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Purification and characterization of two high molecular mass snake venom metalloproteinases (P-III SVMPs), named SV-PAD-2 and HR-Ele-1, from the venom of *Protobothrops elegans* (Sakishima-habu)

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ABSTRACT

We herein identified two high molecular mass metalloproteinases, named SV-PAD-2 and HR-Ele-1, in the venom of *Protobothrops elegans*. HR-Ele-1 appeared as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) regard under reducing and non-reducing conditions, and the molecular mass of this protease was approximately 60 kDa under reducing conditions. On the other hand, the molecular masses of SV-PAD-2 on SDS-PAGE were 110 kDa under the non-reducing condition and 52 kDa under the reducing condition. These SVMPs exhibited fibrinogenolytic and enzymatic activities against synthetic substrates for matrix metalloproteinases (MMPs) and the insulin B-chain, and were both inhibited by EDTA. SV-PAD-2 inhibited ADP- and collagen-induced platelet aggregation, with IC_{50} values of 240 nM and 185 nM, respectively. HR-Ele-1 exhibited hemorrhagic activity, and its minimum hemorrhagic dose (MHD) was 0.05 μg in the guinea pig.

1. Introduction

Snake venom metalloproteinases (SVMPs) are widely distributed in viperid and crotalid venoms. SVMPs are primarily responsible for hemorrhagic activity and the induction of local and systemic bleeding. They also possess diverse functions such as the disruption of hemostasis mediated by procoagulant or anticoagulant effects, platelet aggregation, and apoptotic or pro-inflammatory activities (Fox and Serrano, 2005). SVMPs range in size from 20 to 100 kDa and have been classified into three groups, P-I–P-III, according to their domain organization (Bjarnason and Fox, 1995). Class P-I SVMPs contain the smallest members that have a metalloproteinase (M) domain. Class P-II SVMPs contain a canonical disintegrin (D) domain connected by a sort spacer region to the carboxy terminal of the M domain. Class P-III SVMPs contain a cysteine-rich (C) domain carboxy to the D domain, and single chain proteins have been subclassified as P-IIIa including HR1A and HR1B from *Protobothrops* (*Trimeresurus*) *flavoviridis* venom (Kishimoto and Takahashi, 2002; Takaya et al., 1990). P-III SVMPs have been further divided into subclasses based on their distinct post-translation modifications, such as dimerization (P-IIIc) or proteolytic processing (P-IIIb). P-IIIc has been reported on HV1 from *Protobothrops flavoviridis* venom, VAP1 from *Crotalus atrox* venom, VLAIPs from *Vipera lebetina* venom, and TSV-DM from *Protobothrops stejnegeri* venom, and most these proteins were shown to induce apoptosis in vascular endothelial cells (Masuda et al., 2001, 2000, 1997; Samel et al., 2012; Trummal et al., 2005; Wan et al., 2006). The heterotrimeric subclass of SVMPs are now included in the P-III group as a subclass (P-IIId), which represents another post-translational modification of canonical P-IIIa SVMPs. RVV-X, belonging to the P-IIId SVMP class, has an additional disulfide-linked C-type lectin domain. Hemorrhagic SVMPs are responsible for the lethal activity of viperid and crotalid venoms. The hemorrhagic activities of P-III SVMPs are generally stronger than those of P-I SVMPs; however, P-III SVMPs are more closely related to ADAM (a disintegrin and metalloproteinase) family proteins and ADAMTSs (ADAM with thrombospondin type-1 motif).

We previously identified a unique protein named SV-PAD-1, which we found inhibited ADP-induced platelet aggregation and promptly dissociated ADP-induced platelet aggregation (Oyama et al., 2009). In the present, we purified two P-III SVMPs, named

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SV-PAD-2 and HR-Ele-1, which have anti-coagulant activities, from the venom of *Protobothrops elegans*, and also clarified the partial characterization of these SVMPs.

2. Materials and methods

Lyophilized *P. elegans* venom was obtained from the Japan Snake Institute (Gunma, Japan). Sephadex G-100, DEAE, CM-Sepharose Fast Flow, and Mono S were obtained from GE Healthcare (UK, Ltd). Bovine fibrinogen was purified using the technique of Doolittle et al. (1967). Immun-Blot PVDF Membrane and Precision Protein Standards were obtained from Bio-Rad Laboratories (Richmond, U.S.A), type I collagen from bovine calf was obtained from Nippi Co. (Tokyo, Japan), and type IV collagen from human placenta, and horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG were obtained from Sigma Chemical (St. Louis, MO, USA). The sources of the other materials used were as follows: ADP and collagen were from Meiji Yakuhin Co., Ltd. (Toyama, Japan); and rabbits (Jla: JW) and guinea pigs (Jla: Hartley) were obtained from Japan Laboratory Animals, Inc. (Tokyo, Japan). The other reagents and chemicals used were analytically pure.

2.1. Purification of SV-PAD-2 and HR-Ele1 from P. elegans venom

SVMPs was purified from *P. elegans* venom by gel-filtration using Sephadex G-100 and ion-exchange chromatography with DEAE Sepharose and SP-Sepharose. The crude venom (1.0 g) was gel-filtrated on a Sephadex G-100 column (2.7 × 85 cm). The high molecular mass fraction was pooled and dialyzed with 0.02 M Tris–HCl buffer (pH 8.0) at 4 °C overnight. The fraction was then ion-exchange chromatographed on a Q-Sepharose Fast Flow column (2.7 × 10 cm). Proteins were eluted with a linear concentration gradient of NaCl from 0 to 0.3 M (each 250 ml), and a 5-ml fraction was collected per tube. Fraction I, which contained SV-PAD-2, was dialyzed with 0.02 M acetate buffer (pH 6.0) and applied to an SP-Sepharose Fast Flow column (1.8 × 8 cm). Proteins were eluted with a linear concentration gradient of NaCl from 0 to 0.3 M (each 250 ml), and a 5 ml fraction was collected per tube. Fraction III, which contained HR-Ele-1, was dialyzed with 0.02 M acetate buffer (pH 6.0) and was applied to an SP-Sepharose Fast Flow column (1.8 × 8 cm). Proteins were eluted with a linear concentration gradient of NaCl from 0 to 0.3 M (each 250 ml; a fraction tube was collected at 5 ml/tube) and was also eluted with 0.5 M NaCl (a 10-ml fraction was collected per tube). The procedures described above were performed at 4 °C. SV-PAD-2 and HR-Ele-1 were detected by western blotting using an anti-HR1A polyclonal antibody.

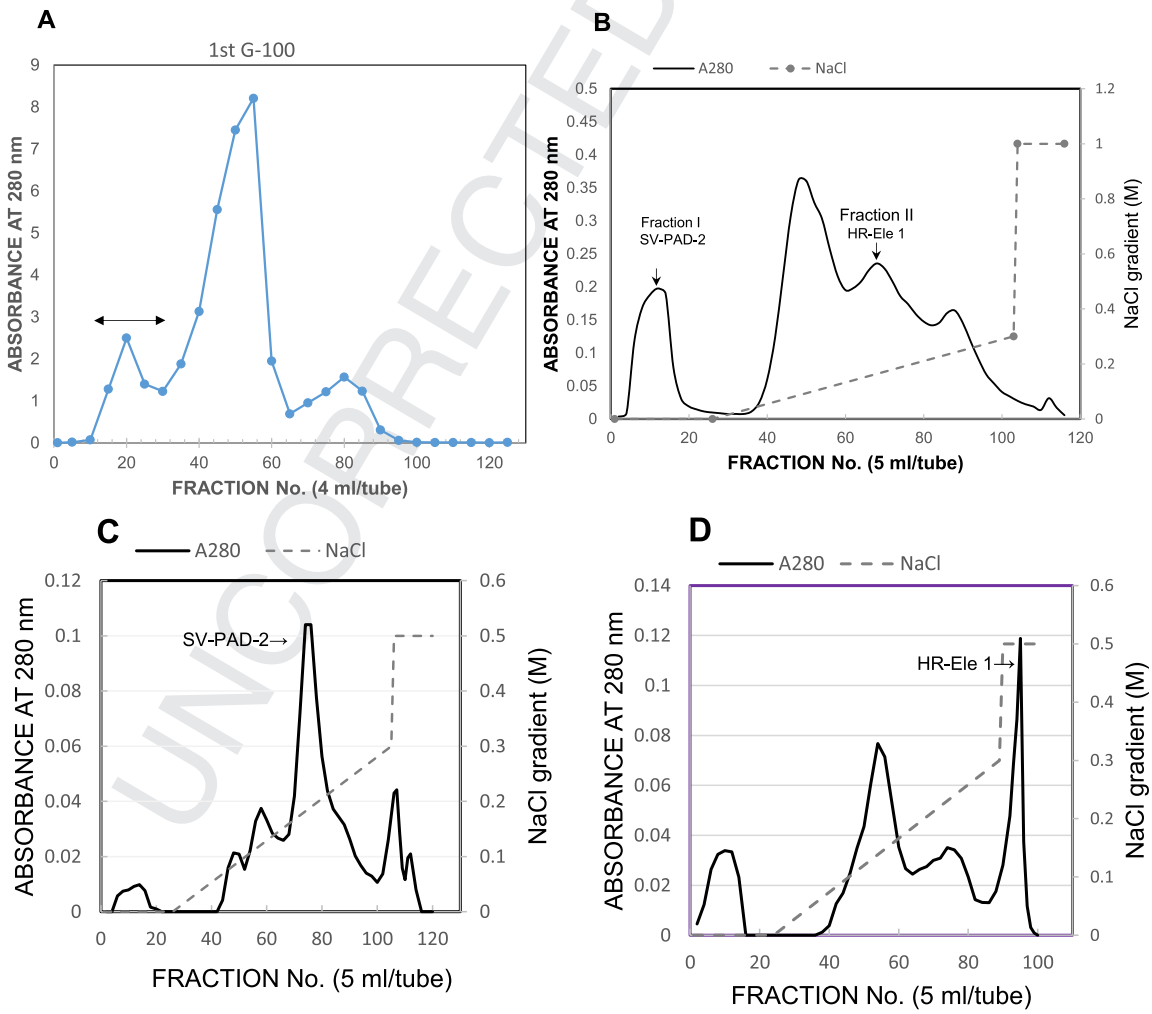

Fig. 1. Purification of SV-PAD-2 and HR-Ele-1 from the venom (1.0 g) of *Protobothrops elegans*. (A) Gel-filtration using Sephadex G-100 of SVMPs. The arrow shows the high molecular fraction containing the target proteins. (B) Q-Sepharose Fast Flow chromatography of the high molecular fraction obtained from gel-filtration. Fraction I contained SV-PAD-2 while fraction II contained HR-Ele-1. SP-Sepharose Fast Flow chromatographies of fraction I containing SV-PAD-2 (C) and fraction II containing HR-Ele-1 (D).

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