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A 28 kDa-protein with disintegrin-like structure (jararhagin-C) purified from *Bothrops jararaca* venom inhibits collagen- and ADP-induced platelet aggregation

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Summary : A 28 kDa-protein with inhibitory activity on collagen- and ADP-induced platelet aggregation was purified from the venom of the snake *Bothrops jararaca*. Its complete amino acid sequence corresponded to the carboxyl-terminal region consisting of disintegrin-like and cysteine-rich domains of jararhagin, a high molecular weight hemorrhagic metalloprotease. Sequence homology of the protein to other disintegrins and disintegrin-like proteins from various snake venoms is also presented.

Many snake venoms, including those from *Bothrops jararaca*, contain hemorrhagic factors (hemorrhagins) which cause severe bleeding in pit viper-envenomed human being and mammals [1]. Pathologically, hemorrhagins appear to cause disruption of collagenous components in the vascular subendothelial matrix [2]. However, recent studies indicate that bleeding by hemorrhagins is attributable to their structural elements; high molecular weight metalloproteases with disintegrin-(like) domains [3]. Disintegrins are a family of Arg-Gly-Asp (RGD)-containing proteins from snake venoms with a high affinity to integrins which participate in cell adhesion or platelet aggregation [4].

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Paine et al. [5] recently reported purification and cDNA cloning of a high molecular weight hemorrhagic metalloprotease (jararhagin) from the *B. jararaca* venom. Jararhagin consists of three structural domains, N-terminal metalloprotease-like, central disintegrin-like, and C-terminal cysteine-rich domains. In the disintegrin-like domain, however, the RGD sequence is replaced by Glu-Cys-Asp (ECD) [5].

We now report purification and characterization of a 28 kDa-protein from the *B. jararaca* venom that specifically inhibits collagen- or ADP-induced platelet aggregation, without affecting on platelet glycoprotein Ib-dependent aggregation. Amino acid sequence analysis revealed that it is identical to residue 210 through the C-terminal end (Ile210-Tyr421) of jararhagin [5]. Thus, we propose to give the name jararhagin-C to this 28 kDa-protein.

Materials and Methods

Materials : Crude venom of *B. jararaca* was purchased from Sigma. Mono Q HR5/5 was obtained from Pharmacia. TSK G2000SW column was from Tosoh (Tokyo). SynChropak RP-8 and RP-4 columns were from SynChrom. Cosmosil 5C18 column was from Nacalai Tesque (Kyoto).

Purification of jararhagin-C : *B. jararaca* venom was dissolved in 84 mM imidazole-HCl buffer (pH 7.4) containing 0.02% NaN_3 and 2 mM benzamidine-HCl. The insoluble material was removed by centrifugation at 7,000×g for 20 min. The supernatant was applied to a DEAE-Sepharose CL-6B column (2.6 × 40 cm) equilibrated with the same buffer. The column was washed with the same buffer and eluted with a linear gradient from 0 to 1 M NaCl. The fraction with jararhagin-C activity was applied to a hydroxyapatite column (2.6 × 18 cm) equilibrated with 0.005 M sodium phosphate buffer (pH 6.8) and eluted with a linear gradient from 0.005 to 0.2 M phosphate. The active fraction was applied to a Mono Q HR5/5 column equilibrated with 50 mM Tris-HCl buffer (pH 8.0) and eluted with a linear gradient from 0 to 1 M NaCl.

Platelet aggregation studies : Platelet-rich plasma from normal individuals was prepared as described [6]. Platelet aggregation was assayed with a NKK Hematracer.

SDS-PAGE : SDS-PAGE was performed by the method of Laemmli [7].

Reduction and S-pyridylethylation : The purified protein was reduced and S-pyridylethylated (PE) as described [8].

Enzymatic digestion and chemical cleavage : The PE-protein was cleaved with cyanogen bromide [9]. Fragments were primarily separated by size-exclusion HPLC on a column of TSK G2000SW (7.5 × 600 mm). Further

purification of fragments was achieved by RP-HPLC on a SynChropak RP-8 column. The PE-protein was digested with *Achromobacter* protease I (a gift from Dr. T. Masaki) or arginyl-endopeptidase (Takara Shuzo, Kyoto) at 37°C overnight in 50 mM Tris-HCl (pH 9.0) in the presence of 2 M urea. Digestion of the PE-protein with *Staphylococcus aureus* V8 protease (Miles, Naperville, IL) was carried out at 37°C overnight in 0.1 M sodium phosphate buffer (pH 7.8) containing 1.5 mM EDTA in the presence of 2 M urea. Cleavage of the PE-protein with 12 N HCl was performed at room temperature overnight. Peptides were separated by RP-HPLC on a SynChropak RP-8, SynChropak RP-4 (4.1 × 250 mm) or Cosmosil 5C18 (4.6 × 100 mm) with gradients of acetonitrile into dilute aqueous trifluoroacetic acid.

Amino acid analysis and sequence determination : Samples were hydrolyzed in 6 N HCl containing 1% phenol at 110°C for 24 h by the vapor-phase method. Amino acid analysis was carried out on a Hitachi model L8500 amino acid analyzer or by the Dabsyl-Cl method [10]. Sequence determination was carried out with an Applied Biosystems model 470A protein sequencer on-line connected to a model 120A PTH analyzer.

Mass spectrometry : Ion-spray mass spectral analysis was performed with a PE-Sciex API-III biomolecular mass analyzer (triple-stage quadrupole mass spectrometer) (PE-Sciex, Ontario, Canada) [11].

Sequence homology search : Sequence homology was searched in the protein sequence database (SWISS-PROT) using DNASIS (Hitachi Software Engineering).

Results

Purification of jararhagin-C : Fig. 1 shows separation of jararhagin-C on a Mono Q column. The purified protein showed an apparent molecular mass of 28,000 before and 32,000 after reduction on SDS-PAGE, indicating that the protein is composed of a single subunit (Fig. 1, inset).

Inhibition of collagen- or ADP-induced platelet aggregation : Collagen or ADP was used at a final concentration of 2 μg/ml or 10 μM in the platelet aggregation studies (Fig. 2). Jararhagin-C exhibited the complete inhibition at a final concentration of 200 nM on collagen-induced platelet aggregation, but 80% inhibition of ADP-induced aggregation even at a final concentration of 300 nM. The reduced and S-carboxyamidomethylated protein inhibited neither collagen- nor ADP-induced platelet aggregation (data not shown).

Amino acid sequence of jararhagin-C : Sequence analysis of the intact PE-protein (ca. 500 pmol) yielded the N-terminal sequence of 39 residues.

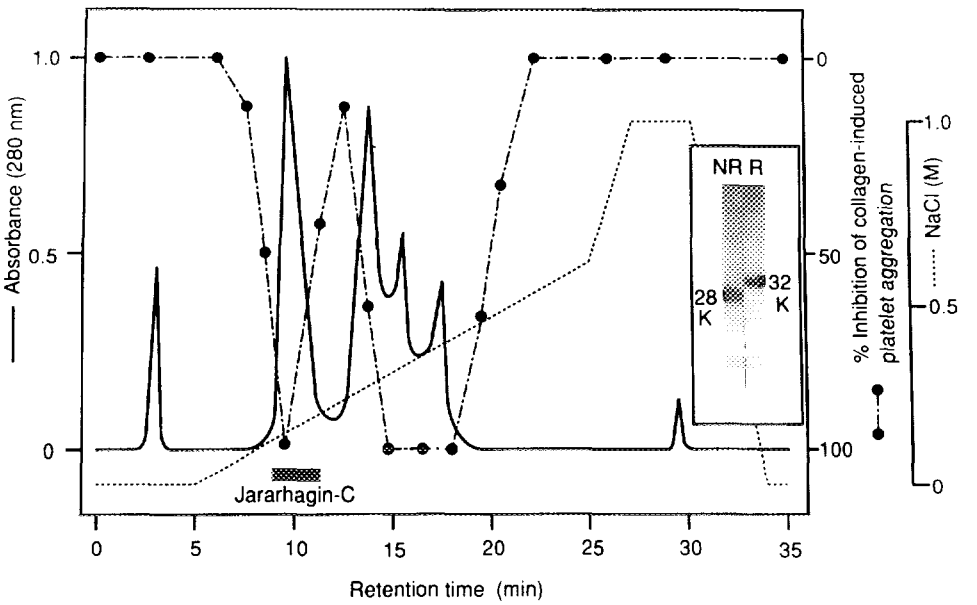

Figure 1. Mono Q column chromatography of jararhagin-C. A Mono Q HR5/5 column was euilibrated with 50 mM Tris-HCl buffer (pH 8.0) and eluted with a linear gradient from 0 to 1 M NaCl. (Inset) SDS-PAGE of purified jararhagin-C under reduced (R) and non-reduced (NR) conditions.

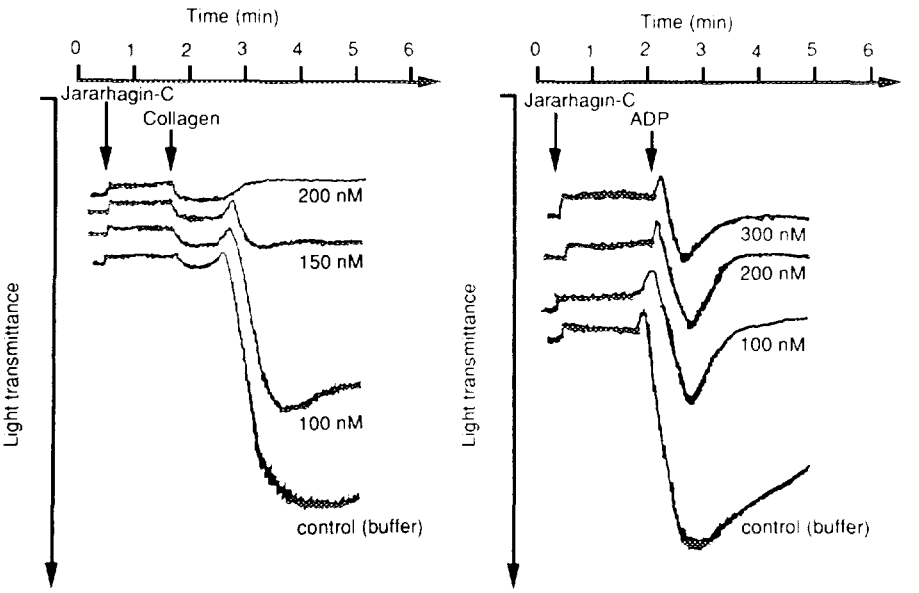

Figure 2. Inhibition of collagen- or ADP-induced platelet aggregation by jararhagin-C. Collagen- (left) or ADP- (right) induced platelet aggregation studies were carried out at a final platelet count of $3 \times 10^5/\mu l$ in the absence or presence of jararhagin-C at various final concentrations indicated.

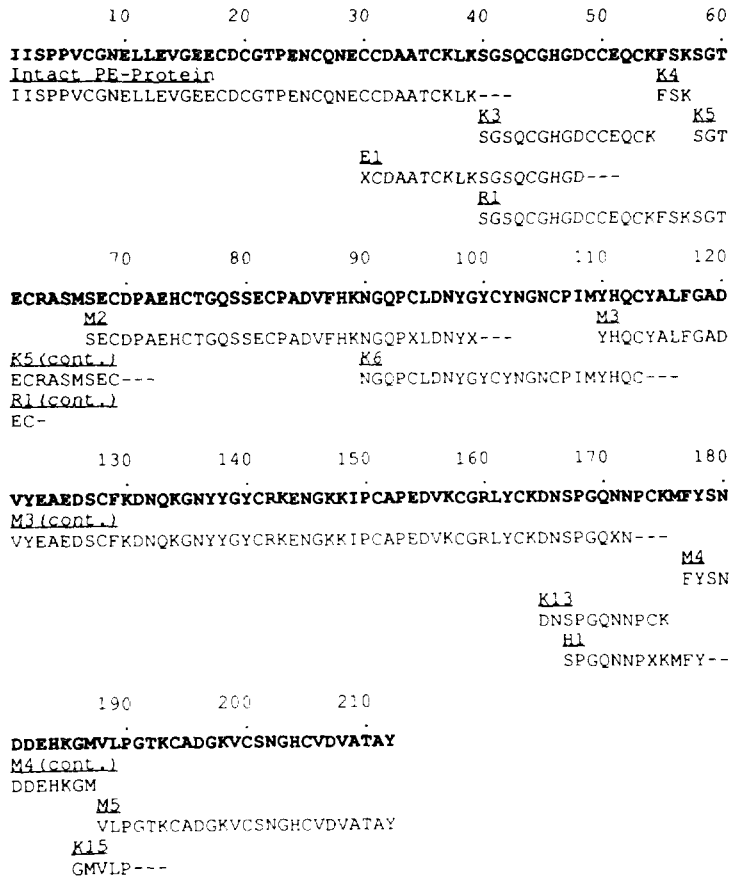

Figure 3. Summary of the sequence proof of jararhagin-C. The proven sequence of specific peptides (underlined) are given in one-letter code below the summary sequence (bold type). Prefixes E, K, M, and R denote peptides generated by cleavage of the PE-protein at glutamyl (with *Staphylococcus aureus* V8 protease), lysyl (with *Achromobacter* protease I), methionyl (with cyanogen bromide) and arginyl bonds (with arginylendopeptidase), respectively. Prefix H denotes a peptide generated by cleavage of the PE-protein with 12 N HCl. The products of lysyl or methionyl cleavage are numbered from the N-terminus toward the C-terminus of the protein. Peptide sequences written in upper case letters are proven by Edman degradation; those in lower case letters indicate tentative identifications. Unidentified residues are shown by dashes.

Cyanogen bromide cleavage of the PE-protein (ca. 5 nmol) yielded five fragments (M1-M5) by size-exclusion and RP-HPLC. These fragments were subjected to amino acid analysis and sequence determination. Fragment M5 was assumed to be derived from the C-terminus by the amino acid and sequence analyses. Digestion of the PE-protein (ca. 3 nmol) with

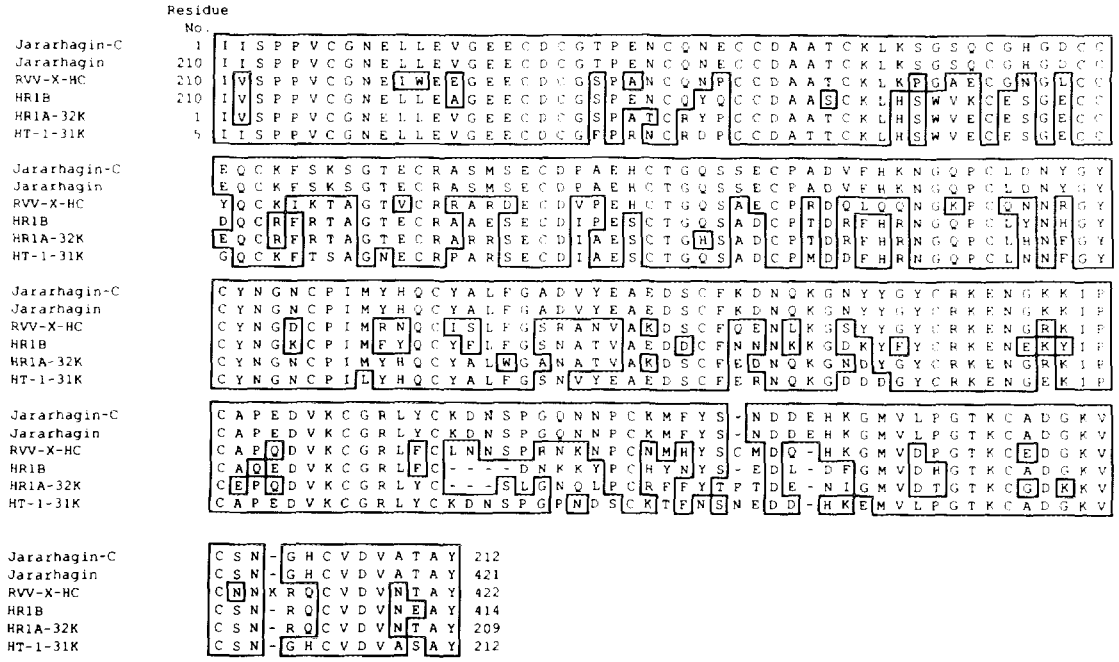

Figure 4. Amino acid sequence homology between jararhagin-C and other known snake venom proteins. Residue numbers indicate positions of amino acid residues from the N-terminus. Gaps have been inserted to maximize homology. Residues identical to those of jararhagin-C are boxed.

Achromobacter protease I yielded thirteen peptides (K1-K17) by RP-HPLC. Peptides K5, K6 and K15 provided overlaps of cyanogen bromide fragments M1-M2, M2-M3 and M4-M5, respectively. Peptide K17 lacked lysine and corresponded to the C-terminus of fragment M5. The remaining overlaps were provided by peptides R1, E1, and H1 isolated from digests with arginylendopeptidase, V8 protease and 12 N HCl, respectively (Fig. 3). Ion-spray mass spectral analysis of the intact protein yielded a MH^+ value of 23,056±5.1 which was in good agreement with the molecular mass of 23,060.90 calculated from the complete amino acid sequence. On the basis that all cysteinyl residues participate in intrachain disulfides.

Sequence homology : The amino acid sequence of jararhagin-C was shown to be identical to the disintegrin-like and cystein-rich domains of jararhagin [5] and also highly homologous to RVV-X-HC from *Vipera russelli* [3], HR1B from

Trimeresurus flavoviridis [12], HR1A-32K from *T. flavoviridis* and HT-1-31K from *Crotalus ruber ruber* [13] (Fig.4).

Discussion

We purified a 28 kDa-protein with a potent inhibitory activity on collagen-induced platelet aggregation from the *B. jararaca* venom and determined the complete amino acid sequence, which revealed that it is derived from the C-terminal region (residues 210-421) of a high molecular weight hemorrhagic metalloprotease jararhagin, already characterized by Paine et al. [5]. Thus we named this protein "jararhagin-C". Jararhagin-C may also be identical to "one-chain botrocetin" that we previously isolated as a byproduct of two-chain botrocetin from the *B. jararaca* venom [14]. Jararhagin-C is a cysteine-rich protein in which 28 out of 212 residues are cysteinyl residues (13.2%) and all of these residues seem to be occupied in intrachain disulfide bridges (data not shown). The protein consists of two domains, the N-terminal disintegrin-like and the C-terminal cysteine-rich domain. Although a protein consisted of only the metalloprotease domain of jararhagin [5] has not yet been isolated from the snake venom, it is presumed that jararhagin-C is produced by autolysis at a single bond between residues 209 and 210 of jararhagin.

The disintegrin-like domain of jararhagin or jararhagin-C shows a high degree of sequence homology to other snake venom proteins such as RVV-X-HC from *V. russelli*, HR1B and HR1A-32K from *T. flavoviridis*, and HT-1-31K from *C. ruber ruber.* All these venom proteins have ECD in place of the RGD sequence observed in normal disintegrins [15-20]. Of these venom proteins, only RVV-X-HC with the CRRARDECD sequence was shown to have a disintegrin-like activity. However, such activity has not yet been proved in jararhagin with the CRASMSECD sequence.

We showed here that jararhagin-C with a disintegrin-like structure inhibits both collagen- and ADP-induced platelet aggregation. However, it should be noted that this activity is totally disappeared after reduction and S-carboxyamidomethylation of the protein as above described. These results

suggest that the activity expressed by jararhagin-C is highly conformation-dependent, different from the RGD sequence [21].

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