Purification, Cloning, and Synthesis of a Novel Salivary Anti-thrombin from the Mosquito *Anopheles albimanus*†

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Received April 1, 1999; Revised Manuscript Received June 1, 1999

ABSTRACT: An anti-thrombin peptide (anophelin) was isolated from the salivary glands of the mosquito *Anopheles albimanus* through molecular sieving and reverse-phase high-performance liquid chromatography. The purified peptide inhibited thrombin-induced platelet aggregation, thrombin esterolytic activity on a synthetic substrate, and thrombin cleavage of fibrinogen. The purified anti-thrombin had a molecular mass of 6342.4 Da. Its amino terminus was blocked, but internal sequence yielded three peptide sequences, which were used to design oligonucleotide probes for polymerase chain reaction amplification of salivary gland cDNA and isolation of the full-length clone. Analysis of the sequence of anophelin shows no similarities to any other anti-thrombin peptides. Anophelin was successfully synthesized and characterized to be a tight-binding, specific, and novel inhibitor of thrombin.

The blood feeding habit has evolved several times in invertebrates (Annelidae, Arachnidae, and insects), as well as in mammal bats. Within the insects, it has evolved at least in five different orders, and possibly more than once within some orders (*1*). Not surprisingly, the salivary glands of such animals have a great compositional diversity of compounds. These compounds antagonize the redundant hemostatic response of the bloodsucker's hosts, allowing fast and successful blood feeding (*2, 3*).

Several antihemostatic agents from blood-sucking animals have been characterized, including anti-thrombins and factor Xa inhibitors (*4*−*6*). The leech anti-thrombin hirudin is a very potent and specific peptide (*5*). It belongs to a family of leech antihemostatic peptides (*7*) containing a disulfide structural motif (*8*). Anti-factor Xa peptides from ticks have no homology to other anticlotting agents, although they have a similar molecular weight and a similar number of cysteine residues as does hirudin (*9, 10*). The hematophagous hemiptera *Rhodnius prolixus* has a typical Kazal-type anti-thrombin (*11*) of 11 kDa. Recently, a 48 kDa factor Xa inhibitor from the mosquito *Aedes aegypti* has been purified, cloned, expressed, and shown to be a member of the serpin family of serine protease inhibitors (*12*). Interestingly, anopheline mosquitoes had salivary anti-thrombin (*13*), not an anti-factor Xa inhibitor. In this paper, we purified, cloned, and synthesized the salivary anti-thrombin of the mosquito *Anopheles albimanus*, which is a peptide with no homologies to proteins of known function in GenBank and is a specific and tight-binding inhibitor of thrombin.

EXPERIMENTAL PROCEDURES

Insect Rearing. Anopheles albimanus (Santa Tecla strain) were reared in the Medical Entomology Section of the Laboratory of Parasitic Diseases under the expert direction of Andre Laughinghouse. Adults were offered cotton swabs containing 10% Karo syrup (CPC International Inc., Englewood Cliffs, NJ). Salivary glands from female mosquitoes at least 3 days old were dissected in groups of 20 pairs in 20 μL of phosphate-buffered saline (10 mM sodium phosphate at pH 7.0 with 150 mM NaCl) and kept at −75 °C until needed. These glands were used for purification of the salivary anti-thrombin. Homogenization of the salivary glands was done by sonication (*22*). For cDNA library construction, pools of female salivary glands were obtained at the day of adult emergence and at 1 day following emergence.

Measurement of Thrombin Activity. Human thrombin and the chromogenic substrate benzoyl-Phe-Val-Arg-pNA were obtained from Calbiochem (San Diego, CA); fibrinogen was obtained from Sigma (St. Louis, MO). The activity of thrombin was measured by two assays: chromogenic assay and fibrinogen assay. Cleavage of the chromogenic substrate by thrombin was measured spectrophotometrically at 405 nm on a Thermomax microplate reader (Molecular Devices, Menlo Park, CA). Briefly, 10 μL of thrombin (20, 10, or 5 nM) were mixed with 60 μL of 10 mM Hepes, pH 7.4, + 150 mM NaCl + 200 μg/mL bovine serum albumin, and the mixture was preincubated for 2 min at 37 °C. Then, 20 μL of chromogenic substrate (0.5 mg/mL) was added to the mixture and the sample was read at 405 nm with the temperature controlled at 37 °C. Thrombin activity on fibrinogen was measured by using 10 μL of thrombin (20, 10, or 5 nM) and 30 μL of 10 mM Hepes buffer, pH 7.0, and the reaction was started by adding 60 μL of fibrinogen

† The anti-thrombin sequence reported in this paper has been submitted to GenBank Accession Number AF125095.

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10.1021/bi990761i
Published on Web 08/05/1999

(2 mg/mL). Anophelin (2 μM) specificity was tested by 10-min incubation of the inhibitor with different enzymes at 37 °C, diluted in Hepes−BSA and 0.15 M NaCl, except when indicated otherwise. Enzyme and substrate concentrations were as follows: factor Xa (12.8 nM, Calbiochem), trypsin (0.66 nM, Sigma Chemical Co.), and chymotrypsin (250 nM, Calbiochem) activities were detected by addition of 200 μM chromogenic substrate for factor Xa, trypsin or chymotrypsin (Chromogenix; Boehringer Mannhein Co., Indianapolis, IN). Activated protein C (64 nM) activity was started by addition of 600 μM chromogenic substrate for aPC (Calbiochem). Plasmin activity (0.02 unit/mL) and elastase activity (10 nM) were tested by addition of 200 μM Val-Leu-Arg-p-Na (Sigma Chemical Co.) or chromogenic substrate for elastase (Calbiochem), respectively. Reptilase activity (*Bothrops atrox* thrombin-like enzyme; Diagnostica Stago, France) was assayed by addition of 250 μM S-2238 to 0.3125 BU (batroxobin units)/mL of enzyme. Papain (Calbiochem) was diluted to 0.2 mg/mL in PBS containing 3 mM DTT and 2 mM EDTA, followed by addition of 200 μM chromogenic substrate for papain (Calbiochem). To test the effects of anophelin on factor IXa activity, factor Xa generation by intrinsic Xase complex was performed with Coatest factor VIII (Chromogenix, Sweden), according to manufacturer's instructions, except that factor VIII (monoclonal antibody affinity purified, Baxter, CA) at 1:15 000 dilution was used instead of plasma. The activity of thrombin (on fibrinogen) was measured spectrophotometrically at 405 nm (measures turbidity increase following formation of fibrin clot) on a microplate reader at 37 °C.

Measurement of Human Plasma Clotting Activity. Blood-clotting activity was measured by the recalcification time of human citrated plasma with a Thermomax microplate reader. Briefly, 30 μL of citrated human platelet-poor plasma and 30 μL of 10 mM Hepes, pH 7.4, + 150 mM NaCl were mixed in 96-well flat-bottom plates (Falcon 3912, Becton and Dickinson, Oxnard, CA) for 2 min at 37 °C, followed by addition of 30 μL of 25 mM $CaCl_2$. The plate was mixed and maintained at 37 °C by the apparatus mixer and heating system, and absorbance readings at 650 nm were taken at 11-s intervals. A fast and sharp increase in the absorbance after a lag phase indicated clotting. We chose the time taken for reaching a 0.06 or 0.03 absorbance value (onset OD) as a measure of clotting time.

Platelet Aggregation Assays. These were performed on a Thermomax microplate reader as previously described with further modifications (*23*). Briefly, 50 μL of 10 mM Hepes, pH 7.4, + NaCl (150 mM), were mixed in 96-well flat-bottom plates (Falcon 3912, Becton and Dickinson, Oxnard, CA) in the presence of thrombin (2.5 units/mL) and the peptide GPRP amide (1 mM). The aggregation was initiated by the addition of 50 μL of human citrated (0.38%) platelet-rich plasma (*24*). The plate was stirred on a microplate mixer (Cole Palmer Series 4732, Vernon Hills, IL) for 5 s before being transferred to the microplate reader.

Purification and Sequencing of A. albimanus anti-thrombin. A. albimanus salivary gland homogenate proteins were separated by molecular sieving HPLC, followed by reverse-phase HPLC. Chromatographic protocols were performed with a CM4100 pump and a SM4100 dual wavelength detector (ThermoSeparation Products, Riviera Beach, FL). Molecular sieving HPLC was performed on an TSK 2000 SW column (600 × 7.5 mm) with 10 mM Hepes at pH 7.0 and 150 mM NaCl at a flow rate of 1 mL/min. Fractions were collected at 0.4 min intervals. Active (anti-thrombin) fractions were pooled and injected into a reverse-phase, nonporous, polymer-based column (PRP-infinity, Hamilton) and eluted with a gradient from 10% to 60% acetonitrile and 0.1% trifluoroacetic acid in 60 min at a flow rate of 0.5 mL/min. Eluates were monitored at 220 and 280 nm. Fractions were collected at 1 min intervals. An aliquot of each fraction was dried in the presence of 10 μL of BSA (1 mg/mL), resuspended in 30 μL of 100 mM Hepes saline buffer (pH 7.0), and tested for anti-thrombin activity. Amino acid composition, mass spectroscopy, Asp-N digestion, and internal sequencing of the Asp-N-digested anti-thrombin were performed at the Harvard University microchemistry facility (Cambridge, MA) under the direction of Dr. William S. Lane.

Salivary Gland cDNA Library. A. albimanus salivary gland mRNA was isolated from 260 gland pairs by using the Micro-FastTrack mRNA isolation kit (Invitrogen, San Diego, CA) yielding a total of 250 ng of poly(A)+ mRNA. The PCR-based cDNA library was made following the instructions for the SMART cDNA library construction kit (Clontech, Palo Alto, CA). The mRNA was reverse-transcribed to cDNA using Superscript II RNase H^- reverse transcriptase (Gibco−BRL, Gaithersburg, MD) and the CDS/3′ primer (Clontech) for 1 h at 42 °C. Second-strand synthesis was obtained by a PCR-based protocol with the SMART primer (Clontech) as the sense primer and the CDS/3′ primer as anti-sense primer on a Perkin-Elmer 2400 thermal cycler with Klen-Taq DNA polymerase (Clontech). Conditions were the following: 94 °C for 2 min; 22 cycles of 94 °C for 15 s and 68 °C for 5 min. *Eco*RI adapters were ligated to the obtained cDNA. The cDNA was fractionated on columns provided by the manufacturer (Clontech). Fractions of cDNA of more than 400 bp were pooled and ligated into Lambda-ZAP II vector (Stratagene). The unamplified library obtained had a complexity of 5.7×10^6 recombinants.

For PCR cloning of *Anopheles* anti-thrombin, mRNA from 60 pairs of *A. albimanus* salivary glands was isolated with the Micro-FastTrack mRNA isolation kit (Invitrogen). The mRNA was then reverse-transcribed to cDNA using Superscript II RNase H^- reverse transcriptase (Gibco−BRL, Gaithersburg, MD) and the CDS/3′ primer (Clontech) for 1 h at 42 °C. Second-strand synthesis was obtained as described above. To obtain the partial DNA sequence of *A. albimanus* anti-thrombin, cDNA obtained by PCR amplification containing the SMART sequence was used as a template for the PCR reaction. Primers used in this reaction were the 5′ primer that recognizes the SMART sequence (Clontech) and primers designed from two internal peptides obtained by Asp-N digestion of the purified anti-thrombin (ATRI, 5′- TT-(AG) TT(TC) TC(ATCG) AC(I) A(AG)(I) TT(AG) TC- 3′; ATRII, 5′- GG(TC) TT(I) (GC)(AT)(I) CC(TC) TC(I) CC-(I) A(AG)- 3′). Conditions were as follows: 1 min at 95 °C; 5 cycles of 1 min at 94 °C, 30 s at 40 °C, and 45 s at 68 °C; 20 cycles of 1 min at 94 °C, 30 s at 45 °C, and 45 s at 68 °C.

PCR products were separated on a 1.0% agarose gel, excised, and purified by using the Sephaglas Bandprep Kit (Pharmacia) and cloned into a PCRscript vector (Stratagene) by using the PCRscript cloning system (Stratagene). Competent cells were transformed following the manufacturer's

protocol, and white colonies were isolated and grown overnight in Luria broth medium containing ampicillin (100 μg/mL) at 37 °C. Plasmids from two independent clones were isolated by using the Wizard Miniprep kit (Promega, Madison, WI). The clones were sequenced by using dye terminator reactions according to the manufacturer's instructions (DNA sequencing kit, part number 402079, Perkin-Elmer Applied Biosystems, Foster City, CA) and were analyzed by an automated ABI sequencer (ABI prism, 377 DNA sequencer, Perkin-Elmer).

After confirming that the sequence of the PCR product contained the predicted sequence of two internal peptides of the *Anopheles* anti-thrombin, the PCR insert was digested with *Eco*RI from the plasmid, gel-purified, and cleaned as described above. The PCR insert was then labeled with dUTP-digoxigenin with specific forward and reverse primers under the following PCR conditions: 75 °C for 5 min; 94 °C for 2 min; 25 cycles of 1 min at 94 °C, 1.5 min at 42 °C, and 1 min at 72 °C; and finally 5 min at 72 °C. The reaction mixture included the PCR insert as a template, 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris, pH 8.3, 0.01% gelatin, 0.2 mM each dNTP, DNA labeling mix (Genius system; Boehringer Mannheim, Indianapolis, IN), and 2 units of Ampli-Taq polymerase (Gibco−BRL). The 300-bp PCR clone labeled with dUTP-digoxigenin was used to screen the *A. albimanus* salivary gland cDNA library. Phage plaques were lifted with a Hybond-N nylon membrane (Amersham, Arlington Heights, IL) and hybridized with the digoxigenin-labeled PCR probe by the plaque hybridization protocol of the Genius system (Boehringer Mannheim). Positive plaques were picked and plated again for a secondary screening. Well-isolated positive plaques were selected, and the phagemid carrying the peroxidase clone was isolated from the phage with the in vivo excision protocol from the UNI-ZAP vector manual (Stratagene).

White colonies that originated from the phagemid excision protocol were isolated and grown overnight in Luria broth plus ampicillin (100 μg/mL) at 37 °C. Plasmid isolation was performed with the Wizard Miniprep kit (Promega). The insert of the isolated plasmid was sequenced as described above with the M13 and M13 reverse primers.

Sequence Analysis. Analysis of the predicted protein sequence was done with the BLAST programs (http://www.ncbi.nlm.gov/BLAST/) (*14*) and the Sequence analysis services programs (http://molbio.info.nih.gov) at the computational molecular biology Internet site from The National Institutes of Health.

Synthesis of A. albimanus Anti-thrombin Peptide. Peptide synthesis of the predicted anti-thrombin sequence generated from the anti-thrombin cDNA clone was performed at the Peptide Synthesis Laboratory, Structural Biology Section, NIAID (Rockville, MD) by Dr. Jan Lukszo. A 61 amino acid peptide was synthesized with the inclusion of an amide linked to the proline at the carboxy-terminal end. The synthetic peptide was subjected to mass spectroscopy to verify its size and to reverse-phase HPLC for purification and concentration of the sample.

Characterization of Synthetic Anti-thrombin. Synthetic peptide was reconstituted in 10 mM Hepes saline buffer and injected into a molecular sieving HPLC column. Chromatographic conditions were as described in the purification protocol of the native protein. Eluted fractions were tested for anti-thrombin activity and the amount of protein with anti-thrombin activity was estimated by measuring the area under the absorbance/time graphs standardized with bovine serum albumin. Synthetic peptide was tested as an anti-thrombin molecule by a chromogenic assay, fibrinogen assay, and recalcification time assay and by a platelet aggregation assay as described for the characterization of the native salivary anti-thrombin.

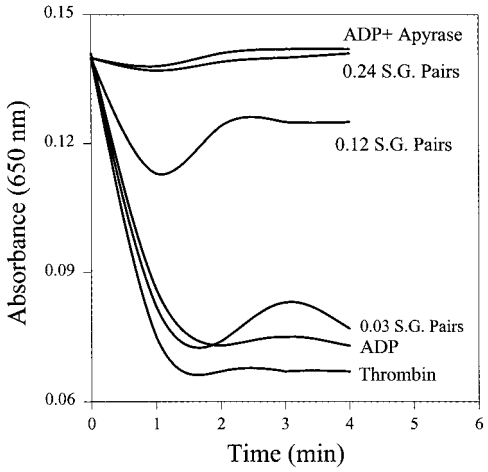

FIGURE 1: Inhibition of thrombin-induced platelet aggregation by salivary gland homogenate of *A. albimanus*. Platelet-rich plasma was incubated with 2 μM adenosine diphosphate and potato apyrase (ADP + apyrase) to demonstrate that platelet aggregation in our assay conditions was not dependent on ADP. Platelet aggregation induced by thrombin (2.5 units/mL) in the presence of potato apyrase was inhibited with 0.03, 0.12, and 0.24 salivary gland pairs of *A. albimanus*.

RESULTS

Effect of A. albimanus Salivary Gland Homogenate on Thrombin-Induced Platelet Aggregation. To test whether *A. albimanus* salivary gland homogenate inhibited platelet aggregation induced by thrombin, we incubated salivary homogenates with platelet-rich plasma and added thrombin as an agonist of platelet aggregation. Because salivary gland apyrase (*15, 16*) could interfere with the results, we also added an excess of potato apyrase to the reaction and confirmed its effect by showing the inability of ADP (2 μM) to induce platelet aggregation in the presence of the enzyme (Figure 1). In these conditions, where thrombin-induced platelet aggregation is independent of the ADP pathway, we observed inhibition of aggregation by thrombin with 0.03 homogenized salivary gland pair (each pair of glands have approximately 1 μg of total protein), and complete inhibition was observed with 0.24 salivary gland pair per 0.1 mL assay (Figure 1). Inhibition of thrombin by a chromogenic assay, a fibrinogen assay, and a delay in the recalcification time was also observed with the salivary gland homogenate (not shown). These results indicate that salivary gland homogenates of *A. albimanus* have a potent inhibitor of thrombin-induced platelet aggregation.

Purification of A. albimanus Salivary Anti-Thrombin. Salivary gland homogenate (1000 salivary gland pairs) was injected into a molecular sieving HPLC column (Figure 2A) and eluted fractions were tested for anti-thrombin activity by a colorimetric assay with a synthetic thrombin substrate (Figure 2B). Fractions rich in anti-thrombin activity also

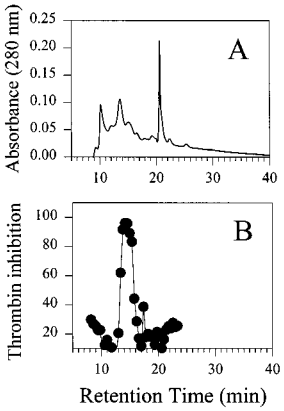

FIGURE 2: Purification of *A. albimanus* salivary anti-thrombin by molecular sieving HPLC. Salivary gland homogenate (1000 salivary gland pairs) was eluted with 10 mM Hepes buffer, pH 7.0, and 150 mM NaCl. Eluted proteins were detected at 280 nm (A) and tested for anti-thrombin activity on a chromogenic assay (B).

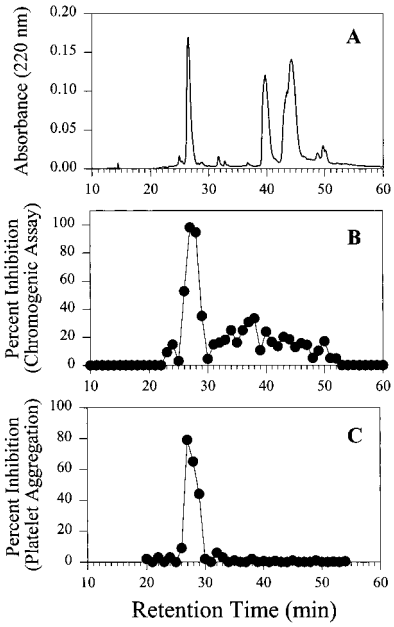

FIGURE 3: Second purification step on *A. albimanus* salivary anti-thrombin by reverse-phase HPLC. Active anti-thrombin fractions eluted from a molecular sieving column were injected into a reverse-phase PRP-infinity column. The eluent was monitored at 220 nm (A). Eluted fractions were dried in the presence of BSA and tested for anti-thrombin activity on a thrombin chromogenic assay (B) and on a thrombin-induced platelet aggregation assay (C).

inhibited thrombin on a fibrinogen assay, delayed the recalcification time of human citrated plasma, and inhibited thrombin-induced platelet aggregation (not shown). The active anti-thrombin fractions were further injected into a reverse-phase HPLC column, resulting in the separation of three major peaks (Figure 3A). Aliquots from the reverse-phase fractions were dried in the presence of 10 μL of BSA (1 mg/mL) and reconstituted in Hepes saline buffer. The 220 nm absorbing peak eluting at minute 27 (Figure 3A) inhibited thrombin activity in a chromogenic assay (Figure 3B) and inhibited thrombin-induced platelet aggregation with approximately 0.74 ng of peptide (Figure 3C). Purified material also inhibited thrombin in a fibrinogen assay (not shown) and delayed the recalcification time of human citrated plasma (not shown). Mass spectroscopy of the purified material resulted in a peak of 6342.4 Da (Figure 4). N-Terminal

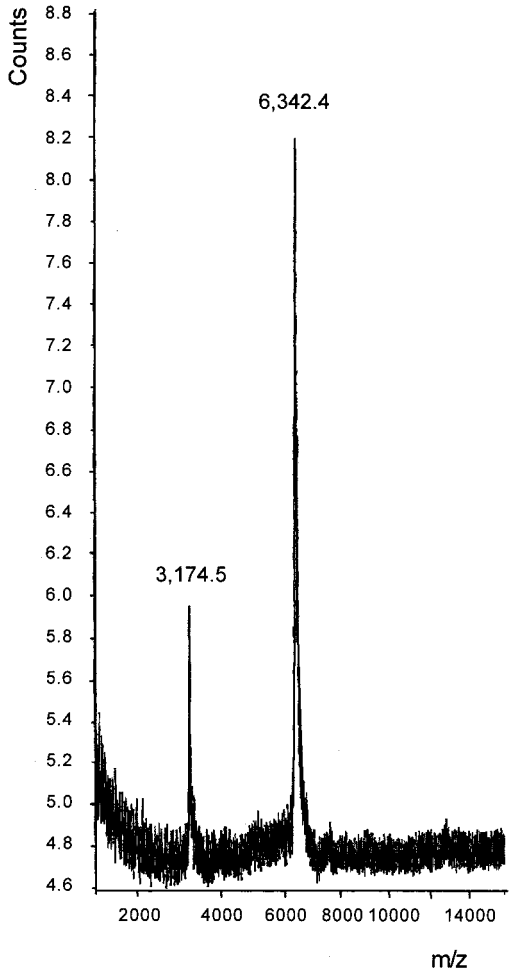

FIGURE 4: Mass spectroscopic analysis of purified *A. albimanus* anti-thrombin.

sequence of the peptide was unsuccessful. The purified anti-thrombin was then enzymatically cleaved with Asp-N, and three internal peptides were obtained: (1) DPGRRLGEGSKP, (2) TFNT, and (3) DKLVENN.

Isolation of A. albimanus Anti-thrombin cDNA Clone. To obtain full sequence information of the salivary anti-thrombin of *A. albimanus*, the amino acid sequence of the internal peptides were used to design oligonucleotide primers (see Experimental Procedures) for a PCR reaction, using as a template cDNA of *A. albimanus* salivary glands. A single PCR product of 300 bp was thus obtained, and its sequence was found to contain the sequence of the three internal peptides obtained with Asp-N digestion of the purified anti-thrombin. This PCR product was then labeled by digoxigenin and used to screen the *A. albimanus* salivary gland cDNA library. A positive clone of 484 bp in length was obtained and sequenced.

Analysis of *A. albimanus Anti-thrombin*. We sequenced 484 bp of the isolated anti-thrombin clone. This clone is hereby named AlbieAT. The cDNA clone contains an open reading frame of 249 base pairs that codes for a protein of 83 amino acids (Figure 5A). The sequences of all three internal peptides of *A. albimanus* anti-thrombin were represented in the deduced protein (Figure 5A, underlined amino acids). The first 22 amino acids were predicted to be the signal peptide by the SignalP program (*17*) and the remaining 61 amino acids were predicted to constitute the mature

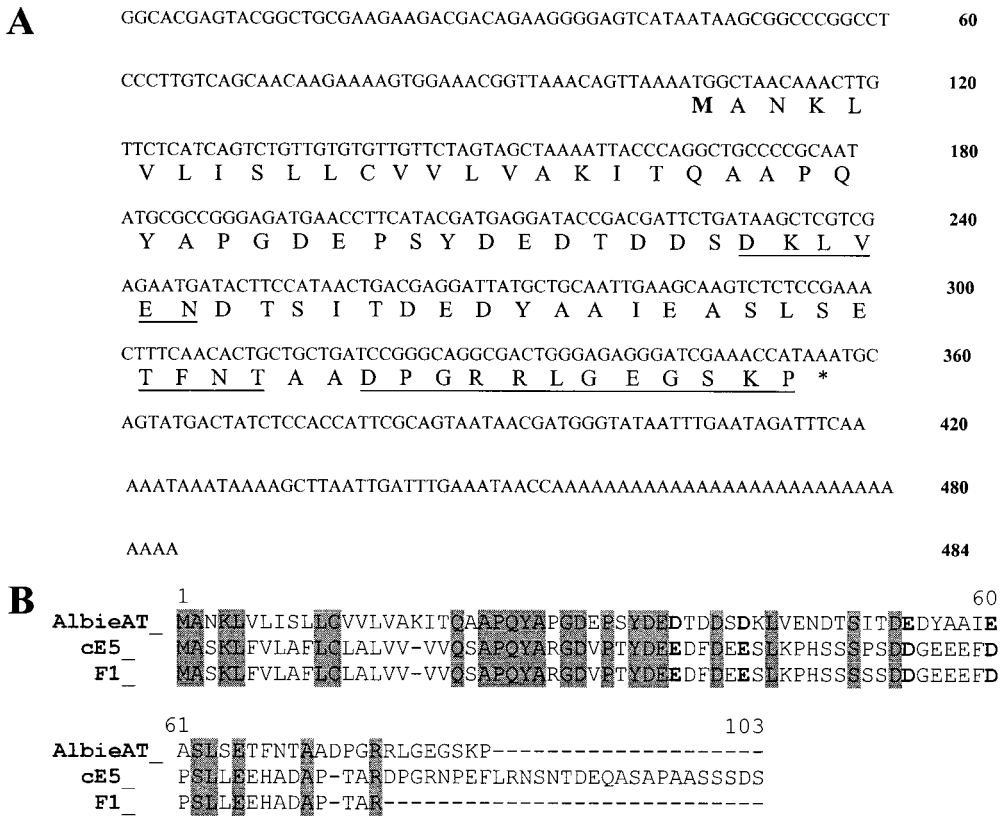

FIGURE 5: (A) DNA sequence and predicted protein sequence of AlbieAT clone. Underlined amino acids represent sequences obtained by Edman degradation of Asp-N digestion products from purified anti-thrombin. Accession number of albieAT: AF125095. (B) Sequence alignment of *A. albimanus* anti-thrombin and two cDNA clones from *Anopheles gambiae*: cE5 (Y17717) and F1 (AJ000038). Identical amino acids within the three sequences are shaded. Boldface type indicates a substitution of a charged amino acid by another charged amino acid.

protein. Analysis of the predicted processed protein indicated a molecular mass of 6538.74 Da and an acidic isoelectric point of 3.52 with −13.07 charge units at pH 7.0. There are 17 strongly acidic amino acids in the sequence: 10 aspartic acids and 7 glutamic acids. These two amino acids represent one-third of the total amino acids of the protein.

Search of the nonredundant protein database at the National Center for Biotechnology Information (Bethesda, MD) using the gapped BLASTP program (*14*) found high homology with two hypothetical proteins from cDNA clones of *Anopheles gambiae* salivary gland: cE5 protein (Y17717) with a smallest sum probability (*P*) of 3.0×10^{-5} and F1 (AJ000038) with a smallest sum probability of 0.00055. Alignment of the *A. albimanus* anti-thrombin sequence with cE5 and F1 sequences from *A. gambiae* (Figure 5B) and comparison of their predicted p*I* (*A. albimanus* p*I*, 3.52; cE5, 4.04; F1, 4.05) suggest that these two hypothetical salivary proteins are putative anti-thrombins from *A. gambiae*. The alignment shows highly conserved regions at the predicted N-terminal site (APQYA), conserved negatively charged amino acids (D_8, D_{13}, E_{14}, D_{18}, D_{31}, and E_{43}) and a conserved arginine (R_{53}) at the carboxy-terminal region.

Synthesis of A. albimanus Salivary Anti-thrombin. Because the sequence of *A. albimanus* anti-thrombin was not similar to those of other anti-thrombin proteins or of any other proteins with known function in all the databases searched, a peptide with sequence based on the predicted mature product of AlbieAT cDNA was synthesized to confirm the isolation of the anti-thrombin cDNA clone. Figure 6 shows the MALDI spectra of the synthetic peptide, indicating a

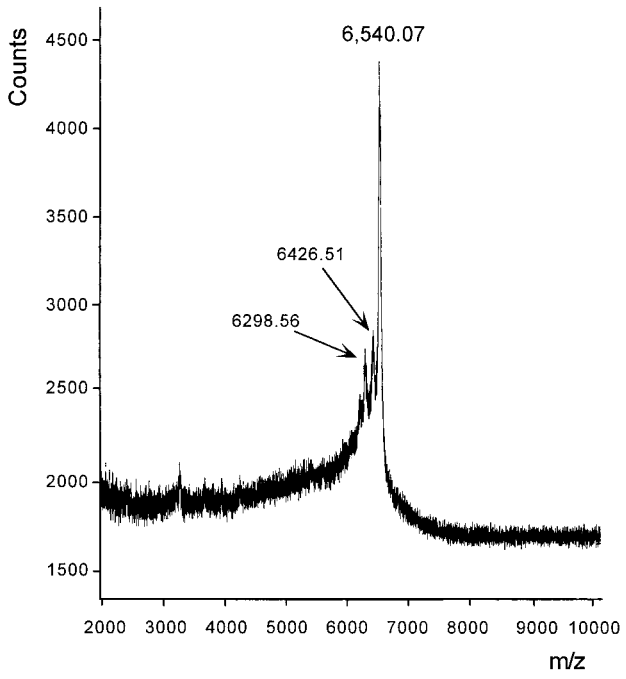

FIGURE 6: Mass spectra of synthetic anophelin.

main ion of 6540 (expected: 6539 + H^+ = 6540 Da). Impurities of lower molecular mass may be due to the lack of addition of Asp or Glu or Ile/Leu, as indicated by the contaminants of masses 6426.5 and 6298.6. HPLC fractionation of the synthetic product showed a single major peak and also two minor impurities (less than 5% total area) with

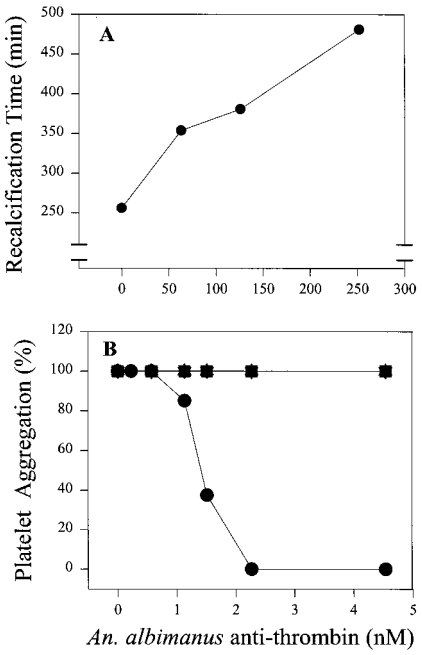

FIGURE 7: Effect of anophelin on the recalcification time assay (A) and on the thrombin-induced platelet aggregation assay (B). (A) Human citrated plasma was incubated with various concentrations (0, 60, 120, and 240 nM) of synthetic anophelin and clotting was initiated with 8.3 mM $CaCl_2$. The time of clotting (recalcification time) was measured as described under Experimental Procedures. (B) Platelet-rich plasma was incubated with different concentrations of synthetic anophelin, and aggregation of platelets was initiated with 0.83 nM thrombin (●) or 1.4 μM of U46619 (□), 10 μg/mL of collagen (△), and 10 μM of ADP (▽).

molecular masses corresponding to the acid hydrolysis of the Asp−Pro bond between amino acids 50 and 51 (not shown). The synthetic peptide doubled the recalcification time of human citrated plasma when added in the range of 250 nM (Figure 7A) and inhibited thrombin-induced platelet aggregation in the range of 1 nM. Synthetic anophelin had no effect on platelet aggregation induced by ADP (10 μM), collagen (10 μg/mL), and the thromboxane analogue U46619 (1.4 μM) indicating that the inhibition on the platelet aggregation assay is solely by the thrombin pathway (Figure 7B). Additionally, the synthetic peptide (hereby named anophelin) displayed anti-thrombin activity by a chromogenic substrate assay in the range of 0.6 nM (Figure 8A, ■), and by the fibrinogen assay in the range of 0.2 nM (Figure 8A, ●) in the nominal presence of 1.05 nM thrombin.

To investigate whether anophelin was a tight inhibitor of thrombin, we measured the activity of thrombin at three different concentrations (0.52, 1.05, and 2.1 nM) as a function of the concentration of anophelin. The resulting curve (Figure 8B) shows a parallel increase in the doses of anophelin needed to give the same inhibition of thrombin. When the same data is replotted as the inhibition of thrombin against the concentration of anophelin divided by the concentration of thrombin (Figure 8B, inset), the lines now overlap. According to Henderson (*18*), this type of graph indicates the concentration of enzyme to be larger than 100 times the K_i value and is indicative of a tight-binding inhibitor.

The specificity of anophelin (2 μM) to thrombin is indicated by its inability to inhibit a number of serine proteases including activated factor X, activated factor IXa

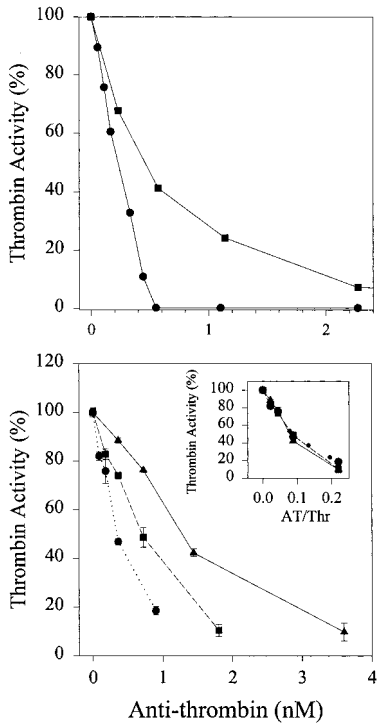

FIGURE 8: (A) Effect of synthetic anophelin on a thrombin chromogenic substrate assay (■) and on a thrombin fibrinogen assay (●). Each data point indicates the average ± SEM of triplicate experiments. (B) Anophelin interaction with thrombin. The effect of various concentrations of anophelin was measured in the presence of 0.52 nM (●), 1.05 nM (■), and 2.1 nM (▲) thrombin on a chromogenic substrate assay as described under Experimental Procedures. The inset shows thrombin activity as a function of the concentration of anophelin divided by the concentration of thrombin used in each data point. (Each data point is the average ± SEM of triplicate experiments.

Table 1: Specificity of Anophelin to Thrombin[a]

enzymes[b]	residual activity[c] (%)
thrombin (2 nM)	1.7 ± 1.50
trypsin (0.66 nM)	106 ± 3.16
chymotrypsin (250 nM)	97.8 ± 0.87
activated factor X (12.8 nM)	103 ± 1.09
activated protein C (64 nM)	97 ± 1.03
IXa (intrinsic Xase)	105 ± 0.96
reptilase (0.3125 BU/mL)	103 ± 0.98
plasmin (0.02 unit/mL)	92 ± 7.70
papain (0.2 mg/mL)	99.6 ± 4.13
elastase (10 nM)	98.2 ± 1.36

[a] Anophelin (2 μM) was incubated with 2 nM thrombin, 0.66 nM trypsin, 250 nM chymotrypsin, 12.8 nM factor Xa, 64 nM activated protein C, IXa (intrinsic Xase), 0.3125 batroxobin unit (BU)/mL of reptilase, 0.02 unit/mL plasmin, 0.2 mg/mL papain, 10 nM neutrophil elastase for 10 min, at 37 °C, before addition of chromogenic substrate specific for each enzyme, as described under Experimental Procedures. Reactions were followed for 15 min, and the effect of anophelin was estimated by setting the initial velocity obtained in the presence of enzyme alone (without inhibitor) as 100%. [b] Assay conditions are described under Experimental Procedures. [c] Enzyme activities in the absence of inhibitor were set as 100%. Data are the mean ± SE of triplicate experiments.

(detected by factor Xa generation by intrinsic Xase), activated protein C, trypsin, chymotrypsin, neutrophil elastase, and plasmin. In addition, anophelin does not affect the proteolytic activity of reptilase (a thrombin-like enzyme from *Bothrops atrox*) or the thiol-protease activity of papain (Table 1).

DISCUSSION

The salivary anti-thrombin of *A. albimanus* (anophelin) is shown in this paper to be a novel, specific, and tight-binding inhibitor of thrombin. Anophelin is novel because it has no sequence similarities to any known anti-protease or known peptide. Anophelin has no cysteines, a common finding in leech and tick anticoagulant peptides (*7*, *9*). The presence of many cysteines complicates folding of the recombinant or synthetic protein (*19*), a problem that should not be found with anophelin. Synthetic anophelin has a very high affinity for thrombin, as indicated by the shape of the dose−response curve of anophelin in different concentrations of thrombin (*18*). The need for larger concentration of peptide required to inhibit the recalcification time of plasma is possibly due to the "explosive" formation of thrombin following factor X activation. This phenomenon is also observed with hirudin, the medicinal leech (*20*). Finally, anophelin is not a generic serine protease inhibitor, as it does not inhibit factor Xa, active protein C, trypsin, chymotrypsin, factor IXa, plasmin, and elastase. It also does not affect the proteolytic activity of *B. atrox* thrombin-like enzyme (reptilase) and the thiol protease activity of papain.

Although there are no obvious sequence analogies between hirudin and anophelin, both peptides are rich in negatively charged amino acids. However, hirudin has a higher density of negatively charged amino acids in the carboxy-terminal region, whereas anophelin's amino-terminal portion has a higher negative charge density. In the case of hirudin, these negatively charged residues are important to interact with thrombin's anion-binding exosite (a positively charged cleft in the thrombin molecule, near the active site, where part of the fibrinogen substrate fits). The steeper anophelin dose−inhibition curve when fibrinogen is the substrate when compared to the similar curve for the small synthetic substrate indicates a possible interaction of anophelin with the thrombin anion binding exosite.

The discrepancy between the molecular mass obtained from the native antithrombin (6342.2 Da) and the predicted mass of the native peptide resulting from the albieAT cDNA (6538 Da) can be explained by a posttranslational modification of the protein. It is possible that the first amino acid of the secreted protein is actually glutamine, the third amino acid on the predicted mature peptide. Glutamine can be converted to pyroglutamic acid (*21*), resulting in the observed blocked peptide sequence when we attempted to perform Edman degradation of the native peptide. The predicted mass of this modified peptide would be 6370.55, still 28.35 mass units above the value of 6342.2 found for the native peptide. The 28 mass difference could result from a single formylation of an amino acid residue. However, the resolution for externally calibrated MALDI analysis is on the order of 0.25%, and the 28 mass difference represents 0.4% of the predicted mass. Accordingly, we thus cannot decide whether some other minor modification of the native molecule exists. Synthetic anophelin, however, can be abundantly produced and its mechanism of interaction with thrombin can thus be further studied.

ACKNOWLEDGMENT

We thank Drs. Louis Miller and Helge Zieler for critical review of the manuscript.

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BI990761I