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# *Acasp,* a Gene Encoding a Cathepsin D-like Aspartic Protease from the Hookworm *Ancylostoma caninum*

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Intestinal infection with the zoonotic hookworm *Ancylostoma caninum* can provoke human eosinophilic enteritis. A cDNA was isolated from *A. caninum,* using an oligonucleotide primer designed to hybridize to the region encoding the consensus, catalytic site residues $D_{32}$TGSSNLW of aspartic proteases. This novel cDNA encoded an aspartic protease zymogen of 422 amino acids, exhibiting 47% identity to the lysosomal aspartic protease of *Aedes aegypti,* 46% identity to the aspartic protease of *Schistosoma japonicum,* and 48.5% to human cathepsin D. Its deduced structure differed from that of cathepsin D in the loop 2 ''flap,'' which holds the substrate at the active site, and by the presence of a COOH-terminal extension of ~30 residues. 

Infections with the anthropophilic hookworms *Ancylostoma duodenale* and *Necator americanus* affect more than one fifth of the human population (1warren). Clinically, hookworms cause iron-deficiency anemia as the direct result of the feeding activities of the parasites - they not only ingest blood, but also secrete anticoagulants that exacerbate host blood loss. Subclinically, hookworm infection reduces the growth, development, and general well-being of individuals (1,2). Recently, the common hookworm of dogs, *Ancylostoma caninum,* previously believed to host specific, has been confirmed not only to develop in the human gut, but also provoke the potentially serious disease eosinophilic enteritis (3-5). While expanding the list of important zoonoses, this finding also raises new and pertinent questions about hookworm biology, host-parasite relationships, and the pathogenesis of enteric infections.

Eosinophilic enteritis is presumed to be an allergic response to hookworm secretions (6,7). Given that proteases are components of hookworm secretions, and that asthma can be triggered by dust mite and cockroach proteases, hookworm proteases may be responsible for triggering the intestinal allergic response. In particular, the Der pI allergen of the dust mite is a cysteine protease (8) and the Bla gI allergen of the German cockroach (9) has been shown to be an aspartic protease. We have begun to characterize the genes encoding hookworm proteases, since proteases from other invertebrates are clearly capable of potent allergenicity.

Recently, we have cloned cDNAs that encode two novel cysteine proteases, at least one of which is expressed in the anterior secretory glands of the adult parasite, and is a component of the excretory/secretory products released in vitro (10). Subsequently we undertook a search for gene(s) encoding hookworm aspartic proteases using an homology PCR approach. After comparing the amino acid sequences a panel of aspartic proteases from other organisms including mammalian pepsinogens, renins, and cathepsin Ds, and fungi (11), we designed a consensus primer to recognize the active site region of eukaryotic aspartic proteases. Using this primer in homology PCR, we amplified a gene fragment from an adult *A. caninum* cDNA

Sequences herein have been assigned the GenBank accession number U34888.



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| **Active site residues** | **D33** | **D231** |
|---|---|---|
| **Gastric enzymes** | | |
| pig pepsinogen | FTVIFDTGSSNLWVPSV | QAIVDTGTSLLT |
| human pepsinogen | FTVVFDTGSSNLWVPSV | QAIVDTGTSLLT |
| monkey pepsinogen | FTVIFDTGSSNLWVPSV | QAIVDTGTSLLT |
| chicken pepsinogen | FSVIFDTGSSNLWVPSI | QAIVDTGTSLLV |
| human progastricsin | FLVLFDTGSSNLWVPSV | QAIVDTGTSLLT |
| monkey progastricsin | FLVLFDTGSSNLWVPSV | QAIVDTGTSLLT |
| bovine prochymosin | FTVLFDTGSSDFWVPSI | QAILDTGTSKLV |
| **Renins** | | |
| human renin | FKVVFDTGSSNVWVPSS | LALVDTGASYIS |
| murine renin: kidney | FKVIFDTGSANLWVPST | AVVVDTGSSFIS |
| :submaxillary gland | FKVIFDTGSANLWVPST | EVVVDTGSSFIS |
| **Cathepsin D** | | |
| pig cathepsin D | FTVVFDTGSSNLWVPSI | EAIVDTGTSLIV |
| human procathepsin D | FTVVFDTGSSNLWVPSI | EAIVDTGTSLMV |
| **Fungal enzymes** | | |
| rhizpuspepsinogen | FNLDFDTGSSDLWIAST | DGILDTGTTLLI |
| penicillopepsin | LNLNFDTGSADLWVFST | SGIADTGTTLLL |
| endothiapepsin | LNLDFDTGSSDLWVFSS | DGIADTGTTLLY |
| pro-protease A | FKVIFDTGSSNLWVPSN | GAAIDTGTSLIT |
| **Consensus** | DTGSSNLW | DTGTSLL |

**napD** 5' **TT GAY ACN GGN TCA TCA AAY CTN TGG** G 3'

translation (F) **D** T G S S N L W (V)

**FIG. 1.** Design of aspartic protease gene-specific, consensus primer, napD. The flanking regions around the active site residues ($D_{33}$ and $D_{231}$, human cathepsin D numbering)(12) of 16 aspartic proteases (11) are shown with the consensus sequences. The consensus amino acid sequence around the first active site, being the most highly conserved region, was used to design a degenerate primer set based using the codon bias table of *Caenorhabditis elegans* (13). The resultant 27 mer oligonucleotide primer, named napD, is 256-fold degenerate.

library, and subsequently isolated the cDNA sequence. We now report the molecular cloning, characterization and expression of this hookworm aspartic protease gene, *Acasp*.

## MATERIALS AND METHODS

*Consensus, aspartic protease gene primer.* The highly conserved regions around the first aspartic acid residue of the catalytically active site of sixteen eukaryotic aspartic proteases were aligned (11). From this alignment, we obtained the consensus amino acid sequence $D_{33}$TGSSNLW in which D was the first active site aspartic acid residue $D_{33}$, using the numbering system for human cathepsin D (12). Based on $D_{33}$TGSSNLW, we designed and synthesized a 27 mer oligonucleotide primer 5′ TTGAYACNGGNTCATCAAAYCTNTGG, that incorporated the codon bias of the nematode *Caenorhabditis elegans* (13). We named this oligonucleotide napD, for nematode aspartic protease $D_{33}$ (Fig. 1).

*Homology PCR.* Primer napD and λ phage-specific primer M13 -20 (5′-GTAAAACGACGGCCAGT) were used

in polymerase chain reaction (PCR) experiments where an adult *A. caninum* cDNA library (10) constructed in the vector, λ Uni-Zap XR, was employed as the template. The PCR involved denaturation for 5 min at 94°C, annealing at 55°C for 1 min, and extension at 72°C for 1 min, for 30 cycles, followed by a final extension at 72°C for 10 min.

*Isolation of full-length aspartic protease gene.* The complementary 5′-end of the *A. caninum* gene fragment was amplified from the cDNA library using a gene-specific 3′ primer, 5′-GACACGGTACACGTATCA, paired with the vector-specific M13 reverse primer, 5′-GGAAACAGCTATGACCATG, with an annealing temperature of 55°C. In addition, the nematode spliced leader sequence, 5′-GGTTTAATTACCCAAGTTTGAG (14), was used in combination with the gene specific 3′ primer to locate the extreme 5′-terminus of the *A. caninum* aspartic protease transcript (see below). A complete *A. caninum* gene was constructed by complementary hybridization of both 5′ and 3′ amplified gene fragments across the region of overlap. Briefly, double stranded DNA was end filled using T4 DNA polymerase. Gene fragments were heated to 100°C in water for 3 min to denature, the reaction tube was then chilled on wet ice for 5 min, and subsequently allowed to equilibrate to room temperature over 30 min. Buffer, dNTPs and T4 polymerase were added and the reaction mixture incubated for 1 h at 12°C. Two $\mu$l of this 'polished' DNA mixture was used as a template for a PCR and the entire hybrid product was amplified using primers M13-20 and M13 reverse primer, using 1 min denature, 1 min at 60°C anneal, 1 min 72°C polymerization, and 25 cycles of PCR.

*DNA cloning and nucleotide sequencing.* PCR products were isolated and cloned as described (15). Briefly, PCR products were separated on low melting point 1% agarose gel (NuSieve GTG:SeaKem ME, FMC Bioproducts, Rockland, ME) by electrophoresis, excised from the gel, purified by centrifugation through Whatman filter paper (0.2 mm thickness) and ligated into pGEM-T vector (Promega, Madison, WI) using T4 DNA ligase. The ligation products were used to transform *E. coli* strain XL1-Blue cells (Stratagene, La Jolla, CA) by electroporation. Transformed cells were cultured on LB agar supplemented with ampicillin, X-gal and IPTG. Maxipreps (Qiagen Inc., Chatsworth, CA) of recombinant pGEM-T plasmids were prepared from bacterial cultures. Nucleotide sequences of both strands of insert plasmid DNA were determined by the dideoxy method using the Prism cycle system (Applied Biosystems, Foster City, CA) and an ABI model 373A automated sequencer.

*Sequence analysis.* A comparison of aspartic proteases was made using the program PileUp (16), which aligns sequences by the method of Needleman and Wunsch (17). Since Tang and Wong (11) consider that all aspartic proteases are derived from a common, primordial enzyme, a clustering strategy represented by a phenogram (18) was constructed to predict the sequence relationship between members of the sub classes of aspartic proteases. A protein hydropathy plot was performed using the program PepWindow (19) according to the method of Kyte and Doolittle (20). The curve represents an average of a residue specific hydropathy index over a window of nine residues.

*Expression of recombinant enzyme.* The plasmid vector pET3a (21) was used to express the recombinant hookworm aspartic protease. The pET vector confers ampicillin resistance in conjunction with the BL21 *E. coli* strain. The plasmind pET3a was linearized with *Bam*HI and dephosphorylated using calf intestinal alkaline phosphatase. The transcript was subcloned by using the unique restriction site, *Bam*HI, which was incorporated by PCR using the primers 5′-CGCGGATCCCTCGCACTATTTACCCTAGC and 5′-GCGGATCCTTATAGAGTGAATACATCATC (*Bam* HI sites are underlined). Ligation products were used to transform *E. coli* strain BL21[Dec3]pLysS (Novagene, Madison, WI). The plasmid pLysS, which confers chloramphenicol resistance, produces lysozyme under the T7 promoter. This lysozyme inhibits T7 polymerase, to prevent 'leaky' expression, and also cleaves the *E. coli* cellular peptidoglycan layer, to release recombinant proteins from induced cells (21). Positive clones with inserts in the correct orientation were assessed by PCR analysis using a specific gene primer and a directional universal vector primer (T7 promoter 5′-TAATACGACTCACTATAGGG and T7 terminator 5′-GCTAGTTATTGCTCAGCGG nucleotide sequences). Transformed cells were grown in LB media supplemented with ampicillin at 100 mg $ml^{-1}$ until an $OD_{600}$ of 0.6 was achieved. Protease gene expression was induced by the addition of IPTG to 2 mM, and after which the cells were cultured for a further 4 h. Recombinant protein was purified and refolded by the rapid dilution method (22) and incubated at a range of pH (100 mM sodium acetate at pH5.5, pH 4.5, pH 3.5) to induce autoactivation. The purified polyprotein was separated by SDS-PAGE (10% acrylamide) and stained with Coomassie Blue. SDS-PAGE separated protein was electrotransferred to a nitrocellulose membrane by standard methods, after which the membrane was probed with a commercial monoclonal antibody which recognizes the pET3a T7 $NH_2$-terminal MASMTGGQQMG residues (Novagene) in order to identify the nascent recombinant polypeptide.

## RESULTS AND DISCUSSION

*Protease gene amplified by homology PCR.* When oligonucleotide napD was paired with the vector specific M13-20 primer in the PCR with adult *A. caninum* cDNA phage library as the template, an ethidium-stainable band of ~1.4 kb was amplified. When cloned and sequenced, this PCR product was found to encode a long, but truncated, open reading frame. Accordingly, a reverse-directed 5′-GACACGGTACACGTATCA was designed from the region encoding the open reading frame and, when paired with the vector-specific, M13 reverse primer, resulted in a PCR product of ~1 kb. The sequence of this fragment was found to

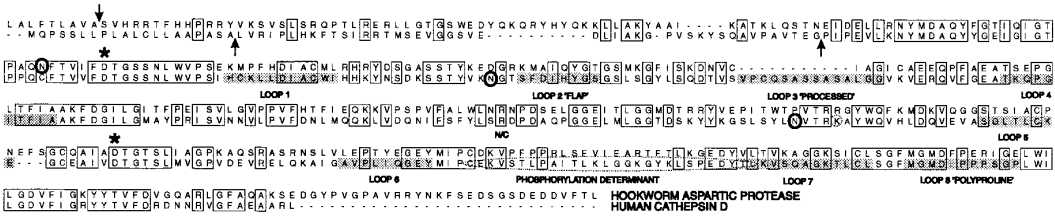


**FIG. 2.** Comparison of the amino acid sequence of the hookworm aspartic protease. Alignment of the hookworm (Acasp) aspartic protease with human cathepsin D using the PileUp algorithm (16,17). The putative signal cleavage points (29) and mature cleavage position are marked with arrows, but the mature cleavage position cannot be positively predicted. Potential Asn-glycosylation sites are circled and the two active sites are marked with asterisks. Loops characteristic of aspartic proteases are shaded and numbered (30). Human cathepsin D phosphorylation determinants (33) are indicated. The Acasp sequence contains a COOH-terminal extension of ~30 residues.

overlap that of the original 1.4 kb clone (above) unambiguously over ~800 bp (Figure 2 below), and it was clear that together these two fragments spanned a full-length transcript. Subsequently, in order to reconstruct this full-length cDNA, the two fragments were hybridized, and then amplified by PCR. The hybrid PCR product of ~1.6 kb was then cloned into plasmid vector pGEM-T (Promega) and its nucleotide sequence determined.

*Hookworm aspartic protease gene.* The hybrid cDNA was 1413 nucleotides in length, contained an open reading frame coding for 442 amino acids which was terminated by a TTA (ochre) stop codon, and ended in a poly A stretch with a putative polyadenylation-like signal GATAAA (23,24) at 12 bp upstream from the start of poly $A^+$ tail. Based on the alignment of deduced amino acid sequences and on the Kyte-Doolittle plot (20)(see below), it appeared that the transcript was truncated by a few nucleotide residues. Repeated screening of the library by PCR-based strategies employing the spliced leader sequence of nematodes (14) failed to locate a longer fragment that could encode the missing nucleotides.

The best matches for identity of the open reading frame were 47 % with the lysosomal aspartic protease of the mosquito *Aedes aegypti* (25), 46% with the cathespin D-like aspartic protease (Sjpasp) from the human blood fluke *Schistosoma japonicum* (26), and 47-50% with mammalian cathespin D (12). Accordingly, it was clear that our homology PCR using the aspartic protease gene-specific primer napD performed well, resulting in the isolation of a cDNA encoding a novel aspartic protease. While we are unaware of a similar homology PCR strategy for isolating aspartic protease genes from parasitic helminths, a similar strategy has been employed to characterize aspartic protease genes expressed by human pituartary cells (27).

*Structural and functional motifs.* We have named the gene encoding this novel hookworm aspartic protease *Acasp*, for *Ancylostoma caninum* aspartic protease, and named the enzyme Acasp. The deduced amino acid sequence of Acasp (442 residues) had a predicted molecular size of 49,304 daltons (Fig. 2). Figure 2 presents an alignment of Acasp with human cathepsin D (12). Aspartic proteases are synthesized as pro-enzymes and activated in lysosomes and other sites by cleavage of the pro-enzyme (28). A comparison of the deduced amino acid sequence of Acasp with the $NH_2$-termini of Sjpasp and other proteases in the SwissProt data base suggested that 64 amino acid residues of Acasp were a putative pro-enzyme sequence (Fig. 3). Despite the similarity in primary amino acid sequence with Sjpasp, mammalian cathepsin D, and the lysosomal aspartic protease of *A. aegypti*, the deduced amino acid sequence of *Acasp* showed substantial divergence from other aspartic proteases, and in particular had an atypical carboxyl terminal extension of ~30 amino acid residues. The mature, sub-unit form of Acasp contained 369 amino acid residues with a predicted mass of 40,722 daltons

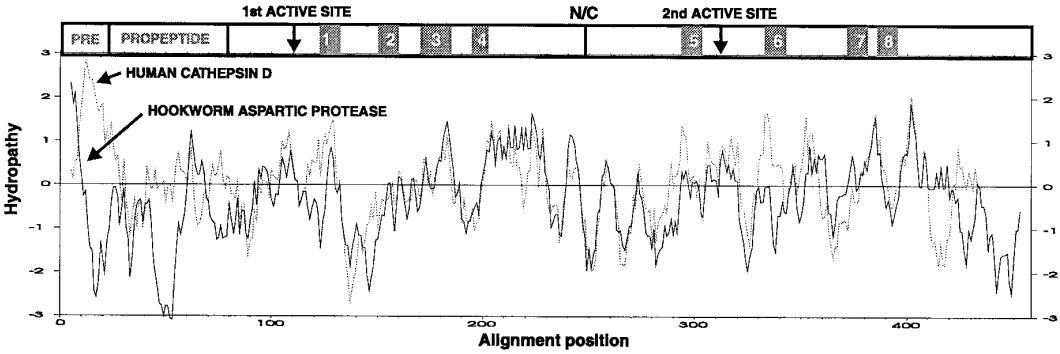


**FIG. 3.** Hydropathy plots of the deduced amino acid sequences of Acasp and human cathepsin D. The abscissa indicates amino acid residues and the ordinate shows the hydropathy index obtained using the Kyte and Doolittle (20) algorithm. The plots clearly show that the Acasp zymogen has a hydrophobic domain at the $NH_2$-terminus, which is representative of a signal peptide sequence. In the top panel, pre and propeptide regions are indicated. The active site regions and the N domain/C domain boundary (N/C) are indicated and structural loop regions are shaded. The alignment position is identical to that for Figure 2.

and pI 4.79. However, the zymogen lacked a recognizable autoactivation site (Kay, personal communication). An hydropathy plot of the deduced amino acid sequence of Acasp was compared to that of human cathepsin D (12)(Figure 3). The $NH_2$-terminal portion of the Acasp zymogen was highly hydrophobic. This is a typical feature of a signal or prepeptide sequence, and according to the $(-3, -1)$ rule for signal cleavage sites (29), contained a 9 amino acid residue signal. In addition, the hydropathicity comparison showed high homology between the two proteases outside the propeptide and carboxy terminus regions.

Acasp exhibited dual active-site aspartic acid residues, and more specifically both exhibited two catalytic DTG active-site motifs, which are the hallmarks of aspartic proteases, together with the conserved regions flanking these catalytic sites (11). Residue Gln-14 in Acasp, as numbered from the cleavage point of the pro-region and mature protease, is likely to be pivotal in determining S3 subsite interactions with substrates, and was positioned within a block of conserved residues, QYFG (30). The P2 determinant, MGMD, was present in loop 8 (figure 2) and, like cathepsin D molecules, the hookworm enzyme did not exhibit positively-charged residues at this position (30).

Eight regions of primary amino acid sequence in Acasp corresponded to the structural loops characteristic of mature aspartic proteases (31); the positions of these loop residues are marked in the respective positions on Fig. 2 and Fig 3. Loop 3 comprises a $\beta$-hairpin which in many cathepsin D zymogens contains a small extended structure that bears cleavage sites for the heavy and light chains of the active enzyme (11). This $\beta$-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 cathepsin D (28). However, neither Acasp nor Sjpasp, the only other helminth aspartic protease so far reported (26), exhibit this $\beta$-hairpin. Aspartic proteases that lack the $\beta$-harpin have been shown to be processed in other ways; the aspartic protease of barley and that of *A. aegypti* are cleaved at other sites to generate hetero- and homo-dimeric structures, respectively (25). Acasp appeared to be unique in that cysteine residues flank this region and possibly form a disulfide connection across this $\beta$-hairpin region. The termini of the Acasp ''flap'' (loop 2), which covers the catalytic pocket of the enzyme and holds the substrate within it's catalytic cleft during hydrolysis (Fig. 2), showed marked divergence from those of the flap of human cathepsin D. The changes included, from Phe to Met at the $NH_2$-terminus of the flap, a change from

Leu to Met at its carboxyl end, and a Ser to Lys difference, pointing outwards. In addition, there was a Val to Ala substitution in the P2 binding pocket, which can be predicted to influence the substrate specificity of Acasp. These differences were also reflected as structural changes predicted in a pilot 3D homology model of mature Acasp, based upon the crystal co-ordinates of human cathepsin D (HUC1) from the Brookhaven Protein Database (Bur and Harrop, unpublished).

Of the six cysteine residues which are present in many aspartic proteases (one pair each at loops one and five and one adjacent to each of loops six and seven) (32), five were present in Acasp, with a methionine present at the homologous position of loop one. As mentioned, an extra pair of cysteine residues also flanked loop 3. The putative S3 subsite region of Acasp appeared relatively conserved and since this region interacts directly with loop four, we examined it and found moderate conservation with other aspartic proteases. We next considered whether the Acasp sequence would support targeting to the lysosomes via glycosylation and phosphorylation. A single potential Asn-glycosylation site was present in Acasp at a homologous position with those of human and guinea pig cathepsin E and monkey pepsin (SwissProt codes: CATE_CAVPO, CATE_HUMAN, PEP4_MACFU). Glycosylation of this residue and addition of mannose-6-phosphate would facilitate entry of the nascent protein into the endocytic pathway and subsequent lysosomal localization (33). However, the equivalent of the critical phosphorylation determinant, Lys 203 on human cathepsin D (Figure 2), was not present on Acasp, and the $\beta$-strand phosphorylation determinant region (Figure 2) of human cathepsin D was not conserved in the Acasp sequence. Since only one potential Asn-glycosylation site was present on the Acasp molecule, and given that cathepsin E is not lysosome-associated (34), Acasp may not be a lysosomal enzyme.

Acasp appears to be longer than aspartic proteases from most other animals due to the presence of a carboxyl terminal extension of $\sim$30 amino acids. The aspartic protease of *Eimeria acervunila* (35) contains a similar feature, which is, however, markedly divergent. This feature, which is present on at least two aspartic proteases, is reminiscent of the COOH-terminal extensions of the trypanosome cysteine proteases, congopain and cruzipain (36). The aspartic protease cyprosin which functions in the maturation of the flower of the cardoon, *Cynara cardunculus*, bears a long C-domain inclusion, not an extension, of around 100 residues (37).

A primary sequence alignment of 49 aspartic proteases (from the SwissProt database), Sjpasp (26) and the aspartic protease of *E. acervulina* (35) which bear the highest identity scores with respect to Acasp, is shown in the phenogram (Figure 4). Interestingly, Sjpasp and Acasp, both from parasitic helminths, were very similar (43%) and were closely grouped with the mosquito lysosomal protease and vertebrate cathepsin D. These other enzymes are considered to be lysosomal-associated, aspartic proteases (25,33). The malarial aspartic proteases, plasmepsin I and II (22,30), bore only 32 % identity to Acasp. Major groupings within the phenogram include cathepsin D, pepsins, cathepsin E, renin, and enzymes from plants and fungi.

*Recombinant Acasp polypeptide.* A time course of the induction of Acasp revealed significant expression 2 h after the addition of IPTG. SDS-PAGE analysis of recombinant Acasp polypeptide revealed two major protein bands at $\sim$50 kDa and $\sim$30 kDa, both of which were recognized by a T7-Tag antibody on Western blot. The $\sim$50 kDa band had an apparent molecular weight consistent with that predicted for recombinant Acasp fusion product, while the lower band may be the zymogen after COOH-terminal processing or may be the mature protease We were unable to detect proteolytic activity in preparations of recombinant Acasp (not shown).

*The role of Acasp.* It is known that aspartic proteases are activated by acids after local denaturation of the pro-peptide and function optimally in an acid pH range (34). Their functions include processing of hormones, growth factors and proteolytic enzymes such as procathepsin B (a cysteine protease zymogen) (34). Interestingly, the two cathepsin B-like molecules from *A. caninum* have been expressed, but to date no enzymatically active recombinant molecules

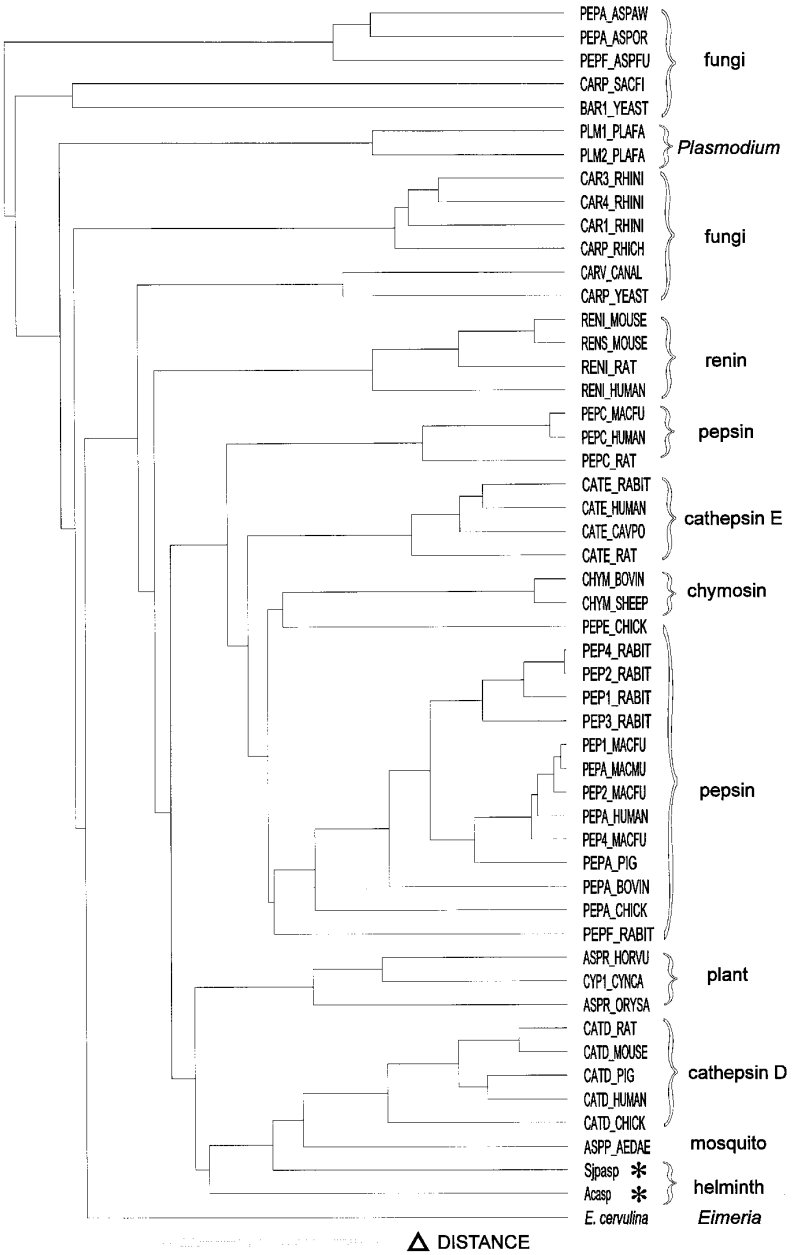


**FIG. 4.** Phenogram of multiple pairwise alignments of deduced amino acid sequences of aspartic proteases from *Ancylostoma caninum* (Acasp) and 51 aspartic proteases to produce clusters of similar sequences. Similarities were obtained using the Fasta and PileUp programs (16,17). Acasp has high identity (47%) to mammalian cathepsin D and to *Aedes aegypti* protease (25) and 45–50% identity to mammalian cathepsin D. In addition, Acasp shows 43% identity to Sjpasp. The abscissa is in amino acid distance scale Δ using uncorrected amino acid differences. Major identity groupings are indicated on the right. Sjpasp and *Eimeria* have GenBank accession numbers L41346 and Z24676, respectively, and others are denoted by their SwissProt database codes.

have been obtained (10). It is possible that Acasp may be required for the activation of these cathepsin B zymogens (34). Since hookworms must employ proteolytic enzymes to digest hemoglobin from blood that they suck, and since aspartic proteases are involved in digestion

of hemoglobin by schistosomes (26) and by malaria parasites (38,22), a role for Acasp in hemoglobin digestion should be investigated. Irrespective of their functional roles, secreted hookworm proteases may be allergenic in humans and contribute to the segmental inflammation of the bowel characteristic of eosinophilic enteritis (6). Proteases from other parasitic worms, and from arthropods, are potent allergens, and are reputed to trigger $T_H2$-type immune responses requisite for the manifestation of immediate-type hypersensitivity reactions (39).

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