This study describes the first isolation of hemorrhagic metalloproteinase HR1b from the venom of Okinawa habu, and its cDNA cloning. The substrate specificity of isolated HR1b definitely differed from that of HR1a, further supporting the presence of a metalloproteinase distinguishable from HR1a in the venom of Okinawa habu. The deduced amino acid sequence of HR1b showed 99.67% identity with HR1b of Amami habu, with only two amino acid residue replacements.

**Key words:** hemorrhagic metalloproteinase; HR1b; *Protobothrops flavoviridis*; Okinawa habu

Hemorrhage is a major complication resulting from envenomation by snakebite. It is a bleeding symptom caused by disruption of the vascular basement membrane. The primary component responsible for this bleeding has been shown to be endopeptidase, belonging to the family of snake venom metalloproteinases (SVMPs). It has been found that the composition of venom protein shows regional variations even in the same snake species. Two closely related metalloproteinases, HR1a and HR1b, were purified from the venom of *Protobothrops flavoviridis* (Amami habu), but it has been reported that the venom of Okinawan *Protobothrops flavoviridis* (Okinawa habu) lacks HR1b. The hemorrhagic activities of HR1a and HR1b were found to be 10 times higher than that of HR2a or HR2b. It is thus important to identify and characterize the full spectrum of SVMPs in snake venom.

Purification were undertaken at 4 °C, unless otherwise stated. Fluorescence-quenching peptide, (Nma)-Ser-Pro-Met-Leu-Lys-(Dnp) rr-NH₂, was synthesized and used as a substrate throughout this study. One g of crude *Protobothrops flavoviridis* venom was dissolved in 10 ml of 0.01 M sodium phosphate buffer (pH 6.8, unless otherwise stated). The venom solution was centrifuged at 10,000 × g for 20 min to remove insoluble material. The supernatant liquid was applied to a Sephacryl S-200 column (2.6 × 90 cm) pre-equilibrated with the same buffer. The proteins were eluted at a flow rate of 60 ml/h, and 10 ml fractions were collected. The protein content of each fraction was monitored at 280 nm with a spectrophotometer. The resulting metalloproteinase fractions were combined and loaded on a Chelating Sepharose Fast Flow (1.5 × 15 cm) column that had been immobilized with Zn²⁺, and equilibrated with 0.01 M sodium phosphate buffer containing 0.5 M NaCl. The column was washed with the same buffer, and the venom metalloproteinase was eluted by a linear 0–30 mM imidazole gradient in 0.01 M sodium phosphate buffer. After dialysis against 0.01 M sodium phosphate buffer containing 0.5 M NaCl. The column was washed with the same buffer, and the venom metalloproteinase was eluted by a linear 0–30 mM imidazole gradient in 0.01 M sodium phosphate buffer containing 0.5 M NaCl. After dialysis against 0.01 M sodium phosphate buffer, the venom metalloproteinase solution was loaded onto a Poros S (Sulfoethyl) column (4.6 × 100 mm, Applied Biosystems, Foster City, CA) which had been equilibrated with 0.01 M sodium phosphate buffer. The column was washed and eluted with a linear 0–500 mM NaCl gradient in 0.01 M sodium phosphate buffer. Figure 1 shows the elution profile of Poros S column chromatography. The venom metalloproteinases were separated into two fractions: non-bound fraction I (HR1a) and bound fraction II (HR1b). These two fractions were found to be electro-
Phoretically homogenous, and they were dialyzed against 0.01 M Tris–HCl buffer (pH 8.0). Treatment of HR1b with reducing agent only slightly decreased its mobility on SDS–PAGE, suggesting that this protein is monomeric (Fig. 1, inset).

Fraction II, expected to be HR1b, was separated on SDS–PAGE, electrotransferred onto a polyvinylidene difluoride membrane, and excised for N-terminal amino acid sequencing. In the case of internal amino acid sequencing, the protein band of SDS–PAGE gel was in-gel digested with lysyl endopeptidase in digestion buffer (pH 8.5) for 20 h at 35°C. The peptides liberated by the digestion were separated by reversed-phase HPLC and used for amino acid sequencing. In-gel digestion, peptide separation by reversed-phase HPLC, and Edman degradation sequencing were outsourced, and performed by APRO Life Science Institute (Naruto, Japan).

Cysteine was detected as Cys-S-propionamide after reduction and alkylation. The N-terminal amino acid sequencing was unsuccessful, probably due to blockage of the N-terminal amino acid residue, but some of internal amino acid sequencing was successful. The sequences were determined to be QRSHDCAILLT, AYYQTFLTXHK, CPIMFYQXYFXF, and TDHVXPV. The comparison of these partial sequences with those from a database revealed that fraction II was identical with HR1b of Amami habu, with low homology to HR1a. This finding clearly indicates the occurrence of HR1b in the venom of Okinawa habu, as is the case for Amami habu. Hence, the substrate specificity of HR1b was compared with that of HR1a to characterize further its proteolytic property.

The substrate specificities of HR1a and HR1b from Okinawa habu venom were examined by fluorescence resonance energy transfer (FRET) combinatorial libraries (Peptide Institute, Osaka, Japan), as shown below.

\[
d^-A2pr(Nma)-Gly-Zaa-Yaa-Xaa-Ala-Phe-Pro-Lys(Dnp)-d-Arg-d-Arg
\]

Xaa (P1) stands for 19 natural amino acids, excluding Cys. A mixture of five amino acid residues (P, Y, K, I, and D) was incorporated at the Yaa (P2) position along with another mixture of five amino acid residues (F, A, V, E, and R) at the Zaa (P3) position for each Xaa. This yielded 25 peptide mixtures for each 19 Xaa series, resulting in a combinatorial library of a total 475 peptide substrates.

Primary screening of the favored Xaa was conducted as described previously. The metalloproteinase was reacted with each 19 series of FRET libraries in 10 mM Tris–HCl buffer (pH 8.0) for 60 min at room temperature. The fluorescence intensities were measured at \( \lambda_{ex} = 340 \) nm and \( \lambda_{em} = 440 \) nm. The resulting hydrolysate was analyzed by LC-MS, and the peptides cleaved were identified on the basis of their MS spectra.

Selection of favored Xaa revealed that HR1b preferred Tyr, Leu, Arg, Met, and Phe at the P1 position, in descending order (Fig. 2 A-1). Contrasting with this observation, HR1a preferred Ala, His, Pro, Met, and Tyr in descending order at this position (Fig. 2 A-2).

A second screening of the favored amino acid residue at the P2 and P3 positions was performed with the FRETS-25Tyr for HR1b, and FRETS-25Ala for HR1a as the best Xaa substrate (Fig. 2B). HR1b showed equal preference for the sequences of Ala-Asp and Arg-Ile at the P3-P2 position with different enzyme cleavage sites across the P1 position: the N-terminus side for Ala-Asp and the C-terminus side for Arg-Ile (Fig. 2 B-1). HR1a

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**Fig. 1.** Separation of HR1b from HR1a on Poros S Column.

Metalloproteinase fractions eluted from a Chelating Sepharose Fast Flow column were separated with a Poros S column, as described in the text. The figure inset shows the SDS–PAGE profile of HR1b and HR1a with and without treatment with a reducing agent (2-mercaptoethanol).
predominantly preferred Val and Asp at the P3 and P2 positions respectively (Fig. 2 B-2). Thus the substrate specificity of HR1b definitely differed from that of HR1a.

In order to confirm further the occurrence of HR1b in the venom of Okinawan habu, cDNA cloning of HR1b was attempted. RNA ligase-mediated rapid amplification of cDNA ends was performed using the GeneRacer kit (Invitrogen Corp., Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA was purified from the venom glands of a Protobothrops flavoviridis (Okinawa habu) using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA), and ligated to the GeneRacer RNA oligo adapter (5'-CGACUGGAGCACGAGGACACTGA-3'). The GeneRacer Oligo (dT) primer [5'-GCTGTCAACGATACGCTACGTAACG-3'] was then used to prime first-strand cDNA synthesis in the reverse-transcription reaction. The 5' ends of cDNA were amplified with the GeneRacer 5' primer (5'-CGACTG-GAGCAGGAGGACACTGA-3') and the gene-specific primer (5'-CCAAACAAAATAAGATTACCGTGCAGTCAG-3'). The 3' ends were amplified with the GeneRacer 3' primer (5'-GCTGTCAACGATACGCTACGTAACG-3') and the gene-specific primer (5'-GGAAA-CCACCCAAGTGACAGGAAATTTCAG-3'). Gene-specific primers for HR1b were synthesized based on the sequence of the HR1b from Protobothrops flavoviridis (Amami habu).11) PCR products were gel-purified and cloned into pCR4-TOPO vector (Invitrogen Corp., Carlsbad, CA, USA) for sequencing. Figure 3 shows the deduced amino acid sequences of Okinawa habu HR1b. The open reading frame (ORF) consisted of 1,845 bp. It encodes signal peptides and a mature HR1b polypeptide of 423 amino acids. HR1b of Okinawa habu shows significant similarity to HR1b of Amami habu: 99.67% identity in amino acid sequence. Only two amino acid residues are different: Val-34 and Cys-313.

In this study, we determined the occurrence of HR1b in the venom of Okinawan Protobothrops flavoviridis (Okinawa habu), as the case of the same species living on Amami Island (Amami habu). We also found that the concentration of HR1b in the venom of Okinawa habu (0.49 mg/g crude venom) was much lower than that of Amami habu (3.8 mg/g crude venom), as shown in Fig. 1, which probably has been the major obstacle to the isolation of this protein. HR1b manifested its characteristics in a different substrate specificity from
HR1a. The presence of HR1b in even small quantities can broaden the number of substrates to be cleaved, and can potentiate the hemorrhagic activity of the snake venom.

References


