Purification and partial characterization of draculin, the anticoagulant factor present in the saliva of vampire bats (Desmodus rotundus)

Purification and Partial Characterization of Draculin, the Anticoagulant Factor Present in the Saliva of Vampire Bats *(Desmodus rotundus)*

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Summary

From the saliva of the vampire bat *Desmodus rotundus*, we isolated an unknown anticoagulant protein which we have named *draculin*. Its molecular mass as determined by non-reduced SDS-PAGE is about 83 kDa. The reduced polypeptide shows a slower migration. HPLC in a molecular sieve matrix yields a single, symmetrical peak corresponding to 88.5 kDa. Isoelectric focusing shows an acidic protein with pI = 4.1–4.2. Aminoacid analysis is compatible with a single chain polypeptide of about 80 kDa. Cyanogen bromide cleavage yields a single 16-aminoacid peptide, corresponding to the amino-terminus of the native molecule. Draculin inhibits the activated form of coagulation factors IX and X. It does not act on thrombin, trypsin, chymotrypsin and does not express fibrinolytic activity. The inhibition is immediate and not readily reversible, with a stoichiometry of about two molecules of draculin per molecule of factor IXa or Xa. Surprisingly, the inhibitory activity against either factor is not affected by the presence of the other. Draculin binds quantitatively to either immobilised factor Xa or factor IXa. Our preliminary interpretation is that there are two forms of draculin that hardly differ in structure. Both bind to factor Xa and to factor IXa but one form inhibits factor Xa and the other inhibits factor IXa.

When added to plasma, draculin increases the lag phase as well as the height of the peak of thrombin generation.

Introduction

The anticoagulant properties of vampire bat saliva have been intuitively known for many years as is witnessed by their name. *Vampir*, since the early middle ages or even before indicated blood drinking "semi-dead" in eastern European folklore. The name evidently has been extended to haematophagous bats after these have become known to Europeans, i. e. after 1492. One of the first documented accounts on the anticoagulant effect of saliva from an haematophagous bat was done in 1932 by Bier, and some years later by Romana (1, 2). After these, very few papers have appeared on this subject. At present, the best characterized factor from vampire bat saliva is a recently isolated plasminogen activator from extracts of vampire salivary glands (3). It is not clear if this corresponds to the plasminogen activator from vampire bat saliva previously described by Hawkey (4) and Cartwright (5).

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Here, we report results on the isolation and partial characterization of the anticoagulant factor present in vampire bat saliva and we determined its site of action in the coagulation mechanism. We have purified the anticoagulant factor to apparent homogeneity and show that it is an acidic (pI = 4.15) 88.5 kDa glycoprotein which interacts with purified factor IXa and Xa and inhibits thrombin generation in human plasma.

Materials and Methods

Sephacryl S-200 (Pharmacia, Sweden), hydroxyapatite (BioGel HTP), Affi-Gel-15, and acrylamide (BioRad Labs), Activated Thrombofax, and rabbit brain partial Thromboplastin (Ortho Diagnostic Systems, Raritan, NJ, USA) were obtained from commercial sources. Coagulation factors were purified by established procedures by Dr. Rob Wagenvoord (Dept. of Biochemistry, Univ. of Limburg, Maastricht, The Netherlands). Chromogenic substrates were obtained from Kabi (Sweden). Thiobenzyl Benzyloxicarbonyl-L-lysinate · HCl and p'-nitrophenyl-p-guanidobenzoate · HCl were from Sigma. All other reagents used were also of the highest quality available. The buffers used were A: 0.05 M Tris-HCl, 0.1 M NaCl pH 7.35, with 0.5% of egg albumin (Sigma) and B: Buffer A at pH 7.9 with 20 mM EDTA.

Animals

Vampire bats *(Desmodus rotundus)* were regularly captured from wild colonies living in a cave in the northwest part of Venezuela (State of Falcón) in a region where rabies is reportedly absent. Twenty vampires were currently kept in captivity, in individual cages of the metabolic type (Acme Metal Products, Chicago, IL, USA), under controlled light and temperature (25° C). The animals were maintained on bovine blood anticoagulated with 3.2% sodium citrate at a ratio of 1:9. Food was given every 24 h, always in the late afternoon. Water was given ad libitum (6).

Saliva Collection

For saliva collection, the vampires were anaesthetized with a mixture of 2.5% 2-bromo-2-chloro, 1,1,1-trifluorethane (Halothan, Hoechst), 30% nitrous oxide, in oxygen (5, 6). Once anaesthetized, 20 µl of 1% pilocarpine (Isopto Carpin, Alcon Labs., Inc., Ft. Worth, TX, USA) was placed in the mouth in order to stimulate salivation. It was observed that incidentally wounding of the gingivae with the plastic pipette tip that was used to apply the pilocarpine caused a very minor bleeding that stopped almost immediately. Saliva was collected in plastic microcentrifuge tubes, placed in ice. The saliva collection took 30 to 40 min, with a regular yield of about 0.5 ml/animal. Individual samples were kept at –30° C until use.

Isolation of the Anticoagulant Factor from Vampire Bat Saliva

The protocol routinely used was as follows: Saliva (≈5 ml, 15 mg of protein) was thawed and dialysed for six hours against 10 litres of distilled

water. The dialysate was centrifuged at 48,000 ×g during 20 min and the clear supernatant was lyophilized and redissolved in 1.5 ml of distilled water. This was loaded on a Sephacryl S-200 column (2.5 × 30 cm) and eluted with distilled water at a rate of 0.5 ml/min at room temperature. Fractions of 2 ml were collected and the relative protein concentration as well as the anti-Xa activity were measured every two fractions. Anti-Xa activity of the fractions was assayed using a micro-plate adaptation of the FXa assay based on the hydrolysis of the chromogenic substrate S-2222 as follows: Each microplate well contained 6.25 nM FXa, 50 mM Tris pH 7.35 and an aliquot of the fractions, in a total volume of 150 µl. Reaction was started with 20 µl of 4 mM S-2222 and after 10 min of incubation at 37° C the absorbency at 405 nm was recorded in a micro-plate reader (Bio-Tek, Model EL312e). Fractions with anti-Xa activity were pooled and lyophilized. The lyophilized material was dissolved in 1 mM NaCl (3–5 ml) and the pH adjusted to 7.2 with NaOH. The sample was loaded on a hydroxyapatite column (1 × 7.5 cm, Biogel HTP) equilibrated with 1 mM NaCl, pH 7.2. After all of the sample was loaded, the column was washed with 6 column volumes of 1 mM NaCl (pH 7.2) followed by 2 column volumes of 200 mM potassium phosphate, pH 6.8. At this point, a phosphate gradient from 200 mM to 1 M (total volume = 30 ml) was initiated. One ml fractions were collected at a rate of 0.3 ml/min. The anti-Xa activity appeared at about 0.3 M phosphate, while most of the protein eluted with the initial washing and the 200 mM phosphate. The active fractions were pooled and concentrated by ultrafiltration through an Amicon XM-50 filter.

A high degree of purification can also be obtained after chromatography of crude saliva (dialysed against 1 mM NaCl) on hydroxyapatite, with omisson of the Sephacryl step. Draculin obtained by this single step procedure is always slightly contaminated by a lower molecular weight polypeptide which elutes from the hydroxyapatite column at the same phosphate concentration as draculin.

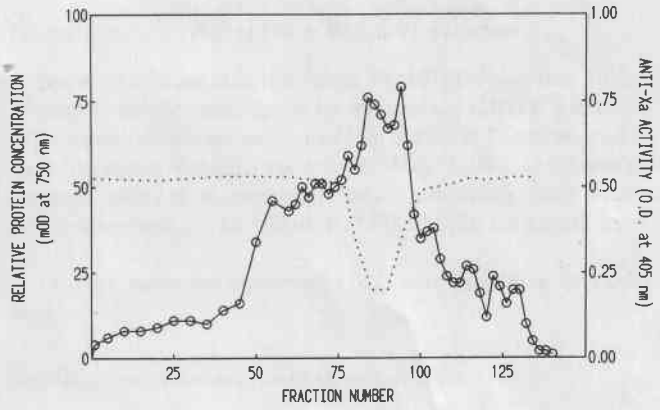

Fig. 1 Protein and anti-FXa activity profile from fractions obtained after molecular sieve of lyophilised vampire bat saliva through sephacryl S-200. Column size was 2.5 × 30 cm; equilibration and elution was done with deionized water and 2 ml fractions were collected. Dashed line corresponds to the inhibitory activity assayed with the microplate system described in Methods and expressed as the decrease of absorbency measured at 405 nm. Solid line corresponds to the relative protein concentration determined by Bradford's method

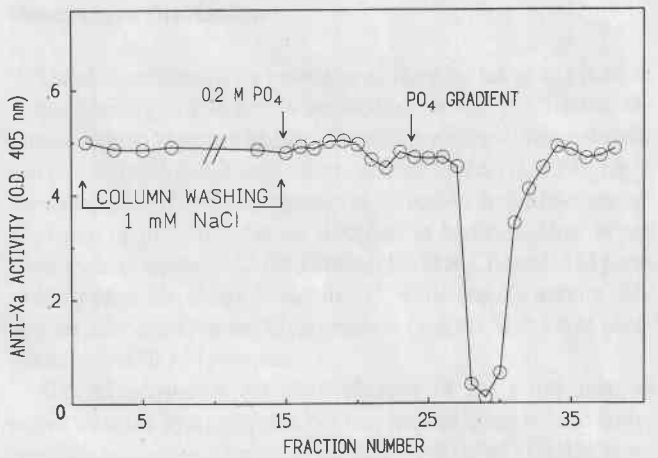

Fig. 2 Anti-FXa activity profile of material eluted from a hydroxyapatite column. Column (1 × 7.5 cm) was loaded with active material obtained from Sephacryl S-200. Fractions volume was 1.0 ml. Inhibitory activity expressed as in Fig. 1. See Methods section for details

Coagulation Assays

a) *Human whole blood coagulation time* (WBCT) was measured in one ml samples collected after discarding the first 2 ml of blood. Coagulation was allowed to proceed at 37° C, in glass tubes containing 50 µl of EACA (114 mg/ml).

b) *Activated partial thromboplastin time* (aPTT) was measured using a commercial partial Thromboplastin (Activated Thrombofax, Ortho Diagnostic Systems, Raritan, NJ, USA), which contains Elagic acid as activator.

c) *Prothrombin time* (PT) was measured using a commercial Brain Thromboplastin/calcium chloride reagent (Ortho).

d) *Thrombin time* (TT) was determined with bovine thrombin (Sigma Chem., St. Louis, MO, USA). Experimental conditions were chosen in order to obtain a TT of 30 seconds for the control.

All coagulation times were read manually by tilting the tubes.

Factor Xa Activity

Factor Xa was assayed by its activity on the chromogenic substrate S-2337 (Kabi Diagnostica, Sweden). Usually 10 µl samples were put in 0.490 ml of buffer B containing 4 mM of the substrate. The reaction was followed at 37° C, at 405 nm in a home made fixed dual-wavelength photometer (Biochemistry and Instrumental Dept., Univ. of Limburg), and all relevant parameters were calculated by a dedicated software developed for that instrument (Laudy, P., Instrumental Department, Univ. of Limburg). Factor Xa concentration was determined by titration with p'-N-p-guanidobenzoate as described by Jameson et al. (7).

Factor IXa Activity

Factor IXa was assayed according to the principles worked out by van Dieyen et al. (8) as further elaborated by Wagenvoord et al. (9). We measured the enzymatic activity on factor X activation in a system where the non-factor IXa components of the tenase complex are present in excess: factor VIIIa (10 nM), phospholipid (PS/PC 20/80, 1 µM), and Ca^{++} (5 mM). The samples contained up to 1 nM of factor IXa mixed with up to 2 nM draculin. Factor IXa and draculin were incubated for at least one minute before the other components of tenase were added, except for the experiment of Fig. 7. The reaction was initiated by addition of purified factor X (200 nM). The production of factor Xa was followed on subsamples taken into EDTA buffer, containing the chromogenic substrate S-2337, as described above. Typically 1 nM of factor IXa will cause a reaction velocity of 200 nM of factor Xa being formed per minute in this system. Because never more than 2 nM of draculin was added per nM of factor IXa, the inihibitory action of draculin on factor Xa would not significantly interfere with the outcome of the factor IXa determination.

Additionally, FIXa amidolytic activity was tested on thiobenzyl benzylocarbonyl-L-lysinate as described by Green and Shaw (10).

Determination of Other Proteases

The following proteases were tested on the chromogenic substrates indicated: thrombin-S2238; trpysin-S2160; chymotrypsin-S2586; plasmin-S2160;

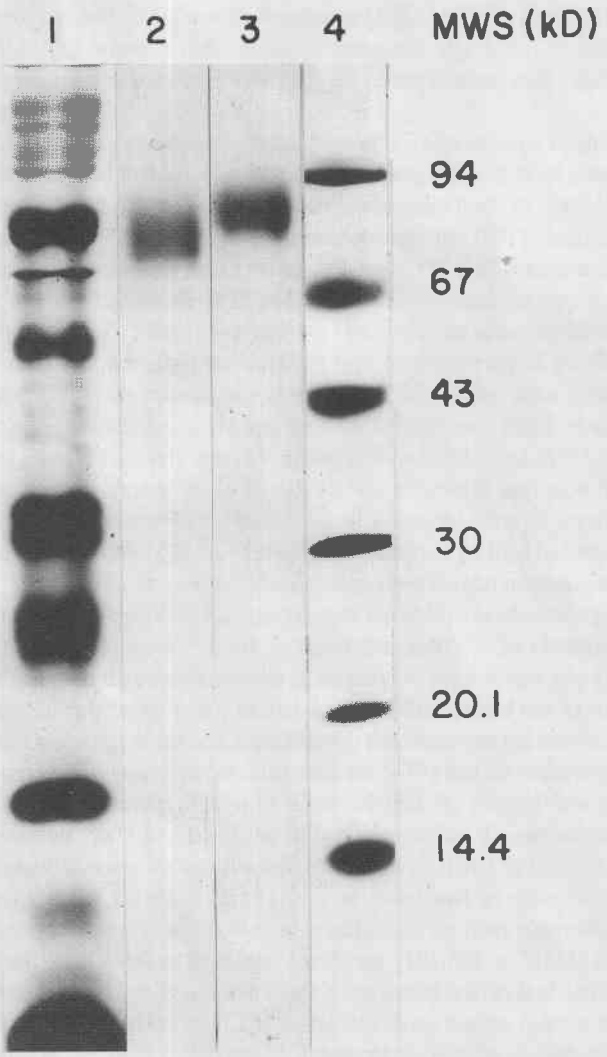

Fig. 3 SDS-Polyacrylamide gel electrophoresis of draculin. Lane 1: crude saliva; lane 2: non-reduced draculin (10 μg), lane 3: reduced draculin (10 μg); Lane 4 corresponds to the low molecular weight markers from Pharmacia. Protein staining with Coomassie Blue

plasma kallikrein-S2302. Their activity was tested in the presence and in the absence of roughly equimolar as well as up to tenfold excess amounts of draculin.

Protein determination was done using the method described by Bradford (11).

SDS-Polyacrylamide gel electrophoresis was done as described by Shagger and von Jagow (12). Periodic acid-Schiff staining for glycoproteins was done as described (13).

Isoelectric focusing was performed on agarose in a Multiphor LKB (Sweden) electrophoresis chamber as described by Vesterberg and Gramstrup-Christensen (14), with minor modifications. Ampholine (pH 3.5–10, LKB, Sweden) was added to obtain a final concentration of 5%.

HPLC of purified draculin was performed in a Shimadzu HPLC equipment (Shimadzu Corp., Japan) using a silica based molecular sieve column (10 μm Protein Pak 300 sw,Waters, Millipore Corp., USA). Equilibration of the column and elution of the sample was done with 250 mM potassium phosphate, pH 6.8. Calibration of this column was done with molecular weight markers from Pharmacia (Sweden).

Immobilization of FIXa and FXa on AffiGel-15 was done as indicated in the manufacturer brochure, using 1.2 mg of each purified factor for coupling to 0.5 ml of gel (bed volume).

Effect of Immobilized FIXa or FXa on the Activity of Draculin

Draculin was incubated for two minutes with 150 μl of inactivated AffiGel-15 (control) and after centrifugation for one minute at 12,000 × g an aliquot of the supernatant was assayed for inhibition of FIXa or FXa in the standard assay. The amount of draculin was chosen to obtain 95–100% inhibition of the enzymatic activity of the corresponding factors. Identical amounts of draculin were incubated with a) FXa-AffiGel, or b) FIXa-AffiGel and similarly tested.

Thrombin generation experiments (TGT) were executed as previously described (15).

Aminoacid Composition and Partial Aminoacid Sequence

Draculin was further purified, prior to protein chemistry, by reverse phase HPLC on a C-18 column (Vydac 201 TP, The Separations Group, Hesperia CA, or Asahipak ODP-50, from Anspec Co., Ann Arbor, MI) equilibrated in 0.1% trifluoroacetic acid and developed with a gradient (0.8% per minute) of acetonitrile. Each column yielded a single peak. Amino acid composition was determined by derivatisation with phenyl isothiocyanate (PITC) after vapour phase acid hydrolysis for 16 h at 110° C ("Picotag" methodology, Milligen Corp., Waters Division).

Results and Discussion

The anticoagulant factor present in vampire bat saliva was purified to homogeneity as described in the Methods section. Fig. 1 shows the protein separation pattern and anti-Xa activity obtained after molecular sieve of dialysed, lyophilized saliva, through Sephacryl S-200. Fig. 2 shows results of the chromatography of the pooled, active fractions, on a column of hydroxyapatite as described in Methods. Most of the protein comes through with the washing (1 mM NaCl) and 0.2 M phosphate (pattern not shown in the figure), while anti-Xa activity only appears after establishment of the gradient from 0.2 M to 1.0 M phosphate, at about 0.3 M phosphate.

SDS-polyacrylamide gel electrophoresis of the active material eluted from the hydroxyapatite column, showed a single band corresponding to a molecular mass in the range of 80 to 85 kDa. Under reducing conditions, this band migrates as an apparently slightly higher molecular mass component, suggesting the presence of intra-chain disulphide bonds (Fig. 3, lanes 2 and 3). The protein band gives a strong positive reaction for carbohydrates with the periodic acid-Schiff stain (not shown).

HPLC of the purified native draculin showed a single, symmetrical protein peak eluting at a position corresponding to a molecular mass of 88.5 kDa (Fig. 4). This result was obtained using 0.25 M phosphate for elution of the column. Several trials using lower ionic strength eluent resulted in apparent binding of the protein to the column matrix, which markedly retarded elution of the protein. Fig. 5 shows that isolelectric focusing of draculin in an agarose based gel resulted in a single polypeptide band which immobilizes at pI = 4.1–4.2.

The amino acid composition of non-reduced (native) and reduced, alkylated (S-pyridilethyl) draculin is shown in Table 1. In SPE draculin, seven moles of SPE cysteine per mole of protein was also recovered. Form the amino acid composition a molecular weight of approximately 80 Da can be calculated, which is in agreement with the molecular mass obtained from SDS-PAGE. The contribution of the carbohydrate moiety seems to amount to about 10% of the polypeptide weight, as indicated by the molecular mass obtained from HPLC molecular sieve of native draculin (88.5 kDa). Cleavage of draculin with cyanogen bromide yields a single peptide with apparent molecular

weight around 7000 Da, with the sequence NH_2-ARRRGVRWCTISK-PEA. This sequence contains the 16-aminoacid sequence from intact draculin, indicating that it represents the amino-terminus of the molecule.

Preliminary experiments on the effect of draculin on blood coagulation indicated that draculin prolonged the coagulation time of whole human blood (WBCT), as well as the prothrombin time (PT, extrinsic pathway) or the activated partial thromboplastin time (aPTT, instrinsic pathway) of citrated human plasma. About 50 nM final concentration of draculin doubled the aPTT and the WBCT. The same concentration prolonged the PT from 14 (control) to 18 s. Under the same conditions, draculin did not affect the thrombin time or the activity of purified thrombin on the chromogenic substrate S-2238. Neither did it inhibit trypsin, chymotrypsin, plasmin or plasma kallikrein. These results suggested a relatively specific anticoagulant effect located in the coagulation cascade at the level of factor X, or factor X activation. To further define the mechanism(s) of the anticoagulant effect of draculin we investigated its effect on the catalytic activity of purified factors.

Fig. 6 shows the progress of the hydrolysis of the chromogenic substrate S-2337 by factor Xa, expressed as the change in absorbency at 405 nm. Addition of draculin to the system results in an immediate inhibition of the catalytic activity of the enzyme, suggesting a very fast interaction between factor Xa and draculin. We repeated this experiment at a series of factor Xa and draculin concentrations but inhibition always was apparently complete within the 5–10 s that are required for addition and mixing of draculin in the solution. No stopped flow experiments could be carried out in our laboratories. To our surprise draculin also inhibits the activation of factor X catalysed by factor IXa. If 0.3, 0.5 or 1.0 nM of factor IXa is incubated with an equimolar or excess amount of draculin, then, after addition of the other components of the tenase system in excess, i. e. factor VIIIa (10 or 20 nM) and phospholipid (2 or 20 μM), no factor X converting activity is observed. This is illustrated in Fig. 7. This figure also shows that the order of addition of the reaction components influences the velocity of inhibition. This means that factor IXa in the tenase complex system is to a certain degree protected from draculin action and that fast inhibition is only achieved when free factor IXa interacts with draculin. This suggests

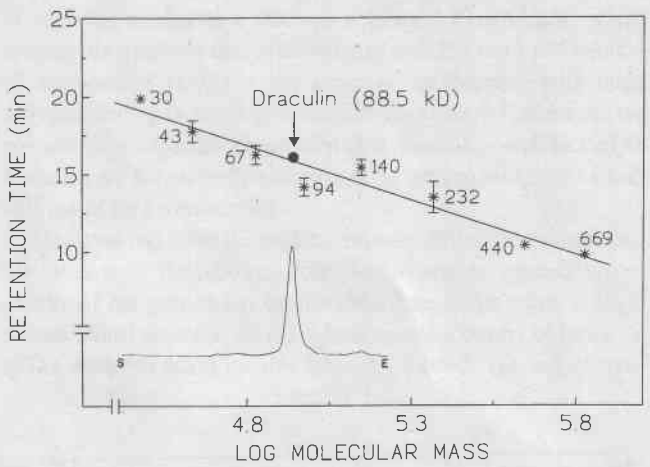

Fig. 4 HPLC of purified draculin. A Protein Pak 300sw column (7.8 × 300 mm), was equilibrated and eluted with 250 mM potassium phosphate, pH 6.8. Figures (kDa) correspond to molecular weight markers from Pharmacia used for column calibration (n = 5, ± SEM). No difference in retention time of the markers was observed with Tris or HEPES buffers. The insert corresponds to a typical run of 10 μg of draculin (S = start, E = end of the run)

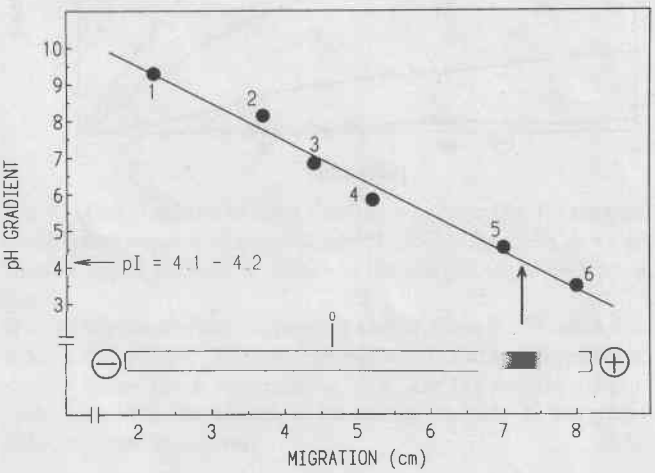

Fig. 5 Isoelectric focusing of purified draculin. Fifteen μg of protein was applied. Insert shows the actual agarose gel after staining with Coomassie Blue (0 indicates original position of the sample). Isolelectric point markers used were: 1, Trypsinogen (9.3); 2, Lentil Lectin (8.15); 3, Horse myoglobin (6.85); 4, β-lactoglobulin A (5.2); 5, Soybean trypsin inhibitor (4.55); 6, Amyloglucosidase (3.5)

Table 1 Amino acid composition of non-reduced (native) and reduced, alkylated (s-pyridylethyl, SPE) draculin

Amino acid	SPE	Native
ASX	78.1	78.9
GLX	75.0	79.6
SER	51.1	53.2
GLY	70.5	69.4
HIS	8.3	9.0
ARG	46.2	45.4
THR	37.0	34.4
ALA	75.9	71.8
PRO	36.8	34.6
TYR	25.6	22.5
VAL	45.4	43.7
MET	5.9	6.8
ILE	17.9	16.5
LEU	72.2	69.7
PHE	32.7	33.7
LYS	47.1	46.4

The figures indicate residues per mole.

that the inhibition observed is basically due to the effect of draculin on free factor IXa. Comparable protection of the amidolytic activity of factor Xa on S-2337 is observed when the components of the prothrombinase system (factor Va and phospholipids) are added (results not shown).

The dose-response for the interaction between sub-equimolar amounts of draculin and factor IXa or Xa is depicted in Fig. 8. As can be seen, the inhibitory effect is dose-dependent and shows a tendency towards complete inhibition when about two moles of draculin (MW = 88.5 kDa) are added to one mole of either factor. A similar conclusion can be drawn from the results shown in the insert, which describe the effect of draculin on the activity of factor IXa on the chromogenic sub-

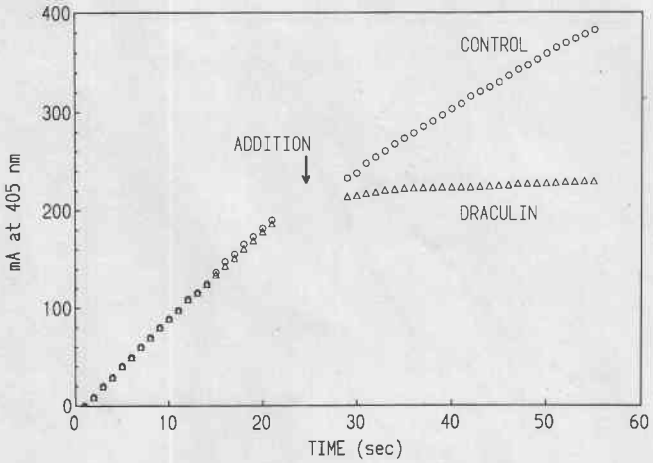

Fig. 6 Inhibition of factor Xa amidolytic activity by draculin. Progress curve for the hydrolysis of the chromogenic substrate S-2337 by purified coagulation factor Xa (25 nM), expressed as the change in absorbency at 405 nm. After 22 seconds, buffer (Control) or draculin (50 nM) was added and the reaction was allowed to proceed for another 30 s

strate Thiobenzyl Benzyloxycarbonyl-L-lysinate (Z-Lys-SBzl · HCl). Since this is not a good substrate for factor IXa, relatively high concentrations of enzyme must be used in order to have a reasonable rate to hydrolysis. Due to this handicap, only two molecular ratios of draculin : factor IXa were examined, however, their inhibitory effect clearly falls within the expected range assuming a molar ratio of 2:1 and a molecular mass for draculin of 88.5 kDa (dashed line). This inhibition of the activity of FIXa on an artificial substrate confirms that the inhibitory activity of draculin observed in the tenase system is indeed due to a direct effect of draculin on factor IXa.

Results from dose-response experiments done with six batches of purified draculin, show a variability in the stoichiometry that ranges between 1.8 and 2.7. This variability could be related to a variable degree of microheterogeneity, e. g. caused by different degrees of glycosylation, of native draculin. At present we do not have experimental evidence on the possible dependence of the biological activity of draculin and the level of glycosylation.

Table 2 shows that the effect of draculin is specific for the activated form of factor X. As can be seen, the inhibitory effect of draculin on the activity of FXa is not affected by the presence of FX or a chemically modified FXa (Dansyl-Glu-Gly-Arg-chloromethyl ketone-FXa) which is catalytically inactive. Table 2 also shows a surprising lack of competition between FIXa and FXa in their interaction with draculin. Incubation of draculin with an excess of FIXa does not affect the inhibition of FXa by draculin. Table 3 indicates that interaction of draculin with either factor IXa or Xa which have been immobilized to a matrix (AffiGel-15), also immobilizes the inhibitory activity of the protein against the other factor. We attribute the weak residual inhibition observed after incubation with FIXa-AffiGel to a less than optimal coupling of this factor to the matrix.

At the moment we cannot propose a detailed mechanism of action of draculin on factor Xa and/or factor IXa. From its size one might think draculin to be related to one of the many known serpins but the N-terminal amino acid sequence does not show homology with this group of proteins. Also the inhibition is much faster than that obtained with most serpins. Compared to the typical anticoagulant serpin antithrombin we see that the specificity is higher (no inhibition of thrombin) and the action is much faster [the pseudo first order decay constant of thrombin-antithrombin interaction being 1.49 $min^{-1}/\mu M$, which accounts for a half-life time of thrombin of over 25 s in a 1 μM solution of antithrombin (16), the interaction of antithrombin with other activated clotting factors is even considerably slower]. Furthermore the completely unexpected observation that factor Xa- and factor IXa inhibition are not mutually exclusive is, to out knowledge, not found with any of the known serpins.

This latter fact is for the moment the most difficult to explain. One has to reconcile the following four observations: a) apparent homogeneity of our preparation by the usual criteria, inclusive a single aminoterminal sequence, b) a 2:1 binding stoichiometry of factor Xa or IXa inhibition that is variable between 1.8 and 2.7, c) lack of com-

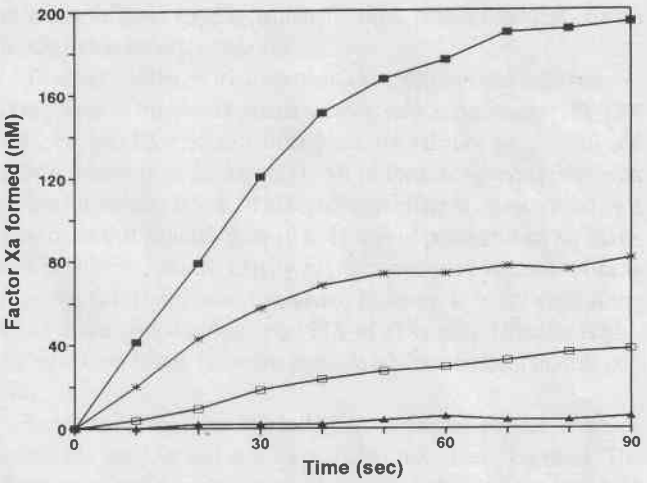

Fig. 7 Effect of draculin on factor X activation by factor IXa. The complete tenase system was used, as described in Methods. This figure also shows the effect of varying the order of addition of draculin (DRAC) to the reaction system.

■ Control without draculin; * sequence of addition: factor IXa, phospholipids, factor VIIIa, draculin; □ sequence of addition: factor IXa, phospholipids, draculin, factor VIIIa. ▲ Sequence of addition: factor IXa, draculin, phospholipids, factor VIIIa. The substrate of the reaction, i. e. factor X, was always added last to start the reaction

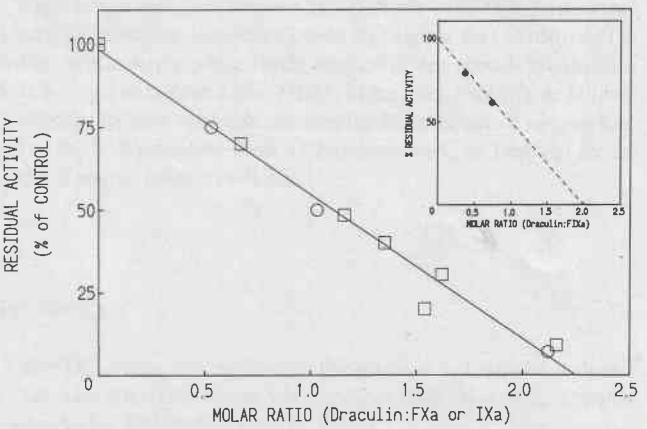

Fig. 8 Stoichiometry of draculin-clotting factor interaction. Stoichiometry of the inhibition of purified factor IXa (circles) and purified factor Xa (squares) by draculin (standard assay). Insert shows results obtained for the effect of draculin on purified factor IXa using Z-Lys-SBzl · HCl as substrate (filled circles). The dashed line corresponds to the theoretical inhibition that will be obtained assuming a 2:1 stoichiometry

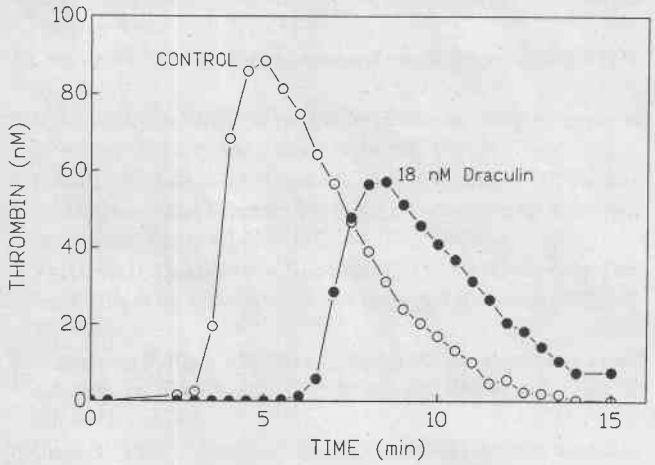

Fig. 9 Effect of draculin on intrinsic thrombin generation in human, defibrinated plasma. Experimental protocol and analysis of the data, as described in Methods and Ref. 13. Open circles, control; closed circles, 18 nM draculin

Table 2 Effect of the addition of FX, modified FXa and FIXa on the anti-Xa activity of draculin

Addition*	FXa activity (mA/min)
FXa	610
FXa + draculin	330
FXa + FX + draculin	327
FXa + mFXa + draculin	339
FXa + FIXa + draculin	332

*FXa, mFXa and FIXa were 20 nM. Draculin concentration was chosen to produce about 50% inhibition (≈ 20 nM based on a MW = 80.5 kDa). mFXa is factor Xa of which the active site is blocked with dansyl-Glu-Gly-Arg-Chloromethylketone.

Table 3 Effect of immobilized factor IXa and Xa on the activity of draculin

	Anti-Xa	Anti-IXa
Draculin + blocked AffiGel	100	100
Draculin + IX-AffiGel	20	25
Draculin + Xa-AffiGel	4	2

An aliquot of a draculin solution was incubated with the AffiGel preparation. Then the AffiGel was removed by centrifugation. The inhibitory capacity of the supernatant is expressed as a percentage of that of the original draculin solution

petition between the two substrates factor Xa and factor IXa and d) binding of both activities to immobilised factor Xa or factor IXa. The minimal hypothesis seems to be that two very similar protein-chains are present in our preparation, one (drac-Xa) that inhibits factor Xa and the other that inhibits factor IXa (drac-IXa). To explain the retention on gel we need the extra assumption of either an interaction between the two forms of draculin (heterodimer) or a non inhibitory interaction between factor Xa and drac-IXa and vice versa. More complex interactions, such as binding of drac-IXa to the factor Xa-drac-Xa complex remain possible. This problem is at present under investigation.

The effect of draculin on the generation of thrombin mediated by the components of the tenase complex (intrinsic thrombin generation) is shown in Fig. 9. As can be seen, thrombin generation is both retarded and inhibited by draculin. The increase of the lag phase is brought about by factor Xa inhibition, while the decrease of the peak value is due to factor IX inhibition (17).

To summarize, in this paper we report the isolation from vampire bat saliva of a new anticoagulant glycoprotein with a molecular mass of 88.5 kDa and isoelectric point of 4.1–4.2. It appears as a single chain polypeptide. However, from the functional and binding studies we have to accept the possibility of a microheterogeneity. This polypeptide, which we named draculin, seems to be highly specific for the *activated* forms of coagulation factors IX and X and its inhibitory effect is immediate. The stoichiometry (2:1) of inhibition and the interaction with immobilised activated factor Xa and IXa as well as the lack of competition between FIXa and FXa for draculin, suggests the existence of two specific inhibitory sites, possibly present in two hardly different polypeptide chains.

Draculin is different from other anti-Xa molecules that have been recently isolated from other animal sources, such as Antistasin (18), TAP (19), the anti-FXa isolated from black fly salivary glands (20) and anticoagulants from leeches (21). All of them are polypeptides with molecular weights below 20 kDa and their effect is characterized by a slow onset that requires from 10 to 15 min of preincubation for maximal inhibition. Anti-IX activity has been observed in crude extracts from the tick *Ornithodorus moubata*, however, it is not clear if the effect of the anticoagulant is on FIX or FIXa (22). Draculin is also different from Ecotin the serine protease inhibitor isolated from *E. coli* (23).

To our knowledge, draculin is the first natural polypeptide for which immediate anti-IXa and anti-Xa activities have been described. The discovery of this new anticoagulant provides a new tool for studies of the coagulation mechanism(s), and the possibility of design of substrates for the assay of factor IXa. Further, it opens a new approach to the strategy in the search for new anticoagulants with therapeutic potential. The observation that vampires are immune to the anticoagulant action of their own saliva suggests that there are interesting differences between the clotting system of these mammals and others, that remain to be investigated.

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