Hookworm cathepsin D aspartic proteases: contributing roles in the host-specific degradation of serum proteins and skin macromolecules

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SUMMARY

Cathepsin D aspartic proteases of hookworms were recently implicated in the host-specific digestion of haemoglobin by adult parasites. *Ac*-APR-1 from the dog hookworm, *Ancylostoma caninum* and *Na*-APR-1 from the human hookworm, *Necator americanus*, were shown to be expressed in the infective larval stage (L3) as well as adult worms. We now show that both proteases degraded skin macromolecules and serum proteins, some of which were cleaved more readily from permissive definitive hosts as opposed to non-permissive hosts. *Na*-APR-1 degraded human collagens more efficiently than did *Ac*-APR-1, and *Ac*-APR-1 degraded canine serum albumin more efficiently than did *Na*-APR-1. On the other hand, both enzymes degraded human serum proteins (albumin and fibrinogen) with approximately equal efficiency under the conditions of our assays *in vitro*. Molecular models of these 2 orthologous, aspartic proteases showed that, despite having active site clefts with identical primary sequences, residues in the S3 pocket adopted different conformations, likely accounting for different substrate preferences reported previously. Antisera raised to both proteases partially inhibited (16–26%) migration of hookworm L3 through hamster skin *in vitro*, further implying a connective tissue invasive role for these enzymes in addition to digestion of serum and erythrocyte proteins for nutrition.

Key words: aspartic protease, haemoglobin, albumin, collagen, hookworm, host specificity.

INTRODUCTION

Hookworms are voracious blood feeders that infect more than 1 billion people (Crompton, 1999). Proteolytic enzymes of various mechanistic classes are abundant in larval and adult hookworm extracts and secretions, and some of these activities are associated with digestion of haemoglobin (Hb) (Williamson *et al*. 2002; Brown *et al*. 1999). We recently reported the cloning and expression of orthologous cathepsin D-like aspartic proteases from the canine hookworm *Ancylostoma caninum* and the human hookworm *Necator americanus* (Williamson *et al*. 2002). Aspartic proteases play a pivotal role in the digestion of Hb by schistosomes (Brindley *et al*. 2001) and *Plasmodium* (Francis, Sullivan & Goldberg, 1997), and we showed that the 2 forms of hookworm cathepsin D, *Ac*-APR-1 from *A. caninum* and *Na*-APR-1 from *N. americanus*, rapidly degrade Hb in a host-specific fashion i.e. recombinant *Ac*-APR-1 was twice as efficient as *Na*-APR-1 at digesting dog Hb, and conversely, recombinant *Na*-APR-1 was twice as efficient as *Ac*-APR-1 at digesting human Hb (Williamson *et al*. 2002).

A. caninum will not sexually mature in humans and *N. americanus* will not develop to patency in dogs. As such, the elevated species-specific affinity of hookworm cathepsin D for human or canine Hb provides molecular evidence for the co-evolution of a parasite enzyme and its host protein substrate. A contribution to the evolution of the host species range of hookworms (and indeed of other haematophagous parasites) is an implication of this finely tuned interplay between haemoglobin-degrading proteases of hookworms and their natural substrates, mammalian haemoglobins (Brinkworth *et al*. 2000).

Helminth proteases facilitate the invasion of host tissues (Salter *et al*. 2000), digest host proteins as a food source (Brindley *et al*. 2001; Lipps, Fullkrug & Beck, 1996; Williamson *et al*. 2002) and selectively skew the host immune response (O'Neill, Mills & Dalton, 2001; Prowse *et al*. 2002). Unlike their vertebrate, lysosomal counterparts, many acidic proteases of parasitic worms are excretory/secretory (ES)

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products, and function outside of the cell where they interact with host tissues. Infective 3rd-stage larvae (L3) of hookworms secrete hydrolytic enzymes that are thought to facilitate skin penetration and tissue migration. During the transition from free-living larvae to parasitism, host cues induce *A. caninum* L3 to release specific proteins including the cysteine-rich *Ancylostoma* Secreted Proteins, ASP-1 and -2 (Hawdon & Hotez, 1996) and at least 2 hydrolytic enzymes including hyaluronidase (Hotez *et al.* 1992) and a zinc metalloprotease (*Ac*-MTP-1) (Zhan *et al.* 2002). These enzymes are thought to facilitate skin penetration, tissue migration, or act as an ex-sheathing enzyme since metalloprotease activity has been shown to mediate exsheathment in *Haemonchus contortus* (Gamble, Purcell & Fetterer, 1989) and moulting in *Brugia pahangi* (Hong *et al.* 1993). Hookworm larvae also secrete aspartic protease activity that is capable of digesting skin macromolecules. Furthermore, inhibition of aspartic protease activity with the inhibitor pepstatin A, decreases larval migration through skin (Brown *et al.* 1999). As such, inhibition of their function *in vivo* offers a potentially useful strategy for the development of effective vaccines and chemotherapeutic agents against hookworm infection.

Ac- and *Na*-APR-1 mRNAs were expressed in L3 and adult *A. caninum* and *N. americanus* (Williamson *et al.* 2002), indicating that the proteases likely play roles other than feeding during the hookworm life-cycle. The aim of this study was to evaluate the ability of hookworm forms of recombinant cathepsin D to degrade skin macromolecules and serum proteins from permissive and non-permissive host species and determine whether the proteases cleaved substrates other than Hb in a host-specific manner. We also explored the molecular basis of the host-specific cleavage of substrates using comparative modelling of the active site clefts of the 2 forms of the enzyme.

MATERIALS AND METHODS

Parasites

Third-stage infective *A. caninum* larvae were isolated from vermiculite coprocultures (Hawdon *et al.* 1995) maintained at George Washington University (GWU), and washed in phosphate-buffered saline (PBS), pH 7·2, before use. *N. americanus* L3 were isolated from charcoal coprocultures at GWU and stored in BU buffer (50 mM Na_2HPO_4, 22 mM KH_2PO_4, 70 mM NaCl, pH 6·8) at 22 °C until use.

cDNAs, recombinant proteins and antisera

Ac-apr-1 (Harrop, Prociv & Brindley, 1996) and *Na-apr-1* (Williamson *et al.* 2002) cDNAs were amplified from cDNA libraries by PCR using a degenerate primer designed to bind to the region of the active site of eukaryotic aspartic proteases (Harrop, Prociv & Brindley, 1996) and the pBluescript-derived primer M13 Reverse as described (Williamson *et al.* 2002).

The regions spanning the pro-domain to the C-terminus of both *Ac*-APR-1 and *Na*-APR-1 were expressed as secreted pro-enzymes in *Trichoplusia ni* High 5 cells using the baculovirus system as described (Williamson *et al.* 2002). Recombinant proteins were auto-processed at low pH, affinity purified using pepstatin A-agarose, and assessed for proteolytic activity by fluorometry with a defined aspartic protease-specific peptide as described (Williamson *et al.* 2002). Antisera were raised to the recombinant enzymes as described (Williamson *et al.* 2002).

Cleavage of skin macromolecules and albumin by recombinant Ac-*APR-1 and* Na-*APR-1*

Human collagens (I, III, IV and V), canine and human serum albumin, and canine and human fibrinogen were all obtained from Sigma. Ten micrograms of each substrate were incubated with recombinant *Ac*-APR-1 and *Na*-APR-1 (1·0 μg) in 0·1 M sodium formate, pH 5·5, for up to 4 h at 37 °C. Hydrolysates of skin macromolecules and serum proteins were examined after electrophoresis in non-denaturing 15% SDS–PAGE gels stained with Coomassie Brilliant Blue (CBB).

Effect of antisera to hookworm aspartic proteases on L3 migration

Skin from a freshly killed hamster was shaved, scrubbed with gauze and washed with PBS, then stretched over a glass migration tube filled with PBS so that the buffer was in contact with the underside of the skin (Clegg & Smithers, 1972). *N. americanus* and *A. caninum* L3 (300 L3/group) were incubated in 50 μl of one of the following solutions for 30 min at 37 °C. (1) PBS, pH 7·2; (2) neat normal mouse serum (NMS); (3) neat mouse antiserum to *Ac*-APR-1; (4) neat mouse antiserum to *Na*-APR-1. Each group of L3 were then placed on the hamster skin (in 1·0 ml of PBS) and allowed to migrate for 30 min at RT. L3 that migrated through the skin were collected from the media (PBS) in the collection tube and counted, as were L3 that remained on the outer surface of the skin.

Modelling of substrate binding clefts

Ac- and *Na*-APR-1 proteins were modelled using the X-ray crystal structure of human cathepsin D (1LYA) as a template. Homology models were generated in optimize mode and assessed using the Swiss Model server and predicted structures were viewed using Swiss pdbViewer v. 3.6.

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Western blots

Western blots were conducted using standard methodologies and developed using chemiluminescence (ECL, Amersham). Infection sera (used at a 1 : 1000 dilution) from naturally infected humans (collected from Daocong Village, Hainan Province, P.R. China) and from dogs experimentally infected with *A. caninum* L3 at GWU, were kindly provided by Dr Bin Zhan, GWU.

RESULTS

Production of recombinant protein

The optimal conditions for *Ac*-APR-1 protein production using High 5 cells was at a density of 1×10^6 cells/ml with a multiplicity of infection (MOI) of 1·0. The optimal conditions for production of recombinant *Na*-APR-1 was at a cell density of 1×10^6 cells/ml with an MOI of 0·5. Following 4 days incubation at 27 °C, the baculovirus supernatant was harvested and analysed by SDS–PAGE and Western blotting for the expression of recombinant *Ac*-APR-1 and *Na*-APR-1. Analysis of the supernatants (before low pH activation) showed the overexpression of bands representative of *Ac*-APR-1 and *Na*-APR-1 pro-enzymes were evident at ~49 kDa (not shown). *Ac*-APR-1 and *Na*-APR-1 were purified to homogeneity on pepstatin A–agarose, resulting in a single band when electrophoresed under reducing and denaturing conditions in SDS–PAGE (Williamson *et al*. 2002). Purified *Ac*-APR-1 and *Na*-APR-1 were recognized by rabbit antiserum to recombinant *Schistosoma japonicum* (SjCathD) (Brindley *et al*. 2001) in Western blots (not shown). The amount of protease purified from a 500 ml culture varied between 2·0–8·0 mg/l for *Ac*-APR-1 and 1·0–6·0 mg/l for *Na*-APR-1.

Degradation of collagen and fibrinogen by recombinant hookworm cathepsins D

Hydrolysates of skin macromolecules were examined in non-denaturing 15% SDS–PAGE gels and stained with CBB. Degradation of human collagens (types I, III, IV and V) was evident after 10 min when incubated with recombinant *Na*-APR-1, with complete degradation evident after 4 h at 37 °C; *Ac*-APR-1 only partially degraded human collagen over the same time-period (Fig. 1A). Dog collagens were not commercially available and therefore not analysed in this study. Both proteases degraded human fibrinogen at similar rates, whereas *Ac*-APR-1 was more efficient than *Na*-APR-1 at degrading dog fibrinogen (not shown). Degradation of collagen and fibrinogen was completely inhibited by the addition of pepstatin A.

Degradation of serum albumin by recombinant hookworm cathepsins D

Ac-APR-1 preferentially degraded canine serum albumin (CSA) with partial degradation evident after 10 min and complete hydrolysis of the intact protein after 4 h at 37 °C. *Na*-APR-1 exhibited only partial degradation of CSA after 4 h (Fig. 1B). *Ac*-APR-1 and *Na*-APR-1 degraded human serum albumin (HSA) at approximately the same rate (Fig. 1B). Degradation of albumin was completely inhibited by the addition of pepstatin A.

Molecular model of the active site cleft of hookworm cathepsins D

Given the absolute conservation of residues that form the active site clefts of *Na*- and *Ac*-APR-1, and the considerable differences observed in substrate cleavage patterns (Williamson *et al*. 2002), we constructed molecular models of the hookworm proteases based upon the crystal structure of human cathepsin D (Baldwin *et al*. 1993). Except for a loop inserted at positions 215–218 in *Ac*-APR-1, which is distant from the active site cleft, the 2 hookworm proteases were very similar (Fig. 2). However, the orientation of a number of key active site amino acids, notably Gln-16 and Leu-115 in the S3 subsite, was informative. Leu-115 in *Na*-APR-1 faces away from the binding site, opening up a larger S3 pocket that would be able to accommodate the bulky side chains of aromatic residues. By contrast, Leu-115 of *Ac*-APR-1 has rotated toward the substrate-binding cleft, where it likely excludes larger residues.

Effect of antisera to Ac-*APR-1 and* Na-*APR-1 on L3 migration*

In total, 95% of L3 that were incubated in PBS or NMS successfully migrated through hamster skin. Migration of L3 incubated in anti-*Ac*-APR-1 or anti-*Na*-APR-1 serum was inhibited by 16–26% (Table 1). Each antiserum was more efficient at inhibiting penetration of larvae of the homologous species; i.e. anti-*Ac*-APR-1 inhibited 26% of *A. caninum* and 21% of *N. americanus* L3 from penetrating hamster skin. Likewise, anti-*Na*-APR-1 inhibited 26% of *N. americanus* and 16% of *A. caninum* L3 from penetrating hamster skin.

Naturally infected hosts recognize Ac-*APR-1 and* Na-*APR-1*

Purified, recombinant *Ac*-APR-1 and *Na*-APR-1 were probed with sera from dogs experimentally infected with *A. caninum*, and humans naturally infected with *N. americanus*. Antibodies were detected to both proteins by sera from infected hosts by Western blotting (Fig. 3). Serum from uninfected

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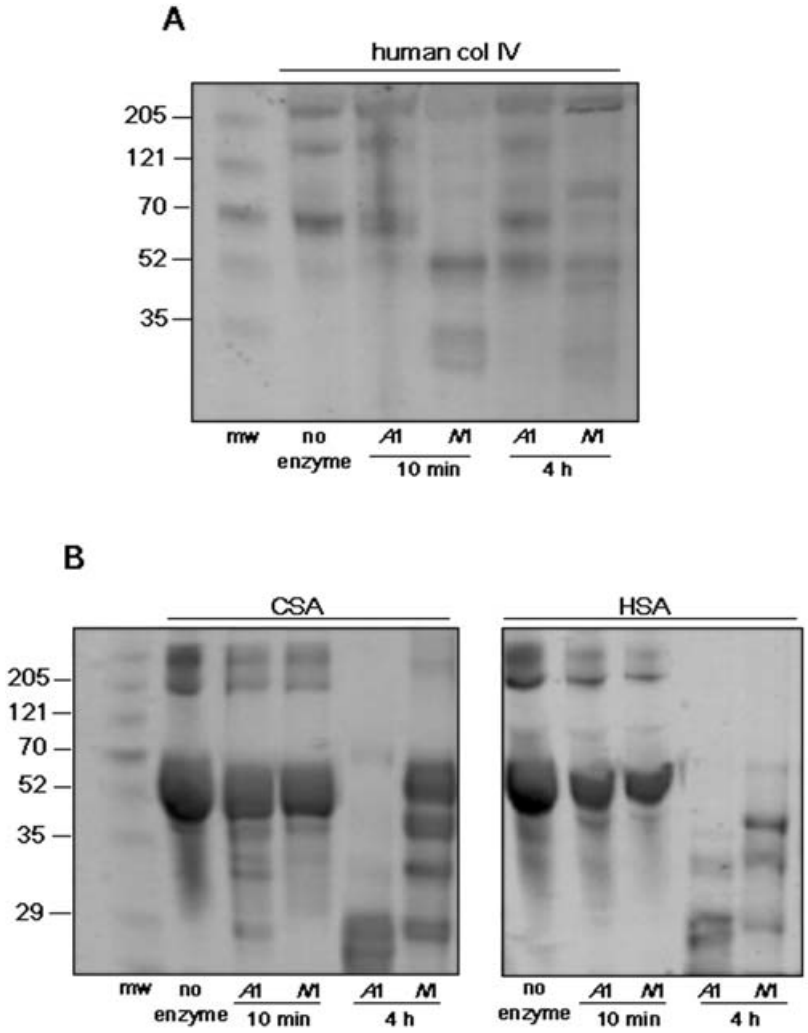

Fig. 1. (A) Hydrolysates of human collagens after cleavage with hookworm forms of cathepsin D resolved in a non-denaturing 15% SDS–PAGE gel. mw, Molecular weight markers (kDa); *A*1, 1·0 μg *Ac*-APR-1 + 10 μg human col IV; *N*1, 1·0 μg *Na*-APR-1 + 10 μg human col IV. Incubation times are listed beneath the lanes. Similar results were seen for all types of collagen (I–IV). (B) Hydrolysates of canine serum albumin (CSA) and human serum albumin (HSA) after cleavage with 1·0 μg of recombinant *Ac*- or *Na*-APR-1 and electrophoresis in non-denaturing 15% SDS–PAGE gels. mw, Molecular weight markers (kDa); *A*1, 1·0 μg *Ac*-APR-1 + 10 μg either CSA (left panel) or HSA (right panel); *N*1, 1·0 μg *Na*-APR-1 + 10 μg either CSA (left panel) or HSA (right panel). Incubation times are listed beneath the lanes.

dogs and uninfected humans did not recognize either of these proteins.

DISCUSSION

In vitro studies have shown that hookworm extracts and secretory products digest a panel of skin macromolecules and serum proteins (Brown *et al*. 1995, 1999; Hotez *et al*. 1990) as well as class-specific, peptide substrates (Dowd *et al*. 1994). We recently reported for the first time, host-specific cleavage of haemoglobin by recombinant hookworm proteases, *Ac*- and *Na*-APR-1 (Williamson *et al*. 2002). We now show that these cathepsin D-like aspartic proteases cleave skin macromolecules as well as serum proteins, suggesting that they play roles in penetration and migration by larvae in addition to a digestive role in blood-feeding adult parasites. We also provide a potential explanation for the different substrate preferences (of Hb at least) of *Ac*- and *Na*-APR-1 using molecular modelling to highlight differences in the orientation of residues lining the S3 pockets of the enzymes.

In earlier work, we showed that *Ac*- and *Na*-APR-1 were expressed in the gut lumen and excretory glands of hookworms and that the recombinant proteins cleaved Hb, the major food source of the adult parasites (Williamson *et al*. 2002). Moreover, cleavage of Hb was host specific and we suggested that this was a contributing factor to the range of hosts in which adult hookworms can develop and reproduce. In this study, we have shown that the 2 orthologous forms of cathepsin D degrade skin macromolecules and serum proteins from permissive and non-permissive hosts, and that some of these molecules were cleaved in a host-specific fashion.

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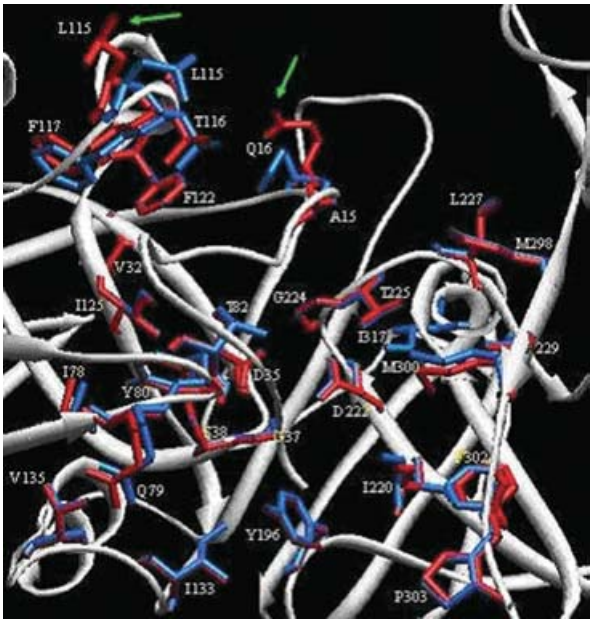

Fig. 2. Hookworm forms of cathepsin D have identical sequences at their active site clefts but some S3 subsite residues adopt distinct orientations. Molecular model of *Ac*-APR-1 (blue) superimposed on *Na*-APR-1 (red). The ribbon backbone of *Na*-APR-1 only is shown for clarity purposes, and side chains are shown for only the active site residues. Note the rotation of L115 and Q16 (green arrows) of *Na*-APR-1 away from the substrate binding pocket, possibly accounting for the acceptability of large, aromatic residues at this site in the *Necator* but not the *Ancylostoma* enzyme.

The *Ac*- and *Na-apr-1* mRNAs were detected in L3 as well as adult worms, suggesting that they play roles other than feeding, such as skin penetration and tissue migration. *Na*-APR-1 was more efficient than *Ac*-APR-1 at cleaving human collagen; however, since dog collagen was unavailable to perform the reciprocal experiment, it is difficult to draw any major conclusions from this finding. Hookworms can penetrate the skin of a wide range of mammalian hosts but once beneath the epidermis, migratory routes are presumably compromised in non-permissive hosts. This can be attributed, in part, to molecular incompatibilities between ligand-receptor interactions (including protease–substrate relationships), where subtle amino acid changes, or even protein folds in the absense of sequence variation, can result in dramatic differences in kinetic values.

Despite having identical residues lining their active site clefts, both *Ac*- and *Na*-APR-1 showed dramatically different cleavage profiles for Hb and Hb-derived synthetic peptides (Williamson *et al*. 2002) as well as some serum proteins. Indeed, molecular modelling of the 2 hookworm proteases showed that the active site residues superimposed very closely except those lining the S3 pockets. The conformations of Gln-16 and Leu-115 differed, making the S3 pocket of *Na*-APR-1 more accessible to large residues. Mapping of the APR-1 cleavage sites within

Table 1. Effect of antisera to *Ac*-APR-1 and *Na*-APR-1 on L3 migration

(Mean percentage decrease in migration relative to the control (PBS or NMS) groups are shown in parentheses.)

L3 group (300 L3/group)	No. of L3 to penetrate skin*
A. caninum L3 + PBS	285 ± 3 (5%)
A. caninum L3 + normal mouse serum	285 ± 4 (5%)
A. caninum L3 + α-*Ac*-APR-1 serum	210 ± 7 (26%)
A. caninum L3 + α-*Na*-APR-1 serum	240 ± 5 (16%)
N. americanus L3 + PBS	285 ± 3 (5%)
N. americanus L3 + normal mouse serum	285 ± 3 (5%)
N. americanus L3 + α-*Na*-APR-1 serum	210 ± 4 (26%)
N. americanus L3 + α-*Ac*-APR-1 serum	225 ± 7 (21%)

* Values are represented as the means and range of triplicate experiments.

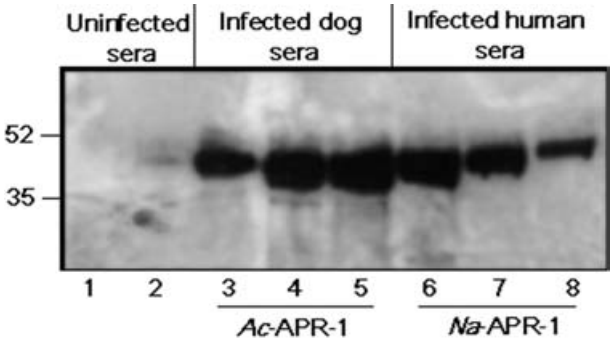

Fig. 3. Immunological recognition of recombinant *Ac*-APR-1 and *Na*-APR-1 by sera from dogs experimentally infected with *Ancylostoma caninum* (*Ac*-APR-1, [lanes 3–5]), and humans naturally infected with *Necator americanus* (*Na*-APR-1, [lanes 6–8]). Sera from an uninfected dog (*Ac*-APR-1, [lane 1]) and an uninfected human (*Na*-APR-1, [lane 2]) did not strongly recognize the recombinant proteases. Molecular weight markers are shown in kDa at the left.

Hb showed that *Na*-APR-1 had a preference for bulky, aromatic residues (His) while *Ac*-APR-1 only accommodated smaller P3 residues (Williamson *et al*. 2002). The *S. mansoni* cathepsin D is also thought to be involved in Hb degradation (Brindley *et al*. 1997, 2001). Interestingly, molecular modelling of this enzyme and subsequent comparison of the predicted structure with the X-ray crystal structure of human cathepsin D revealed nearly identical residues but different rotamers in the S3 pockets of the 2 enzymes (Silva *et al*. 2002). Furthermore, the authors attributed different specificities for P3 substrate residues to different conformations of Gln-14 (equivalent to Gln-16 in the hookworm proteases); the schistosome proteases, like *Na*-APR-1, accommodated bulky P3 residues such as His, while the human enzyme (where Gln-14 is directed into the binding pocket) is only capable of binding P3 residues with a short side chain (Silva *et al*. 2002).

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We suggest that co-evolution of *A. caninum* with its dog host has resulted in a fine-tuning of the molecular relationships between host and parasite proteins and this is evident in the preference of *Na*-APR-1 for cleaving human over dog collagens. On a more global scale, this could be expected to manifest as more efficient penetration and navigation of *A. caninum* L3 through the tissues of permissive rather than non- or less-permissive hosts.

Despite molecular (and probably mechanical) barriers inhibiting developing hookworms from reaching the gut of non-permissive hosts, some do reach the intestine and attempt to feed (Prociv & Croese, 1990; Yoshida *et al*. 1960). Protease–substrate relationships further influence the development of the parasite at this point; incompatibilities between digestive enzymes and food substrate proteins come into play (Williamson *et al*. 2002) and we have provided further evidence to support this here with the host-specific cleavage of some serum proteins. Hookworms ingest whole blood and probably rely on abundant serum proteins as well as Hb to survive. *Ac*-APR-1 was more efficient at cleaving CSA than HSA. *Na*-APR-1, however, did not display host-specific differences in the cleavage of canine and human serum albumins, degrading both substrates with approximately equal efficiency. This might reflect differences in the feeding patterns and fecundity of the hookworms: ancylostomatids are voracious blood feeders and produce large numbers of eggs, while *Necator* causes less blood loss and produces fewer eggs. The high energy life-style and associated protein demand of *A. caninum* might have resulted in a broader repertoire of food (protein) sources including serum proteins as well as Hb to obtain sufficient nutrition to feed and reproduce. This might have resulted in complementarity between *A. caninum* gut proteases and a range of food substrate proteins other than Hb. *Necator*, on the other hand, with a putatively lower metabolism, might not have undergone such extensive evolutionary pressure to match its digestive proteases with a wide range of substrate proteins. Schistosome cathepsin D has also been shown to degrade human serum albumin into numerous small fragments, indicating that it too may play a role in the degradation of host serum proteins ingested as part of the bloodmeal (Verity *et al*. 2001).

The hypothesis that the hookworm cathepsins D play several roles during the hookworm life-cycle was further supported by the partial inhibition of L3 migration *in vitro* in the presence of anti-*Ac*-APR-1 and -*Na*-APR-1 sera. Previous studies have shown that *N. americanus* larval secretions are capable of degrading collagen types I, III, IV and V, fibronectin, laminin and elastin (Brown *et al*. 1999). Furthermore, all the skin macromolecules tested were hydrolysed by protease activity that was inhibited by pepstatin A, suggesting that aspartic proteases were responsible. These studies also demonstrated that the penetration of L3 through hamster skin was significantly inhibited (54·8%) by pepstatin A, confirming the importance of aspartic protease activity during the skin penetration process (Brown *et al*. 1999).

Antisera from naturally infected definitive hosts recognized both cathepsin D proteases, indicating that they are exposed to the immune system *in vivo* during natural hookworm infections. Other nematodes express a suite of related aspartic proteases (Geier *et al*. 1999) and at least 6 different clusters of *N. americanus* expressed sequence tags encoding aspartic proteases are present in the public databases (http:///www.nematode.net/), implying that a multigene family exists and antibodies to one protease are likely to cross-react with others.

Regardless of their specific functions *in vivo*, these hookworm proteases play at least dual roles; tissue migration (also supported by the degradation of collagen) in L3, and Hb and serum protein degradation in adults. Hence, inhibiting these molecules may prove an effective control measure against canine and anthropophilic hookworms. Moreover, the aspartic proteases both cleaved substrate molecules in a host-specific manner, further supporting the theory that the complementarity between parasite proteases and host substrate molecules may be a contributing factor in determining host range.

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