Isolation and characterization of an anticoagulant present in the salivary glands of the bont-legged tick, *Hyalomma truncatum*

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ABSTRACT

A low molecular mass anticoagulant (17 kDa) was isolated from the salivary glands of prefed female *Hyalomma truncatum* ticks by means of reverse phase and anion-exchange HPLC. Trypsin digestion and amino acid analysis confirmed the protein nature of the anticoagulant. The inhibitor appears to be uncompetitive with a K_i of 6.9×10^{-10}M. The target of the anticoagulant is factor Xa at the junction of the extrinsic and intrinsic pathways. This may be crucial for the survival of the tick, making it feasible to investigate the possibility of vaccination with this antihaemostatic against tick feeding. In addition, tick anticoagulants may possibly have therapeutic application in controlling thrombosis.

Key words: Haematophageous, tick, *Hyalomma truncatum*, anticoagulant, factor Xa.

INTRODUCTION

Haematophagous ectoparasites obtain nourishment by feeding on hosts and have to maintain the flow of blood during this process. Saliva of blood-sucking insects, leeches, bats and ticks contain anticoagulants that maintain the blood in a fluid state in their guts (Waxman and Connolly, 1993). It is assumed that the role of anticoagulants and other antihaemostatic factors in tick saliva is to inhibit extravascular coagulation arising from procoagulants released during feeding and tissue damage at the site of a tick bite.

Blood coagulation comprises a series of proteolytic reactions which terminate in the thrombin-catalysed conversion of soluble fibrinogen to an insoluble fibrin clot. Activation of the coagulation cascade through either its intrinsic or extrinsic pathways results in the formation of factor Xa, which subsequently catalyses the formation of thrombin (Neeper *et al.*, 1990).

Bringing blood into contact with negatively charged surfaces such as glass or kaolin, initiates clotting via the intrinsic pathway (Margolis, 1958). *In vivo*, collagen and platelet membranes have the same effect. The contact system of the intrinsic pathway consists of four glycoproteins: Hageman factor (XII), prekallikrein, plasma thromboplastin antecedent (XI) and high molecular mass kininogen (HMK). In damaged tissues, the proteins kininogen and kallikrein activate factor XII, which in turn activates factor XI and the cascade of reactions proceeds. This is called the intrinsic pathway, because all of its protein components are contained in the blood. Alternatively, damage to blood vessels leads to the release of tissue factor and activation of factor VII, starting the extrinsic pathway, so-called because one of its important components occurs in the tisssues (Voet and Voet, 1990). The two pathways merge in the activation of factor X, which will proteolyse and thereby activate prothrombin (Mathews and van Holde, 1990).

According to Limo *et al.* (1991), the possible role of tick anticoagulants in induction of host immune response to tick infestation should be evaluated, since antibodies capable of blocking the antihemostatic properties of the saliva anticoagulant, would interfere with tick feeding and disease transmission.

The presence of anticoagulants in whole tick extracts was first demonstrated in 1898 by Sabbatini and has since been confirmed in a variety of tick species. Waxman *et al.* (1990) reported the purification of a low molecular mass (6.850 kDa) serine protease inhibitor (TAP) from extracts of the soft tick *Ornithodoros moubata*. The 60 amino acid single-chain acidic peptide is a slow tight-binding inhibitor, specific for factor Xa of the blood clotting cascade, with a pI of 4.5. The potency and specificity of TAP in the inhibition of factor Xa, suggests that it may be effective in the treatment or prophylaxis of thrombosis. Limo *et al.* (1991) purified and characterized an inhibitor of factor Xa from the ixodid tick, *Rhipicephalus appendiculatus*. The anticoagulant has a relative molecular weight of 65 kDa and a pI between 8.0 and 8.5. Gordon and Allen (1991) reported anticoagulant activities in the saliva of *Dermacentor andersoni* directed against both coagulation factors V and VII. These activities attained their highest levels when ticks were fed to a weight of approximately 250 mg.

This paper describes the isolation and characterization of an anticoagulant present in the salivary glands of the bont-legged tick, *H. truncatum*. The purified anticoagulant could be useful for controlling tick infestation and furthermore, it may be useful on a therapeutic level in patients suffering from thrombosis.

MATERIALS AND METHODS

Dissection, storage and preparation of salivary gland extracts

Salivary glands were dissected from unfed and prefed female *H. truncatum* ticks. The latter were fed in the presence of males on sheep for 5–7 days at which time they reached a body mass range of 15–40 mg. Dissection was performed under a

binocular stereomicroscope and the material suspended in physiological saline (0.9% NaCl). The glands were frozen in liquid nitrogen immediately after dissection and then stored at –70°C. Salivary glands were sonicated by means of a Branson sonifier B-30 (Branson Sonic Power Company) for 10 pulses in the buffer appropriate for the given experiment. The samples were centrifuged in an Eppendorf microcentrifuge at 8000*g* for 10 minutes and the supernatants used for determination of protein concentration and inhibition of the intrinsic and extrinsic pathway as well as factor Xa.

Determination of protein concentration
The protein content of salivary glands was determined by Coomassie blue staining (Bradford, 1976). Bovine serum albumin (BSA) was used as standard.

Bio-assay for the inhibition of the intrinsic blood coagulation pathway
Determination of the intrinsic coagulation time was conducted using the Coagulen activated partial thromboplastin time (APTT) kit which is a two-component reagent kit composed of lyophilized partial thromboplastin and a buffer containing optimal concentrations kaolin, stabilizers and preservatives. After recalcification of plasma and in the presence of a contact activator (kaolin) and partial thromboplastin, a solid fibrin clot forms in a specific time.
Salivary gland extracts were prepared as described above using PBS (phosphate buffered saline), pH 7.0 (0.135 M NaCl, 2.5 mM KCl, 1.5 mM KH_2PO_4, 7.5 mM Na_2HPO_4). Plasma was obtained by centrifugation of a mixture of 3.6 ml human blood and 0.4 ml of an 0.11M trisodiumcitrate solution at 2700*g* for 10 minutes. Crude salivary gland extracts (50 µl) were added to the plasma (50 µl) and incubated for 3 minutes at 37°C. Coagulen APTT (50 µl) (Lennon Diagnostics) was added and the mixture was incubated for another 3 minutes at 37°C. Finally, 0.05M $CaCl_2$ (50 µl, pre-warmed at 37°C) was added and the clotting time recorded. For determination of the control time, the experiment was performed by using PBS (50 µl) instead of crude salivary gland extracts.

Bio-assay for the inhibition of the extrinsic blood coagulation pathway
The extrinsic pathway is initiated by the proteolysis of proconvertin (factor VII), a process that can be catalysed by activated Hageman factor (factor XIIa) as well as thrombin. Activated proconvertin mediates the activation of factor X and its rate is enhanced by the presence of phospholipid membrane, Ca^{2+} and tissue factor/thromboplastin (factor III). Determination of the extrinsic coagulation time was done using the Coagulen PT kit which composed of tissue thromboplastin and a buffer containing optimal concentrations of Ca^{2+} ions, stabilizers and preservatives. The clotting time for this test is normally shorter than that for the intrinsic clotting pathway probably because of the more reactions involved in the latter cascade.

Crude salivary gland extracts of 10 salivary glands of prefed *H. truncatum* female ticks, were prepared as described above and used for determination of inhibition of the extrinsic pathway of blood coagulation. Plasma (50 μl) was incubated with 50 μl of PBS or crude salivary gland extract supernatant for 6 minutes at 37°C. Coagulen PT (100 μl, pre-warmed at 37°C) was added and clotting time was determined.

Bio-assay for inhibition of factor Xa using plasma deficient factor Xa
Salivary gland extracts were prepared as described above in PBS. Normal plasma (50 μl) was added to the factor X deficient plasma (50 ml) and salivary gland extract or PBS (50 μl). The plasma was obtained from a donor congenitally deficient in factor X (Lennon Diagnostics). The mixture was incubated for 6 minutes at 37°C. PT (100 μl, pre-warmed at 37°C), was added to determine clotting time.

Bio-assay for inhibition of factor Xa using a synthetic substrate
Salivary gland extracts of prefed *H. truncatum* female ticks were prepared as described above in 20 mM Tris-HCl, 0.15M NaCl, 0.1% BSA buffer, pH 7.4. The enzyme, factor Xa (Boehringer Mannheim) was diluted in 20 mM Tris-HCl, 0.15M NaCl, 0.1% BSA buffer, pH 7.4 to 0.2 U ml^{-1}. The substrate, Chromozym X (N-methoxycarbonyl-D-norleucyl-glycyl-arginine-4-nitranilide acetate, Boehringer Mannheim) was solubilized in sterile water to a concentration of 1 μmol ml^{-1}. A dilution series of salivary glands (50 μl) was prepared and added to 20 mM Tris-HCl, 0.15M NaCl, 0.1%BSA buffer, pH 7.4 (100 μl) and factor Xa (50 μl). The mixture was incubated at room temperature whereafter 50 μl substrate was added and the reaction rate monitored over 10 minutes at 405 nm.

Reverse phase chromatography
A C_{18} column (Bondclone 300 × 3.9 mm, Phenomenex) was equilibrated with buffer B (60% acetonitrile in 0.1% TFA) and then with buffer A (0.1% TFA and 0.1% acetonitrile). Extracts of forty salivary glands of female ticks (prefed for 4–5 days) were prepared as described above in 100 μl of buffer A. The supernatant was filtered through a 0.22 μm membrane (Millipore) and applied onto the HPLC column.

A gradient of TFA/acetonitrile (0–60%) was used over 110 minutes. The flow rate was 1 $mlmin^{-1}$. and the absorbance monitored at 230 nm. Fractions of 3 ml each were collected. The samples were dried in a speedy-vac (Bachofer, vacuum concentrator) and assayed for inhibition of factor Xa using a synthetic substrate as described above. A blank separation, performed without injection of sample, was also assayed to serve as control.

Anion-exchange chromatography
Fractions collected from the HPLC reverse phase column which showed anticoagulant activity were resuspended in 100 μl of 20mM Tris-HCl, pH 8.0. The

samples were filtered through a 0.22 μm membrane (Millipore) and applied onto the HPLC anion-exchange column (TSK-GEL, DEAE 5PW, 7.5 cm × 7.5 mm, TosoHaas) which had been equilibrated with 20 mM Tris-HCl, 0.5 M NaCl, pH 8.0 and then with 20 mM Tris-HCl, pH 8.0. A gradient of NaCl was applied over 10 minutes. The flow rate was 1 $mlmin^{-1}$ and the absorbance monitored at 280 nm. Fractions (1 ml) were collected and assayed for factor Xa inhibition. A blank run was also assayed to serve as control. The samples which tested positive for factor Xa inhibition were desalted using Sephadex G-25 PD10 columns (Pharmacia) and lyophilized.

Capillary zone electrophoresis

Buffer A (Beckman Instruments) containing Tris, boric acid and EDTA, pH 8.3 was used for separation on the Beckman P/ACE System 2000 instrument. Silica capillary columns (50 cm × 50 μm, Beckman Instruments) were used and absorbances were monitored at 214 nm. Separations were completed within 14 minutes with a current of 36.5 mA and a voltage of 30kV and analyses was done using Beckman System Gold software.

Capillary gel electrophoresis

SDS Gelbuffer 200 (Beckman Instruments) was used as buffer for the separation on the Beckman P/ACE System 2000. The desalted, lyophilized anticoagulant fraction was resuspended in 20 μl of 20 mM Tris-HCl, pH 7.0. Sample buffer (20 μl) containing 0.12M Tris-HCl, 1% SDS, pH 6.6, an internal standard containing 0.1% Orange G dye (5 μl) and 5 μl mercaptoethanol were added to the sample. The sample was heated at 100°C for 5 minutes. Blank fractions collected from IEC were treated in the same way to serve as controls. An U100P gel column (65 cm × 100 mm, Beckman Instruments) was used and separations were completed within 30 minutes at 14.1 kV and a current of 25–30 mA. Analyses was done using Beckman System Gold chromatography software.

Amino acid analysis

A sample of the purified anticoagulant was resuspended in 20 mM Tris-HCl, pH 7.0 (100 μl) and subjected to amino acid analysis (PICO.TAG Immobilon Tech, 1989). Blank fractions obtained from a separation performed without a sample injection into the PICO.TAG column (3.9 mm × 15 cm, Microcep) were treated in the same way to serve as controls.

Determination of K'_m *and* V'_{max} *of factor Xa in the presence of the inhibitor*

A Chromozym X stock solution of 71 μM in 20mM Tris-HCl, 0.15M NaCl, 0.1% BSA, pH 7.4 was prepared. Substrate concentrations (50 μl aliquots) used were from 0-to 71 μM. Factor Xa was diluted to 9×10^{-5} U/50 μl. The anticoagulant was resuspended in the latter buffer to a concentration 0.27 pmol/well. After

incubation of the inhibitor and factor Xa (15 minutes at room temperature), the different substrate concentrations were added and the reaction rate monitored over 10 minutes as described above.

Determination of the inhibition constant (K_i)
Since the kinetic data indicated an uncompetitive inhibition a Dixon plot was used to determine the K_i (Segal, 1976). The substrate concentration (50 µl aliquots) was kept constant at 71 µM. The inhibitor concentration was varied between 0.04 and 1.4 µM by diluting it with 20 mM Tris-HCl, 0.15 M NaCl, 0.1% BSA, pH 7.4. Factor Xa was also diluted with the latter buffer to a concentration of 0.002 U ml^{-1}. Inhibitor (50 µl aliquots at various concentrations) was added to 100 µl of 20 m M Tris-HCl, 0.15M NaCl, 0.1% BSA, pH 7.4 and 50 µl of the diluted enzyme. Substrate was added and the reaction rate was monitored over 10 minutes as described previously.

RESULTS

Anticoagulation activity of crude salivary gland extracts
Various amounts of the salivary glands of prefed *H. truncatum* ticks were prepared and assayed for inhibition of the intrinsic and extrinsic coagulation pathways respectively, to determine the potency of the anticoagulants present in their glands (Figures 1 and 2). Both pathways were prolonged in a concentration dependent manner. The minimum protein per assay to achieve two-fold prolongation in clotting time was approximately 26 µg protein for the intrinsic and 52 µg for the extrinsic pathway. Unfed females exhibited very low protein concentration and virtually no inhibiton of the intrinsic blood coagulation cascade (Figure 1 and Table 1).

In order to determine the inhibition of factor Xa by salivary glands obtained from prefed ticks, two assay methods were used. The first assay was done using factor Xa deficient plasma (Table 2). The second method which is more sensitive, employed factor Xa and its substrate, Chromozym X (Table 3). Results obtained for both methods showed that prefed *H. truncatum* salivary gland extracts inhibit factor Xa. It was observed that the equivalent of 0.17 gland, representing approximately 14 µg protein, caused almost 90% inhibition (Table 3).

TABLE 1

Protein content of salivary glands (s.g.) of prefed and unfed female *Hyalomma truncatum* ticks. BSA was used as standard. Results are the average of eight aliquots.

Sample	Protein concentration (µg/s.g.)
Female prefed	83.3
Female unfed	3.2

Fig. 1. A graph showing the intrinsic coagulation time vs. salivary gland protein per assay of prefed (□) and unfed (○) ; one concentration only) female *Hyalomma truncatum* ticks. Results are the average of replicate aliquots. The control time is shown as a dotted line.

TABLE 2

Inhibition of factor X by salivary gland extracts of prefed female *Hyalomma truncatum* ticks, using factor X deficient plasma. Results are the average of quadruplicate aliquots.

Number of salivary glands	Clotting time (s)
2.5	145
1.25	40.0
0	18.9

Isolation of anticoagulant

Separation of salivary gland proteins was performed by means of reverse phase chromatography (Figure 3). The anticoagulant which inhibited factor Xa eluted at 20–23% acetonitrile with a retention time of 51–54 minutes. Capillary zone electrophoresis was performed to establish the purity of the inhibitor after anion-exchange chromatography. The inhibitor appeared to be virtually homogeneous (Figure 4). According to capillary gel electrophoresis the relative molecular mass of the anticoagulant is 17 kDa (Figure 5).

To establish whether the inhibitor was a protein, proteolytic digestion was con-

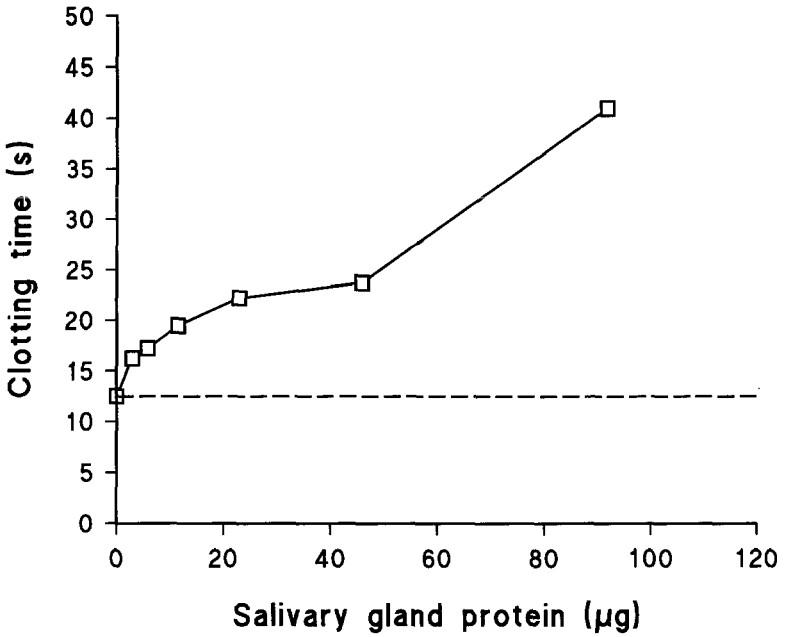

Fig. 2. A graph showing the extrinsic coagulation time vs. salivary gland protein per assay of prefed *Hyalomma truncatum* ticks. Results are the average of replicate aliquots. The control time is shown as a dotted line.

TABLE 3

Percentage inhibition of factor Xa by salivary gland extracts of prefed *Hyalomma truncatum* ticks using factor Xa and a synthetic substrate. Results are the average of replicate aliquots.

Number of salivary glands	% FXa inhibition
2.50	100
1.25	95.9
0.63	95.2
0.30	94.6
0.17	87.8
0.08	71.5
0.04	66.7

ducted by incubation of the inhibitor with trypsin attached to polyacrylamide and an overnight incubation at 30°C. Factor Xa inhibition was totally abolished after the proteolysis (data not shown). It can thus be concluded that the anticoagulant is a protein protease inhibitor. A further indication of the protein nature was obtained through amino acid analysis (Table 4). Since the amount of sample used

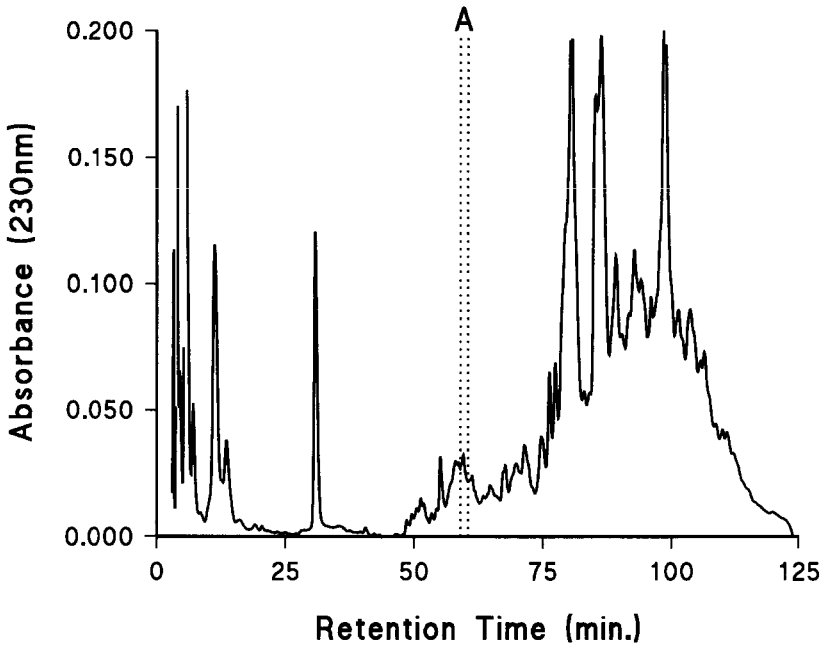

Fig. 3. Reverse phase chromatography of prefed *Hyalomma truncatum* salivary gland extracts. A gradient of acetonitrile (0.1–60%) was formed over 110 minutes and 3 ml fractions collected. The inhibitor eluted at 20–23% acetonitrile. A: activity with respect to inhibition of factor Xa using synthetic substrate.

TABLE 4

Amino acid analysis of the isolated inhibitor from *Hyalomma truncatum*. All ratios were calculated relative to leucine.

Amino acid	Inhibitor
Asp	1.74
Glu	2.33
Ser	1.91
Gly	2.00
His	0.27
Arg	0.55
Thr	1.36
Ala	1.32
Tyr	0.15
Pro	1.41
Ile	2.40
Leu	1
Phe	0.49
Lys	1.92

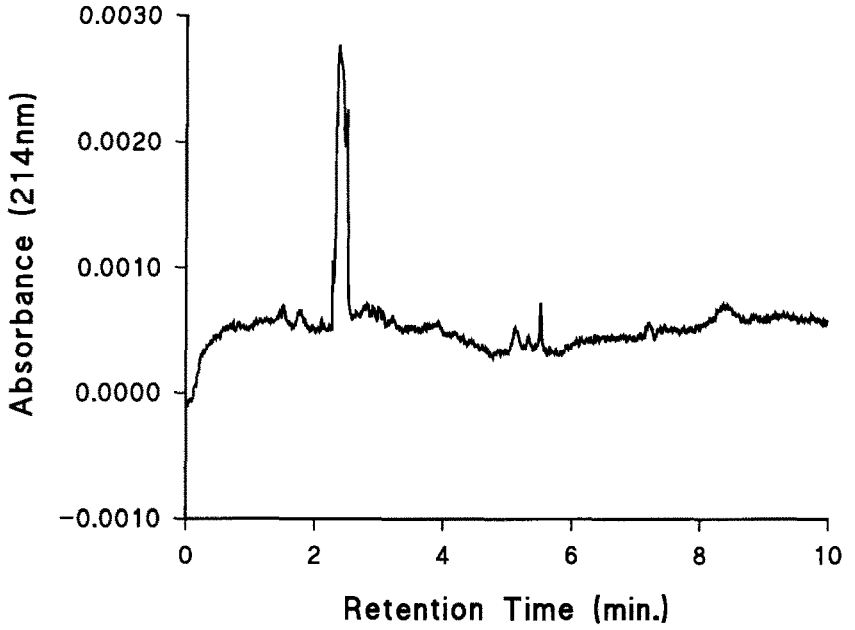

Fig. 4. Capillary zone electrophoresis of the anticoagulant from *Hyalomma truncatum* after ion-exchange HPLC.

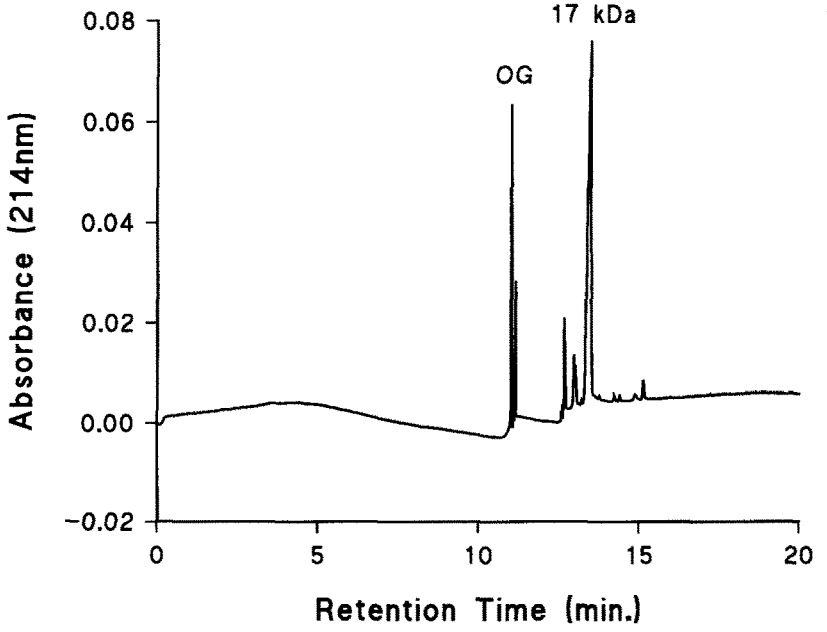

Fig. 5. Capillary gel electrophoresis of the anticoagulant from *Hyalomma truncatum* after ion-exchange HPLC. OG: Orange G dye.

for amino acid analysis was not known, it was not possible to calculate the composition on a percentage basis.

The K_m, V_{max}, K'_m and V'_{max} of human factor Xa with respect to Chromozym X was determined in the absence and presence of the inhibitor obtained after reverse phase chromatography since insufficient activity was obtained after anion exchange chromatography. According to Limo *et al.* (1991) this may be due to loss of a cofactor. The K_m and V_{max} was found to be 5.1×10^{-4} M and 51.4 μmol $l^{-1}min^{-1}$ respectively. K'_m and V'_{max} was determined as 6.5×10^{-5}M and 2.7 μmol l^{-1} min^{-1} by means of a Lineweaver–Burk plot (Figure 6). The anticoagulant appears to be uncompetitive and was confirmed by repetition of this experiment without preincubation of factor Xa and the inhibitor. An experiment was conducted using a fixed concentration of substrate and varying the inhibitor concentration to prepare a Dixon plot in order to determine the inhibition constant (K_i). Deviations of duplicates were negligible (Figure 7). The K_i was found to be 6.9×10^{-10}M by means of computer curve fitting.

DISCUSSION

The anticoagulant isolated from prefed female *H. truncatum* salivary glands appears to be a potent inhibitor of factor Xa. The inhibitor may be isolated in small

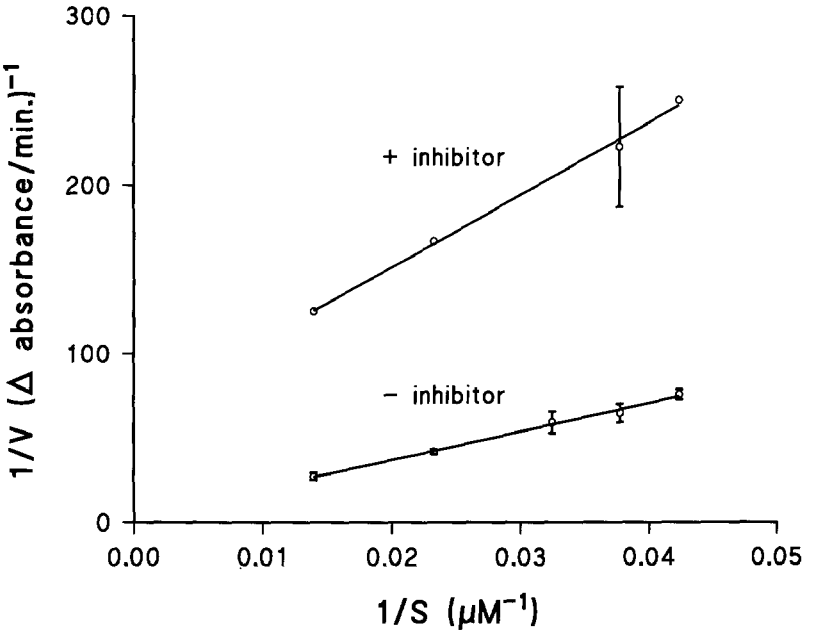

Fig. 6. Determination of K'_m and V'_{max} for factor Xa with Chromozym X as substrate in the presence of inhibitor by means of a Lineweaver-Burk plot. Results are the average of quadruplicate values ± SD.

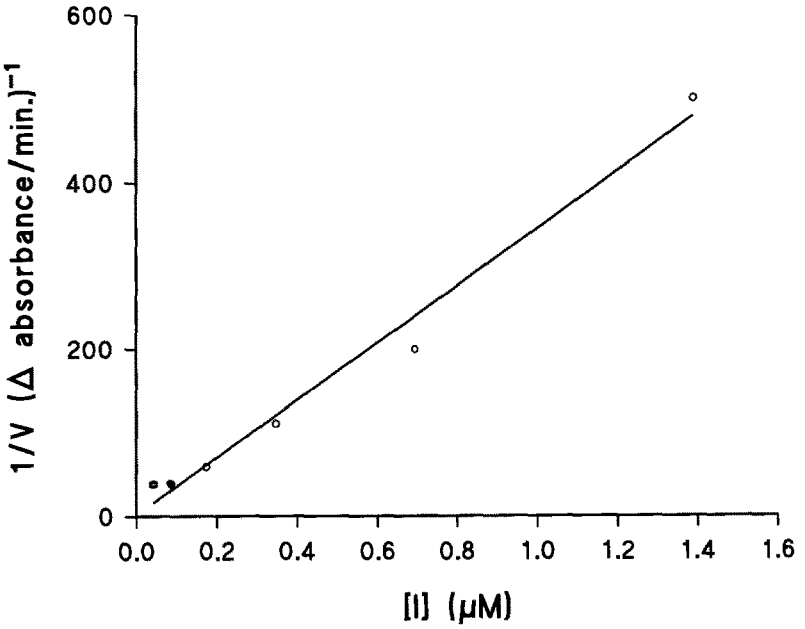

Fig. 7. Determination of the K_i of the inhibitor for the ES complex by means of a Dixon plot. Results are the average of duplicate values ± SD. The K_i was determined as 6.9×10^{-10}M by means of computer curve fitting.

quantities by reverse phase and ion-exchange HPLC. A large enrichment was obtained after the first HPLC step. Little further purification was obtained after the second HPLC step.

The inhibitor was kinetically characterized as an uncompetitive inhibitor with a K_i of about 7×10^{-10}M calculated from the Dixon plot by means of computer curve fitting. For this type of inhibition preincubation of factor Xa and the inhibitor was not necessary as the inhibitor binds only the ES complex, thus after the enzyme has bound to the substrate. K'_m and V'_{max} were determined with and without preincubation of factor Xa and the inhibitor. In both cases, uncompetitive inhibition was observed. The non-parallel Lineweaver–Burk plot observed in this study may be ascribed to experimental deviation. According to Cornish-Bowden (1986) uncompetitive inhibition is rare, because it is not particularly plausible in general to suppose that an inhibitor exerts an effect on the ES complex and not on the free enzyme. Literature has shown that a K_i of 10^{-10}M indicates a potent inhibitor with a high affinity of the inhibitor for the enzyme or in this case the ES complex. The competitive inhibitor, tick anticoagulant peptide (TAP) isolated from *O. moubata*, binds to factor Xa with an inhibition constant of 5×10^{-10}M (Vlasuk *et al.*, 1991), while the recombinant TAP has a K_i of 1.8×10^{-10}M (Jordan *et al.*, 1991) and was

shown to be a reversible, slow tight-binding inhibitor of factor Xa. Effects of recombinant TAP and heparin were compared in an anaesthetized baboon model with respect to arterial thrombosis and data suggested that controlling thrombin generation through inhibition of factor Xa, may be a novel and effective pharmacological approach in the prevention of arterial thrombosis (Meyer *et al.*, 1992). Recombinant antistasin isolated from the salivary glands of the Mexican leech, *Haementeria officinalis* and recombinant TAP, have been shown to be as effective as conventional anticoagulant treatment with heparin in preventing venous thrombosis in rabbits (Vlasuk *et al.*, 1991).

Different concentrations of the inhibitor from *Hyalomma truncatum* and substrate should be employed in the Lineweaver–Burk and Dixon plot respectively for verification of the uncompetitive nature of the anticoagulant. These experiments were not performed due to low availability of the inhibitor. Because the inhibitor is of the uncompetitive type, it is unlikely that the inhibitor will also inhibit other serine proteases as binding of the inhibitor takes place at a different location than the active site of the enzyme. Proteases might be similar in the active site regions, but chances of conserved inhibitor binding sequences existing elsewhere in factor Xa and other serine proteases are unlikely.

For further characterization, the anticoagulant activity should be tested for sensitivity to DTT treatment, to tell whether disulphide bonds are essential for activity. EDTA treatment may suggest whether the inhibitor is a metallo-protein protease inhibitor. Neuramidase treatment would indicate whether neuramic acid moieties are implicated in the anticoagulant activity. Determination of the amino acid sequence would make it possible to establish to what family of inhibitors it is related and whether it is unique, or conserved among hard and soft ticks. The sequence would also aid in the cloning and expression of its gene.

The anticoagulant is probably important for successful tick feeding, because it has the capacity to inhibit the clotting cascade at the junction of the extrinsic and intrinsic pathway (factor Xa) and may thus be crucial for the survival of the tick. Expression of the recombinant gene therefore appears to be a worthwhile venture for obtaining sufficient quantities of the inhibitor for exploring alternative methods of tick control and for evaluation as an therapeutic agent.

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