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Jararhagin ECD-Containing Disintegrin Domain: Expression in *Escherichia coli* and Inhibition of the Platelet–Collagen Interaction

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Jararhagin, a hemorrhagin from *Bothrops jararaca* venom, is a soluble snake venom component comprising metalloproteinase and disintegrin cysteine-rich domains and, therefore, is structurally closely related to the membrane-bound A Disintegrin And Metalloproteinase (ADAMs) protein family. Its hemorrhagic activity is associated with the effects of both metalloproteinase and disintegrin domains; the metalloproteinase enzymatically damages the endothelium and the disintegrin domain inhibits platelet–collagen interactions. The expression of whole jararhagin or its disintegrin domain has never been attempted before. The aim of this study was to investigate whether we could express the disintegrin domain of jararhagin and to verify whether this domain displays an inhibitory effect on the platelet–collagen interaction. Therefore, the cDNA fragment coding for the disintegrin plus cysteine-rich domains of jararhagin was cloned into the pET32a vector, used to transform the *Escherichia coli* AD494(DE3)pLysS strain. The thioredoxin–disintegrin fusion protein was recovered from the soluble extract of the cells, yielding up to 50 mg/liter culture. The fusion protein was isolated using polyhistidine binding resin which resulted in a main band of 45 kDa recognized by anti-native jararhagin antibodies. Antibodies raised in rabbits against the fusion protein had high enzyme-linked immunosorbent assay titers against native jararhagin and detected a band of 52 kDa on Western blots of whole *B. jararaca* venom demonstrating that these antibodies recognize the parent jararhagin molecule. Treatment of the fusion protein with enterokinase, followed by further capture of the enzyme, resulted in a band of 30 kDa, the expected size for jararhagin-C. Further purification of the cleaved disintegrin using FPLC Mono-Q columns resulted in one fraction capable of efficiently inhibiting collagen-induced platelet aggregation in a dose-dependent manner (IC_{50} of 8.5 μg/ml).

Key Words: **disintegrin; metalloproteinase; expression in *E. coli*; venom; platelet.**

The action of snake venom toxins implies binding to a variety of critical physiological targets interfering with important processes such as neuromuscular transmission or hemostasis. One particularly important group of snake venom toxins is the hemorrhagins. These are found mainly in viper venoms and are responsible for the often massive hemorrhage due to the disruption of blood vessels as well as for inhibition of platelet aggregation (1). Hemorrhagins are soluble zinc-dependent metalloproteinases possessing an additional disintegrin-like domain extended by a cysteine-rich carboxy terminus, characteristic of the P-III snake venom metalloproteinases (2). The molecular mechanisms related to the activity of the metalloproteinase domain are well understood and resemble those of the matrixins (3). However, little is known about the action of the disintegrin domain of the metalloproteinases. The most frequently studied disintegrins are the P-II snake venom metalloproteinases known as RGD-disintegrins (4). These are coded as precursor molecules

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with a similar domain orientation to the P-III metalloproteinases. However, the mature proteins are short peptide chains comprising only the disintegrin domain. They bind to the platelet $\alpha_{IIb}\beta_3$ integrin with nanomolar affinity, blocking fibrinogen binding and thus inhibiting platelet aggregation by this route (4). Other functional activities include interference with cell metastasis in experimental models (5). These activities are directly related to the presence of an RGD motif, which is present on the surface of the molecule in a disulfide bond-stabilized loop (6). The P-II and P-III disintegrins share a high amount of sequence similarity. However, the latter possess a carboxy-terminal extension rich in cysteines and they have distinct RSECD motifs, which replace the RGD sequence (7).

Venom metalloproteinases and disintegrins share strong sequence similarity to the recently characterized ADAM[2] (A Disintegrin And Metalloproteinase) family of mammalian proteins (8). The ADAMs have the same domain organization as venom metalloproteinases added to a transmembrane domain and a potential signaling cytoplasmic tail. These proteins have been ascribed adhesive and antiadhesive properties; adhesive properties include sperm–egg fusion as well as muscle fusion and antiadhesive properties such as inhibition of neural cell fate at different stages of embryogenesis and axonal extension in *Drosophila* (8). Therefore, the soluble venom metalloproteinases would be expected to possess antiadhesive properties inhibiting intercellular communication. With native jararhagin we have shown that $\alpha_2\beta_1$ integrin is the selective target of this enzyme (1). However, knowledge is lacking on the structure of the ECD-containing disintegrin domain, which could explain the specificity of the integrin binding. It appears therefore that the P-III disintegrins may bind to different types of cell surface receptors; snake venom peptides could act as antagonists of the mammalian ADAMs or even agonists in integrin-mediated cell activation.

Production of recombinant P-III disintegrins is therefore crucial for characterizing the molecular mechanisms of action of these peptides. In this paper we report the successful expression of the disintegrin domain of jararhagin, a P-III snake venom metalloproteinase isolated from *Bothrops jararaca* venom (9) using *Escherichia coli* expression vectors. We also show that the recombinant protein is functional in the inhibition of collagen-induced platelet aggregation.

EXPERIMENTAL

Reagents. Unless otherwise indicated, standard protocols of molecular biology were used (10). Restriction enzymes, *Taq* DNA polymerase, and T4 ligase were supplied by Boehringer-Mannheim. pET32 vector, *E. coli* strains, enterokinase and an enterokinase capture kit, and poly-histidine-binding resin were purchased from Novagen (Cambridge, UK). Oligonucleotides were synthesized by Genosys (Cambridge, UK).

pET32-JD9. The cDNA sequence coding for the disintegrin plus cysteine-rich domains of jararhagin was amplified by PCR using the complete original clone (BJD_4) of jararhagin cDNA as template (9; EMBL X68251). A *Sac*I site and codons for the amino acid sequence LGTDIISP flanked the amplification product at the 5′ end. The 3′ end consisted of the anticodons for VATAY amino acids followed by a stop codon and a *Not*I site. These amino acid sequences comprise respectively the N-terminal residues of the disintegrin domain and the C-terminal residues of the cysteine-rich domain. The PCR products were digested by *Sac*I and *Not*I and ligated to the same sites of the linearized pET32a vector. The resulting vector, pET32-JD9, predicts a protein with 109 amino acids of thioredoxin A, a histidine tag, a thrombin site, and an enterokinase site, followed by the disintegrin plus cysteine-rich extension of jararhagin. Correct cloning was confirmed by partial sequence of the 5′ end of 300 bp, which confirmed the complete sequence of the disintegrin domain.

E. coli cultures and protein expression. pET32-JD9 was transfected into *E. coli* AD494(DE3)pLysS and cultured at 37°C in Luria–Bertani medium, containing the appropriated antibiotics, to A_{600} = 0.500. The T7 RNA polymerase under *lac*UV5 promoter was induced with 1 mM isopropyl-thio-β-D-galactopyranoside (IPTG).

Protein isolation. Bacteria from cultures were collected by centrifugation, suspended in 1/20 vol of 50 mM Tris/HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9, and sonicated. The soluble intracellular fraction was added to a histidine-binding nickel–Sepharose resin. Elution was performed after extensive washing with 60 mM imidazole by addition of 1 M imidazole, both in the same Tris buffer. The eluted sample was dialyzed against 20 mM Tris/HCl, 50 mM NaCl, 2 mM $CaCl_2$, pH 7.4, and the recombinant disintegrin released from the thioredoxin peptide by digestion with enterokinase (1 U/100 μg fusion protein). The cleavage enzyme was then removed using a capture kit according to the manufacturer's instructions. In some experiments, the cleaved fusion protein was further chromatographed in FPLC Mono-Q columns (Pharmacia) equilibrated with 20 mM Tris/HCl, pH 8.0. Retained proteins were eluted with a linear gradient of 0–1 M NaCl in the same buffer. Protein concentration was estimated using Bradford reagent (Amresco, U.S.A.).

Immunochemical analyses. ELISA and Western blotting were carried out using standard protocols (11). Antibodies against the native jararhagin (JAR) isolated from *B. jararaca* venom (9), the recombinant fusion protein (Trx-JD9), and the expression product of the wild vector (Trx) were raised in rabbits by immunization with 500 μg of each protein in Freund's complete adjuvant. After 4 weeks, the animals received two boosters of 100 μg of samples in saline at 15-day intervals. The antibody titers were assayed by ELISA using peroxidase-labeled anti-rabbit IgG conjugates (Sigma). For SDS–PAGE (12), pellets from 0.5-ml cultures or 10 μg of purified proteins (4 μg for pool 6) were diluted in 25 μl of sample buffer (125 mM Tris/HCl, 2.5% SDS, 2 mM EDTA) and applied to the 12% acrylamide gels under nonreducing conditions or in the presence of 0.025% 2-mercaptoethanol (2-ME). For Western blotting, SDS–PAGE bands were transferred to nitrocellulose membranes, which were subsequently blocked by 5% nonfat milk in Tris/NaCl (50 mM Tris/HCl, 150 mM NaCl, pH 7.4). Membranes were incubated with 1:1000 diluted antibodies for 2 h at room temperature followed by washing with Tris/HCl, 150 mM NaCl, pH 7.4 buffer, incubation with 1:1000 diluted peroxidase-labeled anti-rabbit IgG for 2 h at room temperature, and addition of α-chloronaphthol (Merck) plus H_2O_2 as enzyme substrates.

Collagen-induced platelet aggregation. Fresh citrated human blood obtained from healthy donors was centrifuged for 10 min at

[2] Abbreviations used: ADAM, A Disintegrin And Metalloproteinase; IPTG, isopropyl-thio-β-D-galactopyranoside; ELISA, enzyme-linked immunosorbent assay; PRP, platelet-rich plasma.

room temperature at 125*g* to obtain the supernatant platelet-rich plasma (PRP). Platelets were isolated from PRP by gel filtration on Sepharose 2B (Pharmacia) as described previously (13). They were then resuspended to a final concentration of 3×10^8/ml in Tyrode's–Hepes buffer (138 mM NaCl, 3 mM KCl, 1 mM $MgCl_2$, 1 mM glucose, 0.5 mM NaH_2PO_4, 20 mM Hepes, pH 7.4) containing 0.35% bovine serum albumin. The effects of native and recombinant proteins were studied by incubation with platelets for 10 min at 37°C before the addition of 1 μg/ml collagen (Horm Chemie). The extent of aggregation was estimated as the percentage increase in light transmission taking as 100% the value obtained for platelets incubated with control buffer and stimulated with collagen.

RESULTS AND DISCUSSION

Production of the recombinant protein. The choice of *E. coli* vectors that combines both cytoplasmic expression and solubility of the recombinant protein encouraged us to use the pET32 system which is based on the *E. coli* thioredoxin (*trx*A) as a gene fusion partner. This system has been used to obtain several interleukins with complete preservation of biological activity, despite unsuccessful expression in other *E. coli* vectors (14). The production of the recombinant protein in *E. coli* strains such as AD494 should allow disulfide bond formation in the cytoplasm (15) and should increase the possibility of obtaining the soluble proteins with preserved biological activity. To clone the ECD-containing disintegrin domain of jararhagin into pET32, two primers were designed from 5′ and 3′ ends of the jararhagin cDNA coding for the disintegrin and cysteine-rich domains, added by the restriction sites for *Sac*I and *Not*I, respectively. This allowed directional ligation into the pET32-linearized vector. The *E. coli* HB101 strain was then transformed with the resulting ligation reaction and clone Trx-JD9 was selected showing the in-frame disintegrin and cysteine-rich cDNA sequences (not shown). Clone Trx-JD9 was used subsequently to transform the *E. coli* AD494(DE3)pLysS strain. These cells were cultured and a major band of about 45 kDa was clearly detectable on SDS–PAGE after induction with IPTG (Fig. 1, lane 2). This size matched the expected value calculated for the disintegrin protein fused to thioredoxin (109 amino acids). The overproduced protein was fully soluble in the cytoplasm (Fig. 1, lane 3), very little of this band being detectable either in the insoluble fraction or in the periplasm of the cells (not shown). Purification was carried out from supernatants of the total lysate of the cell pellet obtained by sonication. The 45-kDa Trx-JD9 band was completely bound to nickel–Sepharose columns and specifically eluted by 1 M imidazole (Fig. 1, lane 5). Few high-molecular-weight contaminants were consistently observed together with the Trx-JD9 protein. Cleavage with enterokinase efficiently released the disintegrin (JD9) from the thioredoxin (Trx) peptide (Fig. 1, lane 6). The yield observed was very high, varying from 20 to above 50 mg/liter culture medium.

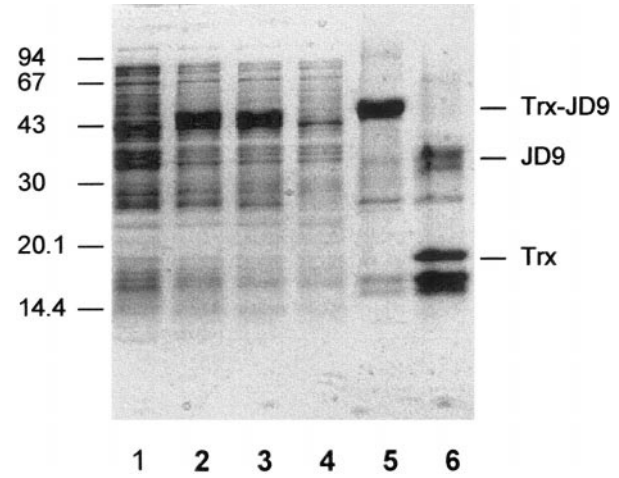

FIG. 1. SDS–PAGE of the recombinant Trx-JD9 expressed in *E. coli*. *E. coli* AD494(DE3)pLysS strain was transformed with pET32-Trx-JD9 and cultured either as a noninduced control (lane 1) or induced with 1 mM IPTG for 4 h (lane 2). Induced cultures were pelleted and submitted to sonication. The sonicated cells were centrifuged, the soluble extract (lane 3) was applied to a histidine-binding column, nonretained proteins (lane 4) were washed with 60 mM imidazole, and the recombinant protein was eluted with 1 M imidazole (lane 5). The fusion protein was cleaved with enterokinase (lane 6). Samples were run on 12% acrylamide gels and stained with Coomassie blue. Numbers on the left indicate migrations of the molecular weight markers.

The recombinant proteins were then analyzed for their potential in the inhibition of collagen-dependent platelet aggregation. The JD9 disintegrin inhibited platelet aggregation in a dose-dependent manner (micromolar level). However, inhibition was less than 30% using doses as high as 5 μM (Fig. 2). No inhibition of collagen-induced platelet aggregation could be observed using the product obtained from the induction of cells carrying the wild plasmid (Trx) or the fusion proteins not treated with enterokinase (Trx-JD9).

The relatively low activity suggests that only a small proportion of the expressed molecules were correctly folded. Refolding protocols were therefore performed after reduction of the protein with dithiothreitol using extensive dialysis against cysteine/cystine urea-containing buffers; however, these measures failed to improve the quality of the protein as the biological activity of the protein was not enhanced. We therefore attempted to isolate the correctly folded population of molecules responsible for the biological activity from the incorrectly folded population. Following fractionation using FPLC Mono-Q columns at pH 8.0, enterokinase-treated proteins were recovered in six pools (Fig. 3). Pools 1 and 2 contained the Trx fragment; pool 3 contained the Trx fragment plus JD9; the intensity of the JD9 band increased in pools 4 and 5. Pool 6 presented a very low protein concentration (85 μg/ml) corresponding mainly to a JD9 band with slightly

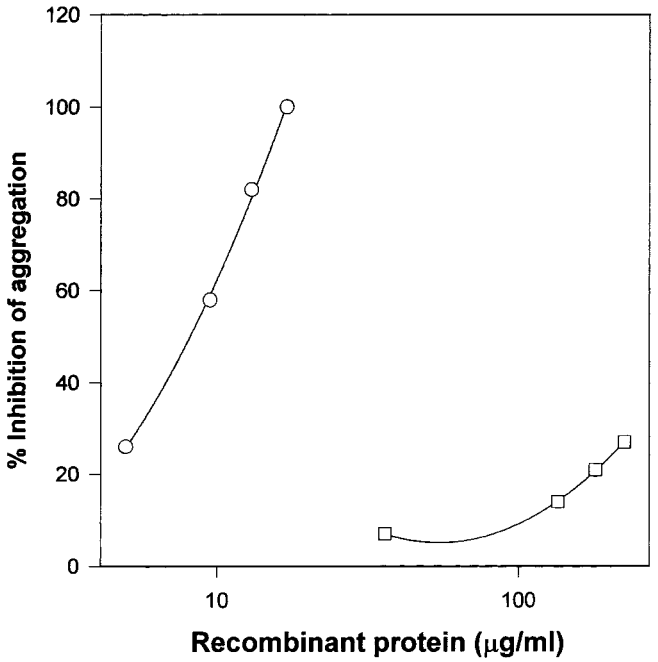

FIG. 2. Collagen-induced aggregation of platelets treated with the jararhagin recombinant disintegrin domain. The recombinant disintegrin domain was produced in AD494(DE3)pLysS/pET32-Trx-JD9, isolated using a histidine-binding column, cleaved with enterokinase, and further isolated by anion-exchange chromatography. Enterokinase-cleaved product (□) was relatively ineffective until fractionated into a highly active pool, P6 (○). Gel-filtered platelets (3×10^8/ml) were incubated for 10 min with increasing volumes of the samples. One microgram of collagen was then added and aggregation was recorded as percentage inhibition of the increase in light transmission in comparison with platelets incubated only with collagen. Data were confirmed in two other experiments, one with the product of *E. coli* BL21(DE3) strain.

lower electrophoretic mobility (Figs. 3B and 3C). The yield of pool 6 was 850 μg/liter culture. Inhibition of collagen-induced platelet aggregation could be detected only in fractions eluted with NaCl concentrations above 0.6 M (pools 5 and 6). Pool 5 (60 μg/test) demonstrated a 20% inhibition and pool 6 inhibited aggregation in a dose-dependent manner with an IC_{50} of 8.5 μg/ml (Fig. 2). Pool 6 still contained a few contaminants together with JD9. However, considering that the purity of JD9 is between 50 and 70% (each protein has different staining properties), the molar ratio of inhibition would be between 140 and 200 nM. The specific activity obtained for pool 6 was close to the levels currently reported for native jararhagin. Both native and 1,10-phenanthroline-inactivated jararhagin inhibited platelet responses to collagen with estimated IC_{50} values of 40 and 140 nM, respectively (16). Apart from the buffer control, five units of enterokinase was incubated with platelets as an additional control; such treatment did not interfere with collagen-induced platelet aggregation.

Our data are in agreement with earlier work in which the successful production of the soluble recombinant κ-bungarotoxin, using the same system, was reported (17). An important observation was that a significant part of our recombinant protein entered a native folding pathway, resulting in conservation of the biological activity of the disintegrin as shown by inhibition of collagen-induced platelet aggregation. This has also been observed when other proteins in which the S—S bonds are important were overproduced in the same expression system. For example, it has been shown that only part of a Fab antibody fragment (18) and of recombinant glutamate decarboxylase GAD65 (19) were folded in the native configuration. Purification of this group of molecules is achievable by ion-exchange chromatography, as shown here, or by more direct methods such as immunoaffinity chromatography using antibodies against conformational epitopes of the native protein.

Immunochemical properties of Trx-JD9. Antigenicity of jararhagin was clearly preserved in the fusion proteins. The Trx-JD9 band was recognized by antibodies raised against native jararhagin. Despite the fact that anti-native jararhagin serum recognized bacterial proteins, a band of 45 kDa was clearly detected by this antiserum only in the soluble extracts of induced cultures (Fig. 4, lanes 2 and 3). The isolated Trx-JD9 protein (Fig. 4, lane 5) was also revealed, together with high-molecular-weight contaminants and bands of smaller sizes which were not detected in bacterial extracts. Following cleavage with enterokinase, the band corresponding to Trx-JD9 was completely digested, generating the JD9 band (30 kDa) and the Trx fragment. The JD9 band was recognized by anti-jararhagin antibodies with almost the same intensity as the fusion protein Trx-JD9 (Fig. 4, lane 6). It is important to point out that the Trx fragment was not recognized by the antibodies when cleaved from Trx-JD9, although its presence could be clearly detected in the Coomassie-stained gels (Fig. 1, lane 6). After fractionation by Mono-Q chromatography, a component of pool 6 was also detected by the antibodies as a band of approximately 30 kDa corresponding to JD9 (Fig. 4, lane 7). A 66-kDa band was consistently revealed in all samples, suggesting that this comprises a bacterial contaminant, which is strongly recognized by the anti-native jararhagin serum. These observations clearly identify the 45-kDa Trx-JD9 fusion protein and 30-kDa JD9 bands as immunologically related to native jararhagin.

Production of soluble recombinant antigens in *E. coli* may be useful for antivenom preparation or even for producing antibodies against chimeric molecules which could be important for understanding the mechanisms of the action of individual venom toxins. Rabbits were therefore immunized with the fusion protein Trx-JD9;

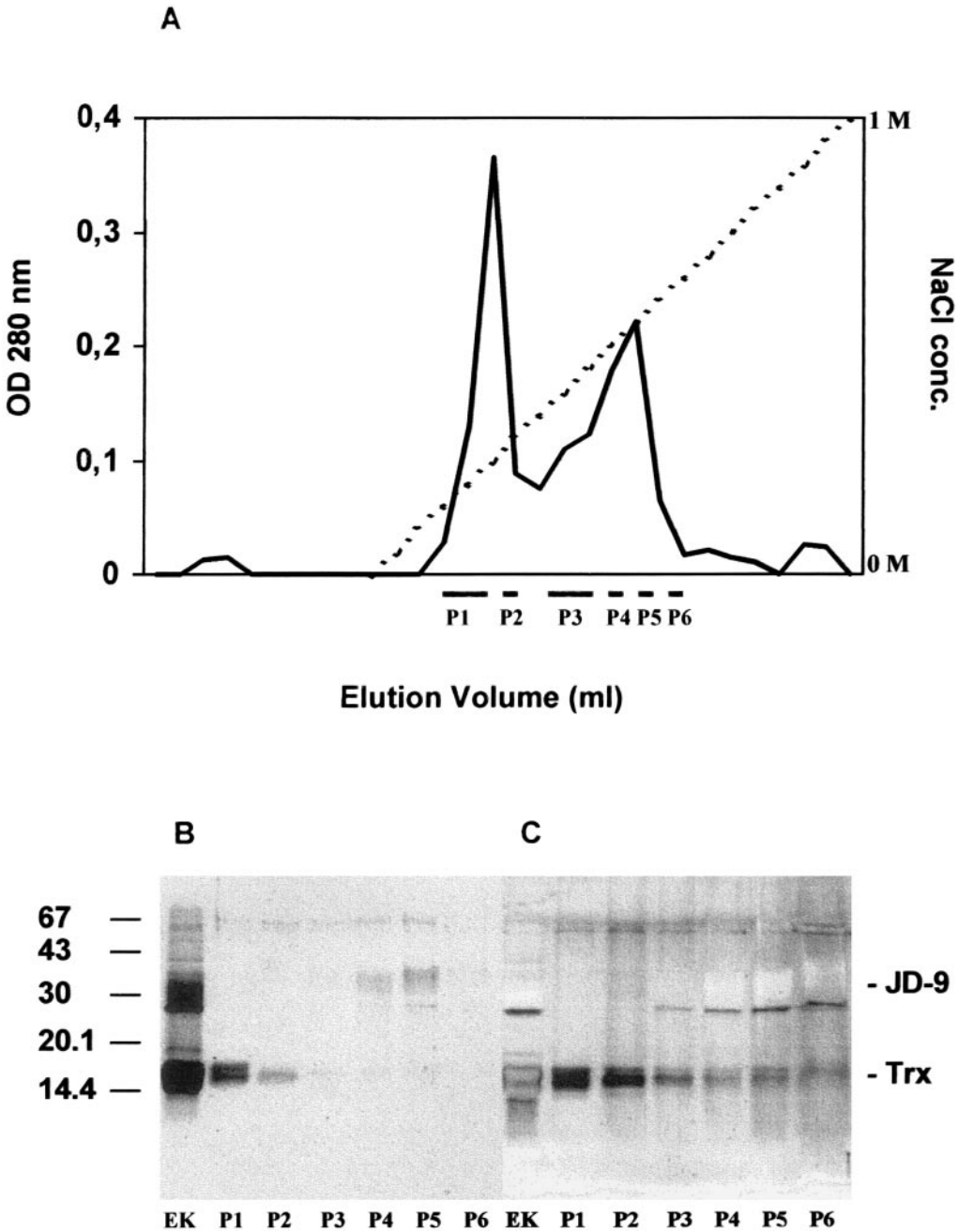

FIG. 3. Isolation of enterokinase-treated Trx-JD9 by anion-exchange chromatography. The fusion protein product of *E. coli* AD494(DE3)pLysS/pET32-Trx-JD9 strain was isolated by Hys-binding resin and cleaved with enterokinase. Two milligrams of the final product was chromatographed on FPLC Mono-Q columns equilibrated with 20 mM Tris/HCl, pH 8.0. Retained proteins were eluted with a linear gradient of 0–1 M NaCl in the same buffer at a flow rate of 1 ml/min. Fractions of 1 ml were collected. Chromatography was monitored by A_{280} (A). Fractions were pooled as indicated (P1–P6) and analyzed by SDS–PAGE followed by Coomassie blue (B) or silver (C) staining, compared to enterokinase-digested Trx-JD9 (EK). Numbers on the left indicate migrations of the molecular weight markers.

antibodies were produced which were capable of reacting strongly with the native jararhagin in ELISAs. The antibody levels induced by immunization with the product of the wild vector (Trx) were very low, comparable to those obtained for normal rabbit sera. The recognition pattern on Western blots of anti-Trx-JD9 serum was very similar to the bands recognized by anti-native jararhagin serum comprising bands of approximately 52, 47, 44, and 30 kDa in whole venom (Fig 5, lane 1). The main band of 52 kDa corresponds to jararhagin (9) while the other detected bands may be correlated to members of the metalloproteinase family

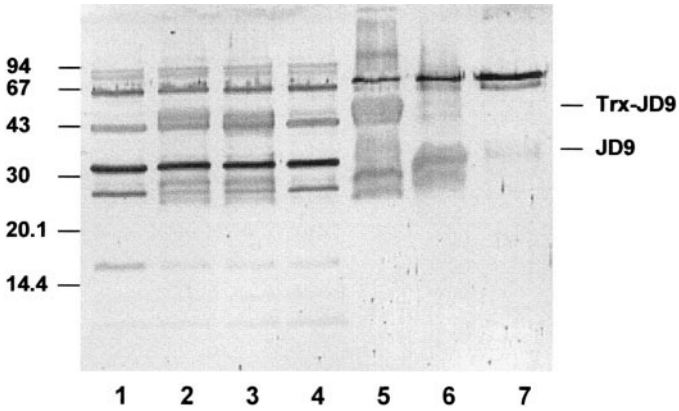

FIG. 4. Western blotting of Trx-JD9 with anti-native jararhagin antibodies. *E. coli* AD494(DE3)pLysS strain was transformed with pET32-Trx-JD9 and either cultured as a noninduced control (lane 1) or induced with 1 mM IPTG for 4 h (lane 2). Induced cultures were pelleted and subjected to sonication. The sonicated cells were centrifuged, and the soluble extract (lane 3) was applied to histidine-binding resin. After collecting the nonadsorbed proteins (lane 4), the column was washed with 60 mM imidazole, and the recombinant protein was eluted with 1 M imidazole (lane 5). The fusion protein was cleaved with enterokinase (lane 6) and further purified by FPLC to generate pool 6 (lane 7). Samples were subjected to SDS–PAGE conditions; the resulting bands were transferred to nitrocellulose membranes which were subsequently blocked by nonfat milk in Tris/NaCl (50 mM Tris/HCl, 150 mM NaCl, pH 7.4). Membranes were incubated with 1:1000 diluted antibodies for 2 h at room temperature followed by washing with Tris/NaCl buffer, incubation with 1:1000 diluted peroxidase-labeled anti-rabbit IgG for 2 h at room temperature, and addition of α-chloronaphthol (Merck) plus H_2O_2 as enzyme substrates. Numbers on the left indicate migrations of the molecular weight markers.

with different molecular sizes, including the 28-kDa native jararhagin-C (20). The recombinant proteins were also similarly recognized by both antisera (Fig. 5, lanes 4 and 5). The main differences between antiserum against native jararhagin and anti-Trx-JD9 sera consisted in the nonrecognition of the Trx band by the former (Fig. 5A, lane 3) and the stronger reaction of the latter with several bands of native jararhagin (Fig. 5B, lane 2) that may correspond to the products of autolysis of the protease, including jararhagin-C.

CONCLUSION

The production of recombinant snake toxins is important for understanding their mechanisms of action, for engineering the molecules for use as high-affinity biological tools, and for the eventual production of antibodies suitable for use in serum therapy. However, recombinant bioactive toxins are very difficult to obtain. This is because they are very rich in cysteines and generally suffer posttranslational modifications; their affinity may also depend upon interaction with receptors and this depends on the strict preservation of natural folding. Using the pET32 thioredoxin fusion vector we were able to produce the cysteine-rich disintegrin domain of jararhagin in a completely soluble form and in a high yield. The recombinant disintegrin domain of jararhagin showed good immunogenicity and the expected biological activity of inhibition of collagen-induced platelet aggregation. At least some of the recombinant proteins had a very high inhibitory activity (nanomolar magnitude), with levels comparable to those of the native protein (16) or to the RGD-containing peptides (4). Two other recombinant venom P-III disintegrins have been obtained recently using a baculovirus expression system; Zhou *et al.* (21) reported the expression of the complete catrocollastatin molecule (metalloproteinase, disintegrin, and cysteine-rich extension) and Jia *et al.* (22) the expression of the disintegrin and cysteine-rich domains of catrocollastatin. However, the yields of expression in both studies were very low. Biological activity was detected in these expression products, however at higher concentrations than the native proteins. Unfortunately, data on the structural characterization of the native and recombinant proteins are not available in the literature to explain the differences in protein activity.

We have shown recently that jararhagin is a potent inhibitor of collagen-induced platelet aggregation, by interacting with the platelet $\alpha_2\beta_1$ integrin followed by proteolytic cleavage of the β_1 subunit of the integrin collagen receptor on the platelet surface (16). Jararhagin-C isolated from *B. jararaca* venom inhibits collagen-induced platelet aggregation (20) and here we show that the recombinant protein is also active. The

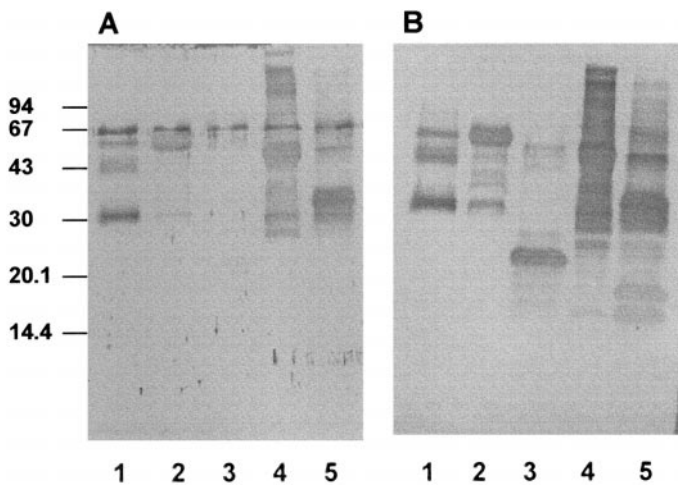

FIG. 5. Comparison of Western blotting recognition pattern of anti-native jararhagin and anti-Trx-JD9 antibodies. Samples of *Bothrops jararaca* venom (lane 1), jararhagin (lane 2), Trx (lane 3), Trx-JD9 (lane 4), and enterokinase-cleaved JD9 (lane 5) were subjected to SDS–PAGE and Western blotting analysis was carried out. Blots were revealed with 1:1000 diluted antibodies anti-native jararhagin (A) and anti-Trx-JD9 (B), followed by incubation with peroxidase-labeled anti-rabbit IgG and enzyme substrates. Numbers on the left indicate migrations of the molecular weight markers.

binding of jararhagin to the platelet collagen receptor has been localized to the disintegrin-containing SECDPA motif as the synthetic peptide containing this sequence also had the same biological effects on platelets (23). Our data therefore provide more evidence to support the importance of the disintegrin domain of jararhagin in the binding to platelet $\alpha_2\beta_1$ integrin. Further work using site-directed mutagenesis might help in the elucidation of the structural features of these ECD-containing disintegrins.

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