Purification and characterization of prolixin S (nitrophorin 2), the salivary anticoagulant of the blood-sucking bug *Rhodnius prolixus*

José M. C. RIBEIRO,*‡ Marcelo SCHNEIDER* and Jorge A. GUIMARÃES*†

*Department of Entomology and Center for Insect Sciences, University of Arizona, Forbes Building, Room 410, Tucson, AZ 85721, U.S.A., and †Department of Medical Biochemistry-ICB, Federal University of Rio de Janeiro, Rio de Janeiro, RJ, Brazil

The salivary anticoagulant of the blood-sucking bug *Rhodnius prolixus* was purified to homogeneity using a protocol consisting of weak cation-exchange, DEAE, hydrophobic-interaction and octadecyl reverse-phase chromatography, yielding a protein with the same N-terminal sequence as nitrophorin 2, one of the four NO haem protein carriers present in the salivary glands of *Rhodnius* with a molecular mass of 19689 Da [D. Champagne, R. H. Nussenzveig and J. M. C. Ribeiro, (1995) J. Biol. Chem. **270**, in the press]. To exclude the possibility of the nitrophorin being a contaminant, another chromatographic protocol was performed, consisting of chromatofocusing followed by strong-cation-exchange chromatography. Again the anticoagulant was eluted with nitrophorin 2. Nitrophorin 2 inhibits coagulation Factor VIII-mediated activation of Factor X and accounts for all the anti-clotting activity observed in *Rhodnius* salivary glands.

INTRODUCTION

Blood-sucking animals have developed a sophisticated array of salivary antihaemostatic compounds to counteract their host's defence against blood loss. Indeed, anti-clotting, anti-platelet and vasodilatory substances have been characterized in various arthropods, nematodes and vampire bats [1–5]). Anti-clotting agents have been recognized in the salivary glands of blood-feeding ticks and insects for about 80 years [6], but only more recently a few of these compounds have been characterized biochemically from organisms such as leeches [7–8], *Ancylostoma* hookworms[9], the soft tick *Ornithodorus moubata* [10–11], the blackfly *Simmulium vittatum* [12] and the triatomine bug *Rhodnius prolixus* [13].

Hellmann and Hawkins [14] have identified and named two anti-clotting activities from the blood-sucking bug *R. prolixus*. Prolixin S was postulated to be an anti-(Factor VIII) and found in salivary glands, and prolixin G, a thrombin inhibitor found in the anterior midgut of the insect. Recently Friedrich et al. [13] have purified and cloned a thrombin inhibitor found in whole-body homogenates of *R. prolixus*. Although Friedrich et al. [13] presumed a salivary origin for this inhibitor, it is possible that it represented the purification of prolixin G and not prolixin S, if not from foreign source, since whole blood-fed insects were used.

According to Hellmann and Hawkins [14], prolixin S did not retard a one-stage prothrombin time, and greatly increased the kaolin/cephalin time, suggesting its action was before Factor Xa activation in the intrinsic pathway. The effect of salivary homogenates did not differ according to whether they were added before or after activation of the contact factors in the kaolin/cephalin assay, excluding effect on Factors XI and XII. Finally, reconstitution assays with Factor VIII- or IX-deficient plasmas indicated that the main activity of prolixin S was on Factor VIII. Evidence for the protidic nature of prolixin S was given by its sensitivity to trypsin and precipitation by acetone. No further characterization of prolixin S was pursued.

In the present study we purified and obtained the partial sequence of prolixin S. The pure salivary anti-clotting substance is a salivary haem protein formerly identified as nitrophorin 2 [15], which inhibits the action of Factor VIII activity and shows no sequence similarity to the thrombin inhibitor isolated by Friedrich et al. [13].

MATERIAL AND METHODS

Insect rearing

R. prolixus was reared and maintained in the central insectary facility of the Center for Insect Sciences. All instars fed on an anaesthetized rabbit. Salivary glands from unfed fifth-instar nymphs were dissected under PBS [10 mM sodium phosphate (pH 7.0)/150 mM NaCl], transferred to 1.5 ml conical plastic tubes containing 100 μl of 10 mM Tris/HCl, pH 7.4, and stored, usually 100 pairs/vial, at −75 °C until needed. Salivary homogenates were prepared by homogenizing the glands in the conical tubes by using a plastic pestle (Kontes pellet pestle, obtained from Fisher Scientific, Pittsburg, PA, U.S.A.). After centrifugation at 1400 *g* for 5 min, the supernatant was collected and used for the experiments.

Protein purification

HPLC was performed by using either a CM4000 or CM4100 pump from Thermoseparation Products (Riviera Beach, FL, U.S.A.) and either a SM4000 or a SM4100 dual-wavelength detector from the same company. The detector output was connected to the serial port of a 80286- or a 80386-based microcomputer with math co-processor, and the absorbance recorded 50 times/min with a home-made computer program written in QUICKBASIC. Weak-cation-exchange chromato-

Abbreviation used: NP, nitrophorin; aPTT, partial activated thromboplastin time.

‡ To whom correspondence should be addressed.

graphy was used with a Alltech (Deerfield, IL, U.S.A.) WCX macrosphere column 25 cm × 4.3 mm. Buffer and gradient conditions were: Solution A, 20 mM sodium Hepes buffer, pH 6.9; Solution B, Solution A + 2 M NaCl. A linear gradient from 100% A to 75% A and 25% B in 30 min was used. Weak-anion-exchange chromatography was used with a TSK-DEAE column (15 cm × 7.5 mm) obtained from Bio-Rad (Hercules, CA, U.S.A.). Buffer and gradient conditions were Solution A, 10 mM Tris/HCl, pH 8.3; Solution B, Solution A + 2 M NaCl. A linear gradient from 100% A to 75% A and 25% B in 30 min was used. Hydrophobic-interaction chromatography was used with a phenyl-TSK column (15 cm × 7.5 mm) obtained through Bio-Rad. Buffer and gradient conditions were: Solution A, 2 M $(NH_4)_2SO_4$ + 20 mM sodium Hepes, pH 7.0; Solution B, 20 mM sodium Hepes, pH 7.0. A linear gradient from 100% A to 100% B in 60 min was used. Reverse-phase chromatography was used with a Hamilton PRP-∞ octadecyl column (10 cm × 4.3 mm) obtained through Alltech. Buffer and gradient conditions were: Solution A, 20% acetonitrile in water + 0.1% trifluoroacetic acid; Solution B, 60% acetonitrile in water + 0.1% trifluoroacetic acid. A linear gradient from 100% A to 100% B in 60 min was used. Chromatofocussing was used with a Pharmacia (Uppsala, Sweden) Mono P column (20 cm × 0.5 cm) in the range of pH 8.3–7.0 according to the manufacturer's instructions. Solution A was 25 mM triethanolamine/HCl, pH 8.3; solution B was Polybuffer 96 (Pharmacia) diluted 1:10 in water, pH 7.0. After equilibration of the column in Solution A, 2 ml of salivary homogenate sample, previously dialysed against several changes of water for 12 h, was diluted with concentrated triethanolamine to give 25 mM triethanolamine/HCl, pH 8.3, and injected into the column. The column was eluted with Solution B. Strong-cation-exchange was used in an Alltech SCX macrosphere column (25 cm × 4.3 mm). Buffer and gradient elution conditions were: Solution A, 20 mM sodium acetate, pH 5.0; Solution B, 20 mM sodium acetate, pH 5.0, plus 1 M NaCl. A linear gradient from 100% A to 100% B in 60 min was used. All chromatographic procedures were done at room temperature and at an elution rate of 0.5 ml/min; fractions were collected at 1 min intervals.

Amino acid sequencing

The N-terminal sequence of the purified anticoagulants were performed at the Biotechnology facility of the University of Arizona under Dr. Wallace Clark's supervision. Laser-desorption MS, Lys-C digestion, peptide separation by C_{18} reverse-phase chromatography and N-terminal sequencing of the resulting peptides were performed in the Harvard Microchemical Laboratory, Cambridge, MA, U.S.A. under the direction of Dr. William Lane.

Clotting assays

Recalcification times and partial activated thromboplastin time (aPTT) were assessed using a Thermomax microplate ELISA reader (Molecular Devices, Menlo Park, CA, U.S.A.) having a kinetic module. Flat-bottom well plates (96 wells; Falcon 3912; Beckton and Dickinson, Oxnard, CA, U.S.A.) were used. For the recalcification time, 50 μl of human citrated platelet-poor plasma and 50 μl of 0.15 M NaCl/10 mM sodium Hepes, pH 7.4, containing or not the sample to be tested for anti-clotting activity, were mixed for 2 min at 37 °C, followed by addition of 50 μl of pre-warmed (37 °C) 25 mM $CaCl_2$. An eight-channel multipipette was used for delivering portions of $CaCl_2$. After the addition of the $CaCl_2$, the plate was mixed and maintained at 37 °C using the apparatus mixer and heating system and absorbance readings at 650 nm taken at 10 s intervals. A fast and sharp increase in the absorbance after a lag phase indicated clotting. We chose the time taken for reaching a 0.1 or 0.05 absorbance value (onset absorbance) as a measure of clotting time, using the 'time to selected absorbance' module included in the instrument's software. With this procedure, all the fractions resulting from a whole chromatographic procedure could be analysed quickly and simultaneously for anti-clotting activity. The aPTT tests were performed according to the method of Proctor and Rapaport [16] using a kaolin suspension in the presence of rabbit brain cephalin (Sigma Diagnostics, St. Louis, MO, U.S.A.) as indicated by the manufacturer, but using the ELISA reader kinetic module described above. To 50 μl of citrated human plasma, 50 μl of different dilutions of crude salivary homogenates or purified fractions in Hepes/saline buffer (10 mM Hepes/0.15 M NaCl, pH 7.4) were mixed inside the plate wells, which contained 25 μl of kaolin/cephalin mixture (equal volumes of 8% kaolin/cephalin working suspension in the Hepes/saline buffer). After incubating at 37 °C, with shaking for 3 min, 25 μl of pre-warmed (37 °C) 50 mM $CaCl_2$ was added by using an eight-channel multipipette. The plate was then shaked once and maintained at 37 °C, with the onset absorbance at 0.05 unit. The absorbance readings at 650 nm were recorded at 10 s intervals. For both recalcification and aPTT assays, clotting times were referred to the plasma control samples assayed under the same conditions, but in the absence of salivary homogenates or their purified fractions. One unit of anti-coagulant activity was defined as the amount of protein able to double the clotting time of normal plasma controls.

Thrombin and Factor Xa assays

Thrombin- and Factor Xa-inhibiting activity were done with the chromogenic substrates benzoyl-Phe-Val-Arg *p*-nitroanilide hydrochloride and benzoyl-Ile-Glu-Gly-Arg *p*-nitroanilide trifluoroacetate (S-2222) respectively, obtained from Calbiochem (San Diego, CA, U.S.A.).

Anti-(Factor VIII) activity

Anti-(Factor VIII) activity was assayed using COATEST Factor VIII kit from Helena Laboratories (Beaumont, TX, U.S.A.), according to the recommendations of the manufacturer. This test measures the activation of Factor X (determined by the chromogenic substrate S-2222) in the presence of Factor IXa, Ca^{2+}, phospholipid and I-2581 (a thrombin inhibitor), the activation of Factor X being dependent on the amount of added Factor VIII. In the absence of Factor VIII, activation of Factor X is delayed. Whenever needed, platelet-poor human plasma from one of us (J. M. C. R) was used.

RESULTS

Preliminary experiments using whole salivary-gland homogenates

Preliminary experiments confirmed previously obtained results [14], namely that homogenates from *R. prolixus* salivary glands increased human recalcification time and aPTT, and affected neither the prothrombin time nor the direct thrombin or Factor Xa activities towards their chromogenic substrates. The anti-clotting activity of the crude homogenate accounted for 10.3 ± 0.54 units per pair of salivary glands (mean ± S.E.M., $n = 3$). The use of a kit to detect decrease in Factor VIII activity indicated that adding the salivary homogenate to human platelet poor plasma prevented activation of Factor X to Factor Xa

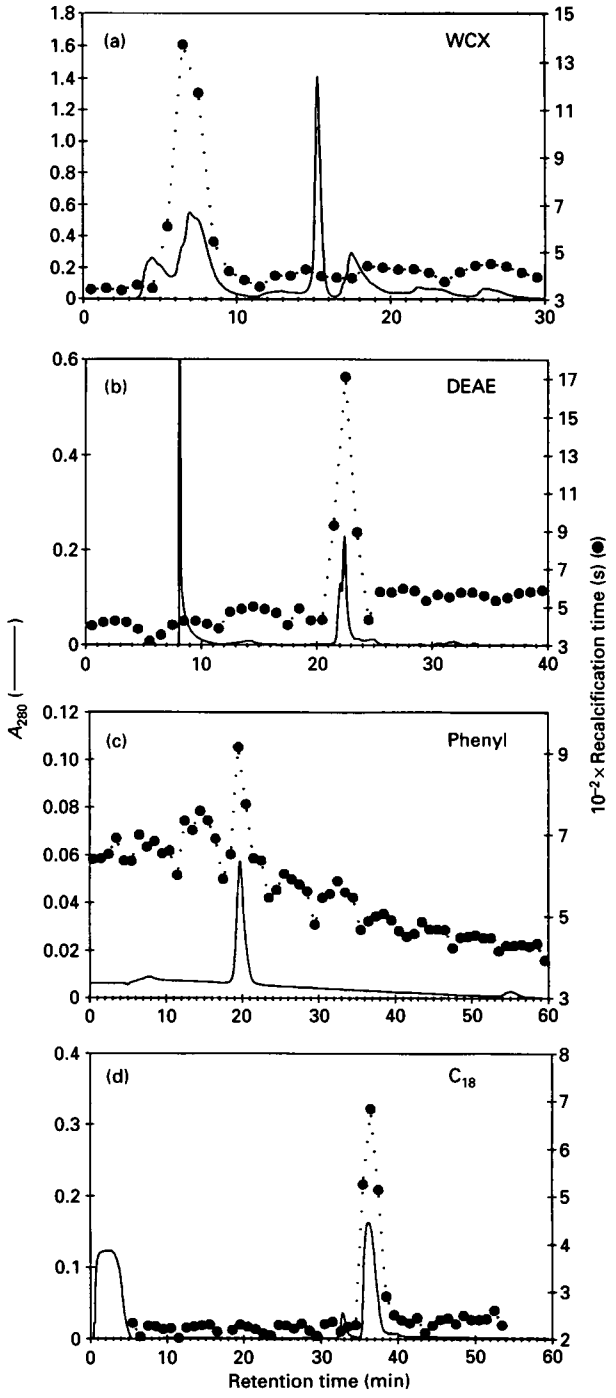

Figure 1 Purification of the salivary anticoagulant from *R. prolixus* by sequential chromatographic fractionation on weak-cation-exchange (WCX) (a) weak-anion-exchange (DEAE) (b), hydrophobic-interaction (Phenyl) (c) and by reverse-phase (C_{18}) (d) chromatography

A total of 100 homogenized salivary glands from fifth-instar bugs were applied to the weak-cation-exchange column. Active fractions containing anticoagulant activity were applied to each subsequent column. Flow rates were 0.5 ml/min, and fractions were collected at 1 min intervals. Absorption at 280 or 220 nm was recorded. Aliquots of 10 μl (weak-cation-exchange and DEAE columns) or 1 μl (phenyl) were used for the anti-clotting assay. For monitoring the anti-clotting activity from the C_{18} column, 100 μl of each fraction was mixed with 10 μl of BSA at 1 mg/ml, followed by drying and reconstitution to 100 μl of Hepes-buffered saline and using 50 μl of this solution for the anti-clotting assay. Aliquots from each fraction were used to test for inhibition of clotting of citrated plasma by the recalcification time assay. For more details, see the Materials and methods section.

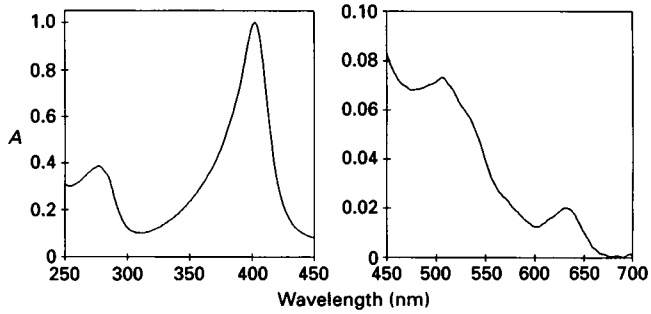

Figure 2 UV and visible spectra of the anticoagulant principle obtained from the hydrophobic-interaction column, after dialysis, freeze-drying and reconstitution of the fractionated material in 0.1 M sodium phosphate buffer, pH 7.0

through the intrinsic pathway (results not shown). These results are in contrast with those of Friedrich et al. [13].

Purification of the salivary gland anti-clotting activity

To purify prolixin S we submitted 100 pairs of homogenized glands (approx. 10 mg of total protein) to chromatography on a weak cation-exchange (WCX) column (Figure 1a). A 10 μl portion of each fraction were used to assay anti-clotting activity on a recalcification time assay, indicating that fractions eluted between 7 and 9 min contained anti-clotting activity. The two fractions were combined (1 ml), and diluted with water to 2 ml, followed by chromatography in a TSK-DEAE column. A recalcification time assay of 10 μl from each fraction indicated further purification of the anti-clotting activity (Figure 1b). The active fractions eluted between 22 and 25 min (1.5 ml) were pooled, satd. $(NH_4)_2SO_4$ was added to 2 M final concentration, and the fractions subjected to hydrophobic-interaction chromatography (phenyl-TSK gel). Because the high salt content of this column's eluate leads to interference with the clotting assay, we used only 1 μl from each fraction to locate the anti-clotting activity. Despite a relatively weak signal, the activity was co-eluted with the main eluted peak absorbing at 280 nm (Figure 1c). Throughout this whole procedure we observed that the anticoagulant activity was co-eluted with a reddish-brown pigment. The anticlotting fractions resulting from the hydrophobic-interaction column displayed the typical spectra of a haem protein (Figure 2). To desalt the fractions eluted from the phenyl-TSK column, as well as to obtain a further indication of the purity of the anticoagulant, we submitted the active fractions to reverse-phase chromatography. The fractions from hydrophobic-interaction chromatography eluted from 19 to 22 min were pooled (1.5 ml); acetonitrile and trifluoroacetic acid were added to give final concentrations of 20% and 0.1% (v/v) respectively, and the fractions injected into an octadecyl reverse-phase column. From each fraction of volume 0.5 ml, 0.1 ml was removed, 10 μl of 1% BSA was added, and the buffer evaporated by centrifugation *in vacuo*. A major peak absorbing at 220 nm contained the anti-clotting activity (Figure 1d). This four-step purification procedure yielded a 5.4% recovery of the initial activity and a purification index of 23-fold. The N-terminal sequence of 30 amino acid residues of the protein resulting from the reverse-phase column:

DCSTNISPKQGLDKAKYFSGKKYVTHFLDK

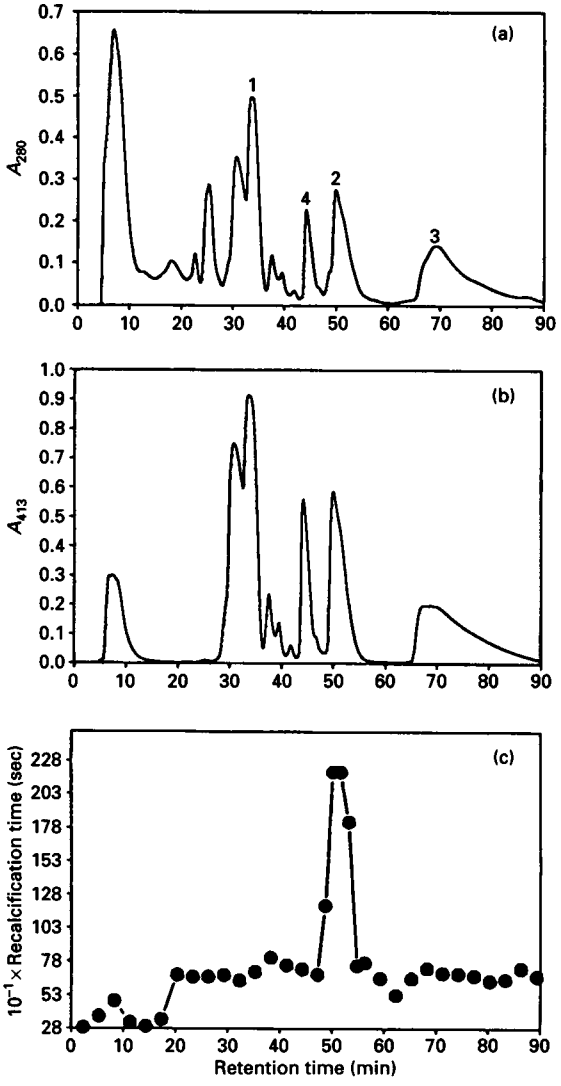

Figure 3 Chromatofocusing of 100 pairs of salivary glands from fifth-instar nymphs of *R. prolixus* on a Mono P column (Pharmacia), running a gradient from pH 8.0 to 7.0

The Arabic numerals above the peaks in the top panel indicate the nomenclature used for nitrophorins 1–4 [15]. Absorbance at 280 (**a**) and 413 (**b**) nm were recorded in a dual-wavelength detector. A 5 μl portion from each fraction was used in the plasma recalcification clotting assay (**c**).

indicated complete identity with the sequence of our previously isolated nitrophorin 2 [15].

Confirmation of anti-clotting activity of nitrophorin 2

Because the isolated haem protein could be a major contaminant and responsible for the observed anticoagulant activity, we decided to purify the haem proteins following our protocol for nitrophorin purification [15]. A total of 300 pairs of salivary glands were chromatofocused on a Mono P column eluted from pH 8.3 to 7.0. Anticoagulant activity (using 5 μl of each column fraction/assay) co-eluted with the nitrophorin 2 (Figure 3). Further chromatographic fractionation of the pooled active fractions into a strong-cation exchanger (SCX column) led to

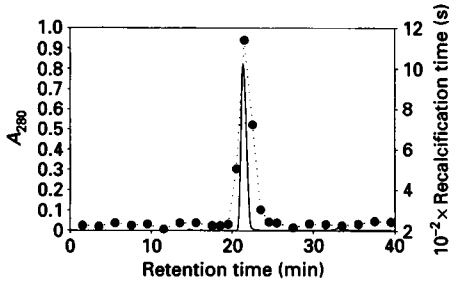

Figure 4 Strong-cation-exchange chromatography of the active material eluted from the chromatofocusing column (shown in Figure 3)

The flow rate was 0.5 ml/min, and fractions were collected at 1 min intervals. Absorbance at 280 nm was recorded. A 5 μl portion from each fraction was used in the plasma recalcification clotting assay.

our recovering only a single major haem-protein peak containing the anticoagulant activity (Figure 4). The N-terminal sequence from ten residues of the pooled fractions from the strong-cation-exchange column (after dialysis and freeze-drying) yielded:

DCSTNISPKQ

a sequence identical with that of the purified anti-clotting peptide obtained by the above procedure. This two-step purification protocol for the salivary anticoagulant led to a higher yield of the activity (58.7%) when compared with the four-step protocol described above, and a similar purification index of 25 (Table 1), indicating that the anticoagulant represents approx. 4% of the total salivary-gland protein. It is therefore concluded that nitrophorin 2 obtained by two independent procedures expresses potent and unique anticoagulant activity.

Partial sequence of nitrophorin 2 and comparison with nitrophorin 1

After dialysis and freeze-drying of the strong-cation-exchanger-column fractions, the pure protein was submitted to digestion with Lys-C, and the resulting peptides were separated by reverse-phase (C_{18}) HPLC. Three additional peptidic fragments were obtained. One of them was:

YVTHFLDKDPQVTDQYESSFTPR

of which the first eight amino acids matched the last eight residues of the 30-amino-acid sequence reported above (singly underlined part of the sequence). Accordingly, the two fragments are tentatively shown as one sequence in Figure 5(a), having as a guide the best alignment with nitrophorin 1. The two other peptides matched sequences near the C-terminal region of nitrophorin I (Figure 5a and 5b). A search on combined databanks (PDB + SwissProt + PIR + SPUpdate + GenPep + GPUpdate) using the BLAST program from GCG (University of Wisconsin Genetics Computer Group) software revealed no substantial homologies with other recorded sequences.

Characterization of the anti-clotting activity of nitrophorin 2

Pure nitrophorin 2 delayed activation of Factor X by Factor IXa in the presence of Ca^{2+} and phospholipids, a step accelerated by

Table 1 Purification of nitrophorin 2 from 300 pairs of salivary glands from *R. prolixus*

Step	Protein (μg)	Yield (%)	Anticoagulant activity (units*)	Yield (%)	(units/μg)	Purification index
Homogenate	12396†	100	2757	100	0.22	1.0
CMTF‡	1240†	10	2340	85	0.73	3.3
SCX§	293‖	4	1617	59	5.52	25.1

* One unit of anti-clotting activity is defined as the amount doubling the recalcification time.
† Estimates based on the absorption coefficient using BSA as an standard.
‡ CMTF, chromatofocusing purification step.
§ SCX, strong-cation-exchange purification step.
‖ Estimated from the absorption coefficient for pure NP2 at 280 nm as 2.92 for a solution of 1 mg/ml (D. Champagne, R. H. Nussenzveig and J. M. C. Ribeiro, unpublished work).

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(a)
NP2     1 DCSTNISPKQGLDKAKYFSGK.KYVTHFLDKDP.QVTDQYESSFTP 44
          .|..|  :. |::|.|||.|. .|||.:|| :| :|...| ..:.:
NP1    24 KCTKNALAQTGFNKDKYFNGDVWYVTDYLDLEPDDVPKRYCAALAA 69

(b)
NP2     1 EGSKDLGDLYTVLTHQK 17
          .|.|||||||.||.:.|
NP1   148 KGNKDLGDLYAVLNRNK 164

(c)
NP2     1 SAVTQAGLQLSQFVGTK 17
          :|||.|:|.:|:|::||
NP1   174 GAVTAASLKFSDFISTK 190
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Figure 5 N-terminal and peptidic fragments of nitrophorin 2 (NP2) as compared with nitrophorin 1 (NP2)

(**a**) N-terminal sequence of nitrophorin 2 and its best alignment with nitrophorin 1 fragment 24–69. The percentage similarity is 54.55 and the percentage identity 36.36. The first 23 amino acids of nitrophorin 1 are part of a signal peptide. The N-terminal region of the mature protein starts at amino acid 24. (**b**) Peptide fragment of nitrophorin 2 and its similarity to nitrophorin 1 sequence 148–164. The percentage similarity is 88.24 and the percentage identity 52.94. (**c**) Peptide fragment of nitrophorin 2 with homologous nitrophorin 1 sequence 174–190. The percentage similarity is 70.59 and the percentage identity 64.71. Comparisons were made with the BESTFIT module of the GCG software. Explanation of symbols: |, identity of amino acid residue; ., conserved replacement; :, partially conserved replacement.

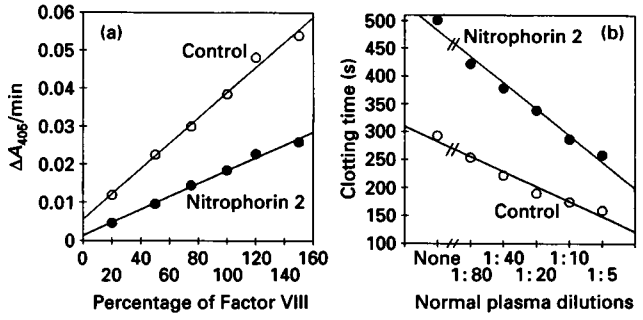

Figure 6 (a) Anti-(Factor VIII) activity of pure nitrophorin 2 and (b) effect of nitrophorin 2 on the recalcification time assay of Factor VIII-deficient plasma

(**a**) Factor IXa, Factor X, phospholipids, buffer and nitrophorin 2 were mixed for 2 min at 37 °C, then various amounts of Factor VIII were added (as a percentage of the normal plasma equivalent), in a final volume of 75 μl. After further incubation for 5 min, 25 μl of 25 mM $CaCl_2$ was added and the mixture incubated for an additional 5 min, after which 50 μl of the substrate mixture was added. Activation of Factor X was measured by the ability of Factor Xa to cleave the synthetic substrate S-2222 in the presence of I-2581, a thrombin inhibitor, producing various rates of colour change at 405 nm. This assay is based on the COATEST kit (Helena Laboratories). (**b**) Nitrophorin 2 was present at 0.2 μg per assay.

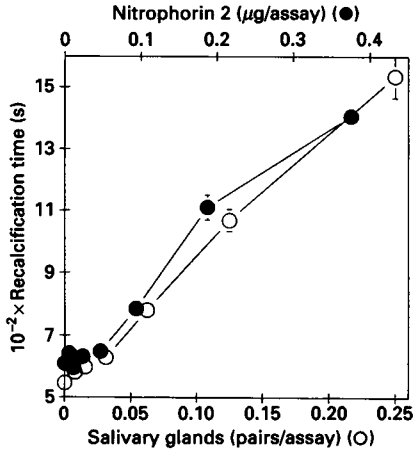

Figure 7 Increased recalcification time in the presence of nitrophorin 2 or salivary-gland homogenates

The scale representing amounts of nitrophorin 2 added per assay is made in proportion with the expected amount of nitrophorin 2 per pair of glands.

Factor VIII, thus confirming its anti-(Factor VIII) activity postulated 30 years ago by Hellmann and Hawkins [14] (Figure 6a). Accordingly, correction of the clotting time of Factor VIII-deficient plasma by normal plasma dilutions was consistently inhibited by nitrophorin 2 (Figure 6b). In order to estimate to whether all the salivary anticoagulant activity in *R. prolixus* salivary glands could be attributed to nitrophorin 2, a parallel plasma recalcification assay using increasing concentrations of salivary-gland homogenates or nitrophorin 2 was performed (Figure 7). Taking into consideration the molar absorption coefficients for each nitrophorin [15] and the amount of protein estimated by measuring the area under the 280 nm absorption curve of the chromatofocusing column (Figure 3a) for each nitrophorin, we arrived at a total nitrophorin content of 325 pmol per pair of glands, and close to the value of 282 pmol of NO per pair of glands reported previously [17]. Of this total, 20% is accounted for by nitrophorin 2, or 65 pmol per pair of glands or 1.28 μg of nitrophorin 2 per pair of glands based on the molecular mass of 19689 Da obtained by laser-desorption MS [15]. Also, from clotting experiments with pure nitrophorin 2, we estimated an anti-clotting activity of 5534 ± 615 units/mg of protein (mean $\pm$ SEM, $n = 5$), close to what was estimated for the crude

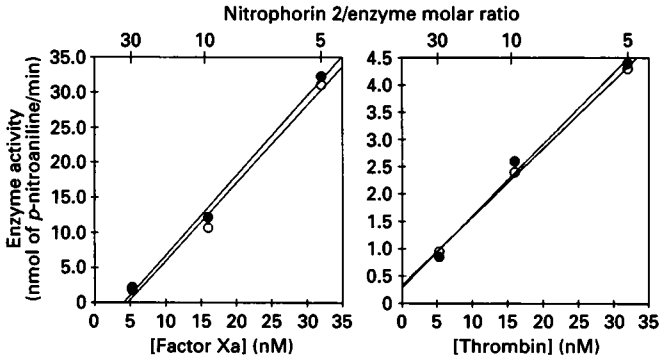

Figure 8 Lack of effect of nitrophorin 2 on Factor Xa and thrombin amidolytic activities

Factor Xa and thrombin were assayed with the synthetic substrate S-2222 and benzoyl-Phe-Val-Arg *p*-nitroanilide respectively by measuring the increase in absorbance at 405 nm and using a value of 10 m*A*/min as representing an enzyme activity of 1 nmol/min. Open symbols represent controls without nitrophorin 2 and closed symbols represent experiments performed with the indicated amounts of nitrophorin 2.

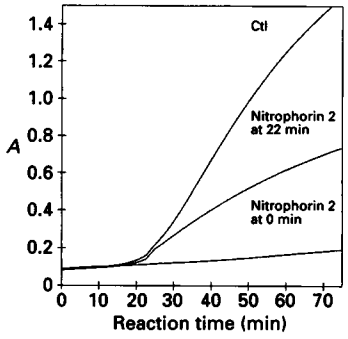

Figure 9 Effect of time of addition of nitrophorin 2 on the Factor VIIIa-mediated activation of Factor X

The COATEST kit (Helema Laboratories) was used for these experiments; 100% plasma units (as defined by the manufacturer) were used. Ctl indicates reaction in the absence of nitrophorin 2. Other curves indicates addition of nitrophorin 2 (0.48 μg/assay) at the beginning of the reaction, or at 22 min, after the lag phase necessary for assembly of the Factor Xa with activated Factor VIII and phospholipids. Other reaction conditions were similar to those of Figure 6. Similar results were obtained in three other experiments.

homogenate. Results comparing nitrophorin 2 with whole homogenates on the blood plasma recalcification time indicate that nitrophorin 2 alone can explain all anticoagulant activity of whole-salivary-gland homogenates.

Since Factor VIII is liable to activation by thrombin and Factor Xa, a detailed experiment using pure nitrophorin 2 was carried out to test its activity against the amidolytic activities of these two clotting Factors. No inhibitory activity was found, even when molar ratios were 30 times larger in favour of nitrophorin 2 (Figure 8). To investigate further the effect of nitrophorin 2 on the Factor VIIIa-dependent activation of Factor X, nitrophorin 2 was added to the Factor IXa–Factor VIII–phospholipid complex provided by the COATEST kit, at the beginning of the reaction or after the lag phase necessary for the formation of activated Factor VIII. While almost complete inhibition of the reaction occurred if nitrophorin 2 is added at zero time, significant inhibition still occurs if nitrophorin 2 is added after formation of the complex and activation of Factor VIIIa (Figure 9).

DISCUSSION

Purification of the salivary anticoagulant activity of *R. prolixus* by two independent chromatographic protocols led to the identification of nitrophorin 2 [15] as the anti-(Factor VIII) molecule present in *R. prolixus* glands. Nitrophorins are ferric haem proteins found in the salivary glands of *R. prolixus* ([15, 17] which carry NO from the salivary glands of the bug to the skin of its vertebrate host, accounting for most of the salivary vasodilatory activity found in *R. prolixus* glands [17]. The role of NO in the anti-clotting activity can be dismissed on the basis of the fact that the purified haem protein has no bound NO, as shown by the Soret absorption band of the purified protein [15, 17], as well as the lack of activity of the other haem proteins.

It is interesting to note the acquisition of multiple functions in nitrophorins. Recently we described the antihistamine activity of nitrophorins [17], another function that might help *R. prolixus* to feed. Indeed the order Hemiptera (bugs) is evolutionarily old, having appeared at the Carboniferous period. *Rhodnius* and its ancestors may have been in the habit of blood feeding on reptiles much before mammals appeared (50 million years ago), giving ample time to accumulate multiple functions in proteins that are important in the process of obtaining a blood meal.

The anti-(Factor VIII) activity of nitrophorin 2 may reside in a relatively small stretch of the total molecule not related to the NO binding, because the other three nitrophorins in *R. prolixus* glands do not display anticoagulant activity, but have similar NO-carrying functions. In particular, nitrophorin 3 shows more sequence similarity at the N-terminal end with nitrophorin 2 than to either nitrophorin 1 and nitrophorin 4 [15], but only nitrophorin 2 displays anti-clotting activity. On the other hand, Factor VIII is a very large protein containing two subunits which interact with Factor IXa, phospholipids and Ca^{2+}, thus behaving as a cofactor to mediate and to enhance the activity of Factor IXa on Factor X to produce Factor Xa [19]. Because nitrophorin 2 does not inhibit thrombin and Factor Xa (Figure 8), the observed anti-(Factor VIII) activity of nitrophorin 2 cannot be due to inhibition of the activation of Factor VIII by these two enzymes. It appears that nitrophorin 2 is a specific inhibitor of Factor VIII and/or Factor VIIIa. No specific inhibitors of Factor VIII have been reported so far, although proteinases such as protein C, Factor IXa or Factor Xa can inactivate Factor VIII by proteolysis, and anti-(Factor VIII) antibodies can also deplete plasma of functional Factor VIII [19]. Further characterization of nitrophorin 2 (prolixin S) may yield insights on the molecular organization of the Factor X-activating complex and of the role of Factor VIII in this process.

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