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A Broad Spectrum Kunitz Type Serine Protease Inhibitor Secreted by the Hookworm *Ancylostoma ceylanicum**

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Although blood-feeding hookworms infect over a billion people worldwide, little is known about the molecular mechanisms through which these parasitic nematodes cause gastrointestinal hemorrhage and iron deficiency anemia. A cDNA corresponding to a secreted Kunitz type serine protease inhibitor has been cloned from adult *Ancylostoma ceylanicum* hookworm RNA. The translated sequence of the *A. ceylanicum* Kunitz type inhibitor 1 (AceKI-1) cDNA predicts a 16-amino acid secretory signal sequence, followed by a 68-amino acid mature protein with a molecular mass of 7889 daltons. Recombinant protein (rAceKI-1) was purified from induced lysates of *Escherichia coli* transformed with the rAceKI-1/pET 28a plasmid, and *in vitro* studies demonstrate that rAceKI-1 is a tight binding inhibitor of the serine proteases chymotrypsin, pancreatic elastase, neutrophil elastase, and trypsin. AceKI-1 inhibitory activity is present in soluble protein extracts and excretory/secretory products of adult hookworms but not the infective third stage larvae. The native AceKI-1 inhibitor has been purified to homogeneity from soluble extracts of adult *A. ceylanicum* using size exclusion and reverse-phase high pressure liquid chromatography. As a potent inhibitor of mammalian intestinal proteases, AceKI-1 may play a role in parasite survival and the pathogenesis of hookworm anemia.

Intestinal hookworms, which currently infect billion people worldwide, are a leading cause of iron deficiency anemia in developing countries (1–3). The adult blood-feeding stage of this nematode parasite is responsible for nearly all of the clinically relevant sequelae of infection, most of which are directly attributable to gastrointestinal hemorrhage (4, 5). Using specialized teeth or cutting plates, the adult worm attaches to the intestinal mucosa, lacerating capillaries in the superficial mucosa and sucking blood into its buccal capsule (6, 7). Chronic gastrointestinal blood loss at or near the site of attachment leads to iron deficiency anemia and protein malnutrition, which over time may cause growth retardation, cognitive impairment, and even death (2, 8–10).

It has been demonstrated that adult hookworms produce a number of substances that potentially aid in host invasion and/or parasite survival, thus contributing directly or indirectly to the pathogenesis of hookworm anemia (11, 12). Most of these so-called "virulence factors" have been isolated from the canine hookworm *Ancylostoma caninum*, including the two major anticoagulant serine protease inhibitors from the adult blood-feeding stage. These related compounds, called *A. caninum* anticoagulant peptides 5 and 2, inhibit the proteolytic activities of coagulation factor Xa and the factor VIIa-tissue factor complex, respectively (13, 14). Other bioactive molecules isolated from *A. caninum* include a hyaluronidase (15) and metalloprotease (16) presumed to aid in tissue invasion, neutrophil inhibitory factor (17), and a hookworm platelet inhibitor (18) that targets cell surface integrins GPIIb/IIIa and GPIa/IIa. In contrast to the canine hookworm, very little is known about comparable inhibitory activities in hookworm species that routinely infect humans.

Ancylostoma ceylanicum is a hookworm species capable of completing its natural life cycle in a number of mammalian hosts, including humans, dogs, cats, and hamsters (19–21). Although not as prevalent worldwide as *Ancylostoma duodenale* or *Necator americanus*, *A. ceylanicum* has been reported to cause anemia in parts of India and Southeast Asia (19). Using a combined molecular and biochemical approach, we have identified and characterized a novel Kunitz type serine protease inhibitor from *A. ceylanicum* that blocks the activity of chymotrypsin, pancreatic elastase, neutrophil elastase, and trypsin. The inhibitory spectrum of *A. ceylanicum* Kunitz type inhibitor 1 (AceKI-1)[1] is unique among all previously characterized members of the Kunitz family of serine protease inhibitors.

Based on its broad biologic activity, this multifunctional inhibitor may play an important role in parasite survival within the intestine and thus contribute to the pathogenesis of hookworm anemia. The identification of AceKI-1, the first serine protease inhibitor isolated from a major human hookworm, represents an important step in the characterization of the molecular mechanisms of parasite survival within the human

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF172651.

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[1] The abbreviations used are: AceKI-1, *A. ceylanicum* Kunitz type inhibitor 1; rAceKI-1, recombinant AceKI-1; ES, excretory/secretory; PCR, polymerase chain reaction; RT-PCR, reverse-transcription PCR; HPLC, high pressure liquid chromatography; rpHPLC, reverse-phase HPLC; MALDI-MS, matrix-assisted laser desorption ionization mass spectroscopy.

host. Ultimately, AceKI-1 may serve as a potential molecular target in an overall strategy aimed at interfering with the worm's ability to survive within the intestine, thereby limiting the duration of infection and reducing the cumulative effect of chronic blood loss.

EXPERIMENTAL PROCEDURES

Hookworms—-Infective third stage (L_3) *A. ceylanicum* larvae (22, 23) were generously provided by John Hawdon and Peter Hotez at The Yale University School of Medicine, and the parasite life cycle was maintained as described previously (24). Three-week-old Syrian hamsters (LVG strain) were infected orally by gavage with 100–150 L_3 per animal. At 21–28 days postinfection, the hamsters were euthanized, and the live adult hookworms were harvested manually from the intestinal mucosa. Adult *A. caninum* were obtained from the intestines of laboratory-infected dogs using a similar protocol (18, 25).

Hookworm Extracts and Excretory/Secretory Products—Soluble protein extracts of adult hookworms were prepared by manually homogenizing adult worms in 50 mM Tris-HCl, pH 7.5, using a glass homogenizer (26). Soluble protein extracts, which represented the starting material for the purification and characterization of AceKI-1, were obtained by centrifugation at 10,000 × *g*. Protein concentrations of each lot of hookworm extracts were determined using the BCA reagent from Pierce. Extracts of infective larval stage (L_3) hookworms cultured from the feces of infected hamsters (24) were prepared similarly to the adult extracts. Adult hookworm excretory/secretory (ES) products were prepared by incubating live, freshly harvested adult worms in RPMI media with hamster serum (5% *v*/*v*) for 16 h at 37 °C in 5% CO_2 (18). The worms were removed, and the ES products were clarified by centrifugation at 10,000 × *g* prior to use.

Cloning of the AceKI-1 cDNA—Fifty live adult *A. ceylanicum* were suspended in 1.0 ml of Trizol (Life Technologies, Inc.), and total RNA was isolated according to the manufacturer's protocol. The RNA pellet was washed with 75 and 100% ethanol, air-dried for 10 min, and resuspended in 40 μl of diethyl pyrocarbonate-treated water.

Reverse transcription-polymerase chain reaction (RT-PCR) of total adult *A. ceylanicum* RNA was utilized to amplify *A. ceylanicum* cDNA (27). All primers (Fig. 1) were synthesized by the William Keck Foundation Biotechnology Resource Laboratory at Yale University School of Medicine. First strand cDNA was synthesized by incubating approximately 1 μg of total RNA, 10 mM dithiothreitol, and 100 ng (final concentration approximately 2 μM) of the degenerate antisense strand primer 3′-CKDGFYRD in first strand reaction buffer (final concentration 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3) (Life Technologies). The degenerate primer 3′-CKDGFYRD was based on a conserved amino acid sequence located near the carboxyl terminus of a family of serine protease inhibitors previously identified from the hookworm *A. caninum* (14, 28) and the nonblood-feeding nematode parasite *Ascaris suum* (29, 30). The solution was heated for 90 s at 90 °C and cooled on ice. Each of four deoxynucleotides (final concentration 1 mM; dATP, dCTP, dGTP, dTTP; New England Biolabs, Beverly, MA) was added along with 40 units of RNasin (Promega, Madison, WI) and 200 units of reverse transcriptase enzyme (Superscript II; Life Technologies). This mixture was incubated for 1 h at 42 °C and cooled on ice. The entire first strand cDNA mixture was combined with PCR primers 5′-SLXHO and 3′-CKDGFYRD (100 ng each), reaction buffer (final concentration 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), and 1 mM (final concentration) of each of the deoxynucleotides. The sense strand primer 5′-SLXHO corresponds to the nematode spliced leader, a conserved 22-nucleotide sequence present at the 5′-end of many nematode mRNAs (31–33). The reaction mixture was brought to a 49-μl total volume with diethyl pyrocarbonate water. *Taq* polymerase enzyme (5 units; Perkin-Elmer) was then added, and samples were placed in a thermal cycler (PCR Sprint; Hybaid, Middlesex, United Kingdom) for 40 cycles (94 °C for 15 s of denaturation, 55 °C for 5 s of annealing, and 72 °C for 30 s of extension). The reaction mixture was subjected to 1% agarose gel electrophoresis, and the PCR products were visualized by ethidium bromide staining.

The resulting PCR products were ligated into the pCR2.1 (Invitrogen, Carlsbad, CA) cloning vector by incubating 10 ng of PCR product, 50 ng of pCR 2.1 vector, T4 DNA ligase, and ligation buffer overnight at 14 °C. One Shot *E. coli* INVαF′ cells (Invitrogen) were transformed with the ligation product as per the manufacturer's protocol. Samples were plated onto LB agar plates containing 30 μg/ml kanamycin (Life Technologies) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) (Life Technologies) and incubated for 12–14 h at 37 °C. White appearing colonies that grew on the selective media were screened for the appropriate sized insert by direct colony PCR using vector-specific primers (T7 Promoter, M13 Reverse), and by restriction enzyme digest (*Eco*RI; New England Biolabs) of isolated plasmid DNA. Miniprep plasmid DNA (Spin Miniprep Kit; QIAGEN, Valencia, CA) from positive colonies was sent to the Keck Foundation Laboratory at Yale for nucleotide sequencing.

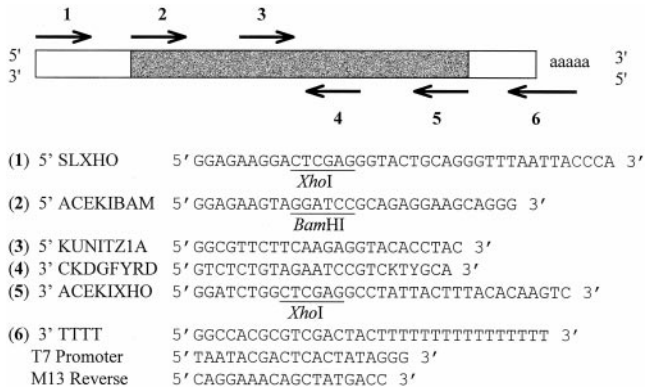

FIG. 1. **Oligonucleotide primers and general strategy for cloning AceKI-1.** Schematic representation of the AceKI-1 cDNA with the relative location of each of the oligonucleotide primers used to isolate the full-length cDNA. A partial cDNA sequence was amplified from adult *A. ceylanicum* total RNA by RT-PCR using a 5′ oligonucleotide primer (*primer 1*) corresponding to the nematode spliced leader sequence (31–33). The 3′ primer (*primer 4*) was a degenerate oligonucleotide corresponding to a conserved amino acid sequence (CKDGFYRD) near the C terminus of *Ascaris* type serine protease inhibitors (28–30). A 3′-rapid amplification of cDNA ends protocol was utilized to clone the 3′-end, using a nondegenerate internal primer (*primer 3*) derived from the sequence of the initial PCR product and an oligo(dT) primer (*primer 6*). A single cDNA corresponding to the predicted mature AceKI-1 protein was obtained using RT-PCR with nondegenerate AceKI-1-specific primers (*primers 2* and *5*).

The partial cDNA sequence obtained above was then used to design a nondegenerate internal primer (5′-KUNITZ1A, Fig. 1) in order to isolate the remainder of the AceKI-1 cDNA using a 3′-rapid amplification of cDNA ends protocol (34). First strand cDNA was amplified by RT-PCR, as above, using 1 μg of total RNA and an antisense primer, 3′-TTTT. The amplified cDNA mixture was then used as template for PCR with the primers 3′-TTTT and 5′-KUNITZ1A.

Sequence Analysis—cDNA and translated protein sequences were analyzed for homology to other known genes and proteins using the BLAST algorithm through the National Center for Biotechnology Information (available on the World Wide Web). The translated full-length amino acid sequence of AceKI-1 was analyzed using the SignalP Program for prediction of signal sequence cleavage sites (available on the World Wide Web) (35, 36).

Expression of Recombinant AceKI-1 (rAceKI-1)—In order to produce recombinant protein, the AceKI-1 cDNA corresponding to the predicted mature protein sequence was cloned into the pET 28a prokaryotic expression vector (Novagen, Madison, WI). The ligated pET28a plasmid containing the AceKI-1 cDNA was transformed into ultracompetent *E. coli* strain BL21 (DE3) cells (Stratagene, La Jolla, CA), and samples were plated onto LB/kanamycin plates and incubated for 12–16 h at 37 °C. Colonies were screened for the appropriate sized insert by direct colony PCR using a 5′ vector-specific primer (T7 Promoter) and a 3′ AceKI-1-specific primer (3′-ACEKIXHO), as well as by restriction enzyme digest of plasmid DNA. Isolated plasmid DNA from rAceKI-1-positive clones was subcloned into *E. coli* strain BL21(DE3) pLysS cells (Stratagene) for optimization of expression. Cells were plated onto LB/kanamycin plates, and individual colonies were screened by PCR for the presence of AceKI-1 cDNA.

A single colony of transformed BL21(DE3) pLysS *E. coli* containing the rAceKI-1:pET28a construct was incubated with shaking in LB/kanamycin medium, and protein expression by the log phase culture (A_{600} = 0.6–1.0) was induced by the addition of 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (Labscientific Inc., Livingston, NJ). The time course of rAceKI-1 expression was followed by SDS-polyacrylamide gel electrophoresis (37) with Coomassie staining and Western blotting of aliquots at 1–4 h postinduction. The antibody used for detection of rAceKI-1 is a horseradish peroxidase-labeled monoclonal antibody raised against the T7 epitope present in the pET 28a N-terminal fusion tag (Novagen).

For large scale purification of recombinant protein, a 2-liter culture of log phase BL21(DE3) pLysS cells containing the pET28a:rAceKI-1 construct was induced as above with 1 mM isopropyl-β-D-thiogalactopyranoside. At 3 h postinduction, the cells were harvested and resuspended in one-tenth the original culture volume of binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9). The solution was sonicated and centrifuged at 13,000 × *g* for 20 min at 4 °C to separate soluble and insoluble fractions. The soluble supernatant from the induced cell lysate was applied to a Hi-Trap chelating Sepharose column (Amersham Pharmacia Biotech) (5-ml bed volume) charged with 50 mM Ni_2SO_4 at 25 °C and equilibrated with binding buffer. The column was washed with binding buffer containing 60 mM imidazole, and the bound protein eluted with buffer containing 1 M imidazole. Following affinity chromatography, the partially purified recombinant protein was subjected to reverse-phase HPLC using a C_{18} column (Vydac, Hesperia, CA). The bound protein was eluted with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. Individual peaks of protein as detected by absorbance at 214 nm were collected and assayed for serine protease inhibitory activity as described below.

Following rpHPLC, the purified rAceKI-1 was subjected to electrospray ionization mass spectrometry (38), using a Micromass Q-Tof mass spectrometer, by the Keck Foundation Laboratory at Yale. Quantitative amino acid analysis was utilized to determine the molar concentration (39) of rpHPLC-purified rAceKI-1.

Assays of Protease Inhibition—Single stage chromogenic kinetic assays were used to characterize the inhibitory activity of r-AceKI-1 against six serine proteases (13, 40). rAceKI-1 was preincubated with each of the enzymes listed below for 15 min at 25 °C followed by the addition of the appropriate chromogenic substrate. In a total volume of 200 μl in individual wells of a 96-well microtiter plate, the concentrations (expressed as final concentrations) of enzyme/substrate were as follows: porcine pancreatic elastase (1.5 nM; Sigma Aldrich)/Suc-Ala-Ala-Pro-Ala-*p*-nitroanilide (250 μM; Bachem, Torrence, CA); human neutrophil elastase (7.5 nM; Calbiochem)/elastase substrate 1 (250 μM; Calbiochem); bovine pancreatic α−chymotrypsin (3 nM; Sigma)/Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide (200 μM; Bachem); bovine pancreatic trypsin (4 nM; Sigma)/S2302 (250 μM; DiaPharma, West Chester, OH); human coagulation factor Xa (1.0 nM; Enzyme Research Laboratories, South Bend, IN)/S2765 (250 μM; DiaPharma); human α−thrombin (1.0 nM; Enzyme Research Laboratories)/S2238 (250 μM; DiaPharma). Absorbance changes at 405 nm were monitored over 5 min using a kinetic microplate reader (MRX HD; Dynex Laboratories, Chantilly, VA). Preliminary results were expressed as percentage of inhibition of rAceKI-1 activity using the following formula: percentage of inhibition = (1 − inhibited rate/uninhibited rate) × 100 (13, 40). The reaction rates were computed by the microplate reader software.

A more detailed kinetic analysis was performed as follows to obtain apparent inhibition constants, K_i^* (41, 42). In each microtiter well, varying rAceKI-1 concentrations were incubated for 15 min with a fixed enzyme concentration, as above. Following the addition of the appropriate chromogenic substrate, the changes in absorbance were monitored at 405 nm. This assay was repeated using increasing amounts of inhibitor and the ratio of inhibited velocity (V_i) to uninhibited velocity (V_o) was plotted against the corresponding rAceKI-1 concentration. All experiments were performed in triplicate. The raw experimental data (absorbance *versus* time) were analyzed using the computer program *PlateKi* version 1.02 (BioKin Ltd., Madison, WI; available on the World Wide Web). To determine the initial reaction velocities, the program fits reaction progress curves either to a straight line or to the quadratic parabola using an automatic model selection procedure, based on the sequential modified givens orthogonal transformation method (43). In the second stage of the analysis, the program uses an automatic algorithm described recently (44) to fit the initial velocities to the Morrison equation (Equation 1) to determine the apparent inhibition constants K_i^*.

$$V = V_o \frac{[E] - [I] - K_i^* + \sqrt{([E] - [I] - K_i^*)^2 + 4K_i^*[E]}}{2[E]} \qquad \text{(Eq. 1)}$$

To ascertain the influence of substrate concentration on the apparent inhibition constant, the K_i^* measurements were repeated at substrate concentrations ranging from 0.2 to 10.0 × K_m. Assuming the competitive mechanism of inhibition, the true inhibition constants were computed from the apparent inhibition constant using the formula $K_i = K_i^*/(1 + [S]/K_m)$ (45).

Purification of Native AceKI—Soluble extracts from 200 adult *A. ceylanicum* were applied to a 300 × 7.5-mm size exclusion chromatography column (Bio-Sil TSK-125; Bio-Rad) equilibrated with 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl. Individual fractions were tested for serine protease inhibitory activity, as described above, and active fractions were pooled. The molecular mass of the presumed native AceKI-1 was estimated by extrapolation from a standard curve constructed from the elution profile of a mixture of protein standards (Bio-Rad) with known molecular masses (18). Following size exclusion chromatography, the pooled active fractions were subjected to reverse-phase HPLC using a C_{18} column (Vydac; Hesperia, CA). The bound protein was eluted with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. Individual peaks of protein as detected by absorbance at 214 nm were collected and assayed for serine protease inhibitory activity.

Native Protein Analysis—The molecular mass of the rpHPLC-purified native AceKI-1 was determined by the Keck Foundation Laboratory at Yale using matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS) with a VG TOFspec SE instrument (46). In order to confirm that the purified native inhibitor was identical to the protein encoded by the AceKI-1 cDNA, a sample was sent to the Keck Facility for NH_2-terminal amino acid sequencing using an Applied Biosystems sequencer equipped with an on-line HPLC system (39).

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-30  TTACCCAAGTTTGAGAAGTCGTCGCCGTAAATGAAGGGTCTTCTGGTGGT
 21  GCTGCTTTGTGTTGCGATCGCCTATTGCGCAGAGGAAGCAGGGAAGAAGC
 71  TGACAGACGAGGAGAGATGTAATGCTCCGACTCACCTAGATGGACCACAA
121  TGCATGGCGTTCTTCAAGAGGTACACCTACAACAAGGAAAAGAAGCAATG
171  CGAAGAATTCGTTTATGGAGGATGCCGCCCATCTCCAAACAACTTTGAGA
221  CGATGGAGGAGTGCAAGAAGACTTGTGTAAAGTAATAGGCAACGGCTCTG
271  GTAGAACGCAGTACAAGAAGAGGTCTTCGACTGCTTGAAAAACTATTATT
321  CCTTCTCAACATTATGCTGTAAGCTCTTCATTGTTTGCTCGTGAGATACC
371  ATTGCGTATAAATGGCAGAGTAGTGCAAAAAAAAAAAAAAAAAAA

                Signal Sequence

     LPKFEKSSP*MKGLLVVLLCVAIAYCAEEAGKKLTDEERCNAPTHLDGPQ
     CMAFFKRYTYNKEKKQCEEFVYGGCRPSPNNFETMEECKKTCVK*
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FIG. 2. **Nucleotide and deduced amino acid sequence of AceKI-1.** The 252-base pair AceKI-1 cDNA (*top*, in *boldface type*) predicts a translated protein sequence of 84 amino acids (*below*). Initiation of translation was based on the first ATG downstream of the nematode spliced leader sequence. The translated protein has a 16-amino acid secretory signal sequence (*shaded*) with cleavage predicted between residues Cys^{16} and Ala^{17}. NH_2-terminal amino acid sequencing of purified native AceKI-1 confirmed identity of the first eight amino acids (*underlined*). The complete cDNA was submitted to the GenBank™ data base under accession no. AF172651.

RESULTS

Cloning of AceKI-1 cDNA Sequence—A partial cDNA corresponding to AceKI-1 was initially amplified from adult *A. ceylanicum* RNA using RT-PCR. This 250-base pair partial cDNA contained the nematode spliced leader sequence and the 5′ ATG initiation codon but not the 3′ poly(A) tail. The remainder of the AceKI-1 cDNA was subsequently amplified from adult *A. ceylanicum* RNA using a combination of RT-PCR and 3′-rapid amplification of cDNA ends. This yielded a 300-base pair fragment that contained identical overlapping sequence with the initial cDNA clone.

The full-length AceKI-1 cDNA contains an open reading frame of 252 nucleotides, corresponding to a translated protein of 84 amino acids (Fig. 2). Analysis using the SignalP program predicted a 16-amino acid secretory signal sequence, indicating cleavage of the protein between residues Cys^{16} and Ala^{17} of the translated full-length AceKI-1 clone. Based on this cleavage site, the translated sequence of the AceKI-1 cDNA predicts a mature protein of 68 amino acids with a molecular mass of 7889 Da and a pI of 6.79.

Expression and Purification of Recombinant AceKI-1—The cDNA fragment corresponding to the mature 68-amino acid AceKI-1 was cloned in-frame into the pET28a prokaryotic expression vector. Induced cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and Western blotting using a monoclonal antibody against the pET28a fusion protein T7 tag. Although minimal protein expression was detected by Coomassie staining, Western blotting confirmed the presence of

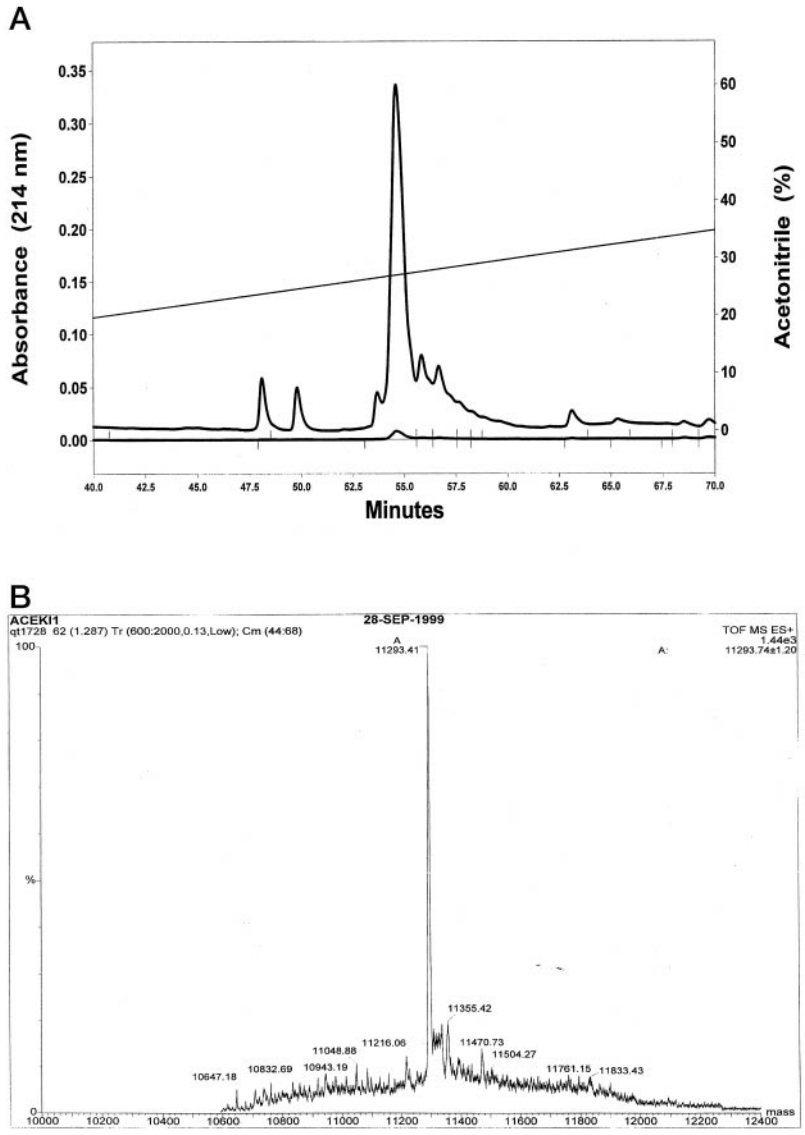


FIG. 3. **Purification of recombinant AceKI-1.** *A*, following nickel resin chromatography, the partially purified recombinant protein was subjected to reverse-phase HPLC using a C_{18} column. The bound protein was eluted with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. Individual peaks were tested for inhibition of chymotrypsin and elastase. The rAceKI-1 eluted at an acetonitrile concentration of approximately 26%. *B*, electrospray ionization mass spectrometry of purified rAceKI-1 following rpHPLC. Results show a predominant peak at a molecular mass of 11,293 Da., compared with a predicted mass of 11,451 Da, based on translation of the pET28:AceKI-1 cDNA construct.

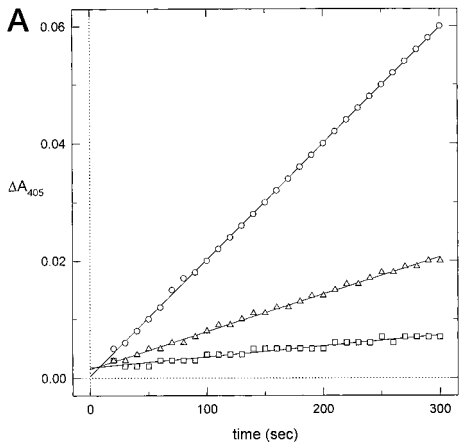
the rAceKI-1 fusion protein in the soluble fraction (not shown).

The rAceKI-1 was purified from induced cell lysates using nickel resin affinity chromatography, followed by rpHPLC. The bound protein was eluted from the C_{18} rpHPLC column under a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid (Fig. 3*A*). Samples from individual peaks were tested for serine protease inhibition. Based on characterization of inhibitory activity, the rAceKI-1 eluted at an acetonitrile concentration of approximately 26%. Electrospray mass spectrometry determined that the molecular mass of rAceKI-1 following rpHPLC is 11,293 Da (Fig. 3*B*). This compares to a predicted molecular mass of 11,451 daltons (mature protein (7889 Da) + fusion tag (3562 Da)), a difference of 158 Da or 1.4%. Based on quantitative amino acid analysis of the rpHPLC-purified protein, it is estimated that each liter of induced *E. coli* yielded approximately 0.5–1 mg of purified recombinant protein.

Serine Protease Specificity and Inhibitory Spectrum of rAceKI-1—The selectivity of rAceKI-1 was examined against 6 serine proteases, and inhibitory activity was demonstrated against chymotrypsin, pancreatic elastase, neutrophil elastase, and trypsin. At a molar ratio of 2:1 (inhibitor/enzyme), there was no detectable inhibition of the human coagulation serine proteases factor Xa or thrombin.

A single stage chromogenic assay was used to measure the rate of enzyme hydrolysis of chromogenic substrate in the presence of increasing concentrations of rAceKI-1. Preliminary results of these kinetic assays showed that rAceKI-1 is a "tight binding" (41) inhibitor ($K_i^* < [E]$) of chymotrypsin, pancreatic elastase, neutrophil elastase, and trypsin. To exclude the possibility that the enzyme activity might be changing over time due to "slow binding" (42), our kinetic studies were performed with 15-min preincubation of the inhibitor and the enzymes, followed by the addition of a relatively small volume of substrate. Under these conditions, the reaction progress curves showed no change of enzyme activity (reaction velocity) over time, as is illustrated in Fig. 4*A*. Progress curves were obtained with 3.0 nM chymotrypsin and 0.0 nM (*circles*), 2.0 nM (*triangles*), or 3.0 nM (*squares*) rAceKI-1, respectively. Fig. 4*A* illustrates that all reaction progress curves are linear, which eliminates the possibility of "slow binding" or conversely "slow dissociation" of the enzyme-inhibitor complex. These results show that the equilibrium between the enzyme(s) and the inhibitor is established practically instantaneously on the time scale of the experiment.

To determine the apparent inhibition constants for rAceKI-1 binding to these four serine proteases, the initial velocity data were analyzed by nonlinear least-squares fit. A representative set of initial velocity *versus* inhibitor concentration data for bovine pancreatic elastase is shown in Fig. 4*B*. The dose-response curves for chymotrypsin, neutrophil elastase, and tryp-



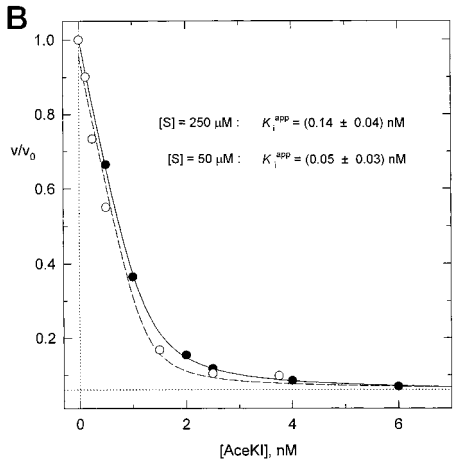


FIG. 4. *A*, time course of substrate hydrolysis. Chymotrypsin (3.0 nM) was assayed in the presence of rAceKI-1 (0.0 nM (*circles*), 2.0 nM (*triangles*), or 3.0 nM (*squares*)). The raw data were fit to a straight line model (least-squares method) by using the computer software program *PlateKi*. *B*, concentration-dependent inhibition of pancreatic elastase by recombinant AceKI-1. Recombinant AceKI-1 (rAceKI-1) was incubated with pancreatic elastase, followed by the addition of chromogenic substrate (250 μM (*filled circles*) or 50 μM (*open circles*)). The ratio of velocities of substrate hydrolysis in the presence of rAceKI-1 (V) and the absence of rAceKI-1 (V_o) is plotted, showing the relative inhibition by the recombinant protein. The smooth curves represent the best nonlinear least squares fit to Equation 1 using the software program *PlateKi*.

sin followed the same pattern (data not shown). These data were fit to an equation (41, 42) describing the kinetics of tight binding inhibitors, generating apparent equilibrium dissociation inhibitory constants (K_i*) for pancreatic elastase, chymotrypsin, neutrophil elastase, and trypsin (Table I). In order to account for the effect of substrate concentration on the K_i* derivation, the true K_i (43) was also calculated (Table I).

rAceKI-1 Is a Competitive Inhibitor—The dose-response behavior for rAceKI-1 suggests that the inhibitor is kinetically competitive with the protease substrates, as is illustrated in Fig. 4*B* for pancreatic elastase. The *full circles* in Fig. 4*B* represent a dose-response curve obtained at a final substrate concentration of 250 μM, yielding a K_i* = 140 ± 40 pM. At a final substrate concentration of 50 μM, the apparent inhibition constant was 50 ± 30 pM. According to the theory of tight binding enzyme inhibition (41), the apparent inhibition constant for a competitive inhibitor should increase with substrate concentration, following the linear relationship K_i* = K_i (1 + [S]/K_m), where K_i is the true inhibition constant and K_m is the Michaelis constant of the substrate (in this case, [S]/K_m = 5.0). Thus, the competitive inhibition constant $K_i = K_i$*/(1 + [S]/K_m) calculated from the two dose-response curves in Fig. 4*B* are in good agreement as predicted by the kinetic theory (23 and 25 pM, respectively).

TABLE I
Inhibitory kinetics of rAceKI-1

Enzyme	[S]/K_m	K_i*[a]	K_i[b]
		nM	*nM*
Pancreatic elastase	5.0	0.14 ± 0.04	0.023
Chymotrypsin	6.9	0.49 ± 0.10	0.061
Neutrophil elastase	4.0	1.06 ± 0.43	0.21
Trypsin	4.1	21.0 ± 3.8	4.1

[a] Inhibitory data similar to that illustrated in Fig. 4*B* were used to derive the apparent equilibrium dissociation inhibitory constants (K_i*) for purified rAceKI-1 against pancreatic elastase, chymotrypsin, neutrophil elastase, and trypsin. Each experiment was performed in triplicate. The notation "±" represents formal S.E. values of estimated parameters computed by the nonlinear least-squares regression software, using Equation 15.6.4 in Ref. 66. The velocity of substrate hydrolysis in the presence and absence of rAceKI-1 was measured as described under "Materials and Methods." The observed velocity data (Fig. 4*B*) were fitted to Equation 1 (41, 42).

[b] In order to account for the effect of substrate concentration on the K_i*, the actual equilibrium dissociation inhibitory constants (K_i) were derived using the following formula: $K_i = K_i$*/(1 + [S]/K_m) (45).

Characterization of Native AceKI-1 Activity from A. ceylanicum—Soluble protein extracts from adult *A. ceylanicum* hookworms were shown to contain an inhibitory activity that is similar to that described for the purified rAceKI-1 protein (Fig. 5). Using the single stage chromogenic assays described above, soluble extracts substantially inhibited the catalytic activity of chymotrypsin, pancreatic elastase, and trypsin. This activity was also present in extracts of *A. caninum* (data not shown), suggesting that specific inhibition of these intestinal proteases is conserved across individual species of the hookworm genus *Ancylostoma*. Whether all of the inhibitory activity contained in soluble extracts of *A. ceylanicum* is due to a single protein inhibitor is not yet known.

In order to determine whether AceKI-1 is secreted by the worm at the time of mucosal attachment and feeding, ES products from live, freshly harvested adult *A. ceylanicum* were also tested for AceKI-1 inhibitory activity (Fig. 5). Adult hookworm ES products contain an inhibitory activity that is similar to AceKI-1, effectively inhibiting pancreatic elastase, chymotrypsin, and trypsin compared with control RPMI media/hamster serum alone. Soluble protein extracts and ES products from *A. ceylanicum* also inhibited human neutrophil elastase (not shown).

Life Cycle Stage Specificity—Equal amounts of soluble protein extracts from both infective third stage (L_3) larvae and adult *A. ceylanicum* were tested for inhibitory activity against pancreatic elastase, human neutrophil elastase, trypsin, and chymotrypsin. Using the single stage chromogenic assay, adult *A. ceylanicum* extracts (2 μg) inhibited each of the four serine proteases by 79–87%. An identical amount (2 μg) of L_3 stage hookworm extracts showed only minimal inhibitory activity against pancreatic elastase, trypsin, and chymotrypsin (4–16%), with slightly greater activity against human neutrophil elastase (59% inhibition). These data suggest that, based on a per mg of soluble protein basis, AceKI-1 activity is specific for the adult parasite stage.

Purification of Native AceKI—In order to confirm that the inhibitory activity in soluble hookworm extracts corresponds to the AceKI-1 cDNA gene product, the native inhibitor was purified to homogeneity from adult *A. ceylanicum*. Soluble extracts from *A. ceylanicum* were applied to a size exclusion

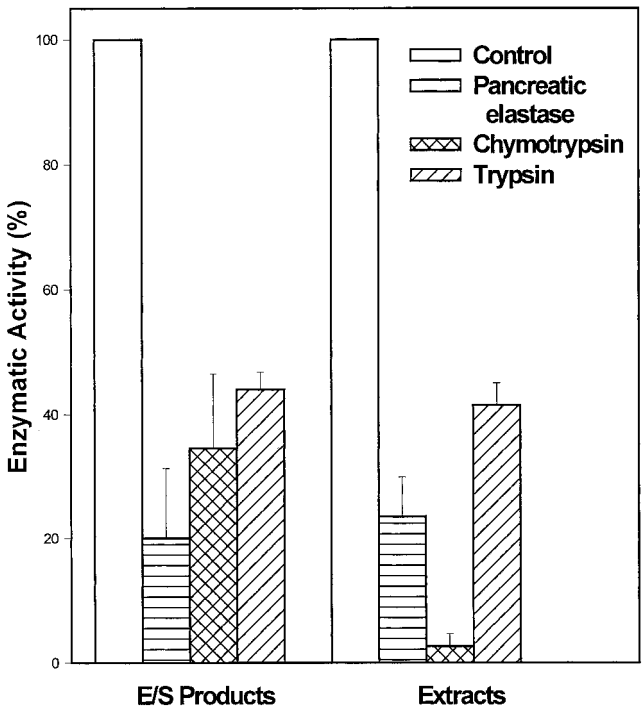


FIG. 5. **Inhibitory activity of native AceKI-1.** Soluble protein extracts and ES products from adult *A. ceylanicum* were tested for AceKI-1 activity using pancreatic elastase (1.5 nM), trypsin (4 nM), and chymotrypsin (3 nM). The data are presented as percentage of activity (1% inhibition) compared with substrate hydrolysis in the absence of hookworm proteins. Based on the inhibition of all three enzymes tested, these data suggest that an activity similar to AceKI-1 is present in adult *A. ceylanicum* extracts and is secreted by the adult worm. Comparable inhibitory activity was also detected in extracts and ES products against human neutrophil elastase (not shown).

column, and individual elution fractions (0.5 ml) were tested for inhibitory activity against chymotrypsin and elastase. Based on extrapolation from a standard curve constructed with proteins of known molecular mass, the activity eluted at an estimated MW of 5–10 kDa, comparable with the molecular mass of the predicted mature protein (7.9 kDa) based on translation of the AceKI-1 cDNA. The pooled fractions containing the chymotrypsin and elastase inhibitory activity were then subjected to rpHPLC using a C_{18} column. All of the anti-chymotrypsin and anti-elastase activity eluted in a single protein peak at an acetonitrile concentration of approximately 26% (Fig. 6*A*), comparable with the acetonitrile concentration at which the recombinant AceKI-1 fusion protein eluted from the same C_{18} column (26%) (Fig. 4*A*).

MALDI-MS of the purified native AceKI-1 revealed a predominant peak at a mass of 7882 daltons (Fig. 6*B*), compared with the predicted molecular mass of 7889 Da based on the translated sequence of the AceKI-1 cDNA. This difference of 7 Da (0.08%) is well within the range of error for the MALDI-MS measurements of protein mass. In order to confirm that the native protein was, in fact, AceKI-1, the purified inhibitor was submitted to the Keck Foundation Laboratory at Yale for NH_2-terminal amino acid sequencing. The first eight NH_2-terminal amino acid residues from the purified protein (AEEAGKKL) matched the first eight amino acids of the translated AceKI-1 cDNA (Fig. 2), strongly suggesting that the inhibitor purified from soluble extracts of *A. ceylanicum* represents the native AceKI-1. These data also confirmed the signal sequence cleavage site prediction generated by the SignalP computer software program.

DISCUSSION

Hookworms are blood-feeding intestinal nematodes that currently infect more than one billion people worldwide. As part of an overall strategy aimed at identifying important hookworm virulence factors, we have previously isolated a number of bioactive molecules from adult *A. caninum* hookworms, including a family of anticoagulant serine protease inhibitors (13, 14), as well as the major inhibitor of platelet integrin function (18). These antithrombotic compounds most likely facilitate hookworm blood feeding by preventing the formation of clots at the site of attachment, thereby contributing to the pathogenesis of iron deficiency anemia. Other compounds identified from *A. caninum* include proteases (16, 47, 48), a hyaluronidase (16), and a potent inhibitor of neutrophil function (17). While much has been discovered about these important virulence factors from *A. caninum*, very little is known about the evolutionary survival strategies of the human the hookworm parasites, *A. duodenale* and *A. ceylanicum*.

Utilizing a molecular strategy designed specifically to identify serine protease inhibitors from *A. ceylanicum*, we have successfully amplified by RT-PCR four cDNAs whose translated amino acid sequences show homology to members of two distinct inhibitor families. These cDNAs were isolated using a 5′ oligonucleotide primer (Fig. 1) corresponding to the nematode spliced leader (31–33), and a 3′ degenerate primer derived from a conserved internal amino acid sequence of a family of serine protease inhibitors originally isolated from the non-blood-feeding intestinal nematode *A. suum* (14, 29, 30). While three of the four cDNAs isolated from *A. ceylanicum* RNA encoded proteins belonging to the *Ascaris* family of serine protease inhibitors, the fourth, reported here, is a unique member of the Kunitz type inhibitor family.

To our knowledge, AceKI-1 is the first Kunitz type serine protease inhibitor identified from a blood-feeding hookworm of humans. The translated sequence of the AceKI-1 cDNA predicts a mature protein of 68 amino acids with a molecular mass of 7889 Da. The AceKI-1 cDNA was cloned into a prokaryotic expression vector and rAceKI-1 was purified using nickel resin affinity and rpHPLC chromatographies. Data from single stage chromogenic enzyme assays have indicated that rAceKI-1 is a competitive, tight binding inhibitor of the serine proteases pancreatic elastase (K_i = 23 pM), chymotrypsin (K_i = 61 pM), neutrophil elastase (K_i = 210 pM), and trypsin (K_i = 4.1 nM). The inhibitor demonstrates no detectable activity against human coagulation proteases factor Xa and thrombin. As further confirmation that this inhibitor does not contain anticoagulant activity, rAceKI did not prolong the clotting times of normal human plasma when evaluated using standard prothrombin time and activated partial thromboplastin time assays. After identifying the same inhibitory activity in soluble extracts of adult worms, the native AceKI-1 (nAceKI-1) protein was purified from *A. ceylanicum* using size exclusion and rpHPLC chromatographies. Mass spectrometry and NH_2-terminal protein sequencing confirmed the size and sequence identity of the native and recombinant inhibitors.

The translated amino acid sequence of the AceKI-1 cDNA demonstrates homology to members of the Kunitz type family of serine protease inhibitors, including a trypsin inhibitor from *A. caninum* called catrin (49), a chymotrypsin inhibitor from the silkworm *Bombyx mori* (50), and multiple domains of human and rat tissue factor pathway inhibitor (51) (Fig. 7). The results of data base and scientific literature searches indicate that AceKI-1 is the first Kunitz type serine protease inhibitor identified from *A. ceylanicum*.

A review of the published data on Kunitz type inhibitors suggests that the inhibitory spectrum of AceKI-1 is unique

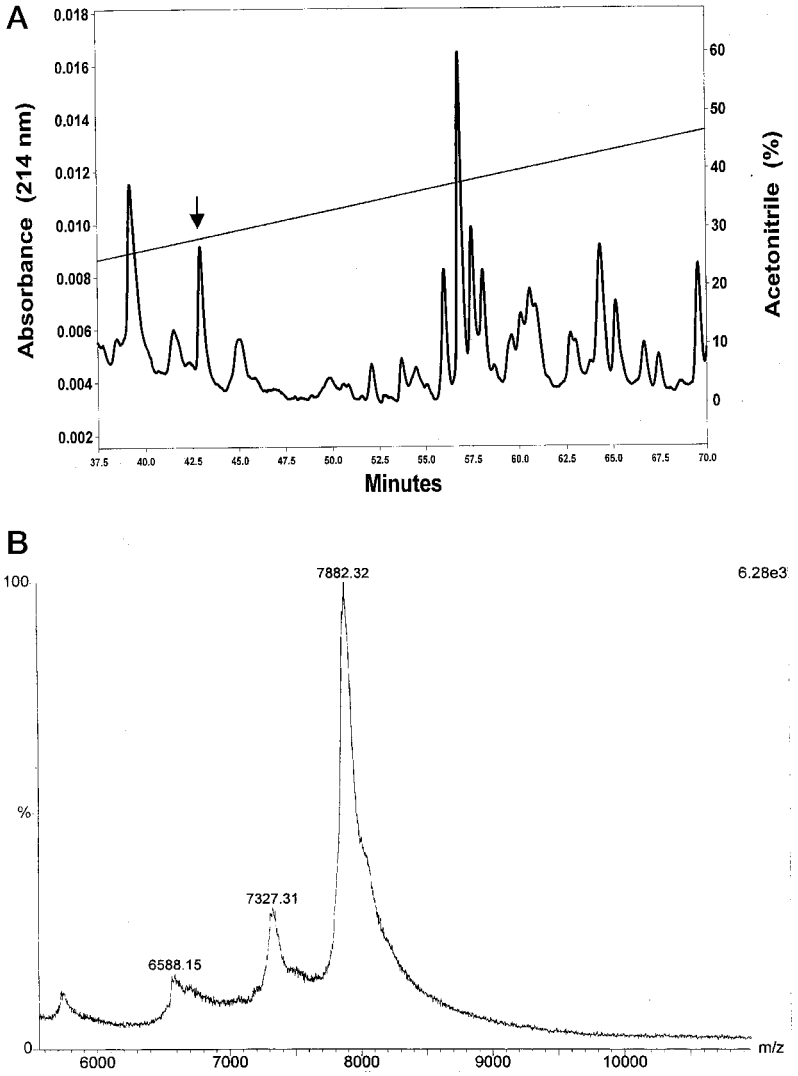


FIG. 6. **Purification of native AceKI-1.** *A*, following size exclusion chromatography, the partially purified native AceKI-1 protein was subjected to reverse-phase HPLC using a C_{18} column. The bound protein was eluted with a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid. Individual peaks were tested for inhibition of chymotrypsin and elastase. The native AceKI-1 eluted at an acetonitrile concentration of approximately 26% (*black arrow*). *B*, MALDI-MS of native AceKI-1 following rpHPLC. There is a predominant peak with a molecular mass of 7882 Da, compared with a molecular mass of 7889 Da based on translation of the cDNA corresponding to the predicted mature AceKI-1 protein.

FIG. 7. **Partial amino acid sequence comparison of AceKI-1 with other Kunitz type inhibitors.** The translated amino acid sequence of AceKI-1 is shown aligned with other members of the Kunitz type family of serine protease inhibitors. Listed are catrin (a trypsin inhibitor from *A. caninum*) (48); a chymotrypsin inhibitor from the silkworm *B. mori* (*ISC2*; GenBank™ accession no. S01803) (49); bovine pancreatic trypsin inhibitor (*BPTI*; AR040666); human tissue factor pathway inhibitor (*TFPI*; AF021834) (50); human inter-α-trypsin inhibitor, domain 1 (*ITI/H*; X63652); black mamba venom basic protease inhibitor, *Dendroaspis polylepis polylepis* (*IVBB*; P00983); and long-nosed viper chymotrypsin inhibitor, *Viper ammodytes* (LNVChyI, P00992). The six conserved cysteine residues at positions 14, 25, 41, 48, 61, and 65 of AceKI-1, which presumably form three intramolecular disulfide bonds, are *shaded* in *light gray*. The predicted P1 inhibitory reactive site residue for each of the Kunitz inhibitors is shown in *boldface type*. Compared with other members of the Kunitz type inhibitor family, AceKI-1 contains an Asn at amino acid residue 15 and an additional Gln (amino acid 24) located at the P2 residue of the inhibitory reactive site.

among members of this ubiquitous family of low molecular weight compounds. In fact, we have not identified any previously described Kunitz inhibitors with activity against chymotrypsin, pancreatic elastase, neutrophil elastase, and trypsin. Evaluation of the reactive site amino acid sequences perhaps offers insight into the broad spectrum and mechanisms of action of this hookworm inhibitor. As expected for an inhibitor of chymotrypsin and elastase, AceKI-1 has a methionine (Met^{26}) at its putative P1 inhibitory reactive site. Chymotrypsin is selective for peptide bonds on the carboxyl side of large hydrophobic or aromatic residues, due in part to the presence of a large S1 cleft with a neutral serine that interacts well with the aromatic side chain of phenylalanine or the hydrophobic amino acid methionine (52). However, when aligned with other Kunitz type inhibitors, AceKI-1 is shown to contain an additional two amino acid residues between the first two cysteines (Cys^{14} and Cys^{25}), located near the NH_2-terminal of the mature protein (Fig. 7). AceKI-1 contains an additional asparagine (Asn^{15}) and an additional glutamine (Glu^{24}) occupying the P12 and P3 positions, respectively, of the inhibitory reactive site. Although the P1 residue confers much of the target protease specificity, it has been suggested that other residues, particularly between P3 and P10, may influence binding interactions of Kunitz type inhibitors with individual proteases (53–55). Work is currently under way to determine the role of specific amino acid residues within the inhibitor's reactive site in defining the specificity of AceKI-1 for various serine proteases.

Based on the broad spectrum of activity of AceKI-1 against a number of important proteolytic enzymes, it is intriguing to speculate as to the biologic role of this inhibitor *in vivo*. The mammalian small intestine contains significant amounts of the

digestive enzymes chymotrypsin, pancreatic elastase, and trypsin, three of the proteases against which AceKI-1 demonstrates substantial inhibitory activity *in vitro*. Since we have demonstrated that AceKI-1 is secreted by the adult stage of the parasite, it is possible that the hookworm releases the inhibitor in order to neutralize the potentially damaging effects of these digestive proteases within its immediate environment. In fact, other parasitic intestinal nematodes have been shown to produce potent inhibitors of chymotrypsin, pancreatic elastase, and/or trypsin, including *A. suum* (29, 30), *Trichuris suis* (56), and the zoonotic nematode *Anisakis simplex* (57). The fact that the comparable inhibitors from *A. suum* and *A. simplex* belong to the *Ascaris* trypsin inhibitor family, rather than the Kunitz type, suggest that these activities have arisen in various nematodes by convergent evolution, *i.e.* without a common precursor.

Interestingly, AceKI-1's additional inhibitory activity against human neutrophil elastase suggests that this molecule may also be involved in host immune evasion. Because the adult worm is capable of surviving for months to years attached to the intestinal mucosa of its mammalian host, the ability to inhibit nonspecific immune mediators may confer some survival benefit. Because neutrophils are recruited to sites of tissue damage, which occurs at the site of hookworm attachment (6, 7), the parasite would clearly be exposed to any substances produced by these inflammatory cells. Interestingly, it has already been reported that adult *A. caninum* hookworms produce a potent inhibitor of neutrophil function, called neutrophil inhibitory factor, or NIF (17). This 41-kDa glycoprotein blocks the neutrophil integrin receptor CD11b/CD18, thereby inhibiting neutrophil activation. Together, hookworm inhibitors of neutrophil activation (NIF) and proteolysis (AceKI-1) would provide an effective means of preventing the host inflammatory response to parasite-mediated tissue damage in the intestine. Further characterization of these hookworm inhibitors should offer insight into their specific role in parasite survival.

Iron deficiency anemia, the most significant clinical sequela of hookworm infection, is a direct result of chronic gastrointestinal blood loss caused by the adult worm. Women and children, particularly in the developing world, frequently have lower total body iron stores and are therefore most susceptible to developing hookworm anemia. Most current public health strategies targeting hookworm disease rely primarily on the administration of oral anthelminthic agents, particularly benzimidazoles (61, 62). However, because reinfection occurs quickly among individuals living in endemic areas (60, 61), such chemotherapeutic interventions may need to be repeated at frequent intervals in order to prevent recurrence of disease. Such mass treatment strategies may also encourage the development of anthelminthic resistance, which has recently been documented in human hookworm species (63, 64). In addition, there are relatively few data on the potential toxicities of repeated anthelminthic treatments, particularly among children and pregnant women. Because of these concerns, there remains considerable interest in the development of vaccines against hookworm anemia (65). Ultimately, targeting specific parasite virulence factors like AceKI-1 may serve as a novel therapeutic strategy to alleviate hookworm disease in endemic areas.

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