

Research paper

P-III hemorrhagic metalloproteinases from Russell's viper venom: Cloning, characterization, phylogenetic and functional site analyses[☆]

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Received 25 December 2007; accepted 14 May 2008

Available online 23 May 2008

Abstract

Two homologous P-III hemorrhagic metalloproteinases were purified from Russell's viper venoms from Myanmar and Kolkata (eastern India), and designated as daborhagin-M and daborhagin-K, respectively. They induced severe dermal hemorrhage in mice at a minimum hemorrhagic dose of 0.8–0.9 µg. Daborhagin-M specifically hydrolyzed an A α -chain of fibrinogen, fibronectin, and type IV collagen *in vitro*. Analyses of its cleavage sites on insulin chain B and kinetic specificities toward oligopeptides suggested that daborhagin-M prefers hydrophobic residues at the P₁, P₁' and P₂' positions on the substrates. Of the eight *Daboia* geographic venom samples analyzed by Western blotting, only those from Myanmar and eastern India showed a strong positive band at 65 kDa, which correlated with the high risk of systemic hemorrhagic symptoms elicited by *Daboia* envenoming in both regions. The full sequence of daborhagin-K was determined by cDNA cloning and sequencing, and then confirmed by peptide mass fingerprinting. Furthermore, molecular phylogenetic analyses based on 27 P-IIIs revealed the co-evolution of two major P-III classes with distinct hemorrhagic potencies, and daborhagin-K belongs to the most hemorrhagic subclass. By comparing the absolute complexity profiles between these two classes, we identified four structural motifs probably responsible for the phylogenetic subtyping and hemorrhagic potencies of P-III SVMPs.

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Keywords: metalloproteinase; Substrate specificity; Geographic variation; Phylogenetic analysis; *Daboia russelii*; *Daboia siamensis*

1. Introduction

Local and systemic hemorrhages are prominent symptoms of envenoming by snakes of the Viperidae family [1]. Symptoms observed are caused mainly by the actions of snake venom metalloproteinases (SVMPs), which belong to the repolysin subfamily of metzincins [2]. SVMP-induced hemorrhage may result from fibrinogenolysis that impairs coagulation, and the degradation of basement membrane proteins,

which damages blood vessels [3,4]. SVMPs are categorized into P-I, P-II, and P-III groups according to the extension of several structural domains [2]. P-I enzymes comprise only the metalloproteinase domain, P-II enzymes contain a disintegrin domain after the metalloproteinase domain, and P-III enzymes (50–70 kDa) are usually glycoproteins that contain an additional Cys-rich C-terminal domain. In general, the P-III SVMPs are more hemorrhagic than the P-I SVMPs, which indicate that the additional domains of P-IIIs might contribute to their hemorrhagic potencies.

In addition to hemorrhage, other versatile functions have also been reported for P-III SVMPs, e.g. activating prothrombin [5,6], inducing endothelial cell apoptosis [7], cleaving integrins [3], and inhibiting platelet functions [8], which implies that more structural diversities exist between P-III SVMPs. Based on the position of the seventh cysteinyl residue in the metalloproteinase domain, three new P-III subclasses were

Abbreviations: MHD, minimum hemorrhagic dose; MMP, matrix metalloproteinase; PMF, peptide mass fingerprinting; SVMPs, snake venom metalloproteinases.

[☆] The nucleotide sequence of daborhagin-K was deposited in GenBank with the accession number DQ137798.

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revealed [2,9]: P-IIIa, which undergoes autoproteolysis to release a ~30-kDa fragment with disintegrin- and Cys-rich domains; P-IIIb, which forms a dimeric structure; and P-IIIc, which contains the seventh cysteinyl residue at position 100 in its metalloproteinase domain. However, the structural elements attributed to their functional variations and hemorrhagic potencies remain elusive [2].

Two species of Russell's vipers, *Daboia siamensis* and *Daboia russelii*, are medically the most important Viperinae in South and Southeast Asia [10], and distributed in eastern and western ranges of their habitat, respectively [11]. Consumptive coagulopathy resulting in spontaneous and systemic bleeding are the main causes of fatal envenoming by the viper [10]. The venom's phospholipases A₂, hemorrhagins, and pro-coagulant enzymes, including Factors X and V protease activators, are presumed to be responsible for these clinical manifestations [10,12]. However, striking variations in the clinical symptoms of envenomed victims [12,13] appeared to reflect some geographical differences in venom components [13].

Relative to other regions, *Daboia* snakebites in Myanmar and eastern India are known to cause severe internal bleeding and higher mortality [14,15]. Although the hemorrhagic protease VRR-73 was isolated from *D. russelii*, its properties and contribution to hemorrhage have not been clarified [16]. In the present study, we purified and characterized the corresponding hemorrhagins (namely daborhagin) of *Daboia* venoms from both regions and solved its full sequence. We also examined the occurrences of P-IIIs in the venoms from various true-viper species or subspecies, and did phylogenetic analyses to trace the evolution of daborhagin and other P-III SVMs.

2. Materials and methods

2.1. Venom and reagents

Daboia venoms from Myanmar and eastern India were gifts from Professors Yu-Yen Shu and Antony Gomes [17]. Three individual *D. siamensis* venom samples from southern Myanmar were kindly given by Professor R. David G. Theakston. Venoms of *Daboia russelii pulchella* (Sri Lanka), *D. russelii* (Pakistan), *D. siamensis* (Taiwan), *Echis leucogaster*, *Echis sochureki*, *Echis pyramidium*, *Vipera lebetina turanica*, *Vipera lebetina mauritanica*, and *Vipera ammodytes montandoni* were purchased from Latoxan (Rosans, France). Venom of *D. siamensis* (Thailand) was from the Thailand Red-Cross, Bangkok, and venom of *D. siamensis* (Indonesia) was from Venom Supplies (Adelaide, Australia). *Vipera ammodytes* and *Vipera berus* venoms were from Sigma–Aldrich Co. (St Louis, MO, USA). Western Indian *D. russelii* venom was obtained from the Haffkine Institute (Mumbai, India). Other venoms used were from the Miami Serpentarium Laboratory (Punta Gorda, FL, USA).

Peptide:N-glycosidase F (PNGase F) and Endo H were from New England Biolabs (Beverly, MA, USA). Modification enzymes, restriction enzymes, broad-range protein markers,

sequencing-grade modified trypsin, and pGEM-T vector were purchased from Promega Corp. (Madison, WI, USA). Protein substrates, insulin chain B, and other chemicals were from either Sigma–Aldrich or Merck (Darmstadt, Germany). Three fluorogenic substrates, (7-Methoxycoumarin-4-yl)acetyl-Pro-Leu-Gly-Leu-(2,4-dinitrophenyl)-Ala-Arg-NH₂ (FS-1), (7-Methoxycoumarin-4-yl)acetyl-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys- $\{N^{\beta}$ -3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl}-NH₂ (NFF-2), and (7-Methoxycoumarin-4-yl)acetyl-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys- $\{N^{\beta}$ -3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl}-NH₂ (NFF-3), were from the Peptide Institute Inc. (Osaka, Japan).

2.2. Purification of P-III SVMs

Approximately 20 mg of the crude venoms of *D. siamensis* (Myanmar) or *D. russelii* (Kolkata) were dissolved in 200 μ l of 0.1 M ammonium acetate (pH 6.7) and then centrifuged at 12,000 \times g for 5 min. The supernatant was then loaded to a Superdex G-75 column (10/300 GL; Pharmacia, Uppsala, Sweden) on an FPLC apparatus. The column was eluted at a flow rate of 1.0 ml/min, and fractions of 0.5 ml were collected. Fractions showing caseinolytic activities based on a colorimetric assay [18] were collected. Pooled sample was lyophilized, redissolved, injected into a Mono Q column (5/50 GL; Pharmacia) pre-equilibrated with 50 mM Tris–HCl buffer (pH 8.0), and then eluted with a 0–0.6 M NaCl gradient in the same buffer. Finally, the sample was purified using a Mono S column (HR 16/10; Pharmacia) pre-equilibrated with 10 mM sodium phosphate (pH 6.7) and eluted with a 0–0.3 M NaCl gradient. Likewise, another two other P-IIIs were purified from *C. vipera* and *E. leucogaster* venoms, and designated as CVHRa (99 kDa) and ECLV-DM (97 kDa), respectively. The other three P-IIIs, Acurhagin, BHRa, and TSV-DM, were also purified according to published methods [18–20]. Their purity and molecular mass were confirmed by SDS–PAGE.

2.3. Protein quantification

Soluble crude venom and purified proteins were quantified using a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA) with bovine serum albumin as a standard.

2.4. Hemorrhage test

The hemorrhagic activity of samples was measured by the published methods [21] with minor modifications. ICR mice (28–30 g) were shaved on their dorsal regions and then subcutaneously injected with samples in 100 μ l of PBS buffer. After 24 h, the mice were sacrificed with an overdose of CO₂, and the hemorrhagic spots were measured from the inside of the moved skins. The minimal hemorrhagic dose (MHD), defined as the amount of toxins causing a 10 mm skin-lesion in 24 h, was determined from a plot of lesion diameters against the doses of toxins injected. Experiments involving mice and rabbits were done under the approval and followed the guidelines

of the Animal Experiment Review Committee of the Academia Sinica, Taiwan.

2.5. Mass spectrometry

The precise molecular weight of daborhagin-M was analyzed in linear mode using the MALDI-TOF mass spectrometer (4700 Proteomics Analyzer; Applied Biosystems, Foster City, CA, USA) equipped with an Nd:YAG laser (355-nm wavelength and 200-Hz repetition rate). One thousand shots were accumulated in positive ion mode. The sample was dissolved in 50% acetonitrile with 0.1% formic acid and pre-mixed with a 5 mg/ml matrix solution of sinapic acid in 70% acetonitrile with 0.1% formic acid for spotting onto the target plate.

2.6. Caseinolytic activity assay and inhibitor study

Caseinolytic activity was measured colorimetrically as previously described [18]. The 100 μ l reaction mixture, containing 0.1 μ g of purified daborhagin, 0.5 mg of azocasein in 50 mM Tris–HCl buffer (pH 8.0), was incubated at 37 °C for 90 min. Reactions were quenched by adding 200 μ l of 5% trichloroacetic acid at room temperature. After spinning at 1000 \times g for 5 min, 150 μ l of the supernatant was mixed with an equal volume of 0.5 M NaOH to halt the reaction, and absorbance was determined at 450 nm. One unit of proteolytic activity was defined as the amount of enzyme required to cause a 1.0 increase in absorbance at 450 nm per minute. Specific activity was expressed as units/mg protein. For the inhibitor study, 5 mM of metal ions, chelators, or protein inhibitors were added to each reaction, separately.

2.7. Hydrolysis of native proteins and oligopeptide substrates

Fibrinogen and basement membrane proteins (collagen, laminin, and fibronectin) were incubated with daborhagin-M in 100 mM Tris–HCl buffer (pH 8.0) at 37 °C. The reactions were terminated by adding reducing buffer, and boiled at 95 °C for 5 min. The hydrolyzed products were then subjected to SDS–PAGE. The gels were stained with Coomassie Brilliant blue G-250 (Gelcode Blue Stain; Pierce Chemical).

Oxidized insulin chain-B (715 μ M) was incubated with 1.0 μ M daborhagin-M at 37 °C in 50 mM Tris buffer (pH 8.0). At various times, the reaction was halted by adding 25 mM EDTA. Peptide products were separated using reverse phase HPLC on a Vydac C₁₈-column, and monitored with the absorbance at 214 nm. The peak fractions were collected and dried in a vacuum-centrifuge device (Labconco Corp., Kansas City, MO, USA). Each sample was analyzed using MALDI-TOF or electrospray ionization (ESI) mass spectrometry.

Stock solutions of synthetic fluorogenic substrates (FS-1, NFF-2, NFF-3) [22,23] were prepared in dimethyl sulfoxide. The P-III enzymes used for the kinetic study were freshly purified and kept active; their concentrations were determined based on their molecular weights. The assay was done by

incubating a 1-ml mixture containing 10 nM enzymes and 1 μ M substrate in 0.1 M Tris–HCl (pH 7.5) with 0.1 M NaCl, 10 mM CaCl₂, and 0.05% (w/v) Brij35. The increase in fluorescence intensity, in relative fluorescence units (RFU), was measured on a fluorescence spectrophotometer (F-3010; Hitachi Koki Co. Ltd., Tokyo, Japan), with excitation at 325 nm and emission at 393 nm. The first-order rate constant obtained in each experiment was divided by the enzyme concentration to calculate its specificity index: k_{cat}/K_m .

The individual kinetic parameters, K_m and k_{cat} , for the NFF-2 substrate were also determined by analyzing experimental data with Lineweaver–Burk plots. The assay mixture contained 10–20 nM P-III enzymes and the concentration of the substrate was 0.2–5.0 μ M.

2.8. Antiserum and immunoblotting

Approximately 250 μ g of purified daborhagin-M in phosphate-buffered saline was thoroughly mixed with an equal volume of Freund's complete adjuvant for the first injection, or Freund's incomplete adjuvant for the second and third injections. It was subcutaneously injected every two weeks into the back of a male rabbit biweekly. Ten days after the third injection, blood was taken from the rabbit's ear vein. The serum was harvested using centrifugation after it had clotted and been stored at 4 °C. For Western blotting, 100 ng of crude venoms was separated using 8% SDS–PAGE under non-reducing conditions. After the samples had been blotted onto a PVDF membrane, they were probed using anti-daborhagin-M antiserum (1:1000 dilution) and horseradish peroxidase-conjugated second antibody (1:2000 dilution). Immunoreactive bands were detected using the NiCl₂ enhancement method [24].

2.9. N-terminal sequencing

To determine their N-terminal sequences, purified daborhagins (10–20 μ g/well) were electrophoresed on a 1.0 mm thick 8% SDS–PAGE under reducing condition. The protein bands were blotted to a PVDF membrane. After the samples had been stained with Amido Black (0.2% in 7% acetic acid), the bands were cut out and sequenced using a gas-phase amino acid sequencer (Procise 492; Applied Biosystems).

2.10. Cloning and sequencing

RNA and cDNA were prepared from a pair of venom glands obtained from a *D. russelii* specimen from eastern India [17]. PCR amplification of the daborhagin-K cDNA was done using specific primers: a sense degenerate 17-mer complementing the N-terminal residues 7–12 (NRYFNP), and an antisense 18-mer designed according to the conserved sequences of far 3'-UTR of SVMPs [25]. To clone the 5' upstream region, one primer based on the 5'-end conserved sequences and the other based on the amino acid sequence "AIDLNGL" of the protease domain were used. PCR conditions were as follows: initial denaturation for 2 min at 94 °C, followed by 35 extension cycles (denaturation for 1 min at 94 °C, annealing for

1 min at 52 °C, and elongation for 1 min at 72 °C), and a terminal extension step of 72 °C for 10 min. The products were cloned into a pGEM-T vector, and the plasmids were transformed to *Escherichia coli* strain JM 109. White transformants were selected and positive clones were subjected to DNA sequencing using Taq-Dye-Deoxy terminator cycle sequencing kit (Applied Biosystems).

2.11. Peptide mass fingerprinting

For peptide mass fingerprinting (PMF), gel bands of native and deglycosylated daborhagins were excised separately and cut into pieces. Each sample was dehydrated with acetonitrile for 10 min, dried, and then dissolved in 25 mM NH_4HCO_3 (pH 8.5) containing 100 mM dithioerythritol at 37 °C for 1 h. Its Cys-residues were alkylated with 65 mM iodoacetamide at 27 °C for 1 h in the dark. The protein was washed twice with 50% acetonitrile, dried, and hydrolyzed with 25 ng of modified trypsin (Promega) in 25 mM NH_4HCO_3 (pH 8.5) at 37 °C for 16 h. The digest was twice extracted with 50% acetonitrile (containing 5% formic acid) for 15 min each and then dried. The resultant peptides were analyzed using MALDI-TOF/TOF with a detecting mass range of 800–4000 Da.

2.12. Phylogenetic analysis

Amino acid sequences of 26 P-III SVMPs were retrieved using BlastP search. Their sequences were aligned using the Vector NTI program (Invitrogen Corp., Carlsbad, CA, USA). A phylogenetic tree was generated using the neighbor-joining methodology of the PHYLIP program, with an RVV-X heavy chain as an out-group. The degree of confidence was determined using bootstrap analyses of 1000 replicates [26].

2.13. Absolute complexity plot and homologous modeling

The absolute complexity plot shows the average of pairwise alignment scores of each residue using the substitution matrix blosum62mt2. Individual plots were generated using the alignment of the members from each class with the AlignX module of the Vector NTI program.

The 3D-model of daborhagin was built using the Modeller program (<http://salilab.org/modeller>), with the crystal structure of crotocollastatin/VAP2B (PDB code 2DW0) from *Crotalus atrox* venom [27] as a template. Model geometry was analyzed using the PROCHECK program. The ribbon diagrams were generated using the PyMOL program (<http://pymol.sourceforge.net>).

3. Results

3.1. Purification and characterization of daborhagin

The crude venom of *D. siamensis* (Myanmar) was separated into several fractions using a Superdex G-75 column (Fig. 1A). The first fraction, which showed the strongest caseinolytic

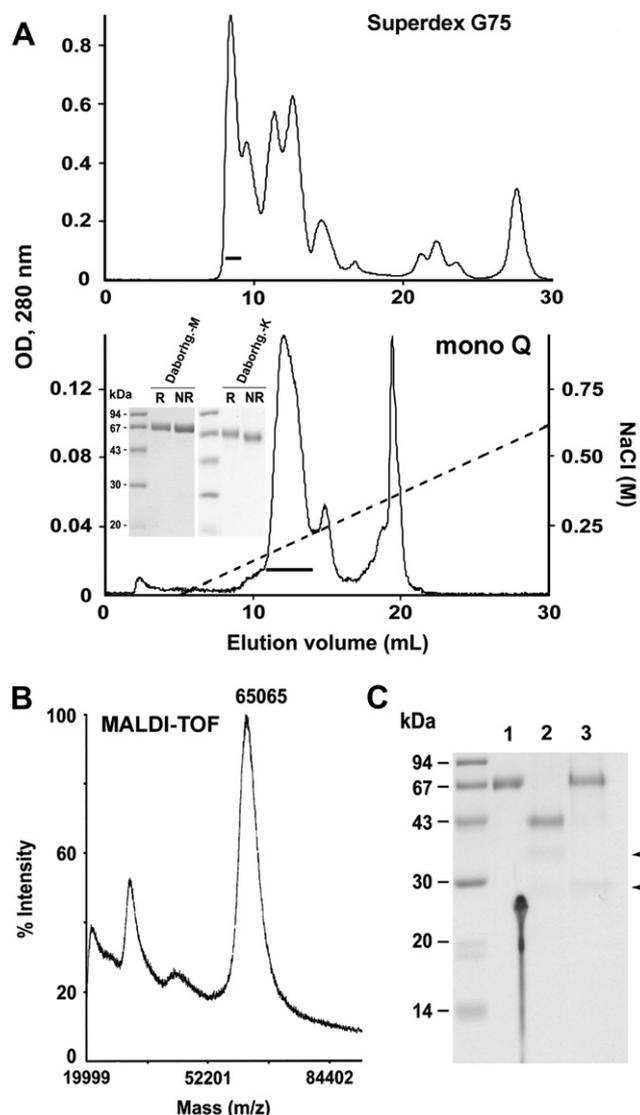


Fig. 1. Purification and characterization of daborhagins. (A) Myanmar *Daboia siamensis* venom was separated using a Superdex G-75 column equilibrated with 0.1 M ammonium acetate (pH 6.7), and then a Mono Q column with a 0–0.6 M NaCl gradient in 50 mM Tris–HCl (pH 8.0). The active fractions are indicated by bars. The inset indicates the SDS–PAGE patterns of purified daborhagin-M and -K under reducing (R) and non-reducing (NR) conditions. (B) The precise molecular weight of native daborhagin-M was analyzed using MALDI-TOF. (C) Daborhagin-M (4 µg) was incubated with buffer only (lane 1) or with 1 unit of PNGase F (lane 2) or 5 units of Endo H (lane 3) at 37 °C for 3 h. Samples were then subjected to SDS–PAGE under reducing conditions. Arrowheads indicate deglycosylation enzymes.

activity, was collected. After it had been desalted and concentrated, it was further partitioned using ion-exchange chromatography on Mono Q (Fig. 1A) and Mono S (data not shown) columns. The purified active component induced hemorrhage with a minimum hemorrhagic dose (MHD) of 0.86 µg when subcutaneously injected into mice. Based on the species origin (*Dabo-*), hemorrhagic activity (-rhagin), and geographic region (Myanmar), we designated it daborhagin-M. Using the same procedures, we purified another hemorrhagin from the venom of *D. russelii* (Kolkata, eastern India) and designated it daborhagin-K. Its hemorrhagic potency with a MHD of 0.82 µg

was similar to that of daborhagin-M. The amounts of daborhagin-M and daborhagin-K in the crude venoms were estimated to be 5.5% and 0.8% (w/w), and their specific activities toward azocasein were 7.1 and 7.3 units/mg, respectively.

SDS-PAGE analysis of both daborhagins revealed a single band with an apparent mass of 62 and 66 kDa under non-reducing and reducing conditions, respectively (Fig. 1A, inset). We further determined the mass of daborhagin-M using MALDI-TOF mass spectrometry. We identified a single-charged monomer with an average molecular mass of 65,065 Da and a double-charged monomer (m/z 31,627) (Fig. 1B). PNGase F treatment reduced the mass to 43 kDa, but Endo H treatment did not affect it (Fig. 1C), which suggested that daborhagin-M contains 4~5 complex type *N*-linked glycans. To examine its general proteolytic activities, we used various divalent metal ions and proteinase inhibitors in regular azocasein assays (data not shown). Adding 5 mM of Ca^{2+} or Mg^{2+} increased the caseinolytic activities of the purified daborhagin-M by 5–23%, and adding 5 mM of EDTA, EGTA, or 1,10-phenanthroline strongly inhibited its activities by 84–94%. However, serine protease inhibitor, e.g. PMSF, was not inhibitory. These results suggested that daborhagin-M is a high molecular weight metalloproteinase.

3.2. Proteolytic activity toward plasma and basement membrane proteins

The specificities of daborhagin-M were studied using potential plasma and matrix proteins as substrates. At a low enzyme concentration of 75 nM, daborhagin-M cleaved the A α -chain of human fibrinogen specifically within minutes, but it had no apparent effect on B β - and γ -chains (Fig. 2A). Thus, daborhagin-M is a new α -fibrinogenase. Notably, it completely degraded high molecular weight subunits (>200 kDa) of type IV

collagen in 2 h, and partially degraded a 250 kDa main chain of fibronectin into five fragments of 83–225 kDa. By contrast, we barely detected laminin hydrolysis after 24 h (data not shown).

3.3. Cleavage sites on insulin chain-B

We also used oxidized insulin chain-B to examine the proteolytic specificity of daborhagin (Fig. 2B). After oxidized insulin chain-B had been hydrolyzed by 1.0 μM daborhagin-M at 37 °C, its products at various incubation times (10 min–24 h) were isolated using reverse-phase HPLC, and then each purified oligopeptide was analyzed using MALDI-MS spectrometry (data not shown). We found that daborhagin-M had cleaved chain-B at four sites (e.g. X-Leu and X-Phe), similar to other SVMPs [28]. The fast cleavages were at Ala¹⁴–Leu¹⁵ and Tyr¹⁶–Leu¹⁷, followed by those at His¹⁰–Leu¹¹, and the slow cleavages were at Phe²⁴–Phe²⁵ (Fig. 2B).

3.4. Kinetic study using fluorogenic substrates

The fluorogenic peptide substrates FS-1 (cleaving at Gly-Leu), NFF-2 (cleaving at Ala–Nva), and NFF-3 (cleaving at Glu–Nva) were originally developed to measure matrix metalloproteinase (MMP) activity [22,23]. To study the relationship between substrate specificity and hemorrhagic potency, we compared the kinetic specificities of daborhagin-M and the other five P-IIIs toward these substrates. The specificity index ($k_{\text{cat}}/K_{\text{m}}$) of daborhagin-M toward NFF-2 was about 3–4 times higher than those toward FS-1 or NFF-3 (Table 1). Other strong venom hemorrhagins—BHRa of *Bitis arietans* venom [19], CVHRa of *C. vipera* venom, and acurhagin of *Deinagkistrodon acutus* venom [18]—also showed higher $k_{\text{cat}}/K_{\text{m}}$ values toward NFF-2 or NFF-3 than the weak hemorrhagins, e.g. TSV-DM of *Trimeresurus stejnegeri* venom [20] and ECLV-

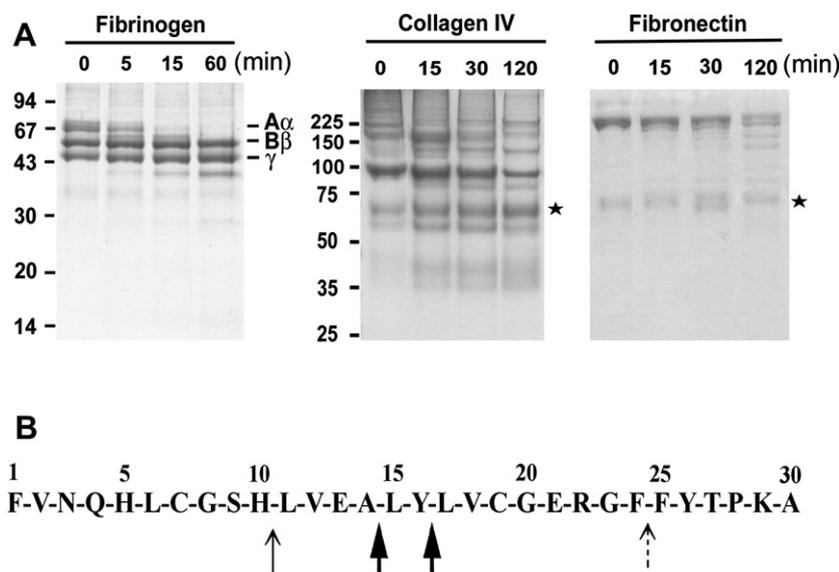


Fig. 2. Substrate specificities of daborhagin-M. (A) Fibrinogen and basement membrane proteins (1 mg/ml) in 100 mM Tris–HCl (pH 8.0) were hydrolyzed by 75 nM daborhagin-M (for fibrinogen) or 750 nM daborhagin-M (for other substrates) at 37 °C. Reaction time is shown above the gel lanes; the daborhagin-M band is marked with an asterisk. (B) Hydrolysis of oxidized insulin chain B using 1.0 μM daborhagin-M at 37 °C. Fast, moderate, and slow cleavages are marked by thick arrows, thin solid-line arrow and thin dashed-line arrow, respectively.

Table 1
Kinetic specificities of P-III SVMPs toward three synthetic oligopeptides in 0.1 M Tris-HCl (pH 7.5) at 25 °C

Class	P-III enzymes	$k_{\text{cat}}/K_m \times 10^{-3}, \text{M}^{-1} \text{s}^{-1}$			k_{cat} (s^{-1})	K_m (μM^{-1})
		FS-1	NFF-3	NFF-2		
HH	Daborhagin-M	30.6	41.2	125.0	0.88	7.0
	BHRa	3.2	128.7	23.7	0.13	5.5
	CVHRa	11.0	38.4	96.4	1.26	13.0
	Acurhagin	18.2	41.9	34.2	0.32	9.4
NH	ECLV-DM	0.4	3.8	3.2	0.05	16.1
	TSV-DM	1.9	46.1	1.2	0.03	25.0
Other	RVV-X	0.2	5.7	0.2	— ^a	—

Substrate structures are shown in Section 2.

^a —, not determined.

DM of *E. leucogaster* venom (Table 1). By contrast, RVV-X barely hydrolyzed these synthetic substrates.

We also determined the K_m and k_{cat} values of all the above P-IIIs for substrate NFF-2. The data suggested that lower K_m and much higher k_{cat} values accounted for the faster hydrolysis of NFF-2 by the strong hemorrhagic P-IIIs than the weak or non-hemorrhagic P-IIIs (Table 1).

3.5. Occurrence of daborhagin-like enzymes in *Daboia* and other viperid venoms

Anti-daborhagin antiserum was prepared by immunizing one rabbit with native daborhagin-M; the antiserum reacted similarly to both daborhagins and easily recognized them below 1.0 ng in Western blot analysis (Fig. 3A). To avoid false-positive results, only 100 ng of each venom sample was loaded into the gel. Daborhagins were easily detected in Myanmar and eastern India *Daboia* venoms, with estimated levels of 8.0% and 1.5% (w/w of total soluble venom proteins) (Fig. 3B), respectively. A faint 60-kDa band was also detected in western India *D. russelii* venom. However, no antigen was detected in the *Daboia* venoms collected from Pakistan, Sri Lanka, Thailand, Indonesia, and Taiwan. Thus, daborhagin is found in *Daboia* venoms only in particular geographical regions. Western blotting also revealed that three of the *Daboia* venom samples collected from southern Myanmar contained abundant daborhagins (data not shown).

To further explore the distribution of similar P-III enzymes in other viperid venoms, ten available venom samples under the genera *Vipera*, *Cerastes*, and *Echis* were also tested. The results suggested that various P-III enzymes are simultaneously present in most viperid venoms (Fig. 3C). Notably, *C. vipera* venom is especially rich in P-III enzymes, whereas European viper venoms such as *V. ammodytes* and *V. berus* had low P-III levels.

3.6. cDNA cloning and the predicted sequence of daborhagin-K

The cDNA of daborhagin-K was cloned and sequenced. The open reading frame of the daborhagin-K precursor

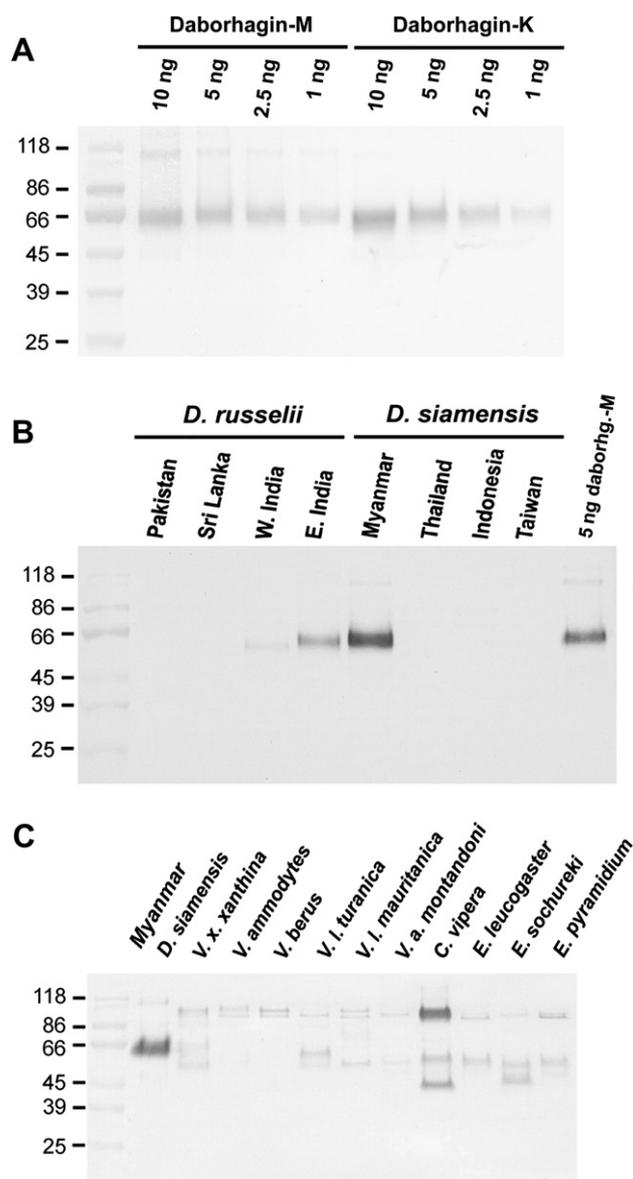


Fig. 3. Detection of daborhagin-like or P-III SVMPs in *Daboia* and other viperid venoms by Western blot analysis. (A) Serological cross-reactivity of both daborhagins with anti-daborhagin-M polyclonal antibodies. (B) Analysis of eight *Daboia* geographical samples. W. India and E. India denote the venoms from Mumbai and Kolkata, respectively. (C) Analysis of ten venom samples from other Viperinae species. In (B) and (C), each sample containing 100 ng of soluble crude venom proteins was separated using SDS-PAGE (8%) under non-reducing conditions. After blocking, the samples on blotting membranes were probed with anti-daborhagin-M polyclonal antibodies.

encoded 615 amino acid residues, including a highly conserved 18-residue signal peptide and a 171-residue proenzyme domain (data not shown). *N*-terminal sequences of predicted mature enzyme nicely matched that of purified daborhagin-K, which were determined as VATSERNRYFNPSYV by automatic sequencer. The deduced daborhagin-K contained 426 residues (with a calculated molecular mass of 48,041 Da) including three classic structural domains of P-IIIs (Fig. 5). Additionally, four potential *N*-glycosylation sites at Asn 74, 80, 189, and 339 were found, which accorded with our observation of a 17-kDa mass increase due to glycosylation (Fig. 1C).

To verify the sequence, native and deglycosylated daborhagin-K were digested in gel with trypsin, and the resulting peptides were analyzed using MALDI-TOF/TOF. The resultant 23 peptide fragments matched those predicted, and they represented more than 70% coverage of the entire sequence (Table 2). Similarly, 11 peptide fragments of daborhagin-M also matched those predicted from the digestion of daborhagin-K. Thus, daborhagin-K and -M shared a high degree of identity in their primary structures.

3.7. Molecular phylogeny and classification of P-IIIs

A phylogenetic tree was constructed based on 27 amino acid sequences of P-III SVMPs; some of them were predicted from cDNA clones and their functions have not been characterized. The topology of the tree reveals three major clusters of P-III enzymes (Fig. 4). Based on their MHD values (shown in parentheses), one cluster containing highly hemorrhagic P-III members is designated as the HH class, and another cluster containing mainly weakly or non-hemorrhagic enzymes is designated as the NH class. The other small cluster is comprised of prothrombin activators derived from *Echis* venom. Notably, daborhagin-K is associated with other strong hemorrhagins, e.g. HR1a (from *Protobothrops flavoviridis*) and HF3 (from *Bothrops jararaca*) within the HH class, but not with the P-IIla and P-IIlc members [2,9].

3.8. Sequence alignment and comparison between the HH and NH classes

Protein sequences of daborhagin-K and the other eight P-III enzymes selected from each lineage of the phylogeny tree were aligned (Fig. 5). Their *pI* values, potential *N*-glycosylation sites, numbers of Cys-residues, and classification subtypes [2,9] were also listed for comparison. Like other P-IIIs, daborhagin contains 17 intramolecular disulfide bonds; its zinc-chelating motif, Met-turn (CIM), and three Ca²⁺ binding sites [29] are highly conserved. In addition, its disintegrin domain contains a DECD sequence instead of the RGD or KGD found in P-II members [2]. Two hydrophobic ridges (HR) at positions 332–333 and 356–357, which possibly create a novel interaction surface with the hyper-variable region (HVR) at 383–410 [29], were also present.

To elucidate the molecular features responsible for distinguishing the HH and NH classes, we further constructed their individual absolute complexity profiles (Fig. 6A). By examining the superimposed topographies of both profiles, we found that most regions were similar. However, four structural elements (designated as M1, M2, C1, and C2) were found to bear significantly higher absolute complexity in HH than in NH members. The consensus sequences of M1, M2, C1, and C2 in HH members were ¹³⁷YSPINLV¹⁴³, ¹⁷⁶PVISxxPSKF¹⁸⁶ (x represents a less-conserved residue), ³⁵³KGNY³⁵⁶, and

Table 2
Match between the calculated (MS_c) and the experimental (MS_e) masses of tryptic fragments of daborhagins

Tryptic peptides of daborhagin-K	Position	MS _c ^a	MS _e	
			Daborhagin-K	Daborhagin-M
VATSERNR	1–8	932.49	932.47	— ^b
NRYFNPYSYVELIITVDHSMVTK	7–29	2805.39	2805.33	—
YFNPYSYVELIITVDHSMVTK	9–29	2535.25	2535.22	—
YKNDLTAIR	30–38	1093.60	1093.59	1093.60
IHDNSQLLTAIDLNGLTIGMAYVSTMCQSK	99–128	3326.60	3326.43	—
YSVGVVQDHSKINLR	129–143	1714.92	1714.90	—
YFSNCSYNQYR	185–195	1501.62	1501.75	—
YFSNCSYNQYRR	185–196	1657.72	1657.79	—
RFLTEHNPECIINPPLR	196–212	2106.09	2106.07	2106.06
FLTEHNPECIINPPLR	197–212	1949.99	1949.98	1949.99
TDIVSPPACGNELLER	213–228	1770.87	1770.86	1770.84
LHSWVECESGKCCNQCR	252–268	2209.90	2209.92	2209.92
RAGTECRPARDECDKAEQCTGR	271–292	2623.15	2623.17	—
AGTECRPARDECDKAEQCTGR	272–292	2467.05	2467.05	2467.06
SANCPVDFEHENGRPCLHNFYCYNGK	293–319	3242.36	3242.23	—
CPIMYHQCHALFGQNVTVGVQDSCFQYNR	320–347	3446.49	3446.32	—
LGVYYAYCR	348–356	1164.55	1164.54	—
LGVYYAYCRK	348–357	1292.65	1292.63	—
ENGRKIPCAPKDEK	358–371	1584.82	1584.67	1584.68
LYCSYKSPGNQIPCLPYIIPSDENK	375–399	3006.40	—	3006.36
CGRLYCSYK	372–380	1206.54	1206.58	—
SPGNQIPCLPYIIPSDENK	381–399	2192.03	2192.00	2192.00
SPGNQIPCLPYIIPSDENKGMVDHGT	381–407	3033.40	3033.27	3033.38
CGDGKVCNSNGQCVDLNIAY	408–426	2129.90	2129.98	2129.93

All Cys residues have been transformed to *S*-carbamidomethylated. The MS_c calculation was based on monoisotopic masses of amino acids, assuming the peptide mass as [M + H]⁺.

^a The calculated masses (MS_c) of tryptic peptides are based on predicted daborhagin-K sequence.

^b —, not found in the experimental MS data.

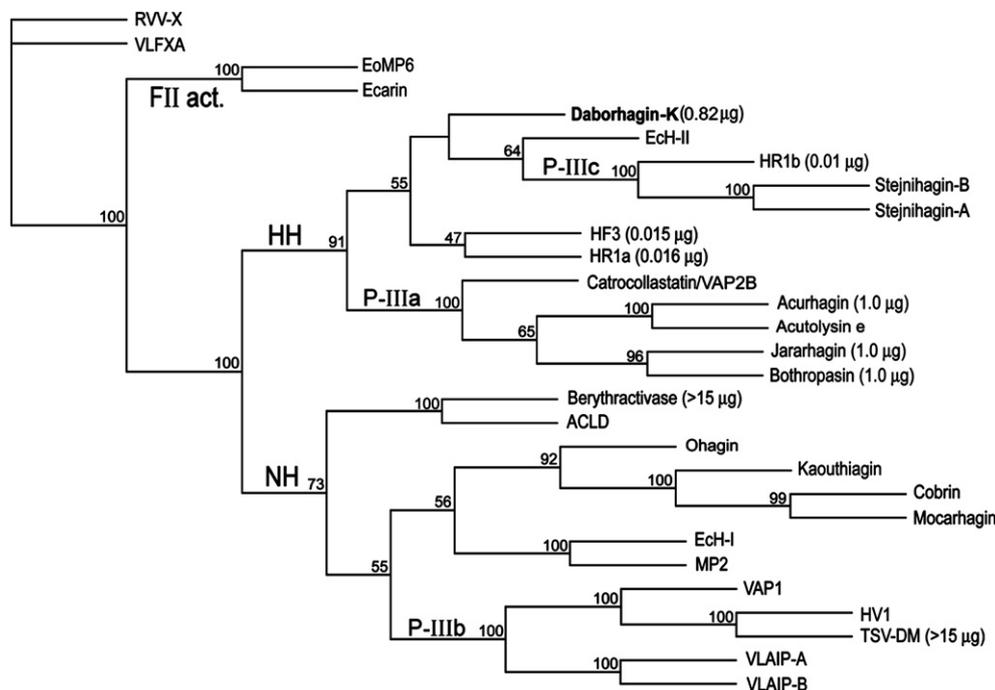


Fig. 4. Phylogenetic tree of P-III SVMPs based on amino acid sequences. The RVV-X heavy chain (AAB22477) was the out-group. Bootstrap values are shown at each node and MHD values are shown in parentheses. Abbreviations: FII act., prothrombin activators; HH, highly hemorrhagic class; NH, non-hemorrhagic class. The accession numbers and species are: Daborhagin-K (DQ137798), *Daboia russelii*; ACLD (AAC18911), *Agkistrodon contortrix laticinctus*; Acurhagin [18] (AAS57937) and Acutolysin e (AAD27891), *Deinagkistrodon acutus*; MP-2 (AAX86634), *Bitis arietans*; Bothropasin (AAC61986), HF3 [49] (AAG48931), and Jararhagin [50] (CAA48323), *Bothrops jararaca*; Berythreactivase [6] (AAL47169), *Bothrops erythromelas*; Catrocollastatin/VAP2B [27] (AAC59672) and VAP1 [29] (BAB18307), *Crotalus atrox*; Ecarin [5] (Q90495), *Echis carinatus*; EoMP6 (AAP92424), *Echis ocellatus*; EcH-I (CAA55565) and EcH-II (CAA55566), *Echis pyramidum leakeyi*; Ohagin (ABM87941), *Ophiophagus hannah*; Kaouthiagin [51] (P82942), *Naja kaouthia*; Cobrin (AAF00693), *Naja naja*; Mocarhagin (AAM51550), *Naja mossambica*; HR1a (BAB92013), HR1b [52] (BAB92014), and HV1 (BAB60682), *Protobothrops flavoviridis*; TSV-DM [20] (ABC73079), Stejnihagin-A [9] (ABA40760), and Stejnihagin-B [9] (ABA40759), *Trimeresurus stejnegeri*; VLFXA HC (AAQ17467), VLAIP-A [53] (AAX38182), and VLAIP-B [53] (AAX38182), *Macrovipera lebetina*.

⁴⁰¹KYFY⁴⁰⁴ (Fig. 6B). The corresponding locations of these four regions are shown in the 3D model of daborhagin (Fig. 6C).

4. Discussion

To address the problem of highly hemorrhagic symptoms elicited by Myanmar and eastern India *Daboia* envenoming [13–15], we isolated the active P-III protease, daborhagin, from both *Daboia* venoms (Fig. 1). When subcutaneously injected into dorsal skin of mice, daborhagins caused severe hemorrhage with a MHD of 0.8–0.9 µg. Thus, daborhagins are highly hemorrhagic (MHD < 1 µg) toxins [30]. We also showed that daborhagin is a potent α -fibrinogenase, similar to the VaH1 hemorrhagic P-III from *V. ammodytes* venom [31], but it has no effect on collagen- or ADP-induced platelet aggregation (data not shown). Daborhagin thus is able to interfere with the homeostatic system by degrading plasma fibrinogen. Furthermore, it has highly proteolytic activities toward type IV collagen and fibronectin, corroborating that the enzymatic hydrolysis of matrix components is a key event of SVMP-induced microvessel disruption [3,30,32]. A recent study [4] reported that jararhagin (P-III class) was more potently hemorrhagic than BaP1 (P-I class) because it selectively cleaved key peptide bonds in mouse nidogen. Therefore,

daborhagin appears to be another good model for the biochemical study of hemorrhagic mechanisms induced by SVMPs.

Western blot analyses showed that only the *Daboia* venoms from Myanmar and eastern India have high levels of daborhagin (Fig. 3), and, indeed, that the venom of *Daboia* from Myanmar contains 6–7 times more daborhagin than that from eastern India. Such results are consistent with the fact that *Daboia* snakebites in both regions are particularly lethal and hemorrhagic, and that Myanmar's viper envenoming frequently causes gastrointestinal or respiratory tract bleeding as well as pituitary infarction [13–15]. Thus, daborhagins are most likely critical in these syndromes. Recently, we found that the PLA₂ isoforms isolated from *Daboia* venom of both regions share 97–100% sequence identities [17]. All these venom similarities strongly support that these two *Daboia* populations are closely related and represent a special lineage of this genus, which may explain why Myanmar antivenom for Russell's viper was not effective against Thailand and Sri Lanka Russell's viper venoms [33,34]. This is useful information for *Daboia* antivenom production and the clinical management of *Daboia* envenomed victims.

Daborhagin-M and -K are orthologous genes in two *Daboia* species, but they have some microheterogeneities imbedded in their sequences. First, both enzymes were isolated from *Daboia* venoms using similar steps and had comparable

Metalloprotease domain

	10	20	30	40	50	60	70	80	90	100	
Daborhagin	VATSERNRVFNPSYSVELIITVDHSMVTYKNDLTAIRTWVFLVNTINEIFKYLYIRVPLVGLIEWKNRDLINVTSAANVTLDLFGWEWRKSYLLPRKIHDSQLL										
HF3	EQRY. H. K. I. V. LA. YR. NNG. GK. . K. IY. I. IL. R. IA. I. S. A. S. LS. DD. AS. T. GTV. K. S. A.										
HR1b	QQ—RF. RR. IK. A. V. GI. HHGN. KK. K. IYQ. N. YRS. N. L. A. Y. SKONK. T. Q. S. D. E. V. KQRS. CA.										
Jararhagin	EQRYD. K. I. FFVV. QGT. NNG. DK. KARMY. A. IV. R. MH. A. S. G. K. T. KPVDVY. NS. A. TD. T. K. A.										
Acurhagin	EQQLYD. FK. TVVV. KA. NG. DK. K. KMY. AA. NM. MYR. MFF. VM. I. TEE. K. T. KPVDVY. NA. A. T. AE. K. A. I										
VAP1	QQ. L. AKK. K. FLVA. YI. YL. GRN. V. RMYDI. V. TP. YHRMN. H. A. S. T. K. T. Q. S. D. AK. ATD. S. S. A.										
VLAIP-B	QRT. LKSKK. V. VA. YI. FW. DRS. ST. RIY. I. L. V. YRF. N. YIA. AV. SKG. S. YD. S. ERD. N. R. A.										
Berythtract	QQA. LDAKK. FVVVL. G. YK. D. DK. KRRIY. I. M. M. IP. N. A. T. SKG. K. ESWF. I. TN. GAD. K. S. A.										
Mocarhagin	TN. P. QD. LQAKK. I. FVVV. NV. YR. TGK. HV. TRR. Y. M. AL. TMYRR. NFHIA. I. S. GNE. Q. DVQA. ENK. RN. A.										
	110	120	130	140	150	160	170	180	190	200	210
Daborhagin	TAIDLNGLTIGMAYVSTMCQSKYSVGVVQDHSKINLRVAVTMAHEIGHNGLGTHDGVYCTCGGYSCTMSAVLGDQPSKYFNSNCSYQVRRFLTEHNPECIINPP										
HF3	FD. Q. I. NIAS. NQNK. M. Y. P. V. I. M. IN. TG. S. S. APEIS. L. KQA. Q. YINYK. Q. L. E.										
HR1b	FD. P. K. TAS. DP. R. I. Y. P. V. I. T. M. IP. NS. FF. PMIS. P. EL. KAY. QT. D. K. Q. L. A.										
Jararhagin	F. P. Y. IGS. HP. R. I. Y. P. V. I. M. IH. TG. S. S. D. F. GPTIS. NE. F. I. QWD. IMN. E.										
Acurhagin	FR. SI. Y. IGS. HP. R. II. Y. P. VL. I. M. IH. DG. Y. F. GPSIS. PE. F. I. QWD. IMN. D. E.										
VAP1	GNF. P. A. LG. LGGI. NTM. A. I. HHL. IA. I. M. MD. KDT. TRF. V. RGA. S. TEA. FL. D. QKDH. E. IKNM. Q. LKK.										
VLAIP-B	GNF. PSA. RGF. GR. P. I. Y. L. SA. M. MD. RID. AK. GI. R. ET. YL. D. REEH. KY. INKM. Q. L. K.										
Berythtract	NT. FD. S. R. HIGS. HPYL. II. Y. PV. L. S. M. MH. NDT. AP. V. A. AIS. KD. L. QEYQ. KY. IKNR. Q. LL. K.										
Mocarhagin	STEF. T. T. LG. IGSL. SP. K. A. STSM. I. QM. MND. RAS. SNK. TKY. YESLSE. S. VQEH. EY. LRDR. Q. L. K.										

Linker and disintegrin domain

	220	230	240	250	260	270	280	290	300
Daborhagin	LRTDIVSPACGNELLERGEEDCGSPENCRDPCDAAACKLHSHWVECESGKCCNCRFKRAGTECRPARDECDKAEQCTGRSANCVPVDEFHE								
HF3	V. M. R. T. E. D. G. A. S. I. S. Q. D. T. D. KR								
HR1b	SK. V. A. QYQ. K. E. D. RT. A. ES. IP. S. Q. D. T. R. R								
Jararhagin	I. V. V. T. QNE. T. K. GSQ. GH. D. E. K. SKS. ASMS. P. H. Q. SE. A. V. K								
Acurhagin	I. L. V. T. QNE. T. K. GSQ. GH. D. E. K. RTS. ASMS. P. H. Q. SE. A. V. K								
VAP1	K. V. AV. YFV. V. RT. TT. RGAQ. AE. L. D. G. A. K. M. DV. E. T. R. QR								
VLAIP-B	AV. YFV. V. A. Q. R. T. RPGAQ. GD. V. Y. K. R. V. NG. VSDL. Q. E. T. Q. QR								
Berythtract	I. V. V. T. N. TT. TPGSQ. VE. L. D. RKT. A. KHD. LP. S. Q. D. M. D. QR								
Mocarhagin	KA. T. V. YFV. D. QNT. T. QHEAQ. D. E. EK. K. G. A. A. KND. FP. L. K. K. S. QR								

Cys-rich domain

	310	320	330	340	350	360	370	380	390	400	410
Daborhagin	NGRPLHNFGYCYNGKCPIMYHQCHALFG										
HF3	Q. Y. N. Y. S. A. VAE. G. EF. EN. DK. F. QS. VN. QE. V. F. HT. KKH. DYK. S-E. PD										
HR1b	Q. Y. H. FY. YF. S. A. VAE. D. NN. KK. DK. F. EKY. QE. V. F. DN. KYP. HYN. S-E. LD										
Jararhagin	Q. D. Y. N. Y. AD. YEAE. KD. QK. N. G. K. E. V. KDN. QNN. KMF. SND. H										
Acurhagin	E. D. Y. N. Y. ADIYEAE. ES. KK. N. G. K. SE. V. KDD. QNN. KMF. SND. H										
VAP1	Q. KN. N. AD. I. T. PGA. VS. A. F. E. NH. G. QNT. A. E. Q. V. FPN. E. KN. NT. S. N. D										
VLAIP-B	Q. QN. K. T. EK. IS. ASA. VA. F. R. NH. G. NT. A. E. V. LDN. S. HKN. QI.										
Berythtract	H. QN. N. T. EN. ID. V. PKA. VAE. KD. QK. ND. G. K. E. Q. V. NDN. QNN. KQI. F. RN. D										
Mocarhagin	H. QN. Q. T. TLTN. AT. W. PGAKMSPGL. ML. WNARS. GL. L. A. V. F. KK. SMI. HPPPSK. P.										

	420	430	441	identity (%)	pI	No. of N-glycan	No. of Cys	Subclass
Daborhagin	KGMVDHGTCKGDGKVCN—NGQCVDLNIAY—			100	6.90	4	34	
HF3	Y. N. A. H. VAT. —			63	5.26	5	34	
HR1b	F. A. A. R. V. E. KS			60	6.02	4	35	PIIIc
Jararhagin	LP. A. H. VAT. —			61	5.07	1	35	PIIIa
Acurhagin	LP. A. H. VTT. —			58	4.94	1	35	PIIIa
VAP1	LP. A. R. A. VTTT. —			57	7.41	1	35	PIIIb
VLAIP-B	P. M. K. VT.			60	7.82	4	35	PIIIb
Berythtract	R. LP. A. RH. VAT. —			56	6.15	4	36	
Mocarhagin	Y. AP. VK. R. KV—			50	8.77	2	36	

Fig. 5. Alignment of amino acid sequences of daborhagin-K with those of representative HH and NH members. The HH sequences are above the dashed lines, and the NH sequences are below. Residues identical to those of daborhagin-K are denoted by dots, and the gaps are marked with hyphens. Potential N-glycosylation sites of daborhagin-K are underlined. Ca²⁺-binding sites and non-conserved Cys-residues are shaded in gray and black, respectively. Conserved zinc-binding sites, Met-turns, and ECD motifs are boxed. Two hydrophobic ridges (HR) and hyper-variable regions (HVR) are also marked.

hemorrhagic potencies. Second, their masses were almost identical according to SDS-PAGE analysis. Third, anti-daborhagin-M antiserum easily detected daborhagin-K in Western blot analysis, which indicated that they are closely related and share common epitopes. Furthermore, tryptic PMF analysis of daborhagin-M and -K confirmed that they both had 11 and 23 unique peptides, which nicely matched those predicted from daborhagin-K cDNA (Table 2). Moreover, the N-terminal sequence (1–25) of daborhagin-M is identical to that of daborhagin-K, except for the replacement of the Pro⁶ with Arg⁶.

Upon obtaining the full primary sequence of daborhagin-M, one may clarify how many heterogeneities exist between them.

The previously reported VRR-73 (MHD = 0.5 μg) [16] showed comparable masses and hemorrhagic potencies with daborhagin-K, and both were derived from eastern India *D. russelii* venom. Like other hemorrhagic SVMPs, daborhagin-K contains one typical zinc-chelating motif and three Ca²⁺ binding sites. Indeed, adding 5 mM Mg²⁺ or Ca²⁺ to daborhagin-K increased the casein hydrolysis rate by 13–23%.

from those of weakly hemorrhagic Ht-c and Ht-d (P-I class), which prefer a small residue at the P₁ position [36]. In support of this idea is the observation that daborhagin hydrolyzed NFF-2 (cleaving at Ala–Nva) more efficiently than it did FS-1 (cleaving at Gly–Leu) or NFF-3 (cleaving at Glu–Nva). Notably, daborhagin cleaved the bond between His¹⁰ and Leu¹¹Val¹², but not the bond between His⁵ and Leu⁶Cys⁷(–SO₃H) in insulin chain-B, which suggested that negatively charged residues at the P₂' position were not favored. Taking all these findings together, daborhagin has a substrate specificity toward hydrophobic and less polar amino acid residues at the P₁, P₁', and P₂' positions.

Molecular phylogeny has been a powerful tool in classifying venom protein families and identifying distinct functional subtypes. Our previous phylogenetic analyses of the P-I (and processed P-II metalloproteinases) clearly revealed three existing subtypes [25]. Recently, two cladograms showed that P-III SVMPs could be divided into P-IIIa, P-IIIb, and P-IIIc subclasses [2,9]. With more P-III sequences, our new phylogenetic tree revealed two major subtypes (designated HH and NH classes). Consistent with its functional assays, daborhagin belongs to the HH class and is associated with several of the strongest hemorrhagins. Remarkably, there is no subclass-specific seventh cysteinyl residue in the metalloproteinase domains of daborhagin, EcH-II, HR1a, or HF3 (Figs. 4 and 5). This led us to hypothesize that they constitute another new hemorrhagic group among the HH members in addition to P-IIIa and P-IIIc. It is noteworthy that these four P-III enzymes and P-IIIc are more hemorrhagic than P-IIIa, and that they contain an average of four *N*-glycosylation sites while P-IIIa contains only one (Fig. 5). Further investigations of whether these glycosylation sites are important to hemorrhagic activity are needed. By contrast, the NH group is more versatile and further divided into three clusters, possibly with different functions, e.g. inducing endothelial cell apoptosis [7].

Our phylogenetic tree also suggests the co-evolution of paralogous HH and NH enzymes in many venom species (Fig. 4), e.g. *P. flavoviridis* (HR1a, HR1b, HV1) and *Crotalus atrox* (Crotocollastatin/VAP2B, VAP1). Recent studies [18,31,37,38] have also reported that at least two types of P-IIIs with different biological functions are expressed in *D. acutus* (Acurhagin, AAV1) and *V. ammodytes* (VaH1, VaH2, Ammodytase) venoms. These paralogous P-IIIs co-expressed in snake venoms might synergistically affect different targets and adapt to the ecology of their prey. Moreover, both P-III paralogs might have evolved before the subfamilies Viperinae and Crotalinae split.

Each member of the P-III SVMPs elicits varying degrees of hemorrhagic potency, and the responsible structural elements are still puzzling [2,39]. Since the tree topology clearly classified the P-III SVMPs into two major classes with distinct hemorrhagic potencies, the motifs or residues that distinguish these two classes in the phylogenetic analysis might also be critical in P-III hemorrhagic potencies. Based on this consideration, we identified four conserved motifs in the HH class, relative to the NH class. Of these, the M1 and M2 motifs are in the metalloproteinase domain and separated by the zinc-binding

region and the Met-turn. Both motifs form loop structures around the catalytic site (Fig. 6C). The M1 motif (positions 137–143) serves as a dimer-interface in the P-IIIb subclass [29], and the formation of dimer probably hinders the entrance of substrates to the active site and thus restricts the enzyme specificity. The M2 motif in the HH class contains a conserved proline bracket [40], ¹⁷⁶PVISxxP¹⁸³, which has been suggested to form one wall of the extended substrate-binding site of SVMPs [41]. The other two motifs, C1 and C2, are in the Cys-rich domain. 3D homologous modeling showed that the C1 motif forms the hydrophobic ridge and that the C2 motif is the central part of the hyper-variable region (HVR). Both the C1 and C2 motifs have been suggested to constitute a potential protein–protein interaction interface [29]. By contrast, the disintegrin domains of both the HH and NH classes are similar in their absolute complexity profiles, which agree with findings that the disintegrin domain might be merely a linker for the other two domains [29]. Collectively, these four structural motifs are probably important determinants for substrate interaction and the P-III hemorrhagic potencies.

The *pI* values of HH-class members (average 5.5) were lower than those of NH-class members (average 7.4). By comparing the surface electro-potential of 3D models between more than 10 representative P-III SVMPs (data not shown), we found that most HH enzymes bore more surface negative charges than NH enzymes did. The importance of this difference remains to be determined. Remarkably, the *N*-glycosylation site at Asn189 (Fig. 6B), on the C-terminal side of the M2 motif, is important for the hemorrhagic potencies of jararhagin [42], and this site is strictly conserved in HH- but not in NH-class members.

Fluorogenic peptide substrates have been successfully used for comparing the specificities of SVMPs [36,43]. The synthetic tetrapeptide Abz-Ala-Gly-Leu-Ala-Nba (cleaving at Gly–Leu) has been examined as a good substrate for weakly hemorrhagic Ht-c and Ht-d toxins [36]. Additionally, a 38-kDa non-hemorrhagic metalloproteinase from *Rhabdophis tigrinus tigrinus* venom has high proteolytic specificities towards FS-1 (cleaving at Gly–Leu) [43]. Here, we found that the highly hemorrhagic P-III enzymes were more active toward three fluorogenic substrates, especially NFF-2 and NFF-3 (Table 1). Further kinetic analyses suggested that the differences between the HH and NH enzymes for NFF-2 are in both *k*_{cat} and *K*_m. Three regions—residues 140–143, 176–181 and 182–185—were found to be related to the active site domains of SVMPs [41,44,45] and located in M1 and M1 motifs. Remarkably, these regions within the HH members are more hydrophobic (including Ile140, Asn141, Leu142, Val143, Val177, and Ile178) and rigid (Pro139, 176, and 183) (Fig. 6B). Presumably, by favorable interactions with hydrophobic and bulky residues in the substrates (e.g. the P₁ and P₁' subsites), HH members might trigger a better induced-fit effect and subsequent transition state stabilization to increase their *k*_{cat}. Notably, the specificity of the HH enzymes for these fluorogenic substrates appears to be similar to that of the vertebrate hemorrhagic MMP3 (Stromelysin 1) [22,23], which

may activate several MMP zymogens [46]. Whether daborhagin or other HH-class members activate endogenous MMPs or inflammatory cytokines, which may contribute to local tissue damage, requires further investigation [47,48].

In summary, we purified and characterized daborhagin, a highly hemorrhagic P-III metalloproteinase of Russell's viper's venom, and solved its sequence. Daborhagin has the high proteolytic activity toward fibrinogen, collagen, and fibronectin; this activity appears to be related to the severe bleeding symptoms of its envenomed victims. Moreover, a phylogenetic analysis unraveled the co-evolution of two paralogous P-III classes with different hemorrhagic potencies in the venoms of Viperidae, and daborhagin apparently belongs to the new, highly hemorrhagic subclass. We identified four regions related to the classifications of the P-III SVMPs; detailed studies of their importance to hemorrhagic activities and functional diversities can now begin. Our findings not only unravel the important toxicology of hemorrhagin in specific populations of *Daboia*, but also provide a good basis for designing rational mutagenesis experiments to study the structure-function relationships of the P-III SVMPs.

Acknowledgments

We thank Professors Antony Gomes (University of Calcutta, India), Yu-Yen Shu (Kuangxi Medical University, China), and R. David G. Theakston (Liverpool School of Tropical Medicine, UK) for their gifts of venom samples, and Mr. S. Lin for proofreading the manuscript. This work was supported by grants from the National Science Council and Academia Sinica of Taiwan.

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