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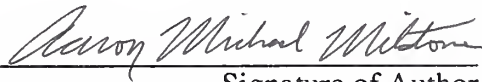A NOVEL KUNITZ-TYPE SERINE PROTEASE INHIBITOR FROM THE
HOOKWORM, *ANCYLOSTOMA CEYLANICUM*

A Thesis Submitted to the
Yale University School of Medicine
In Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Aaron Michael Milstone

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ABSTRACT

A NOVEL KUNITZ-TYPE SERINE PROTEASE INHIBITOR FROM THE HOOKWORM,
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Hookworms, blood feeding intestinal nematodes, infect more than one billion people in the
developing world. Because little is known about the role of protease inhibitors in the pathogenesis of
human hookworm disease, we have recently devised a molecular strategy aimed at isolating cDNAs that
encode related serine protease inhibitors from adult *Ancylostoma ceylanicum,* a human hookworm
parasite. Using a combination of RT-PCR and 3' RACE, we have recently cloned the first Kunitz-type
serine protease inhibitor from *A. ceylanicum*. The translated sequence of the cDNA encoding the mature
Ancylostoma ceylanicum Kunitz-type Inhibitor 1 (AceKI-1) predicts a 68 amino acid protein with a MW
of 7889 Da and a presumed methionine residue at its putative P1 inhibitory reactive site. The AceKI-1
cDNA was cloned into the pET28a expression vector, and recombinant protein (rAceKI-1) was purified
from induced lysates of *E. Coli* transformed with the rAceKI-1/pET 28a plasmid. Using single stage
chromogenic assays, we have determined that rAceKI-1 is a potent inhibitor of the serine proteases
chymotrypsin ($K_i^* = 485$ pM), porcine pancreatic elastase ($K_i^* = 277$ pM), and human neutrophil elastase
($K_i^* = 1.06$ nM). The inhibitor shows weaker inhibitory activity against trypsin ($K_i^* = 21$ nM) and no
activity against coagulation proteases factor Xa and thrombin. Additional studies have revealed that
soluble protein extracts from the hookworms *A. ceylanicum*, *A. caninum* and *Necator americanus* contain
identical inhibitory activities, suggesting that chymotrypsin/elastase/trypsin inhibition is a broadly
conserved evolutionary strategy of adult hookworms. To confirm this biological activity *in vivo*, we have
purified the native inhibitor from soluble extracts of *A. ceylanicum*, using size exclusion and rpHPLC
chromatographies. Work is currently underway to further characterize the unique mechanism of protease
inhibition by AceKI-1, as well as to determine its biologic role in parasite survival and the pathogenesis
of hookworm anemia.

ACKNOWLEDGEMENTS

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Introduction:

Hookworm infection affects up to one billion people in the developing world and is a major cause of gastrointestinal blood loss and iron deficiency anemia. Hookworm disease has plagued mankind for thousands of years. Physicians in ancient China were quoted as describing those with "able to eat but too lazy to work yellow disease" (1). In 1880, an epidemic struck Italian laborers building a railway tunnel in the Swiss Alps, afflicting them with what was termed miner's anemia. In an attempt to rid the Southeastern United States of rampant infection, the Rockefeller Sanitary Commission for the Eradication of Hookworm Disease was launched in 1909. Hookworms quickly gained respect not just as the "germ of laziness", but also as a major cause of clinical disease. John D. Rockefeller hoped his Commission could help "relieve human suffering"(2). Through increased public health awareness, the Commission spawned research to study hookworm biology and the pathogenesis of hookworm disease (3). Almost a century later, hookworm infection continues to plague approximately one fifth of the world's population. We now know, however, that the presence of hookworm in their human host is not the problem; it is disease these hookworms cause that we hope to eliminate.

In human populations the prevalence of hookworm infection depends upon the presence of an environment that is suitable for development and survival of hookworm eggs and larvae, an environment where soil is contaminated with feces, and where humans are in contact with the contaminated soil (4). Consistently, hookworm infection is predominant in parts of the rural tropics where poor sanitary conditions and the use of human feces as fertilizer provide a favorable climate for parasite transmission.

Hookworms infect people of all ages with peak prevalence in late adolescence. An increased rate of infection in older children and adults may result from a greater likelihood of this group contacting contaminated soil. Prevalence of hookworm has been reported as high as 87% in some areas of Kenya (5), but commonly 30% of people in a population will have intense infections, harboring 70% of the total worm burden (6).

Ancylostoma duodenale is the most prevalent human hookworm pathogen and is predominantly found in India, China, and Mediterranean areas of Africa and Europe, and has been described in Latin America as well (7). *A. ceylanicum* is an infrequent parasite of humans in India and Southeast Asia (8,9), but its ability to also affect other animals including dogs, cats, and hamsters makes it one of the easier species to study using animal models (10). *Necator americanus*, the New World hookworm, named because of its widespread distribution in the Caribbean and the Americas, is also found in the Pacific Islands, Sub-Saharan Africa, and Southeast Asia.
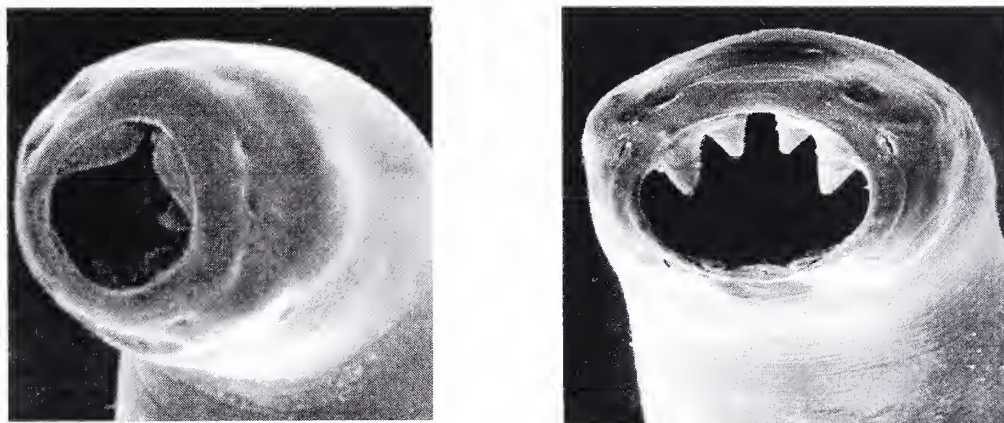
Figure 1: *Necator americanus* (left) and *Ancylostoma duodenale* (right)

Hookworms are nematodes of the family *Ancylostomidae* and are differentiated by their buccal teeth or cutting plates (see figure 1). Two important genera of hookworms, *Ancylostoma* and *Necator,* are responsible for most clinical disease in

humans. Species of *Ancylostoma*, including *A. braziliense* (cat hookworm) and *A. caninum* (dog hookworm) generally invade the human host percutaneously and cause cutaneous larva migrans. *Ancylostoma duodenale*, *A. ceylanicum*, and *Necator americanus* invade percutaneously, but they complete their lifecycle in the host, becoming adult blood feeding worms. Additionally, *Ancylostoma* can be transmitted by oral ingestion of contaminated food or soil (11). Vertical transmission of *A. caninum* has been demonstrated in the colostrum and milk of dogs, and although *A. duodenale* has not been isolated human milk, a high prevalence of infection in infants and young children has led to suggestions that it, too, can be transmitted vertically (12).

Hookworms infect humans by a rather indirect route (see figure 2). Adult female worms in the host intestine produce thousands of eggs that are expelled in the host's feces. These eggs are best suited for damp, sandy, and friable soil in a warm and shady climate, where they will hatch into microscopic larvae, feed on bacteria and organic debris, and undergo two developmental molts (13). Larvae can live for up to a month in the soil before entering a host to continue development. Third stage infective larvae will migrate up in the soil and can exhibit "questing behavior" as they climb up a blade a grass, stand upright, and sway, awaiting contact with a host.

Hookworms contact human skin typically on the feet and legs, where they enter percutaneously by burrowing into the skin (14). In previously exposed individuals, an inflammatory response leads to the accumulation of white blood cells and an often intense itch. The hookworm larva penetrates the epidermis, the basement membrane, the lymphatics and venules in the skin, and is then carried in the circulation to the right side of the heart. After reaching the pulmonary capillaries, the larva penetrates the lung

parenchyma and ascends to the trachea before being coughed up and swallowed by the host. In the small intestine the third stage infective larva undergoes two additional molts, sexually differentiates into a blood feeding adult, and attaches to the intestinal mucosa.
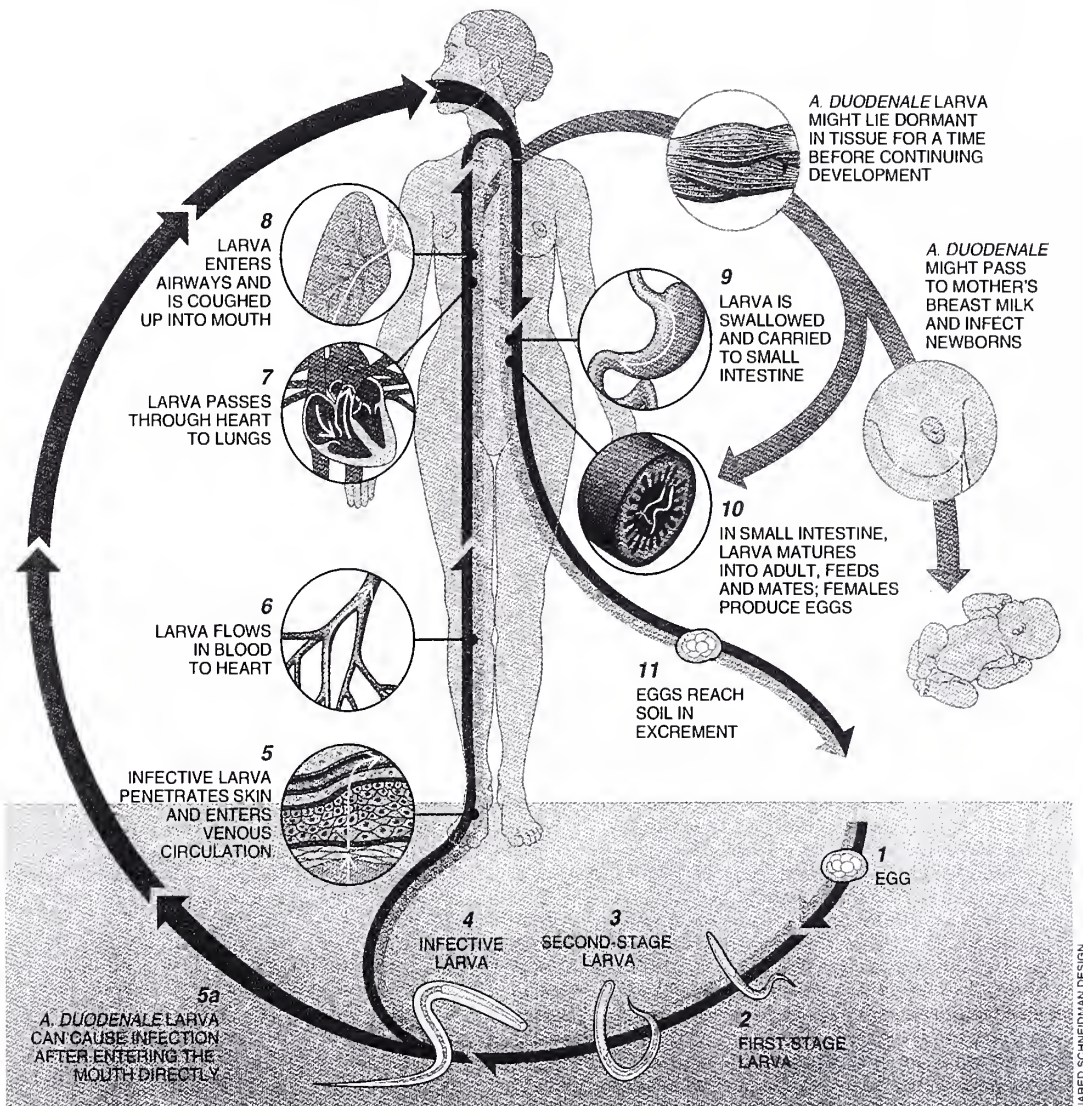
Figure 2: The lifecycle of *A. duodenale*. (13)

The lifecycle begins with development of the egg in the soil (1-4). Infective larvae (L_3) usually penetrate the skin and follow the venous circulation to the lungs, eventually making their way to the intestine (5-9). Mature adults copulate and eggs are excreted in feces to renew the lifecycle in the soil (10,11). *A. duodenale* has been shown to establish infection following oral ingestion (5a). L3 can migrate to tissues and remain dormant and other studies suggest the potential for breast milk transmission.

Within two months of arriving in the host as larvae, adult female worms lay eggs which are passed with feces into the soil to continue the transmission cycle.

Dr. Gerhard Schad identified a divergence from this cycle when he demonstrated *A. duodenale* larvae's ability for arrested development in their host (15). The infective larvae can remain dormant for months in the host, often in skeletal muscle, before maturing to a patent adult. Dormancy may have a selective advantage for hookworms. If eggs are laid into soil that is dry and hot, then they will not survive. Arrested larvae have been shown to resume development with seasonal periodicity, which leads to speculation that larvae develop to peak ova-producing adults by onset of monsoon season, presumably to ensure for egg deposition into moist soil (16).

The presence of a few hookworms in the human intestine is clinically benign, but in cases of heavy worm burden, the resulting hookworm disease, namely iron deficiency anemia, can be lethal (17). While in the small intestine, most often the jejunum (18), the hookworm attaches to the intestinal mucosa by its specialized teeth (*A. duodenale*) or cutting plates (*N. americanus*). The parasite's muscular pharynx creates negative pressure sucking in a plug of mucosal membrane and underlying tissue (see photo to right). Lysis of mucosal cells and localized tissue damage leads to rupture of capillaries in the lamina propria and hemorrhage (19). Primary blood loss occurs as
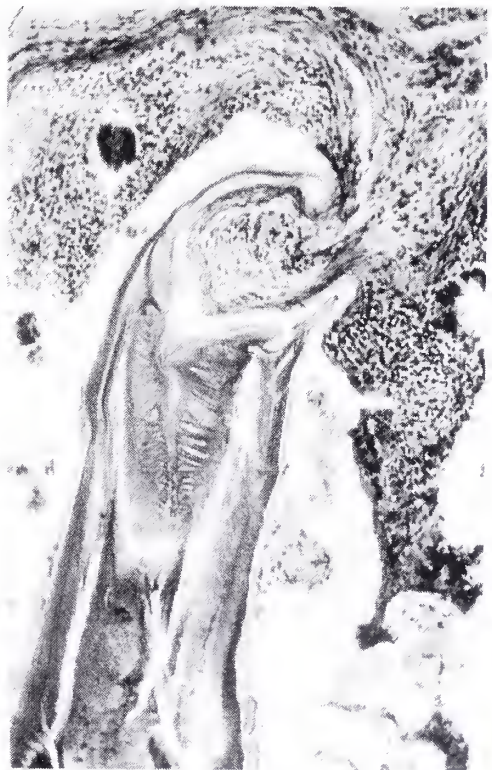
blood passes through the intestine of the worm, and secondarily from leakage around the worm's attachment site (20). Bleeding also occurs at previous attachment locations as worms move to new sites and from occasional extensive intestinal hemorrhage (21). It has been estimated that each adult *A. duodenale* can cause 0.2 - 0.4 cc of blood loss per day (22) while *N. americanus* are less aggressive feeders and result in only ten percent of the blood loss of *A. duodenale*. As mentioned earlier, many infected people harbor few worms, but those with high intensity infection are at risk for clinically significant blood loss. In such cases, studies with *A. duodenale* and *N. americanus* infection have shown daily losses of 4.8 mg iron (23) and 2 mg iron (24), respectively, up to five times the daily median requirement of iron for a healthy school-age child. Plasma protein is also lost in the blood and hypoproteinemia can be seen in heavily infected individuals.

Many studies have tried to correlate intestinal worm load, estimated by fecal egg count, and the resulting anemia, measured by hemoglobin concentration (25). The hemoglobin level, however, only declines when total body iron stores have been depleted. The development of iron deficiency anemia, therefore, is dependent not only upon the intensity and chronicity of infection, but also upon the host's total body stores and dietary iron intake. Children and women usually have the lowest iron stores and are therefore most susceptible to developing iron deficiency anemia, especially in the context of insufficient iron intake and malnutrition. Some studies have estimated that 20-80% of iron and other blood constituents can be reabsorbed further along in the intestine; however, common co-infections with *Ascaris lumbricoides* and *Trichuris trichiura* can impair the absorptive surface of the gut (26). Ultimately, though, if the infection is

treated and there is sufficient iron intake, then the hemoglobin levels will rise and the anemia will resolve (27).

The most significant clinical complication from hookworm infection is the iron deficiency anemia that results from chronic gastrointestinal blood loss. Growth retardation is a sequela of iron deficiency that can be reversed by iron supplementation (28). The effects of iron deficiency on mental performance, however, have been long debated. Observations in the Southeastern United States that had termed hookworms the "germ of laziness" also noted improvements in mental alertness that occurred following treatment of heavily infected children (2). Studies have correlated iron deficiency anemia in children with lower test scores on intelligence tests and decreased scholastic performance. Although the mechanism for this is unknown, it has been shown that as iron stores are depleted in the host, there is a decrease in iron containing enzymes such as monoamine oxidase, an enzyme involved in the production of the neurotransmitters serotonin, dopamine and norepinephrine (29). Overall, Pollitt's in depth review of the often contradictory literature concluded that hookworm infection can cause cognitive impairment (5).

The ideal approach to prevention of hookworm infection relies on improvement in the quality of life with better sanitation and water supplies as demonstrated in the southeastern United States. Better sanitary systems, however, are not imminent in the developing world. Popular literature has argued that wearing shoes would help reduce transmission of hookworm, but arguments to the contrary point out the incidence of reinfection from activated dormant larvae (11). Current strategies targeting hookworm infection involve treatment with anthelmintic agents. A single oral dose of albendazole

or mebendazole can remove adult hookworms from the small intestine. In endemic areas, however, reinfection is common within 4-12 months of treatment. Additionally, anthelminthics do not target dormant larvae, and in order to prevent anemia, multiple courses of treatment are required (30). Although effective and inexpensive (by developed nation standards), these drugs are contraindicated in patients with blood dyscrasias, leukopenia, and liver disease, and concerns remain about embryo toxicity and teratogenicity (7,24). Additionally, as has been seen with many classes of antibiotics, the development of drug resistance is possible with continued widespread usage of anthelminthics (31). The interventions present to target global hookworm disease, are therefore either improbable or have potential risks. Currently no human hookworm vaccine exists mainly due to a lack of understanding of hookworm biology. Further discovery of the requirements for parasite survival and the pathogenesis of hookworm anemia will hopefully lead to the development of a novel treatment strategy to eliminate hookworm disease.

Hookworms secrete various molecules that potentially aid in parasite survival and the pathogenesis of hookworm anemia. Targeting these virulence factors may serve as a new approach to alleviating hookworm disease (32). As a result, killing the worm may not be necessary to prevent disease; neutralizing the virulence factors that enable it to blood feed and evade the host immune response may be sufficient. These substances include anticoagulants, antiplatelet agents, protease inhibitors, proteases, hyaluronidases, and anti-inflammatory agents. These molecules are presumed to enable hookworms to penetrate the skin, travel passively through the blood stream to lodge in the capillaries of

the lungs, invade the intestinal mucosa, and cause sufficient blood loss to induce iron deficiency anemia, all occurring in the absence of an effective host immune response.

Hookworms have evolved the ability to secrete molecules that facilitate tissue invasion. The third stage (L_3) infective larva of *Ancylostoma caninum* secretes a hyaluronidase speculated to break the hyaluronan bridges connecting epidermal keratinocytes and aid in percutaneous invasion (16). Secreted metalloproteases may also play a role in breaking down connective tissue barriers allowing the spread of other secreted proteins (33). A proteolytic enzyme from adult *A. caninum* is produced presumably for degrading and lysing the engulfed bolus of host intestinal mucosa (34). Likewise, *Necator americanus* contains a variety of proteolytic activities *in vitro*, thought to be responsible for digesting host tissues (35).

Hookworms produce proteins, proteases, and protease inhibitors that may aid the parasite in surviving the host environment and evading the immune response. A serine protease inhibitor has been cloned from *Ancylostoma caninum*, named Catrin, with specific inhibitory activity against trypsin (36). Inhibiting trypsin may enable the worm to survive the hydrolytic environment of the gastrointestinal tract. A neutrophil inhibitory factor (NIF) has been cloned from adult *Ancylostoma caninum* excretory and secretory products (ES products), which binds to a CD11/CD18 receptor of neutrophils and inhibits phagocytosis and the release of oxidants (37). A 42 kD protein, named *Ancylostoma* Secreted Protein, has also been cloned from activated infective larval stage *Ancylostoma caninum* ES products (38). This secreted protein has unknown function but is similar in sequence to NIF. Proteases from *A. caninum* also demonstrate homology to cysteine proteases from *H. contortus* that cleave immunoglobulins (39). *N. americanus*

secretes a superoxide dismutase and a glutathione-S-transferase, antioxidants that can neutralize free radicals (40). All these substances are thought to help hookworms evade destruction by interfering with the host environment and immune response.

Hookworms have also evolved the ability to enhance blood feeding by secreting proteins and protease inhibitors that inhibit coagulation and platelet aggregation. This phenomenon was described as early as 1910 by Loeb and Fleisher who described a substance in hookworm homogenates that targeted and inactivated a host protein, thereby inhibiting blood coagulation and facilitating blood loss (41). More recently, distinct serine protease inhibitors from *Ancylostoma caninum* have been cloned and characterized including AcAPc2, which has inhibitory activity against the complex of coagulation factor VIIa and tissue factor (42), and AcAP5, which has activity against coagulation factor Xa (43, 44). *A. caninum* has also been shown to secrete a potent inhibitor of platelet function which blocks platelet binding to immobilized fibrinogen and collagen by the glycoproteins IIb/IIIa and Ia/IIb (45). One can speculate that *Ancylostoma* has evolved this complex strategy to block blood coagulation and platelet interaction to facilitate and enhance blood feeding.

These hookworm proteins, proteases, and protease inhibitors are believed to be essential components in the pathogenesis of human hookworm disease. Michael Cappello's laboratory is working to identify novel protease inhibitors from adult hookworms, which ultimately may contribute to parasite survival within its mammalian host. Some inhibitors may lead to increased blood loss and the development of anemia and others may have different functions. More specifically, Dr. Cappello is targeting serine protease inhibitors from *Ancylostoma ceylanicum* because this human parasite also

infects other animals including dogs, cats, and hamsters, and therefore can be studied as a model for human disease (46).

As mentioned above, a number of serine protease inhibitors have been cloned from adult hookworms. Both AcAP5 and AcAPc2 are members of the *Ascaris* family of protease inhibitors, first identified in the non-blood feeding intestinal nematode *Ascaris suum*. Catrin, also cloned from *A. caninum*, is a trypsin inhibitor that belongs to the Kunitz family. The serine protease inhibitors are divided into families based upon the configuration of disulfide bridges and the sequential location of their reactive site (47) (see discussion). The pattern of intrachain disulfide bridges is conserved within families, helping to categorize newly identified protease inhibitors. Some of the families include the Kunitz (Bovine Pancreatic Trypsin Inhibitor) Family, the Kazal (Pancreatic Secretory Trypsin Inhibitor) Family, the Bowman-Birk Inhibitor Family, the Serpins, and the *Ascaris* Trypsin Inhibitor Family. It has been hypothesized that the AcAP inhibitors facilitate hookworm blood feeding, while Catrin may protect the adult worm from trypsin-mediated proteolysis within the mammalian intestine. Intuitively, one could assume that these inhibitory activities represent a conserved evolutionary strategy for survival in all species of hookworm. In such a case, these virulence factors would serve as suitable targets for either drug or vaccine development. Therefore, Dr. Cappello devised a molecular strategy to identify novel serine protease inhibitors from adult *Ancylostoma ceylanicum*. More specifically, this project started with the goal of identifying a factor Xa inhibitor from adult *A. ceylanicum*, similar to the *Ascaris*-type inhibitor of coagulation factor Xa he had previously isolated from the dog hookworm *Ancylostoma caninum* (42).

Why are we interested in identifying and cloning novel protease inhibitors? We hypothesize that these inhibitors serve as hookworm virulence factors and play a biologic role in parasite survival and the pathogenesis of hookworm anemia. Because of their likely role in parasite survival, interfering with protein virulence factors using chemotherapeutics or immunotherapy can be a new therapeutic strategy at alleviating hookworm disease .

STATEMENT OF PURPOSE:

Protease inhibitors have been identified in multiple species of hookworms. The purpose of this research project was to identify novel serine protease inhibitors from *Ancylostoma ceylanicum* that may act as virulence factors and play a biologic role in parasite survival and the pathogenesis of hookworm anemia. We propose to use gene sequences from previously identified protease inhibitors to facilitate our cloning efforts.

Materials and Methods:

Hookworms: The life cycle of the hookworm *Ancylostoma ceylanicum* was maintained in our laboratory by Dr. Richard Bungiro and Esfir Kruglov, using a previously described hamster model (48). Infective third stage (L_3) *A. ceylanicum* larvae were provided initially by Drs. John Hawdon and Peter Hotez at Yale University School of Medicine. Three week old Syrian hamsters (LVG strain) were infected orally by gavage with 100-150 L_3 per animal. Approximately 21 days post infection, the hamsters were euthanized and the live adult hookworms harvested manually from the intestinal mucosa. Adult *Ancylostoma caninum* were obtained from the intestines of laboratory infected dogs using a similar protocol (49). Adult *Necator americanus* hookworms were harvested from hamsters in the laboratory of Dr. David Pritchard of the Department of Life Sciences at The University of Nottingham (Nottingham, England).

Hookworm Extracts and Excretory and Secretory Products : Extracts of adult hookworms were prepared by manually homogenizing the adult worms in 50 mM Tris-HCl pH 7.5 using a glass homogenizer. Soluble protein extracts, which represented the starting material for the purification and characterization of *Ancylostoma ceylanicum* Kunitz-type Inhibitor-1 (AceKI-1), were obtained by centrifugation at 10,000 x g. Protein concentrations of each lot of hookworm extracts were determined using the BCA reagent from Pierce (Rockford, IL). Extracts of infective larval stage (L_3) hookworms were prepared similarly to the adult extracts. Adult hookworm excretory and secretory products (ES products) were prepared by incubating live, freshly harvested adult worms in RPMI media with or without host serum (5%) overnight at 37° C in 5% CO_2. The

worms were removed and the secretory products clarified by centrifugation at 10,000 x g prior to use.

RNA Extraction: Fifty adult *Ancylostoma ceylanicum* were resuspended in 1.0 ml of Trizol (Gibco/BRL; Gaithersberg, MD); total RNA isolation was carried out according to the manufacturer's protocol. In brief, 50 worms were homogenized in 1.0 ml Trizol and incubated at 25°C for 10 min to allow for complete dissociation of nucleoprotein complexes. Following 5 min incubation with 200 µl of chloroform, the sample was centrifuged and the aqueous phase removed. Total RNA was precipitated by incubating the aqueous phase with 500 µl isopropyl alcohol at 25°C, followed by centrifugation. The RNA pellet was washed with 75% and 100% ethanol, air dried for 10 min, and resuspended in 40 µl diethyl pyrocarbonate (DEPC)-treated water.

Reverse transcription-polymerase chain reaction (RT-PCR) : RT-PCR of total adult *A. ceylanicum* RNA was utilized to obtain the *A. ceylanicum* complementary DNA (50). All primers were synthesized by the William Keck Foundation Biotechnology Resource Laboratory at Yale University School of Medicine (see figure 3).

First strand cDNA was synthesized by incubating approximately 1 µg RNA, 10 mM DTT and 100 ng 3'CKDGFYRD in Superscript 5X first strand reaction buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl$_2$, pH 8.3) (Gibco/BRL). The solution was heated for 90 sec at 90°C and cooled on ice. Each of four deoxynucleotides (1 mM; dATP, dCTP, dGTP,dTTP; New England Biolabs, Beverly, MA) was added along with 40 U RNasin (Promega; Madison, WI) and 200 U reverse transcriptase enzyme (Superscript II; Gibco-BRL). This mixture was incubated for 1 hr at 42°C and cooled on ice.

Figure 3 : Primers for cloning rAceKI-1

(1) 5' SLXHO	5' GGAGAAGGA<u>CTCGAG</u>GGTACTGCAGGGTTTAATTACCCA 3'	
	*Xho*1	
(2) 3' CKDGFYRD	5' GTCTCTGTAGAATCCGTCKTYGCA 3'	
(3) 5' KUNITZ1A	5' GGCGTTCTTCAAGAGGTACACCTAC 3'	
(4) 3' TTTT	5' GGCCACGCGTCGACTACTTTTTTTTTTTTTTTT 3'	
5' ACEKIBAM	5' GGAGAAGTA<u>GGATCC</u>GCAGAGGAAGCAGGG 3'	
	*Bam*H1	
3' ACEKIXHO	5' GGATCTGG<u>CTCGAG</u>GCCTATTACTTTACACAAGTC 3'	
	*Xho*1	
T7Promoter	5' TAATACGACTCACTATAGGG 3'	
M13Reverse	5' CAGGAAACAGCTATGACC 3'	

Initial cDNA sequence was obtained through RT-PCR using a sense strand PCR primer (**1**) which corresponds to the nematode spliced leader sequence present in the 5' region of many nematodes mRNAs (51-53). A degenerate antisense strand primer (**2**) is homologous to a conserved region (CKDGFYRD) observed near the carboxy-terminus of *Ascaris*-type inhibitors (42). To clone the 3' end, RT-PCR with an antisense oligo (dT) primer (**4**) was used along with an internal primer (**3**) designed from initial sequence.

The entire first strand cDNA mixture was combined with : PCR primers 5'SLXHO and 3'CKDGFYRD (100 ng each), PCR buffer (60 mM Tris-HCl, 15 mM NH_4SO_4, 1.5 mM $MgCl_2$, pH 10.0), and 1 mM deoxynucleotides. The reaction mixture was brought to a 49 ml total volume with DEPC water. Amplitaq taq polymerase enzyme (5 U; Perkin-Elmer, Foster City, CA) was then added and samples were placed in a thermal cycler (PCR Sprint, Hybaid; Middlesex, UK) for 40 cycles (94°C for 15 sec denaturation, 55°C for 5 sec annealing, and 72°C for 30 sec extension). The PCR product reaction mixture was subjected to 1% agarose gel electropheresis and visualized by ethidium bromide staining.

3' RACE (Rapid Amplification of cDNA Ends) : RT-PCR was performed, as above, using 1 µg RNA and an antisense oligo (dT) primer, 3' TTTT. The first strand cDNA mixture was added to PCR reagents, including primers 3' TTTT and 5'KUNITZ1A, an internal primer designed to initial sequence obtained (see figure 3). The mixture was placed in the thermal cycler for 40 cycles, as above using same concentration reagents and cycling conditions.

DNA Sequencing : The resulting PCR product was ligated into the pCR2.1 (Invitrogen; Carlsbad, CA) TA cloning vector by incubating ~10 ng PCR product, 50 ng pCR 2.1 vector, T4 DNA ligase, and ligation buffer overnight at 14°C. One Shot *E. coli* INVαF' (Invitrogen) were transformed with the ligation product as per manufacturer's protocol. In brief, after mixing the *E. coli* with 0.5 M B-mercaptoethanol, 10% of the ligation product was added and incubated on ice for 30 min, followed by a 30 sec heat shock at 42°C and then 2 min on ice. SOC medium was added to the transformed bacteria and incubated for 1 hr at 37°C 250 RPM. Samples were plated on LB agar plates with 30 µg/mL kanamycin (GIBCO/BRL) and grown overnight at 37°C. Colonies which 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 (EcoRI; New England Biolabs) of isolated plasmid DNA. Miniprep plasmid DNA (Spin Miniprep Kit, QIAGEN; Valencia, CA) from positive colonies was sent to the William Keck Foundation Biotechnology Resource Laboratory at Yale University School of Medicine for nucleotide sequencing.

Sequence Analysis : Sequences were analyzed for homology to other known sequences using the BLAST algorithm through the National Center for Biotechnology Information

(http://www.ncbi.nlm.nih.gov/BLAST/) (54). The full length translated amino acid sequence was analyzed using SignalP Program used for determination of the signal sequence cleavage site (http://www.cbs.dtu.dk/services/SignalP/index.html) (55,56).

Expression of Recombinant AceKI-1 (rAceKI-1): In order to clone the entire AceKI-1 cDNA corresponding to the predicted mature protein into a prokaryotic expression vector, RT-PCR was performed again, as above, using 3' ACEKIXHO and 5' ACEKIBAM primers. The 5' primer was designed based on the mature protein's predicted NH_2-terminal amino acid residues. Purified rAceKI-1 cDNA (PCR Purification Kit; QIAGEN, Valencia, CA) was digested with *Bam*H1 and *Xho*1 (New England Biolabs) and directionally cloned into the pET28a expression plasmid vector (Novagen; Madison, WI). The pET28a plasmid encodes a polyhistidine fusion protein tag at the amino terminus (AlaSerMetThrGlyGlyGlnGlnMetGlyArg) and a T7 Tag at the carboxy end. 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 on LB/kanamycin plates and grown overnight. Colonies that grew on the selective media were screened for the appropriate sized insert by direct colony PCR using an AceKI-1 specific primer (3'ACEKIXHO) and a vector specific primer (T7 Promoter) and by restriction enzyme digest (*Bam*H1 and *Xho*1) of plasmid DNA. Miniprep plasmid DNA from AceKI-1 positive colonies was subsequently subcloned into *E. coli* strain BL21 (pLysS) cells (Stratagene) for optimization of expression. Cells were plated on LB/kanamycin plates and individual colonies screened by PCR for presence of AceKI-1 cDNA.

A colony of transformed BL21 (pLysS) *E. coli* with the rAceKI-1:pET28a insert was placed in a 50 mL culture of LB/kanamycin media and incubated at 37°C 250 RPM. Log phase cultures (OD_{600} = 0.6 - 1.0) were induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; Labscientific Inc., Livingston, NJ) at a 1 mM final concentration and incubated for 3 hr with continuous shaking at 37°C. One milliliter aliquots were removed at 0, 1, 2, and 3 hr post induction, centrifuged, and resuspended in tricine SDS-PAGE sample buffer (4% SDS, 12% glycerol, 0.5 M Tris-HCl, pH 6.8, 0.01% Coomassie brilliant blue G) for SDS-PAGE and immunoblotting (see below).

SDS-PAGE and Immunoblotting : Aliquots from above protein expression culture were incubated for 15 min 37°C in SDS-PAGE buffer. Approximately 10 µg of protein from each time point was subjected to SDS-PAGE using a 13% polyacrylamide gel. One protein gel was stained with Coomassie stain (40% methanol, 10% acetic acid, and 0.1% Coomassie Brilliant Blue R250 [BIORAD]) for one hour and then destained with a solution of 30% methanol and 10% acetic acid for observation of increased protein expression by induced bacteria. A second protein gel was used for Western blotting via transfer to a 0.2 µm nitrocellulose membranes (BIO-RAD; Hercules, CA) by electro blotting at 100V for 1 hr at 4°C. The membrane was incubated overnight at 4°C with slow agitation in a 1:10,000 dilution of monoclonal HRP labeled antibody to the T7 tag, 5% milk, 0.05% Tween in PBS. After three washes in PBS 0.1% Tween, bands were visualized using a chemiluminescent substrate (SuperSignal West Pico, Pierce; Rockford, IL) and auto radiographic film (Kodak).

Recombinant AceKI-1 Protein Purification : A 2-liter culture of log-phase BL21 (pLysS) cells containing the pET28a:rAceKI-1 plasmid was induced as above with 1 mM

IPTG. At 3 hr post-induction the cells were iced for 5 min, centrifuged at 4°C 5,000 RPM, and resuspended in 1/10 the original culture volume of binding buffer (5 mM Imidazole, 500 mM NaCl, 20 mM Tris-HCl pH 7.9). The solution was sonicated using a Branson 450 Sonifier (50% Duty cycle, output 2) and centrifuged at 13,000 RPM 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 (Amersham Pharmacia Biotech AB; Uppsala, Sweden) 5 mL bed volume nickel resin affinity column charged with 50 mM Ni_2SO_4 at 25°C and equilibrated with binding buffer. The column was washed with a 60 mM imidazole buffer and eluted with 1 M imidazole buffer (Novagen His-Bind Buffer Kit). Approximately 500 ng of partially purified protein and protein standards (GIBCO/BRL) were analyzed by SDS/PAGE and Western blotting, as described above.

Following nickel resin 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% TFA. Individual peaks of protein as detected by absorbance at 214 nm were collected and assayed for serine protease inhibitory activity (as described below).

Recombinant Protein Analysis : An aliquot from rpHPLC was subjected to Electrospray Ionization Mass Spectrometry (ESMS) using a Micromass Q-Tof mass spectrometer by the William Keck Foundation Biotechnology Resource Laboratory at Yale School of Medicine. Electrospray ionization Mass Spectrometry (ESMS) is used to determine the molecular mass and purity of low molar amounts of proteins and peptides (57).

An aliquot from rpHPLC was sent to the William Keck Foundation Biotechnology Resource Laboratory for amino acid analysis. A Beckman Analyzer was used to determine the molar concentration of the purified inhibitor. This molar concentration was used to characterize rAceKI-1's inhibitory kinetics, see below (58).

Assays of Protease Inhibition : Single stage chromogenic kinetic assays were used to characterize the inhibitory activity of r-AceKI-1 against six serine proteases (59). rAceKI-1 was preincubated with each of the enzymes listed below for 15 min at 25°C followed by addition of the appropriate chromogenic substrate. In a total volume of 200 μl in individual wells of a 96-well microtiter plate, the concentration (expressed as final concentrations) of enzyme/substrate were as follows: porcine pancreatic elastase (1.5 nM; Sigma Aldrich, St. Louis, MO)/ Suc-Ala-Ala-Pro-Ala-pNA (250 μM; Bachem, Torrence, CA); human neutrophil elastase (7.5 nM; Calbiochem, La Jolla, CA)/ Elastase Substrate 1 (250 μM; Calbiochem); bovine pancrease α-chymotrypsin (3 nM; Sigma)/ Suc-Ala-Ala-Pro-Phe-pNA (200 μM, Bachem); bovine pancrease trypsin (4 nM; Sigma)/ S2302 (250 μM; DiaPharma, West Chester, OH); factor Xa (1.0 nM, Sigma)/ S2765 (250 μM; DiaPharma); α-thrombin (0.5 nM; Sigma)/ S2238 (250 μM; DiaPharma). The kinetic rate of substrate hydrolysis (mOD/min) at 405 nM was measured over 5 min using a kinetic microplate reader (MRX HD; Dynex Laboratories, Chantilly, VA). Results were expressed as percent inhibition of rAceKI-1 activity using the following formula: percent inhibition = 1 − inhibited rate/uninhibited rate × 100.

By using the single-stage chromogenic assay described above, we then established the apparent equilibrium dissociation inhibitory constant, K_i^* (60,61), for purified rAceKI-1 against pancreatic elastase, human neutrophil elastase, chymotrypsin,

and trypsin. In each microtiter well, varying rAceKI-1 concentrations were incubated for 15 min with a fixed enzyme concentration, as above. Substrate was then added at a final concentration of five times the K_m and the initial rate (mOD/min) of substrate hydrolysis over 5 min was measured 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, and these kinetic data were fit to the Morrison equation for the analysis of tight binding protease inhibitors to derive the K_i^* for rAceKI-1 (62,63).

Purification of native AceKI: In collaboration with Dr. Richard Bungiro at Yale, soluble extracts from 200 adult *A. ceylanicum* (see above preparation) were applied to a 300×7.5 mm size-exclusion chromatography column (Bio-Sil TSK-125; BIO-RAD, Hercules, CA) that was equilibrated with 50 mM Tris-HCl, pH 7.5 and 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 was estimated by extrapolation from a standard curve ($log_{molecular mass}$ vs. retention factor [R_f]) constructed from the elution profile of a mixture of protein standards (BIO-RAD) with known molecular masses.

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% TFA. Individual peaks of protein as detected by absorbance at 214 nm were collected and reassayed for serine protease inhibitory activity.

Native Protein Analysis : An aliquot of rpHPLC purified native AceKI-1 was subjected to matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS) by the William Keck Foundation Biotechnology Resource Laboratory at Yale School of Medicine to determine molecular mass. MALDI-MS uses a research grade, VG TOFspec SE instrument equipped with delayed extraction and a reflectron. Masses can be determined with an average mass accuracy of $\pm 0.1\%$ with external calibration (64).

To confirm that the purified native protein corresponded to rAceKI-1, a sample was sent to the William Keck Foundation Biotechnology Resource Laboratory at Yale School of Medicine for NH_2-terminal amino acid sequencing. The amino terminal sequencing is carried out on an Applied Biosystems sequencer equipped with an on-line HPLC system (58).

Results:

Cloning of AceKI-1 cDNA Sequence : To obtain initial cDNA sequence of AceKI-1 Lisa Harrison used a sense strand PCR primer corresponding to the nematode spliced leader (SL) sequence present in the 5' region of many nematodes mRNAs (51-53) and a degenerate antisense strand primer homologous to a conserved region (CKDGFYRD) located near the carboxy-terminus of *Ascaris*-type inhibitors (42). Using these primers, a cDNA fragment of approximately 250 bp was amplified from total adult *Ancylostoma ceylanicum* RNA. Translated amino acid sequence of the initial AceKI-1 cDNA demonstrated homology to members of the Kunitz family of serine protease inhibitors (see discussion), including tissue factor pathway inhibitor and the silkworm chymotrypsin inhibitor (see Figure 5).

The partial cDNA contained the spliced leader and the 5' ATG initiation codon, but not the 3' poly(A) tail. I began my project by isolating the 3' end of AceKI-1 from adult *A. ceylanicum* using RT-PCR and a 3' RACE technique with a newly designed internal primer and an oligo (dT) primer. This yielded an approximately 300 bp fragment. Three separate 3' end clones were sequenced and contained identical overlapping sequence with the initial cDNA. The full length AceKI-1 cDNA contains an open reading frame of 252 nucleotides (see figure 4). The translated AceKI-1 amino acid sequence was analyzed using the SignalP Program, a signal peptide prediction program. The SignalP Program strongly predicted a 16 amino acid signal sequence with the cleavage site of the translated protein to be located between residues 16 and 17 (C-A) of the full-length clone.

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

                        Putative
                      Signal sequence
      LPKFEKSSP*MKGLLVVLLCVAIAYCAEEAGKKLTDEERCNAPTHLDGPQ
      CMAFFKRYTYNKEKKQCEEFVYGGCRPSPNNFETMEECKKTCVKADNNFT
      QETAMTMITPSLVPSSDPLVTAASVLEFGLAFFKRYTYNKEKKQCEEFVY
      GGCRPSPNNFETMEECKKTCVK**ATALVERSTRRGLRLLEKLLFLLNIM
      L*ALHCLLVRYHCV*MAE*CKKKKKKSSRRVAKPNSADIHHTGGRSSMHL
      EG

Figure 4 : AceKI-1 nucleotide sequence and deduced amino acid sequence.

AceKI-1 (in bold) consists of 252 base pairs with a deduced protein sequence of 82 amino acids. Initiation of translation was based on the first ATG downstream of the nematode SL sequence. The protein has a 16 amino acid signal sequence (lightly shaded) whose cleavage site was predicted between residues 16 (C) and 17 (A)(underlined). The mature protein is 68 amino acids followed by a TAA stop codon (darkly shaded). Interestingly, the AceKI-1 nucleotide sequence corresponding to the initial 3' primer (CKDGFYRD)(circled) has only 14 of 22 matching nucleotides. The complete cDNA was submitted to GenBank™ with accession number AF172651.

The translated sequence of the AceKI-1 cDNA predicts a mature protein of 68 amino acids with a MW of 7889 Da and a pI of 6.79.

Homology of AceKI-1: Analysis of the translated amino acid sequence using the BLAST algorithm revealed AceKI-1's homology to other Kunitz-type inhibitors, including the chymotrypsin inhibitor from the silkworm *Bombyx mori* (65), a trypsin inhibitor from *Ancylostoma caninum* (36), and multiple domains of human and rat tissue factor pathway inhibitor (36,66). The location of cysteine residues in AceKI-1 (see figure 5) suggest that this is the first Kunitz-type serine protease inhibitor identified from this species of human hookworm (see discussion).

SILKWORM CHYMOTRYPSIN INHIBITOR II – *Bombyx mori*
Score = 64.8 bits (155), Expect = 8e-11, Identities = 26/47 (55%), Positives = 33/47 (69%), Gaps = 1/47 (2%)

```
AceKI-1:   8  KLTDEERCNAPTHLDGPQCMAFFKRYTYNKEKKQCEEFVYGGCRPSPNNFETMEECKKTCVK
              K T +  C        GP C A+ K Y+YN++ K+CEEF+YGGC+ + N F+T+ EC++ C+K
SCI-II:    2  KPTTKPICEQAFGNSGP-CFAYIKLYSYNQKTKKCEEFIYGGCKGNDNRFDTLAECEQKCIK
```

CATRIN – Kunitz-type Trypsin Inhibitor – *Ancylostoma caninum*
Score = 60.5 bits (144), Expect = 1e-09, Identities = 30/80 (37%), Positives = 45/80 (55%), Gaps = 9/80 (11%)

```
AceKI-1:   4  LLVVLLCVAIAYCAEEAGKKLTDEERCNAPTHLDGPQCMAFFKRYTYNKEKKQCEEFVYG 63
              +L++ +C AIA  A    + +   E C          +C AF KR+ Y+   + +C+ F+YG
Catrin:   11  VLLLFVCFAIASEARRDIRCVQAVEAC--------KCRAFMKRWAYDVTENKCKPFMYG 61

AceKI-1:  64  GCRPSPNNFETMEECKKTCV 83
              GC + NNFET  ECK+ CV
Catrin:   62  GCGGTDNNFETEAECKRICV 81
```

TISSUE FACTOR PATHWAY INHIBITOR BETA (TFPI)– *Homo sapiens*
Score = 63.6 bits (152), Expect = 2e-10, Identities = 26/48 (54%), Positives = 34/48 (70%), Gaps = 1/48 (2%)

```
AceKI-1:  21  DGPQCMAFFKRYTYNKEKKQCEEFVYGGCRPSPNNFETMEECKKTCVK 68
              DGP C A  KR+ +N   +QCEEF+YGGC + N FE++EECKK C +
TFPI   :  60  DGP-CKAIMKRFFFNIFTRQCEEFIYGGCEGNQNRFESLEECKKMCTR 106

AceKI-1:  25  CMAFFKRYTYNKEKKQCEEFVYGGCRPSPNNFETMEECKKTC 66
              C +   RY YN + KQCE F YGGC  + NNFET+EECK  C
TFPI   : 134  CRGYITRYFYNNQTKQCERFKYGGCLGNMNNFETLEECKNIC 175
```

TISSUE FACTOR PATHWAY INHIBITOR PRECURSOR (TFPI) – *Rattus rattus*
Score = 64.8 bits (155), Expect = 8e-11, Identities = 26/47 (55%), Positives = 33/47 (69%), Gaps = 1/47 (2%)

```
AceKI-1:  21  DGPQCMAFFKRYTYNKEKKQCEEFVYGGCRPSPNNFETMEECKKTCV 67
              DGP C A + Y +N    QCEEF+YGGCR + N F+T+EEC+KTC+
TFPI:     59  DGP-CKAMIRSYYFNMNSHQCEEFIYGGCRGNKNRFDTLEECRKTCI 104

AceKI-1:  25  CMAFFKRYTYNKEKKQCEEFVYGGCRPSPNNFETMEECKKTC 66
              C  F  RY YN + KQCE+F YGGC  + NNFET+EEC+ TC
TFPI:    133  CRGFMTRYFYNNQSKQCEQFKYGGCLGNSNNFETLEECRNTC 174

AceKI-1:  14  CNAPTHLDGPQCMAFFKRYTYNKEKKQCEEFVYGGCRPSPNNFETMEECKKTCVK 68
              C P   D  C A  KR+ YN   +C +F Y GC  + NNF T ++C + C K
TFPI:    222  CLEPA--DSGLCKASEKRFYYNPAIGKCRQFNYTGCGGNNNNFTTKQDCNRACKK 274
```

Figure 5 : AceKI-1 amino acid sequence homology.

Initial BLAST results revealed AceKI-1's homology to Kunitz-type inhibitors including a chymotrypsin inhibitor from the silkworm *Bombyx mori*, Catrin, two domains of human Tissue Factor Pathway Inhibitor, and all three domains of *R. rattus* Tissue Factor Pathway Inhibitor. Catrin, a trypsin inhibitor previously cloned from *A. caninum*, is the only other Kunitz-type protease inhibitor reported to date from adult hookworms. The cysteine residues involved in disulfide bridges are shaded in light gray. The P_1 reactive site residues are bold faced and underlined, along with the postulated P_1 methionine of AceKI-1 (see discussion).

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. Lysates of induced cells were separated using SDS-PAGE under non-reducing conditions. After Coomassie staining, no bands were visualized that would suggest an obvious increase in protein expression following induction. Western blots, however, using a monoclonal antibody against the pET28a fusion protein T7 Tag confirmed the presence of the rAceKI-1 fusion protein in the soluble fraction.

Having established that AceKI-1 was capable of being expressed in *E. coli*, the pET28a-rAceKI-1 fusion protein was induced in large-scale culture at an estimated concentration of 0.5 mg/L. Induced cell lysates were applied to a nickel resin affinity column. The partially purified recombinant protein was subjected to reverse-phase High Pressure Liquid Chromatography using a C_{18} column. The bound protein was eluted with a linear gradient of 0-60% acetonitrile in 0.1% TFA. Individual peaks were tested for serine protease inhibition. Recombinant AceKI-I eluted at an acetonitrile concentration of approximately 26% (see figure 6).

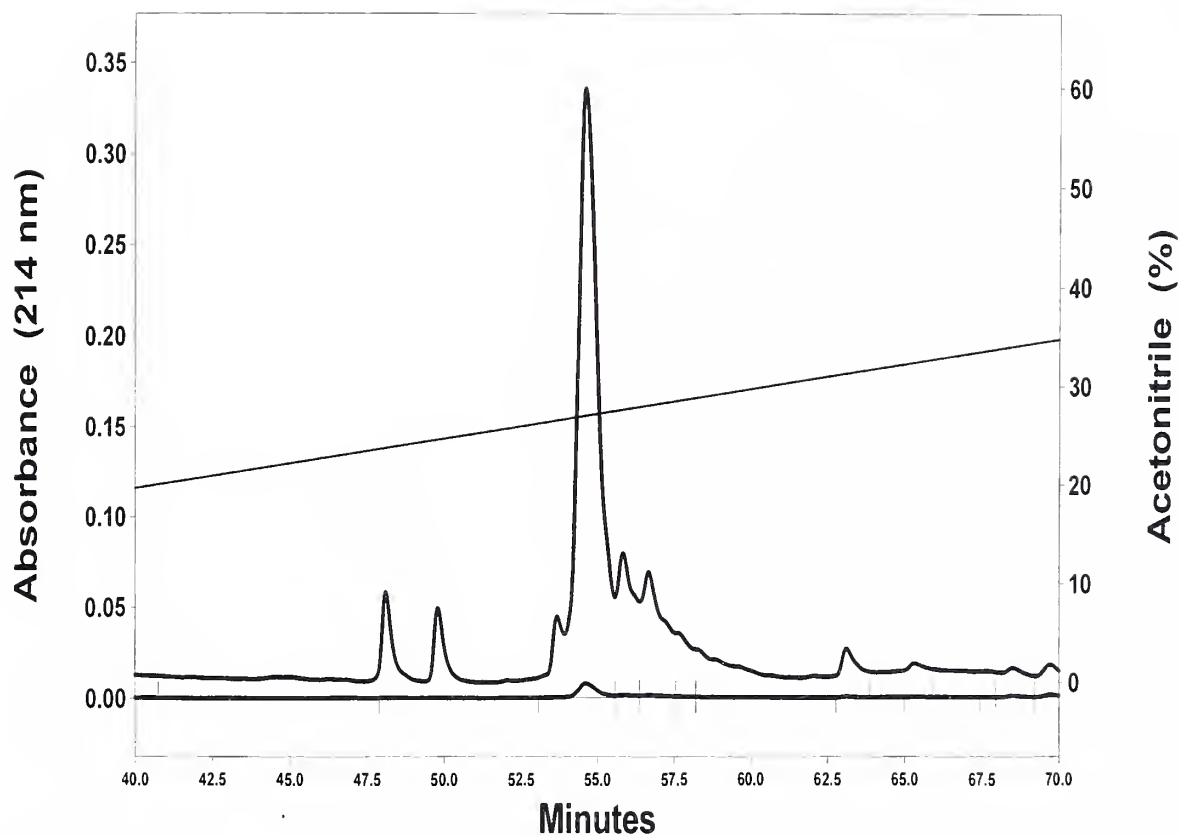Figure 6 : Purification of recombinant AceKI-1 by reversed phase HPLC

Following nickel resin chromatography, the partially purified recombinant protein was subjected to reversed-phase HPLC using a C_{18} column. The bound protein was eluted with a linear gradient of 0-60% acetonitrile in 0.1% TFA. Individual peaks were tested for inhibition of chymotrypsin and elastase. rAceKI-1 eluted at approximately 26%.

The purified recombinant protein was further characterized by ESMS (see Figure 7), and results show a predominant peak with molecular mass of 11,293 Daltons, compared to a predicted molecular mass of 11,451 Daltons [mature protein (7889 Da) + fusion tag (3562 Da)], a 1.4% difference. Quantitative amino acid analysis was performed on the rpHPLC purified protein in order to determine the molar concentration of the protein sample.
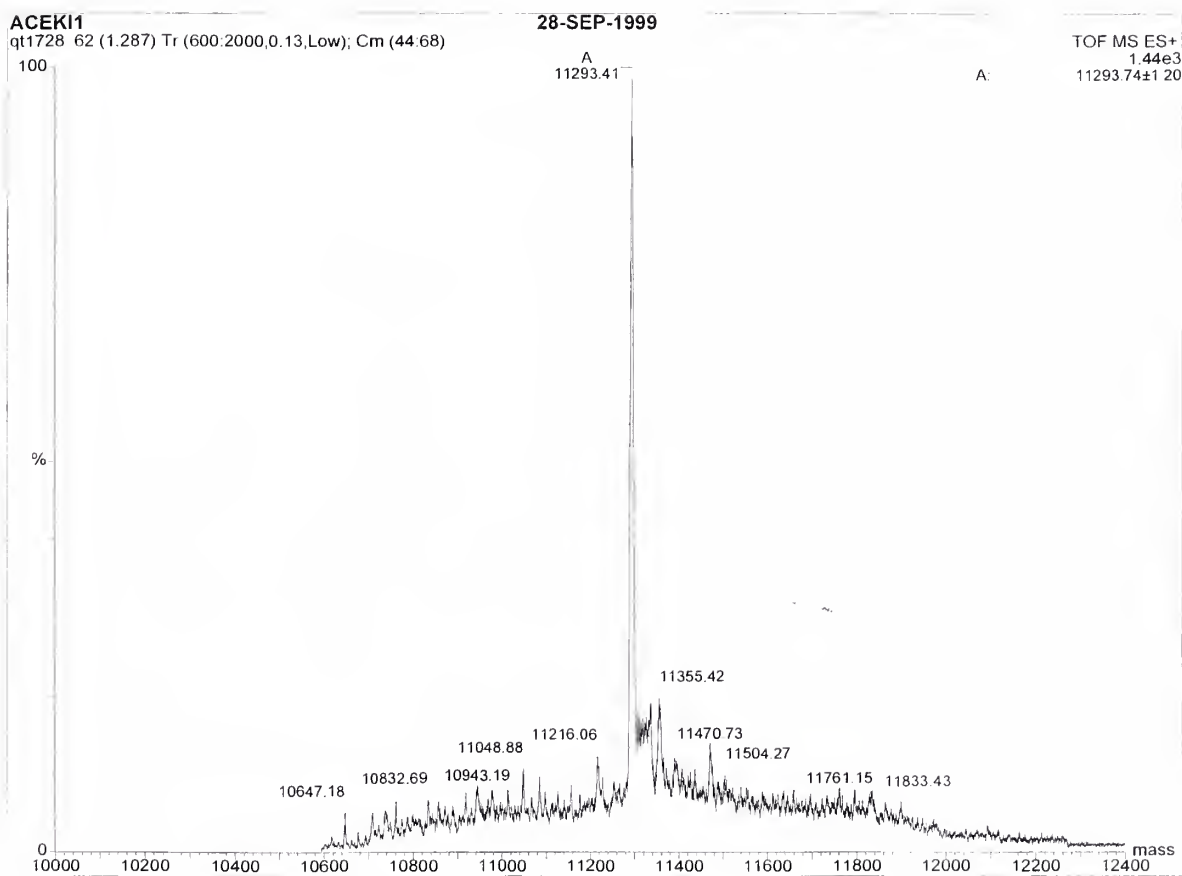

Figure 7 : Electrospray Ionization Mass Spectrometry (ESMS) of recombinant AceKI-1.

The William Keck Foundation Biotechnology Resource Laboratory at Yale School of Medicine subjected an aliquot from rpHPLC representing purified rAceKI-1 to ESMS. Results show a predominant peak with molecular mass of 11,293 Da., compared to a predicted molecular mass of of 11,451 Da.

Serine Protease Specificity : The selectivity of rAceKI-1 was examined against 6 serine proteases, and inhibitory activity was demonstrated against chymotrypsin, pancreatic elastase, human neutrophil elastase, and trypsin. At a molar ratio of 2:1, rAceKI-1 :enzyme, there was no detectable inhibition of factor Xa or thrombin. We then used single stage chromogenic assays to measure the rate of enzyme hydrolysis of chromogenic substrate in the presence of increasing concentrations of rAceKI-1. These kinetic data were fit to the Morrison equation developed for the analysis of tight binding inhibitors, generating an apparent equilibrium dissociation inhibitory constant (K_i^*) for porcine pancreatic elastase chymotrypsin, human neutrophil elastase, and trypsin (see Table 1 and Figures 8).

Table 1

Enzyme	K_i^{*A}
Porcine pancreatic elastase	277 pM +/- 71 pM
Chymotrypsin	485 pM +/- 102 pM
Human neutrophil elastase	1.06 nM +/- 427 pM
Trypsin	21 nM +/- 3.8 nM

[A] Equilibrium Dissociation Inhibitory Constant
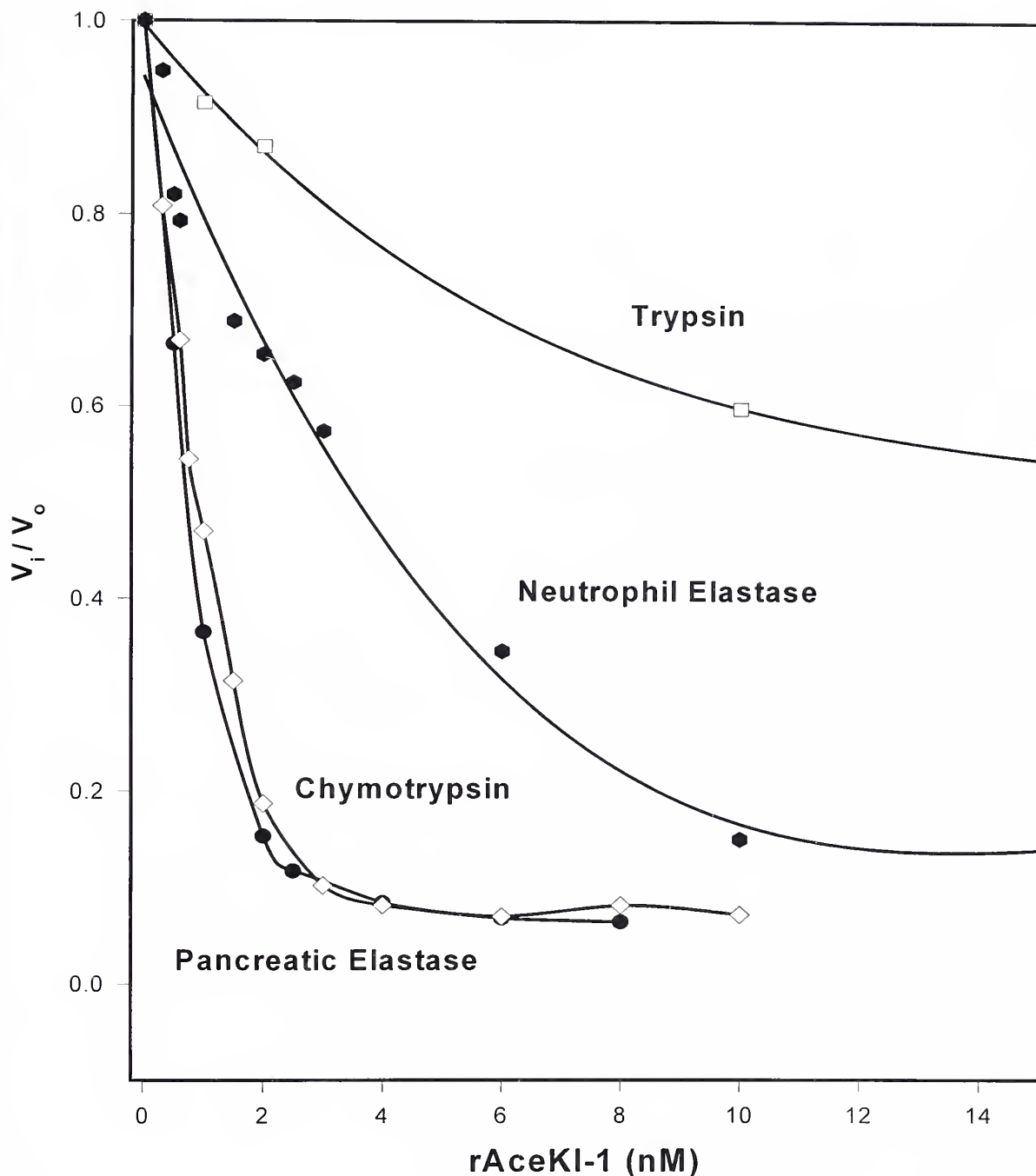
Figure 8 : Inhibitory Kinetics of recombinant AceKI-1.

rAceKI-1 in increasing concentrations was incubated with trypsin, chymotrypsin, pancreatic elastase, and neutrophil elastase followed by addition of chromogenic substrate. The velocity of substrate hydrolysis in the presence of rAceKI-1 (V_i) and the absence of rAceKI-1 (V_o) was then measured as described in the Materials and Methods. The observed velocity data (solid lines) was fit to the Morrison equation for the evaluation of tight binding inhibitors. $V_i/V_o = [E - I - Ki^*] + [(I + Ki^* - E)^2 + 4K_i^*E]^{1/2}/2E$ where E and I are the total amount of enzyme and inhibitor, respectively, and K_i^* is the apparent equilibrium dissociation inhibitory constant for purified rAceKI-1 against pancreatic elastase, chymotrypsin, human neutrophil elastase and trypsin (60,61).

Life cycle Stage Specificity: Having characterized rAceKI-1's inhibitory activity, I wanted to determine whether a similar native activity was present in different developmental stages of *A. ceylanicum*. Soluble protein extracts from both infective third stage (L$_3$) *Ancylostoma ceylanicum* larvae and adult *A. ceylanicum* were therefore tested for inhibitory activity against pancreatic elastase, human neutrophil elastase, trypsin, and chymotrypsin. While adult extracts inhibited all four enzymes, L$_3$ extracts only inhibited human neutrophil elastase (see figure 9). These data suggest that AceKI-1 is not produced in significant amounts by the L$_3$ stage of hookworms (see discussion).

	A. ceylanicum Adult Extracts (2 μg)	*A. ceylanicum* Infective Larval Stage Extracts (2 μg)
Chymotrypsin (3 nM)	87%[A]	4%
Trypsin (4 nM)	80%	14%
Pancreatic Elastase (1.5 nM)	77%	16%
Human Neutrophil Elastase (7.5 nM)	79%	59%

[A] Percent inhibition of each enzyme by stage specific soluble protein extracts

Figure 9 : Lifecycle stage specificity of AceKI-1 activity.

Soluble protein extracts from both adult and L$_3$ *A. ceylanicum* demonstrate inhibitory activity against human neutrophil elastase, but L$_3$ extracts have virtually no significant inhibit activity against chymotrypsin, trypsin and pancreatic elastase. This suggests that AceKI-1 is expressed only during specific stages in the hookworm lifecycle (see discussion).

Excretory and Secretory (ES) Products: After demonstrating a similar inhibitory activity to AceKI-1 in adult *Ancylostoma ceylanicum* extracts, I wanted to determine if a similar activity was present in ES products. Adult *A. ceylanicum* ES products (as described in Methods) were therefore tested for inhibitory activity against pancreatic elastase, trypsin, and chymotrypsin. The results demonstrate that adult ES products effectively inhibited pancreatic elastase (79%), chymotrypsin (66%), and trypsin (56%) compared to RPMI media/hamster sera alone (see Figure 10)
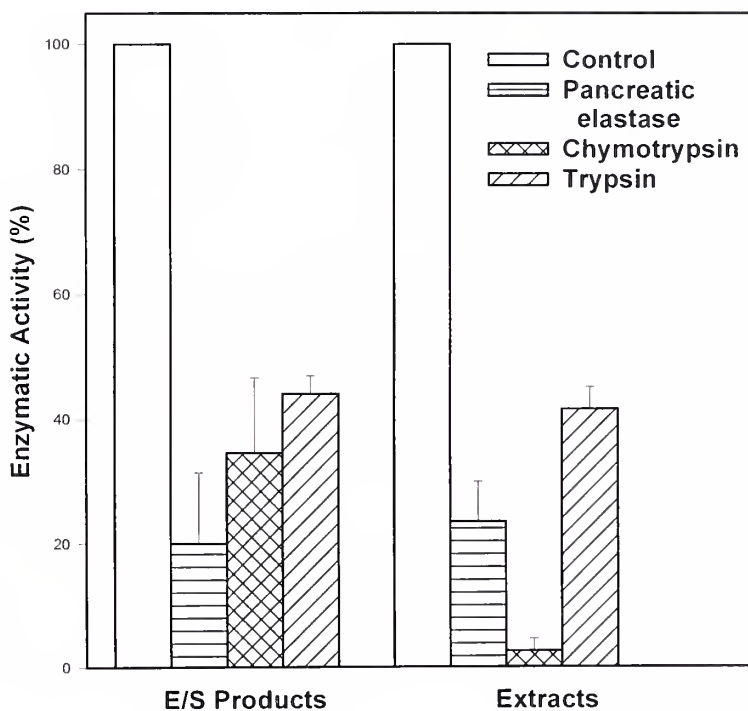
Figure 10 : Inhibitory activity of native AceKI-1.

ES products from adult *A. ceylanicum* are compared to soluble protein extracts of adult *A. ceylanicum* for their respective inhibition of pancreatic elastase (1.5nM), trypsin (4nM), and chymotrypsin (3nM). Both adult *A. ceylanicum* extracts and E/S products inhibit pancreatic elastase, chymotrypsin and trypsin. The inhibitory profile of native AceKI-1 is therefore similar to that of recombinant AceKI-1. Data illustrated as percent activity (1- percent inhibition)

Species Specificity: Equal amounts of soluble protein extracts from adult hookworms *Ancylostoma ceylanicum*, *Ancylostoma caninum*, and *Necator americanus* were screened for inhibitory activity against pancreatic elastase, trypsin, and chymotrypsin. All these hookworm species contained comparable inhibitory activity against these three enzymes (see figure 11). Chymotrypsin was inhibited by *A. ceylanicum* (92%), *A. caninum* (91%), and *N. americanus* (94%); trypsin was inhibited by *A. ceylanicum* (75%), *A. caninum* (88%), and *N. americanus* (90%); pancreatic elastase was inhibited by *A. ceylanicum* (95%), *A. caninum* (93%), and *N. americanus* (89%). Assuming that this inhibitory activity is due to AceKI-1, these data suggest that AceKI-1 is broadly conserved across evolutionary distinct hookworm species.
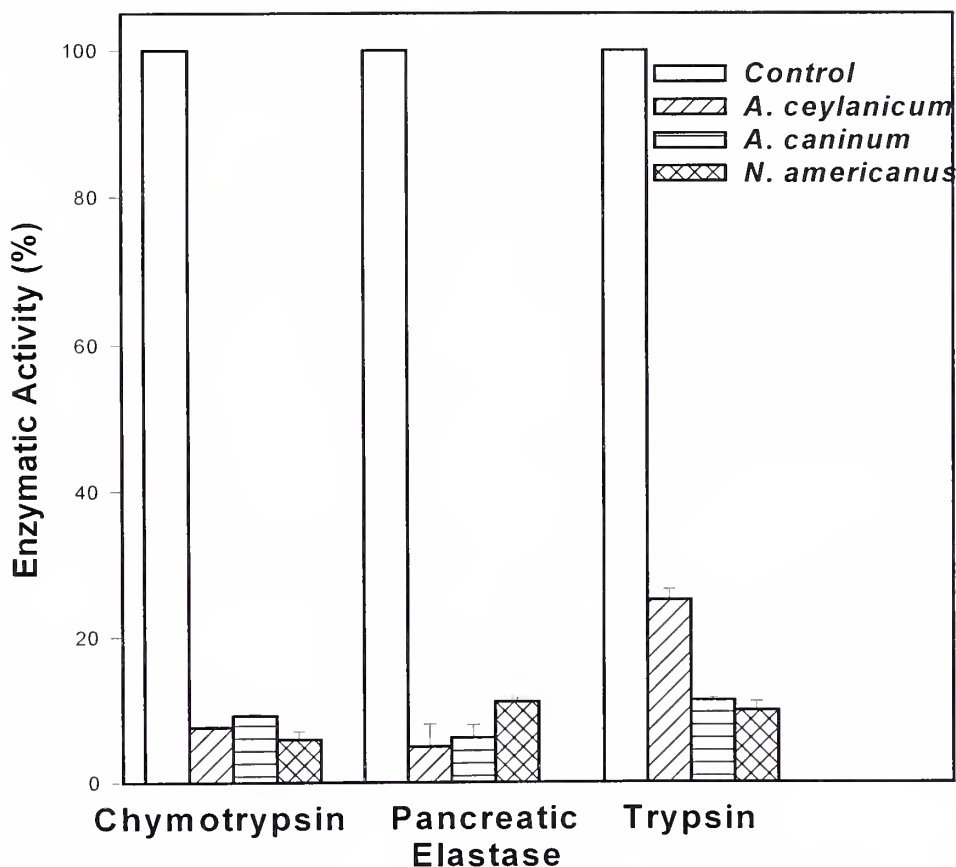
Figure 11 : Conservation of AceKI-1 inhibitory activity across hookworm species.

5 μg of soluble protein extracts from *A. ceylanicum*, *A. caninum*, and *N. americanus* were screened for activity against pancreatic elastase (1.5nM), trypsin (4nM), and chymotrypsin (3nM). Chymotrypsin, trypsin and pancreatic elastase inhibitory activity is present in all three species, suggesting that inhibition of these three serine proteases is broadly conserved.

Purification of native AceKI: In order to prove that the activity in soluble protein extracts corresponds to the AceKI-1 cDNA gene product, with the help of Dr. Richard Bungiro, I purified the native inhibitor from adult *A. ceylanicum*. Fractions from size exclusion chromatography of soluble adult hookworm extracts were measured for inhibitory activity against chymotrypsin and elastase. The activity eluted at an estimated weight of approximately 9 kDa, based on extrapolation from a standard curve constructed with proteins of known molecular mass. The pooled samples of chymotrypsin and elastase inhibitory activity were then subjected to rpHPLC on 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% (see figure 12). Likewise, the rAceKI-1 fusion protein eluted from the same C_{18} column at a concentration of acetonitrile of approximately 26%.
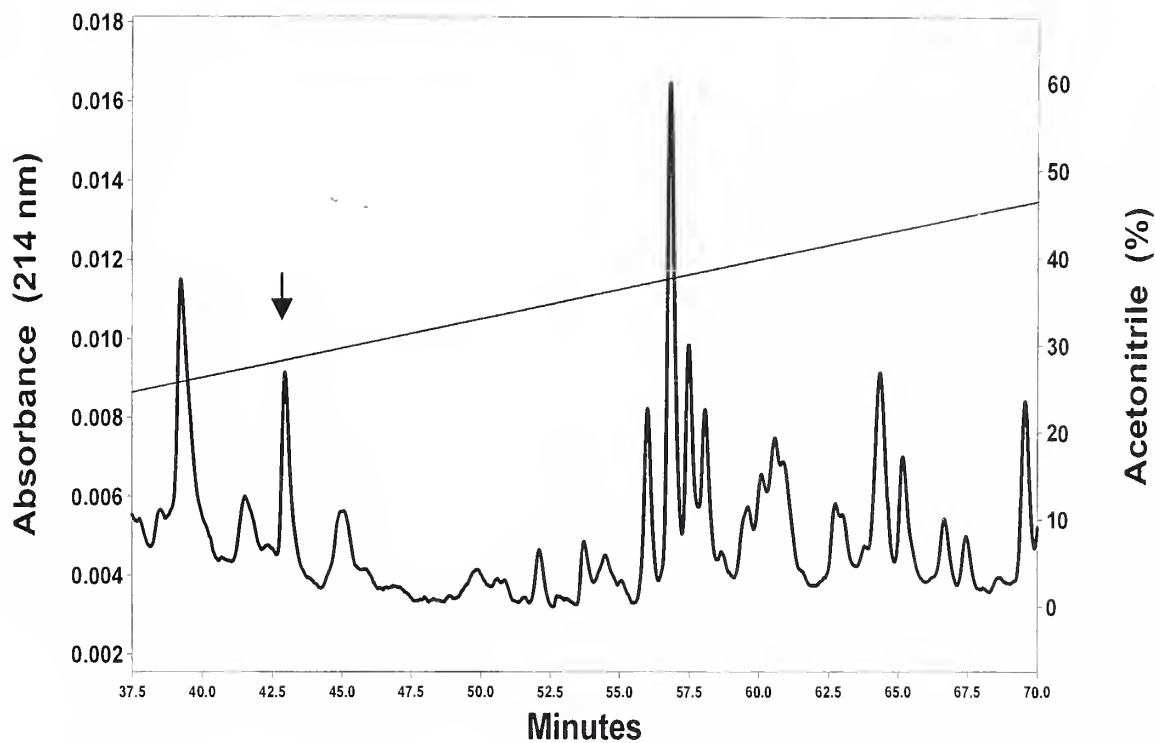
Figure 12 : Purification of native AceKI-1 by reversed phase HPLC

Following size exclusion chromatography, the partially purified protein was subjected to reversed-phase HPLC using a C_{18} column. The bound protein was eluted with a gradient of 0-60% acetonitrile in 0.1% TFA. Individual peaks were tested for inhibition of chymotrypsin and elastase. Native AceKI-1 eluted at approximately 26% (black arrow)

The native protein was further characterized by MALDI-MS (see figure 13), which revealed a single major peak with a molecular weight of 7882 Daltons, compared to the predicted molecular weight of 7889 Da based on the translated sequenceof the AceKI-1 cDNA. This difference of 7 Da (0.08%) is within the range of error for the MALDI-MS measurements of protein mass.
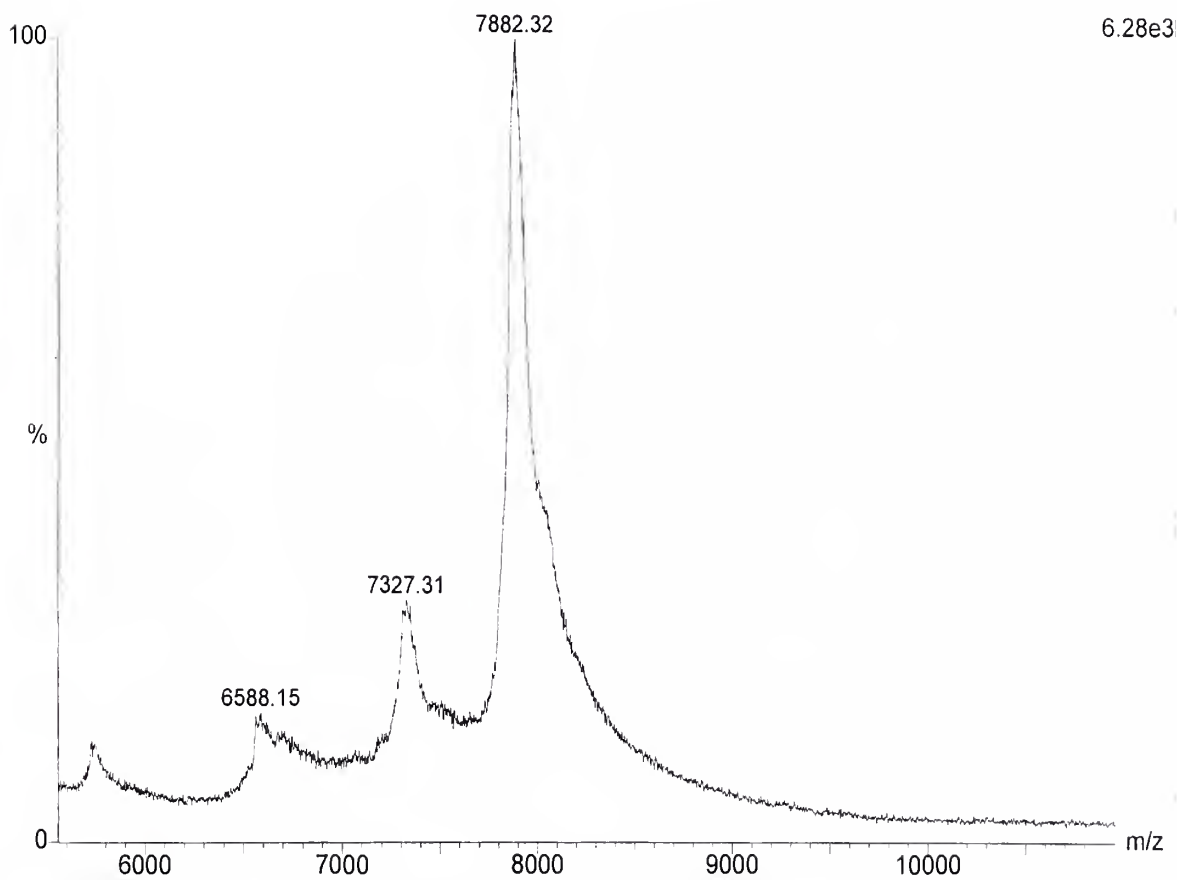
Figure 13 : Matrix-Assisted Laser Desorption Ionization Mass Spectroscopy (MALDI-MS) of native AceKI-1.

The William Keck Foundation Biotechnology Resource Laboratory at Yale School of Medicine subjected an aliquot from rpHPLC representing purified native AceKI-1 to MALDI-MS. Results show a predominant peak with molecular mass of 7,882 Da., compared to a predicted molecular mass of 7889 Da.

In order to confirm that the native protein was, in fact, AceKI-1, the purified inhibitor was submitted to the William Keck Foundation Biotechnology Resource Laboratory at Yale School of Medicine for NH_2-terminal sequence. The first eight NH_2-terminal amino acid residues from the purified protein were identical to the first eight amino acid of the translated AceKI-1 cDNA, confirming identity of the native and recombinant proteins.

Discussion:

Hookworms, blood feeding intestinal nematodes, infect more than one billion people in the developing world. The most significant clinical complication from hookworm infection is iron deficiency anemia, resulting from chronic gastrointestinal blood loss. Women and children often have lower total body iron stores and are therefore most susceptible to developing hookworm anemia. In children, heavy infection is associated with lower scores on intelligence tests and decreased scholastic performance. Current strategies targeting hookworm disease revolve around the use of anthelminthics. With growing concern of drug resistance and drug toxicity, the search for alternative control tactics continues. Dr. Cappello's laboratory is working to identify novel protease inhibitors from adult hookworms that aid in parasite survival and contribute to the pathogenesis of hookworm anemia. Ultimately, interfering with these parasite virulence factors may serve as a novel therapeutic strategy to alleviating hookworm disease.

I report here the first Kunitz-type serine protease inhibitor isolated from adult *Ancylostoma ceylanicum*, a blood feeding hookworm. The translated sequence of the *Ancylostoma ceylanicum* Kunitz-type Inhibitor 1 (AceKI-1) cDNA predicts a mature protein of 68 amino acids with a MW of 7889 Da. The AceKI-1 cDNA was cloned into a prokaryotic expression vector and recombinant AceKI-1 (rAceKI-1) was purified using nickel resin affinity and rpHPLC chromatographies. The molecular weight of rACeKI-1 was confirmed by mass spectroscopy. Single stage chromogenic assays revealed that rAceKI-1 is a potent inhibitor of the serine proteases chymotrypsin ($K_i* = 485$ pM), porcine pancreatic elastase ($K_i* = 277$ pM), and human neutrophil elastase ($K_i* = 1.06$ nM). The inhibitor demonstrates weaker inhibitory activity against trypsin ($K_i* = 21$nM)

and no detectable activity against coagulation proteases factor Xa and thrombin. After identifying this biological activity *in vivo*, we purified native AceKI-1 (nAceKI-1) from soluble extracts of adult *Ancylostoma ceylanicum* using size exclusion and rpHPLC chromatographies. Yale's Keck Foundation Biotechnology Resource Laboratory verified the native protein's molecular weight using mass spectroscopy. NH_2-terminal amino acid sequencing of nAceKI-1's first eight residues confirmed identity of the native and recombinant proteins.

AceKI-1 is the first Kunitz-type protease inhibitor isolated from *Ancylostoma ceylanicum*. As discussed earlier, serine protease inhibitors are divided into families based upon the configuration of disulfide bridges and the sequential location of each reactive site (47). The pattern of intrachain disulfide bridges is conserved within families, helping to categorize newly identified protease inhibitors. Some of the families include the Kunitz (Bovine Pancreatic Trypsin Inhibitor) Family, the Kazal (Pancreatic Secretory Trypsin Inhibitor) Family, the Bowman-Birk Inhibitor Family, the Serpins, and the *Ascaris* Trypsin Inhibitor Family.

Protease inhibitors are ubiquitous in nature, generally functioning to prevent undesired proteolysis. Most protease inhibitors target only proteases belonging to a specific class. Their action relies on presenting an endogenous peptide bond, a reactive site, as a substrate to the target enzyme and forming a classic lock and key interaction. Protease inhibitors are divided into classes by the mechanistic action of their inhibition; some inhibit carboxyl, metalo and sulfhydryl proteinases, and others inhibit serine protease. Serine protease inhibitors are the best characterized and understood, and although there are many heterogeneous subfamilies, they do have a similar mechanism of

action. The reactive site of each inhibitor interacts with the respective active site of its associated enzyme. A stable complex is formed and hydrolysis occurs very slowly as if the coupling was being maintained at equilibrium. Each reactive site residue, denoted as P_1, generally designates the specificity of a given inhibitor for a particular protease (67).

In order to categorize and characterize a novel protease inhibitor we use BLAST searches looking for amino acid sequence homology to previously described inhibitors. All members of the *Ascaris* family, for example, share five intramolecular disulfide bridges (68) and 30-40% amino acid sequence identity (69). Kunitz inhibitors are approximately 60 amino acid residues in length, are generally tight binding inhibitors (70), and their six cysteine residues form three disulfide bridges in the characteristic pattern : 1-6, 2-4, 3-5 (71). rAceKI-1 demonstrates strong homology to members of the Kunitz family based on its spacing pattern of cysteine residues and postulated P_1 reactive site (see figure). By examining the amino acid sequence and comparing it to other protease inhibitors, we can postulate its mechanism of inhibition of chymotrypsin, pancreatic elastase, human neutrophil elastase, and trypsin.

In order to elucidate AceKI-1's mechanism of inhibition, we will first look at the reactive site amino acid residue and the structural interactions of other Kunitz inhibitors. Bovine Pancreatic Trypsin Inhibitor (BPTI) was the first Kunitz inhibitor to be characterized on a structural level and serves as a model for other Kunitz inhibitors. Studies of the molecular structure of BPTI's reactive site and side chain interactions have helped to explain why amino acids in this region determine the specificity by which Kunitz inhibitors bind proteases. The tertiary structure of BPTI suggests the reactive site is an external loop held stable and rigid by interactions with its own core molecule and an

additional minor loop (72). This external loop contains the reactive site, P_1 residue, where primary interaction occurs with the cognate protease's catalytic site, the S_1 principal specificity cavity (73) (see figure 14). This inhibitor:protease complex is stabilized by van der Waals interactions between the carbonyl carbon of the P_1 residue and the nucleophilic oxygen of the catalytic protease side chain (74). Water molecules fill the grooves as permitted, strengthening the interaction with hydrogen bonds. This reactive site loop and active site cleft form a tight interaction with little free energy, thereby requiring a high activation energy for hydrolysis and dissociation (75). The scissile bond of these tight binding inhibitors, therefore, remains intact as it undergoes slow hydrolysis (77). The
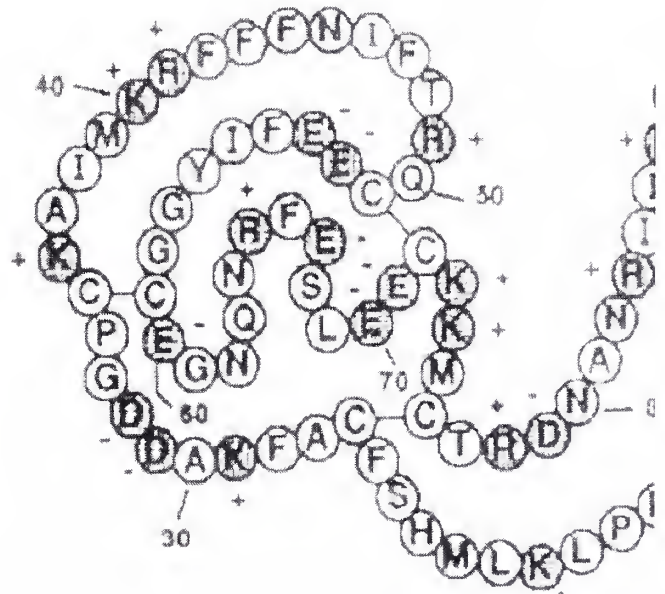
Figure 14 : TFPI Domain 1 (76)

This diagram, based on the secondary structure of BPTI, illustrates the amino acid sequence of TFPI domain 1, which is homologous to AceKI-1. Three cysteine-cysteine disulfide bonds between can be seen as is the protease inhibitor's external loop, residues 30-50. At location 36 is the P_1 lysine. The conserved GPC, P_2 - P_4, should be noted and will be seen discussed below.

interaction between the P_1 residue and the S_1 cleft is determined by the amino acids in the area of the reactive site; specifically, the side chains of the P_1 residue itself and those of its surrounding amino acids, and their ability for polar interaction.

Based on the selective interactions of reactive site residues and their cognate enzyme's catalytic site, we expect proteases to bind preferentially to certain P_1 amino acid residues. For example, chymotrypsin is selective for peptide bonds on the carboxyl

side of large hydrophobic or aromatic residues. Chymotrypsin has a large S1 cleft with a neutral serine and can bond well with aromatic side chains of phenylalanine or a hydrophobic methionine (73). In general, the following P_1 residues commonly interact with the respective proteases: tyrosine, phenylalanine, leucine, and methionine with chymotrypsin; leucine and arginine with trypsin; leucine, methionine, and valine with elastase (78). The nature of interaction between proteases and reactive sites will determine the overall specificity; resultingly, strong inhibition of chymotrypsin and elastase tends not to be seen concurrently with strong inhibition of trypsin. AceKI-1's interaction with chymotrypsin, however, is therefore consistent with its postulated P1 methionine (discussed below).

Understanding how reactive site residues and cognate enzymes interact illustrates why the activity of Kunitz inhibitors depends upon the properties of their P_1 amino acid residues. BPTI, for example, a strong inhibitor of trypsin, has a P_1 lysine that weakly interacts with chymotrypsin. Carboxamidomethylation and reduction of the primary disulfide bond eliminates the chymotrypsin activity while retaining the trypsin activity, presumably by altering bonding interaction between the catalytic sites (79,80). Chymotrypsin inhibitors have been identified from cobra venom of *Naja naja naja*, the silkworm *Bombyx mori*, and venom of *Vipera ammodytes* and their P_1 residues are phenylalanine, phenylalanine, and leucine, respectively (81). A Kunitz-type trypsin inhibitor from *Fasciola hepatica* was identified with P_1 leucine (82). Leucine is not characteristically complexed with trypsin, which might explain why this inhibitor has a high dissociation constant and is not tight binding.

Comparing protease inhibitor homologues from humans and pigs demonstrates the role that the reactive site amino acid residue plays in determining the specificity of interaction between inhibitors and cognate enzymes. Human Inter-α-Trypsin Inhibitor (HITI) is a well-characterized bidomain Kunitz-type inhibitor of trypsin found in many species of mammals. HITI's C-terminal domain, which inhibits trypsin strongly and chymotrypsin weakly, has arginine at its P_1 residue (83). The N-terminal domain has a P_1 methionine, weakly inhibits polymorphonuclear (PMN) granulocyte elastase, and has no activity against chymotrypsin and pancreatic elastase (84). In comparison, bovine Inter-α-Trypsin Inhibitor (BITI)'s C-terminal domain is similar in sequence and activity to HITI; however, its N-terminal P_1 leucine functions as a strong inhibitor of PMN granulocyte elastase, pancreatic elastase and chymotrypsin (85). The N-terminal domain of HITI and BITI exemplify how differences in the reactive site amino acid affect the specificity of individual protease inhibitors.

Other studies demonstrate that substitutions or mutations of the P_1 amino acid residue can alter activity of a homologous protease inhibitor. BPTI derivatives with methionine and valine replacing the P_1 residue lysine are strong inhibitors of chymotrypsin, and chymotrypsin and elastase, respectively (85/72). Mutation of the P_1 lysine in Bovine Trypsin Kallikrein Inhibitor to phenylalanine and tryptophan decreased activity against trypsin and greatly increased inhibition of chymotrypsin (86). Mutagenesis of the P_1 arginine in Alzheimer's β-Amyloid Precursor Protein (APP-KD) to valine changed a trypsin and chymotrypsin inhibitor to a strong inhibitor of human neutrophil elastase (87). Notable in this study was that the small hydrophobic valine did

not confer inhibitory activity against chymotrypsin. Altering the amino acid residue at the reactive site can therefore significantly influence overall inhibitor specificity.

In general, the P_1 residue plays a crucial role in the specificity of cognate enzyme binding. Although we have not determined the active site of rAceKI-1 experimentally, by comparing it to BPTI, human TFPI, and other Kunitz inhibitors, we can assign the methionine as the probable P_1 residue (see figure 15). As mentioned, methionine is a large hydrophobic residue that binds well to chymotrypsin and has been shown to fit into the S_1 cleft of elastase as well (88). Interestingly, rAceKI-1 is also a weak inhibitor of trypsin. Based on the tight binding kinetics of rAceKI-1's protein inhibition, it is surprising that it would have such broad protease inhibitory capacity. There are reported serine protease inhibitors that have a similar broad spectrum of activity. Ecotin, a unique inhibitor of chymotrypsin, elastase, and trypsin from *E. coli* has a Met as its reactive residue (78). Ecotin's unusual primary structure does not demonstrate similarities to those in common families of protease inhibitors. Human α_1-proteinase inhibitor inhibits elastase and chymotrypsin more potently than trypsin, presumably due to the presence of a methionine occupying its P_1 position (89). *Streptomyces* Subtilisin Inhibitor, a homologue of Kazal type inhibitors, has a P_1 methionine and potently inhibits chymotrypsin and trypsin (90). Although such broad activity protease inhibitors exist, AceKI-1 is the first identified from the Kunitz family.

To account for AceKI-1's unique inhibitory properties, it is important to closely compare its amino acid sequence to those of other Kunitz inhibitors (see figure 15). AceKI-1's six cysteine residues clearly align with those of other Kunitz inhibitors, as does the predicted P_1 methionine at position 15. As has been shown above, a methionine

residue at position 15 is consistent with inhibition of chymotrypsin and elastase, but a

large hydrophobic residue does not generally interact with trypsin, and thus does not

explain the weaker trypsin inhibitory activity. To explain this broader inhibitory

activity, we look at other areas of interaction. The amino acid residues 6-13 (P_{10}-P_3) in

Kunitz inhibitors have been shown to play a major role in chemical recognition and

binding interactions with proteases (70). Substitutions in this region rarely affect the

conformation of the external loop but can change the inhibitor's specificity; however,

only the side chains of the P_1 residue, position 15, strongly contact the binding site of the

serine protease. Compared to other Kunitz protease inhibitors, AceKI-1 does have a

unique sequence in the binding domain of residues 12-15, containing a GPQC instead of

the usual GPC. The addition of glutamine at position 13 might play a role in this

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                          P₂P₁P₁'
H-TFPI    C AFKADDGP CKAIMKRFFFNIFTRQCEEFIYGGCEGNQNRFESLEECKKMCTR
Rb-TFPI   C AMKVDDGP CRAYIKRFFFNILAHQCEEFIYGGCEGNENRFESLEECKEKCAR
Rt-TFPI   C AMKAEDGP CKAMIRSYYFNMNSHQCEEFIYGGCRGNKNRFDTLEECRKTCIP
Mo-TFPI   C AMKADDGP CKAMIRSYFFNMYTHQCEEFIYGGCEGNENRFDTLEECKKTCIP
rAceKI-1  CNAPTHLDGPQCMAFFKRYTYNKEKKQCEEFVYGGCRPSPNNFETMEECKKTCVK
BPTI      C LEPPYTGP CKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTC
ITI/H     C QLGYSAGP CMGMTSRYFYNGTSMACETFQYGGCMGNGNNFVTEKECLQTCR
IVBB      C ELIVAAGP CMFFISAFYYSKGANKCYPFTYSGCRGNANRFKTIEECRRTCV
LNVChyI   C YLPADPGR CLAYMPRFYYNPASNKCEKFIYGGCRGNANNFKTWDECRHTCVA
          5            14                30      38          51   55
              Residue     P₄ P₃ P₂ P₁ P₁'P₂'P₃'P₄'
              AceKI-1     P  Q  C  M  A  F  F  K
```

Figure 15 : Alignment of rAceKI-1 amino acid sequence with other Kunitz-type inhibitors.
Human tissue factor pathway inhibitor (TFPI) (66), rabbit TFPI (Rb-TFPI) (66), rat TFPI (Rt-TFPI) (66), mouse TFPI (Mo-TFPI) (91), bovine pancreatic trypsin inhibitor (BPTI) (82), Human inter-alpha-trypsin inhibitor - domain 1 (ITI/H) (84), venom basic protease inhibitor – eastern green mamba (IVBB) (70), and Long-nosed viper chymotrypsin inhibitor – *Viper ammodytes* (LNVChyI) (82). The method for residue localization is demonstrated with rAceKI-1 showing P_4-P_4'. The P_1 residues at position 15, the reactive site, are bold faced. The six cysteine residues at positions 5, 14, 30, 38, 51, and 55, predicted to be involved in disulfide bridges, are shaded in light gray. The underlined amino acid residues 6-13 (P_{10}-P_3) in Kunitz inhibitors have been shown to play a major role in chemical recognition and binding interactions with proteases. On the edge of this binding region the conserved GPC is shadowed, and the additional glutamine residue at position P_3 and asparagine residue at position P_{12} are shaded in dark gray.

inhibitor's more unique chemical recognition and target enzyme specificity.

We have therefore postulated our P_1 reactive site as a methionine and have discovered a unique additional glutamine residue in a generally conserved binding region. We hope to perform site directed mutagenesis at the position 15 methionine measure its inhibitory activity. It will be interesting to determine if, after changing the reactive site from methionine to lysine or arginine, chymotrypsin and elastase activity will be lost and the trypsin activity amplified. We also plan to structurally model this novel protease inhibitor to see if its additional glutamine residue might increase the area of interface in the inhibitor:protease complex providing increased stability and therefore broader activity.

Serine proteases are ubiquitous in nature. As novel inhibitors are identified and their molecular structures and functions are characterized, it is possible to hypothesize as to their presumed role *in vivo*. In studying parasite derived protease inhibitors, similarly, many investigators have questioned what biologic role these proteins play in parasite survival. *E. coli* produce Ecotin, a periplasmic protein that inhibits chymotrypsin, elastase, and trypsin. These pancreatic proteases are all present in high concentrations in the natural environment of the organism leading to the hypothesis that Ecotin is required for *E. coli* to survive in the intestine (92). Similarly, the trypsin inhibitor from *Fasciola hepatica* is presumed to be involved in parasite survival (82). A pancreatic and neutrophil elastase inhibitor from the blood fluke *Schistosoma mansoni* has been suggested to protect the parasite from neutrophil mediated immune attack (93). We can likewise postulate that rAceKI-1 is required for protecting hookworm against host responses, especially in its natural intestinal environment. Interestingly,

immunohistochemistry performed on the intestinal nematode *Ascaris suum*, using antibodies to chymotrypsin and the *Ascaris* chymotrypsin inhibitor, demonstrated host chymotrypsin enzyme and *Ascaris* inhibitor complexes in sections of worm muscle, eggs, sperm, and intestines (94). From these results one could argue either that *Ascaris* are removing host proteases from their environment for neutralization, or that these are endogenous proteases that play no role in parasite survival. We have identified AceKI-1 in both adult hookworm extracts and ES products, implying its role is not simply endogenous to the worm, but it is secreted or excreted into the parasite's surrounding environment. AceKI's inhibition of trypsin and chymotrypsin may therefore enable the parasite to survive the hydrolytic environment of the intestine, and its inhibition of neutrophil elastase might hinder the natural host immune response. By developing a rabbit antibody to rAceKI-1 and using immunohistochemistry to localize its site of production and storage in *A. ceylanicum*, we hope to gain a better understanding of AceKI-1's biologic role.

While characterizing the role of secreted molecules in hookworm survival, scientists have questioned the importance of life cycle or stage specificity. The third stage (L_3) infective larvae of *Ancylostoma*, for example, complete their developmental molts while in the host. It is hypothesized that *Ancylostoma* ES products contain molecules required for completion of these molts, as well as tissue invasion and evasion of the host immune response (95). These activities of tissue invasion and molting are stage specific and the translation of genes encoding such proteins may be up regulated only when developmentally required. We have shown that soluble protein extracts from adult *A. ceylanicum* inhibit chymotrypsin, trypsin, pancreatic elastase, and human

neutrophil elastase. Extracts from infective L_3 *A. ceylanicum* only demonstrate inhibitory activity against human neutrophil elastase. This evidence suggests that AceKI-1 is a stage specific inhibitor expressed in adult *A. ceylanicum* and not the infective L_3 stage. The conserved inhibition of human neutrophil elastase implies the possibility of a second protease inhibitor. In postulating the *in vivo* function of AceKI-1, we would expect an inhibitor of trypsin, chymotrypsin, and pancreatic elastase to be more useful in the adult stage parasite living in the hydrolytic environment of the intestine. We therefore more confidently predict that AceKI-1 has a biologic role in hookworm survival and that such a virulence factor may serve as a target for chemotherapeutic or vaccine development.

As discussed earlier, no human vaccine exists for hookworm. Current hookworm control relies on anthelminthic therapy. With growing concern of drug resistance and prolonged exposure to these potentially toxic chemicals, the search for an alternative therapy, such as a vaccine, continues. Parasites have unique strategies that make vaccine development difficult. Hookworms are able to undergo antigenic changes during development, shed antibody bound surface coats to prevent host recognition, and secrete immunosuppressive molecules (96). These factors may explain why, although hookworms induce a host immune response, this response does not appear to protect against subsequent exposures and development of infection and disease (97). In identifying a vaccine target, it is important to remember the relationship between hookworm infection and hookworm disease. Unlike bacteria and viruses, where eradicating the organism is required for preventing disease, the goal for hookworm vaccine development may be different. Eliminating hookworms may not be necessary to alleviate hookworm anemia; interfering with the parasite's virulence factors and

hindering blood loss might be sufficient (98). We must therefore identify what makes a virulence factor a suitable vaccine target.

Many studies have used natural hookworm antigens in an attempt to induce immunity to hookworms. For example, by administering infective *A. caninum* larvae in numerous small doses, laboratory dogs were protected from challenge with large numbers of larvae (99). Regardless of their potentially protective benefit, however, it is not practical to immunize humans with living hookworm larvae, and additionally, human exposed to repeated hookworm infections have not developed sterile immunity. Many hookworm antigens, however, might serve as targets for inducing protective immunity including somatic antigens and excretory and secretory proteins. Early immunization trials with hookworm extracts were not successful, possibly due to the low concentration of potentially protective antigens and the presence of immunosuppressants in the extracts (96). Another study showed that immunization with either excretory and secretory (ES) product antigens or somatic antigens from *A. ceylanicum* yielded high antibody titers, but ES products provided greater protection (100). Most recently, work in a mouse model has shown that vaccination with recombinant ASP-1, the most abundantly secreted protein following activation of *A. caninum* L_3, can reduce lung migration in mice challenged with infective hookworm larvae (101,102) and pooled antibody from immunized mice can protect naïve mice against hookworm challenge (103). Based on observations that protective immunity can be generated from hookworm secreted antigens (104), we hope to create a genetically engineered vaccine for *A. ceylanicum* from a secreted antigen such as AceKI-1.

We have identified AceKI-1 activity in adult hookworm ES products, but there does not appear to be a natural host immunologic recognition of rAceKI-1. Using our laboratory hamster model, following forty days of infection with *A. ceylanicum*, hamster sera does not recognize recombinant AceKI-1. Hidden antigens are concealed from the immune system and therefore do not induce a normal immune response, and we conclude, therefore, that AceKI-1 is a hidden antigen (105,106). Although natural and overt antigens are more commonly used in vaccine development, hidden antigens can also be utilized to induce protection. The host inherently recognizes natural antigens as foreign and generates a humoral antibody response against them. Since humans for unknown reasons are unable to induce a protective response to natural hookworm antigens, a hidden antigen would be an alternative target. In hookworm, for example, if an antibody is engineered to target a hidden antigen in the parasites intestine, then that antibody will encounter its target during blood feeding. Binding the targeted hidden antigen may interfere with its role in parasite survival as has been demonstrated in *Haemonchus contortus* (105).

We believe that AceKI-1 is a virulence factor that aids hookworm survival within its mammalian host. We have identified a similar inhibitory activity in soluble protein extracts from other species of adult hookworms, including *A. caninum*, and *Necator americanus*. Such a broadly conserved inhibitory activity further suggests that AceKI-1 may be an evolutionary requirement for hookworm survival. By isolating a new serine protease inhibitor from hookworm, we hope to better understand how this specific parasite virulence factor is involved in the pathogenesis of hookworm associated gastrointestinal blood loss. Because the clinically relevant sequelae of hookworm

infection depend upon parasite survival in the host environment, interfering with these protein virulence factors using chemotherapeutics or immunotherapy may represent a novel strategy to ameliorating hookworm disease.

References:

1.   Hoeppli R. 1969. Parasitic Diseases In Africa and the Western Hemisphere. *Verlag fur Recht und Gesellschaft*. Basel, ed. 240.

2.   Ettling J. 1981. *The Germ of Laziness : Rockefeller Philanthropy and Public Health in the New South*. Massachusetts: Harvard University Press. 106-9.

3.   Fee E. 1987. *Disease and Discovery*. The Johns Hopkins University Press. Baltimore.

4.   Gilles H.M. 1985. Selective primary health care: strategies for control of disease in the developing world. XVII. Hookworm infection and anemia. *Reviews of Infectious Diseases*. 7:111-8.

5.   Pollitt E., Watkins W.E. 1997. "Stupidity or Worms": Do Intestinal Worms Impair Mental Performance? *Psychological Bulletin*. 121:171-91.

6.   Warren K.S., Bundy D.A., Anderson R.M., Davis A.R., Henderson D.A., et al. 1993. Helminth Infections. Disease Control Priorities in Developing Countries. Edt. Jamison D.A., Mosley W.H., Measham A.R., Bobadilla J.L. Oxford University Press, New York. pp131-88.

7.   Hotez P.J. 1995. Hookworm Infections. Infections of the Gastrointestinal Tract. Edt Blaser M.J., Smith P.D., Ravdin J.I., Greenberg H.B. and Guerrant R.L. Raven Press, Ltd., New York.

8.   Chowdurry A.B., Schad G.A. 1972. Ancylostoma ceylanicum: a Parasite of Man in Calcutta and Environs. *Amer. J Trop Med Hyg*. 21:300-1.

9.   Carroll S.M., Grove D.I. 1986. Experimental Infection of Humans with *Ancylostoma ceylanicum*: Clinical, Parasitological, Haematological and Immunological Findings. *Trop Geogr. Med*. 38:38-45

10.   Carroll S.M., Grove D.I. 1984. Parasitological, Hematologic, and Immunologic Responses in Acute and Chronic Infections of Dogs with *Ancylostoma ceylanicum*: A Model of Human Hookworm Infection. *J. Inf Dis*. 150:284-294.

11.   Bundy D.A.P., Keymer A.E. 1991. The Epidemiology of Hookworm Infection. *Hookworm Infectious*. 157-75.

12.   Hotez P.J. 1989. Hookworm Disease in Children. *Ped Infect Dis J*. 8:516-20.

13.   Hotez P.J., Pritchard D.J. 1995. Hookworm Infection. *Scient Amer*. *272(6):68-74, 1995 Jun*

14.   Vetter J.C.M., Leegwater V.D., Linden M.E. 1977. Skin Penetration of Infective Hookworm Larvae. *Z. for Parasitenkunde.* 53:263-6.

15.   Schad G.A., Chowdhury A.B., Dean C.G., Kochar V.K., Nawalinski T.A., Thomas J., Tonascia J.A. 1973. Arrested Development in Human Hookworm Infections: an Adaptation to a Seasonally Unfavorable External Environment. *Science.* 180:52-4.

16.   Hotez P.J., Narasimhan S., Haggerty J., Bhopale V., Milstone L., et al. 1992. Hyaluronidase from Infective Ancylostoma Hookworm Larvae and its Possible Function as a Virulence Factor in Tissue Invasion and in Cutaneous Larva Migrans. *Infection and Immunity.* 60:1018-23.

17.   Rep B.H., Van Joost K.S., Vetter J.C.M. 1971. Pathogenicity of *Ancylostoma ceylanicum.* VI. Lethal Blood Loss in Hookworm Infection. *Trop Geogr Med.* 23:184-93.

18.   Santiso R. 1997. Effects of Chronic Parasitosis on Women's Health. *International Journal of Gynecology and Obstetrics.* 58:129-36.

19.   Carroll S.M., Robertson T.A., Papadimitriou J.M., Grove D.I. 1984. Transmission electron microscopical studies of the site of attachment of Ancylostoma ceylanicum to the small bowel mucosa of the dog. *Journal of Helminthology.* 58:313-20.

20.   Banwell J.G., Schad G.A. 1978. Hookworm. *Clinics in Gastroenterology.* 7:129-56.

21.   Zheng-yi W., Xing-zhen W., Yu-fang P., Xiao H., Fang-qing L., et al. 1983. Blood Sucking Activities of Hookworm. *Chinese Med J.* 96:282-6.

22.   Roche M., Torres C.M. 1960. A Method for *in vitro* Study of Hookworm Activity. *Exp Parasitol.* 9:250-6.

23.   Layrisse M., Paz A., Blumenfeld N., Roche M. 1961. Hookworm Anemia: Iron Metabolism and Erythrokinetics. *Blood.* 18:61-72.

24.   Stoltzfus R.J., Dreyfuss M.L., Chwaya H.M., Albonico M. 1997. Hookworm Control as a Strategy to Prevent Iron Deficiency. *Nutrition Reviews.* 55:223-32.

25.   Layrisse M., Roche M. 1964. The Relationship Between Anemia and Hookworm Infection. *Am J Hyg.* 79:279-301.

26.   Robertson L.J., Crompton D.W.T., Sanjur D., Nesheim M.C. 1992. Haemoglobin Concentrations and Concomitant Infections of Hookworm and *Trichuris tichuria* in Poanamanian Primary Schoolchildren. *Trans Roy Soc Trop Med Hyg.* 86:654-6.

27.   Roche M., Layrisse M. 1966. The Nature and Causes of "Hookworm Anemia". *Amer J Trop Med*. 15:1031-1102.

28.   Idjradinata P., Pollitt E. 1993. Reversal of Developmental Delays in Iron-Deficient Anemic Infants Treated with Iron. *The Lancet*. 341:1-4.

29.   Scrimshaw N.S. 1991. Iron Deficiency. *Scientific American*. 265:46-52.

30.   Albonico M., Smith P.G., Ercole E., Hall A., Chwaya H.M., et al. 1995. Rate of reinfection with intestinal nematodes after treatment of children with mebendazole or albendazole in a highly endemic area. *Transactions of the Royal Society of Tropical Medicine & Hygiene*. 89:538-41.

31.   De Clercq D., Sacko M., Behnke J., Gilbert F., Dorny P., et al. 1997. Failure of Mebendazole in Treatment of Human Hookworm Infections in Southeastern Regions of Mali. *Am J Trop Med Hyg*. 57:25-30.

32.   Harrop S.A., Prociv P., Brindley P.J. 1995. Amplification and Characterization of Cysteine Proteinase Genes from Nematodes. *Trop Med Parasitol*. 46:119-22.

33.   Hotez P., Haggerty J, Hawdon J., Milstone L., Gamble R., et al. 1990. Mettalloproteases of Infective *Ancylostoma* Hookworm Larvae and Their Possible Functions in Tissue Invasion and Ecdysis. *Infection and Immunity*. 58:3883-92.

34.   Hotez P.J., Trang N.L., McKerrow J.H., and Cerami A. 1985. Isolation and characterization of a proteolytic enzyme from the adult hookworm *Ancylostoma caninum*. *Journal of Biological Chemistry*. 260:7343-8.

35.   Brown A., Burleigh J.M., Billet E.E., Pritchard D.I. 1995. An Intial Characterization of the Proteolytic Enzymes Secreted By the Adult Stage of the Human Hookworm *Necatur americanus*. *Parasitol*. 110:555-63.

36.   Jespers L.S., Messens J.H., De Keyser A., Eeckhout D., Stanssens P.E., et al. 1995. Surface Expression and Ligand-Based Selection of cDNA Fused to Filamentous Phage Gene VI. *Biotechnology*. 13:378-82.

37.   Moyle M., Foster D.L., McGrath D.E., Brown S.M., Laroche Y., et al. 1994. A hookworm glycoprotein that inhibits neutrophil function is a ligand of the integrin CD11b/CD18. *Journal of Biological Chemistry*. 269:10008-15.

38.   Hawdon J.M., Jones B.F., Hoffman D.R., Hotez P.J. 1996. Cloning and Characterization of *Ancylostoma*-secreted Protein. *J Biol Chem*. 271:6672-8.

39.   Rifkin M., Heng-Fong S., Jackson D., Brown L., Wood P. 1996. Defense against the immune Barrage: Helminth Survival Strategies. *Immunology and Cell Biology*. 74:564-74.

40.   Brophy P.M., Patterson L.H., Pritchard D.I. 1995. Secretory Nematode SOD – Offensive or Defensive? *Int J Parasitol*. 25:865-6.

41.   Loeb L., Fleisher M.S. 1910. The Influence of Extracts of Ancylostoma Caninum on the Coagulation of the Blood and On Hemolysis. *J Inf Dis*. 625-31.

42.   Stanssens P., Bergum P.W., Cappello M, Hotez P., Vlasuk G.P., et al. 1996. Anticoagulant Repertoire of the Hookworm *Ancylostoma caninum*. *Proc Nat Acad Sci*. 93:2149-2154.

43.   Cappello M., Hawdon J.M., Jones B.F., Kennedy W.P., Hotez P.J. 1996. *Ancylostoma canicum* Anticoagulant Peptide: Cloning by PCR and Expression of Soluble, Active Protein in E. coli. *Mol and Biochem Parasit*. 80:113-7.

44.   Cappello M., Clyne L.P., McPhedran P., Hotez P.J. 1993. *Ancylostoma* Factor Xa Inhibitor : Partial Purification and Its Identification as a Major Hookworm-Derived Anticoagulant In Vitro. *J Inf Dis*. 167:1474-7.

45.   Chadderdon, R.C., Cappello M. 1999. The Hookworm Platelet Inhibitor: Functional Blockade of Integrins GPIIb/IIIa and GPIa/Iia Inhibits Platelet Aggregation and Adhesion In Vitro. *J Inf Dis*. 179:1235-41.

46.   Yoshido Y., Okamoto K., Chiu J. 1968. *Ancylostoma ceylanicum* Infection in Dogs, Cats, and Man in Taiwan. *Am J Trop Med Hyg*. 17:378-81.

47.   Bode W., Huber R. 1992. Natural Protein Proteinase Inhibitors and Their Interaction with Proteinases. *Eur. J. Biochem*. 24:433-51.

48.   Garside P., Behnke J.M. 1989. *Ancylostoma ceylamicum* in the Hampster: Observations on the Host-Parasite Relationship During Primary Infection. *Parasitology*. 98:293-9.

49.   Schad G.A. 1979. *Ancylostoma duodenale*: Maintenance Through Six Generations in Helminth-naive Pups. Exp. Parasitol. 47:246-53.

50.   Rico-Hesse R., Harrison L.M., Salas R.A., Tovar D., Nisalak A., et al. 1997. Origins of Dengue Type 2 Viruses Associated with Increased Pathogenicity in the Americas. *Virology*. 230:244-51.

51.   Krause M., Hirsh D. 1987. A Trans-Spliced Leader Sequence on the Actin mRNA in C. elegans. *Cell*. 49:753-61.

52.   Hawdon J.M., Jones B.F., Hotez P.J. 1995. Cloning and Characterization of a cDNA encoding the catalytic subunit of a cAMP-Dependent Protein Kinase from *Ancylostoma caninum* Third Stage Infective Larvae. *Mol Biochem Parasitol*. 69:127-30.

53.   Bektesh S., Van Doren K., Hirsh D. 1988. Presence of the *Caenorhabditis elegans* Spliced Leader on the Different mRNAs and in Different Genera of Nematodes. *Genes Dev*. 2:1277-83.

54.   Bensen D.A., Boguski, M.S., Lipman D.J., Ostell J., Wheeler D.L., et al. 1999. GenBank. *Nucleic Acids Research*. Vol 27.

55.   Nielson H., Engelbrecht J., Brunak S., von Heijne G. 1997. Identification of Prokaryotic and Eukaryotic Signal Peptide and Prediction of their Cleavage Sites. *Protein Engineering*. 10:1-6.

56.   Von Herjne G. 1986. A New Method for Predicting Signal Sequence Cleavage Sites. *Nucl Acids Research*. 14:4683-90.

57.   Stone K.L., DeAngelis R., LoPresti M., Jones J., Papov V., and Williams K. 1997. Use of liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) for routine identification of enzymatically digested proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Electrophoresis*. 19:1046-1052.

58.   Stone K.L., McNulty D.E., LoPresti M.L., Crawford J.M., DeAngelis R. and Williams K.R. 1992. Elution and internal amino acid sequencing of PVDF-blotted proteins. *Techniques in Protein Chemistry III*. R. Angeletti, ed. Academic Press, New York. 23-34.

59.   Cappello M., Li S., Chen X., Chang-Ben L., Harrison L., et al. 1998. Tsetse Thrombin Inhibitor: Bloodmeal-Induced Expression of an Anticoagulant in Salivary Glands and Gut Tissue of *Glossina morsitans morsitans*. *Proc Natl Acad Sci USA*. 95:14290-5.

60.   Cappello M., Bergum P.W., Vlasuk G.P., Pritchard D.I., Aksoy S., et al. 1996. Isolation and Characterization of the Tsetse Thrombin Inhibitor: A Potent Antithrombotic Peptide From the Saliva of Glossina morsitans morsitans. *Am J Trop Med Hyg*. 54:475-80.

61.   Cappello M., Vlasuk G., Hotez P.J. 1995. Ancylostoma caninum Anticoagulant Peptide: A Hookworm Derived Inhibitor of Human Coagulation Factor Xa. *Proc Natl Acad Sci*. 92:6152-6.

62.   Williams J.W., Morrison J.F. 1979. The Kinetics of Reversible Tight-Binding Inhibition. *Methods in Enzymology*. 63:437-67.

63.   Morrison J.F., Walsh C.T. 1988. The Behavior and Significance of Slow-Binding Enzyme Inhibitors. *Advances in Enzymology & Related Areas of Molecular Biology*. 61:201-300.

64.   Williams K.R., Samandar S.M., Stone K.L., Saylor M. and Rush J. 1996. Matrix assisted-laser desorption ionization mass spectrometry as a complement to internal protein sequencing. *The Protein Protocols Handbook*. J.M. Walker, ed. Humana Press, Totowa. 541-555.

65.   Sasaki T., Kobayahi K. 1984.   Isolation of two novel proteinase inhibitors from hemolymph of silkworm larva, *Bombyx mori*. Comparison with human serum proteinase inhibitors. *Journal of Biochemistry*. 95:1009-17.

66.   Enjyoji K., Emi M., Mukai T., Kato H. 1992. cDNA Cloning and Expression of Rat Tisssue Factor Pathway Inhibitor. *J Biochem*. 11:681-687.

67.   Laskowski M. Jr., Kato I. 1980. Protein Inhibitors of Proteinases. *Ann Rev Biochem*. 49:593-626.

68.   Bernard V.D., Peanasky R.J. 1993. The Serine Protease Inhibitor Family from *Ascaris suum* : Chemical Determination of the Five Disulfide Bridges. *Arch Biochem Biophys*. 303:367-76.

69.   Babin D.R., Peansasky R.J., Goos S.M. 1984. The Isoinhibitors of Chymotrypsin/Elastase from Ascaris lumbricoides: The Primary Structure. *Arch of Biochem and Biophys*. 232:143-161.

70.   Pritchard L., Dufton M. 1999. Evolutionary Trace Analysis of the Kunitz/BPTI Family of Proteins: Functional Divergence May Have Been Based on Conformational Adjustment. *J Mol Biol*. 285:1589-1607.

71.   Creighton T.E. 1975 Homology of protein structures: proteinase inhibitors. *Nature*. 255:743-5.

72.   Kraunsoe J.A., Claridge T.D., Lowe G. 1996. Inhibition of human leukocyte and porcine pancreatic elastase by homologues of bovine pancreatic trypsin inhibitor. *Biochemistry*. 35:9090-6.

73.   Lu W., Apostol I., Qasim M., Warne N., Wynn R., et al. 1997. Binding of Amino Acid Side-Chains to S_1 Cavities of Serine Proteases. *J Mol Biol*. 266:441-461.

74.   Perona J.J., Tsu C.A., Craik C.S., Fletterick R.J. 1993. Crystal structures of rat anionic trypsin complexed with the protein inhibitors APPI and BPTI. *Journal of Molecular Biology*. 230:919-33.

75. Huang K., Strynadka N.C.J., Bernard V.D., Peansky R.J., James M. 1994. The Molecular Structure of the Complex of *Ascaris* Chymotrypsin/Elastase Inhibitor with Porcine Elastase. *Structure*. 2:679-89.

76. Broze G.J. Jr. 1995. Tissue Factor Pathway Inhibitor and the Revised Theory of Coagulation. *Annu. Rev. Med*. 46:103-12.

77. Peanasky, R.J. Bentz Y., Homandberg G.A., Minor S.T., Babin D.R. 1984. The Isoinhibitors of Chymotrypsin/Elastase from *Ascaris lumbricoides*: The Reactive Site. *Arch Biochem Biophys*. 232:135-42.

78. McGrath M.E., Hines W.D., Sakanari J.A., Fletterick R.J., Craik C.S. 1991. The Sequence and Reactive Site of Ecotin. *J Biol Chem*. 266:6620-5.

79. Chauvet J., Acher R. 1975. The Reactive Sites of Kunitz Bovine-Trypsin Inhibitor. *Eur. J Biochem*. 54:31-8..

90. Odani S., Ikenaka T. 1977. Studies on soybean trypsin inhibitors. XI. Complete amino acid sequence of a soybean trypsin-chymotrypsin-elastase inhibitor, C-II. *J Biochem*. 82:1523-31.

81. Shafqat J., Zaidi Z.H., Jornvall H. 1990. Purification and Characterization of a Chymotrypsin Kunitz Inhibitor Type of Polypeptide from the Venom of Cobra(*Naja naja, naja*). *FEBS Letters*. 275:6-8.

82. Bozas S.E., Panaccio M., Creaney J., Dosen M., Spithill T.W., et al. 1995. Characterization of a Novel Kunitz-type Molecule rom the Trematode *Fasciola hepatica*. *Mol and Bioch Parasit*. 74:19-29.

83. Diarra-Mehrpour M., Bourguignon J., Sesboue R., Salier J., Leveillard T., et al. 1990. Structural Analysis of the Human Inter-α-Trypsin Inhibitor Light-Chain Gene. *Eur J Biochem*. 191:131-9.

84. Hochstrasser K., Wachter E., Albrecht G.J., Reisinger P. 1985. Kunitz-Type Proteinase Inhibitors Derived by Limited Proteolysis of the Inter-α-Trypsin Inhibitor, X. The Amino-Acid Sequences of the Trypsin-Released Inhibitors from Horse and Pig Inter-α-Trypsin Inhibitors. *Hoppe-Seylers Zeitschrift fur Physiologische Chemie*. 366:473-8.

85. Hochstrasser K., Albrecht G., Schonberger O.L., Wachter E. 1983. Kunitz-type proteinase inhibitors derived by limited proteolysis of the inter-alpha-trypsin inhibitor, VII. Characterization of the bovine inhibitor as double-headed trypsin-elastase inhibitor. *Hoppe-Seylers Zeitschrift fur Physiologische Chemie*. 364:1689-96.

86. Jering H., Tschesche H. 1976. Replacement of Lysine by Arginine, Phenylalanine and Tryptophan in the Reactive Site of the Bovine Trypsin-Kallikrein (Kunitz) and Change of the Inhibitory Properties. *Eur. J. Biochem*. 61:453-63.

87. Sinha S., Knops J., Oltersdorf T., et al. 1991. Conversion of the Alzheimer's B-Amyloid Precursor Protein (APP) Kunitz Domain into a Potent Human Neutrophil Elastase Inhibitor. *J Biol Chem.* 266:21011-3.

88. Papamokos E., Weber E., Bode W., Huber R., Empie M.W., Kato I., Laskowski M. Jr. 1982. Crystallographic refinement of Japanese quail ovomucoid, a Kazal-type inhibitor, and model building studies of complexes with serine proteases. *J Mol Bio.* 158:515-37.

89. Johnson D., Travis J. 1978. Structural evidence for methionine at the reactive site of human alpha-1-proteinase inhibitor. *J Biol Chem.* 253:7142-4.

90. Ikenaka T., Odani S., Sakai M., Nabeshima Y., Sato S., Murao S. 1974. Amino acid sequence of an alkaline proteinase inhibitor (Streptomyces subtilisin inhibitor) from Streptomyces albogriseolus S-3253. *Journal of Biochemistry.* 76:1191-209.

91. Chang J., Monroe D.M., Oliver J.A., Liles D.K., Roberts H. 1998. Cloning, Expression and Characterization of Mouse Tissue Factor Pathway Inhibitor. *Throm Haemost.* 79:306-7.

92. Chung C.H., Ives H.E., Almeda S., Goldberg A.L. 1983. Purification from Escherichia coli of a Periplasmic Protein That is a Potent Inhibitor of Pancreatic Proteases. *J Biol Chem.* 258:11032-8.

93. Ghendler Y., Arnon R., Fishelson Z. 1994. *Schistosoma mansoui*: Isolation and Characterization of Smpi56, a Novel Serine Protease Inhibitor. *Exp Parasitol.* 78:121-31.

94. Martzen M.R., Geise G.L., Hogan B.J., Peanasky R.J. 1985. *Ascaris suum*: Localization by Immunohistochemical and Florescent Probes of Host Proteases and Parsite Proteinase Inhibitors in Cross-Sections. *Exp Parasitol.* 60:139-49.

95. Hawdon J.M., Narasimhan S., Hotez P.J. 1999. *Ancylostoma* Secreted Protein 2: Cloning and Characterization of a Second Member of a Family of Nematode Secreted Proteins from *Ancylostoma caninum*. *Mol Biochem Parasit.* 99:149-65.

96. Meeusen E.N.T. 1996. Rational Design of Nematode Vaccines; Natural Antigens. *Inter J Parasit.* 26:813-8.

97. Behnke J.M. 1987. Do Hookworms Illicit Protective Immunity in Man? *Parasitology Today.* 3:200-6.

98. Hotez P.J., Hawdon J.M., Cappello M., Jones B.F., Pritchard D.I. 1995. Molecular Pathobiology of Hookworm Infection. *Infections Agents and Disease.* 4:71-5.

99.   Hotez P.J., Kashinath G., Hawdon J., Narasimhan S., Jones B., et al. 1997. Vaccine for Hookworm Infection. Ped *Inf Dis J*. 16:935-40.

100.   Khan A.M., Gupta S., Katiyar J.C., Srivastava V.K. 1996. Correlation Between the Degree of Protection and Humoral Antibody Response in Hamsters Immunized with Somatic and Excretory Secretory Antigens of Ancylostoma ceylanicum. *Indian J of Exp Bio*. 34:1015-8.

101.   Hotez P.J., Hawdon J.M., Cappello M., Jones B.F., Ghosh K., et al. 1996. Molecular Approaches to Vaccinating Against Hookworm Disease. *Ped Res*. 40:514-21.

102.   Ghosh K., Hawdon J., Hotez P. 1996. Vaccination with Alum-Precipitated Recombinant *Ancylostoma*-Secreted Protein 1 Protects Mice against Challenge Infections with Infection Hookworm (*Ancylostoma caninum*) Larvae. *J Inf Dis*. 174:1380-3.

103.   Ghosh K., Hotez P.J. 1999. Antibody-Dependent Reductions in Mouse Hookworm Burden After Vaccination with *Ancylostoma caninum* Secreted Protein-1. *J Inf Dis*. 180:1674-81.

104.   Hotez P.J., Trang N.L., Cerami A. 1987. Hookworm Antigens: the Potential for Vaccination. *Parasitology Today*. 3:247-9.

105.   Munn E.A. 1997. Rational Designs of Nematode Vaccine; Hidden Antigens. *Inter J Parasit*. 27:359-366.

106.   Jasmer D.P., McGuire T.C. 1996. Antigens with Application Toward Immune Control of Blood-Feeding Parasitic Nematodes. *Brit Vet J*. 152:251-68.