# Isolation and Characterization of an Anticoagulant Protein Homologous to Botrocetin from the Venom of *Bothrops jararaca*†

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ABSTRACT: We previously isolated a unique anticoagulant protein named IX/X-bp (factor IX/factor X-binding protein) from the venom of the habu snake *Trimeresurus flavoviridis*. We recently determined its primary structure and found that this protein had a structure homologous to the carbohydrate-recognition domains of C-type lectins. Most interestingly, a high homology was found between this protein and botrocetin, an inducer of platelet agglutination found in the venom of the jararaca snake *Bothrops jararaca*. To examine the possible identiy of these proteins, we searched for IX/X-bp-like protein(s) in the venom of *B. jararaca*. When the venom was subjected to DEAE anion-exchange chromatography, such an activity was eluted separately from that of botrocetin. This activity was purified to homogeneity and designated jararaca IX/X-bp. Jararaca IX/X-bp was a disulfide-linked heterodimer consisting of 16- and 15-kDa subunits, being structurally similar to botrocetin. The respective $NH_2$-terminal amino acid sequences were also very similar. Jararaca IX/X-bp had no botrocetin-like activity. However, this protein did have an activity to bind to factors IX and X and protein S in a $Ca^{2+}$-dependent fashion, that resulted in interference with coagulation, while botrocetin did not. The binding to coagulation factors appeared not to be mediated by the lectin-like activity of jararaca IX/X-bp, because a derivative of factor X free of carbohydrates retained the ability to bind. It is concluded, therefore, that the two proteins isolated from the same venom have different biological activities despite the high degree of structural similarity between them.

Snake venoms contain various biologically active substances that are often used as convenient tools in biochemical and physiological investigations. Their effects on blood coagulation/platelet aggregation systems have also been well documented, and a number of coagulant and anticoagulant activities have been identified [for reviews, see Tu (1977) and Pirkle and Markland (1987)]. Recently, we isolated a novel type of anticoagulant protein from the venom of the habu snake *Trimeresurus flavoviridis*. This protein is a disulfide-linked heterodimer consisting of 16- and 15.5-kDa subunits (A and B chains, respectively), and can bind to factors IX and X in a $Ca^{2+}$-dependent fashion. For this reason, it has been designated IX/X-bp[1] (factor IX/factor X-binding protein; Atoda & Morita, 1989). IX/X-bp forms a complex with factors IX or X with a stoichiometry of 1 to 1, and thereby blocks the amplification of the coagulation cascade (Atoda & Morita, 1989).

More recently, we determined the amino acid sequence of IX/X-bp (Atoda et al., 1991) and the position of disulfide bonds in IX/X-bp (Atoda & Morita, 1993). The obtained primary structures revealed that the two chains of IX/X-bp are similar (47% identical) and both belong to the C-type ($Ca^{2+}$-dependent) lectin family: amino acid residues characteristic of the carbohydrate-recognition domain in C-type lectins (Spiess, 1990) are well conserved. Homology with this protein was found in the carbohydrate-recognition domain-like structure of some mammalian proteins such as asialoglycoprotein receptor, proteoglycan core protein, and tetranectin (Atoda et al., 1991). Most interestingly, a recent report by Usami et al. (1993) indicates that botrocetin,[2] a von Willebrand factor (vWF)-dependent inducer of platelet agglutination isolated from the venom of the jararaca snake *Bothrops jararaca* (Read et al., 1978), is also a member of this family and exhibits extremely high homology to IX/X-bp. Botrocetin is also a disulfide-linked heterodimeric protein consisting of 15- and 14.5-kDa subunits (Fujimura et al., 1991). The A chain of IX/X-bp and the $\alpha$ chain of botrocetin are 62% identical, and the B chain and the $\beta$ chain are 52% identical (Atoda et al., 1991; Usami et al., 1993). This unexpectedly high degree of homology led us to the hypothesis that these proteins might be identical proteins of different origin, in other words, that IX/X-bp and botrocetin might have the same biological activity. To examine this hypothesis, we searched for a IX/X-bp-like protein(s) in the venom of *B. jararaca* in the present study, and we found that the jararaca IX/X-bp that we obtained was apparently different in terms of function from botrocetin despite the high degree of structural homology.

## EXPERIMENTAL PROCEDURES

*Materials.* Suppliers of materials used in this study were as follows: lyophilized *B. jararaca* venom, either Latoxan

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[1] Abbreviations: IX/X-bp, factor IX/factor X-binding protein; habu IX/X-bp, IX/X-bp isolated from the venom of the habu snake *T. flavoviridis*; jararaca IX/X-bp, IX/X-bp isolated from the venom of the jararaca snake *B. jararaca*; vWF, von Willebrand factor; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, FPLC, fast protein liquid chromatography; ACD, acid–citrate dextrose; TBS, 20 mM Tris-HCl/140 mM NaCl, pH 7.5; PBS, phosphate-buffered saline, pH 7.5; BSA, bovine serum albumin; Gla, $\gamma$-carboxyglutamic acid; RVV-X, Russell's viper venom factor X-activating enzyme; PVDF, poly(vinylidene difluoride).

[2] Two types of botrocetin have been reported (Fujimura et al., 1991), having one chain and two chains, respectively. Despite their similar biological activities, their structures are very different, and the two-chain type of botrocetin is the one that is homologous to IX/X-bp (Usami et al., 1993). We refer only to the two-chain type of botrocetin, which we designate simply botrocetin in the present study.

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(Rosans, France; lot PA205) or Sigma (lots 119F-0599, 91H-0626, and 22H-0937); lyophilized *T. flavoviridis* venom, The Japan Snake Institute (Gunma, Japan); DEAE-Sepharose Fast Flow, Sephacryl S-200 High Resolution, and Mono-Q 5/5, Pharmacia. Factor IX-Cellulofine was prepared by coupling bovine factor IX to formyl-Cellulofine (Seikagaku Kogyo, Tokyo, Japan) by the method described previously (Atoda & Morita, 1989).

*Proteins.* Habu IX/X-bp was purified from the venom of *T. flavoviridis* as described previously (Atoda & Morita, 1989). The following plasma proteins of bovine origin were purified as described in the cited references: prothrombin, factor IX, factor X, protein C, protein S, and protein Z (Hashimoto et al., 1985); factor VII (Strickland & Castellino, 1981); and vWF (Mascelli et al., 1986). Factor X was activated by Russell's viper venom factor X-activating enzyme (RVV-X) and allowed to stand so that autolysis could proceed, and the resultant factor Xa$\beta$ was freed from its activation peptide and fragment 4 by gel filtration (Morita & Jackson, 1986).

*Enzyme-Linked Immunosorbent Assay.* Antiserum against habu IX/X-bp was obtained by immunizing a rabbit with the purified protein. Microtiter wells were coated with 50 $\mu$L of a solution of 1 $\mu$g/mL factor IX in TBS (20 mM Tris-HCl/140 mM NaCl, pH 7.5) at 4 °C overnight and blocked with 100 $\mu$L of 1% BSA (fatty acid free, Sigma) in TBS for 1 h. Coated wells were then incubated with 50 $\mu$L of a crude preparation of venom appropriately diluted with TBS supplemented with 5 mM $CaCl_2$ and 0.1% Tween-20 for 1 h at ambient temperature, and this buffer was used throughout the subsequent procedures. After the wells were washed, they were reacted with 50 $\mu$L of antiserum (diluted 1:1000) for 1 h, and then with peroxidase-conjugated anti-rabbit IgG (diluted 1:100, from Kirkegaard and Perry Laboratories, Gaithersburg, MD) for 1 h. Reaction with the antigen was visualized by incubation with *o*-phenylenediamine and $H_2O_2$ in 100 $\mu$L of 0.1 M citrate buffer, pH 5.5.

*Purification of Jararaca IX/X-bp and Botrocetin.* One hundred milligrams of crude *B. jararaca* venom (Latoxan) was dissolved in 10 mL of 84 mM imidazole hydrochloride, pH 7.4, containing 2 mM benzamidine, dialyzed against the same buffer, and centrifuged to pellet insoluble materials. The solution was then applied to a column (1.6 × 30 cm) of DEAE-Sepharose Fast Flow preequilibrated with the same buffer, and proteins were eluted with a gradient of NaCl in the buffer (Fujimura et al., 1991). The fractions containing factor IX-binding activity were pooled and dialyzed against TBS. Calcium chloride was added to 5 mM, and the solution was loaded onto a column of factor IX-Cellulofine (1.0 × 15 cm) preequilibrated with TBS containing 5 mM $CaCl_2$. After washing of unbound materials from the column with the same buffer, bound proteins were eluted with TBS containing 5 mM EDTA. The eluate was dialyzed against 20 mM Tris-HCl, pH 8.0, and loaded onto a Mono-Q column equipped to a FPLC system. The column was washed with the same buffer and then developed with a linear gradient of NaCl (0–0.5 M) in the buffer over 50 min at a flow rate of 1 mL/min. Jararaca IX/X-bp was eluted at 0.12 M NaCl. During this step, complete purification was accomplished, and it was then stored at 4 °C. The preparation was stable at least for 6 months.

Botrocetin was purified as follows from the same preparation of venom. The botrocetin-containing fraction from DEAE-Sepharose was concentrated by lyophilization, and subjected to gel filtration on a column of Sephacryl S-200 HR (1.6 × 80 cm) preequilibrated with TBS, and each fraction was assayed for botrocetin activity with platelet agglutination (see below). Active fractions were pooled, dialyzed against 20 mM Tris-HCl, pH 8.0, and subjected to FPLC on a Mono-Q column that was eluted with a linear gradient of NaCl in the buffer (0–1 M) over 50 min at a flow rate of 1 mL/min. Botrocetin was eluted at 0.5 M NaCl as the last peak of protein, and this preparation was apparently homogeneous. The purified botrocetin was dialyzed against saline and stored at 4 °C.

*Amino Acid Sequencing.* Forty micrograms of jararaca IX/X-bp in 50 $\mu$L of TBS containing 1 mM EDTA and 8 M urea was reduced by the addition of 1 mg of dithiothreitol under $N_2$ gas at 50 °C for 2 h; then 10 mg of iodoacetamide was added. Incubation was continued for 30 min at room temperature, and the reaction was terminated by the addition of 2-mercaptoethanol to 10% (v/v). The reduced *S*-acetamidomethylated polypeptides were separated by SDS–PAGE and electroblotted onto a PVDF membrane (Millipore) by the method of Hirano (1989). Protein bands were visualized by Amido Black staining, cut out, and subjected to analysis on an Applied Biosystems protein sequencer Model 473A.

*Preparation of Platelets and Agglutination Assay.* Bovine blood anticoagulated with ACD was obtained from a local slaughterhouse, and platelets were isolated and washed (Nishimura et al., 1975). Washed platelets were suspended in phosphate-buffered saline, pH 7.5 (PBS), fixed with 1% paraformaldehyde for 1 h, washed with PBS, and then stored frozen at −80 °C until use. Platelet agglutination was measured turbidimetrically in a Niko Bioscience Hematracer 601 aggregometer. To 250 $\mu$L of a suspension of fixed platelets ($2 \times 10^8$ platelets/mL) in PBS containing 20 $\mu$g/mL bovine vWF and 1 mg/mL BSA were added the samples to be tested in a minimum volume (less than 10 $\mu$L), and agglutination was monitored.

Alternatively, human venous blood was drawn into 0.1 volume of 3.8% sodium citrate from volunteers who had denied taking any medications, and platelet-rich plasma was obtained by centrifugation. One-tenth volume of ACD was added, and platelets were precipitated by centrifugation, washed with Tyrode's solution [4 mM *N*-(2-hydroxyethyl)piperazine-*N*'-2–ethanesulfonic acid, 137 mM NaCl, 2.7 mM KCl, 3.8 mM $NaH_2PO_4$, and 5.5 mM glucose, pH 7.4] containing 10% ACD, and finally suspended in Tyrode's solution containing 1 mM $CaCl_2$ ($2 \times 10^8$ platelets/mL). Agglutination (and/or aggregation) was carried out in the presence or absence of 10% (v/v) autologous plasma.

*Binding Assay with $^{125}$I-IX/X-bp.* IX-X-bp was labeled with $Na^{125}I$ (Dupont/New England Nuclear) by use of IODOBEADS (Pierce) in accordance with the manufacturer's instruction. Specific activities of labeled proteins were in the range of $1.0 \times 10^6$ to $2.0 \times 10^6$ cpm/$\mu$g. Binding assays were conducted as follows except where otherwise indicated. Wells of breakable microtiter plates (9502-470; Labsystems, Finland) were coated with a solution of 10 $\mu$g/mL protein in 50 $\mu$L of TBS at 4 °C overnight, and the remaining nonspecific binding sites were blocked by incubation with 1% BSA in TBS for 1 h. Each well was incubated with 50 000 cpm of $^{125}$I-IX/X-bp (approximately 0.5 $\mu$g/mL) plus samples to be tested in 50 $\mu$L of TBS containing 5 mM $CaCl_2$ and 1 mg/mL BSA for 2 h at ambient temperature. Wells were then washed twice with 200 $\mu$L of TBS containing $CaCl_2$, cut into pieces, and counted for bound radioactivity in a $\gamma$-counter.

*Other Methods.* SDS–PAGE was carried out by the method of Laemmli (1970). Protein concentrations were determined with the BCA Protein Assay kit (Pierce) with BSA as the standard. Kaolin-induced partial thromboplastin times and factor Xa-induced clotting times were determined by the

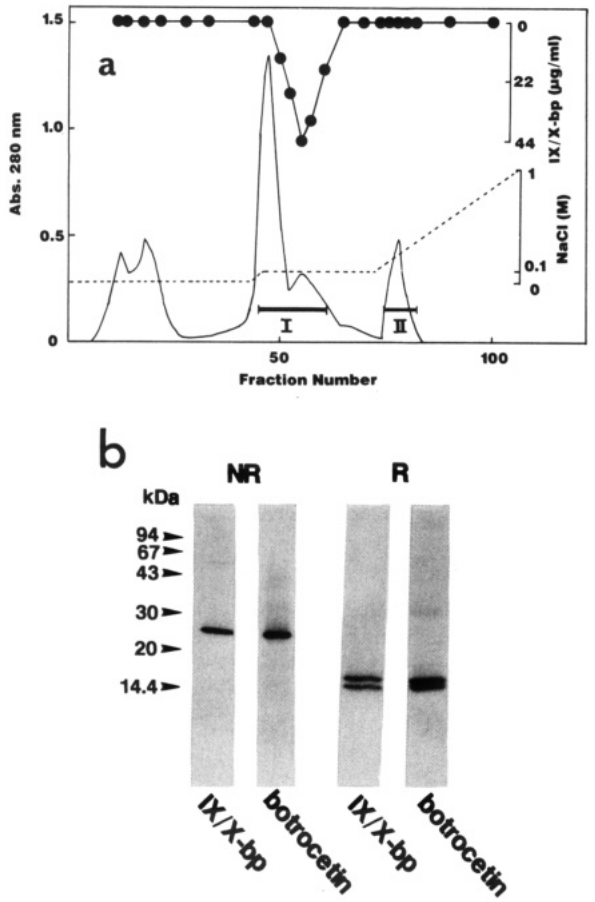


FIGURE 1: Purification of jararaca IX/X-bp. (a) Elution profile of jararaca IX/X-bp from DEAE anion-exchange chromatography. One hundred milligrams of dialyzed crude venom from *B. jararaca* was loaded onto a column of DEAE-Sepharose Fast Flow and eluted with a gradient of NaCl as indicated (dashed line). Five-milliliter fractions were collected and assayed for factor IX-binding activity (closed circles, see text) and botrocetin activity. Fractions containing factor IX-binding activity (bar I) and botrocetin activity (bar II) were pooled separately and subjected to further purification. (b) SDS–PAGE of jararaca IX/X-bp and botrocetin. Purified proteins were analyzed under nonreducing (NR) and reducing (R) conditions on a 13% separation gel and stained with Coomassie Brilliant Blue R-250. Positions of molecular mass markers (Bio-Rad, low range) are shown on the left.

methods of Matchett and Ingram (1965) and Bachmann et al. (1958), respectively, with modifications (Atoda & Morita, 1989).

## RESULTS

We initially confirmed the presence of IX/X-bp-like protein(s) in the venom of *B. jararaca* by means of an enzyme-linked immunosorbent assay, in which microtiter wells coated with factor IX were incubated with the crude venom in the presence of $Ca^{2+}$ ions and then probed with antiserum raised against habu IX/X-bp. It was evident that factor IX-binding protein(s) that could be recognized by the antiserum was (were) indeed present in the venom (data not shown). We also tested several lots of the venom, some from Sigma and one from Latoxan. Such an activity was found in all of them but the highest in that from Latoxan. We decided to isolate this protein(s) using venom from Latoxan as the source.

A dialyzed solution of crude venom was subjected to anion-exchange chromatography on DEAE-Sepharose under identical conditions to those employed by Fujimura et al. (1991) for the initial fractionation of botrocetin (Figure 1a). Each fraction was assayed for factor IX-binding activity by measuring the inhibition of binding of $^{125}$I-habu IX/X-bp to

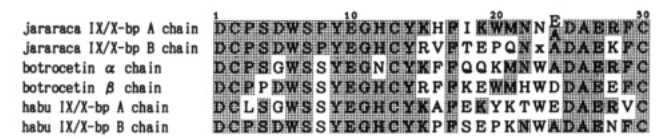


FIGURE 2: $NH_2$-terminal amino acid sequences of the two chains of jararaca IX/X-bp. The determined sequences are aligned with those of botrocetin (Usami et al., 1993) and habu IX/X-bp (Atoda et al., 1991). In cycle 24 with the jararaca IX/X-bp A chain, both Glu and Ala residues were identified, suggesting the presence of polymorphism. The 23rd residue of the B chain was not identified. Residues identical to those in the A chain of jararaca IX/X-bp are shaded.

factor IX and for botrocetin activity by monitoring agglutination of fixed platelets in the presence of vWF. These two activities were clearly resolved by this chromatographic step; factor IX-binding activity was eluted as a single, rather broad peak when the concentration of NaCl was changed from 0 to 0.1 M (Figure 1a, bar I), while botrocetin activity was eluted during a linear gradient of NaCl from 0.1 to 1 M (bar II). The fraction that contained factor IX-binding activity was supplemented with $Ca^{2+}$ ions and loaded onto a column of immobilized factor IX, and bound materials were eluted by depletion of $Ca^{2+}$ with EDTA. The fraction that had bound to factor IX still contained contaminants and was further purified by FPLC on a Mono-Q column (for details, see Experimental Procedures). The isolated protein cross-reacted with the antiserum against habu IX/X-bp (data not shown) and, as described below, had the ability to bind to factor X as well. The protein was designated jararaca IX/X-bp.

Botrocetin was also purified from the same preparation of venom by two additional chromatographic steps: gel filtration of Sephacryl S-200 and subsequent FPLC on Mono-Q (see Experimental Procedures). When jararaca IX/X-bp and botrocetin were analyzed by SDS–PAGE, both gave a single band that corresponded to 27 kDa under nonreducing conditions, and in the presence of a reducing agent, jararaca IX/X-bp gave a doublet of 16 and 15 kDa, while botrocetin gave a doublet of 16 and 15.5 kDa (Figure 1b). Thus, both proteins are disulfide-linked heterodimers, and their structures appear to be similar. Starting with 100 mg of crude venom, we obtained 2 mg of IX/X-bp and 0.2 mg of botrocetin.

The $NH_2$-terminal amino acid sequences of jararaca IX/X-bp chains were determined. A high degree of homology was observed between the 16- and 15-kDa chains (designated the A and B chains, respectively). Moreover, these sequences were highly homologous to those of the subunits of both habu IX/X-bp and botrocetin (Figure 2). It was concluded that jararaca IX/X-bp and botrocetin are structurally related but not identical.

We examined the biological activity of jararaca IX/X-bp. First, platelet agglutinating activity was evaluated with pure proteins in two systems, namely, with fixed bovine platelets plus bovine vWF (Figure 3, upper panel) and with intact human platelets plus autologous plasma (lower panel). Jararaca IX/X-bp failed to induce any agglutination of platelets even at a concentration of 50 $\mu$g/mL in both systems, while botrocetin induced agglutination at much lower concentrations in the presence of vWF. Furthermore, the action of botrocetin was not affected by high concentrations of jararaca IX/X-bp (Figure 3, traces c). The same data were obtained with habu IX/X-bp as with jararaca IX/X-bp (data not shown). It was evident that the IX/X-bps are neither agonists nor antagonists of botrocetin.

We next examined the binding activity of $^{125}$I-jararaca IX/X-bp to $\gamma$-carboxyglutamic acid (Gla)-containing plasma proteins. Factor X and protein S, in addition to factor IX, gave detectable binding among the tested proteins, namely,

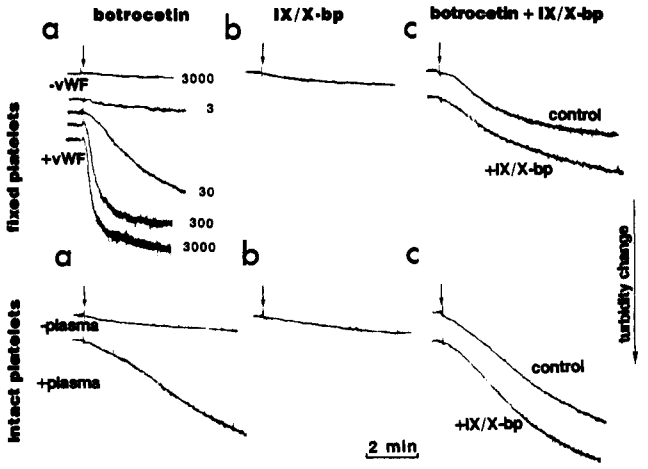


FIGURE 3: Effects of jararaca IX/X-bp and botrocetin on platelets. (Upper panel) Agglutination of fixed bovine platelets. (a) Platelets were stimulated in the absence or presence (20 μg/mL) of vWF by the indicated concentrations of botrocetin (in nanograms per milliliter). (b) Platelets were stimulated by 50 μg/mL jararaca IX/X-bp in the presence of vWF. (c) Platelets were preincubated with or without 50 μg/mL jararaca IX/X-bp in the presence of vWF and then challenged with 30 ng/mL botrocetin. (Lower panel) Aggregation and/or agglutination of intact human platelets. (a) Platelets were stimulated by 3 μg/mL botrocetin in the absence or presence (10%, v/v) of plasma. (b) Platelets were stimulated by 50 μg/mL jararaca IX/X-bp in the presence of plasma. (c) Platelets were preincubated with or without 50 μg/mL jararaca IX/X-bp in the presence of plasma and then challenged with 3 μg/mL botrocetin. Each sample was added at the point indicated by an arrow. Representative traces are shown.

protein C, protein S, protein Z, prothrombin, and factors VII, IX, and X, all of bovine origin (data not shown). The binding was observed only in the presence of physiological concentrations of $Ca^{2+}$ ions (millimolar) and was completely abolished in the absence. $EC_{50}$ values of $Ca^{2+}$ ions for the binding to factors IX and X were 0.7 mM, and maximal binding was observed at about 5 mM $Ca^{2+}$. However, the binding to protein S did not reach a maximum under our experimental condition (up to 30 mM $Ca^{2+}$). The binding was time-dependent and plateaued after a 1-h incubation at ambient temperature (data not shown).

We investigated the dose-dependence of the binding of jararaca IX/X-bp, as well as the effects of habu IX/X-bp and botrocetin on the binding. The binding of $^{125}$I-jararaca IX/X-bp to factors IX and X and protein S was effectively inhibited by raising the concentration of unlabeled jararaca IX/X-bp or of habu IX/X-bp (Figure 4). This result indicates that the binding of jararaca IX/X-bp is specific and saturable and that the two IX/X-bps probably share common binding sites on these coagulation factors. It should be noted that habu IX/X-bp recognized protein S in the present study (Figure 4, right panel). We failed to demonstrate such a binding in a previous study (Atoda & Morita, 1989), and the discrepancy may be due to differences in methodology; we previously employed a gel filtration technique that may be unsuitable for detection of weak associations. Each competition curve for habu IX/X-bp is shifted to the left of that for jararaca IX/X-bp, implying a higher affinity of the proteins for habu IX/X-bp than for jararaca IX/X-bp. On the other hand, botrocetin did not inhibit the binding of $^{125}$I-jararaca IX/X-bp. The biological activities of the IX/X-bps and botrocetin again appear to be different.

Scatchard analysis of the data from the above experiment revealed the presence of a single class of binding sites on each coagulation factor, and apparent $K_d$ values were obtained (Table I). In the presence of $Ca^{2+}$ ions, the highest affinity was seen for factor IX and then for factor X, and the affinity

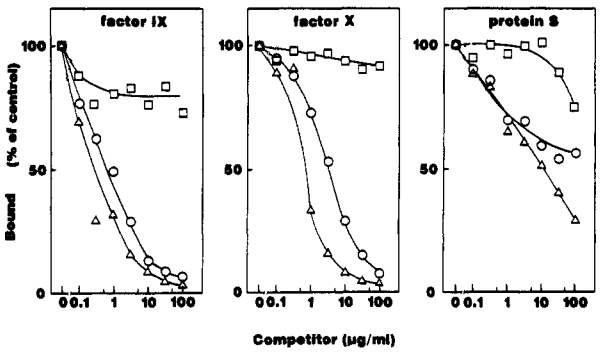


FIGURE 4: Inhibition of binding of $^{125}$I-jararaca IX/X-bp by unlabeled jararaca IX/X-bp, habu IX/X-bp, and botrocetin. Microtiter wells were coated with factor IX (left panel), factor X (middle panel), or protein S (right panel) and incubated with $^{125}$I-jararaca IX/X-bp plus the indicated concentrations of unlabeled jararaca IX/X-bp (circles), habu IX/X-bp (triangles), or botrocetin (squares). Each point represents the mean of duplicate determinations from one of three similar experiments.

Table I: Apparent $K_d$ Values for Binding of Jararaca IX/X-bp to Various Coagulation Factors in the Presence of Different Divalent Cations[a]

| | $K_d$[b] (nM) | | | |
|---|---|---|---|---|
| | $Ca^{2+}$ | $Sr^{2+}$ | $Mg^{2+}$ | $Mn^{2+}$ |
| factor IX | 37 ± 9 | 25 ± 11 | 110 ± 10 | 120 ± 40 |
| factor X | 75 ± 17 | c | c | c |
| protein S | 260 ± 100 | c | c | c |

[a] Each cation was tested at a concentration of 5 mM. [b] The mean ± SEM of four independent determinations. [c] No binding was observed.

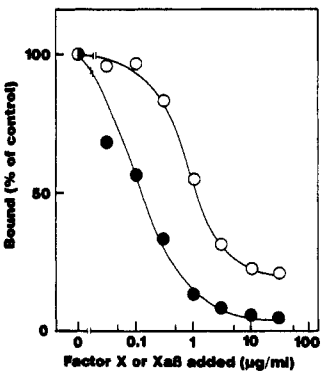


FIGURE 5: Effects of carbohydrate moieties in the factor X molecule on the binding to jararaca IX/X-bp. Microtiter wells were coated with factor X and incubated with $^{125}$I-jararaca IX/X-bp. The fluid phase was supplemented with the indicated concentrations of either intact factor X (open circles) or factor Xaβ (closed circles). Each point represents the mean of duplicate determinations from one of three similar experiments.

for protein S was weaker by 1 order of magnitude. The effects of polyvalent cations other than $Ca^{2+}$ were also investigated. Binding to factor X or protein S had an absolute requirement for $Ca^{2+}$ ions, and other cations tested were without effect. $Sr^{2+}$, $Mn^{2+}$, and $Mg^{2+}$ ions could be substituted for $Ca^{2+}$ ions in binding of factor IX, but their respective potencies were somewhat different from that of $Ca^{2+}$ ions (Table I). $Ba^{2+}$, $Zn^{2+}$, and $La^{3+}$ ions were ineffective in all cases.

Because habu IX/X-bp and botrocetin belong to the C-type lectin family, jararaca IX/X-bp is also likely to be a member of this family. We examined whether the binding of jararaca IX/X-bp was mediated by its lectin-like activity by employing factor Xaβ, a derivative of factor X that is free of carbohydrates. As is shown in Figure 5, both factors X and Xaβ added to the fluid phase effectively inhibited the binding of $^{125}$I-jararaca IX/X-bp to solid-phase factor X. Factor Xaβ

was much more potent in this respect than intact factor X. Nevertheless, the involvement of lectin-like activity in the binding can be ruled out.

As a consequence of its binding to coagulation factors, jararaca IX/X-bp showed anticoagulant activity, and prolongation of clotting time was observed in both factor Xa-induced clotting time and kaolin-induced partial thromboplastin time assays. In factor Xa-induced clotting time (1.5 nM factor Xa), 50% and 80% inhibition was achieved with 3 and 10 μg/mL jararaca IX/X-bp, respectively. By contrast, botrocetin did not have any significant effect in either system (data not shown).

## DISCUSSION

Toxins in snake venoms that affect the coagulation system exhibit considerable heterogeneity in terms of function as well as molecular structure (Tu, 1977; Pirkle & Markland, 1987). Recently, there have been several reports of a novel class of toxins that include the structure of the carbohydrate-recognition domain of C-type lectins; these toxins include botrocetin (Usami et al., 1993), *Crotalus atrox* lectin (Hirabayashi et al., 1991), and RVV-X (Takeya et al., 1992), and habu IX/X-bp also belongs to this group (Atoda et al., 1991). Botrocetin is a well-known toxin, found in the venom of *B. jararaca*, that has been used to study interactions between vWF and platelets (Read et al., 1978, 1989; Fujimura et al., 1987; Andrews et al., 1989). It binds to vWF and changes the conformation of vWF such that "activated" vWF acquires the ability to agglutinate platelets. In the present study, we isolated a protein structurally related to botrocetin from the same venom, jararaca IX/X-bp. However, jararaca (or habu) IX/X-bp neither showed botrocetin-like activity nor interfered with this activity (Figure 3), but did have the ability to bind Gla-containing coagulation factors, namely, factors IX and X and protein S, with resultant interference in blood clotting. By contrast, botrocetin is unlikely to bind to these coagulation factors since it interfered with neither the binding of jararaca IX/X-bp nor coagulation. It appears that the venom of *B. jararaca* contains two highly homologous proteins of which biological activities are quite different. Noteworthily, no botrocetin-like activity was detected in the venom of *T. flavoviridis*. Thus, jararaca IX/X-bp and botrocetin of the same origin may constitute a unique model for the elucidation of protein evolution as well as structure/function relationships of proteins. Identification of the determinants responsible for the functional differences of these two proteins is of interest per se, and, we believe, these studies will be valuable for understanding the physiological function of other proteins that include C-type lectin-like structures [e.g., IgE receptor and asialoglycoprotein receptors (Spiess, 1990)].

Identification of the binding site(s) on coagulation factors is also of interest. The possibility that carbohydrates on the coagulation factors are the site of recognition by jararaca IX/X-bp is excluded by the result of the experiment with factor Xaβ (Figure 5), though this protein should turn out to be a member of a C-type lectin family and the binding required $Ca^{2+}$ ions [C-type lectins recognize carbohydrates only if $Ca^{2+}$ ions are present (Drickamer, 1988; Spiess, 1990)]. Bovine factor X contains three carbohydrate moieties, one at the COOH-terminus of the heavy chain (Titani et al., 1975) and two in its activation peptide portion.[3] Upon activation, the activation peptide is removed, and the active form (factor Xaα) immediately undergoes autolysis, cleaving off its COOH-terminal portion (Fujikawa et al., 1975). The resultant product (factor Xaβ) is, thus, free from carbohydrates. Therefore, since jararaca IX/X-bp recognized factor Xaβ as well as intact factor X, it is clear that its binding site on coagulation factors is distinct from the carbohydrate moieties. It is of interest to note in this context that the action of botrocetin seems also not to be mediated by its lectin-like activity; it does not require $Ca^{2+}$ ions for the recognition of vWF (Read et al., 1978). On the other hand, our previous study with habu IX/X-bp may suggest that the binding site of factor X (or IX) lies within its Gla domain, since factor X without a Gla domain failed to associate with IX/X-bp (Atoda & Morita, 1989). This hypothesis is supported by the present observation that both jararaca and habu IX/X-bps recognize protein S in addition to factors IX and X. The structure of protein S is somewhat different from those of the other two proteins, whereas factors IX and X are very similar along their entire sequences. The only homologous portions are found in the $NH_2$-terminal regions: Gla domains are highly conserved, and epidermal growth factor-like domains are also homologous to some extent (Stenflo, 1991). It is not unreasonable, therefore, to hypothesize that the Gla domain is the site at which IX/X-bp binds. We are currently attempting to prove this hypothesis. It is unclear why IX/X-bp does not recognize other Gla-containing proteins, such as prothrombin. It is also of interest to us to identify the features that determine this rather narrow specificity.

The binding site on the IX/X-bp molecule is also left to be determined, but a recent report by Takeya et al. (1992) about RVV-X may provide a clue. The light chain of this protease exhibits some homology to habu IX/X-bp, and activation of factor X by this protease was inhibited by habu IX/X-bp. It is possible that their recognition sites for coagulation factors exist within the regions that are conserved in these proteins.

In conclusion, we have isolated and characterized a unique anticoagulant protein. This protein, jararaca IX/X-bp, may be useful as a tool for studies of the complex cascade of blood coagulation, and it also may be useful in efforts to elucidate structure/function relationships of proteins, given the high degree of its structural, but not functional, similarity to botrocetin.

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