease and the predicted protein sequences of the overlapping fragments pNPOH2 and pTPZA4 permitted the elucidation of the predicted complete sequence of the mature processed *F. hepatica* cathepsin B protease (Fig. 4, FhCatB1). The predicted protein has 254 amino acids which encode a protease of 28,790 Da. This sequence was aligned with other cathepsin protease sequences and the percentage identity calculated using the program CLUSTAL. The level of identity is greatest with schistosome and mammalian cathepsin B sequences (48–51%). Lower identity was observed with mammalian cathepsin L and the cathepsin L sequences previously identified from *F. hepatica* (19–23%). Heussler and Dobbelaere (1994) have sequenced two incomplete cathepsin B cDNA fragments from adult *F. hepatica.* Comparison of these sequences with the FhCatB1 translated sequence revealed 33% identity with the predicted translation product of the 500-bp fragment (FHCATHPRF) and 60% identity with the 312-bp fragment (FHCATHPRH) (Heussler and Dobbelaere 1994). This suggests that FhCatB1 from NEJ is a separate gene product from the adult cathepsin B fragments. The FhCatB1 sequence exhibits the conserved features of known members of the cathepsin B family including the active site residues Q23, C26, C29, H200, N220, and W222 as well as the occluding loop at residues 104 to 127 (Fig. 4) (Dufour 1988).

## DISCUSSION

Here we describe the isolation, biochemical characterization, and cloning of the major secreted protease of juvenile


L P E S F D A R
Fh Cat B1
Sm Cat B
Hum Cat B
Hum Cat L
Fh Cat L1
30
60
89
119
149
178
207
236
254

*F. hepatica* and show that this protease is a cathepsin B. The activity of NEJ ES cathepsin B and the cathepsin L secreted by adult fluke were found to have many attributes in common. These proteases were found to belong to the cysteine class of proteases, have similar pH optima and activity against the fluorescent substrate Z-Phe-Arg-NMec, were shown to cleave sheep immunoglobulin heavy chain *in vitro,* and showed minimal *in vitro* activity against bovine hemoglobin. The NEJ and adult ES proteases differed in the pattern of cleavage of BSA and their apparent size in SDS–PAGE and gelatin substrate gels.

Amino-terminal sequencing of the NEJ ES protease revealed two similar sequences, of which the major sequence was identical to the somatic cathepsin B sequence identified by Tkalcevic *et al.* (1995). The sequences were also homologous to the mammalian cathepsin B family of proteases and with the cathepsin B-like proteases of *Schistosoma* (Klinkert *et al.* 1989) and *Leishmania mexicana* (Robertson and Coombs 1993). This suggests that NEJ of *F. hepatica* express at least two related cathepsin B sequences.

cDNA fragments partially encoding a cathepsin B protease were obtained by RT-PCR from NEJ RNA and from a 5-week old fluke cDNA library. The sequence obtained from the overlapping cathepsin B transcripts, in combination with the amino-terminal protein sequence, enabled the complete sequence of the mature cathepsin B protease to be deduced. This sequence predicted a protein of 28,790 Da which correlates closely with the observed size on denaturing SDS–PAGE gels of the native protein of 29 kDa. The three-dimensional structure of the *F. hepatica* cathepsin B protease may now be deduced by extrapolation from the coordinates obtained from the X-ray crystallography of human cathepsin B (Musil *et al.* 1991). This structure might then be used to choose active protease inhibitors that could be used as novel anthelmintics specific for the *F. hepatica* cathepsin B.

The two cathepsin B sequences isolated from adult fluke RNA described by Heussler and Dobbelaere (1994) are significantly different to the NEJ FhCatB1 sequence, indicating that these three transcripts are the products of different genes. The results of the present study suggest that cathepsin B is the predominant cathepsin protease released by NEJ. In contrast, cathepsin L is the predominant secretory enzyme of the adult liver fluke (Smith *et al.* 1993b; Yamasaki and Aoki 1993; Wijffels *et al.* 1994a). The demonstration of cathepsin B in the parenchymal tissues of adult fluke may indicate that this class of enzyme has a general role in metabolism of the mature parasite (Creaney 1996). Expression of different classes of secreted cathepsin protein by different developmental stages has been observed in several other parasite species including *Schistosoma* (McKerrow and Doenhoff 1988) and suggests that these secreted enzymes may perform stage-specific functions, such as degradation of different tissue barriers of contrasting protein composition. It has previously been indicated that the two histidine residues of the cathepsin B insertion loop are responsible for the exopeptidase activity of cathepsin B (Musil *et al.* 1991). Analysis of the sequence presented here and the two sequences presented by Heussler and Dobbelaere (1994) reveals that they contain one, zero, and two histidines, respectively. This may represent a functional difference between these molecules in *F. hepatica*.

In a previous study, anti-BoCat B sera detected cathepsin B in electron-dense granules within the gut epithelium of NEJ and within the parenchyma, but *not* the gut, of adult fluke (Creaney *et al.* 1996; Creaney 1995). Cathepsin L-like proteases secreted by adult fluke have previously been localized to granules of the gut epithelia and to the gut lumen (Yamasaki *et al.* 1989; Smith *et al.* 1993b; Yamasaki *et al.* 1992). These granules are morphologically similar to the electron-dense granules which react with anti-BoCat-B in NEJ (Creaney *et al.* 1996). In the adult fluke these granules are not lysosomes (Yamasaki *et al.* 1992) and probably function as secretory organelles in the secretory phase of the adult gut (Thorsell and Bjorkman 1965; Robinson and Threadgold 1975).

In NEJ, the principal function of secreted cathepsin B may be to assist in parasite invasion and migration since cathepsin B proteases secreted by macrophages, osteoclasts, and malignant cancer cells function in invasion of host tissue (Maciewicz *et al.* 1989; Honn *et al.* 1994). The NEJ cathepsin B may not function as a digestive enzyme since the juvenile fluke intestine has only a secretory function, not the dual absorptive/secretory function seen in the adult fluke (Bennett and Threadgold 1973). In addition, NEJ rely upon

**FIG. 4.** Alignment of the deduced *F. hepatica* cathepsin protease sequence (Fh Cat B1) with related cathepsin proteases. Underlined sequence is the amino terminal sequence. □, Active site residues; □, residues involved in disulfide bonding; □, residues of the cathepsin B occluding loop. Numbers refer to the *F. hepatica* cathepsin B sequence. Open triangles denote start and finish of clone pNPOH2. Closed triangle indicates start of clone pTPZA4 which continues until the poly(A)tail. GenBank Accession Nos.: Sm Cat B (*S. mansoni* cathepsin B) M21309; Hum Cat B (Human cathepsin B) L16510; Hum Cat L (Human cathepsin L) M20496; Fh Cat L1 (*F. hepatica* cathepsin L) L33771.

stored glycogen reserves during the early migratory phase rather than upon feeding (Bennett and Threadgold 1973, 1975). Indeed, the gut does not develop into an absorptive organ until the fluke reaches the liver of the host (Bennett and Threadgold, 1975). The presence and gut localization of a similar protease in *Caenorhabditis elegans* (Ray and McKerrow 1992) serves as a reminder that gut-associated proteases were not adapted specifically for parasitism. The NEJ cathepsin B may play a role in immune evasion through the cleavage of host antibodies (Chapman and Mitchell 1982; Smith *et al.* 1993a) and possibly also through the prevention of attachment to NEJ by immune effector molecules as described by Carmona *et al.* (1993). Other possibilities are that the cathepsin B protease aids the sloughing of the tegumental glycocalyx which then acts as an immunological "smoke screen" (Lammas and Duffus 1983) or is involved in excystment of the metacercariae (Chung *et al.* 1995). Proteases secreted by Schistosome cercariae have been shown to promote the sloughing of the glycocalyx which would otherwise render the invading cercariae vulnerable to complement attack (Marikovsky *et al.* 1988).

The high level of expression of the cathepsin B in the mammalian tissue invasive stage of *F. hepatica* indicates that it may be important for successful parasitism. The further study of this protease will reveal valuable insights into the host–parasite interaction and may lead to control of the parasite through the inhibition of protease activity by immunological or chemical means.

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*Note added in proof.* The cloning of two cathepsin L cDNA sequences from adult *F. hepatica* has recently been reported.

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