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Isolation and properties of two forms of thrombin inhibitor from the nymphs of the camel tick *Hyalomma dromedarii* (Acari: Ixodidae)

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Abstract. Two forms of the nymphal thrombin inhibitors (NTI) 3.2 kDa and 14.9 kDa were purified by chromatography on CM-cellulose, Sephacryl S-300 and Sephadex G-50 columns and designated NTI-1 and NTI-2 respectively. The NTI-2 turned out to be homogenous monomeric protein in both native-PAGE and denatured SDS-PAGE with M(r) value of 14.9 kDa approximately and its pI value ranged from 7.2 to 7.5. The NTI-1 and NTI-2 displayed anticoagulant activity since they prolonged both the activated partial thromboplastin time (APTT) and the prothrombin time (PT) of the camel plasma in a concentration-dependent manner. The potency of NTI-1 toward thrombin was 5-fold higher than that toward FXa, while NTI-2 was 3-fold active toward FXa than thrombin. However, both of them did not inhibit any of the other examined proteases. The types of inhibition of thrombin by NTI-1 and NTI-2 were non-competitive and competitive with inhibition constants (Ki) values of 11.7 μM and 211 nM respectively. One binding site was deduced on thrombin for each inhibitor.

Introduction

The ticks are second only to mosquitoes in the medical importance as disease vectors and cause annual multibillion losses to the livestock industry (Fuchsberger et al. 1995). The ticks at all three life stages are obligate blood-feeders. They are relatively long-term feeders compared to other hematophagous animals, such as leeches, bats, horse flies or mosquitoes (Zhu et al. 1997a). Female ixodid ticks may feed on a host for 1 – 2 weeks. Each female adult tick may ingest several milliliters of blood (Zhu et al. 1997b).

The hemostasis is a highly regulated system containing three processes: vasoconstriction, platelet aggregation and blood coagulation which represent significant obstacles to hematophagy (Ribeiro et al. 1995). Indeed, vasodilators, anti-aggregating factors (anti-platelet) and anticoagulants (anti-clotting) have been characterized in various blood-sucking parasites.

Vasodilatory substances have been identified as prostaglandin-like molecules in ticks, nitric oxide-binding proteins in triatomine bugs and novel vasoactive peptides in sandflies and mosquitoes (Champagne 1994).

A platelet anti-aggregating factor has been described as an apyrase activity that functions by inhibiting ADP-stimulated platelet aggregation by the degradation of ADP. The apyrase (ATP-diphosphohydrolase), is an enzyme that hydrolyzes ATP and ADP to a AMP and orthophosphate (Garcia et al. 1994). It appears to be conserved among hematophagous species (Ribeiro et al. 1995).

The blood coagulation is an enzymatic cleavage cascade leading to fibrin formation by the intrinsic and extrinsic pathways. The two pathways converge after the activation of factor X. A prothrombinase (factor Xa, factor Va, Ca^{+2} and phospholipids) catalyses the conversion of prothrombin to thrombin which forms fibrin from fibrinogen. Inhibitors of specific steps in the blood clotting cascade (anticoagulants) have been characterized from various hematophagous parasites (Stark and James 1996). Most of the anticoagulants target thrombin or factor Xa (FXa) in the common pathway of the blood coagulation cascade (Markwardt 1994).

Many thrombin inhibitors were purified from the hematophagous parasites such as, the salivary glands of the tsetse fly, *Glossina morsitans morsitans* (Cappello et al. 1996), and the mosquito *Anopheles albimanus* (Valenzuela et al. 1999) and from the whole body extract of the insect *Rhodnius prolixus* (Friedrich et al. 1993), and the indian leech *Haemadipsa sylvestris* (Strube et al. 1993).

The studied tick anticoagulants might inhibit factor Xa, or thrombin, or both, or other blood coagulation factors. Specific FXa inhibitors have been identified from the whole body extract of the soft tick, *Ornithodoros moubata* (Waxman et al. 1990) and *Argas persicus* (Markwardt 1994), and from the salivary glands of the hard tick *Rhipicephalus appendiculatus* (Limo et al. 1991) and *Hyalomma truncatum* (Joubert et al. 1995). Also specific thrombin inhibitors have been isolated from the whole body extract of the hard tick *Ixodes ricinus* (Hoffmann et al. 1991) and from the salivary glands of the lone star tick *Amblyomma americanum* (Zhu et al. 1997a). An inhibitory activity of both factor Xa and thrombin was detected in the crude extract of the salivary glands of the tick *Ornithodoros savignyi* (Gaspar et al. 1995). Later on, Gaspar et al. (1996) purified a small (7 kDa) specific FXa inhibitor where the inhibition of thrombin was minimal in comparison to the inhibition observed for FXa at similar inhibitor: enzyme ratios. A small peptide (720 Da) inhibitor of both thrombin and FXa was purified from the camel tick *H. dromedarii* embryos (Ibrahim et al. 1998, 2000). Anticoagulant activities directed against both factors V and VII were identified in the saliva of the tick *Dermacentor andersoni* (Gordon and Allen 1991).

The anticoagulants are important for successful tick feeding because they have the capacity to inhibit the clotting cascade. The possible role of the tick anticoagulant in induction of the host immune response to tick infestation is very important, since antibodies capable of blocking the antihaemostatic properties of the anticoagulant may oppose the tick feeding and disease transmission.

The abundance of the nymphal anticoagulants is supposed due to the long-term feeding of the nymphs. Thus, this report aims at isolation and characterization of

the thrombin inhibitor(s) from the whole body extract of the camel tick *H. dromedarii* nymphs.

Materials and methods

Tick material

The engorged nymphs of the camel tick *Hyalomma dromedarii* were collected from the ground of the camel market near Cairo, washed several times with saline solution and stored frozen at −40°C.

Chemicals

Sephacryl S-300 and Sephadex G-50 were purchased from Pharmacia Fine Chemicals Co. Ampholytes pH 3.5 – 10, and pH 3 – 7, isoelectric focusing (IEF) standard markers mixture pI 3.6 – 9.3, thrombin (EC 3.4.21.5) from bovine plasma, factor Xa (EC 3.4.21.6) from bovine plasma, N-*p*-tosyl-gly-pro-arg-*p*-nitroanilide acetate salt, trypsin (EC 3.4.21.4) from bovine pancreas type III 2x crystallized, α-chymotrypsin (EC 3.4.21.1) from bovine pancreas type I-S 3x crystallized, papain (EC 3.4.22.2) from *Papaya latex* 2x crystallized, haemoglobin from bovine blood type I 2x crystallized, azocasein, N-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA), α-N-benzoyl-DL-arginine-*p*-nitro-anilide HCl (BAPNA-HCl), blue dextran, alcohol dehydrogenase (EC 1.1.1.1) from yeast, bovine serum albumin (BSA), ferritin and diethylaminoethyl-cellulose (DEAE-cellulose) were purchased from Sigma Chemical Co. Myoglobin from horse heart was products of BDH Chemicals Ltd. N-benzoyl-ile-glu-gly-arg-*p*-nitroanilide HCl, catalase (EC 1.11.1.6) from bovine liver and subtilisin Carlsberg (EC 3.4.21.14) from *Bacillus subtilis* were purchased from Fluka Chemicals. Pepsin (EC 3.4.23.1) from porcine stomach mucosa was a product of CalBiochem. Céphalit kit for activated partial thromboplastin time (APTT) and thromboplastin with calcium kit for prothrombin time (PT) were purchased from bioMérieux. All of the other chemicals were of analytical grade.

Preparation of the camel plasma

The camel plasma was obtained by centrifugation of a mixture of 900 ml of camel blood and 100 ml of 0.11 M trisodium citrate solution at 2700 Xg for 15 minutes at 4°C. If the plasma was not used immediately, it was dispensed into Eppendorf tubes and stored at −40°C (Joubert et al. 1995).

Enzyme and inhibitor assays

Assay of the thrombin activity

The chromogenic substrate assay of thrombin was carried out in a 96-well microtiter plate at 25°C (Gaspar et al. 1995). Thrombin from bovine plasma was dissolved in buffer A [0.05 M Tris-HCl, 0.227 M NaCl, 0.1% BSA, pH 8.3] at a concentration of 0.08 unit / ml. The substrate N-*p*-tosyl-gly-pro-arg-*p*-nitroanilide acetate salt was dissolved in distilled H_2O at a concentration of 1.98 mM.

The assay reaction mixture of thrombin activity contained in 110 μl total volume: 95 μl of buffer A, 0.4 mU of the enzyme thrombin (5 μl) and 0.18 mM final concentration of the substrate (10 μl). The reaction was initiated by the addition of the substrate and the absorbance was recorded by a microplate reader Bio-Rad model 3550 at 405 nm and compared to the control lacking enzyme after incubation for 1 h at 25°C.

Assay of the activated factor X (FXa) activity

The chromogenic substrate assay of FXa was carried out in a 96-well microtiter plate at 25°C (Gaspar et al. 1995). Activated factor X (FXa) from bovine plasma was dissolved in buffer B [0.02 M Tris-HCl, 0.15 M NaCl, 0.1% BSA, pH 7.4] at a concentration of 0.2 unit / ml. The substrate N-benzoyl-ile-glu-gly-arg-*p*-nitroanilide HCl was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mM.

The assay reaction mixture of FXa activity contained in 125 μl total volume: 75 μl of buffer B, 5 mU of the enzyme FXa (25 μl) and 0.2 mM final concentration of the substrate (25 μl). The reaction was initiated by the addition of the substrate and the absorbance was recorded by a microplate reader Bio-Rad model 3550 at 405 nm and compared to the control lacking enzyme after incubation for 1 h at 25°C.

Amidase activity of trypsin, chymotrypsin and papain

The amidase activities of trypsin and chymotrypsin were determined basically according to the method of Erlanger et al. (1961, 1964) respectively. Trypsin or chymotrypsin was dissolved in 1 ml of 0.001 N HCl then diluted with 4 ml of 0.1 M Tris-HCl buffer, pH 8.0 at a concentration of 1 mg / ml. The substrates BAPNA-HCl and BTPNA were dissolved in dimethylsulfoxide at a concentration of 10 mM and 1 mM respectively. The assay reaction mixture contained 880 μl of 0.1 M Tris-HCl buffer, pH 8.0 and 20 μl of the enzyme (20 μg) in a semi-microcuvette 1.5 ml (1 cm light path). The reaction was initiated by the addition of 100 μl of the substrate at a final concentration of 1 mM BAPNA-HCl for trypsin or 0.1 mM BTPNA for chymotrypsin.

The amidase activity of papain was assayed according to the method of Arnon (1970). The papain was dissolved in 1 ml of 0.001 N HCl then diluted with 4 ml of 50 mM sodium phosphate buffer, pH 6.5 containing 5 mM β-mercaptoethanol and 2 mM EDTA at a concentration of 1 mg / ml. The substrate BAPNA-HCl was dissolved in dimethylsulfoxide at a concentration of 10 mM. The assay reaction mixture contained 880 μl of 50 mM sodium phosphate buffer, pH 6.5 containing 5 mM

β-mercaptoethanol and 2 mM EDTA and 20 μl of papain (20 μg). The reaction was initiated by the addition of 100 μl of the substrate at a final concentration of 1 mM BAPNA-HCl.

Endopeptidase activity of subtilisin

The activity of subtilisin was assayed according to the method of Ryle (1970) using 3%(w/v) azocasein as substrate dissolved in 0.1 M Tris-HCl buffer, pH 8.0. The subtilisin was dissolved in 1 ml of 0.001 N HCl then diluted with 4 ml of 0.1 M Tris-HCl buffer, pH 8.0 at a concentration of 1 mg / ml. The assay reaction mixture was carried out in 395 μl of 0.1 M Tris-HCl buffer, pH 8.0 and 5 μl of subtilisin (5 μg) in an Eppendorf tube. The reaction was started by the addition of 100 μl of azocasein (3 mg) and terminated after incubation for 5 min at 37 °C by addition of 0.5 ml of 10 % trichloroacetic acid (TCA). The precipitate was removed by centrifugation at 8000 Xg for 10 min. The colour of the supernatant was developed by the addition of 0.25 ml of 2.5 N NaOH and the absorbance was recorded at 428 nm.

Endopeptidase activity of pepsin

The activity of pepsin was assayed according to the method of Ryle (1970) using 2% (w/v) acid denatured hemoglobin dissolved in 0.06 N HCl. The pepsin was dissolved in 1 ml of 0.001 N HCl then diluted with 4 ml of 0.1 M KCl-HCl buffer, pH 2.0 at a concentration of 1 mg / ml. The assay reaction mixture was carried out in Eppendorf tube and contained in 0.5 ml total volume: 395 μl of 0.1 M KCl-HCl buffer, pH 2.0, 5 μl of pepsin (5 μg) and 100 μl of hemoglobin (2 mg). The reaction was incubated for 5 min at 37°C and terminated by the addition of 0.5 ml of 10% TCA. The precipitate was removed by centrifugation at 8000 Xg for 10 min. The pepsin activity was determined by measuring the absorbance of the TCA soluble peptides at 280 nm.

The inhibitory activity was assayed by measuring the residual activity of any of the above tested proteinase after pre-incubation of the enzyme with a known amount of the inhibitor for 1 min at room temperature before starting the reaction.

One unit of the inhibitory activity was defined as the amount of the inhibitor which inhibits 50% of the enzyme activity under the described conditions. The specific activity is expressed as units / mg protein.

Bio-assay for the inhibition of the intrinsic blood coagulation pathway

The activated partial thromboplastin time (APTT) measures the clotting time of a plasma at 37°C in the presence of a platelet substitute and an activator. This overall test evaluates the entire intrinsic pathway with the exception of the platelet factors (Becker et al. 1984).

Various concentrations of the isolated thrombin inhibitors in a total volume of 50 μl of 0.05 M sodium phosphate buffer pH 7.2 were added to the camel plasma (50 μl) and incubated for 3 minutes at 37°C. Cèphalite APTT reagent from bioMèrieux (50 μl) was added and the mixture was incubated for another 3 min-

utes at 37°C. Finally, 50 μl of 0.025 M $CaCl_2$ (pre-warmed at 37°C) was added and the clotting time recorded.

Bio-assay for the inhibition of the extrinsic blood coagulation pathway

Prothrombin time (PT) studies the total extrinsic clotting system. It measures the clotting time of a plasma at 37°C in the presence of excess tissue thromboplastin and calcium (Becker et al. 1984).

Various concentrations of the isolated thrombin inhibitors in a total volume of 50 μl of 0.05 M sodium phosphate buffer pH 7.2 were incubated with 50 μl of camel plasma for 6 minutes at 37°C. 100 μl of calcium-thromboplastin reagent from bioMèrieux (pre-warmed at 37°C), was added to determine clotting time (Gaspar et al. 1995).

The control time was determined in both bioassays by using 50 μl of 0.05 M sodium phosphate buffer, pH 7.2 instead of the isolated thrombin inhibitors.

Isolation of thrombin inhibitor from the nymphs of the camel tick H. dromedarii

Preparation of acidic crude nymphal extract

Unless otherwise stated all of the procedures were carried out at 4 – 7°C. The acidic crude nymphal extract was prepared by homogenizing 200 mg of the camel tick nymphs in 2 ml of 0.02 M Na-phosphate buffer, pH 6.0 (1:10 w/v). The homogenate was centrifuged at 5000 Xg for 15 min and the supernatant was saved and designated acidic crude nymphal extract.

Chromatography on CM-cellulose column

The acidic crude nymphal extract was applied to the top of a CM-cellulose column (12 × 1.6 cm i.d.) previously equilibrated with 0.02 M Na-phosphate buffer, pH 6.0. The protein fractions were eluted with stepwise NaCl gradient ranging from 0 to 1 M prepared in the equilibration buffer. 3 ml fractions were collected at a flow rate of 60 ml / h. The fractions exhibiting thrombin inhibitory activity were pooled separately and lyophilized.

Chromatography on sephacryl S-300 column

The lyophilized material which obtained from CM-cellulose was dissolved in H_2O and applied to the top of a Sephacryl S-300 column (95 × 1.6 cm i.d.) equilibrated with 0.02 M Tris-HCl buffer, pH 8.0 containing 0.02% NaN_3. The proteins were eluted with the same buffer at a flow rate of 60 ml / h. Fractions of 3 ml were collected.

A Sephacryl S-300 column was used for molecular weight determination of nymphal thrombin inhibitor-2 (NTI-2) of the camel tick *H. dromedarii* according to the method of Andrews (1964). The above described Sephacryl S-300 column was calibrated with blue dextran (2000 kDa), ferritin (440 kDa), catalase (240 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa) and myoglobin (17.2 kDa).

Chromatography on sephadex G-50 column

A Sephadex G-50 column (92 × 1.6 cm i.d.) equilibrated with 0.02 M Tris-HCl buffer, pH 8.0 containing 0.02% NaN_3 was used for molecular weight determination of nymphal thrombin inhibitor-1 (NTI-1) of the camel tick *H. dromedarii* according to the method of Andrews (1964). The above described Sephadex G-50 column was calibrated with blue dextran (2000 kDa), carbonic anhydrase (29 kDa), trypsin (23.3 kDa), myoglobin (17.2 kDa), cytochrome C (12.4 kDa), protamine sulfate (5 kDa) and cyanocobalamine (1.335 kDa).

Electrophoretic analyses

Native gel electrophoresis was carried out with 7% polyacrylamide gel according to Smith (1969). Denatured gel electrophoresis in the presence of SDS and β-mercaptoethanol as reducing agent was performed with 12% polyacrylamide gel according to Laemmli (1970).

The subunit molecular weight of the purified nymphal thrombin inhibitor-2 (NTI-2) was determined by SDS-PAGE as described by Weber and Osborn (1969), using phosphorylase b (94 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa) and lactalbumin (14.2 kDa) as standard markers.

The isoelectric point (pI) of the NTI-2 was determined on native 5% polyacrylamide vertical slab (10 × 10 × 0.1 cm in size). The gel contained two ampholytes 2% pH 3.5–10 and 0.04% pH 3–7. The electrofocusing was performed at 200 volt per two gels for 1.5 h followed by 400 volt per two gels for 1.5 h (Robertson et al. 1987). A calibration curve was constructed by plotting the distance from anode of the marker proteins versus their isoelectric points (Ubuka et al. 1987). The proteins were stained with 0.25% coomassie brilliant blue R-250.

Protein determination

The protein content was determined by the dye-binding protein assay method of Bradford (1976) using bovine serum albumin as a standard protein.

Results

Isolation of the camel tick nymphal thrombin inhibitor

The purification procedure involved chromatography on a CM-cellulose column (Figure 1), gel filtration on a Sephacryl S-300 column (Figures 2A and B). The chromatography of the acidic crude nymphal extract on CM-cellulose column (Figure 1) revealed the presence of two peaks exhibiting thrombin inhibitor activity eluted in the negatively adsorbed fractions and 0.3 M NaCl fractions and designated nymphal thrombin inhibitor-1 (NTI-1) and nymphal thrombin inhibitor-2

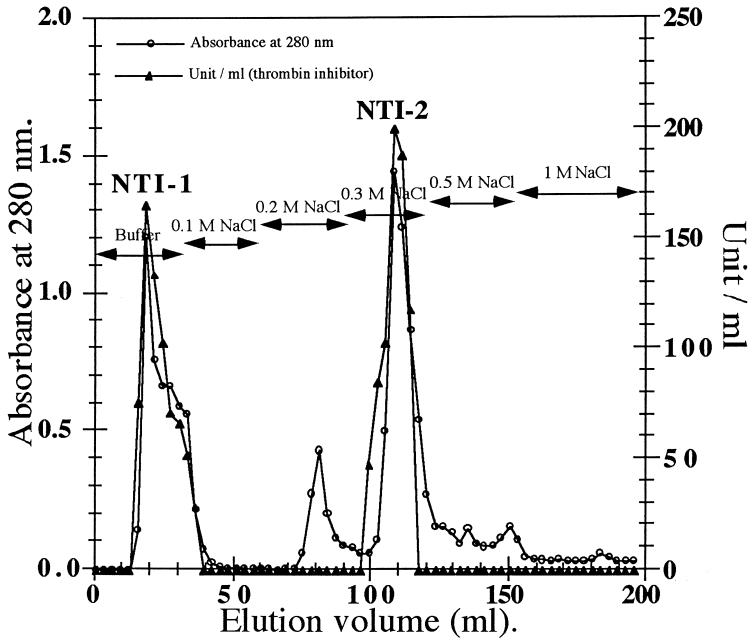

Figure 1. A typical elution profile for the chromatography of the camel tick *H. dromedarii* nymphal extract on CM-cellulose column (12 cm X 1.6 cm i.d.) previously equilibrated with 0.02 M Na-phosphate buffer pH 6.0. The proteins were eluted by a stepwise gradient of NaCl ranging from 0 to 1 M in the equilibration buffer. 3 ml fractions were collected at a flow rate of 60 ml/h.

(NTI-2) respectively. The fractions of the two peaks of the thrombin inhibitors were pooled separately and lyophilized and further purified on the Sephacryl S-300 column (Figures 2A and B). The molecular weights of NTI-1 and NTI-2 were determined by chromatography on Sephadex G-50 (Figure 3) and Sephacryl S-300 columns respectively. The molecular weight for NTI-1 was found to be 3.2 ± 0.06 kDa and that for NTI-2, 14.9 ± 0.4 kDa.

A typical example for the purification scheme of the camel tick nymphal thrombin inhibitors is given in Table 1.

Electrophoretic analyses of the nymphal thrombin inhibitor-2 (NTI-2)

Samples from the different purification steps were analyzed electrophoretically on native 7% polyacrylamide gel (Figure 4). The NTI-2 turned out to be homogenous.

The denatured 12% SDS-PAGE indicated that the NTI-2 is a single polypeptide chain of 14.9 kDa (Figure 5).

The isoelectric point (pI) of the NTI-2 was estimated at pH 7.2 – 7.5 (Figure 6).

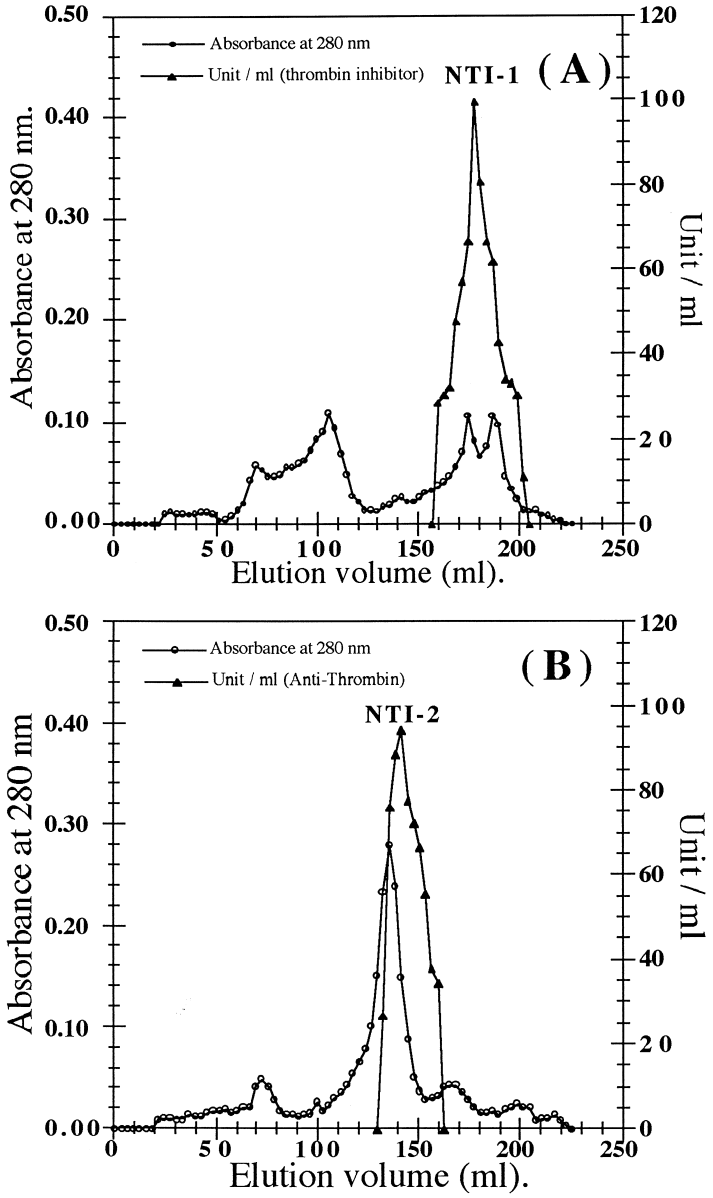

Figure 2. Typical elution profiles for the chromatography of the concentrated CM-cellulose fractions (A) NTI-1 and (B) NTI-2 on Sephacryl S-300 column (95 cm X 1.6 cm i.d.) previously equilibrated with 0.02 M Tris-HCl buffer pH 8.0 containing 0.02% NaN_3. 3-ml fractions were collected at a flow rate of 60 ml / h.

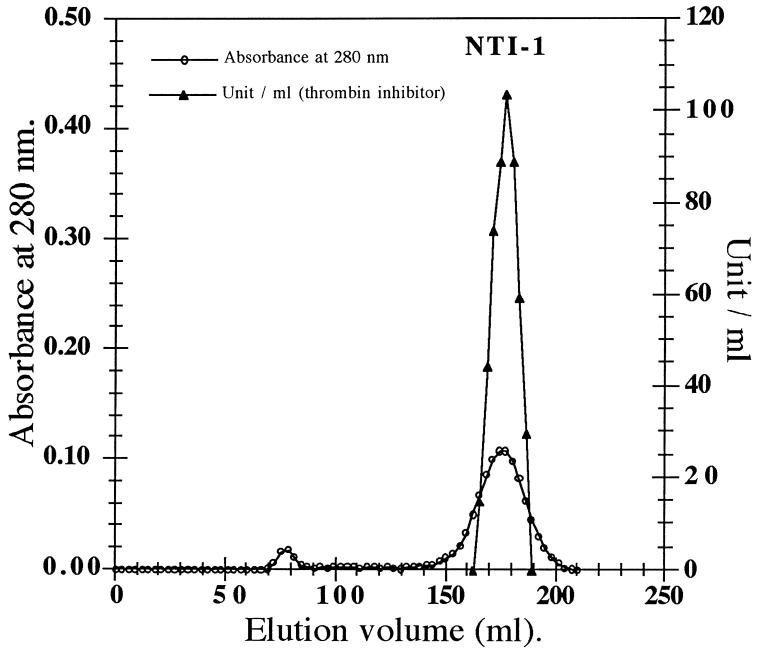

Figure 3. A typical elution profile for the chromatography of the concentrated pooled Sephacryl S-300 fractions of (NTI-1) on Sephadex G-50 column (92 cm X 1.6 cm i.d.) previously equilibrated with 0.02 M Tris-HCl buffer pH 8.0 containing 0.02% NaN_3. 3-ml fractions were collected at a flow rate of 60 ml/h.

Titration of the amidolytic activity of FXa and thrombin with the nymphal thrombin inhibitors (NTI-1 and NTI-2)

The inhibitory effect of thrombin inhibitors (NTI-1 and NTI-2) on the amidolytic activity of both FXa and thrombin are shown in Figures 7A and B. 65 % inhibition of thrombin by NTI-1 was observed at 7.0 μM inhibitor concentration that was found to inhibit 13 % only of the FXa activity. 58 % inhibition of thrombin by NTI-2 was observed at 24 μM inhibitor concentration, while 100 % of the FXa activity was inhibited at 13 μM inhibitor concentration.

Kinetic parameters of thrombin inhibition by the nymphal thrombin inhibitors (NTI-1 and NTI-2)

According to Hill equation (Segel 1993), (log Vi/Vmax-Vi = n log I-log k), when log Vi/Vmax-Vi values are plotted against log of the inhibitor concentrations, a straight line at intermediate inhibitor concentration is obtained with a slope of -n (where Vmax is the enzyme activity in absence of inhibitor and Vi is enzyme activity in presence of inhibitor). The apparent n value determined from the Hill plot approaches the number of inhibitor binding sites. When log Vi/Vmax-Vi values

Table 1. Purification scheme of the nymphal thrombin inhibitors of the camel tick *H. dromedarii*

Purification step	Total protein[1] (mg)	Total units[2]	Specific activity[3]	Recovery %	Fold purification
Crude nymphal extract	37.0	5283	142.8	100.0	1.00
CM-cellulose column:					
Unadsorbed fraction (NTI-1)	10.0	2357	235.7	44.6	1.7
0.3 M NaCl fraction (NTI-2)	14.0	2523	180.0	47.8	1.3
Sephacryl S-300 column:					
(NTI-1)	3.0	2095	698.0	39.7	4.9
(NTI-2)	5.8	2072	357.0	39.2	2.5
Sephadex G-50 column:					
(NTI-1)	2.2	1997	907.8	37.8	6.4

[1] The total protein extracted from 200 mg of nymphs. [2] One unit of the inhibitor activity is defined as the amount of the inhibitor which inhibits 50% of the enzyme activity under the described conditions. [3] The specific activity is expressed as units/mg protein.

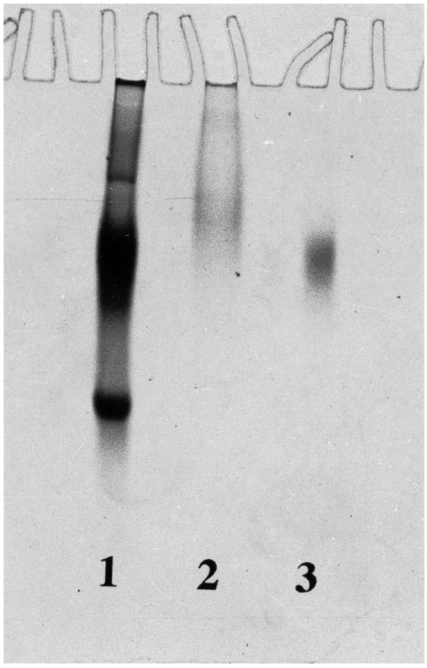

Figure 4. The electrophoretic pattern of the purification steps of the nymphal thrombin inhibitor-2 (NTI-2) on native 7% polyacrylamide gel; (1) Acidic crude nymphal extract, (2) CM-cellulose fraction, (3) Sephacryl S-300 fraction.

were plotted against log [I] of the NTI-1 and NTI-2 (Figures 8A and B), straight lines were obtained with slopes of 1.46 and 0.8 respectively, indicating the presence of one binding site for each inhibitor molecule.

Lineweaver-Burk plot (Figures 9A and B) at three different inhibitor concentrations indicated that the types of inhibition of thrombin by the NTI-1 and NTI-2 are non-competitive and competitive respectively.

The slopes of the lineweaver-Burk plots were plotted against the inhibitor concentrations, and the Ki values were determined directly from the intercept of the X axis to be 11.7 μM and 211 nM for both NTI-1 and NTI-2 (Figures 10A and B) respectively.

Effect of the nymphal thrombin inhibitors (NTI-1 and NTI-2) on various proteases

The effect of the nymphal thrombin inhibitors NTI-1 (2.5 μg) and NTI-2 (20 μg) on various proteases was tested by measuring the inhibition of their activity (Table 2). 2.5 μg / assay of the nymphal thrombin inhibitor-1 (NTI-1) was able to inhibit 65 % of the thrombin activity while 13 % only of the FXa activity was inhibited. 20 μg / assay of the nymphal thrombin inhibitor-2 (NTI-2) which inhibited 34 % of the thrombin activity was able to inhibit the activity of FXa completely

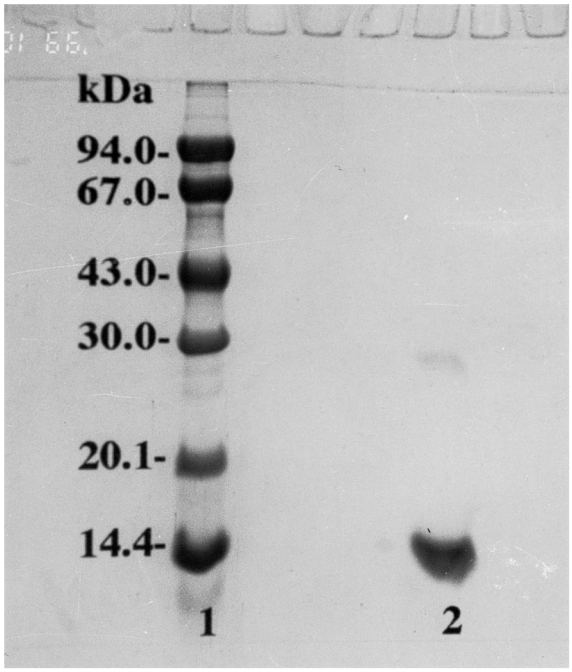

Figure 5. Denatured 12% SDS-polyacrylamide gel electrophoresis for subunit molecular weight determination of the purified nymphal thrombin inhibitor-2 (NTI-2), (1) standard molecular weight markers: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa), (2) Concentrated pooled Sephacryl S-300 fractions of the (NTI-2).

(100 %) and the two inhibitors failed to inhibit significantly the activity of the other proteases.

Effect of the thrombin inhibitors concentration on the intrinsic and extrinsic coagulation pathways

In order to determine the anticoagulation potency of the nymphal thrombin inhibitors NTI-1 and NTI-2, increasing concentrations of their purified forms were assayed for inhibition of the intrinsic (APTT) and extrinsic (PT) coagulation pathways of the camel plasma. Both the APTT and PT were prolonged in a concentration dependent manner. The APTT and PT coagulation pathways were inhibited completely by 3.0 and 12 μg of NTI-1 and NTI-2 (Figures 11A and B) equivalent to 4.56 and 4.02 μM respectively.

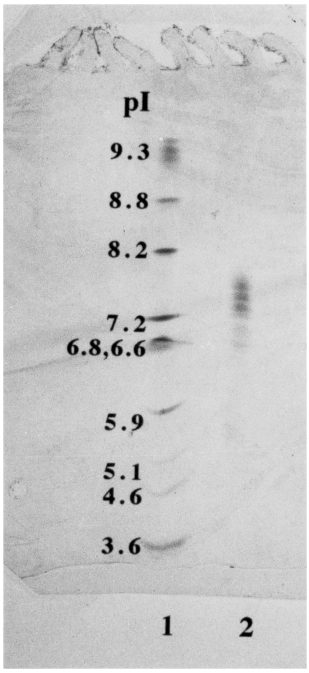

Figure 6. Isoelectric focusing (IEF) on 5 % polyacrylamide gel.

1. pI markers: Trypsinogen (9.3), Lectin (8.8), Lectin (8.2), Myoglobin (7.2), Myoglobin (6.8), Carbonic anhydrase I (6.6), Carbonic anhydrase II (5.9), β-Lactoglobulin A (5.1), Trypsin inhibitor (4.6) and Amyloglucosidase (3.6).
2. Nymphal thrombin inhibitor-2 (NTI-2).

Discussion

The purification of the thrombin inhibitor americanin (16 kDa) from the lone star tick *Amblyomma americanum* was achieved by reversed-phase chromatography followed by anion-exchange chromatography (Zhu et al. 1997a). The purification of both rhodniin, a specific thrombin inhibitor (11 kDa) from *Rhodnius prolixus* (Friedrich et al. 1993) and haemadin (5 kDa) from the indian leech *Haemadipsa sylvestris* (Strube et al. 1993) involved anion-exchange chromatography on Q-Sepharose column followed by affinity chromatography on thrombin-Sepharose column. The haemadin was further purified on reversed-phase HPLC. The purification of tsetse thrombin inhibitor (TTI) (3530 Da), was performed by a combination of size-exclusion chromatography on Superdex HR 10/30 column and reverse-phase, high-performance liquid chromatography (Cappello et al. 1996). Recently, an anti-thrombin (anophelin) with molecular mass of 6342.4 Da was isolated from the salivary glands of the mosquito *Anopheles albimanus* through molecular siev-

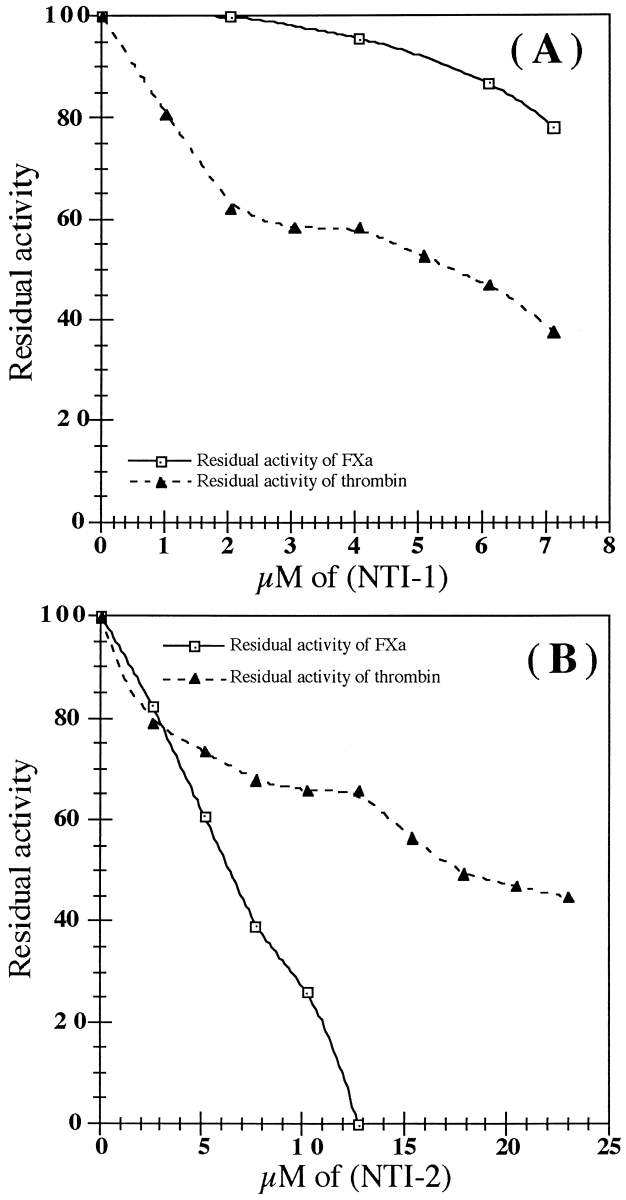

Figure 7. Inhibition of the amidolytic activity of thrombin and FXa by varying concentrations of (A) NTI-1 and (B) NTI-2.

ing and reverse-phase high performance liquid chromatography (Valenzuela et al. 1999).

The present purification procedure of the nymphal thrombin inhibitors is a rapid method consisting of two steps cation-exchange chromatography on CM-cellulose

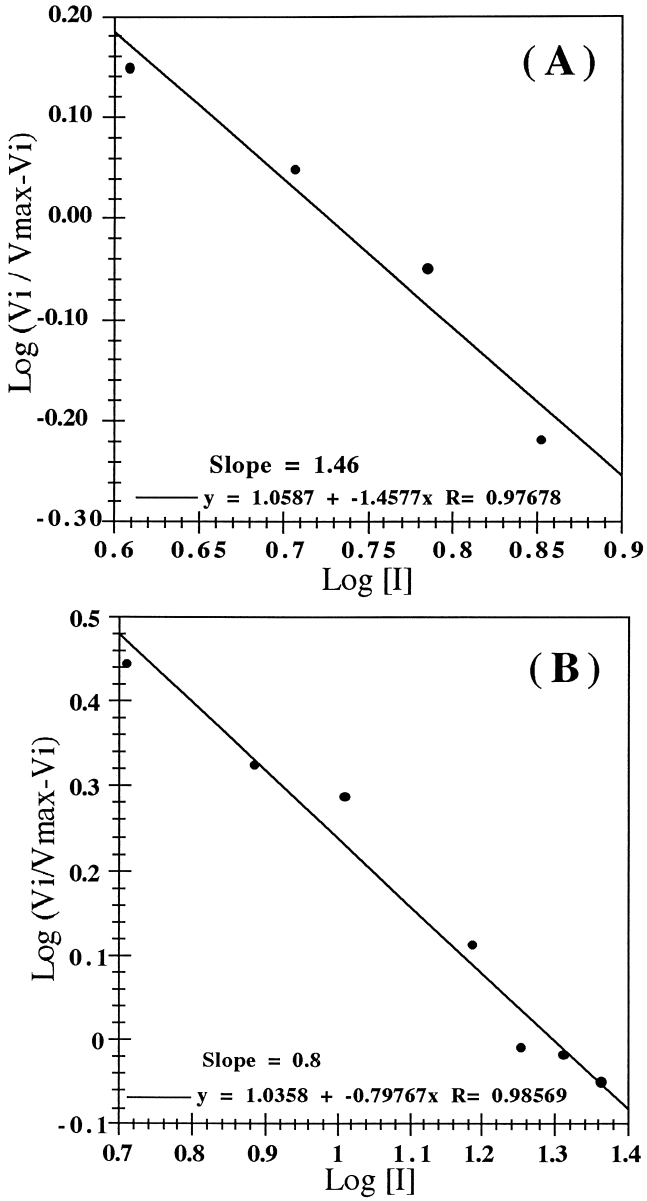

Figure 8. Hill plot for inhibition of the amidolytic activity of thrombin by varying concentrations of (A) NTI-1 and (B) NTI-2.

column (Figure 1) and gel filtration on Sephacryl S-300 column (Figures 2A and B). The chromatography of the acidic crude nymphal extract on CM-cellulose column at pH 6.0 revealed the presence of two types of the nymphal thrombin inhibitors (NTI-1 and NTI-2). NTI-1 did not bind to the CM-cellulose column matrix

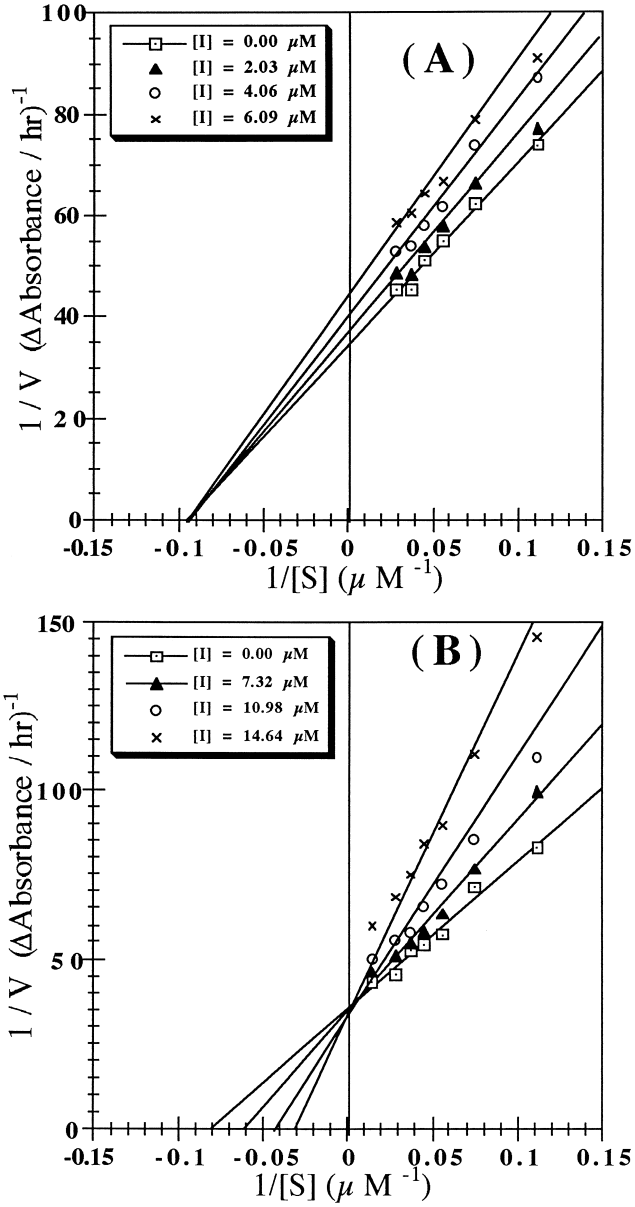

Figure 9. Lineweaver-Burk plots showing the type of inhibition of thrombin by (A) NTI-1 and (B) NTI-2. The amidolytic activity of constant amount of thrombin was measured with varying concentrations of the substrate in absence and presence of three various concentrations of the inhibitor.

while NTI-2 was eluted with 0.3 M NaCl proving that at pH 6.0, NTI-1 is either negatively or slightly positive charged and NTI-2 is highly positively charged. The purification method is very convenient due to the high yield 37.09 % and 39.21 %

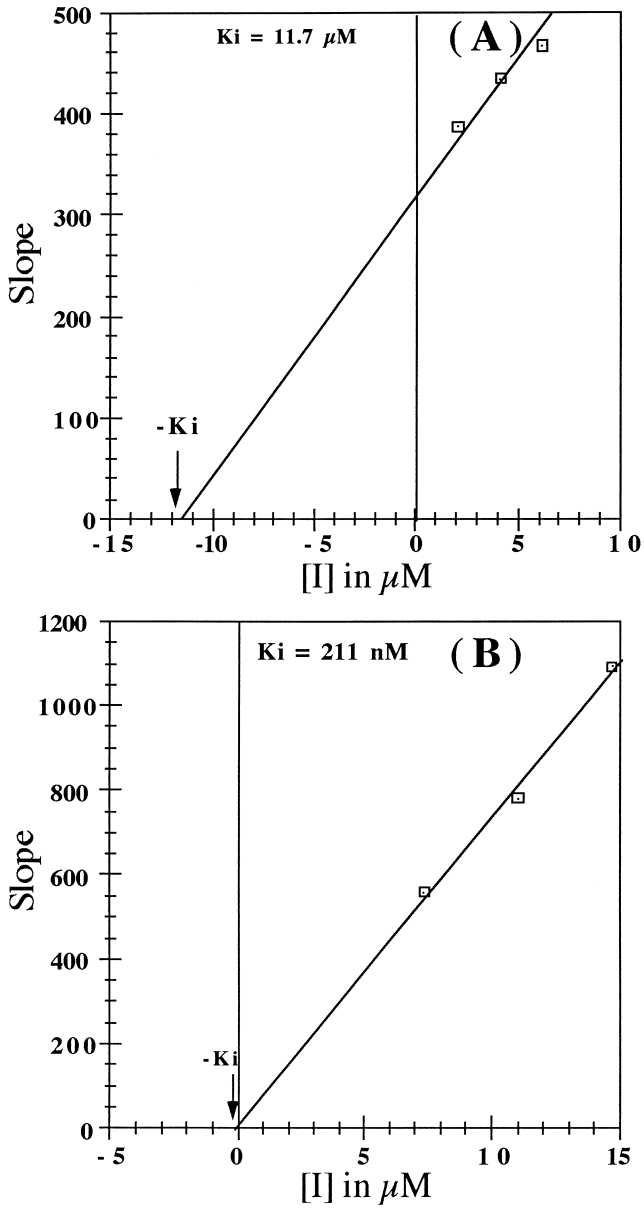

Figure 10. Determination of the inhibition constant (Ki) value for inhibition of thrombin activity by (A) NTI-1 and (B) NTI-2. The plotted slope values were determined from the lines of the reciprocal plots of the different inhibitor concentrations.

for NTI-1 and NTI-2 respectively (Table 1). The molecular weights of the native NTI-1 and NTI-2 were determined by chromatography on Sephadex G-50 column

Table 2. Effect of the nymphal thrombin inhibitors (NTI-1 and NTI-2) on different proteases.

Protease	Inhibition % by thrombin inhibitor-1 (NTI-1) (2.5 μg)	Inhibition % by thrombin inhibitor-2 (NTI-2) (20 μg)
Thrombin	65.0	34.0
Factor Xa	13.0	100.0
Trypsin	1.9	0.0
Chymotrypsin	3.6	2.3
Pepsin	3.1	3.1
Subtilisin	0.0	0.0
Papain	0.8	1.3

(Figure 3) and Sephacryl S-300 column (Figures 2B) to be 3.2 ± 0.06 and 14.9 ± 0.4 kDa respectively.

The preparation of the nymphal thrombin inhibitor-2 (NTI-2) seems to be homogenous as indicated by both native-PAGE (Figure 4). The minor protein band ($\sim$ 30 kDa) appeared in the SDS-PAGE (Figure 5), is about the double of the size of the major band representing a dimer of the NTI-2. This very small amount of the protein dimer could be formed due to the large amount of the applied protein and its incomplete reduction during electrophoresis. The consistency between the molecular weight of the NTI-2 under the reducing conditions (SDS-PAGE) and that of the native protein by gel filtration indicates that the protein is a single polypeptide chain.

Repeated attempts to visualize the purified NTI-1 ($\sim$ 3 kDa) by SDS-PAGE were unsuccessful, despite using the protocol of the Tricine-SDS-PAGE described by Schägger and Von Jagow (1987) for electrophoresis of low molecular weight proteins with coomassie and silver staining. The low molecular weight of NTI-1 may lower its affinity toward the stains and explain the difficulty of its visualization. Similar to the present situation of NTI-1, Cappello et al. (1996) also did not succeed to visualize electrophoretically the tsetse thrombin inhibitor (TTI) (3530 Da).

In the present investigation, the nymphal thrombin inhibitor-2 (NTI-2) has a pI value in the range of 7.2 – 7.5 (Figure 6) different from that of the nymphal factor Xa (FXa) inhibitor (7.7–7.9) (Khalil 2000). This pI value explained the behaviour of NTI-2 on CM-cellulose column, where NTI-2 at pH 6.0 is positively charged and tightly bound to the CM-cellulose matrix and eluted with 0.3 M NaCl (Figure 1). The resolution of the protein into three major bands by isoelectrofocusing indicates the microheterogeneity that may arise from the different degrees of the side chain modifications of the protein.

The isoelectric point pI of the thrombin inhibitor rhodniin (11 kDa) is 4.2 ± 0.5 (Friedrich et al. 1993) lower than that of the NTI-2 (7.2–7.5) of the camel tick *H. dromedarii*.

In the present investigation, the titration curves of NTI-1 and NTI-2 toward the amidolytic activity of thrombin (Figures 7A and B) confirmed the inhibition of

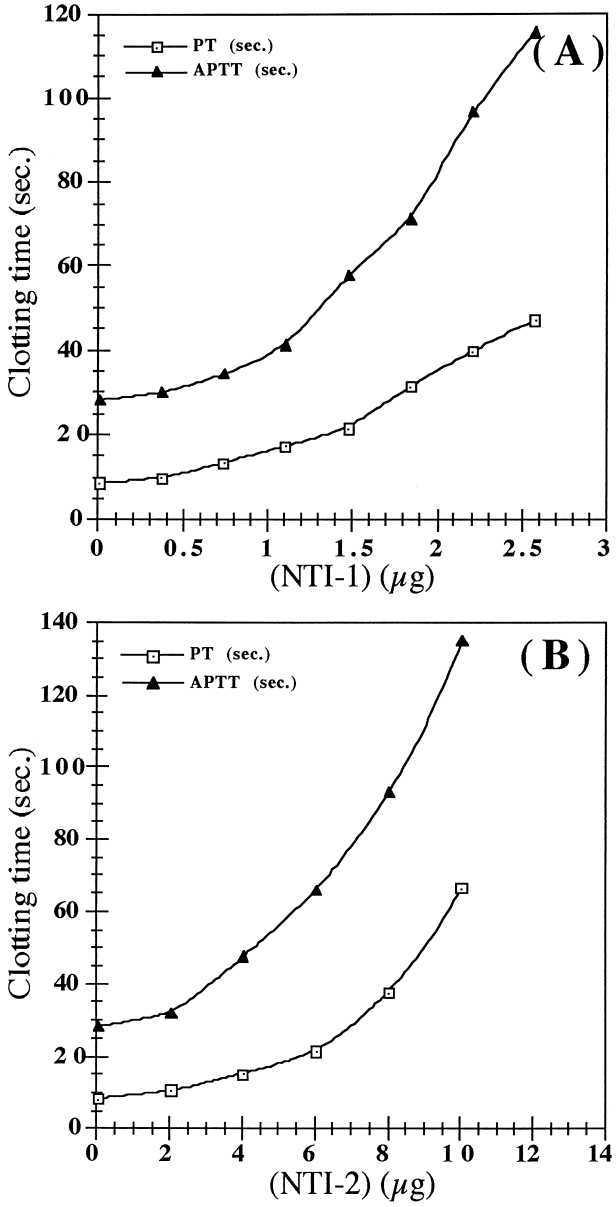

Figure 11. Prolongation of the activated partial thromboplastin time (APTT) and the prothrombin time (PT) of the camel plasma in the presence of various concentrations of the purified (A) NTI-1 and (B) NTI-2 of the camel tick *H. dromedarii*. The control is the clotting time of the camel plasma in absence of the inhibitor.

thrombin and revealed that the 65 % and 58 % inhibition were reached with 7 μM and 24 μM respectively. From the titration curve data, a linear relationships were

observed by constructing the Hill plots for the inhibition of thrombin by NTI-1 and NTI-2 (Figures 8A and B). One binding site is expected for each of the NTI-1 and NTI-2 molecules on thrombin since the slopes of their Hill plots were found to be 1.46 and 0.8 respectively. The presence of the inhibitor NTI-1 did not alter the Km value and decreased the Vmax value (Figures 9A) indicating that the type of inhibition of thrombin by NTI-1 is a non-competitive type. In contrast, the presence of the inhibitor NTI-2 increased the Km value and did not shift the Vmax value (Figures 9B) proving that the type of inhibition of thrombin by NTI-2 is a competitive type. The Ki values were calculated graphically and found to be 11.7 μM and 211 nM respectively (Figures 10A and B). The low Ki value of NTI-2 indicates the higher affinity of this inhibitor toward thrombin than NTI-1. Taking in consideration that NTI-2 is a competitive inhibitor of thrombin. In such type of inhibition, the amounts of inhibition observed will depend on the relative amounts of inhibitor and substrate present. At a fixed concentration of inhibitor, it will be possible to drive all the inhibitor from the enzyme converting it to enzyme substrate complex. Therefore, NTI-2 had the higher affinity for thrombin and showed less inhibition than NTI-1.

The Ki values of thrombin inhibitors from other sources were very low in comparison with those of the camel tick nymphs. The inhibition of thrombin by americanin from the lone star tick *Amblyomma americanum* was a competitive type with an inhibition constant (Ki) value of 0.073 nM (Zhu et al. 1997a). The natural and recombinant rhodniin from the insect *Rhodnius prolixus* both displayed inhibition constants of about 2 X 10^{-13} M (Friedrich et al. 1993). The Ki values have been reported to be 0.02 pM for natural hirudin variant-1 (HV1) from the leech *Hirudo medicinalis* (Braun et al. 1988) and approximately ten times higher for the recombinant desulphated product (Märki and Wallis 1990).

In this study, the two nymphal thrombin inhibitors NTI-1 and NTI-2 inhibited both thrombin and FXa but not other serine proteases (trypsin and chymotrypsin), a cysteine protease (papain), an acid protease (pepsin) or bacterial protease (subtilisin) (Table 2). The NTI-1 was more efficient in inhibiting thrombin than FXa (5-fold higher) while the NTI-2 was more active toward FXa than thrombin (3-fold higher). Since FXa converts the prothrombin to thrombin, the NTI-2 could be a double headed inhibitor mainly toward FXa and subsidiary toward thrombin if any prothrombin is converted to thrombin by FXa. In agreement with the present enzyme specificity, the reported thrombin inhibitors did not exert any inhibitory activity toward other proteases (Talbot 1989; Strube et al. 1993; Cappello et al. 1996; Zhu et al. 1997a).

Both the nymphal thrombin inhibitors NTI-1 and NTI-2 prolonged PT and APTT of the camel plasma in a concentration dependent manner (Figures 11A and B). Complete inhibition of both intrinsic and extrinsic pathways of blood coagulation system was observed by 4.56 and 4.02 μM of NTI-1 and NTI-2 respectively. Similarly, the thrombin inhibitor from tsetse fly (TTI) caused a concentration dependent prolongation of the APTT of normal human plasma (Cappello et al. 1996).

The mammalian plasma contain antithrombin activity with molecular weight of 70 kDa for human (Wunderwald et al. 1982), 58 kDa for porcine, 63 kDa for rabbit

(Koide 1979) and 64 kDa for rat (Takahara and Sinohara 1980). Thus it is obvious that, the mass of the antithrombin molecules present in the mammalian plasma are relatively higher than that of the nymphal thrombin inhibitors of the camel tick *H. dromedarii* [NTI-1 (3 kDa) and NTI-2 (15 kDa)]. Therefore, on the basis of the molecular weight, it could be excluded that the nymphal thrombin inhibitors of the camel tick *H. dromedarii* are originating from the camel plasma of the blood meal.

In conclusion, these two forms of thrombin inhibitors have the capacity to inhibit the blood clotting cascade and may be essential for the survival of the tick. Therefore, raising antibodies toward the purified thrombin inhibitors in the host may block their antihaemostatic properties and interfere with the proper tick feeding.

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