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AUS NEMATODEN EXTRAHIERTE SERINPROTEASE-INHIBITOREN UND DIE KOAGULATION HEMMENDE PROTEINE

PROTEINES ANTICOAGULANTES ET INHIBITEURS DE LA SERINE-PROTEASE EXTRAITS DE NEMATODES

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EP-A- 0 255 771**WO-A-88/09811****WO-A-94/25000**

- **PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA**, vol. 92, no. 13, 20 June 1995, WASHINGTON US, pages 6152-6156, XP000512152 M. CAPELLO ET AL.:

"Ancylostoma caninum anticoagulant peptide: A hookworm derived inhibitor of human coagulation factor Xa"

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- JOURNAL OF INFECTIOUS DISEASES, vol. 167, no. 6, June 1993, pages 1474-1477, XP000569595
M. CAPELLO ET AL.: "Ancylostoma Factor Xa Inhibitor: Partial Purification and Its Identification as a Major Hookworm-Derived Anticoagulant In Vitro"
- CHEMICAL ABSTRACTS, vol. 113, no. 18, 29 October 1990 Columbus, Ohio, US; abstract no. 158738, OKLAHOMA MEDICAL RESEARCH FOUNDATION, USA: "Thrombin-binding polypeptides as antithrombotic agent for artificial organs or other surfaces" XP002002737 & JP,A,02 019 399 (OKLAHOMA MEDICAL RESEARCH FOUNDATION, USA)
- ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 232, no. 1, July 1984, SAN DIEGO, US, pages 143-161, XP000570396 D.R.
BABIN ET AL.: "The Isoinhibitors of Chymotrypsin/Elastase from Ascaris lumbricoides: The Primary Structure"

DescriptionField of the Invention

[0001] The present invention relates to specific proteins as well as recombinant versions of these proteins which are serine protease inhibitors, including potent anticoagulants in human plasma. These proteins include certain proteins extracted from nematodes. In another aspect, the present invention relates to compositions comprising these proteins, which are useful as potent and specific inhibitors of blood coagulation enzymes *in vitro* and *in vivo*, and methods for their use as *in vitro* diagnostic agents, or as *in vivo* therapeutic agents, to prevent the clotting of blood. In a further aspect, the invention relates to nucleic acid sequences, including mRNA and DNA, encoding the proteins and their use in vectors to transfect or transform host cells and as probes to isolate certain related genes in other species and organisms.

Background and Introduction to the Invention

[0002] Normal hemostasis is the result of a delicate balance between the processes of clot formation (blood coagulation) and clot dissolution (fibrinolysis). The complex interactions between blood cells, specific plasma proteins and the vascular surface, maintain the fluidity of blood unless injury occurs. Damage to the endothelial barrier lining the vascular wall exposes underlying tissue to these blood components. This in turn triggers a series of biochemical reactions altering the hemostatic balance in favor of blood coagulation which can either result in the desired formation of a hemostatic plug stemming the loss of blood or the undesirable formation of an occlusive intravascular thrombus resulting in reduced or complete lack of blood flow to the affected organ.

[0003] The blood coagulation response is the culmination of a series of amplified reactions in which several specific zymogens of serine proteases in plasma are activated by limited proteolysis. This series of reactions results in the formation of an insoluble matrix composed of fibrin and cellular components which is required for the stabilization of the primary hemostatic plug or thrombus. The initiation and propagation of the proteolytic activation reactions occurs through a series of amplified pathways which are localized to membranous surfaces at the site of vascular injury (Mann, K.G., Nesheim, M.E., Church, W.R., Haley, P. and Krishnaswamy, S. (1990) Blood 76: 1-16. and Lawson, J.H., Kalafatis, M., Stram, S., and Mann, K.G. (1994) J. Biol. Chem. 269: 23357-23366).

[0004] Initiation of the blood coagulation response to vascular injury follows the formation of a catalytic complex composed of serine protease factor VIIa and the non-enzymatic co-factor, tissue factor (TF) (Rappaport, S.I. and Rao, L.V.M. (1992) Arteriosclerosis and Thrombosis 12: 1112-1121). This response appears to be exclusively regulated by the exposure of subendothelial TF to trace circulating levels of factor VIIa and its zymogen factor VII, following a focal breakdown in vascular integrity. Autoactivation results in an increase in the number of factor VIIa/TF complexes which are responsible for the formation of the serine protease factor Xa. It is believed that in addition to the factor VIIa/TF complex, the small amount of factor Xa which is formed primes the coagulation response through the proteolytic modification of factor IX to factor IX_{alpha} which in turn is converted to the active serine protease factor IXa_{beta} by the factor VIIa/TF complex (Mann, K.G., Krishnaswamy, S. and Lawson, J.H. (1992) Sem. Hematology 29: 213-226.). It is factor IXa_{beta} in complex with activated factor VIIa, which appears to be responsible for the production of significant quantities of factor Xa which subsequently catalyzes the penultimate step in the blood coagulation cascade; the formation of the serine protease thrombin.

[0005] Factor Xa catalyzes the formation of thrombin following the assembly of the prothrombinase complex which is composed of factor Xa, the non-enzymatic co-factor Va and the substrate prothrombin (factor II) assembled in most cases, on the surface of activated platelets which are adhered at the site of injury (Fuster, V., Badimon, L., Badimon, J.J. and Chesebro, J.H. (1992) New Engl. J. Med. 326: 310-318). In the arterial vasculature, the resulting amplified "burst" of thrombin generation catalyzed by prothrombinase causes a high level of this protease locally which is responsible for the formation of fibrin and the further recruitment of additional platelets as well as the covalent stabilization of the clot through the activation of the transglutaminase zymogen factor XIII. In addition, the coagulation response is further propagated through the thrombin-mediated proteolytic feedback activation of the non-enzymatic co-factors V and VIII resulting in more prothrombinase formation and subsequent thrombin generation (Hemker, H.C. and Kessels, H. (1991) Haemostasis 21: 189-196).

[0006] Substances which interfere in the process of blood coagulation (anticoagulants) have been demonstrated to be important therapeutic agents in the treatment and prevention of thrombotic disorders (Kessler, C.M. (1991) Chest 99: 97S-112S and Cairns, J.A., Hirsh, J., Lewis, H.D., Resnekov, L., and Theroux, P. (1992) Chest 102: 456S-481S). The currently approved clinical anticoagulants have been associated with a number of adverse effects owing to the relatively non-specific nature of their effects on the blood coagulation cascade (Levine, M.N., Hirsh, J., Landefeld, S., and Raskob, G. (1992) Chest 102: 352S-363S). This has stimulated the search for more effective anticoagulant agents which can more effectively control the activity of the coagulation cascade by selectively interfering with specific reactions in this process which may have a positive effect in reducing the complications of anticoagulant therapy (Weitz, J., and

Hirsh, J. (1993) J. Lab. Clin. Med. 122: 364-373). In another aspect, this search has focused on normal human proteins which serve as endogenous anticoagulants in controlling the activity of the blood coagulation cascade. In addition, various hematophagous organisms have been investigated because of their ability to effectively anticoagulate the blood meal during and following feeding on their hosts suggesting that they have evolved effective anticoagulant strategies which may be useful as therapeutic agents.

[0007] A plasma protein, Tissue Factor Pathway Inhibitor (TFPI), contains three consecutive Kunitz domains and has been reported to inhibit the enzyme activity of factor Xa directly and, in a factor Xa-dependent manner, inhibit the enzyme activity of the factor VIIa-tissue factor complex. Salvensen, G., and Pizzo, S.V., "Proteinase Inhibitors: α -Macroglobulins, Serpins, and Kunis", "Hemostasis and Thrombosis, Third Edition, pp. 251-253, J.B. Lippincott Company (Edit. R.W. Colman et al. 1994). A cDNA sequence encoding TFPI has been reported, and the cloned protein was reported to have a molecular weight of 31,950 daltons and contain 276 amino acids. Broze, G.J. and Girad, T.J., U.S. Patent No. 5,106,833, col. 1, (1992). Various recombinant proteins derived from TFPI have been reported. Girad, T.J. and Broze, G.J., EP 439,442 (1991); Rasmussen, J.S. and Nordfand, O.J., WO 91/02753 (1991); and Broze, G.J. and Girad, T.J., U.S. Patent No. 5,106,833, col. 1, (1992).

[0008] Antistatin, a protein comprised of 119 amino acids and found in the salivary gland of the Mexican leech, *Haementeria officinalis*, has been reported to inhibit the enzyme activity of factor Xa. Tuszynski et al., J. Biol. Chem, 262:9718 (1987); Nutt, et al., J. Biol. Chem, 263:10162 (1988). A 6,000 daltons recombinant protein containing 58 amino acids with a high degree homology to antistatin's amino-terminus amino acids 1 through 58 has been reported to inhibit the enzyme activity of factor Xa. Tung, J. et al., EP 454,372 (October 30, 1991); Tung, J. et al., U.S. Patent No. 5,189,019 (February 23, 1993).

[0009] Tick Anticoagulant Peptide (TAP), a protein comprised of 60 amino acids and isolated from the soft tick, *Ornithodoros moubata*, has been reported to inhibit the enzyme activity of factor Xa but not factor VIIa. Waxman, L. et al., Science, 248:593 (1990). TAP made by recombinant methods has been reported. Vlausk, G.P. et al., EP 419,099 (1991) and Vlausk, G.P. et al., U.S. Patent No 5,239,058 (1993).

[0010] The dog hookworm, *Ancylostoma caninum*, which can also infect humans, has been reported to contain a potent anticoagulant substance which inhibited coagulation of blood *in vitro*. Loeb, L. and Smith, A.J., Proc. Pathol. Soc. Philadelphia, 7:173-187 (1904). Extracts of *A. caninum* were reported to prolong prothrombin time and partial thromboplastin time in human plasma with the anticoagulant effect being reported attributable to inhibition of factor Xa but not thrombin. Spellman, Jr., J.J. and Nossel, H.L., Am. J. Physiol., 220:922-927 (1971). More recently, soluble protein extracts of *A. caninum* were reported to prolong prothrombin time and partial thromboplastin time in human plasma *in vitro*. The anticoagulant effect was reported to be attributable to inhibition of human factor Xa but not thrombin, Cappello, M, et al., J. Infect. Diseases, 167:1474-1477 (1993), and to inhibition of factor Xa and factor VIIa (WO94/25000; U.S. Patent No. 5,427,937).

[0011] The human hookworm, *Ancylostoma ceylanicum*, has also been reported to contain an anticoagulant. Extracts of *A. ceylanicum* have been reported to prolong prothrombin time and partial thromboplastin time in dog and human plasma *in vitro*. Carroll, S.M., et al., Thromb. Haemostas. (Stuttgart), 51:222-227 (1984).

[0012] Soluble extracts of the non-hematophagous parasite, *Ascaris suum*, have been reported to contain an anticoagulant. These extracts were reported to prolong the clotting of whole blood, as well as clotting time in the kaolin-activated partial thromboplastin time test but not in the prothrombin time test. Crawford, G.P.M. et al., J. Parasitol., 68: 1044-1047 (1982).

Chymotrypsin/elastase inhibitor-1 and its major isoforms, trypsin inhibitor-1 and chymotrypsin/elastase inhibitor-4, isolated from *Ascaris suum*, were reported to be serine protease inhibitors and share a common pattern of five-disulfide bridges. Bernard, V.D. and Peanasky, R.J., Arch. Biochem. Biophys., 303:367-376 (1993); Huang, K. et al., Structure, 2:679-689 (1994); and Grasberger, B.L. et al., Structure, 2:669-678 (1994). There was no indication that the reported serine protease inhibitors had anticoagulant activity.

[0013] Secretions of the hookworm *Necator americanus* are reported to prolong human plasma clotting times, inhibit the amidolytic activity of human FXa using a fluorogenic substrate, inhibit multiple agonist-induced platelet dense granule release, and degrade fibrinogen. Pritchard, D.I. and B. Furmidge, Thromb. Haemost. 73: 546 (1995) (WO95/12615).

Summary of the Invention

[0014] The present invention is directed to isolated proteins having serine protease inhibiting activity and/or anticoagulant activity and including at least one NAP domain. We refer to these proteins as Nematode-extracted Anticoagulant Proteins or "NAPs". "NAP domain" refers to a sequence of the isolated protein, or NAP, believed to have the inhibitory activity, as further defined herein below. The anticoagulant activity of these proteins may be assessed by their activities in increasing clotting time of human plasma in the prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays, as well as by their ability to inhibit the blood coagulation enzymes factor Xa or factor VIIa/TF. It is believed that the NAP domain is responsible for the observed anticoagulant activity of these proteins. Certain of these proteins have

at least one NAP domain which is an amino acid sequence containing less than about 120 amino acid residues, and including 10 cysteine amino acid residues.

[0015] In another aspect, the present invention is directed to a method of preparing and isolating a cDNA molecule encoding a protein exhibiting anticoagulant activity and having a NAP domain, and to a recombinant cDNA molecule made by this method. This method comprises the steps of:

(a) constructing a cDNA library from a species of nematode; (b) ligating said cDNA library into an appropriate cloning vector; (c) introducing said cloning vector containing said cDNA library into an appropriate host cell; (d) contacting the cDNA molecules of said host cell with a solution containing a hybridization probe having a nucleic acid sequence comprising AAR GCi TAY CCi GAR TGY GGi GAR AAY GAR TGG, [SEQ. ID. NO. 94] wherein R is A or G, Y is T or C, and i is inosine; (e) detecting a recombinant cDNA molecule which hybridizes to said probe; and (f) isolating said recombinant cDNA molecule.

[0016] In another aspect, the present invention is directed to a method of making a recombinant protein encoded by said cDNA which has anticoagulant activity and which includes a NAP domain and to recombinant proteins made by this method. This method comprises the steps of: (a) constructing a cDNA library from a species of nematode; (b) ligating said cDNA library into an appropriate cloning vector; (c) introducing said cloning vector containing said cDNA library into an appropriate host cell; (d) contacting the cDNA molecules of said host cell with a solution containing a hybridization probe having a nucleic acid sequence comprising AAR GCi TAY CCi GAR TGY GGi GAR AAY GAR TGG, wherein R is A or G, Y is T or C, and i is inosine [SEQ. ID. NO. 94]; (e) detecting a recombinant cDNA molecule which hybridizes to said probe; (f) isolating said recombinant cDNA molecule; (g) ligating the nucleic acid sequence of said cDNA molecule which encodes said recombinant protein into an appropriate expression cloning vector; (h) transforming a second host cell with said expression cloning vector containing said nucleic acid sequence of said cDNA molecule which encodes said recombinant protein; (i) culturing the transformed second host cell; and (j) isolating said recombinant protein expressed by said second host cell. It is noted that when describing production of recombinant proteins in certain expression systems such as COS cells, the term "transfection" is conventionally used in place of (and sometimes interchangeably with) "transformation".

[0017] In another aspect, the present invention is directed to a method of making a recombinant cDNA encoding a recombinant protein having anticoagulant activity and having a NAP domain, comprising the steps of: (a) isolating a cDNA library from a nematode;

(b) ligating said cDNA library into a cloning vector; (c) introducing said cloning vector containing said cDNA library into a host cell; (d) contacting the cDNA molecules of said host cells with a solution comprising first and second hybridization probes, wherein said first hybridization probe has the nucleic acid sequence comprising AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC GAC TGT GGA ACT CAG AAG CCA TGC GAG GCC AAG TGC AAT GAG GAA CCC CCT GAG GAG GAA GAT CCG ATA TGC CGC TCA CGT GGT TGT TTA TTA CCT CCT GCT TGC GTA TGC AAA GAC GGA TTC TAC AGA GAC ACG GTG ATC GGC GAC TGT GTT AGG GAA GAA GAA TGC GAC CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO. 1], and said second hybridization probe has the nucleic acid sequence comprising AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC GTC TGT GGA ACT AAG AAG CCA TGC GAG GCC AAG TGC AGT GAG GAA GAG GAG GAA GAT CCG ATA TGC CGA TCA TTT TCT TGT CCG GGT CCC GCT GCT TGC GTA TGC GAA GAC GGA TTC TAC AGA GAC ACG GTG ATC GGC GAC TGT GTT AAG GAA GAA GAA TGC GAC CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO. 2]; (e) detecting a recombinant cDNA molecule which hybridizes to said mixture of said probes; and (f) isolating said recombinant cDNA molecule.

[0018] In yet another aspect, the present invention is directed to a method of making a recombinant cDNA encoding a protein having anticoagulant activity and which encodes a NAP domain, comprising the steps of: (a) isolating a cDNA library from a nematode; (b) ligating said cDNA library into an appropriate phagemid expression cloning vector; (c) transforming host cells with said vector containing said cDNA library; (d) culturing said host cells; (e) infecting said host cells with a helper phage; (f) separating phage containing said cDNA library from said host cells; (g) combining a solution of said phage containing said cDNA library with a solution of biotinylated human factor Xa; (h) contacting a streptavidin-coated solid phase with said solution containing said phages containing said cDNA library, and said biotinylated human factor Xa; (i) isolating phages which bind to said streptavidin-coated solid phase; and (j) isolating the recombinant cDNA molecule from phages which bind to said streptavidin-coated solid phase.

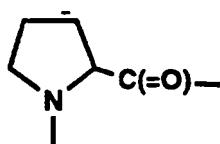
[0019] In one preferred aspect, the present invention is directed to a recombinant cDNA having a nucleic acid sequence selected from the nucleic acid sequences depicted in Figure 1, Figure 3, Figures 7A to 7F, Figure 9, Figures 13A to 13H, and Figure 14.

[0020] The present invention also is directed to NAPs that inhibit the catalytic activity of FXa, to NAPs that inhibit the catalytic activity of the FVIIa/TF complex, and to NAPs that inhibit the catalytic activity of a serine protease, as well as nucleic acids encoding such NAPs and their methods of use.

Definitions.

[0021] The term "amino acid" refers to the natural L-amino acids; D-amino acids are included to the extent that a protein including such D-amino acids retains biological activity. Natural L-amino acids include alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), glycine (Gly), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser), threonine (Thr), tryptophan (Trp), tyrosine (Tyr) and valine (Val).

[0022] The term "amino acid residue" refers to radicals having the structure: (1) -NH-CH(R)C(=O)- , wherein R is the alpha-carbon side-chain group of an L-amino acid, except for L-proline; or (2)



for L-proline.

[0023] The term "peptide" refers to a sequence of amino acids linked together through their alpha-amino and carboxylate groups by peptide bonds. Such sequences as shown herein are presented in the amino to carboxy direction, from left to right.

[0024] The term "protein" refers to a molecule comprised of one or more peptides.

[0025] The term "cDNA" refers to complementary DNA.

[0026] The term "nucleic acid" refers to polymers in which bases (e.g., purines or pyrimidines) are attached to a sugar phosphate backbone. Nucleic acids include DNA and RNA.

[0027] The term "nucleic acid sequence" refers to the sequence of nucleosides comprising a nucleic acid. Such sequences as shown herein are presented in the 5' to 3' direction, from left to right.

[0028] The term "recombinant DNA molecule" refers to a DNA molecule created by ligating together pieces of DNA that are not normally contiguous.

[0029] The term "mRNA" refers to messenger ribonucleic acid.

[0030] The term "homology" refers to the degree of similarity of DNA or peptide sequences.

[0031] The terms "Factor Xa" or "fXa" or "FXa" are synonymous and are commonly known to mean a serine protease within the blood coagulation cascade of enzymes that functions as part of the prothrombinase complex to form the enzyme thrombin.

[0032] The phrase "Factor Xa inhibitory activity" means an activity that inhibits the catalytic activity of fXa toward its substrate.

[0033] The phrase "Factor Xa selective inhibitory activity" means inhibitory activity that is selective toward Factor Xa compared to other related enzymes, such as other serine proteases.

[0034] The phrase "Factor Xa inhibitor" is a compound having Factor Xa inhibitory activity.

[0035] The terms "Factor VIIa/Tissue Factor" or "fVIIa/TF" or "FVIIa/TF" are synonymous and are commonly known to mean a catalytically active complex of the serine protease coagulation factor VIIa (fVIIa) and the non-enzymatic protein Tissue Factor (TF), wherein the complex is assembled on the surface of a phospholipid membrane of defined composition.

[0036] The phrase "fVIIa/TF inhibitory activity" means an activity that inhibits the catalytic activity of the fVIIa/TF complex in the presence of fXa or catalytically inactive fXa derivative.

[0037] The phrase "fVIIa/TF selective inhibitory activity" means fVIIa/TF inhibitory activity that is selective toward fVIIa/TF compared to other related enzymes, such as other serine proteases, including FVIIa and fXa.

[0038] The phrase a "fVIIa/TF inhibitor" is a compound having fVIIa/TF inhibitory activity in the presence of fXa or catalytically inactive fXa derivatives.

[0039] The phrase "serine protease" is commonly known to mean an enzyme, comprising a triad of the amino acids histidine, aspartic acid and serine, that catalytically cleaves an amide bond, wherein the serine residue within the triad is involved in a covalent manner in the catalytic cleavage. Serine proteases are rendered catalytically inactive by covalent modification of the serine residue within the catalytic triad by diisopropylfluorophosphate (DFP).

[0040] The phrase "serine protease inhibitory activity" means an activity that inhibits the catalytic activity of a serine

protease.

[0041] The phrase "serine protease selective inhibitory activity" means inhibitory activity that is selective toward one serine protease compared to other serine proteases.

[0042] The phrase "serine protease inhibitor" is a compound having serine protease inhibitory activity.

[0043] The term "prothrombinase" is commonly known to mean a catalytically active complex of the serine protease coagulation Factor Xa (fXa) and the non-enzymatic protein Factor Va (fVa), wherein the complex is assembled on the surface of a phospholipid membrane of defined composition.

[0044] The phrase "anticoagulant activity" means an activity that inhibits the clotting of blood, which includes the clotting of plasma.

[0045] The term "selective", "selectivity", and permutations thereof, when referring to NAP activity toward a certain enzyme, mean the NAP inhibits the specified enzyme with at least 10-fold higher potency than it inhibits other, related enzymes. Thus, the NAP activity is selective toward that specified enzyme.

[0046] The term "substantially the same" when used to refer to proteins, amino acid sequences, cDNAs, nucleotide sequences and the like refers to proteins, cDNAs or sequences having at least about 90% homology with the other protein, cDNA, or sequence.

[0047] The term "NAP" or "NAP protein" means an isolated protein which includes at least one NAP domain and having serine protease inhibitory activity and/or anticoagulant activity.

Brief Description of the Drawings.

[0048]

Figure 1 depicts the nucleotide sequence of the AcaNAP5 cDNA [SEQ. ID. NO. 3]. The numbering starts at the first nucleotide of the cDNA. Translation starts at the first ATG codon (position 14); a second in frame ATG is present at position 20.

Figure 2 depicts the amino acid sequence of mature AcaNAP5 [SEQ. ID. NO. 4].

Figure 3 depicts the nucleotide sequence of the AcaNAP6 cDNA [SEQ. ID. NO. 5]. The numbering starts at the first nucleotide of the cDNA. Translation starts at the first ATG codon (position 14); a second in frame ATG is present at position 20.

Figure 4 depicts the amino acid sequence of mature AcaNAP6 [SEQ. ID. NO. 6]. Amino acids that differ from AcaNAP5 are underlined. In addition to these amino acid substitutions, AcaNAP6 contains a two amino acid deletion (Pro-Pro) when compared to AcaNAP5.

Figure 5 depicts the amino acid sequence of Pro-AcaNAP5 [SEQ. ID. NO. 7].

Figure 6 depicts the amino acid sequence of Pro-AcaNAP6 [SEQ. ID. NO. 8]. Amino acids that differ from Pro-AcaNAP5 are underlined. In addition to these amino acid substitutions, Pro-AcaNAP6 contains a two amino acid deletion (Pro-Pro) when compared to Pro-AcaNAP5.

Figures 7A through 7F depict the nucleotide sequences of the cDNAs and deduced amino acid sequences of certain NAP proteins isolated from *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, and *Heligmosomoides polygyrus*. Figure 7A depicts sequences for the recombinant cDNA molecule, AceNAP4, isolated from *Ancylostoma ceylanicum* [SEQ. ID. NO. 9]. Figure 7B depicts sequences for the recombinant cDNA molecule, AceNAP5, isolated from *Ancylostoma ceylanicum* [SEQ. ID. NO. 10]. Figure 7C depicts sequences for the recombinant cDNA molecule, AceNAP7, isolated from *Ancylostoma ceylanicum* [SEQ. ID. NO. 11]. Figure 7D depicts sequences for the recombinant cDNA molecule, AduNAP4, isolated from *Ancylostoma duodenale* [SEQ. ID. NO. 12]. Figure 7E depicts sequences for the recombinant cDNA molecule, AduNAP7, isolated from *Ancylostoma duodenale* [SEQ. ID. NO. 13]. Figure 7F depicts sequences for the recombinant cDNA molecule, HpoNAP5, isolated from *Heligmosomoides polygyrus* [SEQ. ID. NO. 14]. The EcoRI site, corresponding to the 5'-end of the recombinant cDNA molecule, is indicated in all cases (underlined). Numbering of each sequence starts at this EcoRI site. AceNAP4 and AduNAP7, each encode a protein which has two NAP domains; all other clones in this Figure code for a protein having a single NAP domain. The AduNAP4 cDNA clone is not full-length, i.e., the recombinant cDNA molecule lacks the 5'-terminal part of the coding region based on comparison with other isoforms.

Figures 8A through 8C depict the nucleotide sequence of the vectors, pDONG61 (Figure 8A) [SEQ. ID. NO. 15], pDONG62 (Figure 8B) [SEQ. ID. NO. 16], and pDONG63 (Figure 8C) [SEQ. ID. NO. 17]. The HindIII-BamHI fragment which is shown is located between the HindIII and BamHI sites of pUC119. The vectors allow the cloning of cDNAs, as SfiI-NotI fragments, in the three different reading frames downstream of the filamentous phage gene 6. All relevant restriction sites are indicated. The AAA Lys-encoding triplet at position 373-375 is the last codon of gene 6. The gene 6 encoded protein is followed by a Gly-Gly-Gly-Ser-Gly-Gly [SEQ. ID. NO. 18] linker sequence.

Figure 9 depicts the nucleotide sequence of the recombinant cDNA molecule, AcaNAPc2 cDNA [SEQ. ID. NO. 19]. The EcoRI site, corresponding to the 5'-end of the cDNA, is indicated (underlined). Numbering starts at this EcoRI

site. The deduced amino acid sequence is also shown; the translational reading frame was determined by the gene 6 fusion partner. The AcaNAPc2 cDNA lacks a portion of the 5'-terminal part of the coding region; the homology with AcaNAP5 and AcaNAP6 predicts that the first seven amino acid residues belong to the secretion signal.

Figures 10A and 10B depict the comparative effects of certain NAP proteins on the prothrombin time (PT) measurement (Figure 10A) and the activated partial thromboplastin time (aPTT) (Figure 10B) of normal citrated human plasma. Solid circles, (•), represent Pro-AcaNAP5; open triangles, (Δ), represent AcaNAP5 (AcaNAP5^a in Table 2); and open circles, (○), represent native AcaNAP5.

Figure 11 depicts the alignment of the amino acid sequences encoded by certain NAP cDNAs isolated from various nematodes. AcaNAP5 [SEQ. ID. NO. 20], AcaNAP6 [SEQ. ID. NO. 21], and AcaNAPc2 [SEQ. ID. NO. 128] were isolated from *Ancylostoma caninum*. AceNAP5 [SEQ. ID. NO. 22], AceNAP7 [SEQ. ID. NO. 23], and AceNAP4 (AceNAP4d1 [SEQ. ID. NO. 24] and AceNAP4d2 [SEQ. ID. NO. 25] were isolated from *Ancylostoma ceylanicum*. AduNAP4 [SEQ. ID. NO. 26] and AduNAP7 (AduNAP7d1 [SEQ. ID. NO. 27] and AduNAP7d2 [SEQ. ID. NO. 28]) were isolated from *Ancylostoma duodenale*. HpoNAP5 [SEQ. ID. NO. 29] was isolated from *Heligmosomoides polygyrus*. The amino acid sequences shown in this figure are as given in Figures 1, 3, 7A through 7F, and 9. The sequences of mature AcaNAP5 [SEQ. ID. NO. 4] and AcaNAP6 [SEQ. ID. NO. 6] (see Figures 2 and 4) are characterized, in part, by ten cysteine residues (numbered one through ten and shown in bold). All of the amino acid sequences in this Figure contain at least one NAP domain. The AceNAP4 cDNA consists of two adjacent regions, named AceNAP4d1 [SEQ. ID. NO. 24] and AceNAP4d2 [SEQ. ID. NO. 25], which encode a first (d1) and second (d2) NAP-domain; similarly, the AduNAP7 cDNA contains two adjacent regions, AduNAP7d1 [SEQ. ID. NO. 27] and AduNAP7d2 [SEQ. ID. NO. 28], encoding a first (d1) and second (d2) NAP-domain. The alignment of the amino acid sequences of all NAP-domains is guided by the cysteines; dashes (---) were introduced at certain positions to maintain the cysteine alignment and indicate the absence of an amino acid at that position. The carboxy-terminal residue of a cDNA encoded protein is followed by the word "end".

Figures 12A and 12B depict a map of the *P. pastoris* pYAM7SP8 expression/secretion vector (Figure 12A) and sequences included in the vector (Figure 12B) [SEQ. ID. NO. 30]. As depicted in Figure 12A, this plasmid contains the following elements inserted between the methanol-induced AOX1 promoter (dark arrow in the 5' AOX untranslated region) and the AOX1 transcription termination signal (3'T): a synthetic DNA fragment encoding the acid phosphatase secretion signal (S), a synthetic 19-amino acid pro sequence (P) ending with a Lys-Arg processing site for the KEX2 protease and a multicloning site. The *HIS4* gene which serves as a selection marker in GS115 transformation was modified by site directed mutagenesis to eliminate the *Stu1* recognition sequence (*HIS4**). pBR322 sequences, including the *Bla* gene and origin (*ori*) for propagation in *E. coli* are represented by a single line. Figure 12B depicts the following contiguous DNA sequences which are incorporated in pYAM7SP8: the acid phosphatase (*PHO1*) secretion signal sequence, pro sequence and multicloning site (MCS) sequence. The ATG start codon of the *PHO1* secretion signal is underlined.

Figures 13A through 13H depict the nucleotide sequences of the cDNAs and deduced amino acid sequences of certain NAP proteins isolated from *Ancylostoma caninum*. Figure 13A depicts sequences for the recombinant cDNA molecule AcaNAP23 [SEQ. ID. NO. 31]. Figure 13B depicts sequences for the recombinant cDNA molecule AcaNAP24 [SEQ. ID. NO. 32]. Figure 13C depicts sequences for the recombinant cDNA molecule AcaNAP25 [SEQ. ID. NO. 33]. Figure 13D depicts sequences for the recombinant cDNA molecules AcaNAP31, AcaNAP42, and AcaNAP46, all of which are identical [SEQ. ID. NO. 34]. Figure 13E depicts sequences for the recombinant cDNA molecule AcaNAP44 [SEQ. ID. NO. 35]. Figure 13F depicts sequences for the recombinant cDNA molecule AcaNAP45 [SEQ. ID. NO. 36]. Figure 13G depicts sequences for the recombinant cDNA molecule AcaNAP47 [SEQ. ID. NO. 37]. Figure 13H depicts sequences for the recombinant cDNA molecule AcaNAP48 [SEQ. ID. NO. 38]. The *EcoRI* site, corresponding to the 5'-end of the recombinant cDNA molecule, is indicated in all cases (underlined). Numbering of each sequence starts at this *EcoRI* site. AcaNAP45 and AcaNAP47, each encode a protein which has two NAP domains; all other clones in this Figure code for a protein having a single NAP domain.

Figure 14 depicts the nucleotide, and deduced amino acid, sequence of the recombinant cDNA molecule NamNAP [SEQ. ID. NO. 39].

Figure 15 presents the antithrombotic activity of AcaNAP5 and Low Molecular Weight Heparin (LMWH; Enoxaparin[™]) evaluated in the FeCl₃ model of arterial thrombosis. Activity data is represented as the percent incidence of occlusive thrombus formation in the carotid artery (circles). Thrombus formation began 150 minutes after subcutaneous (s.c.) administration of test agent. Deep wound bleeding was quantified in a separate group of animals that were treated in an identical manner but without addition of FeCl₃ (squares). Blood loss at a deep surgical wound in the neck was quantified over a total of 210 minutes after subcutaneous compound administration.

Figure 16 presents the alignment of amino acid sequences corresponding to mature NAPs isolated according to the procedures disclosed herein: namely AcaNAP5 [SEQ. ID. NO. 40], AcaNAP6 [SEQ. ID. NO. 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31, 42, 46 [SEQ. ID. NO. 47], AceNAP4d1 [SEQ. ID. NO. 48], AceNAP4d2

[SEQ. ID. NO. 49], AcaNAP45d1 [SEQ. ID. NO. 50], AcaNAP47d1 [SEQ. ID. NO. 51], AduNAP7d1 [SEQ. ID. NO. 52], AcaNAP45d2 [SEQ. ID. NO. 53], AcaNAP47d2 [SEQ. ID. NO. 54], AduNAP4 [SEQ. ID. NO. 55], AduNAP7d2 [SEQ. ID. NO. 56], AceNAP5 [SEQ. ID. NO. 57], AceNAP7 [SEQ. ID. NO. 58], AcaNAPc2 [SEQ. ID. NO. 59], HpoNAP5 [SEQ. ID. NO. 60], and NamNAP [SEQ. ID. NO. 61]. Each NAP domain comprises ten cysteine residues, which are used to align the sequences, and amino acid sequences between the cysteines. A1 through A10 represent the amino acid sequences between the cysteine residues.

Figure 17 depicts the amino acid sequence of mature AceNAP4 [SEQ. ID. NO. 62] having two NAP domains.

Figure 18 depicts the amino acid sequence of mature AcaNAP45 [SEQ. ID. NO. 63] having two NAP domains.

Figure 19 depicts the amino acid sequence of mature AcaNAP47 [SEQ. ID. NO. 64] having two NAP domains.

Figure 20 depicts the amino acid sequence for mature AduNAP7 [SEQ. ID. NO. 65] having two NAP domains.

Detailed Description of the Invention.

[0049] This invention provides a family of proteins, collectively referred to as Nematode-extracted Anticoagulant Proteins (NAPs). These proteins are so designated because the first member originally isolated was extracted from a nematode, the canine hookworm, *Ancylostoma caninum*. However, the designation NAP or NAP domain should not be considered to limit the proteins of the present invention by this or other natural source.

[0050] Individual NAP proteins are characterized by having at least one NAP domain and by having serine protease inhibitory and/or anticoagulant activity. Such anticoagulant activity may be assessed by increases in clotting time in both the PT and aPTT assays described herein, by the inhibition of factor Xa or factor VIIa/TF activity, or by demonstration of activity *in vivo*. Preferably, blood or plasma used in such assays derives from species known to be infected by nematodes, such as pigs, humans, primates, and the like. The NAP domain is an amino acid sequence. It is believed that the NAP domain is responsible for the observed inhibitory and/or anticoagulant activity. Certain representative NAP domains include the amino acid sequences depicted in Figures 11 and 16, particularly the sequences between the cysteines designated as Cysteine 1 and Cysteine 10 in the Figures and the sequence following Cysteine 10. The characteristics broadly defining this family of proteins, as well as the nucleic acid molecules, including mRNAs sequences and DNA sequences which encode such proteins, are provided. Methods of making these proteins, as well as methods of making nucleic acid molecules encoding such proteins, are also provided. The specific examples provided are exemplary only and other members of the NAP family of proteins, as well as nucleic acid sequences encoding them, can be obtained by following the procedures outlined in these examples and described herein.

[0051] The proteins of the present invention include isolated NAPs which comprise proteins having anticoagulant activity and including at least one NAP domain.

[0052] With respect to "anticoagulant activity", the purified proteins of the present invention are active as anticoagulants, and as such, are characterized by inhibiting the clotting of blood which includes the clotting of plasma. In one aspect, the preferred isolated proteins of the present invention include those which increase the clotting time of human plasma as measured in both the prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays.

[0053] In the PT assay, clotting is initiated by the addition of a fixed amount of tissue factor-phospholipid micelle complex (thromboplastin) to human plasma. Anticoagulants interfere with certain interactions on the surface of this complex and increase the time required to achieve clotting relative to the clotting observed in the absence of the anticoagulant. The measurement of PT is particularly relevant for assessing NAP anticoagulant activity because the series of specific biochemical events required to cause clotting in this assay are similar to those that must be overcome by the hookworm in nature to facilitate feeding. Thus, the ability of NAP to act as an inhibitor in this assay can parallel its activity in nature, and is predictive of anticoagulant activity *in vivo*. In both the assay and in nature, the coagulation response is initiated by the formation of a binary complex of the serine protease factor VIIa (fVIIa) and the protein tissue factor (TF) (fVIIa/TF), resulting in the generation of fXa. The subsequent assembly of fXa into the prothrombinase complex is the key event responsible for the formation of thrombin and eventual clot formation.

[0054] In the aPTT assay, clotting is initiated by the addition of a certain fixed amount of negatively charged phospholipid micelle (activator) to the human plasma. Substances acting as anticoagulants will interfere with certain interactions on the surface of the complex and again increase the time to achieve a certain amount of clotting relative to that observed in the absence of the anticoagulant. Example B describes such PT and aPTT assays. These assays can be used to assess anticoagulant activity of the isolated NAPs of the present invention.

[0055] The preferred isolated NAPs of the present invention include those which double the clotting time of human plasma in the PT assay when present at a concentration of about 1 to about 500 nanomolar and which also double the clotting time of human plasma in the aPTT assay when present at a concentration of about 1 to about 500 nanomolar. Especially preferred are those proteins which double the clotting time of human plasma in the PT assay when present at a concentration of about 5 to about 100 nanomolar, and which also double the clotting time of human plasma in the aPTT assay when present at a concentration of about 5 to about 200 nanomolar. More especially preferred are those proteins which double the clotting time of human plasma in the PT assay when present at a concentration about 10 to

about 50 nanomolar, and which also double the clotting time of human plasma in the aPTT assay when present at a concentration of about 10 to about 100 nanomolar.

[0056] Anticoagulant, or antithrombotic, activity of NAPs of the present invention also can be evaluated using the in vivo models presented in Example F. The rat FeCl₃ model described in part A of that Example is a model of platelet dependent, arterial thrombosis that is commonly used to assess antithrombotic compounds. The model evaluates the ability of a test compound to prevent the formation of an occlusive thrombus induced by FeCl₃ in a segment of the rat carotid artery. NAPs of the present invention are effective anticoagulants in this model when administered intravenously or subcutaneously. The deep wound bleeding assay described in part B of Example F allows measurement of blood loss after administration of an anticoagulant compound. A desired effect of an anticoagulant is that it inhibits blood coagulation, or thrombus formation, but not so much as to prevent clotting altogether and thereby potentiate bleeding. Thus, the deep wound bleeding assay measures the amount of blood loss over the 3.5 hour period after administration of anticoagulant. The data presented in Figure 15 show NAP of the present invention to be an effective antithrombotic compound at a dose that does not cause excessive bleeding. In contrast, the dose of low molecular weight heparin (LMWH) that correlated with 0% occlusion caused about three times more bleeding than the effective dose of NAP.

General NAP Domain [FORMULA I]

[0057] With respect to "NAP domain", the isolated proteins (or NAPs) of the present invention include at least one NAP domain in their amino acid sequence. Certain NAP domains have an amino acid sequence having a molecular weight of about 5.0 to 10.0 kilodaltons, preferably from about 7.0 to 10.0 kilodaltons, and containing 10 cysteine amino acid residues.

[0058] Certain NAPs of the present invention demonstrate specificity toward inhibiting a particular component in the coagulation cascade, such as fXa or the fVIIa/TF complex. The specificity of a NAP's inhibitory activity toward a component in the coagulation cascade can be evaluated using the protocol in Example D. There, the ability of a NAP to inhibit the activity of a variety of serine proteases involved in coagulation is measured and compared. The ability of a NAP to inhibit the fVIIa/TF complex also can be assessed using the protocols in Example E, which measure the ability of a NAP to bind fXa in either an inhibitory or noninhibitory manner and to inhibit FVIIa when complexed with TF. AcaNAP5 and AcaNAP6 are examples of proteins having NAP domains that specifically inhibit fXa. AcaNAPc2 is a protein having a NAP domain that demonstrates selective inhibition of the fVIIa/TF complex when fXa, or a catalytically active or inactive derivative thereof, is present.

NAPs having anticoagulant activity, including NAPs having Factor Xa inhibitory activity (FORMULA II)

[0059] Thus, in one aspect NAPs of the present invention also include an isolated protein having anticoagulant activity, including an isolated protein having Factor Xa inhibitory activity, and having one or more NAP domains, wherein each NAP domain includes the sequence:

**Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
Cys-A9-Cys-A10 ("FORMULA II"),**

wherein

- (a) A1 is an amino acid sequence of 7 to 8 amino acid residues;
- (b) A2 is an amino acid sequence of 3 to 5 amino acid residues;
- (c) A3 is an amino acid sequence of 3 amino acid residues;
- (d) A4 is an amino acid sequence of 6 to 19 amino acid residues;
- (e) A5 is an amino acid sequence of 3 to 4 amino acid residues;
- (f) A6 is an amino acid sequence of 3 to 5 amino acid residues;
- (g) A7 is an amino acid;
- (h) A8 is an amino acid sequence of 11 to 12 amino acid residues;
- (i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and
- (j) A10 is an amino acid sequence of 5 to 25 amino acid residues.

[0060] Pharmaceutical compositions comprising NAP proteins according to this aspect, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this aspect also are contemplated by this invention.

[0061] NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are NAPs having

one or two NAP domains. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40] and AcaNAP6 [SEQ. ID. NOS. 6 and 41] have one NAP domain and are preferred NAPs according to this aspect of the invention.

[0062] Thus, according to one preferred aspect, provided are isolated proteins having anticoagulant activity, including isolated proteins having activity as Factor Xa inhibitors, having at least one NAP domain of formula II which includes the following sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ. ID. NOS. 67 and 156; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 157 to 159; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 160 to 173; (d) Cys-A5 is selected from SEQ. ID. NOS. 174 and 175; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 176 to 178; (f) Cys-A7-Cys-A8 is selected from SEQ. ID. NOS. 179 and 180; (g) Cys-A9 is selected from one of SEQ. ID. NOS. 181 to 183; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 184 to 204.

[0063] In another preferred embodiment of this aspect of the invention, A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3_a is selected from the group consisting of Ala, Arg, Pro, Lys, Ile, His, Leu, and Thr, and A3_b is selected from the group consisting of Lys, Thr, and Arg. Especially preferred A3 sequences are selected from the group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and Glu-Thr-Lys.

[0064] In an additional preferred embodiment of this aspect of the invention, A4 is an amino acid sequence having a net anionic charge.

[0065] According to this aspect of the invention, a preferred A7 amino acid residue is Val or Ile.

[0066] Another preferred embodiment of this aspect of the invention is one in which A8 includes the amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68], wherein

- (a) A8_a is the first amino acid residue in A8,
- (b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and
- (c) A8_c through A8_g are independently selected amino acid residues.

[0067] Preferably, A8_c is Gly, A8_d is selected from the group consisting of Phe, Tyr, and Leu, A8_e is Tyr, A8_f is Arg, and A8_g is selected from Asp and Asn. An especially preferred A8_c-A8_d-A8_e-A8_f-A8_g sequence is selected from the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

[0068] An additional preferred embodiment is one in which A10 includes an amino sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

[0069] NAP proteins AcaNAP5 and AcaNAP6 include the amino acid sequence Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74] in A10, and are preferred NAPs according to this embodiment of the invention.

[0070] In one embodiment of this aspect of the invention, a preferred NAP molecule is one wherein

- (a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
- (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A7 is selected from the group consisting of Val and Ile;
- (d) A8 includes an amino acid sequence selected from the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73]; and
- (e) A10 includes an amino sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

[0071] Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins AcaNAP5 and AcaNAP6 have one NAP domain and are preferred NAPs according to this embodiment of the invention.

[0072] In another preferred embodiment, a NAP molecule is one wherein

- (a) A3 is selected from the group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and Glu-Thr-Lys;
- (b) A4 is an amino acid sequence having a net anionic charge;

(c) A7 is Val or Ile;

(d) A8 includes an amino acid sequence selected from the group consisting of A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 78], A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79], A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 80], A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 81], and A8_a-A8_b-Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 82], wherein at least one of A8_a and A8_b is Glu or Asp;

(e) A9 is an amino acid sequence of five amino acid residues; and

(f) A10 includes an amino acid sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77]. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. Preferred are proteins having at least one NAP domain that is substantially the same as that of either AcaNAP5 [SEQ. ID. NO. 40] or AcaNAP6 [SEQ. ID. NO. 41]. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40] and AcaNAP6 [SEQ. ID. NOS. 6 and 41] have one NAP domain and are especially preferred NAPs according to this embodiment of the invention.

[0073] Preferred NAP proteins having anticoagulant activity, including those having Factor Xa inhibitory activity, according to all the embodiments recited above for this aspect of the invention, can be derived from a nematode species. A preferred nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligmosomoides polygyrus*. Particularly preferred are NAP proteins AcaNAP5 and AcaNAP6 derived from *Ancylostoma caninum*.

[0074] This aspect of the invention also contemplates isolated recombinant cDNA molecules encoding a protein having anticoagulant and/or Factor Xa inhibitory activity, wherein the protein is defined according to each of the embodiments recited above for isolated NAP protein having anticoagulant and/or Factor Xa inhibitory activity. Preferred cDNAs according to this aspect of the invention code for AcaNAP5 and AcaNAP6.

[0075] The Factor Xa inhibitory activity of NAPs within this aspect of the invention can be determined using protocols described herein. Example A describes one such method. In brief, a NAP is incubated with factor Xa for a period of time, after which a factor Xa substrate is added. The rate of substrate hydrolysis is measured, with a slower rate compared to the rate in the absence of NAP indicative of NAP inhibition of factor Xa. Example C provides another method of detecting a NAP's inhibitory activity toward factor Xa when it is assembled into the prothrombinase complex, which more accurately reflects the normal physiological function of fXa *in vivo*. As described therein, factor Xa assembled in the prothrombinase complex is incubated with NAP, followed by addition of substrate. Factor Xa-mediated thrombin generation by the prothrombinase complex is measured by the rate of thrombin generation from this mixture.

NAPs having anticoagulant activity, including NAPs having Factor VIIa/TF inhibitory activity (FORMULA III)

[0076] In another aspect, NAPs of the present invention also include an isolated protein having anticoagulant activity, including and isolated, protein having Factor VIIa/TF inhibitory activity and having one or more NAP domains, wherein each NAP domain includes the sequence:

**Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-
Cys-A9-Cys-A10 ("FORMULA III"),**

wherein

(a) A1 is an amino acid sequence of 7 to 8 amino acid residues;

(b) A2 is an amino acid sequence of 3 to 5 amino acid residues;

(c) A3 is an amino acid sequence of 3 amino acid residues; of 6 to 19 amino acid residues;

(d) A4 is an amino acid sequence of 6 to 19 amino acid residues;

(e) A5 is an amino acid sequence of 3 to 4 amino acid residues;

(f) A6 is an amino acid sequence of 3 to 5 amino acid residues;

(g) A7 is an amino acid;

(h) A8 is an amino acid sequence of 11 to 12 amino acid residues;

(i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and

(j) A10 is an amino acid sequence of 5 to 25 amino acid residues.

[0077] Pharmaceutical compositions comprising NAP proteins according to this aspect, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this aspect also are contemplated by this invention. NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. Preferred are proteins having at least one NAP domain substantially the same as that of AcaNAPc2 [SEQ. ID. NO. 59]. NAP protein AcaNAPc2 [SEQ. ID. NO. 59] has one NAP domain and is an especially preferred NAP according to this aspect of the invention.

[0078] Accordingly, in one preferred aspect, provided are NAPs having anticoagulant activity, including factor VIIa/TF inhibitory activity, and having at least one NAP domain of formula III wherein the NAP domain includes the amino acid sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-AS-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ. ID. NOS. 83 and 205; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 206 to 208; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 209 to 222; (d) Cys-A5 is selected from SEQ. ID. NOS. 223 and 224; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 225 to 227; (f) Cys-A7-Cys-A8 is selected from SEQ. ID. NOS. 228 and 229; (g) Cys-A9 is selected from SEQ. ID. NOS. 230 to 232; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 233 to 253.

[0079] In another preferred embodiment according to this aspect of the invention, A3 has the sequence Asp-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3 is Asp-Lys-Lys.

[0080] In an additional preferred embodiment, A4 is an amino acid sequence having a net anionic charge.

[0081] In another preferred embodiment of this aspect of the invention, A5 has the sequence A5_a-A5_b-A5_c-A5_d [SEQ. ID. NO. 84], wherein A5_a through A5_d are independently selected amino acid residues. Preferably, A5_a is Leu and A5_c is Arg.

[0082] According to this aspect of the invention, a preferred A7 amino acid residue is Val or Ile, more preferably Val.

[0083] An additional preferred embodiment of this aspect of the invention is one in which A8 includes the amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68], wherein

- (a) A8_a is the first amino acid residue in A8,
- (b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and
- (c) A8_c through A8_g are independently selected amino acid residues.

[0084] Preferably, A8_c is Gly, A8_d is selected from the group consisting of Phe, Tyr, and Leu, A8_e is Tyr, A8_f is Arg, and A8_g is selected from Asp and Asn. A preferred A8_c-A8_d-A8_e-A8_f-A8_g sequence is Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70].

[0085] In one embodiment, a preferred NAP molecule is one wherein:

- (a) A3 has the sequence Asp-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
- (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A5 has the sequence A5_a-A5_b-A5_c-A5_d, wherein A5_a through A5_d are independently selected amino acid residues; and
- (d) A7 is selected from the group consisting of Val and Ile. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP protein AcaNAPc2 has one NAP domain and is a preferred NAP according to this embodiment of the invention.

[0086] In another preferred embodiment, a NAP molecule is one wherein

- (a) A3 is Asp-Lys-Lys;
- (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A5 has the sequence A5_a-A5_b-A5_c-A5_d [SEQ. ID. NO. 85], wherein A5_a through A5_d are independently selected amino acid residues;
- (d) A7 is Val; and
- (e) A8 includes an amino acid sequence A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79], wherein at least one of A8_a and A8_b is Glu or Asp. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP protein AcaNAPc2 [SEQ. ID. NO. 59] has one NAP domain and is a preferred NAP according to this embodiment of the invention.

[0087] Preferred NAP proteins having anticoagulant activity, including those having Factor VIIa/TF inhibitory activity, according to all the embodiments recited above for this aspect of the invention, can be derived from a nematode species. A preferred nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligmosomoides polygyrus*. Particularly preferred is NAP protein AcaNAPc2 derived from *Ancylostoma caninum*.

[0088] This aspect of the invention also contemplates isolated recombinant cDNA molecules encoding a protein having anticoagulant and/or Factor VIIa/TF inhibitory activity, wherein the protein is defined according to each of the embodiments recited above for isolated NAP protein having anticoagulant and/or Factor VIIa/TF inhibitory activity. A preferred cDNA according to this aspect has a nucleotide sequence [SEQ. ID. NO. 19] and codes for AcaNAPc2 [SEQ. ID. NO. 59].

[0089] The fVIIa/TF inhibitory activity of NAPs within this aspect of the invention can be determined using protocols described herein. Example E describes fVIIa/TF assays. There, the fVIIa/TF-mediated cleavage and liberation of the tritiated activation peptide from radiolabeled human factor IX (³H-FIX) or the amidolytic hydrolysis of a chromogenic peptidyl substrate are measured. Interestingly, NAP fVIIa/TF inhibitors of the present invention require the presence of fXa in order to be active fVIIa/TF inhibitors. However, NAP fVIIa/TF inhibitors were equally effective in the presence of fXa in which the active site had been irreversibly occupied with the peptidyl chloromethyl ketone H-Glu-Gly-Arg-CMK (EGR), and thereby rendered catalytically inactive (EGR-fXa). While not wishing to be bound by any one explanation, it appears that a NAP having fVIIa/TF inhibition activity forms a binary complex with fXa by binding to a specific recognition site on the enzyme that is distinct from the primary recognition sites P₄-P₁, within the catalytic center of the enzyme. This is followed by the formation of a quaternary inhibitory complex with the fVIIa/TF complex. Consistent with this hypothesis is that EGR-fXa can fully support the inhibition of fVIIa/TF by NAPs inhibitory for fVIIa/TF despite covalent occupancy of the primary recognition sites (P₄-P₁) within the catalytic site of fXa by the tripeptidyl-chloromethyl ketone (EGR-CMK).

[0090] The fVIIa/TF inhibitory activity of NAPs also can be determined using the protocols in Example D, as well as the fXa assays described in Examples A and C. There, the ability of a NAP to inhibit the catalytic activity of a variety of enzymes is measured and compared to its inhibitory activity toward the fVIIa/TF complex. Specific inhibition of fVIIa/TF by a NAP is a desired characteristic for certain applications.

[0091] A further aspect of the invention includes an isolated protein having anticoagulant activity, and cDNAs coding for the protein, wherein said protein specifically inhibits the catalytic activity of the fVIIa/TF complex in the presence of fXa or catalytically inactive fXa derivative, but does not specifically inhibit the activity of FVIIa in the absence of TF and does not specifically inhibit prothrombinase. Preferred proteins according to this aspect of the invention have the characteristics described above for an isolated protein having Factor VIIa/TF inhibitory activity and having one or more NAP domains. A preferred protein according to this aspect of the invention is AcaNAPc2.

[0092] NAPs within this aspect of the invention are identified by their fVIIa/TF inhibitory activity in the presence of fXa or a fXa derivative, whether the derivative is catalytically active or not. The protocols described in Examples B, C, and F are useful in determining the anticoagulant activity of such NAPs. The protocol in Example A can detect a NAP's inactivity toward free fXa or prothrombinase. Data generated using the protocols in Example E will identify NAPs that require either catalytically active or inactive fXa to inhibit fVIIa/TF complex.

NAPs having serine protease inhibitory activity (FORMULA IV)

[0093] In an additional aspect, NAPs of the present invention also include an isolated protein having serine protease inhibitory activity and having one or more NAP domains, wherein each NAP domain includes the sequence: Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10, ("FORMULA IV") wherein

- (a) A1 is an amino acid sequence of 7 to 8 amino acid residues;
- (b) A2 is an amino acid sequence of 3 to 5 amino acid residues;
- (c) A3 is an amino acid sequence of 3 amino acid residues;
- (d) A4 is an amino acid sequence of 6 to 19 amino acid residues;
- (e) A5 is an amino acid sequence of 3 to 4 amino acid residues;
- (f) A6 is an amino acid sequence of 3 to 5 amino acid residues;
- (g) A7 is an amino acid;
- (h) A8 is an amino acid sequence of 10 to 12 amino acid residues ;
- (i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and
- (j) A10 is an amino acid sequence of 1 to 25 amino acid residues.

[0094] Pharmaceutical compositions comprising NAP proteins according to this aspect, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this aspect also are contemplated by this invention. NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are NAPs having one or two

NAP domains. Preferred are NAP domains that have amino acid sequences that are substantially the same as the NAP domains of HpoNAP5 [SEQ. ID. NO. 60] or NamNAP [SEQ. ID. NO. 61]. NAP proteins HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61] have one NAP domain and are preferred NAPs according to this aspect of the invention.

[0095] Thus, in one preferred aspect, NAPs exhibiting serine protease activity have at least one NAP domain of Formula IV which includes the amino acid sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ. ID. NOS. 86 and 254; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 255 to 257; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 258 to 271; (d) Cys-A5 is selected from SEQ. ID. NOS. 272 and 273; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 274 to 276; (f) Cys-A7-Cys-A8 is selected from one of SEQ. ID. NOS. 277 to 279; (g) Cys-A9 is selected from one of SEQ. ID. NOS. 280 to 282; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 283 to 307.

[0096] In another preferred embodiment, A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3 is Glu-Pro-Lys.

[0097] In an additional preferred embodiment, A4 is an amino acid sequence having a net anionic charge.

[0098] In another preferred embodiment, A5 has the sequence A5_a-A5_b-A5_c, wherein A5_a through A5_c are independently selected amino acid residues. Preferably, A5_a is Thr and A5_c is Asn. An especially preferred A5 sequence includes Thr-Leu-Asn or Thr-Met-Asn.

[0099] According to this aspect of the invention, a preferred A7 amino acid residue is Gln.

[0100] In one embodiment of this aspect of the invention, a preferred NAP molecule is one wherein

- (a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
- (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A5 has the sequence A5_a-A5_b-A5_c, wherein A5_a through A5_c are independently selected amino acid residues, and
- (d) A7 is Gln. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61] have one NAP domain and are preferred NAPs according to this embodiment of the invention.

[0101] In another preferred embodiment, a NAP molecule is one wherein

- (a) A3 is Glu-Pro-Lys;
- (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A5 is selected from Thr-Leu-Asn and Thr-Met-Asn; and
- (d) A7 is Gln. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61] have one NAP domain and are preferred NAPs according to this embodiment of the invention.

[0102] Preferred NAP proteins having serine protease inhibitory activity, according to all the embodiments recited above for this aspect of the invention, can be derived from a nematode species. A preferred nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligmosomoides polygyrus*. Particularly preferred are NAP proteins HpoNAP5 and NamNAP derived from *Heligmosomoides polygyrus* and *Necator americanus*, respectively.

[0103] This aspect of the invention also contemplates isolated recombinant cDNA molecules encoding a protein having serine protease inhibitory activity, wherein the protein is defined according to each of the embodiments recited above for isolated NAP protein having serine protease inhibitory activity. Preferred cDNAs according to this aspect have nucleotide sequences [SEQ. ID. NO. 14] (HpoNAP5) and [SEQ. ID. NO. 39] (NamNAP) and code for HpoNAP5 [SEQ. ID. NO. 60] and NamNAP [SEQ. ID. NO. 61].

[0104] The serine protease inhibitory activity can be determined using any of the assays disclosed in Examples A through F, or any commonly used enzymatic assay for measuring inhibition of serine protease activity. Procedures for a multitude of enzymatic assays can be found in the volumes of Methods of Enzymology or similar reference materials. Preferred NAPs have serine protease inhibitory activity directed toward enzymes in the blood coagulation cascade or toward trypsin/elastase.

NAPs having anticoagulant activity (FORMULA V)

[0105] In another aspect of the invention. NAPs of the present invention also include an isolated protein having

anticoagulant activity and having one or more NAP domains,

wherein each NAP domain includes the sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 ("FORMULA V"), wherein

- (a) A1 is an amino acid sequence of 7 to 8 amino acid residues;
- (b) A2 is an amino acid sequence of 3 to 5 amino acids;
- (c) A3 is an amino acid sequence of 3 amino acid residues;
- (d) A4 is an amino acid sequence of 6 to 19 amino acid residues;
- (e) A5 is an amino acid sequence of 3 to 4 amino acid residues;
- (f) A6 is an amino acid sequence of 3 to 5 amino acid residues;
- (g) A7 is an amino acid;
- (h) A8 is an amino acid sequence of 11 to 12 amino acid residues;
- (i) A9 is an amino acid sequence of 5 to 7 amino acid residues; AND
- (j) A10 is an amino acid sequence of 5 to 25 amino acid residues.

[0106] Pharmaceutical compositions comprising NAP proteins according to this aspect, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this aspect also are contemplated by this invention. NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. Preferred NAPs include those having at least one NAP domain having an amino acid sequence substantially the same as any of [SEQ. ID. NOS. 40 to 58]. NAP proteins AcaNAP5 [SEQ. ID. NO. 40], AcaNAP6 [SEQ. ID. NO. 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] have one NAP domain and are preferred NAPs according to this aspect of the invention. NAP proteins AceNAP4 [SEQ. ID. NO. 62], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AduNAP7 [SEQ. ID. NO. 65] have two NAP domains and are preferred NAPs according to this aspect of the invention.

[0107] Preferred NAPs of the present invention according to this aspect include isolated proteins having anticoagulant activity and having at least one NAP domain of formula V which includes the following sequence:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10 wherein (a) Cys-A1 is selected from SEQ. ID. NOS. 87 and 308; (b) Cys-A2-Cys is selected from one of SEQ. ID. NOS. 309 to 311; (c) A3-Cys-A4 is selected from one of SEQ. ID. NOS. 312 to 325; (d) Cys-A5 is selected from SEQ. ID. NOS. 326 and 327; (e) Cys-A6 is selected from one of SEQ. ID. NOS. 328 to 330; (f) Cys-A7-Cys-A8 is selected from SEQ. ID. NOS. 331 to 332; (g) Cys-A9 is selected from one of SEQ. ID. NOS. 333 to 335; and (h) Cys-A10 is selected from one of SEQ. ID. NOS. 336 to 356.

[0108] In another preferred embodiment, A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues. More preferably, A3_a is selected from the group consisting of Ala, Arg, Pro, Lys, Ile, His, Leu, and Thr, and A3_b is selected from the group consisting of Lys, Thr, and Arg. Especially preferred A3 sequences are selected from the group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and Glu-Thr-Lys.

[0109] In an additional preferred embodiment, A4 is an amino acid sequence having a net anionic charge.

[0110] According to this aspect of the invention, a preferred A7 amino acid residue is Val or Ile.

[0111] Another preferred embodiment of the invention is one in which A8 includes the amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68], wherein

- (a) A8_a is the first amino acid residue in A8,
- (b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and
- (c) A8_c through A8_g are independently selected amino acid residues.

[0112] Preferably, A8_c is Gly, A8_d is selected from the group consisting of Phe, Tyr, and Leu, A8_e is Tyr, A8_f is Arg, and A8_g is selected from Asp and Asn. A preferred A8_c-A8_d-A8_e-A8_f-A8_g sequence is selected from the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

[0113] Another preferred embodiment is one in which A10 includes an amino sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77]. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40] and AcaNAP6 [SEQ. ID. NOS. 6 and 41] include the amino acid sequence Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74] in A10, and are preferred NAPs according to this embodiment of the invention. NAP protein AcaNAP48 [SEQ. ID. NO. 42] includes the amino acid sequence Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75] in A10 and is a preferred NAP according to this embodiment of the invention. NAP proteins AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45],

AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], and AceNAP4 [SEQ. ID. NO. 48, 49 AND 62] include the amino acid sequence Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76] and are preferred NAPs according to this embodiment of the invention. NAP proteins AcaNAP45 [SEQ. ID. NOS. 50, 53 AND 63], AcaNAP47 [SEQ. ID. NO. 51, 54 AND 64], AduNAP7 [SEQ. ID. NO. 52, 56 AND 65], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] include the amino acid sequence Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77] and are preferred NAPs according to this embodiment of the invention.

[0114] In one embodiment, a preferred NAP molecule is one wherein

- (a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
- (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A7 is selected from the group consisting of Val and Ile;
- (d) A8 includes an amino acid sequence selected from the group consisting of Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69], Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70], Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71], Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73]; and
- (e) A10 includes an amino sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77]. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this aspect of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40], AcaNAP6 [SEQ. ID. NOS. 6 and 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] have one NAP domain and are preferred NAPs according to this embodiment. NAP proteins AceNAP4 [SEQ. ID. NO. 62], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AduNAP7 [SEQ. ID. NO. 65] have two NAP domains and are preferred NAPs according to this embodiment.

[0115] In another preferred embodiment, a NAP molecule is one wherein

- (a) A3 is selected from the group consisting of Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, and Glu-Thr-Lys;
- (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A7 is Val or Ile;
- (d) A8 includes an amino acid sequence selected from the group consisting of A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 78], A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79], A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 80], A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 81], and A8_a-A8_b-Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 82], wherein at least one of A8_a and A8_b is Glu or Asp;
- (e) A9 is an amino acid sequence of five amino acid residues; and
- (f) A10 includes an amino acid sequence selected from the group consisting of Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74], Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75], Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77]. Pharmaceutical compositions comprising NAP proteins according to this embodiment, and methods of inhibiting blood coagulation comprising administering NAP proteins according to this embodiment also are contemplated by this invention. NAP proteins within this embodiment of the invention have at least one NAP domain. Preferred are NAPs having one or two NAP domains. NAP proteins AcaNAP5 [SEQ. ID. NOS. 4 and 40], AcaNAP6 [SEQ. ID. NOS. 6 and 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AduNAP4 [SEQ. ID. NO. 55], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] have one NAP domain and are preferred NAPs according to this embodiment. NAP proteins AceNAP4 [SEQ. ID. NO. 62], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AduNAP7 [SEQ. ID. NO. 65] have two NAP domains and are preferred NAPs according to this embodiment.

[0116] Preferred NAP proteins having anticoagulant activity, according to all the embodiments recited above for this aspect of the invention, can be derived from a nematode species. A preferred nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligmosomoides polygyrus*. Particularly preferred are NAP proteins AcaNAP5 [SEQ. ID. NO. 4 and 40], AcaNAP6 [SEQ. ID. NO. 6 and 41], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AcaNAP31 [SEQ. ID. NO. 47] derived from *Ancylostoma caninum*; AceNAP4 [SEQ. ID. NO. 62], AceNAP5

[SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58] derived from *Ancylostoma ceylanicum*; and AduNAP7 [SEQ. ID. NO. 65] and AduNAP4 [SEQ. ID. NO. 55] derived from *Ancylostoma duodenale*.

[0117] This aspect of the invention also contemplates isolated recombinant cDNA molecules encoding a protein having anticoagulant activity, wherein the protein is defined according to each of the embodiments recited above for isolated NAP protein having anticoagulant activity. Preferred cDNAs according to this aspect include AcaNAP5 [SEQ. ID. NO. 3], AcaNAP6 [SEQ. ID. NO. 5], AcaNAP48 [SEQ. ID. NO. 38], AcaNAP23 [SEQ. ID. NO. 31], AcaNAP24 [SEQ. ID. NO. 32], AcaNAP25 [SEQ. ID. NO. 33], AcaNAP44 [SEQ. ID. NO. 35], AcaNAP31 [SEQ. ID. NO. 34], AduNAP4 [SEQ. ID. NO. 12], AceNAP5 [SEQ. ID. NO. 10], AceNAP7 [SEQ. ID. NO. 11], AceNAP4 [SEQ. ID. NO. 9], AcaNAP45 [SEQ. ID. NO. 36], AcaNAP47 [SEQ. ID. NO. 37], and AduNAP7 [SEQ. ID. NO. 13].

[0118] The anticoagulation activity of NAPs within this aspect of the invention can be determined using protocols described herein. Examples B and F present particularly useful methods for assessing a NAP's anticoagulation activity. The procedures described for detecting NAPs having fXa inhibitory activity (Examples A,C) and fVIIa/TF inhibitory activity (Example E) also are useful in evaluating a NAP's anticoagulation activity.

15 Oligonucleotides

[0119] Another aspect of this invention is an oligonucleotide comprising a sequence selected from

YG109: TCAGACATGT-ATAATCTCAT-GTTGG [SEQ. ID. NO. 88],
 YG103: AAGGCATACC-CGGAGTGTGG-TG [SEQ. ID. NO. 89],
 NAP-1: AAR-CCN-TGY-GAR-MGG-AAR-TGY [SEQ. ID. NO. 90], and
 NAP-4.RC: TW-RWA-NCC-NTC-YTT-RCA-NAC-RCA [SEQ. ID. NO. 91].

These oligonucleotide sequences hybridize to nucleic acid sequences coding for NAP protein.

[0120] The isolated NAPs of the present invention include those having variations in the disclosed amino acid sequence or sequences, including fragments, naturally occurring mutations, allelic variants, randomly generated artificial mutants and intentional sequence variations, all of which conserve anticoagulant activity. The term "fragments" refers to any part of the sequence which contains fewer amino acids than the complete protein, as for example, partial sequences excluding portions at the amino-terminus, carboxy-terminus or between the amino-terminus and carboxy-terminus of the complete protein.

[0121] The isolated NAPs of the present invention also include proteins having a recombinant amino acid sequence or sequences which conserve the anticoagulant activity of the NAP domain amino acid sequence or sequences. Thus, as used herein, the phrase "NAP protein" or the term "protein" when referring to a protein comprising a NAP domain, means, without discrimination, native NAP protein and NAP protein made by recombinant means. These recombinant proteins include hybrid proteins, such as fusion proteins, proteins resulting from the expression of multiple genes within the expression vector, proteins resulting from expression of multiple genes within the chromosome of the host cell, and may include a polypeptide having anticoagulant activity of a disclosed protein linked by peptide bonds to a second polypeptide. The recombinant proteins also include variants of the NAP domain amino acid sequence or sequences of the present invention that differ only by conservative amino acid substitution. Conservative amino acid substitutions are defined as "sets" in Table 1 of Taylor, W.R., J. Mol. Biol., 188:233 (1986). The recombinant proteins also include variants of the disclosed isolated NAP domain amino acid sequence or sequences of the present invention in which amino acid substitutions or deletions are made which conserve the anticoagulant activity of the isolated NAP domain sequence or sequences.

[0122] One preferred embodiment of the present invention is a protein isolated by biochemical methods from the nematode, *Ancylostoma caninum*, as described in Example 1. This protein increases the clotting time of human plasma in the PT and aPTT assays, contains one NAP domain, and is characterized by an N-terminus having the amino acid sequence, Lys-Ala-Tyr-Pro-Glu-Cys-Gly-Glu-Asn-Glu-Trp-Leu-Asp [SEQ. ID. NO. 92], and a molecular weight of about 8.7 kilodaltons to about 8.8 kilodaltons as determined by mass spectrometry.

[0123] Further preferred embodiments of the present invention include the proteins having anticoagulant activity made by recombinant methods from the cDNA library isolated from the nematode, *Ancylostoma caninum*, for example, AcaNAP5 [SEQ. ID. NO. 4 or 40], AcaNAP6 [SEQ. ID. NO. 6 or 41], Pro-AcaNAP5 [SEQ. ID. NO. 7], Pro-AcaNAP6 [SEQ. ID. NO. 8], AcaNAP48 [SEQ. ID. NO. 42], AcaNAP23 [SEQ. ID. NO. 43], AcaNAP24 [SEQ. ID. NO. 44], AcaNAP25 [SEQ. ID. NO. 45], AcaNAP44 [SEQ. ID. NO. 46], AcaNAP31 [SEQ. ID. NO. 47], AcaNAP45 [SEQ. ID. NO. 63], AcaNAP47 [SEQ. ID. NO. 64], and AcaNAPc2 [SEQ. ID. NO. 59]; isolated from the nematode, *Ancylostoma ceylanicum*, for example, AceNAP4 [SEQ. ID. NO. 62], AceNAP5 [SEQ. ID. NO. 57], and AceNAP7 [SEQ. ID. NO. 58]; isolated from the nematode, *Ancylostoma duodenale*, for example, AduNAP4 [SEQ. ID. NO. 55] and AduNAP7 [SEQ. ID. NO. 65]; isolated from the nematode *Heligmosmoides polygyrus*, for example, HpoNAP5 [SEQ. ID. NO. 60]; and the nematode *Necator americanus*, for example, NamNAP [SEQ. ID. NO. 61]. The amino acid sequences of these proteins are shown in Figures

11 and 16 and elsewhere. Each such preferred embodiment increases the clotting time of human plasma in the PT and aPTT assays and contains at least one NAP domain.

[0124] With respect to "isolated proteins", the proteins of the present invention are isolated by methods of protein purification well known in the art, or as disclosed below. They may be isolated from a natural source, from a chemical mixture after chemical synthesis on a solid phase or in solution such as solid-phase automated peptide synthesis, or from a cell culture after production by recombinant methods.

[0125] As described further hereinbelow, the present invention also contemplates pharmaceutical compositions comprising NAP and methods of using NAP to inhibit the process of blood coagulation and associated thrombosis. Oligonucleotide probes useful for identifying NAP nucleic acid in a sample also are within the purview of the present invention, as described more fully hereinbelow.

1. NAP Isolated From Natural Sources.

[0126] The preferred isolated proteins (NAPs) of the present invention may be isolated and purified from natural sources. Preferred as natural sources are nematodes; suitable nematodes include intestinal nematodes such as *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus* and *Heligmosomoides polygyrus*. Especially preferred as a natural source is the hematophagous nematode, the hookworm, *Ancylostoma caninum*.

[0127] The preferred proteins of the present invention are isolated and purified from their natural sources by methods known in the biochemical arts. These methods include preparing a soluble extract and enriching the extract using chromatographic methods on different solid support matrices. Preferred methods of purification would include preparation of a soluble extract of a nematode in 0.02 M Tris-HCl, pH 7.4 buffer containing various protease inhibitors, followed by sequential chromatography of the extract through columns containing Concanavalin-A Sepharose matrix, Poros20 HQ cation-ion exchange matrix, Superdex30 gel filtration matrix and a C18 reverse-phase matrix. The fractions collected from such chromatography columns may be selected by their ability to increase the clotting time of human plasma, as measured by the PT and aPTT assays, or their ability to inhibit factor Xa amidolytic activity as measured in a colorimetric amidolytic assay using purified enzyme, or by other methods disclosed in Examples A to F herein. An example of a preferred method of purification of an isolated protein of the present invention would include that as disclosed in Example 1.

[0128] The preferred proteins of the present invention, when purified from a natural source, such as *Ancylostoma caninum*, as described, include those which contain the amino acid sequence: Lys-Ala-Tyr-Pro-Glu-Cys-Gly-Glu-Asn-Glu-Trp-Leu-Asp [SEQ. ID. NO. 92]. Especially preferred are the purified proteins having this amino acid sequence at its amino terminus, such as shown in Figure 2 (AcaNAP5 [SEQ. ID. NO. 4]) or Figure 4 (AcaNAP6 [SEQ. ID. NO. 6]). One preferred protein of the present invention was demonstrated to have the amino acid sequence, Lys-Ala-Tyr-Pro-Glu-Cys-Gly-Glu-Asn-Glu-Trp-Leu-Asp [SEQ. ID. NO. 92] at its amino-terminus and a molecular weight of 8.7 to 8.8 kilodaltons, as determined by mass spectrometry.

2. NAP Made by Chemical Synthesis.

[0129] The preferred isolated NAPs of the present invention may be synthesized by standard methods known in the chemical arts.

[0130] The isolated proteins of the present invention may be prepared using solid-phase synthesis, such as that described by Merrifield, J. Amer. Chem. Soc., 85:2149 (1964) or other equivalent methods known in the chemical arts, such as the method described by Houghten in Proc. Natl. Acad. Sci., 82:5132 (1985).

[0131] Solid-phase synthesis is commenced from the C-terminus of the peptide by coupling a protected amino acid or peptide to a suitable insoluble resin. Suitable resins include those containing chloromethyl, bromomethyl, hydroxymethyl, aminomethyl, benzhydryl, and t-alkyloxycarbonylhydrazide groups to which the amino acid can be directly coupled.

[0132] In this solid phase synthesis, the carboxy terminal amino acid, having its alpha amino group and, if necessary, its reactive side chain group suitably protected, is first coupled to the insoluble resin. After removal of the alpha amino protecting group, such as by treatment with trifluoroacetic acid in a suitable solvent, the next amino acid or peptide, also having its alpha amino group and, if necessary, any reactive side chain group or groups suitably protected, is coupled to the free alpha amino group of the amino acid coupled to the resin. Additional suitably protected amino acids or peptides are coupled in the same manner to the growing peptide chain until the desired amino acid sequence is achieved. The synthesis may be done manually, by using automated peptide synthesizers, or by a combination of these.

[0133] The coupling of the suitably protected amino acid or peptide to the free alpha amino group of the resin-bound amino acid can be carried out according to conventional coupling methods, such as the azide method, mixed anhydride method, DCC (dicyclohexylcarbodiimide) method, activated ester method (p-nitrophenyl ester or N-hydroxysuccinimide ester), BOP (benzotriazole-1-yl-oxy-tris (diamino) phosphonium hexafluorophosphate) method or Woodward reagent K method.

[0134] It is common in peptide synthesis that the protecting groups for the alpha amino group of the amino acids or peptides coupled to the growing peptide chain attached to the insoluble resin will be removed under conditions which do not remove the side chain protecting groups. Upon completion of the synthesis, it is also common that the peptide is removed from the insoluble resin, and during or after such removal, the side chain protecting groups are removed.

[0135] Suitable protecting groups for the alpha amino group of all amino acids and the omega amino group of lysine include benzyloxycarbonyl, isonicotinylloxycarbonyl, o-chlorobenzyloxycarbonyl, p-nitrophenyloxycarbonyl, p-methoxyphenyloxycarbonyl, t-butoxycarbonyl, t-amylloxycarbonyl, adamantyloxycarbonyl, 2-(4-biphenyl)-2-propyloxycarbonyl, 9-fluorenylmethoxycarbonyl, methylsulfonylethoxycarbonyl, trifluoroacetyl, phthalyl, formyl, 2-nitrophenylsulphenyl, diphenylphosphinothiyl, dimethylphosphinothiyl, and the like.

[0136] Suitable protecting groups for the carboxy group of aspartic acid and glutamic acid include benzyl ester, cyclohexyl ester, 4-nitrobenzyl ester, t-butyl ester, 4-pyridylmethyl ester, and the like.

[0137] Suitable protecting groups for the guanidino group of arginine include nitro, p-toluenesulfonyl, benzyloxycarbonyl, adamantyloxycarbonyl, p-methoxybenzenesulfonyl, 4-methoxy-2,6-dimethylbenzenesulfonyl, 1,3,5-trimethylphenylsulfonyl, and the like.

[0138] Suitable protecting groups for the thiol group of cysteine include p-methoxybenzyl, triphenylmethyl, acetylaminoethyl, ethylcarbamoyl, 4-methylbenzyl, 2,4,6-trimethylbenzyl, and the like.

[0139] Suitable protecting groups for the hydroxy group of serine include benzyl, t-butyl, acetyl, tetrahydropyranyl, and the like.

[0140] The completed peptide may be cleaved from the resin by treatment with liquid hydrofluoric acid containing one or more thio-containing scavengers at reduced temperatures. The cleavage of the peptide from the resin by such treatment will also remove all side chain protecting groups from the peptide.

[0141] The cleaved peptide is dissolved in dilute acetic acid followed by filtration, then is allowed to refold and establish proper disulfide bond formation by dilution to a peptide concentration of about 0.5 mM to about 2 mM in a 0.1 M acetic acid solution. The pH of this solution is adjusted to about 8.0 using ammonium hydroxide and the solution is stirred open to air for about 24 to about 72 hours.

[0142] The refolded peptide is purified by chromatography, preferably by high pressure liquid chromatography on a reverse phase column, eluting with a gradient of acetonitrile in water (also containing 0.1% trifluoroacetic acid), with the preferred gradient running from 0 to about 80% acetonitrile in water. Upon collection of fractions containing the pure peptide, the fractions are pooled and lyophilized to the solid peptide.

3. NAP Made By Recombinant Methods.

[0143] Alternatively, the preferred isolated NAPs of the present invention may be made by recombinant DNA methods taught herein and well known in the biological arts. Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning, A Laboratory Manual, Second Edition, volumes 1 to 3, Cold Spring Harbor Laboratory Press (1989).

[0144] Recombinant DNA methods allow segments of genetic information, DNA, from different organisms, to be joined together outside of the organisms from which the DNA was obtained and allow this hybrid DNA to be incorporated into a cell that will allow the production of the protein for which the original DNA encodes.

[0145] Genetic information encoding a protein of the present invention may be obtained from the genomic DNA or mRNA of an organism by methods well known in the art. Preferred methods of obtaining this genetic information include isolating mRNA from an organism, converting it to its complementary DNA (cDNA), incorporating the cDNA into an appropriate cloning vector, and identifying the clone which contains the recombinant cDNA encoding the desired protein by means of hybridization with appropriate oligonucleotide probes constructed from known sequences of the protein.

[0146] The genetic information in the recombinant cDNA encoding a protein of the present invention may be ligated into an expression vector, the vector introduced into host cells, and the genetic information expressed as the protein for which it encodes.

(A) Preparation of cDNA Library.

[0147] Preferred natural sources of mRNA from which to construct a cDNA library are nematodes which include intestinal nematodes such as *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus* and *Heligmosomoides polygyrus*. Especially preferred as a natural source of mRNA is the hookworm nematode, *Ancylostoma caninum*.

[0148] Preferred methods of isolating mRNA encoding a protein of the present invention, along with other mRNA, from an organism include chromatography on poly U or poly T affinity gels. Especially preferred methods of isolating the mRNA from nematodes include the procedure and materials provided in the QuickPrep mRNA Purification kit (Pharmacia).

[0149] Preferred methods of obtaining double-stranded cDNA from isolated mRNA include synthesizing a sin-

gle-stranded cDNA on the mRNA template using a reverse transcriptase, degrading the RNA hybridized to the cDNA strand using a ribonuclease (RNase), and synthesizing a complementary DNA strand by using a DNA polymerase to give a double-stranded cDNA. Especially preferred methods include those wherein about 3 micrograms of mRNA isolated from a nematode is converted into double-stranded cDNA making use of Avian Myeloblastosis Virus reverse transcriptase, RNase H, and *E. coli* DNA polymerase I and T4 DNA polymerase.

[0150] cDNA encoding a protein of the present invention, along with the other cDNA in the library constructed as above, are then ligated into cloning vectors. Cloning vectors include a DNA sequence which accommodates the cDNA from the cDNA library. The vectors containing the cDNA library are introduced into host cells that can exist in a stable manner and provide an environment in which the cloning vector is replicated. Suitable cloning vectors include plasmids, bacteriophages, viruses and cosmids. Preferred cloning vectors include the bacteriophages. Cloning vectors which are especially preferred include the bacteriophage, lambda gt11 Sfi-Not vector.

[0151] The construction of suitable cloning vectors containing the cDNA library and control sequences employs standard ligation and restriction techniques which are well known in the art. Isolated plasmids, DNA sequences or synthesized oligonucleotides are cleaved, tailored and religated in the form desired.

[0152] With respect to restriction techniques, site-specific cleavage of cDNA is performed by treating with suitable restriction enzyme under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. For example, see the product catalogs of New England Biolabs, Promega and Stratagene Cloning Systems.

[0153] Generally, about 1 microgram of the cDNA is cleaved by treatment in about one unit of a restriction enzyme in about 20 microliters of buffer solution. Typically, an excess of restriction enzyme is used to ensure complete cleavage of the cDNA. Incubation times of about 1 to 2 hours at about 37°C are usually used, though exceptions are known. After each cleavage reaction, the protein may be removed by extraction with phenol/chloroform, optionally followed by chromatography over a gel filtration column, such as Sephadex® G50. Alternatively, cleaved cDNA fragments may be separated by their sizes by electrophoresis in polyacrylamide or agarose gels and isolated using standard techniques. A general description of size separations is found in *Methods of Enzymology*, 65:499-560 (1980).

[0154] The restriction enzyme-cleaved cDNA fragments are then ligated into a cloning vector.

[0155] With respect to ligation techniques, blunt-end ligations are usually performed in about 15 to about 30 microliters of a pH 7.5 buffer comprising about 1 mM ATP and about 0.3 to 0.6 (Weiss) units of T4 DNA ligase at about 14°C. Intermolecular "sticky end" ligations are usually performed at about 5 to 100 nanomolar total-end DNA concentrations. Intermolecular blunt-end ligations (usually employing about 10 to 30-fold molar excess of linkers) are performed at about 1 micromolar total-end DNA concentrations.

(B) Preparation of cDNA Encoding NAP.

[0156] Cloning vectors containing the cDNA library prepared as disclosed are introduced into host cells, the host cells are cultured, plated, and then probed with a hybridization probe to identify clones which contain the recombinant cDNA encoding a protein of the present invention. Preferred host cells include bacteria when phage cloning vectors are used. Especially preferred host cells include *E. coli* strains such as strain Y1090.

[0157] Alternatively, the recombinant cDNA encoding a protein of the present invention may be obtained by expression of such protein on the outer surface of a filamentous phage and then isolating such phage by binding them to a target protein involved in blood coagulation.

[0158] An important and well known feature of the genetic code is its redundancy - more than one triplet nucleotide sequence codes for one amino acid. Thus, a number of different nucleotide sequences are possible for recombinant cDNA molecules which encode a particular amino acid sequence for a NAP of the present invention. Such nucleotide sequences are considered functionally equivalent since they can result in the production of the same amino acid sequence in all organisms. Occasionally, a methylated variant of a purine or pyrimidine may be incorporated into a given nucleotide sequence. However, such methylations do not affect the coding relationship in any way.

(1) Using Oligonucleotide Probes.

[0159] Hybridization probes and primers are oligonucleotide sequences which are complementary to all or part of the recombinant cDNA molecule that is desired. They may be prepared using any suitable method, for example, the phosphotriester and phosphodiester methods, described respectively in Narang, S.A. et al., *Methods in Enzymology*, 68:90 (1979) and Brown, E.L. et al., *Methods in Enzymology*, 68:109 (1979), or automated embodiments thereof. In one such embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage et al., *Tetrahedron Letters*, 22:1859-1862 (1981). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Patent No. 4,458,066. Probes differ from primers in that they are labelled with an enzyme, such as horseradish peroxidase, or radioactive atom, such as ³²P, to facilitate their detection. A synthesized probe is

radiolabeled by nick translation using *E. coli* DNA polymerase I or by end labeling using alkaline phosphatase and T4 bacteriophage polynucleotide kinase.

[0160] Preferred hybridization probes include oligonucleotide sequences which are complementary to a stretch of the single-stranded cDNA encoding a portion of the amino acid sequence of a NAP purified from a nematode, such as the hookworm, *Ancylostoma caninum*. For example, a portion of the amino acid sequence shown in Figure 2 (AcaNAP5) [SEQ. ID. NO. 4] or Figure 4 (AcaNAP6 [SEQ. ID. NO. 6]) can be used. Especially preferred hybridization probes include those wherein their oligonucleotide sequence is complementary to the stretch of the single-stranded cDNA encoding the amino acid sequence: Lys-Ala-Tyr-Pro-Glu-Cys-Gly-Glu-Asn-Glu-Trp [SEQ. ID. NO. 93]. Such hybridization probes include the degenerate probe having the oligonucleotide sequence: AAR GCi TAY CCi GAR TGY GGi GAR AAY GAR TGG [SEQ. ID. NO. 94], wherein R is A or G, Y is T or C, and i is inosine. A preferred recombinant cDNA molecule encoding a protein of the present invention is identified by its ability to hybridize to this probe.

[0161] Preferred hybridization probes also include the pair NAP-1 [SEQ. ID. NO. 90] and NAP-4.RC [SEQ. ID. NO. 91], and the pair YG109 [SEQ. ID. NO. 88] and YG103 [SEQ. ID. NO. 89], both of which are described in Examples 13 and 12, respectively.

[0162] Upon identification of the clone containing the desired cDNA, amplification is used to produce large quantities of a gene encoding a protein of the present invention in the form of a recombinant cDNA molecule.

[0163] Preferred methods of amplification include the use of the polymerase chain reaction (PCR). See, e.g., PCR Technology, W.H. Freeman and Company, New York (Edit. Erlich, H.A. 1992). PCR is an *in vitro* amplification method for the synthesis of specific DNA sequences. In PCR, two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the cDNA of the clone are used. A repetitive series of cycles involving cDNA denaturation into single strands, primer annealing to the single-stranded cDNA, and the extension of the annealed primers by DNA polymerase results in number of copies of cDNA, whose termini are defined by the 5-ends of the primers, approximately doubling at every cycle. *Ibid.*, p.1. Through PCR amplification, the coding domain and any additional primer encoded information such as restriction sites or translational signals (signal sequences, start codons and/or stop codons) of the recombinant cDNA molecule to be isolated is obtained.

[0164] Preferred conditions for amplification of cDNA include those using Taq polymerase and involving 30 temperature cycles of: 1 minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C. Preferred primers include the oligo(dT)-NotI primer, AATTCGCGGC CGC(T)₁₅ [SEQ. ID. NO. 95], obtained from Promega Corp. in combination with either (i) the degenerate primer having the oligonucleotide sequence: AAR GCi TAY CCi GAR TGY GGi GAR AAY GAR TGG [SEQ. ID. NO. 94], wherein R is A or G, Y is T or C, and i is inosine, or (ii) the lambda gt11 primer #1218, GGTGGCGACG ACTCCTGGAG CCCG [SEQ. ID. NO. 96], obtained from New England Biolabs.

[0165] The nucleic acid sequence of a recombinant cDNA molecule made as disclosed is determined by methods based on the dideoxy method of Sanger, F. et al, Proc. Natl. Acad. Sci. USA, 74:5463 (1977) as further described by Messing, et al., Nucleic Acids Res., 9:309 (1981).

[0166] Preferred recombinant cDNA molecules made as disclosed include those having the nucleic acid sequences of Figures 1, 3, 7, 9, 13, and 14.

(2) Using NAP cDNAs As Probes .

[0167] Also especially preferred as hybridization probes are oligonucleotide sequences encoding substantially all of the amino acid sequence of a NAP purified from the nematode, the hookworm, *Ancylostoma caninum*. Especially preferred probes include those derived from the AcaNAP5 and AcaNAP6 genes and having the following nucleic acid sequences (AcaNAP5 gene): AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC GAC TGT GGA ACT CAG AAG CCA TGC GAG GCC AAG TGC AAT GAG GAA CCC CCT GAG GAG GAA GAT CCG ATA TGC CGC TCA CGT GGT TGT TTA TTA CCT CCT GCT TGC GTA TGC AAA GAC GGA TTC TAC AGA GAC ACG GTG ATC GGC GAC TGT GTT AGG GAA GAA GAA TGC GAC CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO. 1], or Figure 3 (AcaNAP6 gene): AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC GTC TGT GGA ACT AAG AAG CCA TGC GAG GCC AAG TGC AGT GAG GAA GAG GAG GAA GAT CCG ATA TGC CGA TCA TTT TCT TGT CCG GGT CCC GCT GCT TGC GTA TGC GAA GAC GGA TTC TAC AGA GAC ACG GTG ATC GGC GAC TGT GTT AAG GAA GAA GAA TGC GAC CAA CAT GAG ATT ATA CAT GTC TGA [SEQ. ID. NO. 2].

[0168] Preferred hybridization probes also include sequences encoding a substantial part of the amino acid sequence of a NAP, such as the PCR fragment generated with the primer couple NAP-1 [SEQ. ID. NO. 90] and NAP-4.RC [SEQ. ID. NO. 91] as described in Example 13.

(3) Using Phage Display.

[0169] Disclosed herein is a method to select cDNAs encoding the proteins of the present invention from whole cDNA libraries making use of filamentous phage display technology. Current display technology with filamentous phage relies

on the in-frame insertion of coding regions of interest into gene 3 or gene 8 which code for the attachment protein and major coat protein of the phage, respectively. Those skilled in the art will recognize that various difficulties are inherent in performing this with a vast mixture of cDNAs of unknown sequence and that the most practical way to obtain functional display of cDNA products would consist of fusing the cDNAs through their 5'-end. Indeed, cDNA libraries of sufficient size may contain several cDNAs which derive from the same mRNA but which are 5'-terminally truncated at various positions such that some of them may be expressed as fusion products. A strategy along this line, which relies on the ability of the leucine zippers Jun and Fos to form heterodimers was recently described. See, Cramer, R. and Suter, M., Gene, 137:69-75 (1993).

[0170] We have found a novel alternative and direct way to covalently link cDNA gene products to the phage surface; the finding is based on the observation that proteins fused to the C-terminus of phage coat protein 6 can be functionally displayed. This observation has led to the development of a phagemid system as described herein which allows the expression of functionally displayed cDNA products, which in turn permits the affinity-selection of phage particles which contain the cDNA required for the production of the displayed cDNA product. This system provides the basis for the isolation of cDNAs which encode a protein of the present invention. Once isolated, recombinant cDNA molecules containing such cDNA can be used for expression of the proteins of the present invention in other expression systems. The recombinant cDNA molecules made in this way are considered to be within the scope of the present invention.

[0171] Recombinant cDNA molecules of the present invention are isolated by preparing a cDNA library from a natural source (as for example, a nematode such as a hookworm), ligating this cDNA library into appropriate phagemid vectors, transforming host cells with these vectors containing the cDNAs, culturing the host cells, infecting the transformed cells with an appropriate helper phage, separating phage from the host cell culture, separating phage expressing a protein of the present invention on its surface, isolating these phage, and isolating a recombinant cDNA molecule from such phage.

[0172] The phagemid vectors are constructed using the pUC119 expression vector described by Vieira, J. and Messing, J., Methods in Enzymology, 153:3-11 (1987). The filamentous phage gene 6 encoding a surface protein of the phage is modified on its 5' and 3' ends by the addition of HindIII and SfiI restriction sites, respectively, by use of three forward primers and one backward primer using PCR. This results in three DNA fragments which are further modified by addition to their 3' ends of NotI and BamHI restriction sites by PCR. After separate digestion of the three DNA fragments with HindIII and BamHI, the three DNA fragments are ligated into the pUC119 to give pDONG61, pDONG62 and pDONG63 expression vectors. These vectors permit the insertion of cDNA as SfiI-NotI fragments into them.

[0173] cDNA libraries are prepared from natural sources, such as nematodes, as described in Examples 2, 9, and 13. Preferred nematodes from which to make such libraries include the intestinal nematodes such as *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus* and *Heligmosomoides polygyrus*.

[0174] A cDNA library as SfiI-NotI fragments may be directly directionally ligated into the phagemid vectors pDONG61, pDONG62 and pDONG63. Alternatively, a cDNA library which has been ligated into the lambda gt11 phage vector as described in Example 2 can be recovered by PCR, followed by isolation with electrophoresis and then directional ligation into these vectors. In the latter approach, preferred conditions for PCR use Taq polymerase; the primers, lambda gt11 primer #1218 having the sequence GGTGGCGACG ACTCCTGGAG CCG (New England Biolabs, Beverly, MA, USA) [SEQ. ID. NO. 96] and the oligo(dT)-NotI primer having the sequence, AATTCGCGGC CGC(T)₁₅, (Promega Corp.) [SEQ. ID. NO. 95]; and 20 temperature cycles of 1 minute at 95°C, 1 minute at 50°C, and 3 minutes at 72°C, followed by 10 minutes at 65°C.

[0175] Host cells are transformed with the pDONG expression vectors containing a cDNA library. Preferred host cells include *E. coli* strains, with strain TG1 being especially preferred. Preferred methods for the transformation of *E. coli* host cells include electroporation.

[0176] The transformed cells are cultured at 37°C in LB medium supplemented with 1% glucose and 100 micrograms/ml carbenicillin until the optical absorbance at 600 nm reaches the value of 0.5 and then are infected with VCSM13 helper phage (Stratagene) at a multiplicity of infection (moi) of 20.

[0177] The phage are separated from the culture by centrifugation, then are purified by precipitations with polyethylene glycol/sodium chloride.

[0178] The phage which express a NAP of the present invention on their surface are isolated by taking advantage of the ability of the NAP to bind to a target protein involved in blood coagulation, for example, Factor Xa.

[0179] Preferred methods of isolating such phage include a method comprising the steps of:

- (1) combining a solution of factor Xa labelled to biotin with a solution of such phage;
- (2) incubating this mixture;
- (3) contacting a solid phase labelled with streptavidin with this mixture;
- (4) incubating the solid phase with the mixture;
- (5) removing the solid phase from the mixture and contacting the solid phase with buffer to remove unbound phage;
- (6) contacting the solid phase with a second buffer to remove the bound phage from the solid phase;

- (7) isolating such phage;
- (8) transforming host cells with such phage;
- (9) culturing the transformed host cells;
- (10) infecting transformed host cells with VCSM13 helper phage;
- (11) isolating the phage from the host cell culture; and
- (12) repeating steps (1) to (11) four more times.

[0180] An especially preferred method of isolating such phage include the method as detailed in Example 10.

[0181] Single-stranded DNA was prepared from the isolated phages and their inserts 3' to the filamentous phage gene 6 sequenced.

[0182] Figure 9 depicts the recombinant cDNA molecule, AcaNAPc2, isolated by the phage display method. The deduced amino acid sequence of the protein of the present invention encoded by AcaNAPc2 is also shown in this figure.

(C) Preparation of Recombinant NAP.

[0183] The recombinant cDNA molecules of the present invention when isolated as disclosed are used to obtain expression of the NAPs of the present invention. Generally, a recombinant cDNA molecule of the present invention is incorporated into an expression vector, this expression vector is introduced into an appropriate host cell, the host cell is cultured, and the expressed protein is isolated.

[0184] Expression vectors are DNA sequences that are required for the transcription of cloned copies of genes and translation of their mRNAs in an appropriate host. These vectors can express either procaryotic or eucaryotic genes in a variety of cells such as bacteria, yeast, mammalian, plant and insect cells. Proteins may also be expressed in a number of virus systems.

[0185] Suitably constructed expression vectors contain an origin of replication for autonomous replication in host cells, or are capable of integrating into the host cell chromosomes. Such vectors will also contain selective markers, a limited number of useful restriction enzyme sites, a high copy number, and strong promoters. Promoters are DNA sequences that direct RNA polymerase to bind to DNA and initiate RNA synthesis; strong promoters cause such initiation at high frequency. The preferred expression vectors of the present invention are operatively linked to a recombinant cDNA molecule of the present invention, i.e., the vectors are capable directing both replication of the attached recombinant cDNA molecule and expression of the protein encoded by the recombinant cDNA molecule. Expression vectors may include, but are not limited to cloning vectors, modified cloning vectors and specifically designed plasmids or viruses.

[0186] Suitable host cells for expression of the proteins of the present invention include bacteria, yeast, mammalian, plant and insect cells. With each type of cell and species therein certain expression vectors are appropriate as will be disclosed below.

[0187] Procaryotes may be used for expression of the proteins of the present invention. Suitable bacteria host cells include the various strains of *E. coli*, *Bacillus subtilis*, and various species of *Pseudomonas*. In these systems, plasmid vectors which contain replication sites and control sequences derived from species compatible with the host are used. Suitable vectors for *E. coli* are derivatives of pBR322, a plasmid derived from an *E. coli* species by Bolivar et al., *Gene*, 2:95 (1977). Common procaryotic control sequences, which are defined herein to include promoters for transcription, initiation, optionally with an operator, along with ribosome binding site sequences, include the beta-lactamase and lactose promoter systems (Chang et al., *Nature*, 198:1056 (1977)), the tryptophan promoter system (Goeddel et al., *Nucleic Acids Res.*, 8:4057 (1980)) and the lambda-derived- P_L promoter and N-gene ribosome binding site (Shimatake et al., *Nature*, 292:128 (1981)). However, any available promoter system compatible with procaryotes can be used. Preferred procaryote expression systems include *E. coli* and their expression vectors.

[0188] Eucaryotes may be used for expression of the proteins of the present invention. Eucaryotes are usually represented by the yeast and mammalian cells. Suitable yeast host cells include *Saccharomyces cerevisiae* and *Pichia pastoris*. Suitable mammalian host cells include COS and CHO (chinese hamster ovary) cells.

[0189] Expression vectors for the eucaryotes are comprised of promoters derived from appropriate eucaryotic genes. Suitable promoters for yeast cell expression vectors include promoters for synthesis of glycolytic enzymes, including those for 3-phosphoglycerate kinase gene in *Saccharomyces cerevisiae* (Hitzman et al., *J. Biol. Chem.*, 255:2073 (1980)) and those for the metabolism of methanol as the alcohol oxidase gene in *Pichia pastoris* (Stroman et al., U.S. Patent Nos. 4,808,537 and 4,855,231). Other suitable promoters include those from the enolase gene (Holland, M.J. et al., *J. Biol. Chem.*, 256:1385 (1981)) or the Leu2 gene obtained from YEp13 (Broach, J. et al., *Gene*, 8:121 (1978)).

[0190] Preferred yeast expression systems include *Pichia pastoris* and their expression vectors. NAP-encoding cDNAs expressed in *Pichia pastoris* optionally may be mutated to encode a NAP protein that incorporates a proline residue at the C-terminus. In some instances the NAP protein is expressed at a higher level and can be more resistant to unwanted proteolysis. One such cDNA, and its expression in *Pichia pastoris*, is described in Example 17.

[0191] Suitable promoters for mammalian cell expression vectors include the early and late promoters from SV40

(Fiers, et al., Nature, 273:113 (1978)) or other viral promoters such as those derived from polyoma, adenovirus II, bovine papilloma virus or avian sarcoma viruses. Suitable viral and mammalian enhancers may also be incorporated into these expression vectors.

[0192] Suitable promoters for plant cell expression vectors include the nopaline synthesis promoter described by Depicker, A. et al., Mol. Appl. Gen., 1:561 (1978).

[0193] Suitable promoters for insect cell expression vectors include modified versions of the system described by Smith et al., U.S. Patent No. 4,745,051. The expression vector comprises a baculovirus polyhedrin promoter under whose control a cDNA molecule encoding a protein can be placed.

[0194] Host cells are transformed by introduction of expression vectors of the present invention into them. Transformation is done using standard techniques appropriate for each type of cell. The calcium treatment employing calcium chloride described in Cohen, S.N., Proc. Natl. Acad. Sci. USA, 69:2110 (1972), or the RbCl method described in Maniatis et al., Molecular Cloning: A Laboratory Manual, p. 254, Cold Spring Harbor Press (1982) is used for procaryotes or other cells which contain substantial cell wall barriers. The transformation of yeast is carried out as described in Van Solingen, P. et al., J. Bacter., 130:946 (1977) and Hsiao, C.L. et al., Proc. Natl. Acad. Sci. USA, 76:3829 (1979). Mammalian cells without much cell wall are transformed using the calcium phosphate procedure of Graham and van der Eb, Virology, 52:546 (1978). Plant cells are transformed by infection with *Agrobacterium tumefaciens* as described in Shaw, C. et al, Gene, 23:315 (1983). Preferred methods of transforming *E. coli* and *Pichia pastoris* with expression vectors include electroporation.

[0195] Transformed host cells are cultured under conditions, such as type of media, temperature, oxygen content, fluid motion, etc., well known in the biological arts.

[0196] The recombinant proteins of the present invention are isolated from the host cell or media by standard methods well known in the biochemical arts, which include the use of chromatography methods. Preferred methods of purification would include sequential chromatography of an extract through columns containing Poros20 HQ anion-ion exchange matrix or Poros20 HS cation exchange matrix, Superdex30 gel filtration matrix and a C18 reverse-phase matrix. The fractions collected after one such chromatography column may be selected by their ability to increase the clotting time of human plasma, as measured by the PT and aPTT assays, or their ability to inhibit factor Xa amidolytic activity as measured in a colorimetric assay, or demonstration of activity in any of the other assays disclosed herein. Examples of preferred methods of purification of a recombinant protein of the present invention are disclosed in Examples 3, 4, 6, 8, 14 and 15.

4. Methods of Using NAP.

[0197] In one aspect, the present invention includes methods of collecting mammalian plasma such that clotting of said plasma is inhibited, comprising adding to a blood collection tube an amount of a protein of the present invention sufficient to inhibit the formation of a clot when mammalian blood is drawn into the tube, adding mammalian blood to said tube, separating the red blood cells from the mammalian plasma, and collecting the mammalian plasma.

[0198] Blood collection tubes include stoppered test tubes having a vacuum therein as a means to draw blood obtained by venipuncture into the tubes. Preferred test tubes include those which are made of borosilicate glass, and have the dimensions of, for example, 10.25 x 47 mm, 10.25 x 50 mm, 10.25 x 64 mm, 10.25 x 82 mm, 13 x 75 mm, 13 x 100 mm, 16 x 75 mm, 16 x 100 mm or 16 x 125 mm. Preferred stoppers include those which can be easily punctured by a blood collection needle and which when placed onto the test tube provide a seal sufficient to prevent leaking of air into the tube.

[0199] The proteins of the present invention are added to the blood collection tubes in a variety of forms well known in the art, such as a liquid composition thereof, a solid composition thereof, or a liquid composition which is lyophilized to a solid in the tube. The amount added to such tubes is that amount sufficient to inhibit the formation of a clot when mammalian blood is drawn into the tube. The proteins of the present invention are added to blood collection tubes in such amounts that, when combined with 2 to 10 ml of mammalian blood, the concentration of such proteins will be sufficient to inhibit clot formation. Typically, this effective concentration will be about 1 to 10,000 nM, with 10 to 1000 nM being preferred.

Alternatively, the proteins of the present invention may be added to such tubes in combination with other clot-inhibiting additives, such as heparin salts, EDTA salts, citrate salts or oxalate salts.

[0200] After mammalian blood is drawn into a blood collection tube containing either a protein of the present invention or the same in combination with other clot-inhibiting additives, the red blood cells are separated from the mammalian plasma by centrifugation. The centrifugation is performed at g-forces, temperatures and times well known in the medical arts. Typical conditions for separating plasma from red blood cells include centrifugation at a centrifugal force of about 100xg to about 1500xg, at a temperatures of about 5 to about 25°C, and for a time of about 10 to about 60 minutes.

[0201] The mammalian plasma may be collected by pouring it off into a separate container, by withdrawing it into a pipette or by other means well known to those skilled in the medical arts.

[0202] In another aspect, the present invention includes methods for preventing or inhibiting thrombosis (clot formation)

or blood coagulation in a mammal, comprising administering to said mammal a therapeutically effective amount of a protein or a pharmaceutical composition of the present invention.

[0203] The proteins or pharmaceutical compositions of the present invention are administered *in vivo*, ordinarily in a mammal, preferably in a human. In employing them *in vivo*, the proteins or pharmaceutical compositions can be administered to a mammal in a variety of ways, including orally, parenterally, intravenously, subcutaneously, intramuscularly, colonically, rectally, nasally or intraperitoneally, employing a variety of dosage forms. Administration is preferably parenteral, such as intravenous on a daily basis. Alternatively, administration is preferably oral, such as by tablets, capsules or elixers taken on a daily basis.

[0204] In practicing the methods of the present invention, the proteins or pharmaceutical compositions of the present invention are administered alone or in combination with one another, or in combination with other therapeutic or *in vivo* diagnostic agents.

[0205] As is apparent to one skilled in the medical art, a therapeutically effective amount of the proteins or pharmaceutical compositions of the present invention will vary depending upon the age, weight and mammalian species treated, the particular proteins employed, the particular mode of administration and the desired affects and the therapeutic indication. Because these factors and their relationship to determining this amount are well known in the medical arts, the determination of therapeutically effective dosage levels, the amount necessary to achieve the desired result of preventing thrombosis, will be within the ambit of one skilled in these arts.

[0206] Typically, administration of the proteins or pharmaceutical composition of the present invention is commenced at lower dosage levels, with dosage levels being increased until the desired effect of preventing *in vivo* thrombosis is achieved which would define a therapeutically effective amount. For the proteins of the present invention, alone or as part of a pharmaceutical composition, such doses are between about 0.01 mg/kg and 100 mg/kg body weight, preferably between about 0.01 and 10 mg/kg, body weight.

5. Utility.

[0207] Proteins of the present invention when made and selected as disclosed are useful as potent inhibitors of blood coagulation *in vitro* and *in vivo*. As such, these proteins are useful as *in vitro* diagnostic reagents to prevent the clotting of blood and are also useful as *in vivo* pharmaceutical agents to prevent or inhibit thrombosis or blood coagulation in mammals.

[0208] The proteins of the present invention are useful as *in vitro* diagnostic reagents for inhibiting clotting in blood drawing tubes. The use of stoppered test tubes having a vacuum therein as a means to draw blood obtained by venipuncture into the tube is well known in the medical arts. Kasten, B.L., "Specimen Collection", Laboratory Test Handbook, 2nd Edition, Lexi-Comp Inc., Cleveland pp. 16-17 (Edits. Jacobs, D.S. et al. 1990). Such vacuum tubes may be free of clot-inhibiting additives, in which case, they are useful for the isolation of mammalian serum from the blood. They may alternatively contain clot-inhibiting additives (such as heparin salts, EDTA salts, citrate salts or oxalate salts), in which case, they are useful for the isolation of mammalian plasma from the blood. The proteins of the present invention are potent inhibitors of blood clotting and as such, can be incorporated into blood collection tubes to prevent clotting of the mammalian blood drawn into them.

[0209] The proteins of the present invention are used alone, in combination of other proteins of the present invention, or in combination with other known inhibitors of clotting, in the blood collection tubes, for example, with heparin salts, EDTA salts, citrate salts or oxalate salts.

[0210] The amount to be added to such tubes, or effective amount, is that amount sufficient to inhibit the formation of a blood clot when mammalian blood is drawn into the tube. The proteins of the present invention are added to blood collection tubes in such amounts that, when combined with 2 to 10 ml of mammalian blood, the concentration of such proteins will be sufficient to inhibit the formation of blood clots. Typically, this effective amount is that required to give a final concentration in the blood of about 1 to 10,000 nM, with 10 to 1000 nM being preferred.

[0211] The proteins of the present invention may also be used to prepare diagnostic compositions. In one embodiment, diagnostic compositions are prepared by dissolving the proteins of the present invention into diagnostically acceptable carriers, which carriers include phosphate buffered saline (0.01 M sodium phosphate + 0.15 M sodium chloride, pH 7.2 or Tris buffered saline (0.05 M Tris-HCl + 0.15 M sodium chloride, pH 8.0). In another embodiment, the proteins of the present invention may be blended with other solid diagnostically acceptable carriers by methods well known in the art to provide solid diagnostic compositions. These carriers include buffer salts.

[0212] The addition of the proteins of the present invention to blood collection tubes may be accomplished by methods well known in the art, which methods include introduction of a liquid diagnostic composition thereof, a solid diagnostic composition thereof, or a liquid diagnostic composition which is lyophilized in such tubes to a solid plug of a solid diagnostic composition.

[0213] The use of blood collection tubes containing the diagnostic compositions of the present invention comprises contacting a effective amount of such diagnostic composition with mammalian blood drawn into the tube. Typically, when

a sample of 2 to 10 ml of mammalian blood is drawn into a blood collection tube and contacted with such diagnostic composition therein; the effective amount to be used will include those concentrations of the proteins formulated as a diagnostic composition which in the blood sample are sufficient to inhibit the formation of blood clots. Preferred effective concentrations would be about 1 to 10,000 nM, with 10 to 1000 nM being especially preferred.

[0214] According to an alternate aspect of our invention, the proteins of the present invention are also useful as pharmaceutical agents for preventing or inhibiting thrombosis or blood coagulation in a mammal. This prevention or inhibition of thrombosis or blood coagulation includes preventing or inhibiting abnormal thrombosis.

[0215] Conditions characterized by abnormal thrombosis are well known in the medical arts and include those involving the arterial and venous vasculature of mammals. With respect to the coronary arterial vasculature, abnormal thrombosis (thrombus formation) characterizes the rupture of an established atherosclerotic plaque which is the major cause of acute myocardial infarction and unstable angina, and also characterizes the occlusive coronary thrombus formation resulting from either thrombolytic therapy or percutaneous transluminal coronary angioplasty (PTCA). With respect to the venous vasculature, abnormal thrombosis characterizes the condition observed in patients undergoing major surgery in the lower extremities or the abdominal area who often suffer from thrombus formation in the venous vasculature resulting in reduced blood flow to the affected extremity and a predisposition for pulmonary embolism. Abnormal thrombosis further characterizes disseminated intravascular coagulopathy which commonly occurs within both vascular systems during septic shock, certain viral infections and cancer, a condition wherein there is rapid consumption of coagulation factors and systemic coagulation which results in the formation of life-threatening thrombi occurring throughout the microvasculature leading to widespread organ failure.

[0216] The NAP proteins of the present invention also are useful immunogens against which antibodies are raised. Antibodies, both monoclonal and polyclonal, directed to a NAP are useful for diagnostic purposes and for the identification of concentration levels of NAP in various biological fluids. Immunoassay utilizing these antibodies may be used as a diagnostic test, such as to detect infection of a mammalian host by a parasitic worm or to detect NAP from a parasitic worm in a tissue of the mammalian host. Also, such immunoassays may be used in the detection and isolation of NAP from tissue homogenates, cloned cells and the like.

[0217] NAP can be used, with suitable adjuvants, as a vaccine against parasitic worm infections in mammals. Immunization with NAP vaccine may be used in both the prophylaxis and therapy of parasitic infections. Disease conditions caused by parasitic worms may be treated by administering to an animal infected with these parasites anti-NAP antibody.

[0218] NAP proteins of this invention having serine protease inhibitory activity also are useful in conditions or assays where the inhibition of serine protease is desired. For example, NAP proteins that inhibit the serine protease trypsin or elastase are useful for treatment of acute pancreatitis or acute inflammatory response mediated by leukocytes, respectively.

[0219] The recombinant cDNA molecules encoding the proteins of the present invention are useful in one aspect for isolating other recombinant cDNA molecules which also encode the proteins of the present invention. In another aspect, they are useful for expression of the proteins of the present invention in host cells.

[0220] The nucleotide probes of the present invention are useful to identify and isolate nucleic acid encoding NAPs from nematodes or other organisms. Additionally, the nucleotide probes are useful diagnostic reagents to detect the presence of nematode-encoding nucleic acid in a sample, such as a bodily fluid or tissue from a mammal suspected of infection by nematode. The probes can be used directly, with appropriate label for detection, to detect the presence of nematode nucleic acid, or can be used in a more indirect manner, such as in a PCR-type reaction, to amplify nematode nucleic acid that may be present in the sample for detection. The conditions of such methods and diagnostic assays are readily available in the art.

[0221] To assist in understanding, the present invention will now be further illustrated by the following examples.

Examples.

Example 1

Isolation of Novel Anticoagulant Protein (NAP) from *Ancylostoma caninum*.

(A) Preparation of the *Ancylostoma caninum* Lysate.

[0222] Frozen canine hookworms, *Ancylostoma caninum*, were obtained from Antibody Systems (Bedford, TX). Hookworms were stored at -80°C until used for homogenate.

[0223] Hookworms were frozen in liquid nitrogen and ground in a mortar followed by a homogenization on ice in homogenization buffer using a PotterS homogenizer with a teflon piston (B.Braun Melsungen AG, Germany). The homogenization buffer contained: 0.02 M Tris-HCl pH 7.4, 0.05 M NaCl, 0.001 M MgCl₂, 0.001 M CaCl₂, 1.0 x 10⁻⁵ M E-64 protease inhibitor (Boehringer Mannheim, Germany), 1.0 x 10⁻⁵ M pepstatin A (isovaleryl-Val-Val-4-amiho-3-hy-

droxy-6-methyl-heptanoyl-Ala-4-amino-3-hydroxy-6-methylheptanoic acid, ICN Biomedicals, CA), 1.0×10^{-5} M chymostatin (Boehringer), 1.0×10^{-5} M leupeptin (ICN), 5×10^{-5} M AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride, ICN), and 5% (v/v) glycerol. Approximately 4 ml of homogenization buffer was used to homogenize each gram of frozen worms (approximately 500 worms). Insoluble material was pelleted by two sequential centrifugation steps: 19,000 $\times g_{\max}$ at 4°C for 30 minutes followed by 110,000 $\times g_{\max}$ at 4°C for 40 minutes. The supernatant solution was clarified by passage through a 0.45 micrometer cellulose acetate filter (Corning, NY) to give *Ancylostoma caninum* lysate.

(B) Concanavalin A Sepharose Chromatography.

[0224] *Ancylostoma caninum* lysate (100 ml) was adsorbed onto 22 ml of Concanavalin A Sepharose (Pharmacia, Sweden) pre-equilibrated with Con A buffer (0.02 M Tris-HCl, pH 7.4, 1 M NaCl, 0.002 M CaCl_2) by loading it onto a 1.6 \times 11 cm column of this gel at a flow rate of 3 ml/minute (90 cm/hour). The column was at ambient temperature while the reservoir of lysate was maintained at ice bath temperature throughout the procedure. The column was subsequently washed with 2 column volumes of Con A buffer. The column flow-through and wash were collected (approximately 150 ml) and stored at -80°C until further processing was done.

(C) Anion-Exchange Chromatography.

[0225] The flow-through and wash of the Concanavalin A Sepharose column was buffered by adding solid sodium acetate to a final concentration of 12.5 mM. The conductivity was reduced by dilution with milliQ water and the pH was adjusted with HCl to pH 5.3. The precipitate formed during pH adjustment was pelleted by centrifugation 15,000 $\times g_{\max}$ at 4°C for 15 minutes. The supernatant solution was clarified by passage through a 0.2 micrometer cellulose acetate filter (Corning, NY).

[0226] This clarified solution (total volume approximately 600 ml) was loaded on to a Poros20 HQ (Perseptive Biosystems, MA) 1 \times 2 cm column pre-equilibrated with Anion buffer (0.05 M Na acetate, pH 5.3, 0.1 M NaCl) at a flow rate of 10 ml/minute (800 cm/hour). The column and the solution added were at ambient temperature throughout this purification step. The column was subsequently washed with 10 column volumes of Anion buffer.

[0227] Material that had inhibitory activity, detected following the procedure below, in the factor Xa amidolytic assay was eluted with Cation buffer containing 0.55 M NaCl at a flow rate of 5 ml/minute (400 cm/hour).

[0228] A sample of solution was tested in a factor Xa amidolytic assay as follows. Reaction mixtures (150 microliters) were prepared in 96-well plates containing factor Xa and various dilutions of the sample in assay buffer (100 mM Tris-HCl pH 7.4; 140 mM NaCl; 0.1% BSA). Human factor X was purchased from Enzyme Research Laboratories (South Bend, IN, USA) and activated with Russell's Viper venom using the procedure of Bock, P. E., Craig, P. A., Olson, S. T., and Singh P., Arch. Biochem. Biophys., 273: 375-388 (1989). Following a 30 minute incubation at ambient temperature, the enzymatic reactions were initiated by addition of 50 microliters of a 1 mM substrate solution in water (N-alpha-benzoyloxycarbonyl-D-arginyl-L-glycyl-L-arginine p-nitroanilide-dihydrochloride; S-2765; Chromogenix, Mölndal, Sweden) to yield final concentrations of 0.2 nM factor Xa and 0.25 mM S-2765. Substrate hydrolysis was monitored by continuously measuring absorbance at 405 nm using a Vmax kinetic plate reader (Molecular Devices, Menlo Park, CA, USA).

(D) Heat Treatment.

[0229] Half of the 0.55 M NaCl elution pool (3 ml) from anion-exchange chromatography was neutralized by adding 1 M Tris-HCl, pH 7.5 to a final concentration of 50 mM, incubated for 5 minutes at 90°C in a glass tube and subsequently cooled rapidly on ice. Insoluble material was pelleted by centrifugation 19,000 $\times g_{\max}$ at 4°C for 20 minutes. The supernatant contained material which inhibited factor Xa in the factor Xa amidolytic assay. About 89% of the factor Xa inhibitory activity was recovered in the supernatant, after this heat treatment after accounting for dilution.

(E) Molecular Sieve Chromatography using Superdex30 (alternative for the heat treatment step).

[0230] Half of the 0.55 M NaCl elution pool (3 ml) from anion-exchange chromatography was loaded on a Superdex30 PG (Pharmacia, Sweden) 1.6 \times 66 cm column pre-equilibrated with 0.01M sodium phosphate, pH 7.4, 0.15 M NaCl at 24°C. The chromatography was conducted at a flow rate of 2 ml/minute. The factor Xa inhibitory activity (determined in the factor Xa amidolytic assay) eluted 56-64 ml into the run (K_{av} of 0.207). This elution volume would be expected for a globular protein with a molecular mass of 14,000 daltons.

(F) Reverse Phase Chromatography.

[0231] Hookworm lysate which was fractionated by chromatography on Concanavalin A Sepharose, anion-exchange

and Superdex30 (or with the alternative heat treatment step) was loaded on to a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia, CA) which was then developed with a linear gradient of 10-35% acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/minute with a rate of 0.625 % change in acetonitrile/minute. FXa inhibitory activity (determined in the factor Xa amidolytic assay) eluted at approximately 30% acetonitrile. The HPLC runs were performed on a Vista 5500 connected with a Polychrom 9600 detector set at 215 nm (Varian, CA). Detector signals were integrated on a 4290 integrator obtained from the same company. Factor Xa inhibitory activity containing fractions were vacuum dried and then redissolved in PBS (0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl).

[0232] These fractions were pooled and then loaded on to a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia, CA) which was developed with a linear gradient of 10-35% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/minute with a slower rate of 0.4% change in acetonitrile/minute. Factor Xa inhibitory activity containing fractions were pooled and subsequently vacuum dried.

(G) Molecular Weight Determination of NAP from

Ancylostoma caninum.

[0233] The estimated mass for NAP isolated as described in this example was determined using electrospray ionisation mass spectrometry.

[0234] A vacuum-dried pellet of NAP was dissolved in 50% (v/v) acetonitrile, 1% (v/v) formic acid. Mass analysis was performed using a VG Bio-Q (Fisons Instruments, Manchester UK).

[0235] The NAP sample was pumped through a capillary and at its tip a high voltage of 4 kV was applied. Under the influence of the high electric field, the sample was sprayed out in droplets containing the protein molecules. Aided by the drying effect of a neutral gas (N₂) at 60°C, the droplets were further reduced in size until all the solvent had been evaporated and only the protein species remained in the gaseous form. A population of protein species arose which differed from each other in one charge. With a quadrupole analyzer, the different Da/e (mass/charge)-values were detected. Calibration of the instrument was accomplished using Horse Heart Myoglobin (Sigma, Missouri).

[0236] The estimated mass of NAP isolated as described in sections A, B, C, D, and F of this example is 8734.60 daltons. The estimated mass of native NAP isolated as described in sections A, B, C, E, and F is 8735.67 daltons.

(H) Amino Acid Sequencing of NAP from *Ancylostoma caninum*.

[0237] Amino acid determination was performed on a 476-A Protein/Peptide Sequencer with On Board Microgradient PTH Analyzer and Model 610A Data Analysis System (Applied Biosystems, CA). Quantification of the residues was performed by on-line analysis on the system computer (Applied Biosystems, CA); residue assignment was performed by visual analysis of the HPLC chromatograms. The first twenty amino acids of the amino-terminus of native NAP were determined to be:

**Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp Asp
Cys Gly Thr Gln Lys Pro [SEQ. ID. NO. 97].**

The cysteine residues were not directly detected in this analysis because the sample was not reduced and subsequently alkylated. Cysteines were assigned to the positions where no specific amino acid was identified.

Example 2

Cloning and Sequencing of NAP from *Ancylostoma caninum*.

(A) Preparation Of Hybridization Probe.

[0238] Full-length cDNA clones encoding NAP were isolated by screening a cDNA library, prepared from the mRNA isolated from the nematode, *Ancylostoma caninum*, with a radiolabeled degenerate oligonucleotide whose sequence was based on the first eleven amino acids of the amino-terminus of NAP from *A. caninum*:

Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp [SEQ. ID. NO. 93]. The 33-mer oligonucleotide hybridization probe, designated YG99, had the following sequence:

AAR GCi TAY CCi GAR TGY GGi GAR AAY GAR TGG [SEQ. ID. NO. 94]

where "R" refers to A or G; "Y" refers to T or C; and "i" refers to inosine. YG99 was radiolabeled by enzymatic 5'-end phosphorylation (5'-end labeling kit; Amersham, Buckinghamshire, England) using gamma-³²P-ATP (specific activity >7000Ci/mmol; ICN, Costa Mesa, CA, USA) and subsequently passed over a NAPTM10 column (Pharmacia, Uppsala, Sweden).

(B) Preparation of cDNA Library.

[0239] A cDNA library was constructed using described procedures (Promega Protocols and Applications Guide 2nd Ed.; Promega Corp., Madison, WI, USA).

[0240] Adult hookworms, *Ancylostoma caninum*, were purchased from Antibody Systems (Bedford, TX). Poly(A⁺) RNA was prepared using the QuickPrep mRNA Purification Kit (Pharmacia). About 3 micrograms of mRNA were reverse transcribed using an oligo(dT)-NotI primer/adaptor, AATTCGCGGCCGC(T)₁₅ [SEQ. ID. NO. 95], (Promega Corp.) and AMV (Avian Myeloblastosis Virus) reverse transcriptase (Boehringer, Mannheim, Germany). The enzymes used for double-stranded cDNA synthesis were the following: *E. coli* DNA polymerase I and RNaseH from Life Technologies (Gaithersburg, MD, USA) and T4 DNA polymerase from Pharmacia.

[0241] EcoRI linkers (pCGGAATTCCG) [SEQ. ID. NO. 98] were ligated onto the obtained cDNA after treatment with EcoRI methylase (RiboClone EcoRI Linker Ligation System; Promega).

[0242] The cDNAs were digested with NotI and EcoRI, passed over a 1.5% agarose gel (all sizeable material was eluted using the GeneClean protocol, BIO101 Inc., La Jolla, CA), and unidirectionally ligated into the EcoRI-NotI arms of the lambda gt11 Sfi-NotI vector (Promega). After *in vitro* packaging (GigapackII-Gold, Stratagene, La Jolla, CA) recombinant phage were obtained by infecting strain Y1090 (Promega).

[0243] The usefulness of the cDNA library was demonstrated by PCR analysis (Taq polymerase from Boehringer; 30 temperature cycles: 1 minute at 95°C; 1 minute at 50°C; 3 minutes at 72°C) of a number of randomly picked clones using the lambda gt11 primer #1218, having the sequence, GGTGGCGACG ACTCCTGGAG CCCG (New England Biolabs, Beverly, MA, USA) [SEQ. ID. NO. 96]; targeting sequences located upstream of the cDNA insert) in combination with the above-mentioned oligo(dT)-NotI primer/adaptor; the majority of the clones was found to contain cDNA inserts of variable size.

(C) Identification of Clones.

[0244] Approximately 1x10⁶ cDNA clones (duplicate plaque-lift filters were prepared using HybondTM-N; Amersham) were screened with the radiolabeled YG99 oligonucleotide using the following pre-hybridization and hybridization conditions: 5x SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate), 5x Denhardt's solution, 0.5% SDS, 100 micrograms/ml sonicated fish sperm DNA (Boehringer), overnight at 42°C. The filters were washed 4 times in 2x SSC, 0.1% SDS at 37°C. After exposure (about 72 hours) to X-ray film, a total of between 350 and 500 hybridization spots were identified.

[0245] Twenty-four positive clones, designated NAP1 through NAP24, were subjected to a second hybridization round at lower plaque-density; except for NAP24, single plaques containing a homogeneous population of lambda phage were identified. The retained clones were analyzed by PCR amplifications (Taq polymerase from Boehringer; 30 temperature cycles: 1 minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C) using the oligo(dT)-NotI primer (AATTCGCGGCCGC(T)₁₅) [SEQ. ID. NO. 95] in combination with either (i) YG99 or (ii) the lambda gt11 primer #1218. The majority of the clones (20 out of 23) yielded a fragment of about 400 bp when the oligo(dT)-NotI/YG99 primer set was used and a fragment of about 520 bp when the oligo(dT)-NotI/#1218 primer couple was used. Nineteen such possibly full-length clones were further characterized.

[0246] The cDNA inserts of five clones were subcloned as SfiI-NotI fragments on both pGEM-5Zf(-) and pGEM-9Zf(-) (Promega). Because the SfiI sites of lambda gt11 and pGEM-5Zf(-) are not compatible with one another, the cloning on this vector required the use of a small adaptor fragment obtained after annealing the following two 5'-end phosphorylated oligonucleotides: pTGGCCTAGCG TCAGGAGT [SEQ. ID. NO. 99] and pCCTGACGCTA GGCCATGG [SEQ. ID. NO. 100]. Following preparation of single-stranded DNA, the sequences of these cDNAs were determined with the dideoxy chain termination method using primer #1233 having the sequence, AGCGGATAAC AATTTACAC AGGA (New England Biolabs) [SEQ. ID. NO. 101]. All five clones were found to be full-length including a complete secretion signal. Clones NAP5, NAP7 and NAP22 were found to have an identical coding region. Clones NAP6 and NAP11 are also identical but differ from the NAP5 type of coding region. Figure 1 depicts the nucleotide sequence of the NAP5 gene and Figure 2 depicts the amino acid sequence of the protein encoded, AcaNAP5. Likewise, Figure 3 depicts the nucleotide sequence of the NAP6 [SEQ. ID. NO. 5] gene and Figure 4 depicts the amino acid sequence of the protein encoded, AcaNAP6 [SEQ. ID. NO. 6].

[0247] Fourteen other possibly full-length clones were subjected to a restriction analysis. The above mentioned 400 bp PCR product obtained with the YG99/oligo(dT)-NotI primer couple, was digested with four different enzymes capable of discriminating between a NAPS- and NAP6-type of clone: Sau96I, Sau3AI, DdeI, and HpaII. The results were consistent

with 10 out of the 14 clones being NAP5-type (e.g. NAP4, NAP8, NAP9, NAP15, NAP16, NAP17, NAP18, NAP20, NAP21, and NAP23) while the remaining four were NAP6-type (e.g. NAP10, NAP12, NAP14, and NAP19).

[0248] These clones were renamed to reflect origin from *Ancylostoma caninum* by placing the letters Aca immediately before the NAP designation. For example, NAP5 became AcaNAP5, NAP6 became AcaNAP6 and so forth.

Example 3

Production and Purification Of Recombinant AcaNAP5 In *P. pastoris*.

(A) Expression Vector Construction.

[0249] The *Pichia pastoris* yeast expression system, including the *E. coli*/*P. pastoris* shuttle vector, pHILD2, has been described in a number of United States Patents. See, e.g., U.S. Patent Nos. 5,330,901; 5,268,273; 5,204,261; 5,166,329; 5,135,868; 5,122,465; 5,032,516; 5,004,688; 5,002,876; 4,895,800; 4,885,242; 4,882,279; 4,879,231; 4,857,467; 4,855,231; 4,837,148; 4,818,700; 4,812,405; 4,808,537; 4,777,242; and 4,683,293.

[0250] The pYAM7SP8 vector used to direct expression and secretion of recombinant AcaNAP5 in *P. pastoris* was a derivative of the pHILD2 plasmid (Despreaux, C.W. and Manning, R.F., Gene 131: 35-41 (1993)), having the same general structure. In addition to the transcription and recombination elements of pHILD2 required for expression and chromosomal integration in *P. pastoris* (see Stroman, D.W. et al., U.S. Patent No. 4,855,231), this vector contained a chimeric prepro leader sequence inserted downstream of the alcohol oxidase (AOX1) promoter. The prepro leader consisted of the *P. pastoris* acid phosphatase (PHO1) secretion signal fused to a synthetic 19-amino acid pro-sequence. This pro-sequence was one of the two 19-aa pro-sequences designed by Clements et al., Gene 106: 267-272 (1991) on the basis of the *Saccharomyces cerevisiae* alpha-factor leader sequence. Engineered immediately downstream from the prepro leader sequence was a synthetic multi-cloning site with recognition sequences for the enzymes StuI, SacII, EcoRI, BglII, NotI, XhoI, SpeI and BamHI to facilitate the cloning of foreign genes. NAP as expressed from pYAM7SP8 in *Pichia pastoris* was first translated as a prepro-product and subsequently processed by the host cell to remove the pre- and pro-sequences.

[0251] The structure of this vector is shown in Figure 12. The signal sequence (S) has the nucleic acid sequence: ATG TTC TCT CCA ATT TTG TCC TTG GAA ATT ATT TTA GCT TTG GCT ACT TTG CAA TCT GTC TTC GCT [SEQ. ID. NO. 102]. The pro sequence (P) has the nucleic acid sequence: CAG CCA GGT ATC TCC ACT ACC GTT GGT TCC GCT GCC GAG GGT TCT TTG GAC AAG AGG [SEQ. ID. NO. 103]. The multiple cloning site (MCS) has the nucleic acid sequence: CCT ATC CGC GGA ATT CAG ATC TGA ATG CGG CCG CTC GAG ACT AGT GGA TCC [SEQ. ID. NO. 104].

[0252] The pGEM-9Zf(-) vector (Promega) containing the AcaNAP5 cDNA was used to isolate by amplification ("PCR-rescue") the region encoding the mature AcaNAP5 protein (using Vent polymerase from New England Biolabs, Beverly, MA; 20 temperature cycles: 1 minute at 94°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The following oligonucleotide primers were used:

YG101: GCTCGCTCTA-GAAGCTTCAG-ACATGTATAA-TCTCATGTTG-G [SEQ. ID. NO. 105]

YG103: AAGGCATACC-CGGAGTGTGG-TG [SEQ. ID. NO. 89]

The YG101 primer, targeting C-terminal sequences, contained a non-annealing extension which included XbaI and HindIII restriction sites (underlined).

[0253] Following digestion with XbaI enzyme, the amplification product, having the expected size, was isolated from gel and subsequently enzymatically phosphorylated (T4 polynucleotide kinase from New England Biolabs, Beverly, MA). After heat-inactivation (10 minutes at 70°C) of the kinase, the blunt-ended/XbaI fragment was directionally cloned into the vector pYAM7SP8 for expression purposes. The recipient vector-fragment from pYAM7SP8 was prepared by StuI-SpeI restriction, and purified from agarose gel. The *E. coli* strain, WK6 [Zell, R. and Fritz, H.-J., EMBO J., 6: 1809-1815 (1987)], was transformed with the ligation mixture, and ampicillin resistant clones were selected.

[0254] Based on restriction analysis, a plasmid clone containing an insert of the expected size, designated pYAM7SP-NAP5, was retained for further characterization. Sequence determination of the clone pYAM7SP-NAPS confirmed the precise insertion of the mature AcaNAP5 coding region in fusion with the prepro leader signal, as predicted by the construction scheme, as well as the absence of unwanted mutations in the coding region.

(B) Expression Of Recombinant AcaNAP5 In *P. pastoris*.

[0255] The *Pichia pastoris* strain GTS115 (his4) has been described in Stroman, D.W. et al., U.S. Patent No. 4,855,231. All of the *P. pastoris* manipulations were performed essentially as described in Stroman, D.W. et al., U.S. Patent No.

4,855,231.

[0256] About 1 microgram of pYAM7SP-NAP5 plasmid DNA was electroporated into the strain GTS115 using a standard electroporation protocol. The plasmid was previously linearized by Sall digestion, which theoretically facilitates the targeting and integration of the plasmid into the his4 chromosomal locus.

[0257] The selection of a AcaNAP5 high-expressor strain was performed essentially as described hereinbelow. His⁺ transformants were recovered on MD plates (Yeast Nitrogen Base without amino acids (DIFCO), 13.4 g/l; Biotin, 400 micrograms/L; D-glucose, 20 g/l; agar, 15 g/l). Single colonies (n=60) originating from the electroporation were inoculated into 100 microliters of FM22-glycerol-PTM1 medium in wells of a 96-well plate and were allowed to grow on a plate-agitator at 30°C for 24 hours. One liter of FM22-glycerol-PTM1 medium contained 42.87 g KH₂PO₄, 5 g (NH₄)₂SO₄, 1 g CaSO₄·2H₂O, 14.28 g K₂SO₄, 11.7 g MgSO₄·7H₂O, 50 g glycerol sterilized as a 100 ml solution, and 1 ml of PTM1 trace mineral mix filter-sterilized. The FM22 part of the medium was prepared as a 900 ml solution adjusted to pH 4.9 with KOH and sterile filtered. One liter of the PTM1 mix contained 6 g CuSO₄·5H₂O, 0.8 g KI, 3 g MnSO₄·H₂O, 0.2 g NaMoO₄·2H₂O, 0.02 g H₃BO₃, 0.5 g CoCl₂·6H₂O, 20 g ZnCl₂, 5 ml H₂SO₄, 65 g FeSO₄·7H₂O, 0.2 g biotin.

[0258] The cells were then pelleted and resuspended in fresh FM22-methanol-PTM1 medium (same composition as above except that the 50 g glycerol was replaced by 0.5 % (v/v) methanol in order to induce expression of the AOX1 promoter). After an additional incubation period of 24 hours at 30°C, the supernatants of the mini-cultures were tested for the presence of secreted AcaNAP5. Two clones that directed a high level of synthesis and secretion of AcaNAP5, as shown by the appearance of high factor Xa inhibitory activity in the culture medium (as measured by the amidolytic factor Xa assay described in Example 1), were selected. After a second screening round, using the same procedure, but this time at the shake-flask level, one isolated host cell was chosen and designated *P. pastoris* GTS115/7SP-NAP5.

[0259] The host cell, GTS115/7SP-NAP5, was shown to have a wild type methanol-utilisation phenotype (Mut⁺), which demonstrated that the integration of the expression cassette into the chromosome of GTS115 did not alter the functionality of the genomic AOX1 gene.

[0260] Subsequent production of recombinant AcaNAP5 material was performed in shake flask cultures, as described in Stroman, D.W. et al., U.S. Patent No. 4,855,231. The recombinant product was purified from *Pichia pastoris* cell supernatant as described below.

(C) Purification of recombinant AcaNAP5.

(1) Cation Exchange Chromatography.

[0261] Following expression, the culture supernatant from GTS115/75SP-NAP5 (100 ml) was centrifuged at 16000 r.p.m. (about 30,000xg) for 20 minutes before the pH was adjusted with 1N HCl to pH 3. The conductivity of the supernatant was decreased to less than 10 mS/cm by adding MilliQ water. The diluted supernatant was clarified by passage through a 0.22 micrometer cellulose acetate filter (Corning Inc., Corning, NY, USA)

[0262] The total volume (approximately 500 ml) of supernatant was loaded on a Poros20 HS (Perseptive Biosystems, MA) 1 x 2 cm column pre-equilibrated with Cation Buffer (0.05 M sodium citrate, pH 3) at a flow rate of 5 ml/minute (400 cm/hour). The column and the sample were at ambient temperature throughout this purification step. The column was subsequently washed with 50 column volumes Cation Buffer. Material that had inhibitory activity in a factor Xa amidolytic assay was eluted with Cation Buffer containing 1M NaCl at a flow rate of 2 ml/minute.

(2) Molecular Sieve Chromatography Using Superdex30.

[0263] The 1M NaCl elution pool containing the inhibitory material (3 ml) from the cation-exchange column was loaded on a Superdex30 PG (Pharmacia, Sweden) 1.6 x 66 cm column pre-equilibrated with 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl at ambient temperature. The chromatography was conducted at a flow rate of 2 ml/minute. The factor Xa inhibitory activity eluted 56-64 ml into the run (K_{av} of 0.207). This is the same elution volume as determined for the native molecule (Example 1, part E).

(3) Reverse Phase Chromatography.

[0264] 1 ml of the pooled fractions from the gel filtration chromatography was loaded on to a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia, CA) which was then developed with a linear gradient of 10-35 % acetonitrile in 0.1 % (v/v) trifluoroacetic acid at 1 ml/minute with a rate of 0.4% change in acetonitrile/minute. Factor Xa inhibitory activity, assayed as in Example 1, eluted around 30-35% acetonitrile and was present in several fractions. HPLC runs were performed on the same system as described in Example 1. Fractions from several runs on this column containing the factor Xa inhibitory activity were pooled and vacuum dried.

(4) Molecular Weight Determination of Recombinant AcaNAP5

[0265] The estimated mass for the main constituent isolated as described in sections (1) to (3) of this example were determined using the same electrospray ionisation mass spectrometry system as described in Example 1.

[0266] The estimated mass of recombinant AcaNAP5 was 8735.69 Daltons.

(5) Amino Acid Sequencing of Recombinant AcaNAP5.

[0267] Following purification by section (1) to (3) of this example, the recombinant AcaNAP5 from *Pichia pastoris* was subjected to amino acid sequence analysis as described in Example 1. The first five amino acids of the amino-terminus of AcaNAP5 were determined to be: Lys-Ala-Tyr-Pro-Glu [SEQ. ID. NO. 106]. The sequence was identical to the native NAP protein (see Example 1).

Example 4Production and Purification Of Recombinant AcaNAP6 In *P. pastoris*.(A) Expression Vector Construction.

[0268] The expression vector, pYAM7SP-NAP6, was made in the same manner as described for pYAM7SP-NAP5 in Example 3.

(B) Expression Of Recombinant AcaNAP6 In *P. pastoris*.

[0269] The vector, pYAM7SP-NAP6, was used to transform the *Pichia* strain GTS115 (his4) as described in Example 3.

(C) Purification of AcaNAP6.

[0270] The recombinant AcaNAP6, expressed from *Pichia* strain GTS115 (his4) transformed with the expression vector, pYAM7SP-NAP6, was purified as described for recombinant AcaNAP5 in Example 3.

[0271] The estimated mass of recombinant AcaNAP6 was determined, as described in Example 3, to be 8393.84 Daltons.

[0272] The majority of the AcaNAP6 preparation had the following amino-terminus: Lys-Ala-Tyr-Pro-Glu [SEQ. ID. NO. 106].

Example 5Expression Of Recombinant Pro-AcaNAP5 In COS Cells(A) Expression Vector Construction.

[0273] The pGEM-9Zf(-) vector (Promega Corporation, Madison, WI, USA) into which the AcaNAP5 cDNA was sub-cloned, served as target for PCR-rescue of the entire AcaNAP5 coding region, including the native secretion signal (using Vent polymerase from New England Biolabs, Beverly, MA, USA; 20 temperature cycles: 1 minute at 95°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The oligonucleotide primers used were: (1) YG101, targeting the 3'-end of the gene encoding a NAP and having the sequence, GCTCGCTCTA GAAGCTTCAG ACATGTATAA TCTCATGTTG G [SEQ. ID. NO. 105], and (2) YG102, targeting the 5'-end of the gene encoding a NAP and having the sequence, GACCAGTCTA GACAATGAAG ATGCTTTACG CTATCG [SEQ. ID. NO. 107]. These primers contain non-annealing extensions which include XbaI restriction sites (underlined).

[0274] Following digestion with XbaI enzyme, the amplification product having the expected size was isolated from an agarose gel and subsequently substituted for the about 450 basepair XbaI stuffer fragment of the pEF-BOS vector [Mizushima, S. and Nagata, S., Nucl. Acids Res., 18: 5322 (1990)] for expression purposes. The recipient vector-fragment was prepared by XbaI digestion and purified from an agarose gel.

[0275] *E. coli* strain WK6 [Zell, R. and Fritz, H.-J., EMBO J., 6: 1809-1815 (1987)] was transformed with the ligation mixture. Thirty randomly picked ampicillin-resistant transformants were subjected to PCR analysis (Taq polymerase from Life Technologies Inc., Gaithersburg, MD, USA; 30 cycles of amplification with the following temperature program: 1 minute at 95°C, 1 minute at 50°C, and 1 minute at 72°C). Oligonucleotide primers used were: (i) YG103 having the sequence, AAGGCATACC CGGAGTGTGG TG [SEQ. ID. NO. 89], and matching the amino-terminus of the region

encoding mature NAP, and (ii) YG60 having the sequence, GTGGGAGACC TGATACTCTC AAG [SEQ. ID. NO. 108], and targeting vector sequences downstream of the site of insertion, i.e., in the 3'-untranslated region of the pEF-BOS expression cassette. Only clones that harbor the insert in the desired orientation can yield a PCR fragment of predictable length (about 250 basepair). Two such clones were further characterized by sequence determination and were found to contain the desired XbaI insert. One of the clones, designated pEF-BOS-NAP5, was used to transfect COS cells.

(B) Transfection of COS Cells.

[0276] COS-7 cells (ATCC CRL 1651) were transfected with pEF-BOS-NAP5, pEF-BOS containing an irrelevant insert or with omission of DNA (mock transfections) using DEAE-dextran. The following media and stock solutions were used with the DEAE-dextran method:

(1) COS-medium: DMEM; 10% FBS (incubated for 30 minutes at 56°C); 0.03% L-glutamine; penicillin (50 I.U./ml) and streptomycin (50 micrograms/ml) (all products from Life Technologies).

(2) MEM-HEPES: MEM medium from Life Technologies Inc., reconstituted according to the manufacturer's specifications; containing a 25 mM final concentration of HEPES; adjusted to pH 7.1 before filtration (0.22 micrometer).

(3) DNA solution: 6 micrograms DNA per 3 ml MEM-HEPES

(4) DEAE-dextran solution: 30 microliters DEAE-dextran stock (Pharmacia, Uppsala, Sweden; 100 mg/ml in H₂O) per 3 ml MEM-HEPES.

(5) Transfection mixture: 3 ml of the DEAE-dextran solution is added to 3 ml of the DNA solution and the mixture is left to stand for 30 minutes at ambient temperature.

(6) Chloroquine solution: a 1:100 dilution of chloroquine stock (Sigma, St.Louis, MO, USA; 10 mM in water; filtered through a 0.22 micrometer membrane) in COS medium.

[0277] Transient transfection of the COS cells was performed as follows. COS cells (about 3.5×10^6), cultured in a 175 cm² Nunc TC-flask (Life Technologies Inc.) were washed once with MEM-HEPES. Six ml of the transfection mixture were pipetted onto the washed cells. After incubation for 30 minutes at ambient temperature, 48 ml of the chloroquine solution were added and the cells were incubated for another 4 hours at 37°C. The cells were washed one time with fresh COS-medium and finally incubated in 50 ml of the same medium at 37°C.

(C) Culturing of Transfected COS Cells.

[0278] Three, four, and five days after transfection a sample of the culture supernatants was tested in a factor Xa amidolytic assay according to the procedure in Example 1. The results clearly demonstrated that factor Xa inhibitory activity was accumulating in the culture supernatant of the cells transfected with pEF-BOS-NAP5.

[0279] The COS culture supernatant was harvested five days after transfection and the NAP protein purified as described in Example 6.

Example 6.

Purification Of Recombinant Pro-AcaNAP5.

(A) Anion Exchange Chromatography.

[0280] The COS culture supernatant containing Pro-AcaNAP5 was centrifuged at 1500 r.p.m. (about 500xg) for 10 minutes before adding solid sodium acetate to a final concentration of 50 mM. The following protease inhibitors were added (all protease inhibitors from ICN Biomedicals Inc, Costa Mesa, CA, USA): 1.0×10^{-5} M pepstatin A (isovaleryl-Val-Val-4-amino-3-hydroxy-6-methyl-heptanoyl-Ala-4-amino-3-hydroxy-6-methylheptanoic acid), 1.0×10^{-5} M leupeptin, 5×10^{-5} M AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride). The pH was adjusted with HCl to pH 5.3. The supernatant was clarified by passage through a 0.2 micrometer cellulose acetate filter (Corning Inc., Corning, NY, USA).

[0281] The clarified supernatant (total volume approximately 300 ml) was loaded on a Poros20 HQ (Perseptive Biosystems, MA) 1 x 2 cm column pre-equilibrated with Anion buffer (0.05 M sodium acetate, pH 5.3, 0.1 M NaCl) at a flow rate of 10 ml/minute (800 cm/hour). The column and the sample were at ambient temperature throughout this purification step. The column was subsequently washed with at least 10 column volumes of Anion buffer. Material that had inhibitory activity in a factor Xa amidolytic assay was eluted with Anion buffer containing 0.55 M NaCl at a flow rate of 5 ml/minute (400 cm/hour) and was collected.

(B) Molecular Sieve Chromatography Using Superdex30.

[0282] The 0.55 M NaCl elution pool (3 ml) from the anion-exchange chromatography was loaded on a Superdex30 PG (Pharmacia, Sweden) 1.6 x 66 cm column pre-equilibrated with 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl at 24°C. The chromatography was conducted at a flow rate of 2 ml/minute. Material which was inhibitory in the Factor Xa amidolytic assay eluted 56-64 ml into the run (K_{av} of 0.207). This was exactly the same elution volume as determined for the native molecule.

(C) Heat Treatment.

[0283] The total pool of fractions having factor Xa inhibitory activity was incubated for 5 minutes at 90°C in a glass tube and subsequently cooled rapidly on ice. Insoluble material was pelleted by centrifugation 19,000 x g_{max} at 4°C for 20 minutes. The supernatant contained all of the factor Xa inhibitory activity.

(D) Reverse Phase HPLC Chromatography.

[0284] The supernatant of the heat-treated sample was loaded onto a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia, CA) which was then developed with a linear gradient of 10-35% acetonitrile in 0.1% (v/v) trifluoroacetic acid at 1 ml/minute with a rate of 0.4% change in acetonitrile/minute. Factor Xa inhibitory activity eluted at approximately 30% acetonitrile. The HPLC runs were performed on the same system as described in Example 1. Factor Xa inhibitory activity-containing fractions were vacuum dried.

(E) Molecular Weight Determination.

[0285] The estimated mass for recombinant Pro-AcaNAP5, isolated as described in sections A-D of this example, was determined using the same electrospray ionisation mass spectrometry system as described in Example 1.

[0286] The estimated mass of recombinant Pro-AcaNAP5 was 9248.4 daltons.

(F) Amino Acid Sequencing.

[0287] Following purification, the recombinant Pro-AcaNAP5 from COS cells was subjected to amino acid analysis to determine its amino-terminus sequence, as described in Example 1. The first nine amino acids of the amino-terminus of Pro-AcaNAP5 was determined to be: Arg Thr Val Arg Lys Ala Tyr Pro Glu [SEQ. ID. NO. 109]. Compared to the native AcaNAP5 protein (see Example 1), Pro-AcaNAP5 possesses four additional amino acids on its N-terminus. The amino acid sequence of Pro-AcaNAP5 is shown in Figure 5.

Example 7Expression Of Recombinant Pro-AcaNAP6 In COS Cells

[0288] Pro-AcaNAP6 was transiently produced in COS cells essentially as described for Pro-AcaNAP5 in Example 5.

[0289] The AcaNAP6 coding region, including the secretion signal, was PCR-rescued with the same two oligonucleotide primers used for AcaNAP5: (1) YG101 targeting the 3'-end of the gene and having the sequence, GCTCGCTCTA GAAGCTTCAG ACATGTATAA TCTCATGTTG G [SEQ. ID. NO. 105], and (2) YG102 targeting the 5'-end of the gene and having the sequence, GACCAGTCTA GACAATGAAG ATGCTTTACG CTATCG [SEQ. ID. NO. 107]. The YG101-primer contains a non-matching nucleotide when used with AcaNAP6 as target (underlined T-residue; compare with Figure 1 and Figure 3); this mismatch results in the replacement an ATT Ile-codon by an ATA Ile-codon. The mismatch did not markedly influence the amplification efficiency.

[0290] The following modification from Example 5 was introduced: twenty-four hours after transfection of the COS cells (which is described in Example 5, section B) the COS-medium containing 10% FBS was replaced with 50 ml of a medium consisting of a 1:1 mixture of DMEM and Nutrient Mixture Ham's F-12 (Life Technologies). The cells then were further incubated at 37°C and the production of factor Xa inhibitory activity detected as described in Example 5.

Example 8Purification Of Recombinant Pro-AcaNAP6.(A) Anion Exchange Chromatography.

[0291] The COS culture supernatant containing Pro-AcaNAP6 was centrifuged at 1500 r.p.m. for 10 minutes before adding solid sodium acetate to a final concentration of 50 mM. The following protease inhibitors were added (all protease inhibitors from ICN Biomedicals Inc, Costa Mesa, CA, USA): 1.0×10^{-5} M pepstatin A (isovaleryl-Val-Val-4-amino-3-hydroxy-6-methyl-heptanoyl-Ala-4-amino-3-hydroxy-6-methylheptanoic acid), 1.0×10^{-5} M leupeptin, 5×10^{-5} M AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride). The pH was adjusted with HCl to pH 5.3. The supernatant was clarified by passage through a 0.2 micrometer cellulose acetate filter (Corning Inc., Corning, NY, USA).

[0292] The clarified supernatant (total volume approximately 450 ml) was loaded on a Poros20 HQ (Perseptive Biosystems, MA) 1 x 2 cm column pre-equilibrated with Anion buffer (0.05 M Na sodium acetate, pH 5.3, 0.1 M NaCl) at a flow rate of 10 ml/minute (800 cm/hour). The column and the sample were at ambient temperature throughout this purification step. The column was subsequently washed with at least 10 column volumes of Anion buffer. Material that had inhibitory activity in a factor Xa amidolytic assay was eluted with Anion buffer containing 0.55 M NaCl at a flow rate of 5 ml/minute (400 cm/hour) and was collected.

(B) Molecular Sieve Chromatography Using Superdex30.

[0293] The 0.55 M NaCl elution pool (3 ml) from the anion-exchange chromatography was loaded on a Superdex30 PG (Pharmacia, Sweden) 1.6 x 66 cm column pre-equilibrated with 0.01 M sodium phosphate, pH 7.4, 0.15 M NaCl at 24°C. The chromatography was conducted at a flow rate of 2 ml/minute. Material which was inhibitory in the Factor Xa amidolytic assay eluted 56-64 ml into the run (K_{av} of 0.207). This was exactly the same elution volume as determined for the native NAP.

(C) Reverse Phase HPLC Chromatography.

[0294] The pooled fractions from the gel filtration were loaded onto a 0.46 x 25 cm C18 column (218TP54 Vydac; Hesperia, CA) which then was developed with a linear gradient of 10-35% acetonitrile in 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/minute with a rate of 0.4% change in acetonitrile/minute. Factor Xa inhibitory activity (assayed according to Example 1) eluted at approximately 30% acetonitrile. The HPLC runs were performed on the same system as described in Example 1. Factor Xa inhibitory activity containing-fractions were vacuum dried.

(D) Molecular Weight Determination.

[0295] The estimated mass for recombinant Pro-AcaNAP6, isolated as described in sections A to C of this example, was determined using the same electrospray ionisation mass spectrometry system as described in Example 1.

[0296] The estimated mass of recombinant Pro-AcaNAP6 was 8906.9 daltons.

(E) Amino Acid Sequencing.

[0297] Following purification, the recombinant Pro-AcaNAP6 from COS cells was subjected to amino acid sequence analysis as described in Example 1. The first five amino acids of the N-terminus of Pro-AcaNAP6 were determined to be: Arg Thr Val Arg Lys [SEQ. ID. NO. 1101. Compared to the native NAP protein (see Example 1), Pro-AcaNAP6 possesses four additional amino acids on its amino-terminus. The amino acid sequence of Pro-AcaNAP6 is shown in Figure 6 [SEQ. ID. NO. 8].

Example 9The Use of NAP DNA Sequences to Isolate Genes Encoding Other NAP Proteins.

[0298] The AcaNAP5 and AcaNAP6 cDNA sequences (from Example 2) were used to isolate related molecules from other parasitic species by cross-hybridization.

[0299] The pGEM-9Zf(-) vectors (Promega) containing the AcaNAP5 and AcaNAP6 cDNAs were used to PCR-rescue the regions encoding the mature NAP proteins (Taq polymerase from Life Technologies; 20 temperature cycles: 1 minute at 95°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The oligonucleotide primers used were: (1) YG109, targeting the

C-terminal sequences of cDNA encoding NAP, and having the sequence, TCAGACATGT-ATAATCTCAT-GTTGG [SEQ. ID. NO. 88], and (2) YG103 having the sequence, AAGGCATACC-CGGAGTGTGG-TG [SEQ. ID. NO. 89]. The YG109 primer contains a single nucleotide mismatch (underlined T-residue; compare with the sequences shown in Figures 1 and 3) when used with AcaNAP6 as target. This did not markedly influence the amplification efficiency. The correctly sized PCR products (about 230 basepairs) were both isolated from a 1.5% agarose gel. An equimolar mixture was radiolabeled by random primer extension (T7 QuickPrime kit; Pharmacia) and subsequently passed over a Bio-Spin 30 column (Bio-Rad, Richmond, CA, USA).

[0300] *Ancylostoma ceylanicum* (Ace), *Ancylostoma duodenale* (Adu), and *Heligmosomoides polygyrus* (Hpo) cDNA libraries were prepared essentially as described for *Ancylostoma caninum* in Example 2.

[0301] *Ancylostoma ceylanicum* and *Heligmosomoides polygyrus* were purchased from Dr. D. I. Pritchard, Department of Life Science, University of Nottingham, Nottingham, UK. *Ancylostoma duodenale* was purchased from Dr. G. A. Schad, The School of Veterinary Medicine, Department of Pathobiology, University of Pennsylvania, Philadelphia, PA, USA.

[0302] In each case, the cDNAs were directionally cloned as EcoRI-NotI fragments in lambda gt11. Approximately 2×10^5 cDNA clones from each library (duplicate plaque-lift filters were prepared using Hybond™-N; Amersham) were screened with the radiolabeled AcaNAP5 and AcaNAP6 fragments using the following prehybridization and hybridization conditions: 5x SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate), 5x Denhardt's solution, 0.5% SDS, 20% formamide, 100 micrograms/ml sonicated fish sperm DNA (Boehringer), overnight at 42°C. The filters were washed 4 times for 30 minutes in 2x SSC, 0.1% SDS at 37°C. After exposure (about 60 hours) to X-ray film, a total of between 100 and 200 hybridization spots were identified in the case of Ace and Adu. A small number of very faint spots were visible in the case of the Hpo cDNA library. For each of the libraries, eight positives were subjected to a second hybridization round at lower plaque-density so as to isolate single plaques.

[0303] The retained clones were further characterized by PCR amplification of the cDNA-inserts using the oligo(dT)-NotI primer (Promega; this is the same primer used to prepare first strand cDNA; see Example 2) [SEQ. ID. NO. 95] in combination with the lambda-gt11 primer #1218 having the sequence, GGTGGCGACG ACTCCTGGAG CCCG [SEQ. ID. NO. 96] (New England Biolabs; primer #1218 targets lambda sequences located upstream of the site of cDNA insertion). PCR amplifications were performed as follows: Taq polymerase from Boehringer; 30 temperature cycles: 1 minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C. Gel-electrophoretic analysis of the PCR products clearly demonstrated that cDNAs of roughly the same size as the AcaNAP5 cDNA (e.g., 400 to 500 bp) were obtained for each species. In addition to these AcaNAP5-sized cDNAs, some Ace and Adu cDNAs were estimated to be about 700 bp long.

[0304] A number of clones, containing either a 500 bp or an 800 bp insert, were chosen for sequence determination. To that end the cDNA inserts were subcloned, as SfiI-NotI fragments, into pGEM-type phagemids (Promega; refer to Example 2 for details) which permit the preparation of single stranded DNA. The sequencing results led to the identification of six different new NAP-like proteins, designated as follows: AceNAP4, AceNAP5, AceNAP7, AduNAP4, AduNAP7, and HpoNAP5. The nucleotide sequences of the cDNAs as well as the deduced amino acid sequences of the encoded proteins are shown in Figure 7A (AceNAP4 [SEQ. ID. NO. 9]), Figure 7B (AceNAP5) [SEQ. ID. NO. 10], Figure 7C (AceNAP7) [SEQ. ID. NO. 11], Figure 7D (AduNAP4) [SEQ. ID. NO. 12], Figure 7E (AduNAP7) [SEQ. ID. NO. 13], and Figure 7F (HpoNAP5) [SEQ. ID. NO. 14]. The AceNAP4 [SEQ. ID. NO. 9] and AduNAP7 [SEQ. ID. NO. 13] cDNAs, each about 700 bp long, each encoded proteins which incorporated two NAP domains; the other cDNAs isolated coded for a protein having a single NAP domain. The AduNAP4 cDNA clone [SEQ. ID. NO. 12] was not full-length, i.e., the clone lacked the 5'-terminal part of the coding region; the correct reading frame could, however, be assigned based on amino acid sequence homology with the NAP family of related molecules.

[0305] The identified cDNA sequences can be used to produce the encoded proteins as disclosed in Examples 3, 4, 5, and 7 using the same or alternative suitable expression systems. Conditioned media or cell lysates, depending on the system used, can be tested as such or after fractionation (using such methodology as outlined in Example 3, 4, 6 and 8) for protease inhibitory and anticoagulant activity. Proteins that are encoded by cDNAs which hybridize to probes derived from fragments of the AcaNAP5 gene (Figure 1) [SEQ. ID. NO. 3] and/or the AcaNAP6 gene (Figure 3) [SEQ. ID. NO. 5] and that possess serine protease inhibitory and/or anticoagulant properties are considered to belong to the NAP family of related molecules.

Example 10

Identification of NAP by Functional Display of cDNA Encoded Proteins.

(A) The pDONG Series of Vectors.

[0306] The nucleotide sequences of the pDONG vectors, pDONG61 [SEQ. ID. NO. 15], pDONG62 [SEQ. ID. NO. 16] and pDONG63 [SEQ. ID. NO. 17], derivatives of pUC119 [Vieira, J. and Messing, J., Methods in Enzymology, 153:3-11

(1987)], are depicted in Figures 8A to 8C respectively.

[0307] To construct these three vectors, HindIII and SfiI restriction sites were added at the 5'-end and 3'-end of the filamentous phage gene 6 by PCR amplification of the M13K07 single stranded DNA [Vieira, J. and Messing, J., *Ibid*] with the G6BACKHIND backward primer and G6FORSFI61, G6FORSFI62 or G6FORSFI63 as forward primers. In a second PCR, the three obtained fragments were re-amplified with G6BACKHIND and G6FORNOTBAMH as forward primer to append NotI and BamHI sites at the 3'-end of the fragments. The sequences of the above mentioned PCR-primers are as follows (restriction sites are underlined):

G6BACKHIND: ATCCGAAGCT TTGCTAACAT ACTGCGTAAT AAG [SEQ. ID. NO. 111]

G6FORSFI61: TATGGGATGG CCGACTTGGC CTCCGCCTGA GCCTCCACCT
TTATCCCAAT CCAAATAAGA [SEQ. ID. NO. 112]

G6FORSFI62: ATGGGATGGC CGACTTGGCC CTCCGCCTGA GCCTCCACCT
TTATCCCAAT CCAAATAAGA [SEQ. ID. NO. 113]

G6FORSFI63: TATGGGATGG CCGACTTGGC CGATCCGCCT GAGCCTCCAC
CTTTATCCCA ATCCAAATAA [SEQ. ID. NO. 114]

GAG6FORNOTBAMH: AGGAGGGGAT CCGCGGCCGC GTGATATGGG
ATGGCCGACT TGGCC [SEQ. ID. NO. 115]

[0308] Finally, the PCR products were gel-purified, individually digested with HindIII and BamHI and inserted between the corresponding sites of pUC119. Sequence determination confirmed that pDONG61, pDONG62, and pDONG63 all contained the intended insert.

[0309] The pDONG series of vectors permit the cloning of cDNAs, as SfiI-NotI fragments. This cloning fuses the cDNAs in each of the three reading (translation) frames to the 3'-end of filamentous phage gene 6 which encodes one of the phage's coat proteins. Infection of a male-specific *E. coli* strain harboring a pDONG-derivative, with VCSM13 helper phage (Stratagene, La Jolla, CA), results in the rescuing of pseudo-virions which encapsidate one specific single strand of the pDONG-derivative and which may also incorporate a recombinant protein 6 (p6) fusion protein in their coat. cDNAs which are such that the encoded protein is functionally displayed on the phage surface as a recombinant p6 fusion protein become identifiable by means of a panning experiment described below.

(B) Transfer of the *Ancylostoma caninum* cDNA Library from Lambda gt11 to the pDONG Series of Vectors.

[0310] A phage lambda preparation of the pooled *A. caninum* cDNA clones (about 1×10^6 plaques, see Example 2) was used to PCR-rescue the cDNA inserts (Taq polymerase from Life Technologies, Gaithersburg, MD, USA; 20 temperature cycles: 1 minute at 95°C, 1 minute at 50°C, and 3 minutes at 72°C followed by 10 minutes at 65°C), with the lambda gt11 primer #1218 having the sequence, GGTGGCGACG ACTCCTGGAG CCCG [SEQ. ID. NO. 96] (New England Biolabs, Beverly, MA, USA; targeting sequences located upstream of the cDNA insert) in combination with the oligo(dT)-NotI primer/adaptor (Promega) used for first strand cDNA synthesis. Following digestion with the restriction enzymes SfiI and NotI, the whole size-range of amplification products were recovered from agarose gel.

[0311] All fragments were directionally cloned into the pDONG61, pDONG62, and pDONG63 vectors. The recipient vector-fragments were prepared by digestion of the CsCl purified vectors with SfiI and NotI and purification with the "Wizard™ PCR Preps DNA Purification System" (Promega Corp, Madison, WI, USA).

[0312] *E. coli* strain TG1 [Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning, A Laboratory Manual*, Second Edition, volumes 1 to 3, Cold Spring Harbor Laboratory Press (1989)] was transformed by electroporation with the pDONG/cDNA ligation mixtures. Electrotransformed cells were incubated 1 hour at 37 °C in SOC medium [Sambrook, J. et al., *Ibid.*] and plated on LB-agar containing 0.1% glucose and 100 micrograms/ml carbenicillin (245x245x25 mm plates; Nunc). 2.2×10^6 , 1.6×10^6 , and 1.4×10^6 carbenicillin resistant transformants were obtained with pDONG61,

pDONG62, and pDONG63, respectively. From each respective library, designated 20L, 21L and 22L, a number of randomly picked transformants were subjected to PCR analysis (Taq polymerase from Life Technologies; 30 cycles of amplification with the following temperature program: 1 minute at 95°C, 1 minute at 50°C, and 1 to 3 minutes at 72°C) using two primers that match with sequences flanking the multiple cloning site of pUC119 (primers #1224 having the sequence, CGCCAGGGTT TTCCCAGTCA CGAC [SEQ. ID. NO. 116], and #1233 having the sequence, AGCGGATAAC AATTTACAC AGGA [SEQ. ID. NO. 101]; New England Biolabs). The results showed that the vast majority of the clones contained a cDNA-insert of variable size.

(C) Factor Xa Based Affinity-Selection of cDNA Clones Encoding a NAP Protein.

[0313] Phage particles from the 20L, 21L and 22L libraries were rescued as follows: each library was scraped from the plates and grown at 37°C in 100 ml LB medium supplemented with 1% glucose and 100 micrograms/ml carbenicillin until the optical absorbance at 600 nm reaches the value of 0.5. After addition of VCSM13 helper phage (Stratagene) at a multiplicity of infection (moi) of 20, the culture was left to stand for 30 minutes at 37°C and then slowly shaken for another 30 minutes. The cells were pelleted by centrifugation and resuspended in 250 ml LB medium supplemented with 100 micrograms/ml carbenicillin and 50 micrograms/ml kanamycin. These cultures were allowed to grow overnight at 30°C under vigorous agitation. The resulting phage particles were purified by two consecutive precipitations with polyethylene glycol/NaCl and resuspended at 1×10^{13} virions per ml in TRIS-buffered saline (0.05M Tris, 0.15M sodium chloride, pH 7.4) (TBS). Equal amounts of phage particles from the 20L, 21L and 22L were then mixed together.

[0314] Human factor Xa (see Example 1 for preparation) was biotinylated with biotin-XX-NHS according to manufacturer's instructions (Pierce). The amidolytic activity of the protease was not affected by this modification as shown by an enzymatic assay using the chromogenic substrate S-2765 (Chromogenix; see Example 1). Streptavidin-coated magnetic beads (Dynal; 1 mg per panning round) were washed three times with TBS and blocked in TBS supplemented with 2% skim milk (Difco) at ambient temperature. After one hour, the magnetic beads were washed twice with TBS before use.

[0315] For the first round of panning, 1×10^{13} phage from the pooled libraries were incubated for 75 minutes at 4°C in 200 microliters of TBS buffer supplemented with 250 nM biotinylated factor Xa, 5 mM CaCl_2 and 2% skim milk. After this time, 1 mg blocked streptavidin-coated magnetic beads, resuspended in 200 microliters of TBS containing 5 mM CaCl_2 and 2% skim milk, was added to the phage solution and incubated for 1 hour at 4°C with gentle agitation. With a magnet (Dynal), the magnetic beads were then rinsed ten times with 500 microliters of TBS containing 0.1% Tween-20. Bound phage were eluted from the magnetic beads by incubating them with 500 microliters of 0.1 M glycine-HCl buffer (pH 2.0) for 10 minutes. The supernatant was neutralized with 150 microliters 1 M Tris-HCl buffer (pH 8.0).

[0316] For phage propagation, *E. coli* strain TG1 [Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning, A Laboratory Manual, Second Edition, volumes 1 to 3, Cold Spring Harbor Laboratory Press (1989)] was grown at 37°C in 10 ml LB medium until the optical absorbance at 600 nm reached the value of 0.5. The culture was infected with 650 microliters of phage eluted from the magnetic beads and briefly incubated at 37°C with no shaking. After centrifugation, the infected cells were resuspended in 2 ml LB medium and plated onto 245x245x25 mm plates filled with LB-agar containing 1% glucose and 100 micrograms/ml carbenicillin. After overnight incubation at 37°C, the cells were scraped from the plates and resuspended in 40 ml LB medium supplemented with 1% glucose and 100 micrograms/ml carbenicillin. A cell aliquot corresponding to 15 optical densities at 600 nm was then used to inoculate 100 ml LB medium containing 1% glucose and 100 micrograms/ml carbenicillin. Phage rescue for the next panning round was done as outlined above.

[0317] For the second panning round, 6×10^{12} phage were incubated during 90 minutes with 1 mg blocked streptavidin-coated magnetic beads in 200 microliters of TBS containing 2.5 mM Ca^{2+} and 2% skim milk (this step was introduced in the procedure to avoid selection of streptavidin-binding clones). After removal of the beads, the same protocol was followed as for round 1. Rounds 3, 4 and 5 were accomplished as round 2, except that the phage input was lowered to 2×10^{12} phage.

[0318] Twenty-four individual carbenicillin resistant clones that were isolated after five rounds of panning against biotinylated factor Xa, were then analyzed by ELISA. Streptavidin-coated 96-well plates (Pierce) were blocked for 1 hour with 200 microliters of TBS containing 2% skim milk per well, then were incubated for 1 hour with 100 microliters of 20 nM biotinylated factor Xa in TBS per well. For each clone, about 10^{10} phage diluted in 100 microliters TBS containing 2% skim milk and 0.1% Tween-20 were added to the wells. After a 2-hour incubation, the wells were rinsed four times with 200 microliters TBS containing 0.1% Tween-20. Bound phage were visualized by consecutively incubating with a rabbit anti-M13 antiserum (see Example 11), an alkaline phosphatase conjugated anti-rabbit serum (Sigma), and p-nitrophenylphosphate as substrate (Sigma). Absorbances were taken at 405 nm after 20 minutes. Out of the 24 clones, five bound strongly to factor Xa. No significant non-specific binding was observed with these phage when tested in the same ELISA with omission of biotinylated factor Xa.

[0319] Single stranded DNA was then prepared from the five positive clones and the inserts 3' to the gene 6 were submitted to automated DNA sequencing using the primer #1224 having the sequence, CGCCAGGGTT TTCCCAGTCA

CGAC [SEQ. ID. NO. 116] (New England Biolabs). All five clones were found to contain the same 470 bp 5'-truncated cDNA fused in frame to gene 6 in pDONG63. The nucleotide sequence of this cDNA as well as the deduced amino acid sequence are depicted in Figure 9 [SEQ. ID. NO. 19]. The cDNA, designated AcaNAPc2, encodes a protein, designated NAP isoform c2, that belongs to the NAP family of related proteins.

Example 11

Preparation of Antiserum Against M13 Phage.

[0320] Antiserum against M13 phage was prepared in rabbits by subcutaneous injections of about 10^{13} M13K07 phage in 500 microliters of PBS (0.01 M sodium phosphate, pH 7.4 + 0.15 M sodium chloride) combined with an equal volume of adjuvant. The M13K07 phage were CsCl-purified essentially as described by Glaser-Wuttke, G., Keppner, J., and Rasched, I., *Biochim. Biophys. Acta*, 985: 239-247 (1989). The initial injection was done with Complete Freund's adjuvant on day 0, followed by subsequent injections with Incomplete Freund's adjuvant on days 7, 14 and 35. Antiserum was harvested on day 42.

[0321] The IgG fraction of the antiserum was enriched by passage over a Protein A-Sepharose column using conditions well known in the art.

Example 12

The Use of AcaNAP5 and AcaNAP6 DNA Sequences to Isolate Additional NAP-Encoding Sequences from *A. caninum*.

[0322] The AcaNAP5 and AcaNAP6 cDNA sequences (from Example 2) were used to isolate related molecules from the same parasitic species by cross-hybridization.

[0323] The pGEM-9Zf(-) vectors (Promega, Madison, WI) containing the AcaNAP5 and AcaNAP6 cDNAs were used to PCR-rescue the regions encoding the mature NAP proteins (Taq polymerase from Life Technologies (Gaithersburg, MD); 20 temperature cycles: 1 minute at 95°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The oligonucleotide primers used were: (1) YG109, targeting the C-terminal-encoding sequences of cDNA encoding AcaNAP5 and AcaNAP6, and having the sequence, TCAGACATGT-ATAATCTCAT-GTTGG [SEQ. ID. NO. 88], and (2) YG103, targeting the N-terminal-encoding sequences of mature AcaNAP5 and AcaNAP6, having the sequence, AAGGCATACC-CGGAGTGT-GG-TG [SEQ. ID. NO. 89]. The YG109 primer contains a single nucleotide mismatch when used with AcaNAP6 as target (underlined T-residue; compare with the sequence shown in Figure 3 [SEQ. ID. NO. 5]). This mismatch did not markedly influence the amplification efficiency. The correctly sized PCR products (about 230 basepairs) for AcaNAP5 and AcaNAP6 were both isolated from a 1.5% agarose gel. An equimolar mixture was radiolabeled by random primer extension (T7 QuickPrime kit; Pharmacia (Sweden) and subsequently passed over a Bio-Spin 30 column (Bio-Rad, Richmond, CA, USA).

[0324] Approximately 750,000 *Ancylostoma caninum* (Aca)cDNA clones (refer to Example 2 (B)); duplicate plaque-lift filters were prepared using Hybond™-N; Amersham (Buckinghamshire, England) were screened with the radiolabeled AcaNAP5 and AcaNAP6 cDNA fragments using the following prehybridization and hybridization conditions: 5x SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate), 5x Denhardt's solution, 0.5% SDS, 20% formamide, 100 micrograms/ml sonicated fish sperm DNA (Boehringer), overnight at 42°C. The filters were washed 4 times for 30 minutes in 2x SSC, 0.1% SDS at 37°C. After exposure to X-ray film, a total of about 300 positives were identified.

[0325] 48 of the 300 positives were subjected to PCR-amplification (Taq polymerase from Boehringer Mannheim, Germany; 30 temperature cycles: 1 minute at 95°C; 1 minute at 50°C; 1.5 minutes at 72°C) using the above mentioned YG109 primer, specific for the C-terminus-encoding sequence of AcaNAP5 and AcaNAP6 cDNAs, and primer #1218 which targets lambda-gt10 sequences located upstream of the site of cDNA insertion (New England Biolabs, Beverly, MA; GGTGCGACG ACTCCTGGAG CCGG [SEQ. ID. NO. 96]). 31 out of the 48 positives yielded a PCR product of a size similar to that expected for a AcaNAP5/6-type cDNA.

[0326] The remaining 17 positives were used as template for amplification with primer #1218 and an AcaNAPc2 specific primer (e.g., LJ189, targeting the AcaNAPc2 C-terminus and having the sequence GTTTCGAGTT CCGGGA-TATA TAAAGTCC [SEQ. ID. NO. 117]; refer to Example 10 and Figure 9). None of the clones yielded a PCR product. All 17 positives were then subjected to a second hybridization round at lower plaque-density; single isolated clones were identified in all cases. The 17 isolated cDNA clones were re-analyzed by PCR using the primer couples #1218/YG109 and #1218/LJ189. Three out of the 17 clones yielded an amplification product with the #1218/YG109 primers.

[0327] The remaining 14 clones were further analyzed by PCR amplification with the primers #1218 and oligo(dT)-Not (Promega, Madison, WI; this is the same primer used to prepare first strand cDNA; see Example 2). All 14 clones yielded a PCR product. Gel-electrophoretic analysis of the PCR products indicated that some cDNAs were considerably longer than the AcaNAP5 cDNA insert.

[0328] Ten clones, including those having the largest cDNA inserts, were chosen for sequence determination. To that end the cDNA inserts were subcloned as SfiI-NotI fragments onto pGEM-type phagemids (Promega, Madison, WI), as described in Example 2. The sequencing identified eight additional NAP protein sequences, designated as follows: AcaNAP23, AcaNAP24, AcaNAP25, AcaNAP31, AcaNAP44, AcaNAP45, AcaNAP47, and AcaNAP48. Two additional cDNA clones, designated AcaNAP42 and AcaNAP46, encoded proteins identical to those encoded by AcaNAP31 [SEQ. ID. NO. 34]. The nucleotide sequences of the cDNAs as well as the deduced amino acid sequences of the encoded proteins are shown in Figure 13A (AcaNAP23 [SEQ. ID. NO. 31]), Figure 13B (AcaNAP24 [SEQ. ID. NO. 32]), Figure 13C (AcaNAP25 [SEQ. ID. NO. 33]), Figure 13D (AcaNAP31 [SEQ. ID. NO. 34]), Figure 13E (AcaNAP44 [SEQ. ID. NO. 35]), Figure 13F (AcaNAP45 [SEQ. ID. NO. 36]), Figure 13G (AcaNAP47 [SEQ. ID. NO. 37]), and Figure 13H (AcaNAP48 [SEQ. ID. NO. 38]). All clones were full-length and included a complete secretion signal. The AcaNAP45 [SEQ. ID. NO. 36] and AcaNAP47 [SEQ. ID. NO. 37] cDNAs, each encode proteins which incorporate two NAP domains; the other cDNAs code for a protein having a single NAP domain.

Example 13

The Use of NAP DNA Sequences to Isolate Sequences Encoding a NAP Protein from *Necator americanus*

[0329] The sequences of AcaNAP5 [SEQ. ID. NO. 3], AcaNAP6 [SEQ. ID. NO. 5], AcaNAPc2 [SEQ. ID. NO. 19], AcaNAP23 [SEQ. ID. NO. 31], AcaNAP24 [SEQ. ID. NO. 32], AcaNAP25 [SEQ. ID. NO. 33], AcaNAP31 [SEQ. ID. NO. 34], AcaNAP44 [SEQ. ID. NO. 35], AcaNAP45 [SEQ. ID. NO. 36], AcaNAP47 [SEQ. ID. NO. 37], AcaNAP48 [SEQ. ID. NO. 38], AceNAP4 [SEQ. ID. NO. 9], AceNAP5 [SEQ. ID. NO. 10], AceNAP7 [SEQ. ID. NO. 11], AduNAP4 [SEQ. ID. NO. 12], AduNAP7 [SEQ. ID. NO. 13], and HpoNAP5 [SEQ. ID. NO. 14] (see Figures 1, 3, 7, and 13) were used to isolate related molecules from the hematophagous parasite *Necator americanus* by PCR-cloning.

[0330] Consensus amino acid sequences were generated from regions of homology among the NAP proteins. These consensus sequences were then used to design the following degenerate PCR primers: NAP-1, 5'-AAR-CCN-TGY-GAR-MGG-AAR-TGY-3' [SEQ. ID. NO. 90] corresponding to the amino acid sequence NH₂-Lys-Pro-Cys-Glu-(Arg/Pro/Lys)-Lys-Cys [SEQ. ID. NO. 118]; NAP-4.RC, 5'-TW-RWA-NCC-NTC-YTT-RCA-NAC-RCA-3' [SEQ. ID. NO. 91], corresponding to the sequence NH₂-Cys-(Val/Ile/Gln)-Cys-(Lys/Asp/Glu/Gln)-(Asp/Glu)-Gly-(Phe/Tyr)-Tyr [SEQ. ID. NO. 119]. These primers were used pairwise to generate NAP-specific probes by PCR using *N. americanus* cDNA as template.

[0331] Adult worms, *N. americanus*, were purchased from Dr. David Pritchard, University of Nottingham. Poly(A⁺) RNA was prepared using the QuickPrep mRNA Purification Kit (Pharmacia, Piscataway, New Jersey). One microgram of mRNA was reverse transcribed using AMV reverse transcriptase and random hexamer primers (Amersham, Arlington Hills, IL). One fiftieth of the single-stranded cDNA reaction product was used as template for -400 pmole of each of NAP-1 and NAP-4.RC, with PCR GeneAmp (Perkin Elmer, Norwalk, CT) reagents, on a Perkin-Elmer DNA thermal cycler. PCR conditions were: cycles 1-3, denaturation at 96 °C for 2 minutes, annealing at 37 °C for 1 minute, and elongation at 72 °C for 3 minutes (ramp time between 37 °C and 72 °C was 2 minutes); cycles 4-5, denaturation at 94 °C for 1 minute, annealing at 37 °C for 1 minute, and elongation at 72 °C for 2 minutes (ramp time between 37 °C and 72 °C was 2 minutes); cycles 6-45, denaturation at 94 °C for 1 minutes, annealing at 37 °C for 1 minute, and elongation at 72 °C for 2 minutes. Elongation times were incremented by 3 seconds/cycle for cycles 6-45.

[0332] PCR amplification of *N. americanus* cDNA with NAP-1 and NAP-4.RC resulted in an approximately 100 bp amplification product. The PCR product was labeled with [α-32P]-dCTP (Amersham) using random primer labeling (Stratagene, La Jolla, CA), and labeled DNA was separated from unincorporated nucleotides using a Chromaspin-10 column (Clontech, Palo Alto, CA).

[0333] A cDNA library was constructed using the following procedure..Double stranded cDNA was synthesized from 1 μg of *N. americanus* poly(A⁺) RNA using AMV reverse transcriptase and random hexamer primers (Amersham, Arlington Hills, IL). cDNA fragments larger than approximately 300 bp were purified on a 6% polyacrylamide gel and ligated to EcoRI linkers (Stratagene, San Diego, CA) using standard procedures. Linkered cDNA was ligated into EcoRI-cut and dephosphorylated lambda gt10 (Stratagene, San Diego, CA) and packaged using a Gigapack Gold II packaging kit (Stratagene, San Diego, CA).

[0334] Prehybridization and hybridization conditions were 6X SSC (SSC: 150 mM NaCl, 15 mM trisodium citrate, pH 7.0), 0.02 M sodium phosphate pH 6.5, 5X Denhardt's solution, 100 μg/ml sheared, denatured salmon sperm DNA, 0.23% dextran sulfate. Prehybridization and hybridization were at 42 °C, and the filters were washed for 30 minutes at 45 °C with 2X SSC after two prewashes with 2X SSC for 20 minutes. The filters were exposed overnight to X-ray film with two intensifying screens at -70 °C.

[0335] Approximately 400,000 recombinant phage of the random primed *N. americanus* library (unamplified) were screened with the NAP-1/NAP-4.RC PCR fragment. About eleven recombinant phage hybridized to this probe, of which four were isolated for nucleotide sequencing analysis. Double stranded sequencing was effected by subcloning the

EcoRI cDNA fragments contained in these phage isolates into pBluescript II KS+ vector (Stratagene, San Diego, CA). DNA was sequenced using the Sequenase version 2.0 kit (Amersham, Arlington Hills, IL)) and M13 oligonucleotide primers (Stratagene, San Diego, CA).

[0336] The four lambda isolates contained DNA that encoded a single 79 amino acid NAP polypeptide that resembles NAP sequences from *Ancylostoma* spp. and *H. polygyrus*. The NAP polypeptide from *N. americanus* has a calculated molecular weight of 8859.6 Daltons. The nucleotide and deduced amino acid sequences are shown in Figure 14.

Example 14.

Expression Of Recombinant AceNAP4 In COS Cells A. Expression

[0337] AceNAP4 was transiently produced in COS cells essentially as described for Pro-AcaNAP5 in Example 5 and Pro-AcaNAP6 in Example 7.

[0338] A pGEM-type phagemid that harbors the AceNAP4 cDNA (from Example 9), served as target for PCR-rescue of the entire AceNAP4 coding region, including the secretion signal, using two XbaI-appending oligonucleotide primers. The primers used were: (1) SHPCR4, targeting the 5'-end of the gene and having the sequence, GACCAGTCTA GACCACCATG GCGGTGCTTT ATTCAGTAGC AATA [SEQ. ID. NO. 120], and (2) SHPCR5, targeting the 3'-end of the gene and having the sequence, GCTCGCTCTA GATTATCGTG AGGTTTCTGG TGCAAAAGTG [SEQ. ID. NO. 121]. The XbaI restriction sites included in the primers are underlined. The primers were used to amplify the AceNAP4 sequence according to the conditions described in Example 5.

[0339] Following digestion with XbaI enzyme, the amplification product, having the expected size, was isolated from an agarose gel and subsequently substituted for the about 450 basepair XbaI stuffer fragment of the pEF-BOS vector [Mizushima, S. and Nagata, S., Nucl. Acids Res., 18: 5322 (1990)]. The protocol described in Example 5 was followed to yield clone pEF-BOS-AceNAP4, which was first shown to harbor the XbaI-insert in the desired orientation by PCR using primers SHPCR4 and YG60, and subsequently confirmed by sequence determination. This clone was used to transfect COS cells according to the methods in Example 5.

[0340] Twenty-four hours after transfection of the COS cells (refer to Example 5, section B) the COS-medium containing 10% FBS was replaced with 50 ml of a medium consisting of a 1:1 mixture of DMEM and Nutrient Mixture Ham's F-12 (Life Technologies (Gaithersburg, MD). The cells were then further incubated at 37°C and the production of EGR-factor Xa dependent TF/factor VIIa inhibitory activity detected as described in Example E.

B. Purification of AceNAP4

1. Anion-exchange chromatography

[0341] The COS culture supernatant from the AceNAP4-expressing cells was centrifuged at 1500 r.p.m. (about 500xg) for 10 minutes before the following protease inhibitors (ICN Biomedicals Inc., Costa Mesa, CA) were added (1.0x 10⁻⁵M pepstatinA (isovaleryl-Val-Val-4-amino-3-hydroxy-6-methyl-heptanoyl-Ala-4-amino-3hydroxy-6-methylheptanoic acid), 1.0x 10⁻⁵M AEBSF (4-(2-amonoethyl)-benzenesulfonyl fluoride). Solid sodium acetate was added to a final concentration of 50mM before the pH was adjusted with 1N HCl to pH 5.3. The supernatant was clarified by passage through a 0.22 micrometer cellulose acetate filter (Corning Inc., Corning, NY, USA).

[0342] The clarified supernatant (total volume aproximately 450ml) was loaded on a Poros20 HQ (Perseptive Biosystems, MA) 1x2cm column preequilibrated with Anion Buffer (0.05M sodium acetate 0.1M NaCl, pH 5.3) at a flow rate of 5ml/minute. The column and the sample were at ambient temperature throughout this purification step. The column was subsequently washed with 10 column volumes of Anion Buffer and 10 column volumes of 50mM sodium acetate, 0.37M NaCl, pH5.3

[0343] Material that had EGR-FXa dependent fVIIa/TF amidolytic inhibitory activity (see Example E) was eluted with 50mM sodium acetate, 1M NaCl, pH5.3 at a flow of 2ml/minute.

2. Reverse-phase chromatography

[0344] An aliquot of the pool of fractions collected after anion exchange chromatography was loaded onto a 0.46x25cm C18 column (218TP54 Vydac; Hesperia, CA) which was then developed with a linear gradient of 10-35% acetonitrile in 0.1% (v/v) trifluoroacetic acid at 1ml/minute with a rate of 0.4% change in acetonitrile/minute. EGR-FXa dependent TF/FVIIa amidolytic inhibitory activity (see Example E) was monitored and fractions containing this inhibitory activity were isolated and vacuum-dried.

3. Characterization of recombinant AceNAP4

[0345] The AceNAP4 compound demonstrated SDS-PAGE mobility on a 4-20% gel, consistent with its size predicted from the sequence of the cDNA (Coomassie stained gel of material after RP-chromatography).

Example 15

Production and Purification Of Recombinant AcaNAPc2 In *P. pastoris*.

A. Expression Vector Construction.

[0346] Expression of the AcaNAPc2 gene in *P. pastoris* was accomplished using the protocol detailed in Example 3 for the expression of AcaNAP5 with the following modifications.

[0347] The pDONG63 vector containing the AcaNAPc2 cDNA, described in Example 10, was used to isolate by amplification ("PCR-rescue") the region encoding mature AcaNAPc2 protein (using Vent polymerase from New England Biolabs, Beverly, MA; 20 temperature cycles: 1 minute at 94°C, 1 minute at 50°C, and 1.5 minutes at 72°C). The following oligonucleotide primers were used:

LJ190: AAAGCAACGA-TGCAGTGTGG-TGAG [SEQ. ID. NO. 122]

LJ191: GCTCGCTCTA-GAAGCTTCAG-TTCGAGTTC-CGGGATATAT-AAAGTCC [SEQ. ID. NO. 123]

[0348] The LJ191 primer, targeting C-terminal sequences, contained a non-annealing extension which included XbaI and HindIII restriction sites (underlined).

[0349] Following digestion with XbaI enzyme, the amplification product, having the expected size, was isolated from gel and subsequently enzymatically phosphorylated (T4 polynucleotide kinase from New England Biolabs, Beverly, MA). After heat-inactivation (10 minutes at 70°C) of the kinase, the blunt-ended/XbaI fragment was directionally cloned into the vector pYAM7SP8 for expression purposes. The recipient vector-fragment from pYAM7SP8 was prepared by StuI-SpeI restriction, and purified from agarose gel. The *E. coli* strain, WK6 [Zell, R. and Fritz, H.-J., EMBO J., 6: 1809-1815 (1987)], was transformed with the ligation mixture, and ampicillin resistant clones were selected.

[0350] Based on restriction analysis, a plasmid clone containing an insert of the expected size, designated pYAM7SP-NAPC2, was retained for further characterization. Sequence determination of the clone pYAM7SP-NAPC2 confirmed the precise insertion of the mature AcaNAPc2 coding region in fusion with the prepro leader signal, as predicted by the construction scheme, as well as the absence of unwanted mutations in the coding region.

B. Expression Of Recombinant AcaNAPc2 In *P. pastoris*.

[0351] The *Pichia* strain GTS115 (his4) has been described in Stroman, D.W. et al., U.S. Patent No. 4,855,231. All of the *P. pastoris* manipulations were performed essentially as described in Stroman, D.W. et al., U.S. Patent No. 4,855,231.

[0352] About 1 microgram of pYAM7SP-NAPC2 plasmid DNA was electroporated into the strain GTS115 using a standard electroporation protocol. The plasmid was previously linearized by SalI digestion, theoretically targeting the integration event into the his4 chromosomal locus.

[0353] The selection of a AcaNAPc2 high-expresser strain was performed as described in Example 3 for NAP isoform 5 (AcaNAP5) using mini-culture screening. The mini-cultures were tested for the presence of secreted AcaNAPc2 using the fVIIa/TF-EGR-fXa assay (Example E) resulting in the selection of two clones. After a second screening round, using the same procedure, but this time at the shake-flask level, one isolated host cell was chosen and designated *P. pastoris* GTS115/7SP-NAPc2.

[0354] The host cell, GTS115/7SP-NAPc2, was shown to have a wild type methanol-utilisation phenotype (Mut⁺), which demonstrated that the integration of the expression cassette into the chromosome of GTS115 did not alter the functionality of the genomic AOX1 gene.

[0355] Subsequent production of recombinant AcaNAPc2 material was performed in shake flask cultures, as described in Stroman, D.W. et al., U.S. Patent No. 4,855,231. The recombinant product was purified from *Pichia pastoris* cell supernatant as described below.

C. Purification of recombinant AcaNAPc2

1. Cation Exchange chromatography

[0356] The culture supernatant (100ml) was centrifuged at 16000 rpm (about 30,000xg) for 20 minutes before the pH was adjusted with 1N HCl to pH 3. The conductivity of the supernatant was decreased to less than 10mS/cm by adding MilliQ water. The diluted supernatant was clarified by passage through a 0.22 micrometer cellulose acetate filter (Corning Inc., Corning, NY, USA).

[0357] The total volume (approximately 500ml) of the supernatant was loaded onto a Poros20HS (Perseptive Biosystems, MA) 1x2cm column pre-equilibrated with Cation Buffer (50mM sodium citrate pH 3) at a flow-rate of 5ml/minute. The column and the diluted fermentation supernatant were at room temperature throughout this purification step. The column was subsequently washed with 50 column volumes Cation Buffer and 10 column volumes Cation Buffer containing 0.1M NaCl. Material that had inhibitory activity in a prothrombinase assay was eluted with Cation Buffer containing 1M NaCl at a flow rate of 2ml/min.

2 Molecular Sieve Chromatography using Superdex30

[0358] The 1M NaCl elution pool containing the EGR-fXa-fVIIa/TF inhibitory material (3ml; see Example C) from the cation-exchange column was loaded onto a Superdex30 PG (Pharmacia, Sweden) 1.6x60cm column pre-equilibrated with 0.1M sodium phosphate pH7.4, 0.15M NaCl at ambient temperature. The chromatography was conducted at a flow-rate of 2 ml/minute. The prothrombinase inhibitory activity (Example C) eluted 56-64ml into the run and was pooled.

3. Reverse Phase Chromatography

[0359] One ml of the pooled fractions from the gel filtration chromatography was loaded onto a 0.46x25 cm C18 column (218TP54 Vydac; Hesperia, CA) which was then developed with a linear gradient 10-30% acetonitrile in 0.1% (v/v) trifluoroacetic acid with a rate of 0.5% change in acetonitrile/minute. The major peak which eluted around 20-25% acetonitrile, was manually collected and displayed prothrombinase inhibitory activity.

4. Molecular Mass Determination

[0360] The estimated mass for the main constituent isolated as described in section (1) to (3) of this example was determined using electrospray ionisation mass spectrometry. The estimated mass of the recombinant AcaNAPc2 was 9640 daltons, fully in agreement with the calculated molecular mass of this molecule derived from the cDNA sequence.

Example 16

Expression of AcaNAP42 in *P. pastoris*.

[0361] The pGEM-9zf(-) vector (Promega) containing the AcaNAP42 cDNA (Example 12) was used to isolate the region encoding the mature AcaNAP42 protein by PCR amplification (using Taq polymerase from Perkin Elmer, Branchburg, New Jersey; 25 temperature cycles: 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72°C). The following oligonucleotide primers were used:

oligo3: 5'GAG ACT TTT AAA TCA CTG TGG GAT CAG AAG3' [SEQ. ID. NO. 124]

oligo2: 5'TTC AGG ACT AGT TCA TGG TGC GAA AGT AAT
AAA3' [SEQ. ID. NO. 125]

[0362] The oligo 3 primer, targeting the N-terminal sequence, contained a non-annealing extension which includes DraI restriction site (underlined). The oligo 2 primer, targeting the C-terminal sequence, contained SpeI restriction site.

[0363] The NAP amplification product, having the expected approximately 250 bp size, was digested with DraI and SpeI enzymes, purified by extraction with phenol: chloroform: iso-amyl alcohol (25:24:1, volume/volume) and precipitated in ethyl alcohol. The recipient vector-fragment from pYAM7SP8 (Example 3) was prepared by StuI- SpeI restriction,

purified by extraction with phenol: chloroform:iso-amyl alcohol (25:24:1, volume/volume) and precipitated in ethyl alcohol. The E.coli strain, XL1-Blue [Bullock, W.O., Fernande, J.M., and Short, J.M. Biotechniques 5: 376-379 (1987)], was transformed with the ligation mixture that contained the above DNA fragments, and ampicillin resistant clones were selected.

[0364] Based on restriction analysis, a plasmid clone containing an insert of the expected size, designated pYAM7SP8-NAP42, was retained for further characterization. Sequence determination of the clone confirmed correct insertion of the mature coding region in fusion with the PHO1/alpha-factor prepro leader signal, as predicted by the construction scheme, as well as the absence of unwanted mutations in the coding region.

[0365] About 10 micrograms of pYAM 7SP-NAP 42 plasmid were electroporated into *Pichia* strain GTS115 (his4), described in Example 3. The plasmid was previously digested by NotI enzyme, targeting the integration event at the AOX1 chromosomal locus.

[0366] The His⁺ transformants were selected as described in Example 3. Single colonies (n=90) from the electroporation were grown in wells of a 96-well plate containing 100 microliters of glycerol-minimal medium for 24 hours on a plate-shaker at room temperature. One liter of the glycerol-minimal medium contained 13.4 g Yeast Nitrogen Base without amino acids (DIFCO); 400 micrograms biotin; 10 ml glycerol; and 10 mM potassium phosphate (pH 6.0).

[0367] The cells were pelleted and resuspended in fresh methanol-minimal medium (same composition as above except that the 10 ml glycerol was replaced by 5 ml methanol) to induce the AOX1 promoter. After an additional incubation period of 24 hours with agitation at room temperature, 10 microliters of culture supernatants were tested by the Prothrombin Time Assay (Example B). The presence of secreted AcaNAP42 was detected by the prolongation of the coagulation time of human plasma.

Example 17

Expression of AcaNAPc2/Proline in *P. pastoris*.

[0368] To enhance stability and the expression level of AcaNAPc2, a mutant cDNA was constructed that encoded an additional proline residue at the C-terminus of the protein (AcaNAPc2/Proline or "AcaNAPc2P"). The expression vector, pYAM7SP8-NAPc2/Proline, was made in the same manner as described in Example 16. The oligo 8 primer is the N-terminal primer with DraI restriction site and the oligo 9 primer is the C-terminal primer containing XbaI site and the amino acid codon, TGG, to add one Proline residue to the C-terminal of the natural form of AcaNAPc2.

oligo 8: 5'GCG TTT AAA GCA ACG ATG CAG TGT GGT G^{3'} [SEQ. ID. NO. 126]

oligo 9: 5'C GCT CTA GAA GCT TCA TGG GTT TCG AGT TCC
GGG ATA TAT AAA GTC^{3'} [SEQ. ID. NO. 127]

[0369] Following digestion of the amplification product (approximately 270 bp) with DraI and XbaI, the amplification product was purified and ligated with the vector-fragment from pYAM7SP8 prepared by StuI-SpeI restriction. A plasmid clone containing the AcaNAPc2/Proline insert was confirmed by DNA sequencing and designated pYAM7SP8-NAPc2/Proline.

[0370] The vector, pYAM7SP8-NAPc2/Proline, was used to transform strain GTS115 (his) as described in Example 16. Transformants were selected and grown according to Example 16. The presence of secreted AcaNAPc2/proline in the growth media was detected by the prolongation of the coagulation time of human plasma (see Example B).

Example 18

Alternative Methods of Purifying AcaNAP5, AcaNAPc2 and AcaNAPc2P

(A) AcaNAP5

[0371] An alternative method of purifying AcaNAP5 from fermentation media is as follows. Cells were removed from a fermentation of a *Pichia pastoris* strain expressing AcaNAP5, and the media was frozen. The purification protocol was initiated by thawing frozen media overnight at 4°C, then diluting it with approximately four parts Milli Q water to lower the conductivity below 8mS. The pH was adjusted to 3.5, and the media was filtered using a 0.22 µm cellulose acetate filter (Corning Inc., Corning, NY).

[0372] The activity of the NAP-containing material was determined in the prothrombin time clotting assay at the

beginning of the purification procedure and at each step in the procedure using the protocol in Example B.

[0373] The filtered media was applied to a Pharmacia SP-Fast Flow column, at a flow rate of 60 ml/min at ambient temperature, and the column was washed with 10 column volumes of 50 mM citrate/phosphate, pH 3.5. Step elution was performed with 100 mM NaCl, 250 mM NaCl, and then 1000 mM NaCl, all in 50 mM citrate/phosphate, pH 3.5. PT activity was detected in the 250 mM NaCl eluate. The total eluate was dialyzed until the conductivity was below 8mS.

[0374] The pH of the material was adjusted to 4.5 with acetic acid, and then applied to a sulfoethyl aspartamide column at ambient temperature. Approximately 10 column volumes of 50 mM ammonium acetate, pH 4.5/40% acetonitrile, were used to wash the column. The column was eluted with 50 mM ammonium acetate, pH 4.5/40% acetonitrile/ 200 mM NaCl, and the eluate was dialyzed or diafiltered as before.

[0375] The eluate was adjusted to 0.1% TFA, applied to a Vydac C18 protein/peptide reverse phase column at ambient temperature, and eluted using 0.1% TFA/ 19% acetonitrile, followed by 0.1% TFA/25% acetonitrile, at a flow rate of 7 ml/min. NAP was detected in and recovered from the 0.1% TFA/25% acetonitrile elution.

(B) AcaNAPc2 and AcaNAPc2P

[0376] AcaNAPc2 or AcaNAPc2P can be purified as described above with the following protocol modifications. After thawing and diluting the media to achieve a conductivity below 8mS, the pH of the AcaNAPc2-containing media was adjusted to pH 5.0 using NaOH. The filtered media was applied to a Pharmacia Q Fast Flow column, at a flow rate of 60 ml/min at ambient temperature, and the column was washed with 10 column volumes of 50 mM acetic acid, pH 5.0. Step elution was performed with 100 mM NaCl, 250 mM NaCl, and then 1000 mM NaCl, all in 50 mM acetic acid, pH 5.0. PT activity was detected in the 250 mM NaCl eluate. The total eluate was dialyzed until the conductivity was below 8mS, and the protocol outlined above was followed using sulfoethyl aspartamide and RP-HPLC chromatography.

Example A.

Factor Xa Amidolytic Assay.

[0377] The ability of NAPs of the present invention to act as inhibitors of factor Xa catalytic activity was assessed by determining the NAP-induced inhibition of amidolytic activity catalyzed by the human enzyme, as represented by K_i^* values.

[0378] The buffer used for all assays was HBSA (10 mM HEPES, pH 7.5, 150 mM sodium chloride, 0.1% bovine serum albumin). All reagents were from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

[0379] The assay was conducted by combining in appropriate wells of a Corning microtiter plate, 50 microliters of HBSA, 50 microliters of the test NAP compound diluted (0.025 - 25nM) in HBSA (or HBSA alone for uninhibited velocity measurement), and 50 microliters of the Factor Xa enzyme diluted in HBSA (prepared from purified human factor X obtained from Enzyme Research Laboratories (South Bend, IN) according to the method described by Bock, P.E. et al., Archives of Biochem. Biophys. 273: 375 (1989). The enzyme was diluted into HBSA prior to the assay in which the final concentration was 0.5 nM). Following a 30 minute incubation at ambient temperature, 50 microliters of the substrate S2765 (N-alpha-benzoyloxycarbonyl-D-argininyl-L-glycyl-L-arginine-p-nitroanilide dihydrochloride, obtained from Kabi Diagnostica (or Kabi Pharmacia Hepar Inc., Franklin, OH) and made up in deionized water followed by dilution in HBSA prior to the assay) were added to the wells yielding a final total volume of 200 microliters and a final concentration of 250 micromolar (about 5-times K_m). The initial velocity of chromogenic substrate hydrolysis was measured by the change in absorbance at 405nm using a Thermo Max[®] Kinetic Microplate Reader (Molecular Devices, Palo alto, CA) over a 5 minute period in which less than 5% of the added substrate was utilized.

[0380] Ratios of inhibited pre-equilibrium, steady-state velocities containing NAP (V_i) to the uninhibited velocity of free fXa alone (V_o) were plotted against the corresponding concentrations of NAP. These data were then directly fit to an equation for tight-binding inhibitors [Morrison, J.F., and Walsh, C.T., Adv. Enzymol. 61:201-300 (1988)], from which the apparent equilibrium dissociation inhibitory constant K_i^* was calculated.

[0381] Table 1 below gives the K_i^* values for the test compounds AcaNAP5 [SEQ. ID. NO. 4], AcaNAP6 [SEQ. ID. NO. 6], and AcaNAPc2 [SEQ. ID. NO. 59], prepared as described in Examples 3, 4, and 15, respectively. The data show the utility of AcaNAP5 and AcaNAP6 as potent *in vitro* inhibitors of human FXa. In contrast, AcaNAPc2 did not effectively inhibit FXa amidolytic activity indicating that it does not affect the catalytic activity of free fXa.

Table 1

Compound	K_i^* (pM)
AcaNAP5	43 ± 5

(continued)

Compound	Ki* (pM)
AcaNAP6	996 ± 65
AcaNAPc2	NI ^a
^a NI=no inhibition; a maximum of 15% inhibition was observed up to 1μM.	

Example B.Prothrombin Time (PT) and Activated Partial Thromboplastin Time (aPTT) Assays.

[0382] The *ex vivo* anticoagulant effects of NAPs of the present invention in human plasma were evaluated by measuring the prolongation of the activated partial thromboplastin time (aPTT) and prothrombin time (PT) over a broad concentration range of each inhibitor.

[0383] Fresh frozen pooled normal citrated human plasma was obtained from George King Biomedical, Overland Park, KS. Respective measurements of aPTT and PT were made using the Coag-A-Mate RA4 automated coagulometer (General Diagnostics, Organon Technica, Oklahoma City, OK) using the Automated aPTT Platelin® L reagent (Organon Technica, Durham, NC) and Simplastin® Excel (Organon Technica, Durham, NC) respectively, as initiators of clotting according to the manufacturer's instructions.

[0384] The assays were conducted by making a series of dilutions of each tested NAP in rapidly thawed plasma followed by adding 200 microliters or 100 microliters of the above referenced reagents to the wells of the assay carousel for the aPTT or PT measurements, respectively. Alternatively, the NAPs were serially diluted into HBSA and 10 μl of each dilution were added to 100μl of normal human plasma in the wells of the Coag-A-Mate assay carousel, followed by addition of reagent.

[0385] Concentrations of NAP were plotted against clotting time, and a doubling time concentration was calculated, i.e., a specified concentration of NAP that doubled the control clotting time of either the PT or the aPTT. The control clotting times (absence of NAP) in the PT and APTT were 12.1 seconds and 28.5 seconds, respectively.

[0386] Table 2 below shows the *ex vivo* anticoagulant effects of AcaNAP5 [SEQ. ID. NO. 4], AcaNAP6 [SEQ. ID. NO. 6], AcaNAPc2 [SEQ. ID. NO. 59], and AceNAP4 [SEQ. ID. NO. 62] and Pro-AcaNAP5 [SEQ. ID. NO. 7] represented by the concentration of each that doubled (doubling concentration) the control clotting time of normal human plasma in the respective PT and APTT clotting assays relative to a control assay where no such NAP was present. The data show the utility of these compounds as potent anticoagulants of clotting human plasma. The data also demonstrate the equivalency of native NAP and recombinant NAP.

Table 2

Compound	Doubling Concentration (nM) in the PT	Doubling Concentration (nM) in the aPTT
AcaNAP5 ^a	43 ± 8	87 ± 4
AcaNAP6 ^a	37 ± 3	62 ± 0
AcaNAPc2 ^a	15 ± 1	105 ± 11
AceNAP4 ^a	40 ± 4	115 ± 12
AcaNAP5 ^b	26.9	76.2
AcaNAP5 ^c	39.2	60.0
Pro-AcaNAP5 ^d	21.9	31.0
^a Made in <i>Pichia pastoris</i> . ^b Native protein. ^c Made in <i>Pichia pastoris</i> (different recombinant batch than (a)). ^d Made in COS cells.		

[0387] Figures 10A and 10B also show NAP-induced prolongation of the PT (Figure 10A) and aPTT (Figure 10B) in

a dose-dependent manner.

Example C

Prothrombinase inhibition assay

[0388] The ability of NAP of the present invention to act as an inhibitor of the activation of prothrombin by Factor Xa that has been assembled into a physiologic prothrombinase complex was assessed by determining the respective inhibition constant, K_i^* .

[0389] Prothrombinase activity was measured using a coupled amidolytic assay, where a preformed complex of human FXa, human Factor Va (FVa), and phospholipid vesicles first activates human prothrombin to thrombin. The amidolytic activity of the generated thrombin is measured simultaneously using a chromogenic substrate. Purified human FVa was obtained from Haematologic Technologies, Inc. (Essex Junction, VT). Purified human prothrombin was purchased from Celsus Laboratories, Inc. (Cincinnati, OH). The chromogenic substrate Pefachrome t-PA (CH_3SO_2 -D-hexahydrotyrosine-glycyl-L-arginine-p-nitroanilide) from Pentapharm Ltd (Basel, Switzerland) was purchased from Centerchem, Inc. (Tarrytown, NY). The substrate was reconstituted in deionized water prior to use. Phospholipid vesicles were made, consisting of phosphatidyl choline (67%, w/v), phosphatidyl glycerol (16%, w/v), phosphatidyl ethanolamine (10%, w/v), and phosphatidyl serine (7%, w/v) in the presence of detergent, as described by Ruf et al. [Ruf, W., Miles, D.J., Rehemtulla, A., and Edgington, T.S. Methods in Enzymology 222: 209-224 (1993)]. The phospholipids were purchased from Avanti Polar Lipids, (Alabaster, Alabama).

[0390] The prothrombinase complex was formed in a polypropylene test tube by combining FVa, FXa, and phospholipid vesicles (PLV) in HBSA containing 3 mM CaCl_2 for 10 min. In appropriate wells of a microtiter plate, 50 μl of the complex were combined with 50 μl of NAP diluted in HBSA, or HBSA alone (for V_o (uninhibited velocity) measurement). Following an incubation of 30 min at room temperature, the triplicate reactions were initiated by the addition of a substrate solution, containing human prothrombin and the chromogenic substrate for thrombin, Pefachrome tPA. The final concentration of reactants in a total volume of 150 μL of HBSA was: NAP (.025-25 nM), FXa (250 fM), PLV (5 μM), prothrombin (250 nM), Pefachrome tPA (250 μM , 5X K_m), and CaCl_2 (3 mM).

[0391] The prothrombinase activity of fXa was measured as an increase in the absorbance at 405 nm over 10 min (velocity); exactly as described in Example A, under steady-state conditions. The absorbance increase was sigmoidal over time, reflecting the coupled reactions of the activation of prothrombin by the FXa-containing prothrombinase complex, and the subsequent hydrolysis of Pefachrome tPA by the generated thrombin. The data from each well of a triplicate were combined and fit by reiterative, linear least squares regression analysis, as a function of absorbance versus time², as described [Carson, S.D. Comput. Prog. Biomed. 19: 151-157 (1985)] to determine the initial velocity (V_i) of prothrombin activation. Ratios of inhibited steady-state initial velocities containing NAP (V_i) to the uninhibited velocity of prothrombinase fXa alone (V_o) were plotted against the corresponding concentrations of NAP. These data were directly fit to the equation for tight-binding inhibitors, as in Example A above, and the apparent equilibrium dissociation inhibitory constant K_i^* was calculated.

[0392] Table 3 below gives the dissociation inhibitor constant (K_i^*) of recombinant AcaNAP5 [SEQ. ID. NO. 4], AcaNAP6 [SEQ. ID. NO. 6] and AcaNAPc2 [SEQ. ID. NO. 59] (all made in *Pichia pastoris* as described) against the activation of prothrombin by human fXa incorporated into a prothrombinase complex. These data show the utility of these compounds as inhibitors of human FXa incorporated into the prothrombinase complex.

Table 3

Compound	K_i^* (pM)
AcaNAP5	144 \pm 15
AcaNAP6	207 \pm 40
AcaNAPc2	2385 \pm 283

[0393] The data presented in Examples A, B, and C suggest that AcaNAP5 and AcaNAP6 may be interacting with FXa in a similar manner that involves directly restricting access of both the peptidyl and macromolecular substrate (prothrombin) to the catalytic center of the enzyme. In contrast, AcaNAPc2 appears to be interacting with FXa in a way that only perturbs the macromolecular interactions of this enzyme with either the substrate and/or cofactor (Factor Va), while not directly inhibiting the catalytic turnover of the peptidyl substrate (see Table 1).

Example DIn vitro Enzyme Assays for Activity Specificity Determination

[0394] The ability of NAP of the present invention to act as a selective inhibitor of FXa catalytic activity or TF/VIIa activity was assessed by determining whether the test NAP would inhibit other enzymes in an assay at a concentration that was 100-fold higher than the concentration of the following related serine proteases: thrombin, Factor Xa, Factor XIa, Factor XIIa, kallikrein, activated protein C, plasmin, recombinant tissue plasminogen activator (rt-PA), urokinase, chymotrypsin, and trypsin. These assays also are used to determine the specificity of NAPs having serine protease inhibitory activity.

(1) General protocol for enzyme inhibition assays

[0395] The buffer used for all assays was HBSA (Example A). All substrates were reconstituted in deionized water, followed by dilution into HBSA prior to the assay. The amidolytic assay for determining the specificity of inhibition of serine proteases was conducted by combining in appropriate wells of a Corning microtiter plate, 50 μ l of HBSA, 50 μ l of NAP at a specified concentration diluted in HBSA, or HBSA alone (uninhibited control velocity, V_o), and 50 μ l of a specified enzyme (see specific enzymes below). Following a 30 minute incubation at ambient temperature, 50 μ l of substrate were added to triplicate wells. The final concentration of reactants in a total volume of 200 μ l of HBSA was: NAP (75 nM), enzyme (750 pM), and chromogenic substrate (as indicated below). The initial velocity of chromogenic substrate hydrolysis was measured as a change in absorbance at 405nm over a 5 minute period, in which less than 5% of the added substrate was hydrolyzed. The velocities of test samples, containing NAP (V_i) were then expressed as a percent of the uninhibited control velocity (V_o) by the following formula: $V_i/V_o \times 100$, for each of the enzymes.

(2) Specific enzyme assays(a) Thrombin Assay

[0396] Thrombin catalytic activity was determined using the chromogenic substrate Pefachrome t-PA (CH_3SO_2 -D-hexahydrotyrosine-glycyl-L-arginine-p-nitroaniline, obtained from Pentapharm Ltd., Basel, Switzerland). The final concentration of Pefachrome t-PA was 250 μ M (about 5-times K_m). Purified human alpha-thrombin was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

(b) Factor Xa Assay

[0397] Factor Xa catalytic activity was determined using the chromogenic substrate S-2765 (N-benzyloxycarbonyl-D-arginine-L-glycine-L-arginine-p-nitroaniline), obtained from Kabi Pharmacia Hepar, Inc. (Franklin, OH). All substrates were reconstituted in deionized water prior to use. The final concentration of S-2765 was 250 μ M (about 5-times K_m). Purified human Factor X was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN) and Factor Xa (FXa) was activated and prepared from Factor X as described [Bock, P.E., Craig, P.A., Olson, S.T., and Singh, P. Arch. Biochem. Biophys. 273:375-388 (1989)].

(c) Factor XIa Assay

[0398] Factor FXIa catalytic activity was determined using the chromogenic substrate S-2366 (L-Pyroglutamyl-L-prolyl-L-arginine-p-nitroaniline, obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2366 was 750 μ M. Purified human FXIa was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

(d) Factor XIIa Assay

[0399] Factor FXIIa catalytic activity was determined using the chromogenic substrate Spectrozyme FXIIa (H-D-CHT-L-glycyl-L-arginine-p-nitroaniline), obtained from American Diagnostica, Greenwich, CT). The final concentration of Spectrozyme FXIIa was 100 μ M. Purified human FXIIa was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

(e) Kallikrein Assay

[0400] Kallikrein catalytic activity was determined using the chromogenic substrate S-2302 (H-D-prolyl-L-phenylala-

nyl-L-arginine-p-nitroaniline, obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2302 was 400 μ M. Purified human kallikrein was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

(f) Activated Protein C (aPC)

[0401] Activated Protein C catalytic activity was determined using the chromogenic substrate Spectrozyme PCa (H-D-lysyl(-Cbo)-L-prolyl-L-arginine-p-nitroaniline) obtained from American Diagnostica Inc. (Greenwich, CT). The final concentration was 400 μ M (about 4 times K_m). Purified human aPC was obtained from Hematologic Technologies, Inc. (Essex Junction, VT).

(g) Plasmin Assay

[0402] Plasmin catalytic activity was determined using the chromogenic substrate S-2366 (L-Pyroglutamyl-L-prolyl-L-arginine-p-nitroaniline, obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2366 was 300 μ M (about 4 times K_m). Purified human plasmin was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN).

(h) Recombinant tissue plasminogen activator (rt-PA)

[0403] rt-PA catalytic activity was determined using the substrate, Pefachrome t-PA (CH_3SO_2 -D-hexahydrotyrosine-glycyl-L-arginine-p-nitroaniline, obtained from Pentapharm Ltd., Basel, Switzerland). The final concentration was 500 μ M (about 3 times K_m). Human rt-PA (Activase®) was obtained from Genentech, Inc. (So. San Francisco, CA).

(i) Urokinase

[0404] Urokinase catalytic activity was determined using the substrate S-2444 (L-Pyroglutamyl-L-glycyl-L-arginine-p-nitroaniline, obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2444 was 150 μ M (about 7 times K_m). Human urokinase (Abbokinase®), purified from cultured human kidney cells, was obtained from Abbott Laboratories (North Chicago, IL).

(j) Chymotrypsin

[0405] Chymotrypsin catalytic activity was determined using the chromogenic substrate, S-2586 (Methoxy-succinyl-L-argininyl-L-prolyl-L-tyrosine-p-nitroaniline, which was obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2586 was 100 μ M (about 8 times K_m). Purified (3X-crystallized; CDI) bovine pancreatic-chymotrypsin was obtained from Worthington Biochemical Corp. (Freehold, NJ).

(k) Trypsin

[0406] Trypsin catalytic activity was determined using the chromogenic substrate S-2222 (N-benzoyl-L-isoleucyl-L-glutamyl [-methyl ester]-L-arginine-p-nitroaniline, which was obtained from Kabi Pharmacia Hepar, Franklin, OH). The final concentration of S-2222 was 300 μ M (about 5 times K_m). Purified human pancreatic trypsin was obtained from Scripps Laboratories (San Diego, CA).

[0407] Table 4 lists the inhibition of the amidolytic activity of FXa and 10 additional serine proteases by either recombinant AcaNAP-5 [SEQ. ID. NO. 4] or recombinant AcaNAP-6 [SEQ. ID. NO. 6] (both expressed in *Pichia pastoris*, as described), expressed as percent of control velocity. These NAPs demonstrate a high degree of specificity for the inhibition of FXa compared to the other, related serine proteases.

Table 4

Enzyme	% Control Velocity	% Control Velocity
	+ AcaNAP5	+AcaNAP6
FXa	1 \pm 1	14 \pm 1
FIIa	104 \pm 5	98 \pm 3
FXIa	34 \pm 12	98 \pm 3
FXIIa	103 \pm 6	100 \pm 4

(continued)

Enzyme	% Control Velocity	% Control Velocity
kallikrein	102 ± 4	101 ± 3
aPC	95 ± 2	98 ± 1
plasmin	111 ± 6	113 ± 12
r-tPA	96 ± 9	96 ± 7
urokinase	101 ± 14	96 ± 2
chymotrypsin	105 ± 0	100 ± 11
trypsin	98 ± 6	93 ± 4

[0408] Table 5 lists the inhibitory effect of recombinant AcaNAPc2 [SEQ. ID. NO. 59] and recombinant AceNAP4 [SEQ. ID. NO. 62] (both expressed in *Pichia pastoris*, as described) on the amidolytic activity of 11 selected serine proteases. Inhibition is expressed as percent of control velocity. These data demonstrate that these NAPs possess a high degree of specificity for the serine proteases in Table 5.

Table 5

Enzyme	% Control Velocity	% Control Velocity
	+ AcaNAPc2	+ AceNAP4
FXa	84 ± 3	76 ± 3
FIIa	99 ± 3	93 ± 3
FXIa	103 ± 4	96 ± 1
FXIIa	97 ± 1	102 ± 2
kallikrein	101 ± 1	32 ± 1
aPC	97 ± 3	103 ± 1
plasmin	107 ± 9	100 ± 1
r-tPA	96 ± 2	108 ± 3
urokinase	97 ± 1	103 ± 4
chymotrypsin	99 ± 0	96 ± 4
trypsin	93 ± 4	98 ± 4

Example E

Assays for measuring the inhibition of the fVIIa/TF complex by NAP

(1) fVIIa/TF fIX activation assay

[0409] This Example measures the ability of NAPs of the present invention to act as an inhibitor of the catalytic complex of fVIIa/TF, which has a primary role in initiation of the coagulation response in the *ex vivo* prothrombin time assay (Example B). Activation of tritiated Factor IX by the rFVIIa/rTF/PLV complex was assessed by determining the respective intrinsic inhibition constant, K_i^* .

[0410] Lyophilized, purified, recombinant human factor VIIa was obtained from BiosPacific, Inc. (Emeryville, CA), and reconstituted in HBS (10 mM HEPES, pH 7.5, 150 mM sodium chloride) prior to use. Purified human Factor X was obtained from Enzyme Research Laboratories, Inc. (South Bend, IN) and Factor Xa (free FXa) was activated and prepared from Factor X as described (Bock, P.E., Craig, P.A., Olson, S.T., and Singh, P. Arch. Biochem. Biophys. 273:375-388 (1989)). Active site-blocked human Factor Xa (EGR-FXa), which had been irreversibly inactivated with L-Glutamyl-L-glycyl-L-arginyl chloromethylketone, was obtained from Haematologic Technologies, Inc. (Essex Junction, VT). Recombinant human tissue factor (rTF) was produced by a baculovirus-expression system, and purified to homo-

geneity by monoclonal antibody affinity chromatography (Corvas International, Inc., San Diego, CA).

[0411] The purified rTF apoprotein was incorporated into phospholipid vesicles (rTF/PLV), consisting of phosphatidyl choline (75%, w/v) and phosphatidyl serine (25%, w/v) in the presence of detergent, as described by Ruf et al. (Ruf, W., Miles, D.J., Rehemtulla, A., and Edgington, T.S. *Methods in Enzymology* 222: 209-224 (1993)). The phospholipids were purchased from Avanti Polar Lipids, (Alabaster, Alabama). The buffer used for all assays was HBSA, HBS containing 0.1% (w/v) bovine serum albumin. All reagents were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated.

[0412] The activation of human ^3H -Factor IX (FIX) by the rFVIIa/rTF complex was monitored by measuring the release of the radiolabelled activation peptide. Purified human fIX was obtained from Haematologic Technologies, Inc. (Essex Junction, VT), and radioactively labelled by reductive tritiation as described (Van Lenten & Ashwell, 1971, JBC 246, 1889-1894). The resulting tritiated preparation of FIX had a specific activity of 194 clotting units/mg as measured in immune-depleted FIX deficient plasma (Ortho), and retained 97% of its activity. The radiospecific activity was 2.7×10^8 dpm/mg. The K_m for the activation of ^3H -FIX by rFVIIa/rTF/PLV was 25 nM, which was equivalent to the K_m obtained for untreated (unlabelled) FIX.

[0413] The assay for K_i^* determinations was conducted as follows: rFVIIa and rTF/PLV were combined in a polypropylene test tube, and allowed to form a complex for 10 min in HBSA, containing 5 mM CaCl_2 . Aliquots of rFVIIa/rTF/PLV complex were combined in the appropriate polypropylene microcentrifuge tubes with EGR-FXa or free FXa, when included, and either the NAP test compound at various concentrations, after dilution into HBSA, or HBSA alone (as V_o (uninhibited velocity) control). Following an incubation of 60 min at ambient temperature, reactions were initiated by the addition of ^3H -FIX. The final concentration of the reactants in 420 μL of HBSA was: rFVIIa [50 pM], rTF [2.7 nM], PLV [6.4 micromolar], either EGR-FXa or free FXa [300 pM], recombinant NAP [5-1,500 pM], ^3H -FIX [200 nM], and CaCl_2 [5mM]. In addition, a background control reaction was run that included all of the above reactants, except rFVIIa.

[0414] At specific time points (8, 16, 24, 32, and 40 min), 80 μL of the reaction mixture was added to an eppendorf tube that contained an equal volume of 50 mM EDTA in HBS with 0.5% BSA to stop the reaction; this was followed by the addition of 160 μL of 6% (w/v) trichloroacetic acid. The protein was precipitated, and separated from the supernatant by centrifugation at 16,000Xg for 6 min at 4°C . The radioactivity contained in the resulting supernatant was measured by removing triplicate aliquots that were added to Scintiverse BD (Fisher Scientific, Fairlawn, NJ), and quantitated by liquid scintillation counting. The control rate of activation was determined by linear regression analysis of the soluble counts released over time under steady-state conditions, where less than 5% of the tritiated FIX was consumed. The background control (<1.0% of control velocity) was subtracted from all samples. Ratios of inhibited steady-state velocities (V_i), in the presence of a NAP, to the uninhibited control velocity of rFVIIa/TF alone (V_o) were plotted against the corresponding concentrations of NAP. These data were then directly fit to an equation for tight-binding inhibitors [Morrison, J.F., and Walsh, C.T., *Adv. Enzymol.* 61:201-300 (1988)], from which the apparent equilibrium dissociation inhibitory constant K_i^* was calculated.

[0415] The data for recombinant AcaNAP5, AcaNAP6, AcaNAPc2, and AceNAP4 (prepared as described) is presented in Table 6 following Section B, below.

(2) Factor VIIa/Tissue factor amidolytic assay

[0416] The ability of NAPs of the present invention to act as an inhibitor of the amidolytic activity of the fVIIa/TF complex was assessed by determining the respective inhibition constant, K_i^* , in the presence and absence of active site-blocked human Factor Xa (EGR-fXa).

[0417] rFVIIa/rTF amidolytic activity was determined using the chromogenic substrate S-2288 (H-D-isoleucyl-L-prolyl-L-arginine-p-nitroaniline), obtained from Kabi Pharmacia Hepar, Inc. (Franklin, OH). The substrate was reconstituted in deionized water prior to use. rFVIIa and rTF/PLV were combined in a polypropylene test tube, and allowed to form a complex for 10 min in HBSA, containing 3 mM CaCl_2 . The assay for K_i^* determinations was conducted by combining in appropriate wells of a Corning microtiter plate 50 μL of the rFVIIa/rTF/PLV complex, 50 μL of EGR-FXa, and 50 μL of either the NAP test compound at various concentrations, after dilution into HBSA, or HBSA alone (for V_o (uninhibited velocity) measurement). Following an incubation of 30 min at ambient temperature, the triplicate reactions were initiated by adding 50 μL of S-2288. The final concentration of reactants in a total volume of 200 μL of HBSA was: recombinant NAP (.025-25 nM), rFVIIa (750 pM), rTF (3.0 nM), PLV (6.4 micromolar), EGR-FXa (2.5 nM), and S-2288 (3.0 mM, 3X K_m).

[0418] The amidolytic activity of rFVIIa/rTF/PLV was measured as a linear increase in the absorbance at 405 nm over 10 min (velocity), using a Thermo Max[®] Kinetic Microplate Reader (Molecular Devices, Palo Alto, CA), under steady-state conditions, where less than 5% of the substrate was consumed. Ratios of inhibited pre-equilibrium, steady-state velocities (V_i), in the presence of NAP, to the uninhibited velocity in the presence of free fXa alone (V_o) were plotted against the corresponding concentrations of NAP. These data were then directly fit to the same equation for tight-binding inhibitors, used in Example E.1., from which the apparent equilibrium dissociation inhibitory constant K_i^* was calculated.

[0419] Table 6 below gives the K_i^* values of recombinant AcaNAPc2 [SEQ. ID. NO. 59], AceNAP4 [SEQ. ID. NO. 62],

AcaNAP5 [SEQ. ID. NO. 4], and AcaNAP6 [SEQ. ID. NO. 6] (prepared in *Pichia pastoris*, as described) in inhibitory assays of rFVIIa/rTF activity. The data shows the utility of AcaNAPc2 and AceNAP4 as potent inhibitors of the human rFVIIa/rTF/PLV complex in the absence and presence of either free FXa or active site-blocked FXa. The *in vitro* activity of AcaNAPc2P (see Example 17) was substantially the same as AcaNAPc2.

Table 6

	Ki* (pM)				
	Amidolytic Assay		³ H-FIX Activation		
NAP Compound	No FXa Addition	Plus FXa	EGR- No FXa Addition	+ free FXa	+ EGR-FXa
AcaNAPc2	NI	36 ± 20	NI	35 ± 5	8.4 ± 1.5
AceNAP4	59,230 ± 3,600	378 ± 37	ND	ND	ND
AcaNAP5 AcaNAP6	NI	NI	NI	NI	NI
	NI	NI	NI	NI	NI
NI=no inhibition ND=not determined					

Example F

In vivo Models of NAP activity

(1) Evaluation of the antithrombotic activity of NAP in the rat model of FeCl₃-induced platelet-dependent arterial thrombosis

[0420] The antithrombotic (prevention of thrombus formation) properties of NAP were evaluated using the established experimental rat model of acute vascular thrombosis.

[0421] The rat FeCl₃ model is a well characterized model of platelet dependent, arterial thrombosis which has been used to evaluate potential antithrombotic compounds. Kurz, K. D., Main, B. W., and Sandusky, G. E., *Thromb. Res.*, 60: 269-280 (1990). In this model a platelet-rich, occlusive thrombus is formed in a segment of the rat carotid artery treated locally with a fresh solution of FeCl₃ absorbed to a piece of filter paper. The FeCl₃ is thought to diffuse into the treated segment of artery and cause de-endothelialization of the affected vessel surface. This results in the exposure of blood to subendothelial structures which in turn cause platelet adherence, thrombin formation and platelet aggregation. The net result is occlusive thrombus formation. The effect of a test compound on the incidence of occlusive thrombus formation following application of FeCl₃ is monitored by ultrasonic flowtometry and is used as the primary end point. The use of flowtometry to measure carotid artery blood flow, is a modification of the original procedure in which thermal detection of clot formation was employed. Kurz, K. D., Main, B. W., and Sandusky, G. E., *Thromb. Res.*, 60: 269-280 (1990).

(a) Intravenous administration

[0422] Male Harlan Sprague Dawley rats (420-450 g) were acclimated at least 72 hours prior to use and fasted for 12 hours prior to surgery with free access to water. The animals were prepared, anesthetized with Nembutal followed by the insertion of catheters for blood pressure monitoring, drug and anesthesia delivery. The left carotid artery was isolated by making a midline cervical incision followed by blunt dissection and spreading techniques to separate a 2 cm segment of the vessel from the carotid sheath. A silk suture is inserted under the proximal and distal ends of the isolated vessel to provide clearance for the placement of a ultrasonic flow probe (Transonic) around the proximal end of the vessel. The probe is then secured with a stationary arm.

[0423] Following surgery the animals were randomized in either a control (saline) or treatment (recombinant AcaNAP5) group. The test compound (prepared in *P. pastoris* according to Example 3) was administered as a single intravenous bolus at the doses outlined in Table 7 after placement of the flow probe and 5 min prior to the thrombogenic stimulus. At t=0, a 3mm diameter piece of filter paper (Whatman #3) soaked with 10 µL of a 35% solution of fresh FeCl₃ (made up in water) was applied to the segment of isolated carotid artery distal to the flow probe. Blood pressure, blood flow, heart rate, and respiration were monitored for 60 minutes. The incidence of occlusion (defined as the attainment of zero blood flow) was recorded as the primary end point.

[0424] The efficacy of AcaNAP5 [SEQ. ID. NO. 4] as an antithrombotic agent in preventing thrombus formation in this in vivo model was demonstrated by the dose-dependent reduction in the incidence of thrombotic occlusion, as shown

in Table 7 below.

Table 7

Treatment Group	Dose (mg/kg)	n	Incidence of Occlusion
Saline	-----	8	8/8
AcaNAP5	0.001	8	7/8
AcaNAP5	0.003	8	5/8
AcaNAP5	0.01	8	3/8*
AcaNAP5	0.03	8	1/8*
AcaNAP5	0.1	8	0/8*
AcaNAP5	0.3	4	0/4*
AcaNAP5	1.0	2	0/2*
*-p≤0.05 from saline control by Fishers test			

[0425] The effective dose which prevents 50% of thrombotic occlusions in this model (ED₅₀) can be determined from the above data by plotting the incidence of occlusion versus the dose administered. This allows a direct comparison of the antithrombotic efficacy of AcaNAP5 with other antithrombotic agents which have also been evaluated in this model as described above. Table 8 below lists the ED₅₀ values for several well known anticoagulant agents in this model compared to AcaNAP5.

Table 8

Compound	ED ₅₀ ^a
Standard Heparin	300 U/kg
Argatroban	3.8 mg/kg
Hirulog TM	3.0 mg/kg
rTAP ^b	0.6 mg/kg
AcaNAP5	0.0055 mg/kg
^a ED ₅₀ is defined as the dose that prevents the incidence of complete thrombotic occlusion in 50% of animals tested b-recombinant Tick Anticoagulant Peptide, Vlasuk et al. Thromb. Haemostas. 70: 212-216 (1993)	

(b) Subcutaneous administration

[0426] The antithrombotic effect of AcaNAP5 compared to Low Molecular Weight heparin (Enoxaparin; Lovenox, Rhone-Poulenc Rorer) after subcutaneous administration was evaluated in rats using the FeCl₃ model. The model was performed in an identical manner to that described above with the exception that the compound was administered subcutaneously and efficacy was determined at two different times: 30 and 150 minutes after administration. To accomplish this, both carotid arteries were employed in a sequential manner. The results of these experiments indicate that AcaNAP5 [SEQ. ID. NO. 4] is an effective antithrombotic agent *in vivo* after subcutaneous administration. The results are shown below in Table 9.

Table 9

Compound	30" ED ₅₀ ^a (mg/kg)	150" ED ₅₀ ^a (mg/kg)
Low Molecular Weight Heparin	30.0	15.0

(continued)

Compound	30" ED ₅₀ ^a (mg/kg)	150" ED ₅₀ ^a (mg/kg)
AcaNAP5	0.07	0.015
^a ED ₅₀ is defined as the dose that prevents the incidence of complete thrombotic occlusion in 50% of animals tested.		

(2) Deep Wound Bleeding Measurement

[0427] A model of deep wound bleeding was used to measure the effect of NAP on bleeding and compare the effect with that of Low Molecular Weight Heparin.

[0428] Male rats were anesthetized and instrumented in an identical manner to those undergoing the FeCl₃ model. However, FeCl₃ was not applied to the carotid artery. The deep surgical wound in the neck that exposes the carotid artery was employed to quantify blood loss over time. Blood loss was measured over a period of 3.5 hours following subcutaneous administration of either AcaNAP5 or LMWH. The wound was packed with surgical sponges which were removed every 30 minutes. The sponges were subsequently immersed in Drabkin's reagent (sigma Chemical Co., St. Louis, MO) which lyses the red blood cells and reacts with hemoglobin in a colorimetric fashion. The colorimetric samples were then quantified by measuring absorbance at 550 nM, which provides a determination of the amount of blood in the sponge.

[0429] The dose response characteristics for both test compounds are shown in Figure 15 along with efficacy data for both compounds. AcaNAP5 [SEQ. ID. NO. 4] was much more potent than Low Molecular Weight heparin in preventing occlusive arterial thrombus formation in this model. Furthermore, animals treated with NAP bled less than those treated with Low Molecular Weight heparin.

[0430] The data presented in Tables 7 and 9 and Figure 15 clearly demonstrate the effectiveness of NAP in preventing occlusive thrombus formation in this experimental model. The relevance of this data to preventing human thrombosis is clear when compared to the other anticoagulant agents, listed in Table 8. These agents were been evaluated in the same experimental models described therein, in an identical manner to that described for NAPs, and in this experimental model and have demonstrated antithrombotic efficacy in preventing thrombus formation clinically, as described in the following literature citations: Heparin-Hirsh, J. N. Engl. J. Med 324:1565-1574 1992, Cairns, J.A. et al. Chest 102: 456S-481S (1992); Argatroban-Gold, H.K. et al. J. Am. Coll. Cardiol. 21: 1039-1047 (1993); and Hirulog™-Sharma, G.V.R.K. et al. Am. J. Cardiol. 72: 1357-1360 (1993) and Lid6n, R.M. et al.. Circulation 88: 1495-1501 (1993).

Example G.Pig Model Of Acute Coronary Artery Thrombosis

[0431] The protocol used in these studies is a modification of a thrombosis model which has been reported previously (Lucchesi, B.R., et al., (1994), Brit. J. Pharmacol. 113:1333-1343).

[0432] Animals were anesthetized and instrumented with arterial and venous catheters (left common carotid and external jugular, respectively). A thoracotomy was made in the 4th intercostal space and the heart was exposed. The left anterior descending (LAD) coronary artery was isolated from the overlying connective tissue and was instrumented with a Doppler flow probe and a 17 gauge ligature stenosis. An anodal electrode also was implanted inside the vessel.

[0433] Baseline measurements were taken and the NAP or placebo to be tested was administered via the external jugular vein. Five minutes after administration, a direct current (300 μ A, DC) was applied to the stimulating electrode to initiate intimal damage to the coronary endothelium and begin thrombus formation. Current continued for a period of 3 hours. Animals were observed until either 1 hour after the cessation of current or the death of the animal, whichever came first.

[0434] Table 10 presents data demonstrating the incidence of occlusion in animals administered AcaNAP5 or AcaNAPc2P (see Example 17) at three increasing doses of NAP. The incidence of occlusion in the animals receiving placebo was 8/8 (100%). Time to occlusion in placebo treated animals was 66.6 ± 7.5 min. (mean \pm sem). Vessels in AcaNAP treated pigs that failed to occlude during the 4 hour period of observation were assigned an arbitrary time to occlusion of 240 minutes in order to facilitate statistical comparisons.

[0435] The data demonstrate AcaNAP5 and AcaNAPc2P were similarly efficacious in this setting; both prolonged the time to coronary artery occlusion in a dose dependent manner. Furthermore, both molecules significantly prolonged in time to occlusion at a dose (0.03 mg/kg i.v.) that did not produce significant elevations in bleeding. These data, and other, suggest AcaNAP5 and AcaNAPc2P have favorable therapeutic indices.

Table 10. Comparison of primary endpoints between AcaNAPc2P and AcaNAP5 after intravenous dosing in the pig model of acute coronary artery thrombosis.

Dose (i.v.) (mg/kg)	Incidence of Occlusion		Time of Occlusion (min)		Total Blood Loss (ml)	
	AcaNAP5	AcaNAPc2P	AcaNAP5	AcaNAPc2P	AcaNAP5	AcaNAPc2P
0.01	6/6	6/6	107 ± 13.0	105 ± 6.2	2.8 ± 0.8	1.6 ± 0.3
0.03	5/6	4/6	150 ± 23.2	159 ± 27	5.6 ± 1.4	4.9 ± 1.4
0.10	4/6	2/6†	187 ± 22.9*	215 ± 25*	43.5 ± 18*	17.6 ± 7.9*
† p<0.05 vs saline (8/8), Fisher's Exact; *p<0.05 vs saline, ANOVA, Dunnett's multiple comparison test.						

[deletion(s)]

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Messens, Joris Hila Lieven

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 Glu Glu Cys Asp Gln His Glu Ile Ile His Val
 40 65 70 75

45 <210> 7
 <211> 81
 <212> PRT
 <213> Ascylostoma caninum

50 <400> 7
 Arg Thr Val Arg Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu
 1 5 10 15
 Asp Asp Cys Gly Thr Gln Lys Pro Cys Glu Ala Lys Cys Asn Glu Glu
 55 20 25 30

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Pro Pro Glu Glu Glu Asp Pro Ile Cys Arg Ser Arg Gly Cys Leu Leu
 35 40 45
 5 Pro Pro Ala Cys Val Cys Lys Asp Gly Phe Tyr Arg Asp Thr Val Ile
 50 55 60
 10 Gly Asp Cys Val Arg Glu Glu Glu Cys Asp Gln His Glu Ile Ile His
 65 70 75 80
 Val

15 <210> 8
 <211> 79
 <212> PRT
 <213> Ascylostoma caninum

20 <400> 8

Arg Thr Val Arg Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu
 1 5 10 15
 25 Asp Val Cys Gly Thr Lys Lys Pro Cys Glu Ala Lys Cys Ser Glu Glu
 20 25 30
 Glu Glu Glu Asp Pro Ile Cys Arg Ser Phe Ser Cys Pro Gly Pro Ala
 30 35 40 45
 Ala Cys Val Cys Glu Asp Gly Phe Tyr Arg Asp Thr Val Ile Gly Asp
 50 55 60
 35 Cys Val Lys Glu Glu Glu Cys Asp Gln His Glu Ile Ile His Val
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40 <210> 9
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 <213> Ancylostoma ceylanicum

45 <220>
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 <222> (21)..(590)

50 <220>
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<400> 9

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      Met Ala Val Leu Tyr Ser Val Ala Ile Ala
5      1      5      10
tta cta ctg gta tca caa tgc agt ggg aaa ccg aac aat gtg atg act  98
Leu Leu Leu Val Ser Gln Cys Ser Gly Lys Pro Asn Asn Val Met Thr
      15      20      25
10     aac gct tgt ggt ctt aat gaa tat ttc gct gag tgt ggc aat atg aag  146
Asn Ala Cys Gly Leu Asn Glu Tyr Phe Ala Glu Cys Gly Asn Met Lys
      30      35      40
15     gaa tgc gag cac aga tgc aat gag gag gaa aat gag gaa agg gac gag  194
Glu Cys Glu His Arg Cys Asn Glu Glu Glu Asn Glu Glu Arg Asp Glu
      45      50      55
20     gaa aga ata acg gca tgc ctc atc cgt gtg tgt ttc cgt cct ggt gct  242
Glu Arg Ile Thr Ala Cys Leu Ile Arg Val Cys Phe Arg Pro Gly Ala
      60      65      70
25     tgc gta tgc aaa gac gga ttc tat aga aac aga aca ggc agc tgt gtg  290
Cys Val Cys Lys Asp Gly Phe Tyr Arg Asn Arg Thr Gly Ser Cys Val
      75      80      85      90
30     gaa gaa gat gac tgc gag tac gag aat atg gag ttc att act ttt gca  338
Glu Glu Asp Asp Cys Glu Tyr Glu Asn Met Glu Phe Ile Thr Phe Ala
      95      100      105
35     cca gaa gta ccg ata tgt ggt tcc aac gaa agg tac tcc gac tgc ggc  386
Pro Glu Val Pro Ile Cys Gly Ser Asn Glu Arg Tyr Ser Asp Cys Gly
      110      115      120
40     aat gac aaa caa tgc gag cgc aaa tgc aac gag gac gat tat gag aag  434
Asn Asp Lys Gln Cys Glu Arg Lys Cys Asn Glu Asp Asp Tyr Glu Lys
      125      130      135
45     gga gat gag gca tgc cgc tca cat gtt tgt gaa cgt cct ggt gcc tgt  482
Gly Asp Glu Ala Cys Arg Ser His Val Cys Glu Arg Pro Gly Ala Cys
      140      145      150
50     gta tgc gaa gac ggg ttc tac aga aac aaa aaa ggt agc tgt gtg gaa  530
Val Cys Glu Asp Gly Phe Tyr Arg Asn Lys Lys Gly Ser Cys Val Glu
      155      160      165      170
55     agc gat gac tgc gaa tac gat aat atg gat ttc atc act ttt gca cca  578
Ser Asp Asp Cys Glu Tyr Asp Asn Met Asp Phe Ile Thr Phe Ala Pro
      175      180      185
60     gaa acc tca cga taaccaaaga tgctacctct cgtacgcaac tccgctgatt gaggtt 636
Glu Thr Ser Arg
      190
65     gattcactcc cttgcatctc aacatttttt ttgtgatgct gtgcatctga gcttaacctg  696

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ataaagccta tggtg

711

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 <211> 425
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 <213> Ancylostoma ceylanicum

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 <222> (10)..(291)

15 <220>
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 <223> Recombinant cDNA Molecule AceNAP5 sequence

<400> 10

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 1 5 10

25 tcg caa tgt aat gga aaa gca ttc ccg aaa tgt gac gtc aat gaa aga 99
 Ser Gln Cys Asn Gly Lys Ala Phe Pro Lys Cys Asp Val Asn Glu Arg
 15 20 25 30

30 ttc gag gtg tgt ggc aat ctg aag gag tgc gag ctc aag tgc gat gag 147
 Phe Glu Val Cys Gly Asn Leu Lys Glu Cys Glu Leu Lys Cys Asp Glu
 35 40 45

35 gac cct aag ata tgc tct cgt gca tgt att cgt ccc cct gct tgc gta 195
 Asp Pro Lys Ile Cys Ser Arg Ala Cys Ile Arg Pro Pro Ala Cys Val
 50 55 60

40 tgc gat gac gga ttc tac aga gac aaa tat ggc ttc tgt gtt gaa gaa 243
 Cys Asp Asp Gly Phe Tyr Arg Asp Lys Tyr Gly Phe Cys Val Glu Glu
 65 70 75

45 gac gaa tgt aac gat atg gag att att act ttt cca cca gaa acc aaa tg 293
 Asp Glu Cys Asn Asp Met Glu Ile Ile Thr Phe Pro Pro Glu Thr Lys
 80 85 90

atgaccgaag cttccacctt tctatacata tcttcactgc ttgacaggct tctcgacaat 353
 ttagaagtgc tgcttgactt tgtctatttg aaattgttca cactaatggg ggaagtaaag 413
 cattttcacg ac 425

55 <210> 11
 <211> 471
 <212> DNA
 <213> Ancylostoma ceylanicum

<220>
 <221> CDS
 <222> (23)..(237)

<220>
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 <223> Recombinant cDNA Molecule AceNAP7 sequence

<400> 11

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                Met Ser Thr Leu Tyr Val Ile Ala Ile Cys
                1           5           10
15  ttg ctg ctt gtt tcg caa tgc aat gga aga acg gtg aag aag tgt ggc      100
    Leu Leu Leu Val Ser Gln Cys Asn Gly Arg Thr Val Lys Lys Cys Gly
                15           20           25
20  aag aat gaa aga tac gac gac tgt ggc aat gca aag gac tgc gag acc      148
    Lys Asn Glu Arg Tyr Asp Asp Cys Gly Asn Ala Lys Asp Cys Glu Thr
                30           35           40
25  aag tgc ggt gaa gag gaa aag gtg tgc cgt tcg cgt gag tgt act agt      196
    Lys Cys Gly Glu Glu Glu Lys Val Cys Arg Ser Arg Glu Cys Thr Ser
                45           50           55
30  cct ggt gcc tgc gta tgc gaa caa gga ttc tac aga gat ccg gct ggc      244
    Pro Gly Ala Cys Val Cys Glu Gln Gly Phe Tyr Arg Asp Pro Ala Gly
                60           65           70
    gac tgt gtc act gat gaa gaa tgt gat gaa tgg aac aat atg gag atc      292
35  Asp Cys Val Thr Asp Glu Glu Cys Asp Glu Trp Asn Asn Met Glu Ile
    75           80           85           90
    att act atg cca aaa cag tagtgcggaag ttcccttctt tctccaaatc tgctccgtg 349
    Ile Thr Met Pro Lys Gln
    95
    ctcaattatc acacacctcc actagttaag attgactgac tctcttgcat tgtagtattt 409
    tcgcttgact ctgtgcattt aagcatgaga tactactagg gagaataaaa attactaact 469
45  ac                                                                 471

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<210> 12
 <211> 396
 <212> DNA
 <213> Ancylostoma duodenale

<220>
 <221> CDS
 <222> (10)..(237)

<220>
 <221> MOD_RES

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<223> Recombinant cDNA Molecule AduNAP4 sequence

<400> 12

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5
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      Lys Cys Pro Thr Asp Glu Trp Phe Asp Trp Cys Gly Thr Tyr
      1          5          10
10
aag cat tgc gaa ctc aag tgc gat agg gag cta act gag aaa gaa gag 99
Lys His Cys Glu Leu Lys Cys Asp Arg Glu Leu Thr Glu Lys Glu Glu
15          20          25          30
15
cag gca tgt ctc tca cgt gtt tgt gag aag tcc gct tgc gta tgc aat 147
Gln Ala Cys Leu Ser Arg Val Cys Glu Lys Ser Ala Cys Val Cys Asn
      35          40          45
20
gac gga tta tac aga gac aag ttt ggc aac tgt gtt gaa aaa gac gaa 195
Asp Gly Leu Tyr Arg Asp Lys Phe Gly Asn Cys Val Glu Lys Asp Glu
      50          55          60
25
tgc aac gat atg gag att att act ttt gca cca gaa acc aaa taatggccta 247
Cys Asn Asp Met Glu Ile Ile Thr Phe Ala Pro Glu Thr Lys
      65          70          75
30
aggttccaaa ccttgctaca caccgtcagt gctttactgt ttcctctacg tgtagtagt 307
tttgcttgac tctgtgtatt taagcattgt ctactaatgg gcaaagtaaa gcattgtaag 367
gacataataa tgagtaaacc ttctgattt 396

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<210> 13

<211> 688

<212> DNA

<213> Ancylostoma ceylanicum

<220>

<221> CDS

<222> (21)..(560)

<220>

<221> MOD_RES

<223> Recombinant cDNA Molecule AduNAP7 sequence

<400> 13

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gaattccggg cggcagaaag atg cga atg ctc tac ctt gtt cct atc tgg 50
Met Arg Met Leu Tyr Leu Val Pro Ile Trp
5 1 5 10
ttg ctg ctc att tcg cta tgc agt gga aaa gct gcg aag aaa tgt ggt 98
Leu Leu Leu Ile Ser Leu Cys Ser Gly Lys Ala Ala Lys Lys Cys Gly
10 15 20 25
ctc aat gaa agg ctg gac tgt ggc aat ctg aag caa tgc gag ccc aag 146
Leu Asn Glu Arg Leu Asp Cys Gly Asn Leu Lys Gln Cys Glu Pro Lys
15 30 35 40
tgc agc gac ttg gaa agt gag gag tat gag gag gaa gat gag tcg aaa 194
Cys Ser Asp Leu Glu Ser Glu Glu Tyr Glu Glu Glu Asp Glu Ser Lys
45 50 55
20 tgt cga tca cgt gaa tgt tct cgt cgt gtt tgt gta tgc gat gaa gga 242
Cys Arg Ser Arg Glu Cys Ser Arg Arg Val Cys Val Cys Asp Glu Gly
60 65 70
25 ttc tac aga aac aag aag ggc aag tgt gtt gca aaa gat gtt tgc gag 290
Phe Tyr Arg Asn Lys Lys Gly Lys Cys Val Ala Lys Asp Val Cys Glu
75 80 85 90
30 gac gac aat atg gag att atc act ttt cca cca gaa gac gaa tgt ggt 338
Asp Asp Asn Met Glu Ile Ile Thr Phe Pro Pro Glu Asp Glu Cys Gly
95 100 105
35 ccc gat gaa tgg ttc gac tac tgt gga aat tat aag aag tgc gaa cgc 386
Pro Asp Glu Trp Phe Asp Tyr Cys Gly Asn Tyr Lys Lys Cys Glu Arg
110 115 120
aag tgc agt gag gag aca agt gag aaa aat gag gag gca tgc ctc tct 434
Lys Cys Ser Glu Glu Thr Ser Glu Lys Asn Glu Glu Ala Cys Leu Ser
40 125 130 135
cgt gct tgt act ggt cgt gct tgc gta tgc aaa gac gga ttg tac aga 482
Arg Ala Cys Thr Gly Arg Ala Cys Val Cys Lys Asp Gly Leu Tyr Arg
45 140 145 150
gac gac ttt ggc aac tgt gtt cca cat gac gaa tgc aac gat atg gag 530
Asp Asp Phe Gly Asn Cys Val Pro His Asp Glu Cys Asn Asp Met Glu
155 160 165 170
50 atc atc act ttt cca ccg gaa acc aaa cat tgaccagagg ctccaactct cgct 584
Ile Ile Thr Phe Pro Pro Glu Thr Lys His

55

EP 0 788 546 B9

175 180
acacaacgtc agggctagaa tggccctct gcgagttagt agttttgctt gactctgctt 644
5 atttgagcac tttctattga tggcgaaaat aaagcattta aaac 688

10 <210> 14
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<212> DNA
<213> Heligmosomoides polygyrus

15 <220>
<221> CDS
<222> (49)..(276)

20 <220>
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<223> Recombinant cDNA Molecule HpoNAP5 sequence
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Met Ile Arg
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30 aag ctc gtt ctg ctg act gct atc gtc acg gtg gtg cta agt gcg aag 105
Lys Leu Val Leu Leu Thr Ala Ile Val Thr Val Val Leu Ser Ala Lys
5 10 15

35 acc tgt gga cca aac gag gag tac act gaa tgc ggg acg cca tgc gag 153
Thr Cys Gly Pro Asn Glu Glu Tyr Thr Glu Cys Gly Thr Pro Cys Glu
20 25 30 35

40 ccg aag tgc aat gaa ccg atg cca gac atc tgt act ctg aac tgc atc 201
Pro Lys Cys Asn Glu Pro Met Pro Asp Ile Cys Thr Leu Asn Cys Ile
40 45 50

45 gtg aac gtg tgt cag tgc aaa ccc ggc ttc aag cgc gga ccg aaa gga 249
Val Asn Val Cys Gln Cys Lys Pro Gly Phe Lys Arg Gly Pro Lys Gly
55 60 65

50 tgc gtc gcc ccc gga cca ggc tgt aaa tagttctcca cctgcccttt cgttggaa 304
Cys Val Ala Pro Gly Pro Gly Cys Lys
70 75

50 caaatggctg tcttttttaca ttctgaatca ataaagccga acggt 349

55 <210> 15
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<212> DNA
<213> Heligmosomoides polygyrus

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<221> CDS

<222> (40).. (393)

<220>

<221> MOD_RES

<223> pDONG61 vector sequence

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                                     Met Pro Val Leu Leu
                                     1           5
ggt att ccg tta tta ttg cgt ttc ctc ggt ttc ctt ctg gta act ttg 102
Gly Ile Pro Leu Leu Leu Arg Phe Leu Gly Phe Leu Leu Val Thr Leu
                                     10           15           20
ttc ggc tat ctg ctt act ttc ctt aaa aag ggc ttc ggt aag ata gct 150
Phe Gly Tyr Leu Leu Thr Phe Leu Lys Lys Gly Phe Gly Lys Ile Ala
                                     25           30           35
att gct att tca ttg ttt ctt gct ctt att att ggg ctt aac tca att 198
Ile Ala Ile Ser Leu Phe Leu Ala Leu Ile Ile Gly Leu Asn Ser Ile
                                     40           45           50
ctt gtg ggt tat ctc tct gat att agc gca caa tta ccc tct gat ttt 246
Leu Val Gly Tyr Leu Ser Asp Ile Ser Ala Gln Leu Pro Ser Asp Phe
                                     55           60           65
gtt cag ggc gtt cag tta att ctc ccg tct aat gcg ctt ccc tgt ttt 294
Val Gln Gly Val Gln Leu Ile Leu Pro Ser Asn Ala Leu Pro Cys Phe
                                     70           75           80           85
tat gtt att ctc tct gta aag gct gct att ttc att ttt gac gtt aaa 342
Tyr Val Ile Leu Ser Val Lys Ala Ala Ile Phe Ile Phe Asp Val Lys
                                     90           95           100
caa aaa atc gtt tct tat ttg gat tgg gat aaa ggt gga ggc tca ggc 390
Gln Lys Ile Val Ser Tyr Leu Asp Trp Asp Lys Gly Gly Gly Ser Gly
                                     105           110           115
gga ggccaagtcg gccatcccat atcacgcggc cgcggatcc 432
Gly

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<210> 16

<211> 433

<212> DNA

<213> Heligmosomoides polygyrus

<220>

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<221> CDS
<222> (40)..(393)

<220>
<221> MOD_RES
<223> pDONG62 vector sequence

<400> 16

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                                     Met Pro Val Leu Leu
                                     1           5
ggt att ccg tta tta ttg cgt ttc ctc ggt ttc ctt ctg gta act ttg 102
Gly Ile Pro Leu Leu Leu Arg Phe Leu Gly Phe Leu Leu Val Thr Leu
          10           15           20
ttc ggc tat ctg ctt act ttc ctt aaa aag ggc ttc ggt aag ata gct 150
Phe Gly Tyr Leu Leu Thr Phe Leu Lys Lys Gly Phe Gly Lys Ile Ala
          25           30           35
att gct att tca ttg ttt ctt gct ctt att att ggg ctt aac tca att 198
Ile Ala Ile Ser Leu Phe Leu Ala Leu Ile Ile Gly Leu Asn Ser Ile
          40           45           50
ctt gtg ggt tat ctc tct gat att agc gca caa tta ccc tct gat ttt 246
Leu Val Gly Tyr Leu Ser Asp Ile Ser Ala Gln Leu Pro Ser Asp Phe
          55           60           65
gtt cag ggc gtt cag tta att ctc ccg tct aat gcg ctt ccc tgt ttt 294
Val Gln Gly Val Gln Leu Ile Leu Pro Ser Asn Ala Leu Pro Cys Phe
          70           75           80           85
tat gtt att ctc tct gta aag gct gct att ttc att ttt gac gtt aaa 342
Tyr Val Ile Leu Ser Val Lys Ala Ala Ile Phe Ile Phe Asp Val Lys
          90           95           100
caa aaa atc gtt tct tat ttg gat tgg gat aaa ggt gga ggc tca ggc 390
Gln Lys Ile Val Ser Tyr Leu Asp Trp Asp Lys Gly Gly Gly Ser Gly
          105           110           115
gga gggccaagtc ggccatccca tatcacgcgg ccgcggatcc 433
Gly

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<210> 17
<211> 434
<212> DNA
<213> Heligmosomoides polygyrus

<220>
<221> CDS
<222> (140)..(291)

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<220>
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 <223> pDONG63 vector sequence

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10                                     Met Pro Val Leu Leu
                                     1           5

ggt att ccg tta tta ttg cgt ttc ctc ggt ttc ctt ctg gta act ttg 102
15 Gly Ile Pro Leu Leu Leu Arg Phe Leu Gly Phe Leu Leu Val Thr Leu
                                     10           15           20

ttc ggc tat ctg ctt act ttc ctt aaa aag ggc ttc ggt aag ata gct 150
Phe Gly Tyr Leu Leu Thr Phe Leu Lys Lys Gly Phe Gly Lys Ile Ala
20                                     25           30           35

att gct att tca ttg ttt ctt gct ctt att att ggg ctt aac tca att 198
Ile Ala Ile Ser Leu Phe Leu Ala Leu Ile Ile Gly Leu Asn Ser Ile
25                                     40           45           50

ctt gtg ggt tat ctc tct gat att agc gca caa tta ccc tct gat ttt 246
Leu Val Gly Tyr Leu Ser Asp Ile Ser Ala Gln Leu Pro Ser Asp Phe
30                                     55           60           65

ggt cag ggc gtt cag tta att ctc ccg tct aat gcg ctt ccc tgt ttt 294
Val Gln Gly Val Gln Leu Ile Leu Pro Ser Asn Ala Leu Pro Cys Phe
35                                     70           75           80           85

tat gtt att ctc tct gta aag gct gct att ttc att ttt gac gtt aaa 342
Tyr Val Ile Leu Ser Val Lys Ala Ala Ile Phe Ile Phe Asp Val Lys
                                     90           95           100

caa aaa atc gtt tct tat ttg gat tgg gat aaa ggt gga ggc tca ggc 390
40 Gln Lys Ile Val Ser Tyr Leu Asp Trp Asp Lys Gly Gly Gly Ser Gly
                                     105           110           115

gga tcggccaagt cggccatccc atatcacgcg gccgcggatc c 434
45 Gly

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<210> 18
 <211> 6
 <212> PRT
 <213> Artificial Sequence

<220>
 <221> misc_feature
 <223> Description of Artificial Sequence: pDONG vector linker sequence

<400> 18

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Gly Gly Gly Ser Gly Gly
1 5

5

<210> 19
<211> 430
<212> DNA
<213> Ancylostoma ceylanicum

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<220>
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<222> (10)..(282)

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<220>
<221> MOD_RES
<223> "w" stands for a or t

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<400> 19

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Leu Val Ser Tyr Cys Ser Gly Lys Ala Thr Met Gln Cys Gly

25

1 5 10
gag aat gaa aag tac gat tcg tgc ggt agc aag gag tgc gat aag aag 99
Glu Asn Glu Lys Tyr Asp Ser Cys Gly Ser Lys Glu Cys Asp Lys Lys

30

15 20 25 30
tgc aaa tat gac gga gtt gag gag gaa gac gac gag gaa cct aat gtg 147
Cys Lys Tyr Asp Gly Val Glu Glu Glu Asp Asp Glu Glu Pro Asn Val

35

35 40 45
cca tgc cta gta cgt gtg tgt cat caa gat tgc gta tgc gaa gaa gga 195

40

45

50

55

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Pro Cys Leu Val Arg Val Cys His Gln Asp Cys Val Cys Glu Glu Gly
5 50 55 60
ttc tat aga aac aaa gat gac aaa tgt gta tca gca gaa gag tgc gaa 243
Phe Tyr Arg Asn Lys Asp Asp Lys Cys Val Ser Ala Glu Asp Cys Glu
65 70 75
10 ctt gac aat atg gac ttt ata tat ccc gga act cga aac tgaacgaaggctc 295
Leu Asp Asn Met Asp Phe Ile Tyr Pro Gly Thr Arg Asn
80 85 90
15 cattcttgct gcacaagatc gattgtctct ccctgcatc tcagtagttt tgctacattg 355
tatatggtag caaaaaatta gcttagggag aataaaatct ttacctatat ttaatcaatg 415
20 aagtattctc tttct 430

<210> 20

<211> 100

25 <212> PRT

<213> Ancylostoma caninum

<400> 20

30 Met Lys Met Leu Tyr Ala Ile Ala Ile Met Phe Leu Leu Val Ser Leu
1 5 10 15
Cys Ser Ala Arg Thr Val Arg Lys Ala Tyr Pro Glu Cys Gly Glu Asn
35 20 25 30
Glu Trp Leu Asp Asp Cys Gly Thr Gln Lys Pro Cys Glu Ala Lys Cys
35 40 45
40 Asn Glu Glu Pro Pro Glu Glu Glu Asp Pro Ile Cys Arg Ser Arg Gly
50 55 60
Cys Leu Leu Pro Pro Ala Cys Val Cys Lys Asp Gly Phe Tyr Arg Asp
65 70 75 80
45 Thr Val Ile Gly Asp Cys Val Arg Glu Glu Glu Cys Asp Gln His Glu
85 90 95
Ile Ile His Val
50 100

<210> 21

<211> 98

<212> PRT

55 <213> Ancylostoma caninum

<400> 21

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Met Lys Met Leu Tyr Ala Ile Ala Ile Met Phe Leu Leu Val Ser Leu
 1 5 10 15
 5 Cys Ser Thr Arg Thr Val Arg Lys Ala Tyr Pro Glu Cys Gly Glu Asn
 20 25 30
 Glu Trp Leu Asp Val Cys Gly Thr Lys Lys Pro Cys Glu Ala Lys Cys
 35 40 45
 10 Ser Glu Glu Glu Glu Glu Asp Pro Ile Cys Arg Ser Phe Ser Cys Pro
 50 55 60
 Gly Pro Ala Ala Cys Val Cys Glu Asp Gly Phe Tyr Arg Asp Thr Val
 15 65 70 75 80
 Ile Gly Asp Cys Val Lys Glu Glu Glu Cys Asp Gln His Glu Ile Ile
 85 90 95
 20 His Val

<210> 22
 <211> 94
 25 <212> PRT
 <213> Ancylostoma ceylanicum

<400> 22

30 Met Arg Thr Leu Tyr Leu Ile Ser Ile Trp Leu Phe Leu Ile Ser Gln
 1 5 10 15
 35 Cys Asn Gly Lys Ala Phe Pro Lys Cys Asp Val Asn Glu Arg Phe Glu
 20 25 30
 Val Cys Gly Asn Leu Lys Glu Cys Glu Leu Lys Cys Asp Glu Asp Pro
 35 40 45
 40 Lys Ile Cys Ser Arg Ala Cys Ile Arg Pro Pro Ala Cys Val Cys Asp
 50 55 60
 Asp Gly Phe Tyr Arg Asp Lys Tyr Gly Phe Cys Val Glu Glu Asp Glu
 45 65 70 75 80
 Cys Asn Asp Met Glu Ile Ile Thr Phe Pro Pro Glu Thr Lys
 85 90

50 <210> 23
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 <213> Ancylostoma ceylanicum

55 <400> 23

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Met Ser Thr Leu Tyr Val Ile Ala Ile Cys Leu Leu Leu Val Ser Gln
1 5 10 15
5 Cys Asn Gly Arg Thr Val Lys Lys Cys Gly Lys Asn Glu Arg Tyr Asp
20 25 30
Asp Cys Gly Asn Ala Lys Asp Cys Glu Thr Lys Cys Gly Glu Glu Glu
35 40 45
10 Lys Val Cys Arg Ser Arg Glu Cys Thr Ser Pro Gly Ala Cys Val Cys
50 55 60
Glu Gln Gly Phe Tyr Arg Asp Pro Ala Gly Asp Cys Val Thr Asp Glu
15 65 70 75 80
Glu Cys Asp Glu Trp Asn Asn Met Glu Ile Ile Thr Met Pro Lys Gln
85 90 95

20
<210> 24
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<213> Ancylostoma ceylanicum
25
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30 1 5 10 15
Cys Ser Gly Lys Pro Asn Asn Val Met Thr Asn Ala Cys Gly Leu Asn
20 25 30
35 Glu Tyr Phe Ala Glu Cys Gly Asn Met Lys Glu Cys Glu His Arg Cys
35 35 40 45
Asn Glu Glu Glu Asn Glu Glu Arg Asp Glu Glu Arg Ile Thr Ala Cys
50 55 60
40 Leu Ile Arg Val Cys Phe Arg Pro Gly Ala Cys Val Cys Lys Asp Gly
65 70 75 80
Phe Tyr Arg Asn Arg Thr Gly Ser Cys Val Glu Glu Asp Asp Cys Glu
45 85 90 95
Tyr Glu Asn Met Glu Phe Ile Thr Phe Ala Pro Glu
100 105

50
<210> 25
<211> 82
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<213> Ancylostoma ceylanicum
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<400> 25

EP 0 788 546 B9

Val Pro Ile Cys Gly Ser Asn Glu Arg Tyr Ser Asp Cys Gly Asn Asp
 1 5 10 15
 5 Lys Gln Cys Glu Arg Lys Cys Asn Glu Asp Asp Tyr Glu Lys Gly Asp
 20 25 30
 Glu Ala Cys Arg Ser His Val Cys Glu Arg Pro Gly Ala Cys Val Cys
 35 40 45
 10 Glu Asp Gly Phe Tyr Arg Asn Lys Lys Gly Ser Cys Val Glu Ser Asp
 50 55 60
 Asp Cys Glu Tyr Asp Asn Met Asp Phe Ile Thr Phe Ala Pro Glu Thr
 15 65 70 75 80
 Ser Arg

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<400> 26

Lys Cys Pro Thr Asp Glu Trp Phe Asp Trp Cys Gly Thr Tyr Lys His
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 20 25 30
 Leu Ser Arg Val Cys Glu Lys Ser Ala Cys Val Cys Asn Asp Gly Leu
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 Tyr Arg Asp Lys Phe Gly Asn Cys Val Glu Lys Asp Glu Cys Asn Asp
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 Met Glu Ile Ile Thr Phe Ala Pro Glu Thr Lys
 40 65 70 75

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<400> 27

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 5 Cys Ser Gly Lys Ala Ala Lys Lys Cys Gly Leu Asn Glu Arg Leu Asp
 20 25 30
 Cys Gly Asn Leu Lys Gln Cys Glu Pro Lys Cys Ser Asp Leu Glu Ser
 10 35 40 45
 Glu Glu Tyr Glu Glu Glu Asp Glu Ser Lys Cys Arg Ser Arg Glu Cys
 50 55 60
 15 Ser Arg Arg Val Cys Val Cys Asp Glu Gly Phe Tyr Arg Asn Lys Lys
 65 70 75 80
 Gly Lys Cys Val Ala Lys Asp Val Cys Glu Asp Asp Asn Met Glu Ile
 85 90 95
 20 Ile Thr Phe Pro Pro Glu
 100

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 25 <211> 78
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<400> 28

Asp Glu Cys Gly Pro Asp Glu Trp Phe Asp Tyr Cys Gly Asn Tyr Lys
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 Ala Cys Leu Ser Arg Ala Cys Thr Gly Arg Ala Cys Val Cys Lys Asp
 40 35 40 45
 Gly Leu Tyr Arg Asp Asp Phe Gly Asn Cys Val Pro His Asp Glu Cys
 50 55 60
 45 Asn Asp Met Glu Ile Ile Thr Phe Pro Pro Glu Thr Lys His
 65 70 75

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 5 Ser Ala Lys Thr Cys Gly Pro Asn Glu Glu Tyr Thr Glu Cys Gly Thr
 20 25 30
 Pro Cys Glu Pro Lys Cys Asn Glu Pro Met Pro Asp Ile Cys Thr Leu
 10 35 40 45
 Asn Cys Ile Val Asn Val Cys Gln Cys Lys Pro Gly Phe Lys Arg Gly
 50 55 60
 Pro Lys Gly Cys Val Ala Pro Gly Pro Gly Cys Lys
 15 65 70 75

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 30 tctgtcttcg cccagccagt tatctccact accgttggtt ccgctgccga gggttctttg 120
 gacaagaggc ctatccgcgg aattcagatc tgaatgcggc cgctcgagac tagtggatcc 180
 35 ttagaca 187

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gtc gct ata tgc tcg ctc ctc att tcg ctg tgt act gga aaa cct tcg 101
Val Ala Ile Cys Ser Leu Leu Ile Ser Leu Cys Thr Gly Lys Pro Ser
10                    10                    15                    20
gag aaa gaa tgt ggt ccc cat gaa aga ctc gac tgt ggc aac aag aag 149
Glu Lys Glu Cys Gly Pro His Glu Arg Leu Asp Cys Gly Asn Lys Lys
                    25                    30                    35
15 cca tgc gag cgc aag tgc aaa ata gag aca agt gag gag gag gat gac 197
Pro Cys Glu Arg Lys Cys Lys Ile Glu Thr Ser Glu Glu Glu Asp Asp
                    40                    45                    50
20 tac gaa gag gga acc gaa cgt ttt cga tgc ctc tta cgt gtg tgt gat 245
Tyr Glu Glu Gly Thr Glu Arg Phe Arg Cys Leu Leu Arg Val Cys Asp
55                    60                    65                    70
25 cag cct tat gaa tgc ata tgc gat gat gga tac tac aga aac aag aaa 293
Gln Pro Tyr Glu Cys Ile Cys Asp Asp Gly Tyr Tyr Arg Asn Lys Lys
                    75                    80                    85
30 ggc gaa tgt gtg act gat gat gta tgc cag gaa gac ttt atg gag ttt 341
Gly Glu Cys Val Thr Asp Asp Val Cys Gln Glu Asp Phe Met Glu Phe
                    90                    95                    100
att act ttc gca cca taaacccaat aatgaccaat gactccatt cttcgtgatcag 398
Ile Thr Phe Ala Pro
35                    105

cgtcggtggt tgacagtctc ccctacatct tagtagtttt gcttgataat gtatacataa 458
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actgtacttt ctgagataga ataaagctct caactac 495

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    <213> Ancylostoma caninum

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5                                1                                5                                10
tcg ctc ctc att tcg ttg tgt act gga aga ccg gaa aaa aag tgc ggt 101
Ser Leu Leu Ile Ser Leu Cys Thr Gly Arg Pro Glu Lys Lys Cys Gly
10                                15                                20                                25
ccc ggt gaa aga ctc gcc tgt ggc aat aag aag cca tgc gag cgc aag 149
Pro Gly Glu Arg Leu Ala Cys Gly Asn Lys Lys Pro Cys Glu Arg Lys
15                                30                                35                                40
tgc aaa ata gag aca agt gag gag gag gat gac tac cca gag gga acc 197
Cys Lys Ile Glu Thr Ser Glu Glu Glu Asp Asp Tyr Pro Glu Gly Thr
20                                45                                50                                55
gaa cgt ttt cga tgc ctc tta cgt gtg tgt gat cag cct tat gaa tgc 245
Glu Arg Phe Arg Cys Leu Leu Arg Val Cys Asp Gln Pro Tyr Glu Cys
60                                65                                70
ata tgc gat gat gga tac tac aga aac aag aaa ggc gaa tgt gtg act 293
25 Ile Cys Asp Asp Gly Tyr Tyr Arg Asn Lys Lys Gly Glu Cys Val Thr
75                                80                                85                                90
gat gat gta tgc cag gaa gac ttt atg gag ttt att act ttc gca cca 341
30 Asp Asp Val Cys Gln Glu Asp Phe Met Glu Phe Ile Thr Phe Ala Pro
95                                100                                105
taaaccaat aatgaccact ggctccatt ctctgtgacc agcgtcgggtg gttgacagtc 401
35 tcccctgcat cttagtagtt ttgcttgata atgtatccat aaacagtact ttctgagata 461

gaataaaagct ctcaact 478
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45 <213> Ancylostoma caninum

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<220>
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      Met Lys Thr Leu Tyr Ile Ile Ala Ile Cys
5      1      5      10
tcg ctg ctc ttt tca ctg tgt act gga aga ccg gaa aaa aag tgc ggt  98
Ser Leu Leu Phe Ser Leu Cys Thr Gly Arg Pro Glu Lys Lys Cys Gly
      15      20      25
10    ccc ggt gaa aga ctc gac tgt gcc aac aag aag cca tgc gag ccc aag  146
Pro Gly Glu Arg Leu Asp Cys Ala Asn Lys Lys Pro Cys Glu Pro Lys
      30      35      40
15    tgc aaa ata gag aca agt gag gag gag gat gac gac gta gag gat acc  194
Cys Lys Ile Glu Thr Ser Glu Glu Glu Asp Asp Asp Val Glu Asp Thr
      45      50      55
20    gat gtg aga tgc ctc gta cgt gtg tgt gaa cgt cct ctt aaa tgc ata  242
Asp Val Arg Cys Leu Val Arg Val Cys Glu Arg Pro Leu Lys Cys Ile
      60      65      70
      tgc aag gat gga tac tac aga aac aag aaa ggc gaa tgt gtg act gat  290
Cys Lys Asp Gly Tyr Tyr Arg Asn Lys Lys Gly Glu Cys Val Thr Asp
25    75      80      85      90
      gat gta tgc cag gaa gac ttt atg gag ttt att act ttc gca cca taaacc 341
Asp Val Cys Gln Glu Asp Phe Met Glu Phe Ile Thr Phe Ala Pro
      95      100      105
30    caataatgac cactggctcc cattcttcgt gatcagcgtc ggtgggttgac agtctcccct  401

      gcattcttagt tgcttttgctt gataatctat acataaacag tactttctga gatagaataa  461
35    aactctcaac t      472

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<211> 487
40    <212> DNA
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45    <222> (57)..(347)

      <220>
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      <223> Recombinant cDNA Molecule AcaNAP31, AcaNAP42 and AcaNAP46 sequence
50    <400> 34

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5                                     1
aag acg ctc tct gct atc cct ata atg ctg ctc ctg gta tcg caa tgc 107
Lys Thr Leu Ser Ala Ile Pro Ile Met Leu Leu Leu Val Ser Gln Cys
10          5          10          15
agt gga aaa tca ctg tgg gat cag aag tgt ggt gag aat gaa agg ctc 155
Ser Gly Lys Ser Leu Trp Asp Gln Lys Cys Gly Glu Asn Glu Arg Leu
          20          25          30
15 gac tgt ggc aat cag aag gac tgt gag cgc aag tgc gat gat aaa aga 203
Asp Cys Gly Asn Gln Lys Asp Cys Glu Arg Lys Cys Asp Asp Lys Arg
          35          40          45
20 agt gaa gaa gaa att atg cag gca tgt ctc aca cgt caa tgt ctt cct 251
Ser Glu Glu Glu Ile Met Gln Ala Cys Leu Thr Arg Gln Cys Leu Pro
          50          55          60          65
cct gtt tgc gta tgt gaa gat gga ttc tac aga aat gac aac gac caa 299
25 Pro Val Cys Val Cys Glu Asp Gly Phe Tyr Arg Asn Asp Asn Asp Gln
          70          75          80
tgt gtt gat gaa gaa gaa tgc aat atg gag ttt att act ttc gcr cca tg 349
30 Cys Val Asp Glu Glu Glu Cys Asn Met Glu Phe Ile Thr Phe Ala Pro
          85          90          95
aagcaaatga cagccgatgg tttggactct cgctacagat cacagcttta ctgttttcct      409
35 tgcatcatag tagtttttgc agatagtgta tatattagca tgatttttctg atagggagaa      469

taaagctttc caattttc      487
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<223> Recombinant cDNA Molecule AcaNAP44 sequence
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5                    1                    5                    10
tcg ctc ctc att tcg ctg tgt act gga aga ccg gaa aaa aag tgc ggt 101
Ser Leu Leu Ile Ser Leu Cys Thr Gly Arg Pro Glu Lys Lys Cys Gly
10                    15                    20                    25
ccc ggt gaa aga ctc gac tgt gcc aac aag aag cca tgc gag ccc aag 149
Pro Gly Glu Arg Leu Asp Cys Ala Asn Lys Lys Pro Cys Glu Pro Lys
15                    30                    35                    40
tgc aaa ata gag aca agt gag gag gag gat gac gac gta gag gaa acc 197
Cys Lys Ile Glu Thr Ser Glu Glu Glu Asp Asp Asp Val Glu Glu Thr
20                    45                    50                    55
gat gtg aga tgc ctc gta cgt gtg tgt gaa cgg cct ctt aaa tgc ata 245
Asp Val Arg Cys Leu Val Arg Val Cys Glu Arg Pro Leu Lys Cys Ile
25                    60                    65                    70
tgc aag gat gga tac tac aga aac aag aaa ggc gaa tgt gtg act gat 293
Cys Lys Asp Gly Tyr Tyr Arg Asn Lys Lys Gly Glu Cys Val Thr Asp
30                    75                    80                    85                    90
gat gta tgc cag gaa gac ttt atg gag ttt att act ttc gca cca taaacc 344
Asp Val Cys Gln Glu Asp Phe Met Glu Phe Ile Thr Phe Ala Pro
35                    95                    100                    105
caataatgac cactggctcc cattcttcgt gatcagcgtc ggtggttgac agtctccct 404
40                    110                    115                    120
gcatcttagt tgctttgctt gataatctat acataaacag tacttttctga gatagaataa 464
45                    125                    130                    135
agctctcaac tac 477
50                    140                    145                    150
<210> 36
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<213> Ancylostoma caninum
55                    155                    160                    165
<220>
<221> CDS
<222> (14)..(556)
50                    165                    170                    175
<220>
<221> MOD_RES
<223> Recombinant cDNA Molecule AcaNAP45 sequence
55                    175                    180                    185
<400> 36

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Met Leu Met Leu Tyr Leu Val Pro Ile Trp Leu Leu
5 1 5 10
ctc att tcg caa tgc agt gga aaa tcc gcg aag aaa tgt ggt ctc aat 96
Leu Ile Ser Gln Cys Ser Gly Lys Ser Ala Lys Lys Cys Gly Leu Asn
10 15 20 25
gaa aaa ttg gac tgt ggc aat ctg aag gca tgc gag aaa aag tgc agc 144
Glu Lys Leu Asp Cys Gly Asn Leu Lys Ala Cys Glu Lys Lys Cys Ser
15 30 35 40
gac ttg gac aat gag gag gat tat aag gag gaa gat gag tcg aaa tgc 192
Asp Leu Asp Asn Glu Glu Asp Tyr Lys Glu Glu Asp Glu Ser Lys Cys
45 50 55 60
cga tca cgt gaa tgt agt cgt cgt gtt tgt gta tgc gat gaa gga ttc 240
Arg Ser Arg Glu Cys Ser Arg Arg Val Cys Val Cys Asp Glu Gly Phe
65 70 75
tac aga aac aag aag ggc caa tgt gtg aca aga gat gat tgc gag tat 288
Tyr Arg Asn Lys Lys Gly Gln Cys Val Thr Arg Asp Asp Cys Glu Tyr
25 80 85 90
gac aat atg gag att atc act ttt cca cca gaa gat aaa tgt ggt ccc 336
Asp Asn Met Glu Ile Ile Thr Phe Pro Pro Glu Asp Lys Cys Gly Pro
30 95 100 105
gat gaa tgg ttc gac tgg tgt gga act tac aag cag tgt gag cgc aag 384
Asp Glu Trp Phe Asp Trp Cys Gly Thr Tyr Lys Gln Cys Glu Arg Lys
35 110 115 120
tgc aat aag gag cta agt gag aaa gat gaa gag gca tgc ctc tca cgt 432
Cys Asn Lys Glu Leu Ser Glu Lys Asp Glu Glu Ala Cys Leu Ser Arg
40 125 130 135 140
gct tgt act ggt cgt gct tgt gtt tgc aac gac gga ctg tac aga gac 480
Ala Cys Thr Gly Arg Ala Cys Val Cys Asn Asp Gly Leu Tyr Arg Asp
145 150 155
45 gat ttt ggc aat tgt gtt gag aaa gac gaa tgt aac gat atg gag att 528

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Asp Phe Gly Asn Cys Val Glu Lys Asp Glu Cys Asn Asp Met Glu Ile
160 165 170
5 atc act ttt cca ccg gaa acc aaa cac tgaccaaagg ctctaactct cgctacat 583
Ile Thr Phe Pro Pro Glu Thr Lys His
175 180
10 aacgtcagtg cttgaattgc ccctttacga gttagtaatt ttgactaaact ctgtgtaatt 643
gagcattgtc tactgatggg gaaaatgaag tgttcaatgt ct 685
15 <210> 37
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20 <220>
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25 <220>
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1 5
35 cct atc tgg ttc ctg ctc att tcg caa tgc agt gga aaa tcc gcg aag 102
Pro Ile Trp Phe Leu Leu Ile Ser Gln Cys Ser Gly Lys Ser Ala Lys
10 15 20
40 aaa tgt ggc ctc aat gaa aaa ttg gac tgt ggc aat ctg aag gca tgc 150
Lys Cys Gly Leu Asn Glu Lys Leu Asp Cys Gly Asn Leu Lys Ala Cys
25 30 35
45 gag aaa aag tgc agc gac ttg gac aat gag gag gat tat ggg gag gaa 198
Glu Lys Lys Cys Ser Asp Leu Asp Asn Glu Glu Asp Tyr Gly Glu Glu
40 45 50 55
gat gag tcg aaa tgc cga tca cgt gaa tgt att ggt cgt gtt tgc gta 246
Asp Glu Ser Lys Cys Arg Ser Arg Glu Cys Ile Gly Arg Val Cys Val
50 60 65 70
tgc gat gaa gga ttc tac aga aac aag aag ggc caa tgt gtg aca aga 294
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Cys Asp Glu Gly Phe Tyr Arg Asn Lys Lys Gly Gln Cys Val Thr Arg
      75                      80                      85
5   gac gat tgc gag tat gac aat atg gag att atc act ttt cca cca gaa 342
    Asp Asp Cys Glu Tyr Asp Asn Met Glu Ile Ile Thr Phe Pro Pro Glu
      90                      95                      100
10  gat aaa tgt ggt ccc gat gaa tgg ttc gac tgg tgt gga act tac aag 390
    Asp Lys Cys Gly Pro Asp Glu Trp Phe Asp Trp Cys Gly Thr Tyr Lys
      105                      110                      115
15  cag tgt gag cgc aag tgc agt gag gag cta agt gag aaa aat gag gag 438
    Gln Cys Glu Arg Lys Cys Ser Glu Glu Leu Ser Glu Lys Asn Glu Glu
      120                      125                      130                      135
20  gca tgc ctc tca cgt gct tgt act ggt cgt gct tgc gtt tgc aac gac 486
    Ala Cys Leu Ser Arg Ala Cys Thr Gly Arg Ala Cys Val Cys Asn Asp
      140                      145                      150
25  gga ttg tat aga gac gat ttt ggc aat tgt gtt gag aaa gac gaa tgt 534
    Gly Leu Tyr Arg Asp Asp Phe Gly Asn Cys Val Glu Lys Asp Glu Cys
      155                      160                      165
30  aac gat atg gag att atc act ttt cca ccg gaa acc aaa cac tgaccaaagg 586
    Asn Asp Met Glu Ile Ile Thr Phe Pro Pro Glu Thr Lys His
      170                      175                      180

ctctagctct cgctacataa cgtcagtgct tgaattgtcc ctttacgtgt tagtaatttt 646

35  gactaactct gtgtatttga gcattgtcta ctaatgggtga aaatgaagct tttcaatgac 706

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40  <210> 38
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ata acg ttg ctc ctg gta tgg caa tgc agt gca aga aca gcg agg aaa 102
Ile Thr Leu Leu Leu Val Trp Gln Cys Ser Ala Arg Thr Ala Arg Lys
10    10                    15                    20
ccc cca acg tgt ggt gaa aat gaa agg gtc gaa tgg tgt ggc aag cag 150
Pro Pro Thr Cys Gly Glu Asn Glu Arg Val Glu Trp Cys Gly Lys Gln
15    25                    30                    35                    40
tgc gag atc aca tgt gac gac cca gat aag ata tgc cgc tca ctc gct 198
Cys Glu Ile Thr Cys Asp Asp Pro Asp Lys Ile Cys Arg Ser Leu Ala
                    45                    50                    55
20    tgt cct ggt cct cct gct tgc gta tgc gac gac gga tac tac aga gac 246
Cys Pro Gly Pro Pro Ala Cys Val Cys Asp Asp Gly Tyr Tyr Arg Asp
                    60                    65                    70
25    acg aac gtt ggc ttg tgt gta caa tat gac gaa tgc aac gat atg gat 294
Thr Asn Val Gly Leu Cys Val Gln Tyr Asp Glu Cys Asn Asp Met Asp
                    75                    80                    85
30    att att atg gtt tca taggggtgac tgaagaatcg aacaaccggt gcacaacttc 349
Ile Ile Met Val Ser
                    90
tatgcttgac tatctctctt gcatcatgca agttagcta gatagtgtat atattagcaa 409
35    gaccccttgg ggagaatgaa gcttcccaac tatattaaat caataacggt ttcgcttcat 469
gtacacgtgc tcagcacatt catatccact cctcacactc catgaaagca gtgaaatgtt 529
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45 <213> Necator americanus
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5 1 5 10
tgc gtc act ttt gca gcg aag aga gat tgt cca gca aat gag gaa tgg 99
Cys Val Thr Phe Ala Ala Lys Arg Asp Cys Pro Ala Asn Glu Glu Trp
10 15 20 25
agg gaa tgt ggc act cca tgt gaa cca aaa tgc aat caa ccg atg cca 147
Arg Glu Cys Gly Thr Pro Cys Glu Pro Lys Cys Asn Gln Pro Met Pro
30 35 40
15 gat ata tgt act atg aat tgt atc gtc gat gtg tgt caa tgc aag gag 195
Asp Ile Cys Thr Met Asn Cys Ile Val Asp Val Cys Gln Cys Lys Glu
45 50 55 60
20 gga tac aag cgt cat gaa acg aag gga tgc tta aag gaa gga tca gct 243
Gly Tyr Lys Arg His Glu Thr Lys Gly Cys Leu Lys Glu Gly Ser Ala
65 70 75
25 gat tgt aaa taagttatca gaacgctcgt tttgtcttac attagatggg tgagctgatg 302
Asp Cys Lys

tatctgtcag ataaactcctt tcttctaaaa aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa 361

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35 <213> Ancylostoma caninum

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40 Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp Asp Cys Gly
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Thr Gln Lys Pro Cys Glu Ala Lys Cys Asn Glu Glu Pro Pro Glu Glu
20 25 30
45 Glu Asp Pro Ile Cys Arg Ser Arg Gly Cys Leu Leu Pro Pro Ala Cys
35 40 45
Val Cys Lys Asp Gly Phe Tyr Arg Asp Thr Val Ile Gly Asp Cys Val
50 50 55 60
Arg Glu Glu Glu Cys Asp Gln His Glu Ile Ile His Val
65 70 75

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<210> 41

<211> 75

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<212> PRT

<213> Ancylostoma caninum

<400> 41

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20 25 30
Pro Ile Cys Arg Ser Phe Ser Cys Pro Gly Pro Ala Ala Cys Val Cys
35 40 45
Glu Asp Gly Phe Tyr Arg Asp Thr Val Ile Gly Asp Cys Val Lys Glu
50 55 60
Glu Glu Cys Asp Gln His Glu Ile Ile His Val
65 70 75

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<211> 74

<212> PRT

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<400> 42

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20 25 30
Cys Arg Ser Leu Ala Cys Pro Gly Pro Pro Ala Cys Val Cys Asp Asp
35 40 45
Gly Tyr Tyr Arg Asp Thr Asn Val Gly Leu Cys Val Gln Tyr Asp Glu
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Cys Asn Asp Met Asp Ile Ile Met Val Ser
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<213> Ancylostoma caninum

<400> 43

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 5 Asn Lys Lys Pro Cys Glu Arg Lys Cys Lys Ile Glu Thr Ser Glu Glu
 20 25 30
 Glu Asp Asp Tyr Glu Glu Gly Thr Glu Arg Phe Arg Cys Leu Leu Arg
 35 40 45
 10 Val Cys Asp Gln Pro Tyr Glu Cys Ile Cys Asp Asp Gly Tyr Tyr Arg
 50 55 60
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 15 65 70 75 80
 Met Glu Phe Ile Thr Phe Ala Pro
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 20 25 30
 35 Asp Asp Tyr Pro Glu Gly Thr Glu Arg Phe Arg Cys Leu Leu Arg Val
 35 40 45
 Cys Asp Gln Pro Tyr Glu Cys Ile Cys Asp Asp Gly Tyr Tyr Arg Asn
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 Glu Phe Ile Thr Phe Ala Pro
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 1 5 10 15
 5 Lys Lys Pro Cys Glu Pro Lys Cys Lys Ile Glu Thr Ser Glu Glu Glu
 20 25 30
 Asp Asp Asp Val Glu Asp Thr Asp Val Arg Cys Leu Val Arg Val Cys
 10 35 40 45
 Glu Arg Pro Leu Lys Cys Ile Cys Lys Asp Gly Tyr Tyr Arg Asn Lys
 50 55 60
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 65 70 75 80
 Phe Ile Thr Phe Ala Pro
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 20 25 30
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 35 35 40 45
 Glu Arg Pro Leu Lys Cys Ile Cys Lys Asp Gly Tyr Tyr Arg Asn Lys
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 20 25 30
 Glu Glu Ile Met Gln Ala Cys Leu Thr Arg Gln Cys Leu Pro Pro Val
 35 40 45
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 50 55 60
 Asp Glu Glu Glu Cys Asn Met Glu Phe Ile Thr Phe Ala Pro
 15 65 70 75

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 35 35 40 45
 Val Cys Phe Arg Pro Gly Ala Cys Val Cys Lys Asp Gly Phe Tyr Arg
 50 55 60
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 Met Glu Phe Ile Thr Phe Ala Pro Glu
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EP 0 788 546 B9

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 20 25 30
 Glu Ala Cys Arg Ser His Val Cys Glu Arg Pro Gly Ala Cys Val Cys
 10 35 40 45
 Glu Asp Gly Phe Tyr Arg Asn Lys Lys Gly Ser Cys Val Glu Ser Asp
 50 55 60
 15 Asp Cys Glu Tyr Asp Asn Met Asp Phe Ile Thr Phe Ala Pro Glu Thr
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 35 35 40 45
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 65 70 75 80
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 35 40 45
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 50 55 60
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 Phe Pro Pro Glu

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 20 25 30
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 35 40 45
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 50 55 60
 40 Val Ala Lys Asp Val Cys Glu Asp Asp Asn Met Glu Ile Ile Thr Phe
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Ala Cys Leu Ser Arg Ala Cys Thr Gly Arg Ala Cys Val Cys Asn Asp
35 40 45
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50 55 60
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35 40 45
Gly Leu Tyr Arg Asp Asp Phe Gly Asn Cys Val Glu Lys Asp Glu Cys
50 55 60
35 Asn Asp Met Glu Ile Ile Thr Phe Pro Pro Glu Thr Lys His
65 70 75

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20 25 30
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35 40 45

EP 0 788 546 B9

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 20 25 30
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 25 35 40 45
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              35              40              45
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              35              40              45
      Asp Cys Val Cys Glu Glu Gly Phe Tyr Arg Asn Lys Asp Asp Lys Cys
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	Glu	Asn	Glu	Glu	Arg	Asp	Glu	Glu	Arg	Ile	Thr	Ala	Cys	Leu	Ile	Arg
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	Val	Cys	Phe	Arg	Pro	Gly	Ala	Cys	Val	Cys	Lys	Asp	Gly	Phe	Tyr	Arg
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15	Asn	Arg	Thr	Gly	Ser	Cys	Val	Glu	Glu	Asp	Asp	Cys	Glu	Tyr	Glu	Asn
	65					70				75					80	
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					85					90					95	
20	Glu	Arg	Tyr	Ser	Asp	Cys	Gly	Asn	Asp	Lys	Gln	Cys	Glu	Arg	Lys	Cys
				100					105					110		
	Asn	Glu	Asp	Asp	Tyr	Glu	Lys	Gly	Asp	Glu	Ala	Cys	Arg	Ser	His	Val
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 15 35 40 45
 Arg Val Cys Val Cys Asp Glu Gly Phe Tyr Arg Asn Lys Lys Gly Gln
 50 55 60
 20 Cys Val Thr Arg Asp Asp Cys Glu Tyr Asp Asn Met Glu Ile Ile Thr
 65 70 75 80
 Phe Pro Pro Glu Asp Lys Cys Gly Pro Asp Glu Trp Phe Asp Trp Cys
 85 90 95
 25 Gly Thr Tyr Lys Gln Cys Glu Arg Lys Cys Ser Glu Glu Leu Ser Glu
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 35 40 45
 15 Val Cys Val Cys Asp Glu Gly Phe Tyr Arg Asn Lys Lys Gly Lys Cys
 50 55 60
 Val Ala Lys Asp Val Cys Glu Asp Asp Asn Met Glu Ile Ile Thr Phe
 20 65 70 75 80
 Pro Pro Glu Asp Glu Cys Gly Pro Asp Glu Trp Phe Asp Tyr Cys Gly
 85 90 95
 25 Asn Tyr Lys Lys Cys Glu Arg Lys Cys Ser Glu Glu Thr Ser Glu Lys
 100 105 110
 Asn Glu Glu Ala Cys Leu Ser Arg Ala Cys Thr Gly Arg Ala Cys Val
 115 120 125
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Claims

1. An isolated protein having anticoagulant activity and having one or more Nematode extracted anticoagulant protein domains (NAP domains), wherein each NAP domain includes the sequence:

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Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10,

wherein

45

- (a) A1 is an amino acid sequence of 7 to 8 amino acid residues;
- (b) A2 is an amino acid sequence of 3 to 5 amino acid residues;
- (c) A3 is an amino acid sequence of 3 amino acid residues;
- (d) A4 is an amino acid sequence of 6 to 19. amino acid residues;
- (e) A5 is an amino acid sequence of 3 to 4 amino acid residues;
- (f) A6 is an amino acid sequence of 3 to 5 amino acid residues;
- (g) A7 is an amino acid residue;
- (h) A8 is an amino acid sequence of 11 to 12 amino acid residues;
- (i) A9 is an amino acid sequence of 5 to 7 amino acid residues; and
- (j) A10 is an amino acid sequence of 5 to 25 amino acid residues.

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55

2. The protein of claim 1 wherein the activity of said protein includes Factor Xa inhibitory activity.

3. The protein of claim 1 or 2, wherein A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues.
- 5 4. The protein of claim 3, wherein A3 has the sequence Glu-A3_a-A3_b, wherein A3_a is selected from the group consisting of Ala, Arg, Pro, Lys, Ile, His, Leu, and Thr, and A3_b is selected from the group consisting of Lys, Thr, and Arg.
- 10 5. The protein of any of claims 3 to 4, wherein A3 is selected from the group consisting of
Glu-Ala-Lys,
Glu-Arg-Lys,
Glu-Pro-Lys,
Glu-Lys-Lys,
Glu-Ile-Thr,
Glu-His-Arg,
Glu-Leu-Lys, and
15 Glu-Thr-Lys.
6. The protein of claim 1, wherein A3 has the sequence Asp-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues.
- 20 7. The protein of claim 1 or 6, wherein A3 is Asp-Lys-Lys.
8. The protein of any of claims 1 to 7, wherein A4 is an amino acid sequence having a net anionic charge.
- 25 9. The protein of any of claims 1 or 6 to 8,
wherein A5 has the sequence A5_a-A5_b-A5_c-A5_d [SEQ. ID. NO. 85] wherein A5_a through A5_d are independently selected amino acid residues.
10. The protein of claim 9, wherein A5_a is Leu and A5_c is Arg.
- 30 11. The protein of any of claims 1 to 10, wherein A7 is selected from the group consisting of Val and Ile.
12. The protein of any of claims 1 to 11, wherein A8 includes an amino acid sequence A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. NO. 68], wherein
35 (a) A8_a is the first amino acid residue in A8,
(b) at least one of A8_a and A8_b is selected from the group consisting of Glu or Asp, and
(c) A8_c through A8_g are independently selected amino acid residues.
- 40 13. The protein of claim 12, wherein
(a) A8_a is Glu or Asp,
(b) A8_b is an independently selected amino acid residue,
(c) A8_c is Gly,
(d) A8_d is selected from the group consisting of Phe, Tyr, and Leu,
45 (e) A8_e is Tyr,
(f) A8_f is Arg, and
(g) A8_g is selected from Asp and Asn.
- 50 14. The protein of claim 12 or 13, wherein
(a) A8_a is an independently selected amino acid residue,
(b) A8_b is Glu or Asp,
(c) A8_c is Gly,
(d) A8_d is selected from the group consisting of Phe, Tyr, and Leu,
55 (e) A8_e is Tyr,
(f) A8_f is Arg, and
(g) A8_g is selected from Asp and Asn.

15. The protein of any of claims 12 to 14, wherein A8_c-A8_d-A8_e-A8_c-A8_g is selected from the group consisting of
 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],
 Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],
 Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],
 Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and
 Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73].

16. The protein of claim 15, wherein A8_c-A8_d-A8_e-A8_f-A8_g is Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70].

17. The protein of any of claims 2 to 16, wherein A10 includes an amino acid sequence selected from the group consisting of
 Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],
 Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],
 Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and
 Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

18. The protein of claim 17, wherein A10 includes the amino acid sequence Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74].

19. The protein of claim 17, wherein A10 includes the amino acid sequence Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75].

20. The protein of claim 19 having a NAP domain with an amino acid sequence having at least 90 % homology to that of AcaNAP48 [Fig. 16].

21. The protein of claim 17, wherein A10 includes the sequence Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76].

22. The protein of claim 21 having a NAP domain with an amino acid sequence having at least 90 % homology to a NAP domain selected from NAP domains of AcaNAP23 [Fig. 16], AcaNAP24 [Fig. 16], AcaNAP25 [Fig. 16], AcaNAP44 [Fig. 16], AcaNAP31 [Fig. 16], and AceNAP4-d1 [Fig. 16] and AceNAP4-d2 [Fig. 16].

23. The protein of claim 17, wherein A10 includes the sequence Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

24. The protein of claim 23 having a NAP domain with an amino acid sequence having at least 90 % homology to a NAP domain selected from NAP domains of AcaNAP45-d1 [Fig. 16], AcaNAP45-d2 [Fig. 16], AcaNAP47-d1 [Fig. 16], AcaNAP47-d2 [Fig. 16], AduNAP7-d1 [Fig. 16], AduNAP7-d2 [Fig. 16], AduNAP4 [Fig. 16], AceNAP5 [Fig. 16], and AceNAP7 [Fig. 16].

25. The protein of claim 1 or 2, wherein

(a) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;

(b) A4 is an amino acid sequence having a net anionic charge;

(c) A7 is selected from the group consisting of Val and Ile;

(d) A8 includes an amino acid sequence selected from the group consisting of

Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 69],

Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 70],

Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 71],

Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 72], and

Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 73]; and

(e) A10 includes an amino sequence selected from the group consisting of

Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],

Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],

Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and

Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

26. The protein of claim 1 or 2, wherein

(a) A3 is selected from the group consisting of

Glu-Ala-Lys,

Glu-Arg-Lys,

Glu-Pro-Lys,
 Glu-Lys-Lys,
 Glu-Ile-Thr,
 Glu-His-Arg,
 Glu-Leu-Lys, and
 Glu-Thr-Lys;

(b) A4 is an amino acid sequence having a net anionic charge;

(c) A7 is Val or Ile;

(d) A8 includes an amino acid sequence selected from the group consisting of

A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. NO. 78],

A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79],

A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. NO. 80],

A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. NO. 81], and

A8_a-A8_b-Gly-Leu-Tyr-Arg-Asp [SEQ. ID. NO. 82],

wherein at least one of A8_a and A8_b is Glu or Asp;

(e) A9 is an amino acid sequence of five amino acid residues; and

(f) A10 includes an amino acid sequence selected from the group consisting of

Glu-Ile-Ile-His-Val [SEQ. ID. NO. 74],

Asp-Ile-Ile-Met-Val [SEQ. ID. NO. 75],

Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. NO. 76], and

Met-Glu-Ile-Ile-Thr [SEQ. ID. NO. 77].

27. The protein of claim 18, 25 or 26 having a NAP domain having at least 90% homology to NAP domains selected from AcaNAP5 [Fig. 16] and AcaNAP6 [Fig. 16].

28. The protein of claim 25 or 26 having a NAP domain having at least 90 % homology to a NAP domain selected from the group consisting of AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAP48 [Fig. 16], AcaNAP23 [Fig. 16], AcaNAP24 [Fig. 16], AcaNAP25 [Fig. 16], AcaNAP44 [Fig. 16], AcaNAP31 [Fig. 16], AceNAP4-d1 [Fig. 16], AceNAP4-d2 [Fig. 16], AcaNAP45-d1 [Fig. 16], AcaNAP45-d2 [Fig. 16], AcaNAP47-d1 [Fig. 16], AcaNAP47-d2 [Fig. 16], AduNAP7-d1 [Fig. 16], AduNAP7-d2 [Fig. 16], AduNAP4 [Fig. 16], AceNAP5 [Fig. 16], and AceNAP7 [Fig. 16].

29. An isolated protein having anticoagulant activity selected from the group consisting of AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAP48 [Fig. 16], AcaNAP23 [Fig. 16], AcaNAP24 [Fig. 16], AcaNAP25 [Fig. 16], AcaNAP44 [Fig. 16], AcaNAP31 [Fig. 16], AceNAP4 [Fig. 16], AcaNAP45 [Fig. 16], AcaNAP47 [Fig. 16], AduNAP7 [Fig. 16], AduNAP4 [Fig. 16], AceNAP5 [Fig. 16], and AceNAP7 [Fig. 16].

30. An isolated protein having Factor Xa inhibitory activity selected from the group consisting of AcaNAP5 [Fig. 16] and AcaNAP6 [Fig. 16].

31. The protein of claim 1, wherein

(a) A3 is has the sequence Asp-A3_a-A3_b, herein A3_a and A3_b are independently selected amino acid residues;

(b) A4 is an amino acid sequence having a net anionic charge;

(c) A5 has the sequence A5_a-A5_b-A5_c-A5_d [SEQ.ID. NO. 85] wherein A5_a through A5_d are independently selected amino acid residues, and

(d) A7 is selected from the group consisting of Val and Ile.

32. The protein of claim 1, wherein

(a) A3 is Asp-Lys-Lys;

(b) A4 is an amino acid sequence having a net anionic charge;

(c) A5 has the sequence A5_a-A5_b-A5_c-A5_d wherein A5_a is Leu, A5_c is Arg, and A5_b and A5_d are independently selected amino acid residues [SEQ. ID. NO. 357].

(d) A7 is Val; and

(e) A8 includes an amino acid sequence A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. NO. 79], wherein at least one of A8_a and A8_b is Glu or Asp.

33. The protein of claim 31 or 32 having a NAP domain with an amino acid sequence having at least 90 % homology

to the NAP domain of AcaNAPc2 [Fig. 16].

34. An isolated protein having Factor VIIa/TF inhibitory activity having a Nematode-extracted anticoagulant protein domain (NAP domain) with an amino acid sequence having at least 90 % homology to the NAP domain of AcaNAPc2 [Fig. 16].

35. An isolated protein according to any one of claims 1 to 34 having anticoagulant activity, wherein said protein specifically inhibits the catalytic activity of the fVIIa/TF complex in the presence of fXa or catalytically inactive fXa derivative, and does not specifically inhibit the activity of FVIIa in the absence of TF and does not specifically inhibit prothrombinase.

36. A protein of claim 35, wherein the protein is AcaNAPc2 [Fig. 16].

37. A protein having anticoagulant activity and having an amino acid sequence having at least 90 % homology to AcaNAPc2 [Fig. 16].

38. An isolated protein having serine protease inhibitory activity and having one or more Nematode-extracted anticoagulant protein domains (NAP domains) with an amino acid sequence having at least 90 % homology to NAP domains selected from HpoNAP5 [Fig. 16] and NamNAP [Fig. 16].
wherein

- (a) A1 is an amino acid sequence of 7 to 8 amino acid residues;
- (b) A2 is an amino acid sequence of 3 to 5 amino acid residues;
- (c) A3 has the sequence Glu-A3_a-A3_b, wherein A3_a and A3_b are independently selected amino acid residues;
- (d) A4 is an amino acid sequence of 6 to 19 amino acid residues having a net anionic charge;
- (e) A5 has the sequence A5_a-A5_b-A5_c, wherein A5_a through A5_c are independently selected amino acid residues;
- (f) A6 is an amino acid sequence of 3 to 5 amino acid residues;
- (g) A7 is Gln;
- (h) A8 is an amino acid sequence of 10 to 12 amino acid residues; and
- (i) A9 is an amino acid sequence of 5 to 7 amino acid residues;
- (j) A10 is an amino acid sequence of 1 to 25 amino acid residues.

39. The protein of claim 38, wherein

- (a) A3 is Glu-Pro-Lys;
- (b) A4 is an amino acid sequence having a net anionic charge;
- (c) A5 is selected from Thr-Leu-Asn and Thr-Met-Asn; and
- (d) A7 is Gln.

40. An isolated protein having serine protease inhibitory activity and a Nematode-extracted anticoagulant protein domain (NAP domain) with an amino acid sequence having at least 90 % homology to NAP domains selected from the group consisting of HpoNAP5 [Fig. 16] and NamNAP [Fig. 16].

41. A protein of any of claims 1, 2, 25, 26, 31, 32, 38 or 39 wherein said protein has two NAP domains.

42. A protein having two Nematode extracted anticoagulant protein (NAP) domains, wherein said protein is selected from the group consisting of AceNAP4 [Fig. 17], AcaNAP45 [Fig. 18], AcaNAP47 [Fig. 19], and AduNAP7 [Fig. 20].

43. The protein of any of claims 1 to 42, wherein said protein is derived from a nematode species which is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligmosomoides polygyrus*.

44. An isolated recombinant cDNA molecule encoding a protein of any of claims 1 to 42.

45. A cDNA molecule encoding the protein of claim 18 or 25 having a nucleotide sequence having at least 90 % homology to that coding for AcaNAP5 [Fig. 1] and AcaNAP6 [Fig. 3].

46. A cDNA molecule encoding the protein having Factor Xa inhibitory activity selected from the group consisting of

proteins having NAP domains having at least 90 % homology to AcaNAP5 [Fig. 16] and AcaNAP6 [Fig. 16].

47. A cDNA molecule encoding the protein of claim 20 having a nucleotide sequence having at least 90 % homology to that coding for AcaNAP48 [Fig. 13h].

48. A cDNA molecule encoding the protein of claim 21 having a nucleotide sequence having at least 90. % homology to that selected from the group consisting of cDNAs coding for AcaNAP23 [Fig. 13a], AcaNAP24 [Fig. 13b], AcaNAP25 [Fig. 13c], AcaNAP44 [Fig. 13e], AcaNAP31 [Fig. 13d], and AceNAP4 [Fig. 7a].

49. A cDNA molecule encoding the protein of claim 23 having a nucleotide sequence having at least 90 % homology that selected from the group consisting of cDNAs coding for AcaNAP45 [Fig. 13f], AcaNAP47 [Fig. 13g], AduNAP7 [Fig. 7e], AduNAP4 [Fig. 7d], AceNAP5 [Fig. 7b], and AceNAP7 [Fig. 7c].

50. A cDNA molecule encoding the protein of claim 26 that is selected from the group consisting of cDNAs coding for AcaNAP5 [Fig. 1], AcaNAP6 [Fig. 3], AcaNAP48 [Fig. 13h], AcaNAP23 [Fig. 13a], AcaNAP24 [Fig. 13b], AcaNAP25 [Fig. 13c], AcaNAP44 [Fig. 13e], AcaNAP31 [Fig. 13d], AceNAP4 [Fig. 7a], AcaNAP45 [Fig. 13f], AcaNAP47 [Fig. 13g], AduNAP7 [Fig. 7e], AduNAP4 [Fig. 7d], AceNAP5 [Fig. 7b], and AceNAP7 [Fig. 7c].

51. A cDNA molecule encoding the protein of claim 38 having a nucleotide sequence having at least 90 % homology to sequences selected from cDNAs coding for HpoNAP5 [Fig. 7f] and NamNAP [Fig. 14].

52. A cDNA molecule encoding a protein having anticoagulant activity selected from the group consisting of cDNAs having at least 90 % homology to cDNAs coding for AcaNAP5 [Fig. 1], AcaNAP6 [Fig. 3], AcaNAP48 [Fig. 13h], AcaNAP23 [Fig. 13a], AcaNAP24 [Fig. 13b], AcaNAP25 [Fig. 13c], AcaNAP44 [Fig. 13e], AcaNAP31 [Fig. 13d], AceNAP4 [Fig. 7a], AcaNAP45 [Fig. 13f], AcaNAP47 [Fig. 13g], AduNAP7 [Fig. 7e], AduNAP4 [Fig. 7d], AceNAP5 [Fig. 7b], and AceNAP7 [Fig. 7c].

53. An isolated recombinant cDNA molecule according to anyone of claims 44 to 52 encoding protein having anticoagulant activity, wherein said protein specifically inhibits the catalytic activity of the fVIIa/TF complex in the presence of fXa or catalytically inactive fXa derivative, and does not specifically inhibit the activity of FVIIa in the absence of TF and does not specifically inhibit prothrombinase.

54. The cDNA molecule of claim 53, wherein the DNA codes for AcaNAPc2 [Fig. 16].

55. An isolated cDNA molecule encoding a protein having anticoagulant activity having at least 90 % homology to AcaNAPc2 [Fig. 9].

56. An isolated recombinant cDNA molecule encoding a protein of claim 32.

57. The cDNA molecule of claim 56 having a nucleotide sequence which codes for an amino acid sequence having at least 90 % homology to AcaNAPc2 [Fig. 16].

58. The cDNA molecule of any of claims 44 to 57 derived from a nematode species.

59. The cDNA molecule of claim 58, wherein said nematode species is selected from the group consisting of *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus*, and *Heligmosomoides polygyrus*.

60. An oligonucleotide comprising a nucleotide sequence selected from NAP-1: AAR-CCN-TGY-GAR-MGG-AAR-TGY [SEQ. ID. NO. 90] and NAP-4.RC TW-RWA-NCC-NTC-YTT-RCA-NAC-RCA [SEQ. ID. NO. 91].

61. Use of a protein of any of claims 1 to 42 for the manufacture of a pharmaceutical composition for inhibiting blood coagulation.

62. The use of claim 61 wherein the protein is selected from the group consisting of AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAPc2 [Fig. 16], HpoNAP5 [Fig. 16], NamNAP [Fig. 16].

63. The use of claim 61 wherein the protein has a NAP domain having at least 90 % homology to NAP domains selected from the group consisting of AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAP48 [Fig. 16], AcaNAP23 [Fig. 16], AcaNAP24 [Fig. 16], AcaNAP25 [Fig. 16], AcaNAP44 [Fig. 16], AcaNAP31 [Fig. 16], AceNAP4-d1 [Fig. 16], AceNAP4-d2 [Fig. 16], AcaNAP45-d1 [Fig. 16], AcaNAP45-d2 [Fig. 16], AcaNAP47-d1 [Fig. 16], AcaNAP47-d2 [Fig. 16], AduNAP7-d1 [Fig. 16], AduNAP7-d2 [Fig. 16], AduNAP4 [Fig. 16], AceNAP5 [Fig. 16], and AceNAP7 [Fig. 16].
64. A pharmaceutical composition comprising a protein of any of claims 1 to 42.
65. The pharmaceutical composition of claim 64 comprising a protein selected from the group consisting of AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAPc2 [Fig. 16], HpoNAP5 [Fig. 16], NamNAP [Fig. 16].
66. The pharmaceutical composition of claim 64 comprising a protein having a NAP domain having at least 90 % homology to a NAP domain selected from the group consisting of AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAP48 [Fig. 16], AcaNAP23 [Fig. 16], AcaNAP24 [Fig. 16], AcaNAP25 [Fig. 16], AcaNAP44 [Fig. 16], AcaNAP31 [Fig. 16], AceNAP4-d1 [Fig. 16], AceNAP4-d2 [Fig. 16], AcaNAP45-d1 [Fig. 16], AcaNAP45-d2 [Fig. 16], AcaNAP47-d1 [Fig. 16], AcaNAP47-d2 [Fig. 16], AduNAP7-d1 [Fig. 16], AduNAP7-d2 [Fig. 16], AduNAP4 [Fig. 16], AceNAP5 [Fig. 16], and AceNAP7 [Fig. 16].
67. An isolated protein having an amino acid sequence of AcaNAPc2 [Fig. 16] having an additional proline residue at the C-terminus.
68. An isolated protein having Factor VIIa/Tissue Factor inhibitory activity and an amino acid sequence having at least 90% homology with the amino acid sequence of AcaNAPc2 [Fig. 16] having an additional proline residue at the C-terminus.
69. An isolated nucleic acid molecule encoding the amino acid sequence of AcaNAPc2 [Fig. 16] having an additional proline residue at the C-terminus.
70. An isolated nucleic acid molecule encoding a protein having Factor VIIa/Tissue Factor inhibitory activity and having at least 90% homology with the amino acid sequence of AcaNAPc2 [Fig. 16] having an additional proline residue at the C-terminus.
71. An isolated nucleic acid molecule encoding a protein Having Factor VIIa/Tissue Factor Inhibitor activity and having at least 90% homology to the nucleic acid sequence encoding the amino acid sequence of AcaNAPc2 [Fig. 16] having an additional proline residue at the C-terminus.

Patentansprüche

1. Isoliertes Protein mit Antikoagulationsaktivität und mit einer oder mehreren aus Nematoden extrahierten Antikoagulationsproteindomänen (NAP-Domänen), wobei jede NAP-Domäne die folgende Sequenz einschließt:

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10,

worin

- (a) A1 eine Aminosäuresequenz mit 7 bis 8 Aminosäureresten ist;
- (b) A2 eine Aminosäuresequenz mit 3 bis 5 Aminosäureresten ist;
- (c) A3 eine Aminosäuresequenz mit 3 Aminosäureresten ist;
- (d) A4 eine Aminosäuresequenz mit 6 bis 19 Aminosäureresten ist;
- (e) A5 eine Aminosäuresequenz mit 3 bis 4 Aminosäureresten ist;
- (f) A6 eine Aminosäuresequenz mit 3 bis 5 Aminosäureresten ist;
- (g) A7 ein Aminosäurerest ist;
- (h) A8 eine Aminosäuresequenz mit 11 bis 12 Aminosäureresten ist;
- (i) A9 eine Aminosäuresequenz mit 5 bis 7 Aminosäureresten ist; und
- (j) A10 eine Aminosäuresequenz mit 5 bis 25 Aminosäureresten ist.

2. Protein nach Anspruch 1, wobei die Aktivität des Proteins Faktor Xa inhibierende Aktivität einschließt.

3. Protein nach Anspruch 1 oder 2, wobei A3 die Sequenz Glu-A3_a-A3_b hat, wobei A3_a und A3_b unabhängig ausgewählte Aminosäurereste sind.

4. Protein nach Anspruch 3, wobei A3 die Sequenz Glu-A3_a-A3_b hat, wobei A3_a ausgewählt ist aus der Gruppe bestehend aus Ala, Arg, Pro, Lys, Ile, His, Leu und Thr, und A3_b ausgewählt ist aus der Gruppe bestehend aus Lys, Thr und Arg.

5. Protein nach einem der Ansprüche 3 bis 4, wobei A3 ausgewählt ist aus der Gruppe bestehend aus
Glu-Ala-Lys,
Glu-Arg-Lys,
Glu-Pro-Lys,
Glu-Lys-Lys,
Glu-Ile-Thr,
Glu-His-Arg,
Glu-Leu-Lys und
Glu-Thr-Lys.

6. Protein nach Anspruch 1, wobei A3 die Sequenz Asp-A3_a-A3_b hat, wobei A3_a und A3_b unabhängig ausgewählte Aminosäurereste sind.

7. Protein nach Anspruch 1 oder 6, wobei A3 Asp-Lys-Lys ist.

8. Protein nach einem der Ansprüche 1 bis 7, wobei A4 eine Aminosäuresequenz mit einer anionischen Nettoladung ist.

9. Protein nach einem der Ansprüche 1 oder 6 bis 8, wobei A5 die Sequenz A5_a-A5_b-A5_c-A5_d [SEQ. ID. Nr. 85] hat, wobei A5_a bis A5_d unabhängig ausgewählte Aminosäurereste sind.

10. Protein nach Anspruch 9, wobei A5_a Leu ist und A5_c Arg ist.

11. Protein nach einem der Ansprüche 1 bis 10, wobei A7 ausgewählt ist aus der Gruppe bestehend aus Val und Ile.

12. Protein nach einem der Ansprüche 1 bis 11, wobei A8 eine Aminosäuresequenz A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ. ID. Nr. 68] einschließt, wobei

- (a) A8_a der erste Aminosäurerest in A8 ist,
- (b) mindestens einer von A8_a und A8_b ausgewählt ist aus der Gruppe bestehend aus Glu oder Asp, und
- (c) A8_c bis A8_g unabhängig ausgewählte Aminosäurereste sind.

13. Protein nach Anspruch 12, wobei

- (a) A8_a Glu oder Asp ist,
- (b) A8_b ein unabhängig ausgewählter Aminosäurerest ist,
- (c) A8_c Gly ist,
- (d) A8_d aus der aus Phe, Tyr und Leu, bestehenden Gruppe ausgewählt ist,
- (e) A8_e Tyr ist,
- (f) A8_f Arg ist und
- (g) A8_g aus Asp und Asn ausgewählt ist.

14. Protein nach Anspruch 12 oder 13, wobei

- (a) A8_a ein unabhängig ausgewählter Aminosäurerest ist,
- (b) A8_b Glu oder Asp ist,
- (c) A8_c Gly ist,
- (d) A8_d aus der aus Phe, Tyr und Leu bestehenden Gruppe ausgewählt ist,
- (e) A8_e Tyr ist,
- (f) A8_f Arg ist und
- (g) A8_g aus Asp und Asn ausgewählt ist.

15. Protein nach einem der Ansprüche 12 bis 14, wobei A8_c-A8_d-A8_e-A8_f-A8_g ausgewählt ist aus der Gruppe bestehend aus
 Gly-Phe-Tyr-Arg-Asp [SEQ. ID. Nr. 69],
 Gly-Phe-Tyr-Arg-Asn [SEQ. ID. Nr. 70],
 Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. Nr. 71],
 Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. Nr. 72] und
 Gly-Leu-Tyr-Arg-Asp [SEQ. ID. Nr. 73].

16. Protein nach Anspruch 15, wobei A8_c-A8_d-A8_e-A8_f-A8_g Gly-Phe-Tyr-Arg-Asn [SEQ. ID. Nr. 70] ist.

17. Protein nach einem der Ansprüche 2 bis 16, wobei A10 eine Aminosäuresequenz einschließt, die ausgewählt ist aus der Gruppe bestehend aus
 Glu-Ile-Ile-His-Val [SEQ. ID. Nr. 74],
 Asp-Ile-Ile-Met-Val [SEQ. ID. Nr. 75],
 Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. Nr. 76] und
 Met-Glu-Ile-Ile-Thr [SEQ. ID. Nr. 77].

18. Protein nach Anspruch 17, wobei A10 die Aminosäuresequenz Glu-Ile-Ile-His-Val [SEQ. ID. Nr. 74] einschließt.

19. Protein nach Anspruch 17, wobei A10 die Aminosäuresequenz Asp-Ile-Ile-Met-Val [SEQ. ID. Nr. 75] einschließt.

20. Protein nach Anspruch 19 mit einer NAP-Domäne mit einer Aminosäuresequenz mit mindestens 90 % Homologie zu derjenigen von AcaNAP48 [Fig. 16].

21. Protein nach Anspruch 17, wobei A10 die Aminosäuresequenz Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. Nr. 76] einschließt.

22. Protein nach Anspruch 21 mit einer NAP-Domäne mit einer Aminosäuresequenz mit mindestens 90 % Homologie zu einer NAP-Domäne ausgewählt aus NAP-Domänen von AcaNAP23 [Fig. 16], AcaNAP24 [Fig. 16], AcaNAP25 [Fig. 16], AcaNAP44 [Fig. 16], AcaNAP31 [Fig. 16] und AceNAP4-d1 [Fig. 16] und AceNAP4-d2 [Fig. 16].

23. Protein nach Anspruch 17, wobei A10 die Aminosäuresequenz Met-Glu-Ile-Ile-Thr [SEQ. ID. Nr. 77] einschließt.

24. Protein nach Anspruch 23 mit einer NAP-Domäne mit einer Aminosäuresequenz mit mindestens 90 % Homologie zu einer NAP-Domäne ausgewählt aus NAP-Domänen von AcaNAP45-d1 [Fig. 16], AcaNAP45-d2 [Fig. 16], AcaNAP47-d1 [Fig. 16], AcaNAP47-d2 [Fig. 16], AduNAP7-d1 [Fig. 16], AduNAP7-d2 [Fig. 16], AduNAP4 [Fig. 16], AceNAP5 [Fig. 16] und AceNAP7 [Fig. 16].

25. Protein nach Anspruch 1 oder 2, wobei

- (a) A3 die Sequenz Glu-A3_a-A3_b hat, wobei A3_a und A3_b unabhängig ausgewählte Aminosäurereste sind;
- (b) A4 eine Aminosäuresequenz mit einer anionischen Nettoladung ist;
- (c) A7 ausgewählt ist aus der Gruppe bestehend aus Val und Ile;
- (d) A8 eine Aminosäuresequenz einschließt, die ausgewählt ist aus der Gruppe bestehend aus:

Gly-Phe-Tyr-Arg-Asp [SEQ. ID. Nr. 69],
 Gly-Phe-Tyr-Arg-Asn [SEQ. ID. Nr. 70],
 Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. Nr. 71],
 Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. Nr. 72], und
 Gly-Leu-Tyr-Arg-Asp [SEQ. ID. Nr. 73]; und

- (e) A10 eine Aminosäuresequenz einschließt, die ausgewählt ist aus der Gruppe bestehend aus:

Glu-Ile-Ile-His-Val [SEQ. ID. Nr. 74],
 Asp-Ile-Ile-Met-Val [SEQ. ID. Nr. 75],
 Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. Nr. 76] und
 Met-Glu-Ile-Ile-Thr [SEQ. ID. Nr. 77].

26. Protein nach Anspruch 1 oder 2, wobei

(a) A3 ausgewählt ist aus der Gruppe bestehend aus:

Glu-Ala-Lys,
Glu-Arg-Lys,
Glu-Pro-Lys,
Glu-Lys-Lys,
Glu-Ile-Thr,
Glu-His-Arg,
Glu-Leu-Lys und
Glu-Thr-Lys;

(b) A4 eine Aminosäuresequenz mit einer anionischen Nettoladung ist;

(c) A7 Val oder Ile ist;

(d) A8 eine Aminosäuresequenz einschließt, die ausgewählt ist aus der Gruppe bestehend aus:

A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ. ID. Nr. 78],
A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. Nr. 79],
A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ. ID. Nr. 80],
A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ. ID. Nr. 81], und
A8_a-A8_b-Gly-Leu-Tyr-Arg-Asp [SEQ. ID. Nr. 82],

wobei mindestens einer von A8_a und A8_b Glu oder Asp ist;

(e) A9 eine Aminosäuresequenz von fünf Aminosäureresten

(f) A10 eine Aminosäuresequenz einschließt, die ausgewählt ist aus der Gruppe bestehend aus:

Glu-Ile-Ile-His-Val [SEQ. ID. Nr. 74],
Asp-Ile-Ile-Met-Val [SEQ. ID. Nr. 75],
Phe-Ile-Thr-Phe-Ala-Pro [SEQ. ID. Nr. 76] und
Met-Glu-Ile-Ile-Thr [SEQ. ID. Nr. 77].

27. Protein nach Anspruch 18, 25 oder 26 mit einer NAP-Domäne mit mindestens 90 % Homologie zu NAP-Domänen ausgewählt aus AcaNAP5 [Fig. 16] und AcaNAP6 [Fig. 16].**28.** Protein nach Anspruch 25 oder 26 mit einer NAP-Domäne mit mindestens 90 % Homologie zu einer NAP-Domäne ausgewählt aus der Gruppe bestehend aus AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAP48 [Fig. 16], AcaNAP23 [Fig. 16], AcaNAP24 [Fig. 16], AcaNAP25 [Fig. 16], AcaNAP44 [Fig. 16], AcaNAP31 [Fig. 16], AceNAP4-d1 [Fig. 16], AceNAP4-d2 [Fig. 16], AcaNAP45-d1 [Fig. 16], AcaNAP45-d2 [Fig. 16], AcaNAP47-d1 [Fig. 16], AcaNAP47-d2 [Fig. 16], AduNAP7-d1 [Fig. 16], AduNAP7-d2 [Fig. 16], AduNAP4 [Fig. 16], AceNAP5 [Fig. 16] und AceNAP7 [Fig. 16].**29.** Isoliertes Protein mit Antikoagulationsaktivität ausgewählt aus der Gruppe bestehend aus AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAP48 [Fig. 16], AcaNAP23 [Fig. 16], AcaNAP24 [Fig. 16], AcaNAP25 [Fig. 16], AcaNAP44 [Fig. 16], AcaNAP31 [Fig. 16], AceNAP4 [Fig. 16], AcaNAP45 [Fig. 16], AcaNAP47 [Fig. 16], AduNAP7 [Fig. 16], AduNAP4 [Fig. 16], AceNAP5 [Fig. 16] und AceNAP7 [Fig. 16].**30.** Isoliertes Protein mit Faktor Xa-inhibierender Aktivität ausgewählt aus der Gruppe bestehend aus AcaNAP5 [Fig. 16] und AcaNAP6 [Fig. 16].**31.** Protein nach Anspruch 1, wobei

(a) A3 die Sequenz Asp-A3_a-A3_b hat, wobei A3_a und A3_b unabhängig ausgewählte Aminosäurereste sind;

(b) A4 eine Aminosäuresequenz mit einer anionischen Nettoladung ist;

(c) A5 die Sequenz A5_a-A5_b-A5_c-A5_d [SEQ. ID. Nr. 85] hat, wobei A5_a bis A5_d unabhängig ausgewählte Aminosäurereste sind, und

(d) A7 ausgewählt ist aus der Gruppe bestehend aus Val und Ile.

32. Protein nach Anspruch 1, wobei

- (a) A3 Asp-Lys-Lys ist;
- (b) A4 eine Aminosäuresequenz mit einer anionischen Nettoladung ist;
- (c) A5 die Sequenz A5_a-A5_b-A5_c-A5_d hat, wobei A5_a Leu ist, A5_c Arg ist, und A5_b und A5_d unabhängig ausgewählte Aminosäurereste sind [SEQ. ID. Nr. 357];
- (d) A7 Val ist; und
- (e) A8 eine Aminosäuresequenz A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ. ID. Nr. 79] einschließt, wobei mindestens eine von A8_a und A8_b Glu oder Asp ist.

33. Protein nach Anspruch 31 oder 32 mit einer NAP-Domäne mit einer Aminosäuresequenz mit mindestens 90 % Homologie zu derjenigen von AcaNAPc2 [Fig. 16].

34. Isoliertes Protein mit Faktor VIIa/TF-inhibierender Aktivität mit einer Nematoden-extrahierten Antikoagulansprotein-domäne (NAP-Domäne) mit einer Aminosäuresequenz mit mindestens 90 % Homologie zu der NAP-Domäne von AcaNAPc2 [Fig. 16].

35. Isoliertes Protein nach einem der Ansprüche 1 bis 34 mit Antikoagulationsaktivität, wobei das Protein die katalytische Aktivität des fVIIa/TF-Komplexes in Gegenwart von fXa oder katalytisch inaktivem fXa-Derivat spezifisch inhibiert und die Aktivität von FVIIa in Abwesenheit von TF nicht spezifisch inhibiert und nicht spezifisch Prothrombinase inhibiert.

36. Protein nach Anspruch 35, wobei das Protein AcaNAPc2 [Fig. 16] ist.

37. Protein mit Antikoagulationsaktivität und mit einer Aminosäuresequenz mit mindestens 90 % Homologie zu AcaNAPc2 [Fig. 16].

38. Isoliertes Protein mit Serinprotease inhibierender Aktivität und mit einer oder mehreren aus Nematoden extrahierten Antikoagulansprotein-domänen (NAP-Domänen) mit einer Aminosäuresequenz mit mindestens 90 % Homologie zu NAP-Domänen ausgewählt aus HpoNAP5 [Fig. 16] und NamNAP [Fig. 16],
worin

- (a) A1 eine Aminosäuresequenz mit 7 bis 8 Aminosäureresten ist;
- (b) A2 eine Aminosäuresequenz mit 3 bis 5 Aminosäureresten ist;
- (c) A3 die Sequenz Glu-A3_a-A3_b hat, wobei A3_a und A3_b unabhängig ausgewählte Aminosäurereste sind;
- (d) A4 eine Aminosäuresequenz mit 6 bis 19 Aminosäureresten mit einer anionischen Nettoladung ist;
- (e) A5 die Sequenz A5_a-A5_b-A5_c hat, wobei A5_a bis A5_c unabhängig ausgewählte Aminosäurereste sind;
- (f) A6 eine Aminosäuresequenz mit 3 bis 5 Aminosäureresten ist;
- (g) A7 Gln ist;
- (h) A8 eine Aminosäuresequenz mit 10 bis 12 Aminosäureresten ist; und
- (i) A9 eine Aminosäuresequenz mit 5 bis 7 Aminosäureresten ist;
- (j) A10 eine Aminosäuresequenz mit 1 bis 25 Aminosäureresten ist.

39. Protein nach Anspruch 38, wobei

- (a) A3 Glu-Pro-Lys ist;
- (b) A4 eine Aminosäuresequenz mit einer anionischen Nettoladung ist;
- (c) A5 ausgewählt ist aus Thr-Leu-Asn und Thr-Met-Asn; und
- (d) A7 Gln ist.

40. Isoliertes Protein mit Serinprotease inhibierender Aktivität und mit einer aus Nematoden extrahierten Antikoagulationsprotein-domäne (NAP-Domäne) mit einer Aminosäuresequenz mit mindestens 90 % Homologie zu NAP-Domänen ausgewählt aus der Gruppe bestehend aus HpoNAP5 [Fig. 16] und NamNAP [Fig. 16].

41. Protein nach einem der Ansprüche 1, 2, 25, 26, 31, 32, 38 oder 39, wobei das Protein zwei NAP-Domänen hat.

42. Protein mit zwei aus Nematoden extrahierten Antikoagulationsprotein- (NAP)-Domänen, wobei das Protein ausgewählt ist aus der Gruppe bestehend aus AceNAP4 [Fig. 17], AcaNAP45 [Fig. 18], AcaNAP47 [Fig. 19] und AduNAP7

[Fig. 20].

43. Protein nach einem der Ansprüche 1 bis 42, wobei das Protein von einer Nematodenspezies abgeleitet ist, die ausgewählt ist aus der Gruppe bestehend aus *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus* und *Heligmosomoides polygyrus*.
44. Isoliertes rekombinantes cDNA-Molekül, das ein Protein gemäß einem der Ansprüche 1 bis 42 kodiert.
45. cDNA-Molekül, welches das Protein gemäß Anspruch 18 oder 25 kodiert, das eine Nukleotidsequenz mit mindestens 90 % Homologie zu derjenigen hat, die für AcaNAP5 [Fig. 1] und AcaNAP6 [Fig. 3] kodiert.
46. cDNA-Molekül, welches das Protein mit Faktor Xa inhibierender Aktivität kodiert, das aus der Gruppe bestehend aus Proteinen mit NAP-Domänen mit mindestens 90 % Homologie zu AcaNAP5 [Fig. 16] und AcaNAP6 [Fig. 16] ausgewählt ist.
47. cDNA-Molekül, welches das Protein gemäß Anspruch 20 kodiert, das eine Nukleotidsequenz mit mindestens 90 % Homologie zu derjenigen hat, die für AcaNAP48 [Fig. 13h] kodiert.
48. cDNA-Molekül, welches das Protein gemäß Anspruch 21 kodiert, das eine Nukleotidsequenz mit mindestens 90 % Homologie zu einer solchen hat, die aus der Gruppe bestehend aus cDNAs ausgewählt ist, die für AcaNAP23 [Fig. 13a], AcaNAP24 [Fig. 13b], AcaNAP25 [Fig. 13c], AcaNAP44 [Fig. 13e], AcaNAP31 [Fig. 13d] und AceNAP4 [Fig. 7a] kodieren.
49. cDNA-Molekül, welches das Protein gemäß Anspruch 23 kodiert, das eine Nukleotidsequenz mit mindestens 90 % Homologie zu einer solchen hat, die aus der Gruppe bestehend aus cDNAs ausgewählt ist, die für AcaNAP45 [Fig. 13f], AcaNAP47 [Fig. 13g], AduNAP7 [Fig. 7e], AduNAP4 [Fig. 7d], AceNAP5 [Fig. 7b] und AceNAP7 [Fig. 7c] kodieren.
50. cDNA-Molekül, welches das Protein gemäß Anspruch 26 kodiert, das ausgewählt ist aus der Gruppe bestehend aus cDNAs, die für AcaNAP5 [Fig. 1], AcaNAP6 [Fig. 3], AcaNAP48 [Fig. 13h], AcaNAP23 [Fig. 13a], AcaNAP24 [Fig. 13b], AcaNAP25 [Fig. 13c], AcaNAP44 [Fig. 13e], AcaNAP31 [Fig. 13d], AceNAP4 [Fig. 7a], AcaNAP45 [Fig. 13f], AcaNAP47 [Fig. 13g], AduNAP7 [Fig. 7e], AduNAP4 [Fig. 7d], AceNAP5 [Fig. 7b] und AceNAP7 [Fig. 7c] kodieren.
51. cDNA-Molekül, welches das Protein gemäß Anspruch 38 kodiert, das eine Nukleotidsequenz mit mindestens 90 % Homologie zu Sequenzen ausgewählt aus cDNAs hat, die für HpoNAP5 [Fig. 7f] und NamNAP [Fig. 14] kodieren.
52. cDNA-Molekül, das ein Protein mit Antikoagulansaktivität kodiert, das aus der Gruppe bestehend aus cDNAs mit mindestens 90 % Homologie zu cDNAs ausgewählt ist, die für AcaNAP5 [Fig. 1], AcaNAP6 [Fig. 3], AcaNAP48 [Fig. 13h], AcaNAP23 [Fig. 13a], AcaNAP24 [Fig. 13b], AcaNAP25 [Fig. 13c], AcaNAP44 [Fig. 13e], AcaNAP31 [Fig. 13d], AceNAP4 [Fig. 7a], AcaNAP45 [Fig. 13f], AcaNAP47 [Fig. 13g], AduNAP7 [Fig. 7e], AduNAP4 [Fig. 7d], AceNAP5 [Fig. 7b] und AceNAP7 [Fig. 7c] kodieren.
53. Isoliertes rekombinantes cDNA-Molekül nach einem der Ansprüche 44 bis 52, das Protein mit Antikoagulationsaktivität kodiert, wobei das Protein die katalytische Aktivität des fVIIa/TF-Komplexes in Gegenwart von fXa oder katalytisch inaktivem fXa-Derivat spezifisch inhibiert und die Aktivität von FVIIa in Abwesenheit von TF nicht spezifisch inhibiert und nicht spezifisch Prothrombinase inhibiert.
54. cDNA-Molekül nach Anspruch 53, wobei die DNA AcaNAPc2 [Fig. 16] kodiert.
55. Isoliertes cDNA-Molekül, das ein Protein mit Antikoagulansaktivität mit mindestens 90 % Homologie zu AcaNAPc2 [Fig. 9] kodiert.
56. Isoliertes rekombinantes cDNA-Molekül, das ein Protein gemäß Anspruch 32 kodiert.
57. cDNA-Molekül nach Anspruch 56 mit einer Nukleotidsequenz, die eine Aminosäuresequenz mit mindestens 90 % Homologie zu AcaNAPc2 [Fig. 16] kodiert.

58. cDNA-Molekül nach einem der Ansprüche 44 bis 57, das von einer Nematodenspezies abgeleitet ist.
59. cDNA-Molekül nach Anspruch 58, wobei die Nematodenspezies ausgewählt ist aus der Gruppe bestehend aus *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus* und *Heligmosomoides polygyrus*.
60. Oligonukleotid, umfassend eine Nukleotidsequenz ausgewählt aus
NAP-1: AAR-CCN-TGY-GAR-MGG-AAR-TGY [SEQ. ID. Nr. 90] und
NAP-4.RC TW-RWA-NCC-NTC-YTT-RCA-NAC-RCA [SEQ. ID. Nr. 91].
61. Verwendung eines Proteins gemäß einem der Ansprüche 1 bis 42 zur Herstellung einer pharmazeutischen Zusammensetzung zur Inhibierung der Blutkoagulation.
62. Verwendung nach Anspruch 61, bei der das Protein ausgewählt ist aus der Gruppe bestehend aus AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAPc2 [Fig. 16], HpoNAP5 [Fig. 16], NamNAP [Fig. 16].
63. Verwendung nach Anspruch 61, bei der das Protein eine NAP-Domäne hat, die mindestens 90 % Homologie zu NAP-Domänen aufweist, die aus der Gruppe bestehend aus AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAP48 [Fig. 16], AcaNAP23 [Fig. 16], AcaNAP24 [Fig. 16], AcaNAP25 [Fig. 16], AcaNAP44 [Fig. 16], AcaNAP31 [Fig. 16], AceNAP4-d1 [Fig. 16], AceNAP4-d2 [Fig. 16], AcaNAP45-d1 [Fig. 16], AcaNAP45-d2 [Fig. 16], AcaNAP47-d1 [Fig. 16], AcaNAP47-d2 [Fig. 16], AduNAP7-d1 [Fig. 16], AduNAP7-d2 [Fig. 16], AduNAP4 [Fig. 16], AceNAP5 [Fig. 16] und AceNAP7 [Fig. 16] ausgewählt sind.
64. Pharmazeutische Zusammensetzung, die ein Protein gemäß einem der Ansprüche 1 bis 42 enthält.
65. Pharmazeutische Zusammensetzung nach Anspruch 64, die ein Protein enthält, das aus der Gruppe bestehend aus AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAPc2 [Fig. 16], HpoNAP5 [Fig. 16], NamNAP [Fig. 16] ausgewählt ist.
66. Pharmazeutische Zusammensetzung nach Anspruch 64, die ein Protein mit einer NAP-Domäne enthält, die mindestens 90 % Homologie zu NAP-Domänen aufweist, die aus der Gruppe bestehend aus AcaNAP5 [Fig. 16], AcaNAP6 [Fig. 16], AcaNAP48 [Fig. 16], AcaNAP23 [Fig. 16], AcaNAP24 [Fig. 16], AcaNAP25 [Fig. 16], AcaNAP44 [Fig. 16], AcaNAP31 [Fig. 16], AceNAP4-d1 [Fig. 16], AceNAP4-d2 [Fig. 16], AcaNAP45-d1 [Fig. 16], AcaNAP45-d2 [Fig. 16], AcaNAP47-d1 [Fig. 16], AcaNAP47-d2 [Fig. 16], AduNAP7-d1 [Fig. 16], AduNAP7-d2 [Fig. 16], AduNAP4 [Fig. 16], AceNAP5 [Fig. 16] und AceNAP7 [Fig. 16] ausgewählt sind.
67. Isoliertes Protein mit einer Aminosäuresequenz von AcaNAPc2 [Fig. 16] mit einem zusätzlichen Prolinrest am C-Terminus.
68. Isoliertes Protein mit Faktor VIIa/Tissue Factor inhibierender Aktivität und einer Aminosäuresequenz mit mindestens 90 % Homologie mit der Aminosäuresequenz von AcaNAPc2 [Fig. 16] mit einem zusätzlichen Prolinrest am C-Terminus.
69. Isoliertes Nukleinsäuremolekül, das die Aminosäuresequenz von AcaNAPc2 [Fig. 16] mit einem zusätzlichen Prolinrest am C-Terminus kodiert.
70. Isoliertes Nukleinsäuremolekül, das ein Protein kodiert, das Faktor VIIa/Tissue Factor inhibierende Aktivität und das eine mindestens 90 % Homologie mit der Aminosäuresequenz von AcaNAPc2 [Fig. 16] mit einem zusätzlichen Prolinrest am C-Terminus hat.
71. Isoliertes Nukleinsäuremolekül, das ein Protein mit Faktor VIIa/Tissue Factor inhibierender Aktivität kodiert und das mindestens 90 % Homologie zu der Nukleinsäuresequenz hat, welche die Aminosäuresequenz von AcaNAPc2 [Fig. 16] mit einem zusätzlichen Prolinrest am C-Terminus kodiert.

Revendications

1. Protéine isolée ayant une activité anticoagulante et ayant un ou plusieurs domaines de protéine anticoagulante

extraite de nématode (domaines NAP), dans laquelle chaque domaine NAP comprend la séquence :

Cys-A1-Cys-A2-Cys-A3-Cys-A4-Cys-A5-Cys-A6-Cys-A7-Cys-A8-Cys-A9-Cys-A10

dans laquelle

- (a) A1 est une séquence d'acides aminés de 7 à 8 résidus d'acide aminé ;
- (b) A2 est une séquence d'acides aminés de 3 à 5 résidus d'acide aminé ;
- (c) A3 est une séquence d'acides aminés de 3 résidus d'acide aminé ;
- (d) A4 est une séquence d'acides aminés de 6 à 19 résidus d'acide aminé ;
- (e) A5 est une séquence d'acides aminés de 3 à 4 résidus d'acide aminé ;
- (f) A6 est une séquence d'acides aminés de 3 à 5 résidus d'acide aminé ;
- (g) A7 est un résidu d'acide aminé ;
- (h) A8 est une séquence d'acides aminés de 11 à 12 résidus d'acide aminé ;
- (i) A9 est une séquence d'acides aminés de 5 à 7 résidus d'acide aminé ;
- (j) A10 est une séquence d'acides aminés de 5 à 25 résidus d'acide aminé.

2. Protéine selon la revendication 1, dans laquelle l'activité de ladite protéine comprend l'activité inhibitrice du facteur Xa.

3. Protéine selon la revendication 1 ou 2, dans laquelle A3 a la séquence Glu-A3_a-A3_b, dans laquelle A3_a et A3_b sont des résidus d'acide aminé choisis indépendamment.

4. Protéine selon la revendication 3, dans laquelle A3 a la séquence Glu-A3_a-A3_b, dans laquelle A3_a est choisi dans le groupe constitué par Ala, Arg, Pro, Lys, Ile, His, Leu et Thr, et A3_b est choisi dans le groupe constitué par Lys, Thr et Arg.

5. Protéine selon l'une quelconque des revendications 3 à 4, dans laquelle A3 est choisi dans le groupe constitué par Glu-Ala-Lys, Glu-Arg-Lys, Glu-Pro-Lys, Glu-Lys-Lys, Glu-Ile-Thr, Glu-His-Arg, Glu-Leu-Lys, et Glu-Thr-Lys.

6. Protéine selon la revendication 1, dans laquelle A3 a la séquence Asp-A3_a-A3_b, dans laquelle A3_a et A3_b sont des résidus d'acide aminé choisis indépendamment.

7. Protéine selon la revendication 1 ou 6, dans laquelle A3 est Asp-Lys-Lys.

8. Protéine selon l'une quelconque des revendications 1 à 7, dans laquelle A4 est une séquence d'acides aminés ayant une charge anionique nette.

9. Protéine selon l'une quelconque des revendications 1 ou 6 à 8, dans laquelle A5 a la séquence A5_a-A5_b-A5_c-A5_d [SEQ ID n° 85], dans laquelle A5_a à A5_d sont des résidus d'acide aminé choisis indépendamment.

10. Protéine selon la revendication 9, dans laquelle A5_a est Leu et A5_c est Arg.

11. Protéine selon l'une quelconque des revendications 1 à 10, dans laquelle A7 est choisi dans le groupe constitué par Val et Ile.

12. Protéine selon l'une quelconque des revendications 1 à 11, dans laquelle A8 comprend une séquence d'acides aminés A8_a-A8_b-A8_c-A8_d-A8_e-A8_f-A8_g [SEQ ID n° 68], dans laquelle

- (a) A8_a est le premier résidu d'acide aminé dans A8,
- (b) au moins un parmi A8_a et A8_b est choisi dans le groupe constitué par Glu ou Asp, et
- (c) A8_c à A8_g sont des résidus d'acide aminé choisis indépendamment.

13. Protéine selon la revendication 12, dans laquelle

- (a) A8 est Glu ou Asp,
- (b) A8_b est un résidu d'acide aminé choisi indépendamment,
- (c) A8_c est Gly,
- (d) A8_d est choisi dans le groupe constitué par Phe, Tyr et Leu,
- (e) A8_e est Tyr,
- (f) A8_f est Arg, et
- (g) A8_g est choisi parmi Asp et Asn.

14. Protéine selon la revendication 12 ou 13, dans laquelle

- (a) A8_a est un résidu d'acide aminé choisi indépendamment,
- (b) A8_b est Glu ou Asp,
- (c) A8_c est Gly,
- (d) A8_d est choisi dans le groupe constitué par Phe, Tyr et Leu,
- (e) A8_e est Tyr,
- (f) A8_f est Arg, et
- (g) A8_g est choisi parmi Asp et Asn.

15. Protéine selon l'une quelconque des revendications 12 à 14, dans laquelle A8_c-A8_d-A8_e-A8_fA8_g est choisie dans le groupe constitué par

- Gly-Phe-Tyr-Arg-Asp [SEQ ID n° 69]
- Gly-Phe-Tyr-Arg-Asn [SEQ ID n° 70]
- Gly-Tyr-Tyr-Arg-Asp [SEQ ID n° 71]
- Gly-Tyr-Tyr-Arg-Asn [SEQ ID n° 72] et
- Gly-Leu-Tyr-Arg-Asp [SEQ ID n° 73]

16. Protéine selon la revendication 15, dans laquelle A8_c-A8_d-A8_e-A8_f, A8_g est Gly-Phe-Tyr-Arg-Asn [SEQ ID n° 70].

17. Protéine selon l'une quelconque des revendications 2 à 16, dans laquelle A10 comprend une séquence d'acides aminés choisie dans le groupe constitué par

- Glu-Ile-Ile-His-Val [SEQ ID n° 74]
- Asp-Ile-Ile-Met-Val [SEQ ID n° 75]
- Phe-Ile-Thr-Phe-Ala-Pro [SEQ ID n° 76] et
- Met-Glu-Ile-Ile-Thr [SEQ ID n° 77]

18. Protéine selon la revendication 17, dans laquelle A10 comprend la séquence d'acides aminés Glu-Ile-Ile-His-Val [SEQ ID n° 74].

19. Protéine selon la revendication 17, dans laquelle A10 comprend la séquence d'acides aminés Asp-Ile-Ile-Met-Val [SEQ ID n° 75].

20. Protéine selon la revendication 19, ayant un domaine NAP avec une séquence d'acides aminés ayant au moins 90 % d'homologie avec celle d'AcaNAP48 [Figure 16].

21. Protéine selon la revendication 17, dans laquelle A10 comprend la séquence Phe-Ile-Thr-Phe-Ala-Pro [SEQ ID n° 76].

22. Protéine selon la revendication 21, ayant un domaine NAP avec une séquence d'acides aminés ayant au moins 90 % d'homologie avec un domaine NAP choisi parmi les domaines NAP AcaNAP23 [Figure 16], AcaNAP24 [Figure 16], AcaNAP25 [Figure 16], AcaNAP44 [Figure 16], AcaNAP31 [Figure 16] et AceNAP4-dl [Figure 16] et AceNAP4-d2 [Figure 16].

23. Protéine selon la revendication 17, dans laquelle A10 comprend la séquence Met-Glu-Ile-Ile-Thr [SEQ ID n° 77].

24. Protéine selon la revendication 23, ayant un domaine NAP avec une séquence d'acides aminés ayant au moins 90 % d'homologie avec un domaine NAP choisi parmi les domaines NAP AcaNAP45-d1 [Figure 16], AcaNAP45-d2 [Figure 16], AcaNAP47-d1 [Figure 16], AcaNAP47-d2 [Figure 16], AduNAP7-d1 [Figure 16], AduNAP7-d2 [Figure 16].

16], AduNAP4 [Figure 16], AceNAP5 [Figure 16] et AceNAP7 [Figure 16].

25. Protéine selon la revendication 1 ou 2, dans laquelle

- (a) A3 a la séquence Glu-A3_a-A3_b, dans laquelle A3_a et A3_b sont des résidus d'acides aminés choisis indépendamment ;
 (b) A4 est une séquence d'acides aminés ayant une charge anionique nette ;
 (c) A7 est choisi dans le groupe constitué par Val et Ile ;
 (d) A8 comprend une séquence d'acides aminés choisie dans le groupe constitué par
 Gly-Phe-Tyr-Arg-Asp [SEQ ID n° 69],
 Gly-Phe-Tyr-Arg-Asn [SEQ ID n° 70],
 Gly-Tyr-Tyr-Arg-Asp [SEQ ID n° 71],
 Gly-Tyr-Tyr-Arg-Asn [SEQ ID n° 72], et
 Gly-Leu-Tyr-Arg-Asp [SEQ ID n° 73]; et
 (e) A10 comprend une séquence d'acides aminés choisie dans le groupe constitué par
 Glu-Ile-Ile-His-Val [SEQ ID n° 74],
 Asp-Ile-Ile-Met-Val [SEQ ID n° 75],
 Phe-Ile-Thr-Phe-Ala-Pro [SEQ ID n° 76], et
 Met-Glu-Ile-Ile-Thr [SEQ ID n° 77]

26. Protéine selon la revendication 1 ou 2, dans laquelle

- (a) A3 est choisi dans le groupe constitué par
 Glu-Ala-Lys,
 Glu-Arg-Lys,
 Glu-Pro-Lys,
 Glu-Lys-Lys,
 Glu-Ile-Thr,
 Glu-His-Arg,
 Glu-Leu-Lys, et
 Glu-Thr-Lys;
 (b) A4 est une séquence d'acides aminés ayant une charge anionique nette ;
 (c) A7 est Val ou Ile ;
 (d) A8 comprend une séquence d'acides aminés choisie dans le groupe constitué par
 A8_a-A8_b-Gly-Phe-Tyr-Arg-Asp [SEQ ID n° 78],
 A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ ID n° 79],
 A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asp [SEQ ID n° 80],
 A8_a-A8_b-Gly-Tyr-Tyr-Arg-Asn [SEQ ID n° 81],
 et
 A8_a-A8_b-Gly-Leu-Tyr-Arg-Asp [SEQ ID n° 82],

dans lesquelles au moins un parmi A8_a et A8_b est Glu ou Asp ;

- (e) A9 est une séquence d'acides aminés de cinq résidus d'acides aminés ; et
 (f) A10 comprend une séquence d'acides aminés choisie dans le groupe constitué par
 Glu-Ile-Ile-His-Val [SEQ ID n° 74],
 Asp-Ile-Ile-Met-Val [SEQ ID n° 75],
 Phe-Ile-Thr-Phe-Ala-Pro [SEQ ID n° 76], et
 Met-Glu-Ile-Ile-Thr [SEQ ID n° 77],

27. Protéine selon la revendication 18, 25 ou 26 ayant un domaine NAP ayant au moins 90 % d'homologie avec les domaines NAP choisis parmi AcaNAP5 [Figure 16] et AcaNAP6 [Figure 16].

28. Protéine selon la revendication 25 ou 26 ayant un domaine NAP ayant au moins 90 % d'homologie avec un domaine NAP choisi dans le groupe constitué par AcaNAP5 [Figure 16], AcaNAP6 [Figure 16], AcaNAP48 [Figure 16], AcaNAP23 [Figure 16], AcaNAP24 [Figure 16], AcaNAP25 [Figure 16], AcaNAP44 [Figure 16], AcaNAP31 [Figure 16], AceNAP4-d1 [Figure 16], AceNAP4-d2 [Figure 16], AcaNAP45-d1 [Figure 16], AcaNAP45-d2 [Figure 16], AcaNAP47-d1 [Figure 16], AcaNAP47-d2 [Figure 16], AduNAP7-d1 [Figure 16], AduNAP7-d2 [Figure 16], AduNAP4

[Figure 16], AceNAP5 [Figure 16] et AceNAP7 [Figure 16].

29. Protéine isolée ayant une activité anti-coagulante choisie dans le groupe constitué par AcaNAP5 [Figure 16], AcaNAP6 [Figure 16], AcaNAP48 [Figure 16], AcaNAP23 [Figure 16], AcaNAP24 [Figure 16], AcaNAP25 [Figure 16], AcaNAP44 [Figure 16], AcaNAP31 [Figure 16], AceNAP4 [Figure 16], AcaNAP45 [Figure 16], AcaNAP47 [Figure 16], AduNAP7 [Figure 16], AduNAP4 [Figure 16], AceNAP5 [Figure 16] et AceNAP7 [Figure 16].

30. Protéine isolée ayant une activité inhibitrice du facteur Xa choisie dans le groupe constitué par AcaNAP5 [Figure 16] et AcaNAP6 [Figure 16].

31. Protéine selon la revendication 1, dans laquelle

- (a) A3 est la séquence Asp-A3_a-A3_b, ici A3_a et A3_b sont des résidus d'acide aminé choisis indépendamment ;
- (b) A4 est une séquence d'acides aminés ayant une charge anionique nette ;
- (c) A5 a la séquence A5_a-A5_b-A5_c-A5_d [SEQ ID n° 85] dans laquelle A5_a à A5_d sont des résidus d'acide aminé choisis indépendamment, et
- (d) A7 est choisi dans le groupe constitué par Val et Ile.

32. Protéine selon la revendication 1, dans laquelle

- (a) A3 est Asp-Lys-Lys ;
- (b) A4 est une séquence d'acides aminés ayant une charge anionique nette ;
- (c) A5 a la séquence A5_a-A5_b-A5_c-A5_d dans laquelle A5_a est Leu, A5_c est Arg, et A5_b et A5_d sont des résidus d'acides aminés choisis indépendamment [SEQ ID n° 357] ;
- (d) A7 est Val ; et
- (e) A8 comprend une séquence d'acides aminés A8_a-A8_b-Gly-Phe-Tyr-Arg-Asn [SEQ ID n° 79], dans laquelle au moins un parmi A8_a et A8_b est Glu ou Asp.

33. Protéine selon la revendication 31 ou 32 ayant un domaine NAP avec une séquence d'acides aminés ayant au moins 90 % d'homologie avec le domaine NAP d'AcaNAPc2 [Figure 16].

34. Protéine isolée ayant une activité inhibitrice du facteur VIIa/TF ayant un domaine de protéine anticoagulante extraite de nématode (domaine NAP) avec une séquence d'acides aminés ayant au moins 90 % d'homologie avec le domaine NAP AcaNAPc2 [Figure 16].

35. Protéine isolée selon l'une quelconque des revendications 1 à 34 ayant une activité anticoagulante, dans laquelle ladite protéine inhibe spécifiquement l'activité catalytique du complexe fVIIa/TF en présence de fXa ou d'un dérivé du fXa catalytiquement inactif, et n'inhibe pas spécifiquement l'activité du FVIIa en l'absence de TF, et n'inhibe pas spécifiquement la prothrombinase.

36. Protéine selon la revendication 35, dans laquelle la protéine est l'AcaNAPc2 [Figure 16].

37. Protéine ayant une activité anticoagulante et ayant la séquence d'acide aminé ayant au moins 90 % d'homologie avec l'AcaNAPc2 [Figure 16].

38. Protéine isolée ayant une activité inhibitrice de sérine protéase et ayant un ou plusieurs domaines de protéine anticoagulante extraite de nématode (domaines NAP) avec une séquence d'acide aminé ayant au moins 90 % d'homologie avec les domaines NAP choisis parmi HpoNAP5 [Figure 16] et NamNAP [Figure 16] dans laquelle

- (a) A1 est une séquence d'acide aminé de 7 à 8 résidus d'acide aminé ;
- (b) A2 est une séquence d'acide aminé de 3 à 5 résidus d'acide aminé ;
- (c) A3 a la séquence Glu-A3_a-A3_b, dans laquelle A3_a et A3_b sont des résidus d'acide aminé choisis indépendamment ;
- (d) A4 est une séquence d'acide aminé de 6 à 19 résidus d'acide aminé ayant une charge anionique nette ;
- (e) A5 a la séquence A5_a-A5_b-A5_c, dans laquelle A5_a à A5_c sont des résidus d'acide aminé choisis indépendamment ;
- (f) A6 est une séquence d'acides aminés de 3 à 5 résidus d'acide aminé ;

- (g) A7 est Gln;
- (h) A8 est une séquence d'acide aminé de 10 à 12 résidus d'acide aminé ; et aminé;
- (j) A10 est une séquence d'acides aminés de 1 à 25 résidus d'acide aminé.

5 39. Protéine selon la revendication 38, dans laquelle

- (a) A3 est Glu-Pro-Lys ;
- (b) A4 est une séquence d'acides aminés ayant une charge anionique nette ;
- (c) A5 est choisi parmi Thr-Leu-Asn et Thr-Met-Asn ; et
- (d) A7 est Gln.

10 40. Protéine isolée ayant une activité inhibitrice de sérine protéase et un domaine de protéine anticoagulante extraite de nématode (domaine NAP) avec une séquence d'acides aminés ayant au moins 90 % d'homologie avec les domaines NAP choisis dans le groupe constitué par HpoNAP5 [Figure 16] et NamNAP [Figure 16].

15 41. Protéine selon l'une quelconque parmi les revendications 1, 2, 25, 26, 31, 32, 38 ou 39, dans laquelle ladite protéine a deux domaines NAP.

20 42. Protéine ayant deux domaines de protéine anticoagulante extraite de nématode (NAP), dans laquelle ladite protéine est choisie dans le groupe constitué par AceNAP4 [Figure 17], AcaNAP45 [Figure 18], AcaNAP47 [Figure 19] et AduNAP7 [Figure 20].

25 43. Protéine selon l'une quelconque des revendications 1 à 42, dans laquelle ladite protéine est dérivée d'une espèce de nématode qui est choisie dans le groupe constitué par *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus* et *Hepigomosomoides polygyrus*.

44. Molécule d'ADNc recombinante isolée codant une protéine selon l'une quelconque des revendications 1 à 42.

30 45. Molécule d'ADNc codant la protéine selon la revendication 18 ou 25 ayant une séquence nucléotidique ayant au moins 90 % d'homologie avec celle codant AcaNAP5 [figure 1] et AcaNAP6 [Figure 3].

35 46. Molécule d'ADNc codant la protéine ayant une activité inhibitrice du facteur Xa choisie dans le groupe constitué par les protéines ayant des domaines NAP ayant au moins 90 % d'homologie avec AcaNAP5 [Figure 16] et AcaNAP6 [figure 16].

47. Molécule d'ADNc codant la protéine selon la revendication 20 ayant une séquence nucléotidique ayant au moins 90 % d'homologie avec celle codant AcaNAP48 [Figure 13h].

40 48. Molécule d'ADNc codant la protéine selon la revendication 21 ayant une séquence nucléotidique ayant au moins 90 % d'homologie avec celle choisie dans le groupe constitué par les ADNc codant AcaNAP23 [Figure 13a], AcaNAP24 [Figure 13b], AcaNAP25 [Figure 13c], AcaNAP44 [Figure 13e], AcaNAP31 [Figure 13d] et AceNAP4 [Figure 7a].

45 49. Molécule d'ADNc codant la protéine selon la revendication 23 ayant une séquence nucléotidique ayant au moins 90 % d'homologie avec celle choisie dans le groupe constitué par les ADNc codant AcaNAP45 [Figure 13f], AcaNAP47 [Figure 13g], AduNAP7 [Figure 7e], AduNAP4 [Figure 7d], AceNAP5 [Figure 7b] et AceNAP7 [Figure 7c].

50 50. Molécule d'ADNc codant la protéine selon la revendication 26 qui est choisie dans le groupe constitué par les ADNc codant AcaNAP5 [Figure 1], AcaNAP6 [Figure 3], AcaNAP48 [Figure 13h], AcaNAP23 [Figure 13a], AcaNAP24 [Figure 13b], AcaNAP25 [Figure 13c], AcaNAP44 [Figure 13e], AcaNAP31 [Figure 13d], AceNAP4 [Figure 7a], AcaNAP45 [Figure 13f], AcaNAP47 [Figure 13g], AduNAP7 [Figure 7e], AduNAP4 [Figure 7d], AceNAP5 [Figure 7b] et AceNAP7 [Figure 7c].

55 51. Molécule d'ADNc codant la protéine selon la revendication 38 ayant une séquence nucléotidique ayant au moins 90 % d'homologie avec les séquences choisies parmi les ADNc codant HpoNAP5 [figure 7f] et NamNAP [figure 14].

52. Molécule d'ADNc codant une protéine ayant une activité anticoagulante choisie dans le groupe constitué par les ADNc ayant au moins 90 % d'homologie avec les ADNc codant AcaNAP5 [Figure 1], AcaNAP6 [Figure 3], AcaNAP48

[Figure 13h], AcaNAP23 [Figure 13a], AcaNAP24 [Figure 13b], AcaNAP25 [Figure 13c], AcaNAP44 [Figure 13e], AcaNAP31 [Figure 13d], AceNAP4 [Figure 7a], AcaNAP45 [Figure 13f], AcaNAP47 [Figure 13g], AduNAP7 [Figure 7e], AduNAP4 [Figure 7d], AceNAP5 [Figure 7b] et AceNAP7 [Figure 7c].

- 5 **53.** Molécule d'ADNc recombinante isolée selon l'une quelconque des revendications 44 à 52, codant une protéine ayant une activité anticoagulante, dans laquelle ladite protéine inhibe spécifiquement l'activité catalytique du complexe FVIIa/TF en présence de fXa ou d'un dérivé de fXa catalytiquement inactif, et n'inhibe pas spécifiquement l'activité du FVIIa en l'absence de TF, et n'inhibe pas spécifiquement la prothrombinase.
- 10 **54.** Molécule d'ADNc selon la revendication 53, dans laquelle l'ADN code AcaNAPc2 [Figure 16].
- 55.** Molécule d'ADNc isolée codant une protéine ayant une activité anticoagulante ayant au moins 90 % d'homologie avec AcaNAPc2 [Figure 9].
- 15 **56.** Molécule d'ADNc recombinante isolée codant une protéine selon la revendication 32.
- 57.** Molécule d'ADNc selon la revendication 56, ayant une séquence nucléotidique qui code une séquence d'acides aminés ayant au moins 90 % d'homologie avec AcaNAPc2 [figure 16].
- 20 **58.** Molécule d'ADNc selon l'une quelconque des revendications 44 à 57, dérivée d'une espèce de nématode.
- 59.** Molécule d'ADNc selon la revendication 58, dans laquelle ladite espèce de nématode est choisie dans le groupe constitué par *Ancylostoma caninum*, *Ancylostoma ceylanicum*, *Ancylostoma duodenale*, *Necator americanus* et *Heligmosomoides polygyrus*.
- 25 **60.** Oligonucléotide comprenant une séquence nucléotidique choisie parmi
NAP-1 : AAR-CCN-TGY-GAR-MGG-AAR-TGY [SEQ ID n° 90] et
NAP-4.RC TW-RWA-NCC-NTC-YTT-RCA-NAC-RCA [SEQ ID n° 91].
- 30 **61.** Utilisation d'une protéine selon l'une quelconque des revendications 1 à 42 pour la fabrication d'une composition pharmaceutique pour inhiber la coagulation du sang.
- 62.** Utilisation selon la revendication 61, dans laquelle la protéine est choisie dans le groupe constitué par AcaNAP5 [Figure 16], AcaNAP6 [Figure 16], AcaNAPc2 [Figure 16], HpoNAP5 [Figure 16], NamNAP [Figure 16].
- 35 **63.** Utilisation selon la revendication 61, dans laquelle la protéine a un domaine NAP ayant au moins 90 % d'homologie avec les domaines NAP choisis dans le groupe constitué par AcaNAP5 [Figure 16], AcaNAP6 [Figure 16], AcaNAP48 [Figure 16], AcaNAP23 [Figure 16], AcaNAP24 [Figure 16], AcaNAP25 [Figure 16], AcaNAP44 [Figure 16], AcaNAP31 [Figure 16], AceNAP4-d1 [Figure 16], AceNAP4-d2 [Figure 16], AcaNAP45-d1 [Figure 16], AcaNAP45-d2 [Figure 16], AcaNAP47-d1 [Figure 16], AcaNAP47-d2 [Figure 16], AduNAP7-d1 [Figure 16], AduNAP7-d2 [Figure 16], AduNAP4 [Figure 16], AceNAP5 [Figure 16] et AceNAP7 [Figure 16].
- 40 **64.** Composition pharmaceutique comprenant une protéine selon l'une quelconque des revendications 1 à 42.
- 45 **65.** Composition pharmaceutique selon la revendication 64, comprenant une protéine choisie dans le groupe constitué par AcaNAP5 [Figure 16], AcaNAP6 [Figure 16], AcaNAPc2 [Figure 16], HpoNAP5 [Figure 16] et NamNAP [Figure 16],
- 50 **66.** Composition pharmaceutique selon la revendication 64, comprenant une protéine ayant un domaine NAP ayant au moins 90 % d'homologie avec un domaine NAP choisi dans le groupe constitué par AcaNAP5 [Figure 16], AcaNAP6 [Figure 16], AcaNAP48 [Figure 16], AcaNAP23 [Figure 16], AcaNAP24 [Figure 16], AcaNAP25 [Figure 16], AcaNAP44 [Figure 16], AcaNAP31 [Figure 16], AceNAP4-d1 [Figure 16], AceNAP4-d2 [Figure 16], AcaNAP45-d1 [Figure 16], AcaNAP45-d2 [Figure 16], AcaNAP47-d1 [Figure 16], AcaNAP47-d2 [Figure 16], AduNAP7-d1 [Figure 16], AduNAP7-d2 [Figure 16], AduNAP4 [Figure 16], AceNAP5 [Figure 16] et AceNAP7 [Figure 16].
- 55 **67.** Protéine isolée ayant une séquence d'acides aminés d'AcaNAPc2 [Figure 16] ayant un résidu proline supplémentaire à l'extrémité C-terminale.

68. Protéine isolée ayant une activité inhibitrice du facteur VIIa/facteur tissulaire et une séquence d'acides aminés ayant au moins 90 % d'homologie avec la séquence d'acide aminés d'AcaNAPc2 [Figure 16] ayant un résidu proline supplémentaire à l'extrémité C-terminale.

5 **69.** Molécule d'acide nucléique isolée codant la séquence d'acides aminés d'AcaNAPc2 [figure 16] ayant une proline supplémentaire à l'extrémité C-terminale.

70. Molécule d'acide nucléique isolée codant une protéine ayant une activité inhibitrice du facteur VIIa/facteur tissulaire et ayant au moins 90 % d'homologie avec la séquence d'acides aminés d'AcaNAPc2 [figure 16] ayant une proline supplémentaire à l'extrémité C-terminale.

10 **71.** Molécule d'acide nucléique isolée codant une protéine ayant une activité inhibitrice du facteur VIIa/facteur tissulaire et ayant au moins 90 % d'homologie avec la séquence d'acides nucléiques codant la séquence d'acides aminés d'AcaNAPc2 [Figure 16] ayant un résidu proline supplémentaire à l'extrémité C-terminale.

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Figure 1

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      1      10      20      30
      *      *      *      *
G AATTCCGCTA CTACTCAACA ATG AAG ATG CTT TAC GCT ATC GCT
  Met Lys Met Leu Tyr Ala Ile Ala

      40      50      60      70
      *      *      *      *
ATA ATG TTT CTC CTG GTA TCA TTA TGC AGC GCA AGA ACA GTG
Ile Met Phe Leu Leu Val Ser Leu Cys Ser Ala Arg Thr Val

      80      90      100      110      120
      *      *      *      *      *
AGG AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC
Arg Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp

      130      140      150      160
      *      *      *      *
GAC TGT GGA ACT CAG AAG CCA TGC GAG GCC AAG TGC AAT GAG
Asp Cys Gly Thr Gln Lys Pro Cys Glu Ala Lys Cys Asn Glu

      170      180      190      200
      *      *      *      *
GAA CCC CCT GAG GAG GAA GAT CCG ATA TGC CGC TCA CGT GGT
Glu Pro Pro Glu Glu Glu Asp Pro Ile Cys Arg Ser Arg Gly

      210      220      230      240
      *      *      *      *
TGT TTA TTA CCT CCT GCT TGC GTA TGC AAA GAC GGA TTC TAC
Cys Leu Leu Pro Pro Ala Cys Val Cys Lys Asp Gly Phe Tyr

      250      260      270      280
      *      *      *      *
AGA GAC ACG GTG ATC GGC GAC TGT GTT AGG GAA GAA GAA TGC
Arg Asp Thr Val Ile Gly Asp Cys Val Arg Glu Glu Glu Cys

      290      300      310      320      330
      *      *      *      *      *
GAC CAA CAT GAG ATT ATA CAT GTC TGA ACGAGAAAGC AACAAATAACC
Asp Gln His Glu Ile Ile His Val

      340      350      360      370      380
      *      *      *      *      *
AAAGGTTCCA ACTCTCGCTC TGCAAAATCG CTAGTTGGAT GTCTCTTTTG

      390      400      410      420      430
      *      *      *      *      *
CGTCCGAATA GTTTTAGTTG ATGTTAAGTA AGAACTCCTG CTGGAGAGAA

      440      450
      *      *
TAAAGCTTTC CAACTCC poly(A)

```

Figure 2

Lys	Ala	Tyr	Pro	Glu	Cys	Gly	Glu	Asn	Glu	Trp	Leu	Asp	Asp
1				5					10				
Cys	Gly	Thr	Gln	Lys	Pro	Cys	Glu	Ala	Lys	Cys	Asn	Glu	Glu
15					20					25			
Pro	Pro	Glu	Glu	Glu	Asp	Pro	Ile	Cys	Arg	Ser	Arg	Gly	Cys
	30					35					40		
Leu	Leu	Pro	Pro	Ala	Cys	Val	Cys	Lys	Asp	Gly	Phe	Tyr	Arg
		45					50					55	
Asp	Thr	Val	Ile	Gly	Asp	Cys	Val	Arg	Glu	Glu	Glu	Cys	Asp
			60					65					70
Gln	His	Glu	Ile	Ile	His	Val							
				75									

Figure 3

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      1      10      20      30
      *      *      *      *
G AATTCGCTA CTACTCAACA ATG AAG ATG CTT TAC GCT ATC GCT
  Met Lys Met Leu Tyr Ala Ile Ala

      40      50      60      70
      *      *      *      *
ATA ATG TTT CTC CTG GTG TCA TTA TGC AGC ACA AGA ACA GTG
Ile Met Phe Leu Leu Val Ser Leu Cys Ser Thr Arg Thr Val

      80      90      100      110      120
      *      *      *      *      *
AGG AAG GCA TAC CCG GAG TGT GGT GAG AAT GAA TGG CTC GAC
Arg Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp

      130      140      150      160
      *      *      *      *
GTC TGT GGA ACT AAG AAG CCA TGC GAG GCC AAG TGC AGT GAG
Val Cys Gly Thr Lys Lys Pro Cys Glu Ala Lys Cys Ser Glu

      170      180      190      200
      *      *      *      *
GAA GAG GAG GAA GAT CCG ATA TGC CGA TCA TTT TCT TGT CCG
Glu Glu Glu Glu Asp Pro Ile Cys Arg Ser Phe Ser Cys Pro

      210      220      230      240
      *      *      *      *
GGT CCC GCT GCT TGC GTA TGC GAA GAC GGA TTC TAC AGA GAC
Gly Pro Ala Ala Cys Val Cys Glu Asp Gly Phe Tyr Arg Asp

      250      260      270      280
      *      *      *      *
ACG GTG ATC GGC GAC TGT GTT AAG GAA GAA GAA TGC GAC CAA
Thr Val Ile Gly Asp Cys Val Lys Glu Glu Glu Cys Asp Gln

      290      300      310      320      330
      *      *      *      *      *
CAT GAG ATT ATT CAT GTC TGA ACGAGAGAGC AGTAATAACC
His Glu Ile Ile His Val

      340      350      360      370      380
      *      *      *      *      *
AAAGGTTCCA ACTTTCGCTC TACAAAATCG CTAGTTGGAT TTCTCCTTTG

      390      400      410      420      430
      *      *      *      *      *
CGTGCGAATA GTTTTAGTTG ATATTAAGTA AAACCTCCTG TTGAAGAGAA

      440
      *
TAAAGCTTTC CAACTTC poly(A)

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Figure 4

Lys Ala Tyr Pro Glu Cys Gly Glu Asn Glu Trp Leu Asp Val
 1 5 10

Cys Gly Thr Lys Lys Pro Cys Glu Ala Lys Cys Ser Glu Glu
 15 20 25

Glu Glu Glu Asp Pro Ile Cys Arg Ser Phe Ser Cys Pro Gly
 30 35 40

Pro Ala Ala Cys Val Cys Glu Asp Gly Phe Tyr Arg Asp Thr
 45 50 55

Val Ile Gly Asp Cys Val Lys Glu Glu Glu Cys Asp Gln His
 60 65 70

Glu Ile Ile His Val
 75

Figure 5

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Arg	Thr	Val	Arg	Lys	Ala	Tyr	Pro	Glu	Cys	Gly	Glu	Asn	Glu
				1				5					10
Trp	Leu	Asp	Asp	Cys	Gly	Thr	Gln	Lys	Pro	Cys	Glu	Ala	Lys
				15					20				
Cys	Asn	Glu	Glu	Pro	Pro	Glu	Glu	Glu	Asp	Pro	Ile	Cys	Arg
25					30					35			
Ser	Arg	Gly	Cys	Leu	Leu	Pro	Pro	Ala	Cys	Val	Cys	Lys	Asp
	40					45					50		
Gly	Phe	Tyr	Arg	Asp	Thr	Val	Ile	Gly	Asp	Cys	Val	Arg	Glu
		55					60					65	
Glu	Glu	Cys	Asp	Gln	His	Glu	Ile	Ile	His	Val			
			70					75					

Figure 6

Arg	Thr	Val	Arg	<u>Lys</u>	Ala	Tyr	Pro	Glu	Cys	Gly	Glu	Asn	Glu
				1				5					10
Trp	Leu	Asp	<u>Val</u>	Cys	Gly	Thr	<u>Lys</u>	Lys	Pro	Cys	Glu	Ala	Lys
				15				20					
Cys	<u>Ser</u>	Glu	Glu	Glu	Glu	Glu	Asp	Pro	Ile	Cys	Arg	Ser	<u>Phe</u>
25						30				35			
<u>Ser</u>	Cys	<u>Pro</u>	<u>Gly</u>	Pro	<u>Ala</u>	Ala	Cys	Val	Cys	<u>Glu</u>	Asp	Gly	Phe
	40					45					50		
Tyr	Arg	Asp	Thr	Val	Ile	Gly	Asp	Cys	Val	<u>Lys</u>	Glu	Glu	Glu
		55					60					65	
Cys	Asp	Gln	<u>His</u>	Glu	Ile	Ile	His	Val					
			70					75					

Figure 7A-1

1	10	20	30	40
*	*	*	*	*
<u>GAATTC</u> ACTA TTATCCAACA ATG GCG GTG CTT TAT TCA GTA GCA				
EcoRI Met Ala Val Leu Tyr Ser Val Ala				
50	60	70	80	
*	*	*	*	
ATA GCG TTA CTA CTG GTA TCA CAA TGC AGT GGG AAA CCG AAC				
Ile Ala Leu Leu Leu Val Ser Gln Cys Ser Gly Lys Pro Asn				
90	100	110	120	
*	*	*	*	
AAT GTG ATG ACT AAC GCT TGT GGT CTT AAT GAA TAT TTC GCT				
Asn Val Met Thr Asn Ala Cys Gly Leu Asn Glu Tyr Phe Ala				
130	140	150	160	170
*	*	*	*	*
GAG TGT GGC AAT ATG AAG GAA TGC GAG CAC AGA TGC AAT GAG				
Glu Cys Gly Asn Met Lys Glu Cys Glu His Arg Cys Asn Glu				
180	190	200	210	
*	*	*	*	
GAG GAA AAT GAG GAA AGG GAC GAG GAA AGA ATA ACG GCA TGC				
Glu Glu Asn Glu Glu Arg Asp Glu Glu Arg Ile Thr Ala Cys				
220	230	240	250	
*	*	*	*	
CTC ATC CGT GTG TGT TTC CGT CCT GGT GCT TGC GTA TGC AAA				
Leu Ile Arg Val Cys Phe Arg Pro Gly Ala Cys Val Cys Lys				
260	270	280	290	
*	*	*	*	
GAC GGA TTC TAT AGA AAC AGA ACA GGC AGC TGT GTG GAA GAA				
Asp Gly Phe Tyr Arg Asn Arg Thr Gly Ser Cys Val Glu Glu				
300	310	320	330	
*	*	*	*	
GAT GAC TGC GAG TAC GAG AAT ATG GAG TTC ATT ACT TTT GCA				
Asp Asp Cys Glu Tyr Glu Asn Met Glu Phe Ile Thr Phe Ala				
340	350	360	370	380
*	*	*	*	*
CCA GAA GTA CCG ATA TGT GGT TCC AAC GAA AGG TAC TCC GAC				
Pro Glu Val Pro Ile Cys Gly Ser Asn Glu Arg Tyr Ser Asp				
390	400	410	420	
*	*	*	*	
TGC GGC AAT GAC AAA CAA TGC GAG CGC AAA TGC AAC GAG GAC				
Cys Gly Asn Asp Lys Gln Cys Glu Arg Lys Cys Asn Glu Asp				
430	440	450	460	
*	*	*	*	
GAT TAT GAG AAG GGA GAT GAG GCA TGC CGC TCA CAT GTT TGT				
Asp Tyr Glu Lys Gly Asp Glu Ala Cys Arg Ser His Val Cys				

Figure 7A-2

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      470      480      490      500
      *      *      *      *
GAA CGT CCT GGT GCC TGT GTA TGC GAA GAC GGG TTC TAC AGA
Glu Arg Pro Gly Ala Cys Val Cys Glu Asp Gly Phe Tyr Arg

      510      520      530      540
      *      *      *      *
AAC AAA AAA GGT AGC TGT GTG GAA AGC GAT GAC TGC GAA TAC
Asn Lys Lys Gly Ser Cys Val Glu Ser Asp Asp Cys Glu Tyr

      550      560      570      580      590
      *      *      *      *      *
GAT AAT ATG GAT TTC ATC ACT TTT GCA CCA GAA ACC TCA CGA
Asp Asn Met Asp Phe Ile Thr Phe Ala Pro Glu Thr Ser Arg

      600      610      620      630      640
      *      *      *      *      *
TAA CCAAAGATGC TACCTCTCGT ACGCAACTCC GCTGATTGAGGTTGATTC

      650      660      670      680      690
      *      *      *      *      *
ACTCCCTTG CATCTCAACATTTTTTTTGTGATGCTGTGCATCTGAGCTTAACCTG

      700      710
      *      *
ATAAAGCCTATGGTG poly(A)

```

Figure 7B

1	10	20	30	40									
*	*	*	*	*									
<u>GAATTC</u> CGC	ATG	CGG	ACG	CTC	TAC	CTC	ATT	TCT	ATC	TGG	TTG		
EcoRI	Met	Arg	Thr	Leu	Tyr	Leu	Ile	Ser	Ile	Trp	Leu		
50	60	70	80										
*	*	*	*										
TTC	CTC	ATC	TCG	CAA	TGT	AAT	GGA	AAA	GCA	TTC	CCG	AAA	TGT
Phe	Leu	Ile	Ser	Gln	Cys	Asn	Gly	Lys	Ala	Phe	Pro	Lys	Cys
90	100	110	120										
*	*	*	*										
GAC	GTC	AAT	GAA	AGA	TTC	GAG	GTG	TGT	GGC	AAT	CTG	AAG	GAG
Asp	Val	Asn	Glu	Arg	Phe	Glu	Val	Cys	Gly	Asn	Leu	Lys	Glu
130	140	150	160										
*	*	*	*										
TGC	GAG	CTC	AAG	TGC	GAT	GAG	GAC	CCT	AAG	ATA	TGC	TCT	CGT
Cys	Glu	Leu	Lys	Cys	Asp	Glu	Asp	Pro	Lys	Ile	Cys	Ser	Arg
170	180	190	200	210									
*	*	*	*	*									
GCA	TGT	ATT	CGT	CCC	CCT	GCT	TGC	GTA	TGC	GAT	GAC	GGA	TTC
Ala	Cys	Ile	Arg	Pro	Pro	Ala	Cys	Val	Cys	Asp	Asp	Gly	Phe
220	230	240	250										
*	*	*	*										
TAC	AGA	GAC	AAA	TAT	GGC	TTC	TGT	GTT	GAA	GAA	GAC	GAA	TGT
Tyr	Arg	Asp	Lys	Tyr	Gly	Phe	Cys	Val	Glu	Glu	Asp	Glu	Cys
260	270	280	290										
*	*	*	*										
AAC	GAT	ATG	GAG	ATT	ATT	ACT	TTT	CCA	CCA	GAA	ACC	AAA	TGA
Asn	Asp	Met	Glu	Ile	Ile	Thr	Phe	Pro	Pro	Glu	Thr	Lys	
300	310	320	330	340									
*	*	*	*	*									
TGACCGAAGC	TTCCACCTTT	CTATACATAT	CTTCACTGCTTGACAGGCTTCT										
350	360	370	380	390	400								
*	*	*	*	*	*								
CGACAATTTAGAAAGTTCTGCTTGACTTTGTCTATTTGAAATTGTTTCACTAATG													
410	420												
*	*												
GGGGAAGTAAAGCATTTTCACGAC poly(A)													

Figure 7C

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      1      10      20      30      40
      *      *      *      *      *
  GAATTCCGCT ACATTTTCAA CA ATG TCG ACG CTT TAT GTT ATC
  EcoRI                               Met Ser Thr Leu Tyr Val Ile

      50      60      70      80
      *      *      *      *
GCA ATA TGT TTG CTG CTT GTT TCG CAA TGC AAT GGA AGA ACG
Ala Ile Cys Leu Leu Leu Val Ser Gln Cys Asn Gly Arg Thr

      90      100      110      120
      *      *      *      *
GTG AAG AAG TGT GGC AAG AAT GAA AGA TAC GAC GAC TGT GGC
Val Lys Lys Cys Gly Lys Asn Glu Arg Tyr Asp Asp Cys Gly

      130      140      150      160
      *      *      *      *
AAT GCA AAG GAC TGC GAG ACC AAG TGC GGT GAA GAG GAA AAG
Asn Ala Lys Asp Cys Glu Thr Lys Cys Gly Glu Glu Glu Lys

      170      180      190      200      210
      *      *      *      *      *
GTG TGC CGT TCG CGT GAG TGT ACT AGT CCT GGT GCC TGC GTA
Val Cys Arg Ser Arg Glu Cys Thr Ser Pro Gly Ala Cys Val

      220      230      240      250
      *      *      *      *
TGC GAA CAA GGA TTC TAC AGA GAT CCG GCT GGC GAC TGT GTC
Cys Glu Gln Gly Phe Tyr Arg Asp Pro Ala Gly Asp Cys Val

      260      270      280      290
      *      *      *      *
ACT GAT GAA GAA TGT GAT GAA TGG AAC AAT ATG GAG ATC ATT
Thr Asp Glu Glu Cys Asp Glu Trp Asn Asn Met Glu Ile Ile

      300      310      320      330      340
      *      *      *      *      *
ACT ATG CCA AAA CAG TAG TCGAAGTTC CCTTCTTTCT CCAAATCTG
Thr Met Pro Lys Gln

      350      360      370      380      390
      *      *      *      *      *
C TCCGTGCTCAATTATCACACACCTCCACTAGTTAAGATTGACTGACTCTCTTG

      400      410      420      430      440      450
      *      *      *      *      *
CATTGTAGTATTTTCGCTTGACTCTGTGCATTTAAGCATGAGATACTACTAGGGA

      460      470
      *      *
GAATAAAAATTACTAACTAC poly(A)

```

Figure 7D

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      1          10          20          30          40
      *          *          *          *          *
      GAATTC CGG AAA TGT CCT ACC GAT GAA TGG TTC GAT TGG TGT
      EcoRI      Lys Cys Pro Thr Asp Glu Trp Phe Asp Trp Cys

          50          60          70          80
          *          *          *          *
      GGA ACT TAC AAG CAT TGC GAA CTC AAG TGC GAT AGG GAG CTA
      Gly Thr Tyr Lys His Cys Glu Leu Lys Cys Asp Arg Glu Leu

          90          100          110          120
          *          *          *          *
      ACT GAG AAA GAA GAG CAG GCA TGT CTC TCA CGT GTT TGT GAG
      Thr Glu Lys Glu Glu Gln Ala Cys Leu Ser Arg Val Cys Glu

          130          140          150          160
          *          *          *          *
      AAG TCC GCT TGC GTA TGC AAT GAC GGA TTA TAC AGA GAC AAG
      Lys Ser Ala Cys Val Cys Asn Asp Gly Leu Tyr Arg Asp Lys

          170          180          190          200          210
          *          *          *          *          *
      TTT GGC AAC TGT GTT GAA AAA GAC GAA TGC AAC GAT ATG GAG
      Phe Gly Asn Cys Val Glu Lys Asp Glu Cys Asn Asp Met Glu

          220          230          240          250
          *          *          *          *
      ATT ATT ACT TTT GCA CCA GAA ACC AAA TAA TGGCCTAAGG TTCC
      Ile Ile Thr Phe Ala Pro Glu Thr Lys

          260          270          280          290          300
          *          *          *          *          *
      AAACCT TGCTACACAC CGTCAGTGCTTTACTGTTTCCTCTACGTGTTAGTAGT

          310          320          330          340          350          360
          *          *          *          *          *          *
      TTTGCTTGACTCTGTGTATTTAAGCATTGTCTACTAATGGGCAAAGTAAAGCATT

          370          380          390
          *          *          *
      GTAAGGACATAATAATGAGTAAACCTTCTGATTT poly(A)

```

Figure 7E-1

1	10	20	30	40
*	*	*	*	*
<u>GAATTC</u> CGGG CGGCAGAAAG ATG CGA ATG CTC TAC CTT GTT CCT				
EcoRI Met Arg Met Leu Tyr Leu Val Pro				
50	60	70	80	
*	*	*	*	
ATC TGG TTG CTG CTC ATT TCG CTA TGC AGT GGA AAA GCT GCG				
Ile Trp Leu Leu Leu Ile Ser Leu Cys Ser Gly Lys Ala Ala				
90	100	110	120	
*	*	*	*	
AAG AAA TGT GGT CTC AAT GAA AGG CTG GAC TGT GGC AAT CTG				
Lys Lys Cys Gly Leu Asn Glu Arg Leu Asp Cys Gly Asn Leu				
130	140	150	160	170
*	*	*	*	*
AAG CAA TGC GAG CCC AAG TGC AGC GAC TTG GAA AGT GAG GAG				
Lys Gln Cys Glu Pro Lys Cys Ser Asp Leu Glu Ser Glu Glu				
180	190	200	210	
*	*	*	*	
TAT GAG GAG GAA GAT GAG TCG AAA TGT CGA TCA CGT GAA TGT				
Tyr Glu Glu Glu Asp Glu Ser Lys Cys Arg Ser Arg Glu Cys				
220	230	240	250	
*	*	*	*	
TCT CGT CGT GTT TGT GTA TGC GAT GAA GGA TTC TAC AGA AAC				
Ser Arg Arg Val Cys Val Cys Asp Glu Gly Phe Tyr Arg Asn				
260	270	280	290	
*	*	*	*	
AAG AAG GGC AAG TGT GTT GCA AAA GAT GTT TGC GAG GAC GAC				
Lys Lys Gly Lys Cys Val Ala Lys Asp Val Cys Glu Asp Asp				
300	310	320	330	
*	*	*	*	
AAT ATG GAG ATT ATC ACT TTT CCA CCA GAA GAC GAA TGT GGT				
Asn Met Glu Ile Ile Thr Phe Pro Pro Glu Asp Glu Cys Gly				
340	350	360	370	380
*	*	*	*	*
CCC GAT GAA TGG TTC GAC TAC TGT GGA AAT TAT AAG AAG TGC				
Pro Asp Glu Trp Phe Asp Tyr Cys Gly Asn Tyr Lys Lys Cys				
390	400	410	420	
*	*	*	*	
GAA CGC AAG TGC AGT GAG GAG ACA AGT GAG AAA AAT GAG GAG				
Glu Arg Lys Cys Ser Glu Glu Thr Ser Glu Lys Asn Glu Glu				
430	440	450	460	
*	*	*	*	
GCA TGC CTC TCT CGT GCT TGT ACT GGT CGT GCT TGC GTA TGC				
Ala Cys Leu Ser Arg Ala Cys Thr Gly Arg Ala Cys Val Cys				

Figure 7E-2

```

      470      480      490      500
      *      *      *      *
AAA GAC GGA TTG TAC AGA GAC GAC TTT GGC AAC TGT GTT CCA
Lys Asp Gly Leu Tyr Arg Asp Asp Phe Gly Asn Cys Val Pro

      510      520      530      540
      *      *      *      *
CAT GAC GAA TGC AAC GAT ATG GAG ATC ATC ACT TTT CCA CCG
His Asp Glu Cys Asn Asp Met Glu Ile Ile Thr Phe Pro Pro

      550      560      570      580      590
      *      *      *      *      *
GAA ACC AAA CAT TGA CCAGAGGCTC CAACTCTCGC TACACAACGT CA
Glu Thr Lys His

      600      610      620      630      640      650
      *      *      *      *      *      *
GGGCTAGAATGGCCCCCTCTGCGAGTTAGTAGTTTTGCTTGACTCTGCTTATTGA

      660      670      680
      *      *      *
GCACTTTCTATTGATGGCGAAAATAAAGCATTTAAAAC poly(A)

```

Figure 7F

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      1      10      20      30      40
      *      *      *      *      *
  GAATTCCGCG CACCTGAGAG GTGAGCTACG CAAGTCTTCG CTGGTACA
  EcoRI

50      60      70      80      90
  *      *      *      *      *
ATG ATC CGA AAG CTC GTT CTG CTG ACT GCT ATC GTC ACG GTG
Met Ile Arg Lys Leu Val Leu Leu Thr Ala Ile Val Thr Val

      100      110      120      130
      *      *      *      *
GTG CTA AGT GCG AAG ACC TGT GGA CCA AAC GAG GAG TAC ACT
Val Leu Ser Ala Lys Thr Cys Gly Pro Asn Glu Glu Tyr Thr

      140      150      160      170
      *      *      *      *
GAA TGC GGG ACG CCA TGC GAG CCG AAG TGC AAT GAA CCG ATG
Glu Cys Gly Thr Pro Cys Glu Pro Lys Cys Asn Glu Pro Met

      180      190      200      210
      *      *      *      *
CCA GAC ATC TGT ACT CTG AAC TGC ATC GTG AAC GTG TGT CAG
Pro Asp Ile Cys Thr Leu Asn Cys Ile Val Asn Val Cys Gln

      220      230      240      250
      *      *      *      *
TGC AAA CCC GGC TTC AAG CGC GGA CCG AAA GGA TGC GTC GCC
Cys Lys Pro Gly Phe Lys Arg Gly Pro Lys Gly Cys Val Ala

260      270      280      290      300
  *      *      *      *      *
CCC GGA CCA GGC TGT AAA TAG TTCTCCACCT GCCCTTTCGT TGGAA
Pro Gly Pro Gly Cys Lys

      310      320      330      340
      *      *      *      *
CAAAT GGCTGTCTTTTTACATTCTGAATCAATAAAGCCGAACGGT poly(A)

```

Figure 8A

1 *	10 *				20 *					30 *				40 *					
AAGCTT	TGCT		AACATA	CTGC	GTAATA	AAGGA	GTCTTA	ATC	ATG	CCA	GTT								
HindIII									Met	Pro	Val								
50 *			60 *				70 *				80 *							90 *	
CTT	TTG	GGT	ATT	CCG	TTA	TTA	TTG	CGT	TTC	CTC	GGT	TTC	CTT						
Leu	Leu	Gly	Ile	Pro	Leu	Leu	Leu	Arg	Phe	Leu	Gly	Phe	Leu						
			100 *				110 *				120 *							130 *	
CTG	GTA	ACT	TTG	TTC	GGC	TAT	CTG	CTT	ACT	TTC	CTT	AAA	AAG						
Leu	Val	Thr	Leu	Phe	Gly	Tyr	Leu	Leu	Thr	Phe	Leu	Lys	Lys						
			140 *				150 *				160 *							170 *	
GGC	TTC	GGT	AAG	ATA	GCT	ATT	GCT	ATT	TCA	TTG	TTT	CTT	GCT						
Gly	Phe	Gly	Lys	Ile	Ala	Ile	Ala	Ile	Ser	Leu	Phe	Leu	Ala						
			180 *				190 *				200 *							210 *	
CTT	ATT	ATT	GGG	CTT	AAC	TCA	ATT	CTT	GTG	GGT	TAT	CTC	TCT						
Leu	Ile	Ile	Gly	Leu	Asn	Ser	Ile	Leu	Val	Gly	Tyr	Leu	Ser						
			220 *				230 *				240 *							250 *	
GAT	ATT	AGC	GCA	CAA	TTA	CCC	TCT	GAT	TTT	GTT	CAG	GGC	GTT						
Asp	Ile	Ser	Ala	Gln	Leu	Pro	Ser	Asp	Phe	Val	Gln	Gly	Val						
			260 *				270 *				280 *							290 *	
CAG	TTA	ATT	CTC	CCG	TCT	AAT	GCG	CTT	CCC	TGT	TTT	TAT	GTT						
Gln	Leu	Ile	Leu	Pro	Ser	Asn	Ala	Leu	Pro	Cys	Phe	Tyr	Val						
			310 *				320 *				330 *							340 *	
ATT	CTC	TCT	GTA	AAG	GCT	GCT	ATT	TTC	ATT	TTT	GAC	GTT	AAA						
Ile	Leu	Ser	Val	Lys	Ala	Ala	Ile	Phe	Ile	Phe	Asp	Val	Lys						
			350 *				360 *				370 *							380 *	
CAA	AAA	ATC	GTT	TCT	TAT	TTG	GAT	TGG	GAT	AAA	GGT	GGA	GGC						
Gln	Lys	Ile	Val	Ser	Tyr	Leu	Asp	Trp	Asp	Lys	Gly	Gly	Gly						
			390 *				400 *				410 *							420 *	
TCA	GGC	GGA	<u>GGCCAAGTCGGCC</u>				ATCCCATATCAC	<u>GCGGCCGC</u>				<u>GGATCC</u>							
Ser	Gly	Gly		SfiI							NotI		BamHI						

Figure 8C

1	10	20	30	40
*	*	*	*	*
<u>AAGCTT</u> TGCT AACATACTGC GTAATAAGGA GTCTTAATC ATG CCA GTT				
HindIII Met Pro Val				
50	60	70	80	90
*	*	*	*	*
CTT TTG GGT ATT CCG TTA TTA TTG CGT TTC CTC GGT TTC CTT				
Leu Leu Gly Ile Pro Leu Leu Leu Arg Phe Leu Gly Phe Leu				
	100	110	120	130
	*	*	*	*
CTG GTA ACT TTG TTC GGC TAT CTG CTT ACT TTC CTT AAA AAG				
Leu Val Thr Leu Phe Gly Tyr Leu Leu Thr Phe Leu Lys Lys				
	140	150	160	170
	*	*	*	*
GGC TTC GGT AAG ATA GCT ATT GCT ATT TCA TTG TTT CTT GCT				
Gly Phe Gly Lys Ile Ala Ile Ala Ile Ser Leu Phe Leu Ala				
	180	190	200	210
	*	*	*	*
CTT ATT ATT GGG CTT AAC TCA ATT CTT GTG GGT TAT CTC TCT				
Leu Ile Ile Gly Leu Asn Ser Ile Leu Val Gly Tyr Leu Ser				
	220	230	240	250
	*	*	*	*
GAT ATT AGC GCA CAA TTA CCC TCT GAT TTT GTT CAG GGC GTT				
Asp Ile Ser Ala Gln Leu Pro Ser Asp Phe Val Gln Gly Val				
	260	270	280	290
	*	*	*	*
CAG TTA ATT CTC CCG TCT AAT GCG CTT CCC TGT TTT TAT GTT				
Gln Leu Ile Leu Pro Ser Asn Ala Leu Pro Cys Phe Tyr Val				
	310	320	330	340
	*	*	*	*
ATT CTC TCT GTA AAG GCT GCT ATT TTC ATT TTT GAC GTT AAA				
Ile Leu Ser Val Lys Ala Ala Ile Phe Ile Phe Asp Val Lys				
	350	360	370	380
	*	*	*	*
CAA AAA ATC GTT TCT TAT TTG GAT TGG GAT AAA GGT GGA GGC				
Gln Lys Ile Val Ser Tyr Leu Asp Trp Asp Lys Gly Gly Gly				
	390	400	410	420
	*	*	*	*
TCA GGC GGA TC <u>GGCCAAGTCGGCC</u> ATCCCATATCAC <u>GCGGCCGC</u> <u>GGATCC</u>				
Ser Gly Gly SfiI NotI BamHI				

Figure 9

```

      1          10          20          30          40
      *          *          *          *          *
      GAATTCCGG CTG GTW TCC TAC TGC AGT GGA AAA GCA ACG ATG
      EcoRI      Leu Val Ser Tyr Cys Ser Gly Lys Ala Thr Met

          50          60          70          80
          *          *          *          *
      CAG TGT GGT GAG AAT GAA AAG TAC GAT TCG TGC GGT AGC AAG
      Gln Cys Gly Glu Asn Glu Lys Tyr Asp Ser Cys Gly Ser Lys

          90          100          110          120
          *          *          *          *
      GAG TGC GAT AAG AAG TGC AAA TAT GAC GGA GTT GAG GAG GAA
      Glu Cys Asp Lys Lys Cys Lys Tyr Asp Gly Val Glu Glu Glu

          130          140          150          160
          *          *          *          *
      GAC GAC GAG GAA CCT AAT GTG CCA TGC CTA GTA CGT GTG TGT
      Asp Asp Glu Glu Pro Asn Val Pro Cys Leu Val Arg Val Cys

          170          180          190          200          210
          *          *          *          *          *
      CAT CAA GAT TGC GTA TGC GAA GAA GGA TTC TAT AGA AAC AAA
      His Gln Asp Cys Val Cys Glu Glu Gly Phe Tyr Arg Asn Lys

          220          230          240          250
          *          *          *          *
      GAT GAC AAA TGT GTA TCA GCA GAA GAC TGC GAA CTT GAC AAT
      Asp Asp Lys Cys Val Ser Ala Glu Asp Cys Glu Leu Asp Asn

          260          270          280          290
          *          *          *          *
      ATG GAC TTT ATA TAT CCC GGA ACT CGA AAC TGA ACGAAGGCTC
      Met Asp Phe Ile Tyr Pro Gly Thr Arg Asn

          300          310          320          330          340
          *          *          *          *          *
      CATTCTTGCT GCACAAGATC GATTGTCTCTCCCCTGCATCTCAGTAGTTTTGC

          350          360          370          380          390          400
          *          *          *          *          *          *
      TACATTGTATATGGTAGCAAAAAATTAGCTTAGGGAGAATAAAATCTTTACCTAT

          410          420          430
          *          *          *
      ATTTAATCAATGAAGTATTCTCTTTCT poly(A)

```

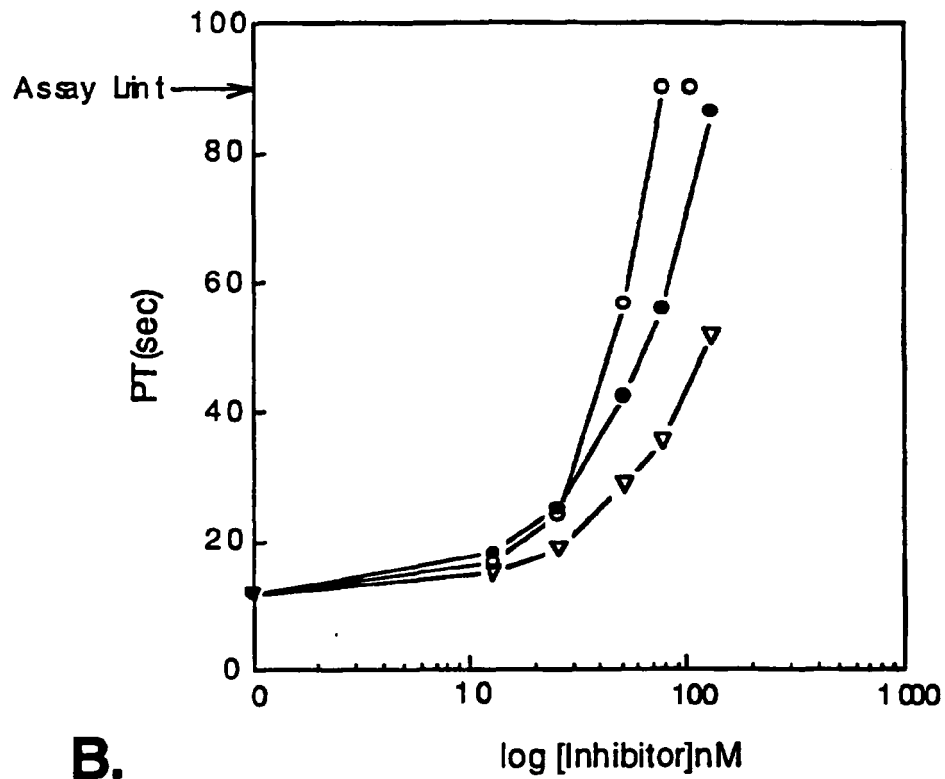
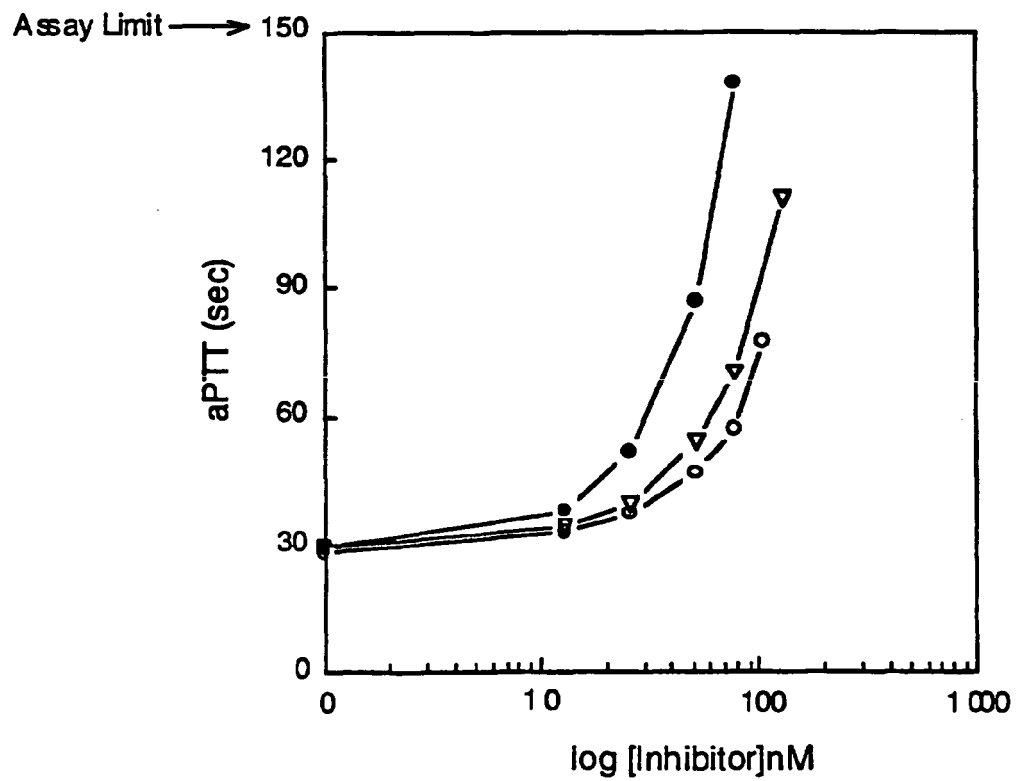
Figure 10**A.****B.**

Figure 11-1

NAP5	Met Lys Met Leu Tyr Ala Ile Ala Ile Met Phe Leu Leu Val
NAP6	Met Lys Met Leu Tyr Ala Ile Ala Ile Met Phe Leu Leu Val
NAPc2	Leu Val
AcNAP5	Met Arg Thr Leu Tyr Leu Ile Ser Ile Trp Leu Phe Leu Ile
AcNAP7	Met Ser Thr Leu Tyr Val Ile Ala Ile Cys Leu Leu Val
AcNAP4d1	Met Ala Val Leu Tyr Ser Val Ala Ile Ala Leu Leu Val
AcNAP4d2	
AduNAP4	
AduNAP7d1	Met Arg Met Leu Tyr Leu Val Pro Ile Trp Leu Leu Ile
AduNAP7d2	
HpoNAP5	Met Ile Arg Lys Leu Val Leu Leu Thr Ala Ile Val Thr

Figure 11-2

NAP5	Ser	Leu	Cys	Ser	Ala	Arg	Thr	Val	Arg	Lys	Ala	Tyr	Pro	Glu
NAP6	Ser	Leu	Cys	Ser	Thr	Arg	Thr	Val	Arg	Lys	Ala	Tyr	Pro	Glu
NAPc2	Ser	TYR	Cys	Ser	Gly	---	---	---	---	Lys	Ala	Thr	Met	Gln
AcenAP5	Ser	Gln	Cys	Asn	Gly	---	---	---	---	Lys	Ala	Phe	Pro	Lys
AcenAP7	Ser	Gln	Cys	Asn	Gly	---	---	---	---	Arg	Thr	Val	Lys	Lys
AcenAP4d1	Ser	Gln	Cys	Ser	Gly	Lys	Pro	Asn	Asn	Val	Met	Thr	Asn	Ala
AcenAP4d2										Val	Pro	Ile		
AduNAP4														Lys
AduNAP7d1	Ser	Leu	Cys	Ser	Gly	---	---	---	---	Lys	Ala	Ala	Lys	Lys
AduNAP7d2													Asp	Glu
HpoNAP5	Val	Val	Leu	Ser	Ala	---	---	---	---	---	---	---	Lys	Thr

Figure 11-3

	¹														²																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
NAP5	Cys	Gly	Glu	Glu	Asn	Glu	Trp	Leu	Asp	Asp	Cys	Gly	Thr	Gln																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								

Figure 11-4

NAP5	Lys	Pro	³ Cys	Glu	Ala	Lys	⁴ Cys	---	---	---	---	Asn	Glu	Glu
NAP6	Lys	Pro	Cys	Glu	Ala	Lys	Cys	---	---	---	---	Ser	Glu	Glu
NAPc2	Glu	---	Cys	Asp	Lys	Lys	Cys	Lys	Tyr	Asp	Gly	Val	Glu	Glu
AcenAP5	Lys	Glu	Cys	Glu	Leu	Lys	Cys	---	---	---	---	---	---	---
AcenAP7	Lys	Asp	Cys	Glu	Thr	Lys	Cys	---	---	---	Gly	---	---	---
AcenAP4d1	Lys	Glu	Cys	Glu	His	Arg	Cys	Asn	Glu	Glu	Glu	Asn	Glu	Glu
AcenAP4d2	Lys	Gln	Cys	Glu	Arg	Lys	Cys	Asn	Glu	Asp	Asp	Tyr	Glu	Lys
AduNAP4	Lys	His	Cys	Glu	Leu	Lys	Cys	Asp	Arg	Glu	Leu	Thr	Glu	Lys
AduNAP7d1	Lys	Gln	Cys	Glu	Pro	Lys	Cys	Ser	Asp	Leu	Glu	Ser	Glu	Glu
AduNAP7d2	Lys	Lys	Cys	Glu	Arg	Lys	Cys	Ser	Glu	Glu	Thr	Ser	Glu	Lys
HpoNAP5	---	Pro	Cys	Glu	Pro	Lys	Cys	---	---	---	---	---	---	---

Figure 11-5

NAP5	Pro	Pro	Glu	Glu	Glu	Asp	Pro	Ile	---	---	⁵ Cys	Arg	Ser	Arg
NAP6	---	---	Glu	Glu	Glu	Asp	Pro	Ile	---	---	Cys	Arg	Ser	Phe
NAPc2	---	Glu	Asp	Asp	Glu	Glu	Pro	Asn	Val	Pro	Cys	Leu	Val	Arg
AceNAP5	---	---	Asp	Glu	Asp	Pro	Lys	Ile	---	---	Cys	---	Ser	Arg
AceNAP7	---	---	Glu	Glu	Glu	---	Lys	---	Val	---	Cys	Arg	Ser	Arg
AceNAP4d1	Arg	---	Asp	Glu	Glu	---	Arg	Ile	Thr	Ala	Cys	Leu	Ile	Arg
AceNAP4d2	Gly	---	Asp	Glu	---	---	---	---	---	Ala	Cys	Arg	Ser	His
AduNAP4	---	---	Glu	Glu	---	---	Gln	---	---	Ala	Cys	Leu	Ser	Arg
AduNAP7d1	Tyr	---	Glu	Glu	Glu	Asp	Glu	Ser	Lys	---	Cys	Arg	Ser	Arg
AduNAP7d2	Asn	---	Glu	Glu	---	---	---	---	---	Ala	Cys	Leu	Ser	Arg
HpoNAP5	---	---	Asn	Glu	Pro	Met	Pro	Asp	Ile	---	Cys	---	Thr	Leu

Figure 11--6

NAP5	Gly	Cys	Leu	Leu	Pro	Pro	Ala	Cys	Val	Cys	Lys	Asp
NAP6	Ser	Cys	Pro	Gly	Pro	Ala	Ala	Cys	Val	Cys	Glu	Asp
NAPc2	Val	Cys	His	Gln	Asp	---	---	Cys	Val	Cys	Glu	Glu
AcenAP5	Ala	Cys	Ile	Arg	Pro	Pro	Ala	Cys	Val	Cys	Asp	Asp
AcenAP7	Glu	Cys	Thr	Ser	Pro	Gly	Ala	Cys	Val	Cys	Glu	Gln
AcenAP4d1	Val	Cys	Phe	Arg	Pro	Gly	Ala	Cys	Val	Cys	Lys	Asp
AcenAP4d2	Val	Cys	Glu	Arg	Pro	Gly	Ala	Cys	Val	Cys	Glu	Asp
AduNAP4	Val	Cys	Glu	Lys	---	Ser	Ala	Cys	Val	Cys	Asn	Asp
AduNAP7d1	Glu	Cys	Ser	Arg	Arg	---	Val	Cys	Val	Cys	Asp	Glu
AduNAP7d2	Ala	Cys	Thr	Gly	Arg	---	Ala	Cys	Val	Cys	Lys	Asp
HpoNAP5	Asn	Cys	Ile	Val	Asn	---	Val	Cys	Gln	Cys	Lys	Pro

Figure 11--7

NAP5	Gly	Phe	Tyr	Arg	Asp	Thr	Val	Ile	Gly	Asp	⁹ Cys	Val	Arg	Glu
NAP6	Gly	Phe	Tyr	Arg	Asp	Thr	Val	Ile	Gly	Asp	Cys	Val	Lys	Glu
NAPC2	Gly	Phe	Tyr	Arg	Asn	Lys	---	Asp	Asp	Lys	Cys	Val	Ser	Ala
AcenAP5	Gly	Phe	Tyr	Arg	Asp	Lys	Tyr	---	Gly	Phe	Cys	Val	Glu	Glu
AcenAP7	Gly	Phe	Tyr	Arg	Asp	Pro	Ala	---	Gly	Asp	Cys	Val	Thr	Asp
AcenAP4d1	Gly	Phe	Tyr	Arg	Asn	Arg	Thr	---	Gly	Ser	Cys	Val	Glu	Glu
AcenAP4d2	Gly	Phe	Tyr	Arg	Asn	Lys	Lys	---	Gly	Ser	Cys	Val	Glu	Ser
AduNAP4	Gly	Leu	Tyr	Arg	Asp	Lys	Phe	---	Gly	Asn	Cys	Val	Glu	Lys
AduNAP7d1	Gly	Phe	Tyr	Arg	Asn	Lys	Lys	---	Gly	Lys	Cys	Val	Ala	Lys
AduNAP7d2	Gly	Leu	Tyr	Arg	Asp	Asp	Phe	---	Gly	Asn	Cys	Val	Pro	His
HpoNAP5	Gly	Phe	Lys	Arg	Gly	Pro	Lys	---	Gly	---	Cys	Val	Ala	Pro

Figure 11-8

NAP5	Glu	Glu	---	¹⁰ Cys	Asp	Gln	His	---	---	Glu	Ile	Ile	His
NAP6	Glu	Glu	---	Cys	Asp	Gln	His	---	---	Glu	Ile	Ile	His
NAPc2	Glu	Asp	---	Cys	Glu	---	Leu	Asp	Asn	Met	Asp	Phe	Tyr
AcenAP5	Asp	Glu	---	Cys	Asn	Asp	---	---	---	Met	Glu	Ile	Thr
AcenAP7	Glu	Glu	---	Cys	Asp	Glu	Trp	Asn	Asn	Met	Glu	Ile	Thr
AcenAP4d1	Asp	Asp	---	Cys	Glu	---	Tyr	Glu	Asn	Met	Glu	Phe	Thr
AcenAP4d2	Asp	Asp	---	Cys	Glu	---	Tyr	Asp	Asn	Met	Asp	Phe	Thr
AduNAP4	Asp	Glu	---	Cys	Asn	Asp	---	---	---	Met	Glu	Ile	Thr
AduNAP7d1	Asp	Val	---	Cys	Glu	Asp	---	Asp	Asn	Met	Glu	Ile	Thr
AduNAP7d2	Asp	Glu	---	Cys	Asn	Asp	---	---	---	Met	Glu	Ile	Thr
HpoNAP5	Gly	Pro	Gly	Cys	Lys	end							

Figure 11-9

NAP5	Val end
NAP6	Val end
NAPc2	Pro Gly Thr Arg Asn end
AcenAP5	Phe Pro Pro Glu Thr Lys end
AcenAP7	Met Pro Lys Gln end
AcenAP4d1	Phe Ala Pro Glu
AcenAP4d2	Phe Ala Pro Glu Thr Ser Arg end
AduNAP4	Phe Ala Pro Glu Thr Lys end
AduNAP7d1	Phe Pro Pro Glu
AduNAP7d2	Phe Pro Pro Glu Thr Lys His end
HponAP5	

Figure 13 A-1 (AcaNAP23)

[illegible]

Figure 13 A-2

```
390          400          410          420          430
  *          *          *          *          *
  CGTGATCAGC GTCGGTGGTT GACAGTCTCC CCTACATCTT AGTAGTTTTG

440          450          460          470          480
  *          *          *          *          *
  CTTGATAATG TATACATAAA CTGTACTTTC TGAGATAGAA TAAAGCTCTC

490
  *
  AACTAC poly(A)
```

Figure 13 B-1 (AcanAP24)

```

      10      20      30      40
      *      *      *      *
  GAATTCGCG GAATTCGCA ACG ATG AAG ACG CTC TAT ATT ATC
  EcoRI                      Met Lys Thr Leu Tyr Ile Ile

      50      60      70      80
      *      *      *      *
  GCT ATA TGC TCG CTC CTC ATT TCG TTG TGT ACT GGA AGA CCG
  Ala Ile Cys Ser Leu Leu Ile Ser Leu Cys Thr Gly Arg Pro

      90     100     110     120
      *      *      *      *
  GAA AAA AAG TGC GGT CCC GGT GAA AGA CTC GCC TGT GGC AAT
  Glu Lys Lys Cys Gly Pro Gly Glu Arg Leu Ala Cys Gly Asn

 130     140     150     160     170
  *      *      *      *      *
  AAG AAG CCA TGC GAG CGC AAG TGC AAA ATA GAG ACA AGT GAG
  Lys Lys Pro Cys Glu Arg Lys Cys Lys Ile Glu Thr Ser Glu

      180     190     200     210
      *      *      *      *
  GAG GAG GAT GAC TAC CCA GAG GGA ACC GAA CGT TTT CGA TGC
  Glu Glu Asp Asp Tyr Pro Glu Gly Thr Glu Arg Phe Arg Cys

      220     230     240     250
      *      *      *      *
  CTC TTA CGT GTG TGT GAT CAG CCT TAT GAA TGC ATA TGC GAT
  Leu Leu Arg Val Cys Asp Gln Pro Tyr Glu Cys Ile Cys Asp

      260     270     280     290
      *      *      *      *
  GAT GGA TAC TAC AGA AAC AAG AAA GGC GAA TGT GTG ACT GAT
  Asp Gly Tyr Tyr Arg Asn Lys Lys Gly Glu Cys Val Thr Asp

      300     310     320     330
      *      *      *      *
  GAT GTA TGC CAG GAA GAC TTT ATG GAG TTT ATT ACT TTC GCA
  Asp Val Cys Gln Glu Asp Phe Met Glu Phe Ile Thr Phe Ala

 340     350     360     370     380
  *      *      *      *      *
  CCA TAA ACCCAATAAT GACCACTGGC TCCCATTCCTT CGTGACCAGC
  Pro

```

Figure 13 B-2

390	400	410	420	430
*	*	*	*	*
GTCGGTGGTT	GACAGTCTCC	CCTGCATCTT	AGTAGTTTTG	CTTGATAATG
440	450	460	470	
*	*	*	*	
TATCCATAAA	CAGTACTTTC	TGAGATAGAA	TAAAGCTCTC	AACT poly(A)

Figure 13 C (AcaNAP25)

```

      10      20      30      40
      *      *      *      *
GAATTCCGTA CTACTCAACG ATG AAG ACG CTC TAT ATT ATC GCT
EcoRI           Met Lys Thr Leu Tyr Ile Ile Ala

      50      60      70      80
      *      *      *      *
ATA TGC TCG CTG CTC TTT TCA CTG TGT ACT GGA AGA CCG GAA
Ile Cys Ser Leu Leu Phe Ser Leu Cys Thr Gly Arg Pro Glu

      90      100      110      120
      *      *      *      *
AAA AAG TGC GGT CCC GGT GAA AGA CTC GAC TGT GCC AAC AAG
Lys Lys Cys Gly Pro Gly Glu Arg Leu Asp Cys Ala Asn Lys

130      140      150      160      170
*      *      *      *      *
AAG CCA TGC GAG CCC AAG TGC AAA ATA GAG ACA AGT GAG GAG
Lys Pro Cys Glu Pro Lys Cys Lys Ile Glu Thr Ser Glu Glu

      180      190      200      210
      *      *      *      *
GAG GAT GAC GAC GTA GAG GAT ACC GAT GTG AGA TGC CTC GTA
Glu Asp Asp Asp Val Glu Asp Thr Asp Val Arg Cys Leu Val

      220      230      240      250
      *      *      *      *
CGT GTG TGT GAA CGT CCT CTT AAA TGC ATA TGC AAG GAT GGA
Arg Val Cys Glu Arg Pro Leu Lys Cys Ile Cys Lys Asp Gly

      260      270      280      290
      *      *      *      *
TAC TAC AGA AAC AAG AAA GGC GAA TGT GTG ACT GAT GAT GTA
Tyr Tyr Arg Asn Lys Lys Gly Glu Cys Val Thr Asp Asp Val

      300      310      320      330
      *      *      *      *
TGC CAG GAA GAC TTT ATG GAG TTT ATT ACT TTC GCA CCA TAA
Cys Gln Glu Asp Phe Met Glu Phe Ile Thr Phe Ala Pro

340      350      360      370      380
*      *      *      *      *
ACCCAATAAT GACCACTGGC TCCCATTCTT CGTGATCAGC GTCGGTGGTT

390      400      410      420      430
*      *      *      *      *
GACAGTCTCC CCTGCATCTT AGTTGCTTTG CTTGATAATC TATACATAAA

440      450      460      470
*      *      *      *
CAGTACTTTC TGAGATAGAA TAAAGCTCTC AACT poly(A)

```

Figure 13 D-1 (AcaNAP31)

```

      10      20      30      40      50
      *      *      *      *      *
GAATTCGGA CTTACTAGTA CTCAGCGAAT CAAATACGAC TTACTACTAC
EcoRI
      60      70      80      90
      *      *      *      *
TCAACG ATG AAG ACG CTC TCT GCT ATC CCT ATA ATG CTG CTC
Met Lys Thr Leu Ser Ala Ile Pro Ile Met Leu Leu

     100     110     120     130
     *     *     *     *
CTG GTA TCG CAA TGC AGT GGA AAA TCA CTG TGG GAT CAG AAG
Leu Val Ser Gln Cys Ser Gly Lys Ser Leu Trp Asp Gln Lys

     140     150     160     170
     *     *     *     *
TGT GGT GAG AAT GAA AGG CTC GAC TGT GGC AAT CAG AAG GAC
Cys Gly Glu Asn Glu Arg Leu Asp Cys Gly Asn Gln Lys Asp

     180     190     200     210
     *     *     *     *
TGT GAG CGC AAG TGC GAT GAT AAA AGA AGT GAA GAA GAA ATT
Cys Glu Arg Lys Cys Asp Asp Lys Arg Ser Glu Glu Glu Ile

    220     230     240     250     260
    *     *     *     *     *
ATG CAG GCA TGT CTC ACA CGT CAA TGT CTT CCT CCT GTT TGC
Met Gln Ala Cys Leu Thr Arg Gln Cys Leu Pro Pro Val Cys

     270     280     290     300
     *     *     *     *
GTA TGT GAA GAT GGA TTC TAC AGA AAT GAC AAC GAC CAA TGT
Val Cys Glu Asp Gly Phe Tyr Arg Asn Asp Asn Asp Gln Cys

     310     320     330     340
     *     *     *     *
GTT GAT GAA GAA GAA TGC AAT ATG GAG TTT ATT ACT TTC GCA
Val Asp Glu Glu Glu Cys Asn Met Glu Phe Ile Thr Phe Ala

     350     360     370     380     390
     *     *     *     *     *
CCA TGA AGCAAATGAC AGCCGATGGT TTGGACTCTC GCTACAGATC
Pro

     400     410     420     430     440
     *     *     *     *     *
ACAGCTTTAC TGTTTCCCTT GCATCATAGT AGTTTTGCTA GATAGTGTAT

```

Figure 13 D-2

450	460	470	480	
*	*	*	*	
ATATTAGCAT	GATTTTCTGA	TAGGGAGAAT	AAAGCTTTCC	AATTTTC

poly(A)

Figure 13 E-1 (AcaNAP44)

```

          10          20          30          40
          *          *          *          *
    GAATTC CGCG GAATTC CGCA ACG ATG AAG ACG CTC TAT ATT ATC
    EcoRI                      Met Lys Thr Leu Tyr Ile Ile

          50          60          70          80
          *          *          *          *
    GCT ATA TGC TCG CTC CTC ATT TCG CTG TGT ACT GGA AGA CCG
    Ala Ile Cys Ser Leu Leu Ile Ser Leu Cys Thr Gly Arg Pro

          90          100          110          120
          *          *          *          *
    GAA AAA AAG TGC GGT CCC GGT GAA AGA CTC GAC TGT GCC AAC
    Glu Lys Lys Cys Gly Pro Gly Glu Arg Leu Asp Cys Ala Asn

    130          140          150          160          170
    *          *          *          *          *
    AAG AAG CCA TGC GAG CCC AAG TGC AAA ATA GAG ACA AGT GAG
    Lys Lys Pro Cys Glu Pro Lys Cys Lys Ile Glu Thr Ser Glu

          180          190          200          210
          *          *          *          *
    GAG GAG GAT GAC GAC GTA GAG GAA ACC GAT GTG AGA TGC CTC
    Glu Glu Asp Asp Asp Val Glu Glu Thr Asp Val Arg Cys Leu

          220          230          240          250
          *          *          *          *
    GTA CGT GTG TGT GAA CGG CCT CTT AAA TGC ATA TGC AAG GAT
    Val Arg Val Cys Glu Arg Pro Leu Lys Cys Ile Cys Lys Asp

          260          270          280          290
          *          *          *          *
    GGA TAC TAC AGA AAC AAG AAA GGC GAA TGT GTG ACT GAT GAT
    Gly Tyr Tyr Arg Asn Lys Lys Gly Glu Cys Val Thr Asp Asp

          300          310          320          330
          *          *          *          *
    GTA TGC CAG GAA GAC TTT ATG GAG TTT ATT ACT TTC GCA CCA
    Val Cys Gln Glu Asp Phe Met Glu Phe Ile Thr Phe Ala Pro

    340          350          360          370          380
    *          *          *          *          *
    TAA ACCCAATAAT GACCACTGGC TCCCATTCTT CGTGATCAGC

          390          400          410          420          430
          *          *          *          *          *
    GTCGGTGGTT GACAGTCTCC CCTGCATCTT AGTTGCTTTG CTTGATAATC

```

Figure 13 E-2

440 450 460 470
* * * *
TATACATAAA CAGTACTTTC TGAGATAGAA TAAAGCTCTC AACTAC
poly(A)

Figure 13 F-1 (AcaNAP45)

10	20	30	40
*	*	*	*
GAATTC	CGGA	AAA	ATG
EcoRI		Met	Leu
CTG	ATG	CTC	TAC
Leu	Met	Leu	Tyr
CTT	GTT	CCT	ATC
Leu	Val	Pro	Ile
TGG			Trp
50	60	70	80
*	*	*	*
TTG	CTA	CTC	ATT
Leu	Leu	Leu	Ile
TCG	CAA	TGC	AGT
Ser	Gln	Cys	Ser
GGA	AAA	TCC	GCG
Gly	Lys	Ser	Ala
AAG	AAA		Lys
Lys			Lys
90	100	110	120
*	*	*	*
TGT	GGT	CTC	AAT
Cys	Gly	Leu	Asn
GAA	AAA	TTG	GAC
Glu	Lys	Leu	Asp
TGT	GGC	AAT	CTG
Gly	Asn	Leu	Lys
AAG	GCA		Ala
130	140	150	160
*	*	*	*
TGC	GAG	AAA	AAG
Cys	Glu	Lys	Lys
TGC	AGC	GAC	TTG
Cys	Ser	Asp	Leu
GAC	TTG	GAC	AAT
Asp	Leu	Asp	Asn
GAG	GAG	GAG	GAT
Glu	Glu	Asp	Tyr
170	180	190	200
*	*	*	*
AAG	GAG	GAA	GAT
Lys	Glu	Glu	Asp
GAG	TCG	AAA	TGC
Glu	Ser	Lys	Cys
CGA	TCA	CGT	GAA
Arg	Ser	Arg	Glu
TGT	AGT		Ser
Cys			
220	230	240	250
*	*	*	*
CGT	CGT	GTT	TGT
Arg	Arg	Val	Cys
GTA	TGC	GAT	GAA
Val	Cys	Asp	Glu
GGA	TTC	TAC	AGA
Gly	Phe	Tyr	Arg
AAC	AAG		Lys
Asn			
260	270	280	290
*	*	*	*
AAG	GGC	CAA	TGT
Lys	Gly	Gln	Cys
GTG	ACA	AGA	GAT
Val	Thr	Arg	Asp
GAT	GAT	TGC	GAG
Asp	Asp	Cys	Glu
TAT	GAC	AAT	
Tyr	Asp	Asn	
300	310	320	330
*	*	*	*
ATG	GAG	ATT	ATC
Met	Glu	Ile	Ile
ACT	TTT	CCA	CCA
Thr	Phe	Pro	Pro
GAA	GAT	AAA	TGT
Glu	Asp	Lys	Cys
GGT	CCC		Pro
Gly			
340	350	360	370
*	*	*	*
GAT	GAA	TGG	TTC
Asp	Glu	Trp	Phe
GAC	TGG	TGT	GGA
Asp	Trp	Cys	Gly
ACT	TAC	AAG	CAG
Thr	Tyr	Lys	Gln
TGT	GAG		Glu
Cys			
380	390	400	410
*	*	*	*
CGC	AAG	TGC	AAT
Arg	Lys	Cys	Asn
AAG	GAG	CTA	AGT
Lys	Glu	Leu	Ser
GAG	AAA	GAT	GAA
Glu	Lys	Asp	Glu
GAG	GCA		Ala
Glu			

Figure 13 F-2

```

          430          440          450          460
          *          *          *          *
TGC CTC TCA CGT GCT TGT ACT GGT CGT GCT TGT GTT TGC AAC
Cys Leu Ser Arg Ala Cys Thr Gly Arg Ala Cys Val Cys Asn

          470          480          490          500
          *          *          *          *
GAC GGA CTG TAC AGA GAC GAT TTT GGC AAT TGT GTT GAG AAA
Asp Gly Leu Tyr Arg Asp Asp Phe Gly Asn Cys Val Glu Lys

          510          520          530          540
          *          *          *          *
GAC GAA TGT AAC GAT ATG GAG ATT ATC ACT TTT CCA CCG GAA
Asp Glu Cys Asn Asp Met Glu Ile Ile Thr Phe Pro Pro Glu

          550          560          570          580
          *          *          *          *
ACC AAA CAC TGA CCAAAGGCTC TAACTCTCGC TACATAACGT
Thr Lys His

          590          600          610          620          630
          *          *          *          *          *
CAGTGCTTGA ATTGCCCCTT TACGAGTTAG TAATTTTGAC TAACTCTGTG

          640          650          660          670          680
          *          *          *          *          *
TAATTGAGCA TTGTCTACTG ATGGTGAAAA TGAAGTGTTT AATGTCT

poly(A)

```

Figure 13 G-1 (AcaNAP47)

```

          10          20          30          40
          *          *          *          *
    GAATTCGCG GAATTCGGT TGGCGGCAGA AAA ATG CTG ATG CTC
    EcoRI                               Met Leu Met Leu

          50          60          70          80
          *          *          *          *
    TAC CTT GTT CCT ATC TGG TTC CTG CTC ATT TCG CAA TGC AGT
    Tyr Leu Val Pro Ile Trp Phe Leu Leu Ile Ser Gln Cys Ser

          90          100          110          120
          *          *          *          *
    GGA AAA TCC GCG AAG AAA TGT GGC CTC AAT GAA AAA TTG GAC
    Gly Lys Ser Ala Lys Lys Cys Gly Leu Asn Glu Lys Leu Asp

130          140          150          160          170
  *          *          *          *          *
    TGT GGC AAT CTG AAG GCA TGC GAG AAA AAG TGC AGC GAC TTG
    Cys Gly Asn Leu Lys Ala Cys Glu Lys Lys Cys Ser Asp Leu

          180          190          200          210
          *          *          *          *
    GAC AAT GAG GAG GAT TAT GGG GAG GAA GAT GAG TCG AAA TGC
    Asp Asn Glu Glu Asp Tyr Gly Glu Glu Asp Glu Ser Lys Cys

          220          230          240          250
          *          *          *          *
    CGA TCA CGT GAA TGT ATT GGT CGT GTT TGC GTA TGC GAT GAA
    Arg Ser Arg Glu Cys Ile Gly Arg Val Cys Val Cys Asp Glu

          260          270          280          290
          *          *          *          *
    GGA TTC TAC AGA AAC AAG AAG GGC CAA TGT GTG ACA AGA GAC
    Gly Phe Tyr Arg Asn Lys Lys Gly Gln Cys Val Thr Arg Asp

          300          310          320          330
          *          *          *          *
    GAT TGC GAG TAT GAC AAT ATG GAG ATT ATC ACT TTT CCA CCA
    Asp Cys Glu Tyr Asp Asn Met Glu Ile Ile Thr Phe Pro Pro

140          350          360          370          380
  *          *          *          *          *
    GAA GAT AAA TGT GGT CCC GAT GAA TGG TTC GAC TGG TGT GGA
    Glu Asp Lys Cys Gly Pro Asp Glu Trp Phe Asp Trp Cys Gly

          390          400          410          420
          *          *          *          *
    ACT TAC AAG CAG TGT GAG CGC AAG TGC AGT GAG GAG CTA AGT
    Thr Tyr Lys Gln Cys Glu Arg Lys Cys Ser Glu Glu Leu Ser

```

Figure 13 G-2

430	440	450	460
*	*	*	*
GAG AAA AAT GAG GAG GCA TGC CTC TCA CGT GCT TGT ACT GGT			
Glu Lys Asn Glu Glu Ala Cys Leu Ser Arg Ala Cys Thr Gly			
470	480	490	500
*	*	*	*
CGT GCT TGC GTT TGC AAC GAC GGA TTG TAT AGA GAC GAT TTT			
Arg Ala Cys Val Cys Asn Asp Gly Leu Tyr Arg Asp Asp Phe			
510	520	530	540
*	*	*	*
GGC AAT TGT GTT GAG AAA GAC GAA TGT AAC GAT ATG GAG ATT			
Gly Asn Cys Val Glu Lys Asp Glu Cys Asn Asp Met Glu Ile			
550	560	570	580
*	*	*	*
ATC ACT TTT CCA CCG GAA ACC AAA CAC TGA CCAAAGGCTC			
Ile Thr Phe Pro Pro Glu Thr Lys His			
590	600	610	620
*	*	*	*
TAGCTCTCGC TACATAACGT CAGTGCTTGA ATTGTCCCTT TACGTGTTAG			
640	650	660	670
*	*	*	*
TAATTTTGAC TAACTCTGTG TATTTGAGCA TTGTCTACTA ATGGTGAAAA			
690	700		
*	*		
TGAAGCTTTT CAATGACT poly(A)			

Figure 13 H-1 (AcaNAP48)

```

          10          20          30          40
          *          *          *          *
    GAATTC CGTA CGACCTACTA CTACTCAACG ATG AAG GCG CTC TAT
    EcoRI                               Met Lys Ala Leu Tyr

          50          60          70          80
          *          *          *          *
    GTT ATC TCT ATA ACG TTG CTC CTG GTA TGG CAA TGC AGT GCA
    Val Ile Ser Ile Thr Leu Leu Leu Val Trp Gln Cys Ser Ala

          90          100          110          120
          *          *          *          *
    AGA ACA GCG AGG AAA CCC CCA ACG TGT GGT GAA AAT GAA AGG
    Arg Thr Ala Arg Lys Pro Pro Thr Cys Gly Glu Asn Glu Arg

    130          140          150          160          170
    *          *          *          *          *
    GTC GAA TGG TGT GGC AAG CAG TGC GAG ATC ACA TGT GAC GAC
    Val Glu Trp Cys Gly Lys Gln Cys Glu Ile Thr Cys Asp Asp

          180          190          200          210
          *          *          *          *
    CCA GAT AAG ATA TGC CGC TCA CTC GCT TGT CCT GGT CCT CCT
    Pro Asp Lys Ile Cys Arg Ser Leu Ala Cys Pro Gly Pro Pro

          220          230          240          250
          *          *          *          *
    GCT TGC GTA TGC GAC GAC GGA TAC TAC AGA GAC ACG AAC GTT
    Ala Cys Val Cys Asp Asp Gly Tyr Tyr Arg Asp Thr Asn Val

          260          270          280          290
          *          *          *          *
    GGC TTG TGT GTA CAA TAT GAC GAA TGC AAC GAT ATG GAT ATT
    Gly Leu Cys Val Gln Tyr Asp Glu Cys Asn Asp Met Asp Ile

    300          310          320          330          340
    *          *          *          *          *
    ATT ATG GTT TCA TAG GGTGACTGA AGAATCGAAC AACCGGTGCA
    Ile Met Val Ser

          350          360          370          380          390
          *          *          *          *          *
    CAACTTCTAT GCTTGACTAT CTCTCTTGCA TCATGCAAGT TTAGCTAGAT

          400          410          420          430          440
          *          *          *          *          *
    AGTGTATATA TTAGCAAGAC CCCTTG GGGGA GAATGAAGCT TCCCAACTAT

          450          460          470          480          490
          *          *          *          *          *
    ATTAAATCAA TAACGTTTTTC GCTTCATGTA CACGTGCTCA GCACATTCAT

```

Figure 13 H-2

500	510	520	
*	*	*	
ATCCACTCCT	CACACTCCAT	GAAAGCAGTG	AAATGTT poly(A)

Figure 14

```

      10      20      30      40
      *      *      *      *
GCC AAC TCT TCG AAC ATG ATT CGA GGC CTC GTT CTT CTT TCT CTC CTG
                      Met Ile Arg Gly Leu Val Leu Leu Ser Leu Leu>

      50      60      70      80      90
      *      *      *      *      *
TTT TGC GTC ACT TTT GCA GCG AAG AGA GAT TGT CCA GCA AAT GAG GAA
Phe Cys Val Thr Phe Ala Ala Lys Arg Asp Cys Pro Ala Asn Glu Glu>

      100     110     120     130     140
      *      *      *      *      *
TGG AGG GAA TGT GGC ACT CCA TGT GAA CCA AAA TGC AAT CAA CCG ATG
Trp Arg Glu Cys Gly Thr Pro Cys Glu Pro Lys Cys Asn Gln Pro Met>

      150     160     170     180     190
      *      *      *      *      *
CCA GAT ATA TGT ACT ATG AAT TGT ATC GTC GAT GTG TGT CAA TGC AAG
Pro Asp Ile Cys Thr Met Asn Cys Ile Val Asp Val Cys Gln Cys Lys>

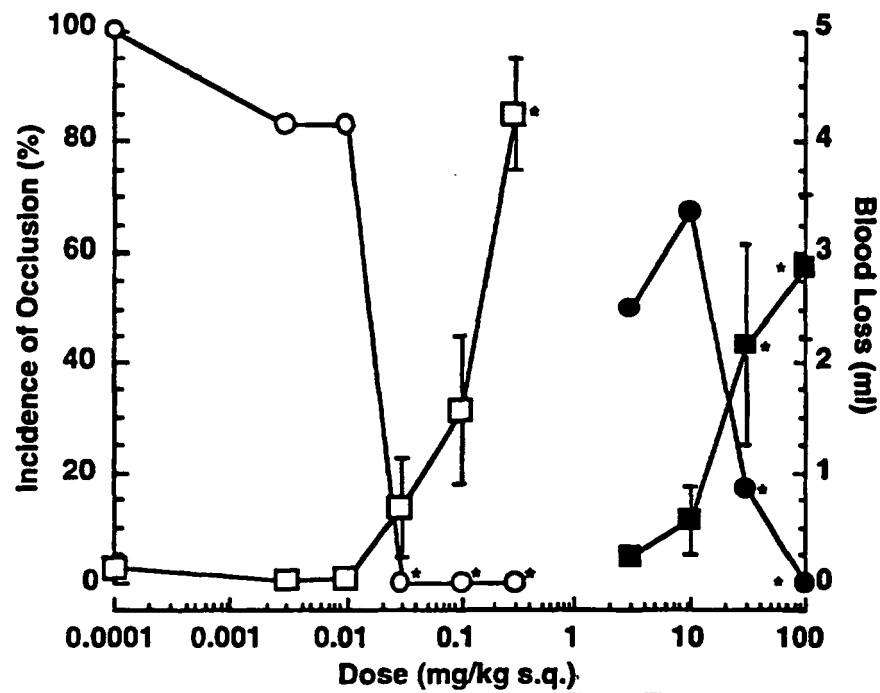
      200     210     220     230     240
      *      *      *      *      *
GAG GGA TAC AAG CGT CAT GAA ACG AAG GGA TGC TTA AAG GAA GGA TCA
Glu Gly Tyr Lys Arg His Glu Thr Lys Gly Cys Leu Lys Glu Gly Ser>

      250     260     270     280
      *      *      *      *
GCT GAT TGT AAA TAA GTT ATC AGA ACG CTC GTT TTG TCT TAC ATT AGA
Ala Asp Cys Lys ***

      290     300     310     320     330
      *      *      *      *      *
TGG GTG AGC TGA TGT ATC TGT CAG ATA AAC TCT TTC TTC TAA AAA AAA

      340     350     360
      *      *      *
AAA AAA AAA AAA AAA AAA AAA AAA A

```

FIGURE 15

Efficacy:		Bleeding:		Estimated 3.5 hr Blood Loss @:	
○	NAP-5	□	NAP-5	ED ₅₀	ED ₁₀₀
●	LMWH	■	LMWH		
* p<0.05 vs saline					
NAP-5		0.3		0.7	
LMWH		1.2		2.9	

FIGURE 16

	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10										
AcaNAP5	KAYPBOGE	NEMLDDC	GTQKP	CEAKC	NEEPPPE	EE	DPIC	RS	RGCL	LPP	ACVCK	D	GFYRD	TV	IGDCVR	E	EBCDQ	H	EIIHV	
AcaNAP6	KAYPBOGE	NEMLDVC	GTQKP	CEAKC	SEEE	EE	DPIC	RS	FSCP	GPA	ACVCE	D	GFYRD	TV	IGDCVK	E	EBCDQ	H	EIIHV	
AcaNAP48	RTARKPPTQGE	NERVEWC	G	KQ	CEITC	DDP	DKIC	RS	LACP	GPP	ACVCD	D	GYTRD	TN	VGLCVQ	Y	DECDN		MDIIMVS	
AcaNAP23	KPSEKQGP	HERLD	C	GNKKP	CERKC	KIETSEEDDYEBGTE	RFR	LL	RUCD	QPY	ECICD	D	GYTRN	K	KGECVT	D	DVCQE		DFMEFITFAP	
AcaNAP24	RPEKKQGP	GERLA	C	GNKKP	CERKC	KIETSEEDDYEBGTE	RFR	LL	RUCD	QPY	ECICD	D	GYTRN	K	KGECVT	D	DVCQE		DFMEFITFAP	
AcaNAP25	RPEKKQGP	GERLD	C	ANKKP	CEPKC	KIETSEEDDDVE	DT	DVRC	LV	RUCE	RPL	KCICK	D	GYTRN	K	KGECVT	D	DVCQE	DFMEFITFAP	
AcaNAP44	RPEKKQGP	GERLD	C	ANKKP	CEPKC	KIETSEEDDDVE	ET	DVRC	LV	RUCE	RPL	KCICK	D	GYTRN	K	KGECVT	D	DVCQE	DFMEFITFAP	
AcaNAP31, 42, 46	KSLMDQKQGE	NERLD	C	GNQKD	CERKC	DKRSE	EI	MQAC	LT	RQCL	PP	VCVCE	D	GFYRN	D	NDQCV	E	EBCN	MEFITFAP	
AceNAP4-d1	KPNVMTNACGL	NEYFABC	GNWKE	CEHRC	NEE	ENEERDE	ER	ITAC	LI	RUCF	RPG	ACVCK	D	GFYRN	R	TGSCVE	E	DDCE	YENMEFITFAPE->	
AceNAP4-d2	VPIQGS	NERYSDC	GNDKQ	CERKC	NED	DYKQ	DEAC	RS	HUCE	RPG	ACVCE	D	GFYRN	K	KGSCVE	S	DDCE		YDNDFITFAPETSR	
AcaNAP45d1	KSARKQGL	NEKLD	C	GNLKA	CEKKC	SDL	DNEEDYKE	ED	ESKC	RS	RECSR	R	VCVCD	E	GFYRN	K	KGQCVT	R	DDCEY	DNMEITFPPE->
AcaNAP47d1	KSARKQGL	NEKLD	C	GNLKA	CEKKC	SDL	DNEEDYKE	ED	ESKC	RS	RECS	R	VCVCD	E	GFYRN	K	KGQCVT	R	DDCEY	DNMEITFPPE->
AduNAP7-d1	KAARKQGL	NERLD	C	GNLKQ	CEPKC	SDL	ESEETEB	ED	ESKC	RS	RECS	R	VCVCD	E	GFYRN	K	KGKQVA	K	DVCED	DNMEITFPPE->
AcaNAP45d2	DKQGP	DEMFDWC	GTQKQ	CERKC	NKE	LSEKD	EEAC	LS	RACTG	R	ACVCN	D	GLYRD	D	FGNCVE	K	DECDN		MEITFPPETKH	
AcaNAP47d2	DKQGP	DEMFDWC	GTQKQ	CERKC	SEE	LSEKN	EEAC	LS	RACTG	R	ACVCN	D	GLYRD	D	FGNCVE	K	DECDN		MEITFPPETKH	
AduNAP4	KCPPT	DEMFDWC	GTQKH	CELKC	DRE	LTEKE	EQAC	LS	RUCE	K	S	ACVCN	D	GLYRD	K	FGNCVE	K	DECDN	MEITFAPEETK	
AduNAP7-d2	DEQGP	DEMFDYC	GNYKK	CERKC	SEE	TSEKN	EEAC	LS	RACT	G	R	ACVCK	D	GLYRD	D	FGNCVP	H	DECDN	MEITFPPETKH	
AceNAP5	KAPFKQGV	NERFEVC	GNLKB	CELKC	D		ED	PKIC	S	RACI	RPP	ACVCD	D	GFYRD	K	YGFCVE	E	DECDN	MEITFPPETK	
AceNAP7	RTVKKQGL	NERVDDC	GNAKD	CEITC	G		EE	EKVC	RS	RECT	SPG	ACVCE	Q	GFYRD	P	AGDCVT	D	EBCDE	WNNMEITMPKQ	
AcaNAPc2	KATNQOGE	NEKYDSC	GSKE	CDKIC	KYDQVEEDDE	EP	NVPC	LV	RVCH	Q	DCVCE	E	GFYRN	K	DDKCVS	A	EDCEL		DNDMFIYQGRN	
HpoNAP5	KTCGP	NEEYTEC	GTP	CEPKC	NEPMPI		C	TLN	CI	VNV	QCK	P	GFYRGPKG		CVA	PGQC	K			
NanNAP	KRDCPA	NEENREC	GTP	CEPKC	NQMPDI		C	TWN	CI	VDV	QCK	E	GYRHEITKG		CLAGE	SADC	K			

NAP = nematode anticoagulant protein

Aca = *Ancyclostoma caninum*
 Ace = *Ancyclostoma ceylanium*
 Adu = *Ancyclostoma duodenale*
 Hpo = *Heligmosomoides polygyrus*
 Asu = *Ascaris suum*
 Nam = *Necator americanus*

Figure 17

Lys	Pro	Asn	Asn	Val	Met	Thr	Asn	Ala	Cys	Gly	Leu	Asn	Glu
1				5					10				
Tyr	Phe	Ala	Glu	Cys	Gly	Asn	Met	Lys	Glu	Cys	Glu	His	Arg
15				20					25				
Cys	Asn	Glu	Glu	Glu	Asn	Glu	Glu	Arg	Asp	Glu	Glu	Arg	Ile
30					35					40			
Thr	Ala	Cys	Leu	Ile	Arg	Val	Cys	Phe	Arg	Pro	Gly	Ala	Cys
		45			50				55				
Val	Cys	Lys	Asp	Gly	Phe	Tyr	Arg	Asn	Arg	Thr	Gly	Ser	Cys
		60					65						70
Val	Glu	Glu	Asp	Asp	Cys	Glu	Tyr	Glu	Asn	Met	Glu	Phe	Ile
				75					80				
Thr	Phe	Ala	Pro	Glu	Val	Pro	Ile	Cys	Gly	Ser	Asn	Glu	Arg
85				90					95				
Tyr	Ser	Asp	Cys	Gly	Asn	Asp	Lys	Gln	Cys	Glu	Arg	Lys	Cys
	100				105					110			
Asn	Glu	Asp	Asp	Tyr	Glu	Lys	Gly	Asp	Glu	Ala	Cys	Arg	Ser
		115					120					125	
His	Val	Cys	Glu	Arg	Pro	Gly	Ala	Cys	Val	Cys	Glu	Asp	Gly
		130					135				140		
Phe	Tyr	Arg	Asn	Lys	Lys	Gly	Ser	Cys	Val	Glu	Ser	Asp	Asp
			145						150				
Cys	Glu	Tyr	Asp	Asn	Met	Asp	Phe	Ile	Thr	Phe	Ala	Pro	Glu
155				160						165			
Thr	Ser	Arg											
	170												

Figure 18

Lys	Ser	Ala	Lys	Lys	Cys	Gly	Leu	Asn	Glu	Lys	Leu	Asp	Cys
1				5					10				
Gly	Asn	Leu	Lys	Ala	Cys	Glu	Lys	Lys	Cys	Ser	Asp	Leu	Asp
15					20					25			
Asn	Glu	Glu	Asp	Tyr	Lys	Glu	Glu	Asp	Glu	Ser	Lys	Cys	Arg
	30					35					40		
Ser	Arg	Glu	Cys	Ser	Arg	Arg	Val	Cys	Val	Cys	Asp	Glu	Gly
		45				50					55		
Phe	Tyr	Arg	Asn	Lys	Lys	Gly	Gln	Cys	Val	Thr	Arg	Asp	Asp
			60					65					70
Cys	Glu	Tyr	Asp	Asn	Met	Glu	Ile	Ile	Thr	Phe	Pro	Pro	Glu
				75					80				
Asp	Lys	Cys	Gly	Pro	Asp	Glu	Trp	Phe	Asp	Trp	Cys	Gly	Thr
85					90					95			
Tyr	Lys	Gln	Cys	Glu	Arg	Lys	Cys	Asn	Lys	Glu	Leu	Ser	Glu
	100					105					110		
Lys	Asp	Glu	Glu	Ala	Cys	Leu	Ser	Arg	Ala	Cys	Thr	Gly	Arg
		115					120				125		
Ala	Cys	Val	Cys	Asn	Asp	Gly	Leu	Tyr	Arg	Asp	Asp	Phe	Gly
			130				135					140	
Asn	Cys	Val	Glu	Lys	Asp	Glu	Cys	Asn	Asp	Met	Glu	Ile	Ile
				145					150				
Thr	Phe	Pro	Pro	Glu	Thr	Lys	His						
155					160								

Figure 19

Lys	Ser	Ala	Lys	Lys	Cys	Gly	Leu	Asn	Glu	Lys	Leu	Asp	Cys
1				5					10				
Gly	Asn	Leu	Lys	Ala	Cys	Glu	Lys	Lys	Cys	Ser	Asp	Leu	Asp
15					20					25			
Asn	Glu	Glu	Asp	Tyr	Gly	Glu	Glu	Asp	Glu	Ser	Lys	Cys	Arg
	30					35					40		
Ser	Arg	Glu	Cys	Ile	Gly	Arg	Val	Cys	Val	Cys	Asp	Glu	Gly
		45			50						55		
Phe	Tyr	Arg	Asn	Lys	Lys	Gly	Gln	Cys	Val	Thr	Arg	Asp	Asp
			60					65					70
Cys	Glu	Tyr	Asp	Asn	Met	Glu	Ile	Ile	Thr	Phe	Pro	Pro	Glu
				75					80				
Asp	Lys	Cys	Gly	Pro	Asp	Glu	Trp	Phe	Asp	Trp	Cys	Gly	Thr
85					90					95			
Tyr	Lys	Gln	Cys	Glu	Arg	Lys	Cys	Ser	Glu	Glu	Leu	Ser	Glu
	100					105					110		
Lys	Asn	Glu	Glu	Ala	Cys	Leu	Ser	Arg	Ala	Cys	Thr	Gly	Arg
		115					120				125		
Ala	Cys	Val	Cys	Asn	Asp	Gly	Leu	Tyr	Arg	Asp	Asp	Phe	Gly
			130			135						140	
Asn	Cys	Val	Glu	Lys	Asp	Glu	Cys	Asn	Asp	Met	Glu	Ile	Ile
				145					150				
Thr	Phe	Pro	Pro	Glu	Thr	Lys	His						
155					160								

Figure 20

Lys	Ala	Ala	Lys	Lys	Cys	Gly	Leu	Asn	Glu	Arg	Leu	Asp	Cys
1				5					10				
Gly	Asn	Leu	Lys	Gln	Cys	Glu	Pro	Lys	Cys	Ser	Asp	Leu	Glu
15					20					25			
Ser	Glu	Glu	Tyr	Glu	Glu	Glu	Asp	Glu	Ser	Lys	Cys	Arg	Ser
	30					35					40		
Arg	Glu	Cys	Ser	Arg	Arg	Val	Cys	Val	Cys	Asp	Glu	Gly	Phe
		45				50					55		
Tyr	Arg	Asn	Lys	Lys	Gly	Lys	Cys	Val	Ala	Lys	Asp	Val	Cys
			60				65						70
Glu	Asp	Asp	Asn	Met	Glu	Ile	Ile	Thr	Phe	Pro	Pro	Glu	Asp
				75					80				
Glu	Cys	Gly	Pro	Asp	Glu	Trp	Phe	Asp	Tyr	Cys	Gly	Asn	Tyr
85					90					95			
Lys	Lys	Cys	Glu	Arg	Lys	Cys	Ser	Glu	Glu	Thr	Ser	Glu	Lys
	100					105					110		
Asn	Glu	Glu	Ala	Cys	Leu	Ser	Arg	Ala	Cys	Thr	Gly	Arg	Ala
		115					120				125		
Cys	Val	Cys	Lys	Asp	Gly	Leu	Tyr	Arg	Asp	Asp	Phe	Gly	Asn
			130				135					140	
Cys	Val	Pro	His	Asp	Glu	Cys	Asn	Asp	Met	Glu	Ile	Ile	Thr
				145					150				
Phe	Pro	Pro	Glu	Thr	Lys	His							
155					160								

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